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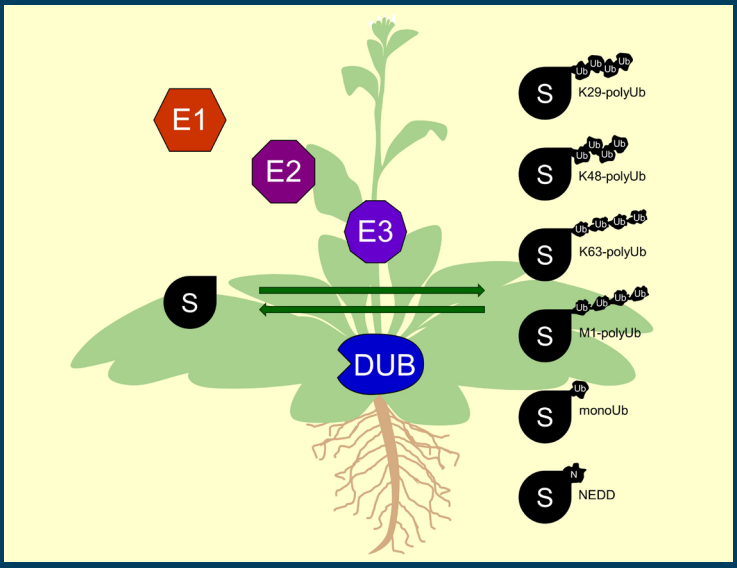

REVERSIBLE UBIQUITYLATION IN PLANT BIOLOGY

Topic Editors
Hongyong Fu, Daphne R. Goring and Pascal Genschik

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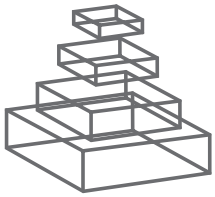

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REVERSIBLE UBIQUITYLATION IN PLANT BIOLOGY

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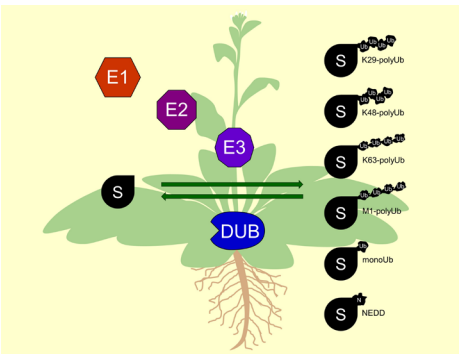

Schematic illustration of the topics covered in the Reversible Ubiquitylation in Plant Biology ebook. Shown are the ubiquitin and ubiquitin-like conjugating enzymes (E1 activating enzyme, E2 conjugating enzyme, E3 ligase), deubiquitinating enzyme (DUB), and the different types of ubiquitin linkages (K29, K48, K63, M1) and the ubiquitin-like protein NEDD.

Reversible ubiquitylation plays an important regulatory role in almost all aspects of cellular and organismal processes in plants. Its pervasive regulatory role in plant biology is primarily due to the involvement of a large set of ubiquitin system constituents (encoded by approximately 6% Arabidopsis genome), the huge number of important cellular proteins targeted as substrates, and various drastic effects on the modified proteins. The major components of the ubiquitin system include a large set of enzymes and proteins involved in ubiquitin conjugation (E1s, E2s, and E3s) and deconjugation (deubiquitinases of different classes) and post ubiquitin conjugation components such as ubiquitin receptors, endocytic machineries, and 26S proteasome. The established substrates include transcriptional activators and repressors, signaling components, key metabolic enzymes, and critical mechanistic components of major cellular processes and regulatory mechanisms. Post-translational modification of proteins by reversible ubiquitylation could drastically affects the modified proteins by proteolytic processing and turnover, altering catalytic activity, subcellular targeting, and protein-protein interaction. Continued efforts are being carried out to identify novel substrates critical for various cellular and organismal processes, to determine effects of reversible ubiquitylation on the modified substrates, to determine signaling determinants triggering reversible ubiquitylation of specific substrates, to illustrate individual components of the ubiquitin system for their in vivo functions and involved mechanistic roles, and to determine mechanistic roles of modification acting on critical components of major cellular processes and regulatory mechanisms. The aim of this special topic is to serve as a platform to report most recent advances on those above listed current research endeavors. We welcome article types including original research, review, mini review, method, and perspective/opinion/hypothesis.

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EDITORIAL
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Reversible ubiquitylation in plant biology

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Keywords: ubiquitin, histone, self-incompatibility, deubiquitination, ubiquitin ligase, abiotic stress, plant innate immunity, NEDD8/RUB

Post-translational modification by ubiquitin plays a critical regulatory function in nearly all aspects of plant biology (Vierstra, 2009). Diverse conjugation enzymes attach monoubiquitin or polyubiquitin, with eight different linkages, as distinct signals to the regulatory and mechanistic components of various cellular processes. This ebook updates the functions, targets, and mechanisms of the conjugation components involved in the monoubiquitination of histones H2A and H2B and the polyubiquitination of all linkage types. Additionally, the roles and mechanisms of E3 ligases in biotic and abiotic stress responses and self-incompatibility (SI) and the regulation of cullin-based ligases (CRLs) by neddylation/deneddylation are updated. Finally, the functional roles of deubiquitination enzymes (DUBs) are reviewed together with a report on the biochemical and phylogenetic analyses of *Arabidopsis* OTU DUBs that support their functional differences.

The topology of the polyubiquitin chain produced by the ubiquitin E3 ligase determines the fate of the conjugated substrate. Here, Walsh and Sadanandom review the functional roles of different ubiquitin linkages in *Arabidopsis* (Walsh and Sadanandom, 2014). The conjugation components involved in the assembly of the K11-, K48-, and K63-linkages are conserved in plants. The K48-linked polyubiquitination targets regulatory factors for proteasomal degradation and is involved in diverse plant functions. The K63 linkage-forming RING E3s, RGLG1/2, are involved in auxin signaling, where they regulate auxin levels by affecting endocytic turnover of the auxin efflux transporter PIN2. In addition, plants overexpressing the K63-linkage-specific E2, UBC13, or harboring *rglg1*/*rglg2* double mutations exhibit bifurcated root hairs, showing an iron deficiency response. Two other reports also detail the importance of the K63 linkage in PIN2 turnover and iron deficiency response (Pan and Schmidt, 2014; Tomanov et al., 2014). While the Bachmair's group extends his discussion on K63-linkage in effector-triggered immunity, the second paper proposes that UBC13 and RGLGs are competed by DNA replication/repair under iron deficient conditions.

Histone H2A and H2B monoubiquitination represents distinct epigenetic marks that repress or activate transcription. Feng and Shen discuss the *Arabidopsis* E2s, E3s, and DUBs responsible for H2B monoubiquitination, which is crucial for the transcriptional activation of key regulators controlling flowering, seed dormancy, clock, photomorphogenesis, and pathogen defense (Feng and Shen, 2014). The mechanisms underlying the targeting of H2B monoubiquitination enzymes and transcriptional activation are updated. Conversely, H2A monoubiquitination, mediated by the polycomb repressive complex PRC1, is a repressive chromatin mark that is important for stem apical meristem maintenance, embryonic cell fate determinacy, and seed germination. The maintenance of gene repression also requires another polycomb complex PRC2, which is responsible for the Lys 27 methylation of histone H3 (H3K27me2/3). Interestingly, findings that challenge the paradigm of PRC2 and PRC1 sequential recruitment are discussed.

Duplan and Rivas update the functional roles of ubiquitin ligases in plant immune signaling (Duplan and Rivas, 2014). Ubiquitin E3 ligases are involved in pathogen perception, where they modulate pathogen-associated molecular pattern receptors at the plasma membrane or intracellular nucleotide-binding leucine-rich repeat-type receptors. These E3s are also involved in signaling responses downstream of pathogen perception through targeting and modulating vesicle trafficking components or transcription factors. Duplan and Rivas also discuss microbial effectors that target host E3s or act as E3s to counteract plant resistance. In parallel, Stone updates the functional roles of E3s in plant responses to abiotic stresses (Stone, 2014). Here E3s are involved in the suppression of stress response activators under non-stress conditions and the inactivation of response suppressors under stress. The roles of E3s in attenuating stress response signaling after stress relief are also discussed. Interestingly, this report outlines how multiple E3s and their targets are involved in the production and signaling of the stress-related hormone abscisic acid (ABA). Additionally, these reviews discuss the importance of the plant U-box armadillo repeat ligases (PUB-ARMs) in biotic and abiotic stress responses. Several PUB-ARMs target the plasma membrane or intracellular trafficking components. Moreover, Vogelmann et al. report that the *Arabidopsis* PUB-ARMs, SAUL1 (SENESCENCE-ASSOCIATED UBIQUITIN LIGASE 1) and its paralogs are plasma membrane (PM)-localized via their C-terminal ARM repeats (Vogelmann et al., 2014). PM-localization is conserved for SAUL1-type PUB-ARM orthologs in land plants, suggesting functional importance; however, their membrane targets have not yet been identified.

E3s are also involved in two major plant SI systems: *S* receptor kinase (SRK)-based and S-RNase-based. ARC1, a PUB-ARM E3, is critical for SRK-based SI in *Brassicaceae* plants. Indriolo and Goring provide updates on the conserved role of SRK-ARC1

signaling in self-pollen rejection in *Brassica* and *Arabidopsis* species and on the role of the ARC1 target, Exo71A1, in secretory activity during compatible pollination on the stigmatic papillae (Indriolo and Goring, 2014). ARC1 is proposed to negatively regulate Exo71A1 to disrupt secretion during incompatible pollination. *S* locus-encoded F-box proteins (SLFs), which are involved in S-RNase-based SI, are the substrate receptors for cullin1-based SCF E3s. Liu et al. provide evidence to support the cytoplasmic localization of pollen and pistil S-factors, SLFs and S-RNase in *Petunia hybrida* pollen tubes (Liu et al., 2014). They report a selective interaction between SLFs and S-RNase and non-self SLF-mediated S-RNase degradation in compatible but not incompatible pollen tubes. All evidence indicates that non-self SLFs mediate cytosolic S-RNase degradation to allow non-self-pollen acceptance. The self-SLF is unable to degrade S-RNase, which acts as a cytotoxin that rejects self-pollen.

CRLs represent the most prominent ubiquitin E3 ligase class, that is critical for specifying substrates, and are regulated by reversible NEDD8/RUB modification of the cullin subunit. Mergner and Schwechheimer update the critical components involved in NEDD8 processing, conjugation, and deconjugation in yeast, animals, and, in particular, plants (Mergner and Schwechheimer, 2014). The update provides insights on the role of cullin neddylation in auxin responses, the role of CSN-mediated cullin deneddylation and CSN-CRL interaction in photomorphogenesis and auxin responses, and the role of substrates, substrate receptors, CAND1, and CSN-associated proteins in CRL regulation. They also discuss possible non-cullin substrates and neddylation functions along with CRL regulation.

Five conserved classes of DUBs play important mechanistic roles in nearly all aspects of eukaryotic cellular processes. Isono and Nagel update the involvement of DUBs of all classes in various aspects of plant biology (Isono and Nagel, 2014). In most cases, the exact molecular mechanisms, targets, and cellular functions of plant DUBs require further investigation. The extent of conservation and divergence on mechanisms and cellular functions also require scrutiny for plant DUBs for which yeast and animal orthologs have been extensively described. In addition, Radjacommare et al. report the characterization of *Arabidopsis* OTU DUBs (Radjacommare et al., 2014), the corresponding mammal DUB class was recently identified as critical for various cellular processes and signaling pathways. Distinct biochemical properties and phylogenetic relationships support the involvement of *Arabidopsis* OTUs in conserved and also plant-specific cellular processes.

It is now clear that reversible ubiquitination is a critical regulatory element of numerous cellular processes and nearly all aspects of plant biology. Mechanistic studies for each ubiquitination or deubiquitination event, including target recognition, assembly and perception of diverse ubiquitin signals, and the regulation of the involved conjugation and deconjugation components could provide bases for better understanding of various aspects of plant biology. The small molecule-mediated substrate recognition of cullin-based CRLs identified in studies of auxin signaling is one excellent example (Santner et al., 2009). Timely updates are necessary in response to the rapid progress and important new discoveries obtained from functional and mechanistic studies of new substrates and the large numbers of conjugation and deconjugation components, such as ubiquitin E3 ligases and DUBs.

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Ubiquitin chain topology in plant cell signaling: a new facet to an evergreen story

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Ubiquitin is a peptide modifier able to form polymers of varying length and linkage as part of a powerful signaling system. Perhaps the best-known aspect of this protein's function is as the driver of targeted protein degradation through the Ubiquitin Proteasome System (UPS). Through the formation of lysine 48-linked polyubiquitin chains, it is able to direct the degradation of tagged proteins by the 26S proteasome, indirectly controlling many processes within the cell. However, recent research has indicated that ubiquitin performs a multitude of other roles within the cell beyond protein degradation. It is able to form 6 other "atypical" linkages though lysine residues at positions 6, 11, 27, 29, 33, and 63. These atypical chains perform a range of diverse functions, including the regulation of iron uptake in response to perceived deficiency, repair of double stranded breaks in the DNA, and regulation of the auxin response through the non-proteasomal degradation of auxin efflux carrier protein PIN1. This review explores the role ubiquitin chain topology plays in plant cellular function. We aim to highlight the importance of these varying functions and the future challenges to be encountered within this field.

Keywords: ubiquitin, plants, signaling pathways, abiotic stress, pathogen

INTRODUCTION

Posttranslational modification (PTM) is a process through which proteins are altered after ribosomal synthesis. This process includes the formation of disulfide bridges, the alteration of amino acids and the addition of new functional groups, thereby changing the proteins from their nascent state to one of full functionality, or changing the function of the mature proteins. Ubiquitin and ubiquitin-like proteins (UBLs) are perhaps the most well-known set of proteins involved in the latter type of PTM.

Ubiquitin is a 76 amino acid protein so named because of its ubiquitous nature. It is part of a powerful signaling system that regulates several processes, the most well-known of which being the degradation of cellular proteins by the 26S proteasome in the Ubiquitin/Proteasome System (UPS). However, the ubiquitin signaling system is versatile and is able to regulate not only the abundance of protein present within the cell by degradation, but it is also able to target proteins to particular organelles (such as the nucleus), initiate membrane receptor recycling, and recruit proteins in the DNA damage repair pathway. Its importance can be seen by the fact that over 6% of the proteins in the *Arabadopsis thaliana* proteome are part of the ubiquitin pathway (Downes and Vierstra, 2005).

Ubiquitin contains 7 lysine residues: K6, K11, K27, K29, K33, K48, and K63. Through these 7 lysine residues and its N-terminal methionine (M1) it is able to form polyubiquitin chains upon a target protein. This provides a huge scope for variation in linkages and thus allows several functions to be encoded by just one peptide tag.

MAKING CHAINS

The ubiquitination of a target protein occurs through an exposed lysine residue. The ε-amino group of the lysine forms a bond with ubiquitin through the carboxyl group of the C-terminal glycine (Pickart, 2004). This tag can then be extended, if required, into a polyubiquitin chain with the sequential ubiquitin moieties connected through lysine-glycine linked isopeptide bonds.

Four enzymes are required for ubiquitin conjugation of the tag to the target protein; a ubiquitin-activating enzyme (E1), a ubiquitin-conjugating enzyme (E2), and two ubiquitin ligases (E3 and E4). Together these enzymes form a cascade, with multiple rounds of repetition giving chain extension. To begin, the E1 activates the Ub moiety using ATP, forming a Ub-adenylate (Schulman and Harper, 2009), which is then bound by the E1. The activated Ub is then transferred to the E2, which correctly orientates the moiety, and the complex recruited by an E3 (Spratt et al., 2012), resulting in the transfer of Ub to the target protein. The chain is then subsequently extended through either E3 or E4 activity (Koegl et al., 1999).

LYSINE 48-LINKED POLYUBIQUITIN CHAINS

Ubiquitin chains connected by "typical" K48-linkages perform one of the most well-known functions of ubiquitin—proteasome targeting. The UPS has been implicated in many aspects of plant function. As plants are sessile organisms, a greater degree of phenotypic plasticity is required to ensure survival in a changing environment. Plants must be able to respond quickly and efficiently to relevant stimuli and this is achieved, in part, by the targeted degradation of proteins by the proteasome.

The function of K48 chains and the UPS has been the subject of several excellent reviews (see Moon et al., 2004; Dreher and Callis, 2007). The UPS plays a major role in plant development, hormone signaling, pollen tube growth, pathogen defense, and the cell cycle.

One specific example is the involvement of the UPS in self-incompatibility (SI). SI is a mechanism by which flowering plants are able to avoid inbreeding due to self-fertilization. In *Brassicaceae sp.*, the UPS plays a role in SI through the U-box-dependant E3 ligase ARM-repeat-containing 1 (ARC1), as shown by the breakdown of SI upon antisense downregulation (Stone et al., 1999). ARC1 is thought to ubiquitinate a compatibility factor in the pistle, leading to its degradation in the proteasome and the rejection of self-pollen (Stone et al., 2003).

Another example of K48-linked polyubiquitin chains and their role in proteasomal degradation is auxin-mediated $SCF^{TIR1/AFB}$ (Skp1, Cullin, F-box receptor-type ubiquitin ligase) pathway. Auxin is a plant hormone that regulates gene expression with respect to plant development. It binds to TIR1 or AFB (F-box) components of the SCF^{TIR1} RING-type E3-ligase complex, enhancing the affinity of SCF^{TIR1} for Aux/IAA transcription regulator proteins. Aux/IAA proteins, such as SHY2 and BDL, form heterodimers with ARF (Auxin Response Factor) transcription factors, resulting in repression of genes controlled by auxin-responsive elements (AuxREs). The SCF^{TIR1} polyubiquitinates these Aux/IAAs and causes their destruction by the 26S proteasome (Maraschin et al., 2009). This results in the binding of ARFs and transcription of specific auxin response genes.

LYSINE 63-LINKED POLYUBIQUITIN CHAINS

K63 polyubiquitin chains have been discovered in both single and multicellular eukaryotes and have been shown to regulate several processes in yeast and mammalian cells in a non-proteolytic manner, such as kinase activation, protein synthesis, DNA repair, and chromosome regulation (Jacobsen et al., 2009). Their function in plants, however, has been less well-characterized, with K63 chains only implicated in apical dominance, DNA repair and iron deficiency mechanisms to date (Li and Schmidt, 2010)

Auxin performs many roles within the plant, as seen above, one of which being apical dominance, where the main central stem exhibits dominance over side shoots. The mutation of two membrane-associated E3 ubiquitin ligases, RGLG1 and RGLG2, exhibits a loss of apical dominance due to reduced cellular auxin concentration and the inability to transcribe auxin-responsive genes upon the application of exogenous auxin (Yin et al., 2007). RGLG1 and RGLG2 have been shown to catalyze the formation of K63 ubiquitin chains *in vitro*, indicating a role for this type of ubiquitin linkage in the regulation of apical dominance.

Unlike the SCF-TIR1 pathway described earlier, RGLG1 and RGLG2 seem to play a role in regulating intracellular auxin levels through PIN (auxin efflux carrier protein) cycling. PIN2 has been shown to undergo K63-linked ubiquitination by RGLG2, with the chain acting as a signal for endocytosis and transport to the vacuole. Analysis of this pathway using an $rglg^{-}$ mutant indicates that ubiquitinated PIN2 plays a role in root gravitropism (Leitner et al., 2012). PIN1 also appears to be a RGLG1/2 target. Evidence indicates that PIN1 abundance is reduced in *rglg1/rglg2* knock out mutants, and an interaction has been demonstrated between RGLG2 and PIN1 in yeast two-hybrid studies (Yin et al., 2007).

K63 linked polyubiquitin chains are also implicated in iron deficiency signaling. Experiments involving the ectopic expression of a cucumber Ubc13 homolog (CsUbc13) in *Arabidopsis* showed the production of bifuricated root hairs, a classical response seen in iron-deficient plants (Li and Schmidt, 2010). Further work by Li and Schmidt indicated an interaction between Ubc13 and RGLG2, with *rglg1/rglg2* double mutants showing constitutively active root hair bifurication, suggesting that auxin directs in morphological responses to iron deficiency (Nagpal et al., 2000).

LYSINE 29-LINKED POLYUBIQUITIN CHAINS

Recent research (Wang et al., 2009) has revealed the role of K29-linked chains in the degradation of DELLA proteins. The DELLA protein family are a group of growth repressors involved in the gibberellic acid (GA) response (Fleet and Sun, 2005). In *Arabidopsis* there are five known DELLA proteins: GAI, RGA, RGL1, RGL2, and RGL3 (Cheng et al., 2004). Involvement of these proteins has been shown in several important environmental responses, such as the light, cold, and salt responses (Achard et al., 2007, 2008; Magome et al., 2008).

DELLA degradation, induced by GA, is an important part of this signaling pathway (Dill et al., 2001). In a cell free system, Wang et al. (2009) were able to show that K29-linked Ub chains were responsible for the targeting of DELLA proteins to the 26S Proteasome. This indicates that the K29 linkage provides a similar function to that of K48.

ATYPICAL LINKAGES (K6, K11, K27, K33, AND M1)

Unlike K48- and K63-linked chains, there has been very little research on other atypical polyubiquitin linkages, with none conducted in plant systems. Experiments in eukaryotes such as *S. cerevisiae* have shown the formation of chains liked via K6, K11, K27, and K33. Research has shown that these atypical linkages play a role in mammalian DNA repair (Wu et al., 2008), cell cycle control through the APC/C complex (Williamson et al., 2009), lysosomal localization of transcription factors (Ikeda and Kerppola, 2008), proteasomal degradation (Xu et al., 2009), and the regulation of signal transduction through the prevention of TCR-ζ and Zap-70 association (Huang et al., 2010), respectively. However, much of the function of these chains remains currently unknown.

As well as chains formed through lysine-glycine linkages, ubiquitin is also able to conjugate through the methionine residue located at the N-terminus (M1) (Emmerich et al., 2011). In doing so, it forms a linear chain which has been shown to play an important role in mammalian signaling pathways, such as those involving tumor necrosis factor (TNF) (Haas et al., 2009) and NF-κB (Tokunaga et al., 2009). To date, there has been no evidence to suggest the formation of M1-type linear chains in *Arabidopsis*.

DETERMINING CHAIN SPECIFICITY

As can be seen from the examples above, ubiquitin signaling is a very versatile system. Through the alteration of chain topology,

the Ub system forms a large part of many, vastly different biological pathways. It is through an integral part of the Ub conjugation pathway, the E2, that this attribute is conferred.

The E2s are a large family of proteins, present in all eukaryotes. They are characterized by both their ability to interact with E1s and E3s and the presence of the UBC motif. The UBC motif consists of a highly conserved catalytic fold of approximately 150–200 amino acids in length (Kim et al., 2004). In this fold sits the catalytic cysteine residue through which the Ub moiety is accepted (Mukhopadhyay and Reizman, 2007).

The E2 family is able to influence the construction of ubiquitin chains of a specific linkage (Ye and Rape, 2009). This includes linear homogenous chains of a single linkage type, heterologous chains (i.e., Ub and SUMO Aillet et al., 2012) and branched/mixed linkage chains. Studies within the field showed that E2s are able to synthesize ubiquitin chains of a particular linkage even whilst not in the presence of an E3 (Hass et al., 1991). Later research identified several E2s in yeast and human cells that appear to predominantly form ubiquitin chains of K48, K63, and K11 linkage; the identified E2s are ScCdc34, ScUBC13/MMS2, and HsUBE2S, respectively, with their mechanisms for preferred lysine selection differing greatly.

Cdc34 is an E2 from *Saccharomyces cerevisiae* that forms ubiquitin chains with predominantly K48 linkages (Petroski and Deshaies, 2005). The specificity for this linkage type appears to be conferred by the interaction between the acceptor Ub and an acidic loop present within the E2 (Li et al., 2007). This selection mechanism for correct Ub orientation differs from that of the predominantly K63-forming UBC13/MMS2 complex, where the active site cysteine residue forms a bond with the donor Ub moiety, with the complexed MMS2 non-covalently binds the acceptor Ub in an optimal position for K63 linkage (Eddins

Table 1 | *Arabidopsis* E2 function.

Gene	Locus	Homolog	Linkage type	Processes involved in	Referenced in
AtUBC11	AT3G08690	ScUBC1	K48	Mediator of selective degradation of abnormal and short-lived proteins. Expression in floral tissues.	Kraft et al., 2005
AtUBC27	AT5G50870	ScUBC1	Predicted K48	Expression in seeds, siliques, pistils, hypocotyls, and leaves.	Kraft et al., 2005
AtUBC28	AT1G64230	ScUBC1	Predicted K48	Expression in seeds, siliques, pistils, hypocotyls, and leaves.	Kraft et al., 2005
AtUBC7	AT5G59300	ScUBC7	K48	Able to ubiquitinate BrARC1 *in vitro*.	van Nocker et al., 1996; Stone et al., 2003; Kraft et al., 2005
AtUBC13	AT3G64460	ScUBC7	Predicted K48	Upregulated by syringolin. Caution: confusion in publications between AtUBC13 and AtUBC35 (AtUBC13A).	van Nocker et al., 1996; Kraft et al., 2005
AtUBC14	AT3G55380	ScUBC7	Predicted K48	Upregulated in the G0 to S phase transition of the cell cycle.	Genschik et al., 1994; van Nocker et al., 1996; Kraft et al., 2005
AtUBC35 (AtUBC13A)	AT1G78870	ScUBC13	K63	Mediator of transcriptional activation of target genes. Involved in UV damage repair. Involved in root development regulation in response to iron availability.	Kraft et al., 2005; Wen et al., 2006; Li and Schmidt, 2010
AtUBC36	AT1G16890	ScUBC13	K63	Partial functional redundancy with AtUBC35	Kraft et al., 2005; Wen et al., 2006; Li and Schmidt, 2010
AtUEV1A (AtMMZ1)	AT1G23260	ScMMS2	K63	Forms heterodimer with AtUBC35 and AtUBC36. Possibly involved in cell cycle control and differentiation.	Yin et al., 2007; Wen et al., 2008
AtUEV1B	AT1G70660	ScMMS2	K63	Forms heterodimer with AtUBC35 and AtUBC36. Possibly involved in cell cycle control and differentiation. May be involved in DNA repair.	Yin et al., 2007; Wen et al., 2008
AtUEV1C	AT2G36060	ScMMS2	K63	Forms heterodimer with AtUBC35 and AtUBC36. Possibly involved in cell cycle control and differentiation. May be involved in DNA repair.	Yin et al., 2007; Wen et al., 2008
AtUEV1D	AT3G52560	ScMMS2	K63	Forms heterodimer with AtUBC35 and AtUBC36. Possibly involved in cell cycle control and differentiation. Involved in DNA repair.	Yin et al., 2007; Wen et al., 2008

Collated data of Arabidopsis thaliana homologs of yeast and human E2s, showing the predicted/observed linkage type for each E2 and the processes in which it is involved.

et al., 2006). It is currently unknown how HsUBE2S orientates Ub moieties to ensure K11-linkage.

In *Arabidopsis thaliana*, 41 E2s have been identified to date (Kraft et al., 2005). In the study conducted by Kraft et al. (2005), a phylogenetic analysis of the identified E2s was conducted. This analysis identified several plant homologs to ScUBC1, ScUBC7, ScUBC13, and ScMMS2. Later research into these homologs, detailed in **Table 1**, has confirmed the role of the E2 in chain topology, with different E2s constructing chains of a distinct linkage (either "typical" K48-linkages or K63-linkages) upon their varied targets.

Aside from the E2s detailed in **Table 1**, the analysis of Kraft et al. (2005) also identified an *Arabidopsis* homolog to the K11-linkage forming HsUBE2S. This homolog, UBC22 (At5g05080), along with other homologs from *Arabidopsis lyrata* (AlUBC18), *Oryza sativa* (Os06g0660700), and *Triticum urartu* (TuUBC22) identified using BLAST (Altschul et al., 1997), showed large areas of conservation in the UBC fold as well as a conserved active site cysteine residue (**Figure 1**). This suggests that AtUBC22 and its homologs may also construct K11-linked chains as seen with HsUBE2S.

LINKAGE ANALYSIS

One inherent obstacle to research into polyubiquitin chain topology is the method of analysis used. Currently there is no direct way of assessing linkages between ubiquitin moieties in a chain. Most information gathered in this area has been obtained through a combination of mutant ubiquitin usage and mass spectrometry, neither of which are without their flaws.

The use of mutant ubiquitin to study chain topology has a number of potential problems associated with it. Creating satisfactory controls for experiments using mutant ubiquitin *in vivo* are difficult. Overexpresson of mutant ubiquitin may alter which substrates are ubiquitinated, and by what type of chain. Also, the mutation of lysine residues to arginine to prevent conjugation may alter the surrounding surface of the protein. An example of this would be the alteration of the K6 residue. K6-sulfosuccinimidobiotin-labled ubiquitin is able to form polyubiquitin chains, but conjugates show a lesser susceptibility to proteasomal degradation. Mutation of the lysine residue (K6W) also gives a similar effect (Shang et al., 2005). This suggests that K6, or the surrounding surface environment, is required for proteasomal degradation through recognition and binding to the 26S proteasome.

Mass spectrometry is one of the most powerful tools available for studying changes in ubiquitin linkages. Often proteins of interest are purified using an affinity matrix, then their linkages analyzed by mass spectrometry. The typical method used involves the detection of a signature tryptic peptide. This peptide is derived from ubiquitin attached through its lysine residue to the—GG or—LRGG of another ubiquitin moiety (Saracco et al., 2009). However, the results may be skewed in favor of chains with a higher relative abundance, making the complexity of less abundant chains harder to determine.

Aside from the more commonly used forms of linkage identification detailed above, it is also possible to determine Ub chain type through the use of deubiquitinating enzymes (DUBs) that specifically cleave ubiquitin moieties from target proteins. Due to structural disparities between multiubiquitin chains of differing linkage, DUBs show binding-site specificity for particular linkages (Reyes-Turcu et al., 2009). For example, the DUB OTU7B shows specificity toward K11-linked chains (Bremm et al., 2010; McGouran et al., 2013). This specificity could be exploited to determine chain linkage *in vitro*.

CONCLUSION

Ubiquitin is an effective peptide modifier that forms part of a powerful, if poorly understood, signaling system. By differing the selection of E1, E2, and E3s used in chain formation, polyubiquitin chains of differing linkage, and thus function, can be formed upon target proteins. The degree of variation of function achieved through these linkages creates a versatile system

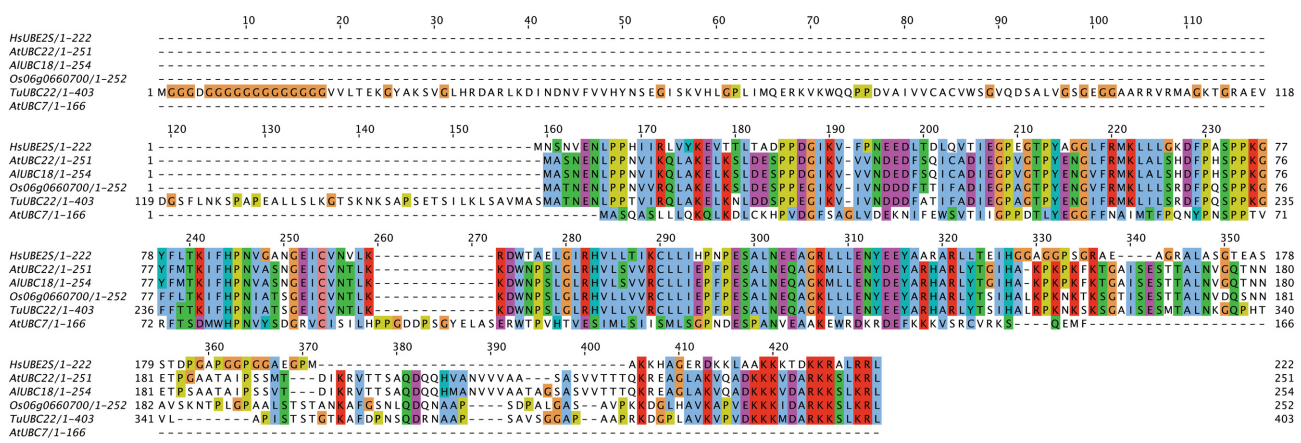

FIGURE 1 | Alignment of HsUBE2S with AtUBC22 homologs and AtUBC7. An alignment showing the amino acid sequence similarity between the K11-chain-forming Human E2, UBE2S, the plant homologs AtUBC22, AlUBC18, Os06g0660700, and TuUBC22, and the K48-chain-forming AtUBC7. The alignment shows large areas of conservation amongst the K11-chain-forming E2s, but little in comparison with UBC7. The active site cysteine (red box) is conserved in all aligned sequences, with the surrounding residues showing very high conservation amongst the K11-chain-forming UBCs.

enabling sessile organisms, such as plants, a greater degree of phenotypic plasticity and thus the ability to adapt to a rapidly changing environment.

Further research into the function of atypical chains in plants is required. However, perhaps the biggest obstacle to overcome in terms of the determination of polyubiquitin linkage in plants is the inability to directly assess topology both *in vivo* and *in vitro*. The development of a direct method of linkage assessment would eliminate the use of mutant ubiquitin as the primary method of chain determination.

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Ubiquitin Lys 63 chains – second-most abundant, but poorly understood in plants

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Covalent attachment of the small modifier ubiquitin to Lys ε-amino groups of proteins is surprisingly diverse. Once attached to a substrate, ubiquitin is itself frequently modified by ubiquitin, to form chains. All seven Lys residues of ubiquitin, as well as its N-terminal Met, can be ubiquitylated, implying cellular occurrence of different ubiquitin chain types. The available data suggest that the synthesis, recognition, and hydrolysis of different chain types are precisely regulated. This remarkable extent of control underlies a versatile cellular response to substrate ubiquitylation. In this review, we focus on roles of Lys63-linked ubiquitin chains in plants. Despite limited available knowledge, several recent findings illustrate the importance of these chains as signaling components in plants.

Keywords: ubiquitin Lys63 chains, cell signaling, vacuolar sorting, endoctosis, iron response, DNA repair, auxin transport, plant defense

Plant cells, similar to animal or fungal cells, have the ability to distinguish between substrates with single ubiquitin moieties attached, and between ubiquitin chains linked via different Lys residues. Mass spectrometric analysis of the *Arabidopsis* proteome showed that linkage of the ubiquitin carboxyl terminus to Lys 48 (K48) of ubiquitin most abundant, followed by K63 and by K11 linkages (Kim et al., 2013). **Figure 1** illustrates that different linkage types lead to differences in three-dimensional structure (for review, see also Kulathu and Komander, 2012). K48 and K11-linked ubiquitin chains result in proteolytic destruction of a substrate by the proteasome. In contrast, K63 chains are believed to have different consequences for the substrate. Although these latter chains can foster proteasomal degradation *in vitro* (Kirkpatrick et al., 2006), the *in vivo* significance of this finding remains to be demonstrated. A case in point is the mammalian "chain editing" enzyme A20, which removes K63 chains from its substrate RIP1 via a ubiquitin protease domain, to subsequently build a K48 chain via its ubiquitin ligase function. Whereas K63 chain-modified RIP1 is stable, the protein is quickly degraded after editing by A20 (Hymowitz and Wertz, 2010). It seems plausible that also in plants, the majority of K63 chains do not target substrates for proteasomal degradation. Plant enzymes and substrates for modification by K63 chains are largely unexplored, but first insights are emerging and, together with a glimpse at fungi and animals, shape expectations regarding conserved and plant-specific features.

Ubiquitin ligases that form K63 chains are not generally conserved across phyla. However, there exists at least one conserved component of K63 chain formation. The ubiquitin conjugating enzyme (Ubc)13 of *Saccharomyces cerevisiae*, together with a Ubc variant called methyl methane sulfonate sensitive (Mms)2, is dedicated to K63 chain formation and has homologs in all eukaryotes. Ubiquitin ligases that associate with this Ubc13/Mms2 heterodimer can form K63 chains. There is no exclusivity, though, because the same E3s may form different linkage types in association with other E2s, and mammalian and yeast E3s exist that form K63 chains without Ubc13/Mms2 participation. Based on interaction with an *Arabidopsis* homolog of Ubc13, a class of plant ubiquitin ligases termed RING DOMAIN LIGASE (RGLG) was identified that forms K63 chains *in vitro* (Yin et al., 2007). Additional candidates for K63 chain forming ligases are plant homologs of yeast Rad5 (Chen et al., 2008), although these enzymes remain to be characterized biochemically. It is possible that a requirement for K63 chains is conserved in certain biological processes, even though this conservation does not extend to the E2/E3 enzymes involved. As summarized in **Figure 2**, we discuss in the following examples of proven or suspected K63 chain requirement, most prominently DNA repair and membrane protein turnover, and additional publications pointing to roles for K63 chains in other processes.

In animals, ubiquitin K63 chains have multiple roles in supporting DNA repair processes (for review, see Jackson and Durocher, 2013). One known contribution is the modification of DNA clamp protein proliferating cell nuclear antigen (PCNA) by a ubiquitin K63 chain during post-replication repair. If the DNA replication fork encounters an obstacle, ubiquitin ligase Rad18 together with E2 Rad6 modifies PCNA by addition of a single ubiquitin moiety, which leads to recruitment of an error-prone DNA polymerase for trans-lesion DNA synthesis. Alternatively, the single ubiquitin moiety is extended into a K63 chain by another ubiquitin ligase (called Rad5 in *S. cerevisiae*), together with the Ubc13/Mms2 heterodimeric E2. The chain fosters error-free repair by facilitating a template switch, so that damages blocking the replication fork can be bypassed using the sister strand

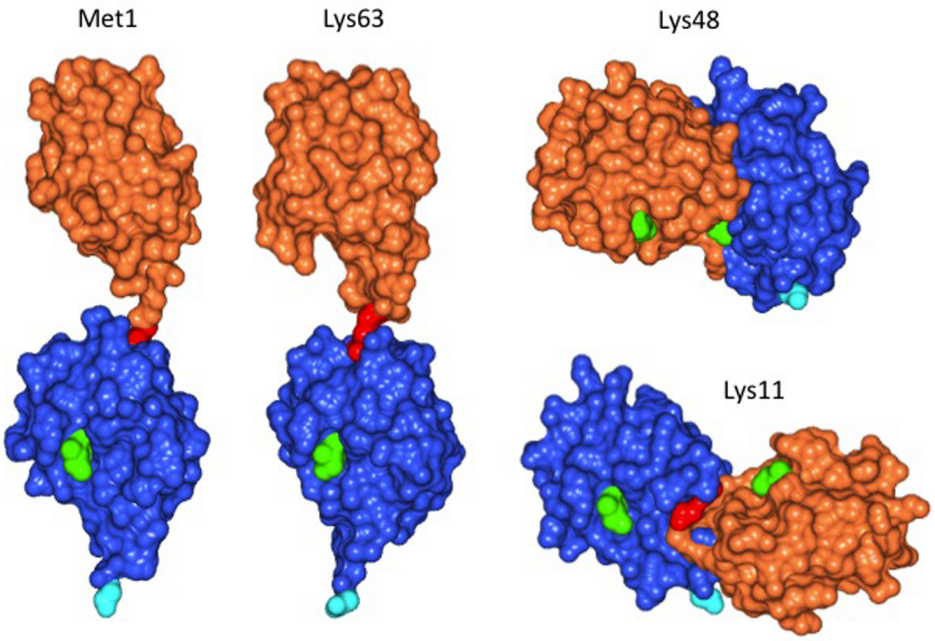

FIGURE 1 | Structure of diubiquitin linked via different Lys residues. Linkage of donor ubiquitin (orange) to Lys63 of the acceptor ubiquitin (blue) results in positioning the donor opposite to the C-terminal Gly of the acceptor (cyan).This structure is similar, but not identical to ubiquitin linked via the amino-terminal Met, which is the co-translational peptide bond linkage of many ubiquitin precursors. In contrast, linkage of the donor to Lys48 gives a more compact structure, with the donor positioned on the side of the acceptor. Similarly, use of Lys11 for linkage positions the donor on the opposite side of the acceptor ubiquitin compared to Lys48 linkages. Longer chains of Lys48 or Lys11 linked ubiquitin are therefore more compact than Lys63 chains and are known as signals for proteasomal degradation, whereas Lys63 chains are believed to have distinct roles, independent of the proteasome. Amino acid Ile44 was colored in light green to highlight the orientation of ubiquitin moieties, the Lys residue of the acceptor and the C-terminal Gly of the donor used for linkage formation are colored in red. Images were withdrawn from RCSB protein data bank [2W9N (M1), 3H7P (K63), 2PE9 (K48), and 2MBO (K11)] and processed with program CCP4 Molecular Graphics.

as a template (Mailand et al., 2013). K63 chains are also essential for double strand break (DSB) repair. Two components of DSB repair, the RING finger domain containing ubiquitin ligases RNF8 and RNF168, associate with the Ubc13/Mms2 heterodimer to build K63 chains. RNF8 acts earlier by binding to phosphorylated histone H2AX, which is an early mark of broken DNA ends. K63 chain formation on as yet poorly defined substrates then allows recruitment of the next layer of the repair machinery, including RNF168, which contains ubiquitin-binding domains. K63 chain deposition by RNF168 is again necessary for recruitment of another set of repair factors. While the ubiquitin ligases RNF8 or RNF168 have no reported plant homologs, Ubc13 and Mms2 homologs of *Arabidopsis* can complement the DNA repair defects of the respective yeast mutants (Wen et al., 2006, 2008). More importantly, *Arabidopsis* mutants in one Mms2 homolog exhibit increased sensitivity to a DNA damaging agent (Wen et al., 2008). Likewise, mutants in an *Arabidopsis* homolog of ubiquitin ligase Rad5 lead to DNA damage sensitivity (Mannuss et al., 2010; Wang et al., 2011), and support a role for template switching in plant post-replication repair (Mannuss et al., 2010). These studies are also consistent with a role for plant RAD5 in DNA repair outside the S-phase (suggesting modification of additional substrates besides PCNA).

Attachment of one ubiquitin moiety to membrane proteins is part of the endocytosis process, and several components of endosomal sorting complexes required for transport (ESCRT complexes) have ubiquitin binding domains (Korbei and Luschnig, 2013). The ubiquitylation of membrane receptors typically occurs after ligand binding. For unclear reasons, some membrane proteins require not only monoubiquitin modification for internalization, but ubiquitin K63 chain attachment. The chain may be short, as shown for the yeast uracil transporter Fur4 (Lauwers et al., 2010), but this short ubiquitin K63 chain is nonetheless essential for endocytosis. The simplest explanation for the requirement is a geometric necessity: if single ubiquitin moieties would be sterically not accessible for ESCRT factors, short ubiquitin K63 chains could act as "honorary monoubiquitin" modifications, promoting substrate endocytosis.

Once a membrane protein is sequestered into a vesicle, productive association with other proteins can continue. For instance, signaling may occur in different or unaltered form compared to the plasma membrane-localized receptor (Jalink and Moolenaar, 2010), and, more important in this context, the role of ubiquitin is not finished. There are many indications that later steps of membrane trafficking require ubiquitylation, and again K63 chains are suspected to play an important part. One of the decisions to be made while a membrane protein is on a vesicle is whether it is re-cycled onto the plasma membrane, or whether it is diverted into the vacuole via multivesicular bodies. In that case, ubiquitin K63 chains can effect proteolytic destruction of

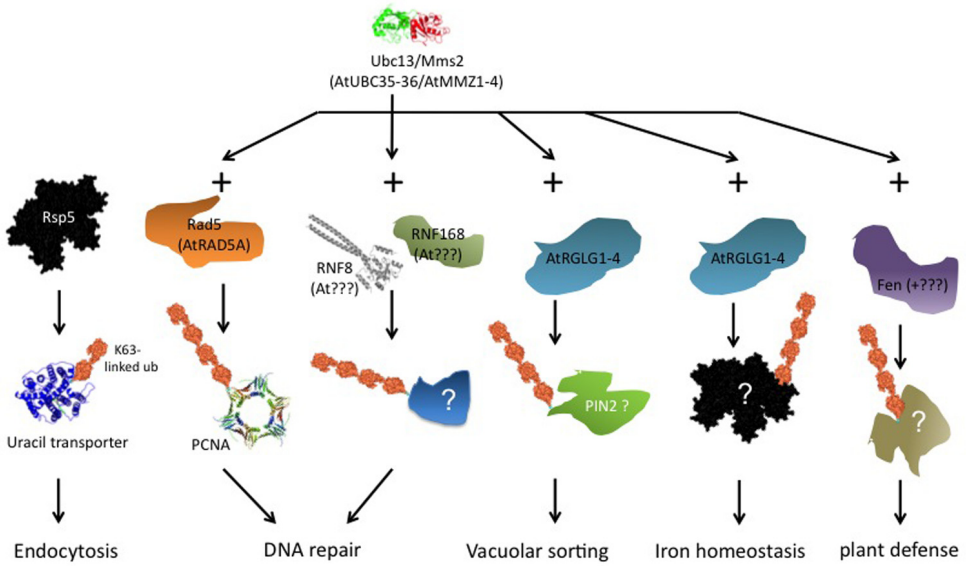

FIGURE 2 | Overview of ubiquitin K63 chain dependent processes. The scheme is a composite of data from different organisms. The leftmost column, endocytosis of uracil permease Fur4 via ubiquitin ligase Rsp5, was investigated in *S. cerevisiae*. Ubiquitin transfer via Rsp5 occurs from a Cys residue in the ligase HECT domain, Ubc13/Mms2 is not involved. RNF8 and RNF168 (next columns) were characterized in mammals, and no plant homologs are known to date. The other schemes refer directly to processes in plants. See text for further details.

substrates, which occurs via vacuolar degradation, rather than by the proteasome.

In mammals, K63 ubiquitin chain-specific enzymes exist that critically influence the vacuolar (lysosomal) versus plasma membrane path of endocytosed membrane proteins (Clague and Urbe, 2010; Wright et al., 2011). Proteins with homology to ubiquitin K63 chain-specific proteases of mammals were investigated in *Arabidopsis* (Isono et al., 2010; Katsiarimpa et al., 2011). Although the plant proteases are not specific for K63 chains, but can also hydrolyze K48 chains, they have a role in vacuolar sorting, just as the mammalian counterparts.

A direct link between ubiquitin K63 chains and plant plasma membrane proteins was established when analyzing turnover of the PIN2 auxin efflux facilitator. Here it turned out that PIN2 decoration by K63 chains functions in efficient endocytic sorting to the vacuole (Leitner et al., 2012). Notably, when testing a *pin2* allele mimicking constitutive mono-ubiquitylation, but lacking further lysines required for its K63-linked ubiquitylation, the fusion protein got stuck *en route* to the vacuole, highlighting an essential function for K63 chains at later steps of PIN2 endocytic sorting. Analysis of PIN2 in an *rglg* mutant combination deficient in K63 chain-forming E3 ligases revealed reduced ubiquitylation levels, which establishes PIN2 as potential RGLG substrate, but direct evidence for such interaction remains to be shown (Yin et al., 2007; Leitner et al., 2012).

An interesting connection was found between ubiquitin K63 chains and iron homeostasis (Li and Schmidt, 2010). Iron deficiency leads to the up-regulation of the Ubc13 homolog in cucumber, which occurs on the protein level without increase of the transcription rate. In line with a role for K63 chains in iron response, a mutation in one of the *Arabidopsis* Ubc13 homologs results in decreased formation of branched root hairs, which are normally formed in response to iron deficiency. Mutations in both Ubc13 homologs lead to a generally decreased density of root hairs. Li and Schmidt (2010) also investigated iron responses in a mutant in two K63 forming ubiquitin ligases, RGLG1 and RGLG2. The double mutant displays, unexpectedly, constitutive formation of forked root hairs, suggesting the existence of additional players in this regulon with impact on K63 chains. For example, this response could involve variations in the abundance of iron transport proteins such as IRON-REGULATED TRANSPORTER1 (IRT1), a key effector of iron availability in *Arabidopsis* (Vert et al., 2002). Ubiquitylation and associated vacuolar targeting of IRT1 has been demonstrated recently, and another E3 ligase termed IRT1 DEGRADATION FACTOR1 (IDF1) has been implicated in such regulation (Barberon et al., 2011; Shin et al., 2013). However, IRT1 appears to be mono-ubiquitylated at one or several sites, whereas modification by K63 chains in a dynamic control of IRT1 turnover has not been demonstrated yet (Barberon et al., 2011). It thus remains unclear whether or not K63 chains could influence iron homeostasis at the level of carrier turnover.

Ubiquitin K63 chains are also important for cytosolic signaling complexes assembled as part of defense responses. Mural et al. (2013) identified Fen-interacting-protein (Fni)3, a tomato Ubc13 homolog, and its cofactor *Solanum lycopersicum* Ubc variant (Suv; an Mms2 homolog) as mediators of the defense response. Fen is a protein kinase involved in recognition of pathogen effector proteins, representing truncated variants of the bacterial E3 ligase AvrPtoB (Abramovitch et al., 2003), and Fen interaction with Fni3 has been suggested to affect Fen-mediated signaling to promote cell death upon *Pseudomonas* infection (Mural et al., 2013). E3 ligases and potential substrates for Fni3/Suv-mediated K63 chain

formation remain to be identified. It should be noted that the *Arabidopsis* plasma membrane-localized flagellin receptor FLS2, and probably other pathogen-associated molecular pattern (PAMP) receptors, represent likely substrate for AvrPtoB-mediated ubiquitylation and degradation (Göhre et al., 2008). It will be interesting to learn if and how all these pathways interact, and how ubiquitin K63 chain formation controls immune responses in higher plants.

Taken together, current knowledge concerning ubiquitin K63 chains in plants is sufficient to recognize their importance, but not yet sufficient to allow, in any individual case, a mechanistic description of how K63 chains are integrated into biological processes. Future research thus holds promise for exciting insights into this topic.

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Functional implications of K63-linked ubiquitination in the iron deficiency response of *Arabidopsis* roots

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Iron is an essential micronutrient that plays important roles as a redox cofactor in a variety of processes, many of which are related to DNA metabolism. The E2 ubiquitin conjugase UBC13, the only E2 protein that is capable of catalyzing the formation of non-canonical K63-linked ubiquitin chains, has been associated with the DNA damage tolerance pathway in eukaryotes, critical for maintenance of genome stability and integrity. We previously showed that UBC13 and an interacting E3 ubiquitin ligase, RGLG, affect the differentiation of root epidermal cells in *Arabidopsis*. When grown on iron-free media, *Arabidopsis* plants develops root hairs that are branched at their base, a response that has been interpreted as an adaption to reduced iron availability. Mutations in *UBC13A* abolished the branched root hair phenotype. Unexpectedly, mutations in *RGLG* genes caused constitutive root hair branching. Based on recent results that link endocytotic turnover of plasma membrane-bound PIN transporters to K63-linked ubiquitination, we reinterpreted our results in a context that classifies the root hair phenotype of iron-deficient plants as a consequence of altered auxin distribution. We show here that UBC13A/B and RGLG1/2 are involved in DNA damage repair and hypothesize that UBC13 protein becomes limited under iron-deficient conditions to prioritize DNA metabolism. The data suggest that genes involved in combating detrimental effects on genome stability may represent essential components in the plant's stress response.

Keywords: auxin, DNA repair, endocytosis, iron deficiency, K63-linked polyubiquitylation, root hairs

INTRODUCTION

Iron participates in a variety of vital processes as a redox cofactor and is an indispensable element for virtually all organisms. In plants, suboptimal iron availability results in decreased yield and reduced quality of edible plant parts, causing severe economic losses. When plants are the major source of dietary iron, a low iron concentration may pose severe health problems. Iron deficiency-induced anemia (IDA) is the most widespread nutritional disorder worldwide. In severe cases, IDA can affect infant development and increase the risk of maternal and child mortality. Due to the limited solubility of iron in aerated soils, mechanisms have evolved that aid in mobilizing otherwise sparingly soluble Fe(III)oxihydroxides, which represent the prevailing form of iron in most soils at neutral or basic pH. Induction of proton-translocating P-type ATPases decreases the rhizospheric pH, thereby increasing the activity of Fe^{3+} by a factor of 1,000 for each unit the pH decreases (Santi et al., 2005; Santi and Schmidt, 2008, 2009). In addition, secretion of iron binding compounds (IBCs) facilitates the mobilization of iron particularly at high pH (Susín et al., 1993; Jin et al., 2007; Fourcroy et al., 2013; Rodríguez-Celma et al., 2013). A further set of supposedly separately regulated responses comprise morphological changes such as the formation of additional root hairs and invaginations of secondary walls in the rhizodermis/hypodermis, responses that were suggested to improve iron acquisition by increasing the absorptive surface area (Schikora and Schmidt, 2001; Schmidt et al., 2003). In contrast to phosphate deficiency, which triggers a nutrient-specific and conserved set of developmental responses including denser and longer root hairs (Ma et al., 2001; Savage et al., 2013) and a dramatically altered root architecture where primary root growth is attenuated and lateral root formation is stimulated (Ticconi et al., 2009), the morphological responses to iron deficiency are less well studied and appear to be less conserved among species. While some plants such as sunflower and cucumber produce very dense root hairs in response to iron deficiency (Landsberg, 1996; Li and Schmidt, 2010), other species such as *Plantago lanceolata* and tomato show only a moderate increase in root hair frequency (Schmidt and Bartels, 1996; Schikora and Schmidt, 2002). Interestingly, both the induction of cell wall invaginations and the formation of extra root hairs are inducible by application of exogenous auxin, suggesting to us that auxin is involved in the induction of these responses (Landsberg, 1996; Schmidt et al., 2003).

In *Arabidopsis*, the morphological responses to iron deficiency are neither pronounced nor uniformly described. Branching of the hairs was described as a major response and interpreted as an alternative to longer and/or denser root hairs as a strategy to increase the surface area of the roots (Müller and Schmidt, 2004). Instead, formation of shorter and misshapen root hairs was described for iron-deficient *Arabidopsis* plants by Dinneny et al.

(2008), indicating that subtle differences in media composition and growth conditions may impact the phenotypic readout.

While the physiological responses to iron deficiency are well explored at the molecular level, not much information is available regarding the mechanisms controlling the morphological alterations typical of iron-deficient plants. So far, the only gene with a putative function in iron deficiency-induced formation of root hairs is the ubiquitin conjugase *UBC13*. UBC13 was identified in the root hair zone of iron-deficient cucumber plants by a proteomic approach and cloned using the CODEHOP strategy (Li and Schmidt, 2010). The sequence of UBC13 is highly conserved among eukaryotes, and its function has been related to the error tolerance branch of DNA repair in yeast and *Arabidopsis* and to the NF-κB signal transduction pathway in mammals (Hofmann and Pickart, 1999; Wen et al., 2006; Wu et al., 2009). In *Arabidopsis*, *UBC13* is encoded by two close sequelogs, *UBC13A* (*UBC35*) and *UBC13B* (*UBC36*). UBC13 is the only known E2 ubiquitin conjugating enzyme that is capable of catalyzing the formation of ubiquitin chains linked to K63, a function that appears to be conserved in eukaryotes (Hofmann and Pickart, 1999). In contrast to the formation canonical of ubiquitin chains linked via K48, which target proteins for degradation via the 26S proteasome, proteins conjugated to K63-linked polyubiquitin chains are involved in signaling and in the coordination of cellular processes such as endocytotic trafficking and DNA repair. UBC13 acts in conjunction with E3 ligases such as the RING domain ligase protein RGLG2 that has been shown to interact with UBC13 in *Arabidopsis* (Yin et al., 2007). Non-canonical ubiquitation through K63-linked ubiquitin chains is required for DNA damage tolerance, a pathway that allows the bypass of lesions in the DNA template during replication. The *AtUBC13* genes complemented the yeast *ubc13* null mutant for sensitivity to DNA damaging agents and for spontaneous mutagenesis, suggesting that in *Arabidopsis* UBC13 proteins are involved in the error-free DNA damage tolerance pathway (Wen et al., 2006). The UBC enzyme variant Mms2 is an E2-like protein that interacts with Ubc13 in eukaryotic cells (ubiquitin conjugating enzyme variant, UEV; Hofmann and Pickart, 1999; VanDemark et al., 2001). The *Arabidopsis* genome harbors four Mms2 homologs, *UEV1A* to *UEV1D*, all of which can form stable complexes with UBC13 from yeast and *Arabidopsis* (Wen et al., 2008). For *AtUEV1D* a function in DNA damage response has been experimentally verified (Wen et al., 2008). We here present evidence that both UBC13 and RGLG1/2 are critical in DNA damage repair and hypothesize that the root hair phenotype of iron-deficient plants is caused by diminished availability of RGLG1/2 protein, probably due to stress-induced movement from the plasma membrane to the nucleus and subsequently changed auxin distribution in epidermal cells. We further speculate that under iron-deficient conditions different E2–E3 complexes are favored in the nucleus to prioritize genome stability.

THE *Arabidopsis* IRON DEFICIENCY ROOT HAIR PHENOTYPE: A CONSEQUENCE OF CHANGES IN AUXIN DISTRIBUTION?

The branched root hair phenotype of iron-deficient plants differs from that of phosphate-deficient plants, which form longer and denser root hairs resulting from restricted longitudinal elongation of root epidermal cells and additional cell fate assignment by increased expression of the Myb-type transcription factor *ETC1* (Ma et al., 2001; Müller and Schmidt, 2004; Savage et al., 2013). Branched root hairs have also been described for auxin-related mutants such as *axr1*, *aux1*, and *axr2*, and for mutants defective in actin filament organization or vesicle transport such as *scn1* or *tip1* (see Guimil and Dunand, 2007 for an overview). Plants harboring mutations in *UBC13A* do not respond to iron deficiency with the formation of branched root hairs. Moreover, *ubc13a ubc13b* double mutants have shorter root hairs compared with the wild type, a trait that was also observed in some auxin-deficient mutants such as *trh1* (Vicente-Agullo et al., 2004; Li and Schmidt, 2010). Transgenic plants overexpressing *UBC13A* (UBC13A OE) showed a phenotype that was essentially similar to that of the wild type when grown on iron-free media, with no further increase in the number of branched hairs. Iron-sufficient UBC13A OE plants are indistinguishable from Col-0 plants, indicating that an iron deficiency signal is required for the induction of root hair branching. Notably, double mutants defective in the expression of the E3 ligases *RGLG1* and *RGLG2* constitutively displayed the branched root hair phenotype (Li and Schmidt, 2010). This was an unexpected result since disruption of the E2/E3 cascade should result in similar phenotypes, regardless of the site of disruption.

Interestingly, the phenotype of *rglg1 rglg2* mutants could be rescued by omitting phosphate from the growth media (Li and Schmidt, 2010). Such a rescue by phosphate deficiency was described for the short root hair phenotype of the auxin transport mutant *trh1* (Müller and Schmidt, 2004). TRH1 is a member of the AtKT/AtKUP/AtHAK family of potassium carriers required for the correct distribution of auxin (Vicente-Agullo et al., 2004). It seems reasonable to speculate that altered auxin metabolism is the cause of the variable phenotype of the mutants grown under the different conditions. In support of this assumption, DR5-GUS reporter lines showed reduced GUS expression under iron-deficient conditions (Lan et al., 2012), indicative of decreased levels of or a diminished responsiveness to auxin. In phosphate-deficient DR5-GUS plants, staining was more intense than under control conditions, indicating increased auxin levels in this growth type. Together these results suggest that compromised auxin transport or metabolism could be the cause for the branched root hair phenotype under iron-deficient conditions.

A MODEL FOR THE FUNCTION OF K63-LINKED UBIQUITINATION IN ROOT HAIR CELLS

The following model would explain the results obtained by us (Li and Schmidt, 2010) and others (**Figure 1**). The model is based on observations made in *Arabidopsis* but may also apply to other species. Under iron-sufficient conditions, RGLG1/2 binds a protein X that, when not captured by RGLG1/2, acts as an inhibitor of proper root hair initiation, probably via a reduction of auxin responsiveness. Protein X may act directly or indirectly on auxin distribution. *AtUBC13* is not induced by iron deficiency (Li and Schmidt, 2010) and not much affected by other stresses[1] and thus

[1]https://www.genevestigator.com/

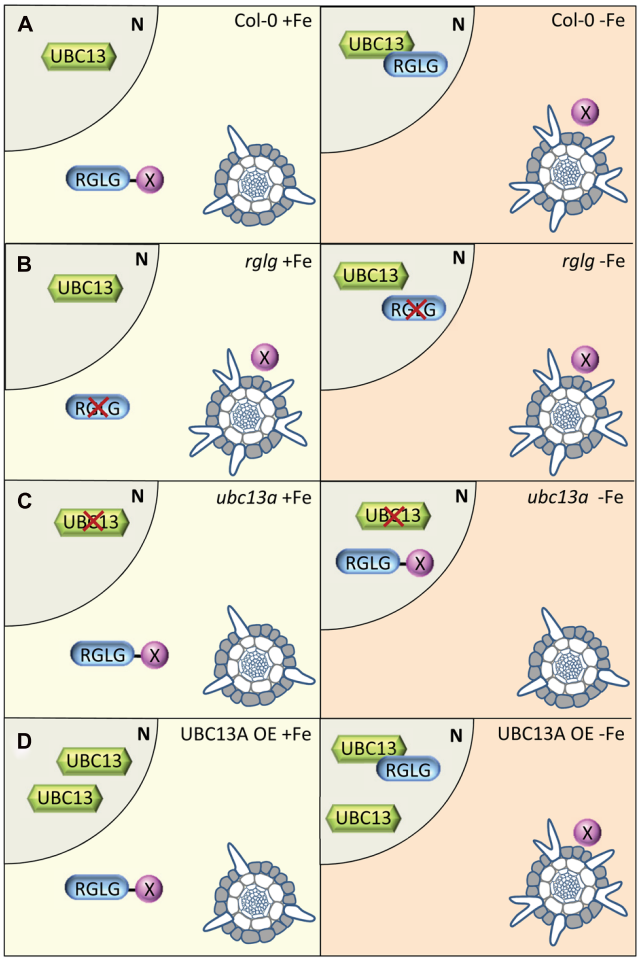

FIGURE 1 | Model depicting putative interactions of UBC13 and RGLG proteins and their effects on root hair differentiation. (A) In wild-type roots, protein X is bound to RGLG proteins, preventing the formation of branched root hairs. UBC13 is present, but only under iron-deficient conditions UBC13 interacts with RGLG proteins which move to the nucleus. The interaction of UBC13 with RGLG1/2 releases protein X which leads to the formation of branched hairs. **(B)** In *rglg* mutants, protein X is free both in the presence and absence of iron, causing root hairs to branch independent on the iron supply. **(C)** In root hair cells of *ubc13* plants, no interaction between UBC13 and RGLG1/2 can occur, leaving protein X inactive under both iron-sufficient and iron-deficient conditions. **(D)** Over-expression of UBC13 increases the level of UBC13 protein but does not affect UBC13-RGLG1/2 interaction, resulting in a phenotype similar to that of the wild type.

likely to be present in iron-sufficient plants in equal amounts. In iron-deficient roots, RGLG1/2 is located to the nucleus where it interacts with UBC13. The UBC13-RGLG1/2 complex formation releases protein X from RGLG, resulting in a decreased auxin concentration (**Figure 1A**). Stress-induced movement of RGLG2 from the plasma membrane to the nucleus has been shown previously (Cheng et al., 2012). In *rglg1 rglg2* mutants, no UBC13-RGLG1/2 interaction is possible, making protein X available both under control and iron-deficient condition. Although K63-linked ubiquitin chain-supporting enzymes such as UBC13 are present in iron-deficient *rglg1 rglg2* plants, they would not affect the abundance of protein X and cause a constitutive branching root hair phenotype (**Figure 1B**). In *ubc13a* plants, branched root hairs are neither formed under iron-sufficient nor under iron-deficient conditions. Adopting the scenario outlined above, protein X remains tightly bound to RGLG1/2, keeping auxin levels (or responsiveness) up and preventing the formation of branched hairs (**Figure 1C**). Over-abundance of UBC13 in UBC13 OE lines is not affecting root hair branching under control conditions (**Figure 1D**). Under iron-deficient conditions, RGLG1/2 moves to the nucleus, releases protein X resulting in a phenotype that is undistinguishable from the wild type.

We can only speculate on the nature of protein X. A possible scenario implies that protein X post-translationally regulates auxin distribution or metabolism. The plasma membrane-localized auxin carrier protein PIN2 is constitutively recycled by endocytosis (Benjamins and Scheres, 2008; Grunewald and Friml, 2010). Recently, RGLG proteins were shown to be involved in the control of the proteolytic turnover of PIN2 via K63-linked ubiquitination (Leitner et al., 2012). Loss of PIN2 ubiquitination interferes with vacuolar targeting, stabilizes PIN2, and alters auxin availability in *Arabidopsis* roots. *rglg1 rglg2* mutants had reduced auxin levels and transgenic *rglg1 rglg2* plants carrying the DR5-reporter construct showed reduced auxin responsiveness (Yin et al., 2007). Translated to the model, PIN2 could represent protein X. A possible scenario is outlined in **Figure 2**. Under iron-sufficient conditions, RGLG proteins mediates K63-linked ubiquitination of PIN2, thereby controlling its proteolytic turnover (**Figure 2A**). Loss of PIN2 polyubiquitination, caused for example by a mutation in *RGLG1/2*, causes an arrest of endocytotic cycling of PIN2, decreased auxin levels and, ultimately, branching of root hairs. Such a scenario may apply to iron-deficient plants in which UBC13 is recruited by RGLG1/2 in the nucleus, compromising the ubiquitination of PIN2, which accumulates in the cell (**Figure 2B**). Recruitment of UBC13 may be facilitated by translocation of RGLG from the plasma membrane to the nucleus.

UBC13 was suggested to be involved in the error-free DNA damage repair pathway (Wen et al., 2006). In yeast, this function is fulfilled by an Ubc13-Mms2-Rad5-mediated polyubiquitination of the homotrimeric protein complex proliferating cell nuclear antigen (PCNA); in humans, PCNA ubiquitination is catalyzed by the Rad5 homologs HLTF and SHPRH (Ulrich, 2007; Chang and Cimprich, 2009). A role for AtRAD5A in DNA repair has been demonstrated (Chen et al., 2008; Mannuss et al., 2010; Wang et al., 2011) and an involvement of UBC13-UEV1 and AtRAD5A in this pathway in *Arabidopsis* represents a likely scenario.

In *ubc13a* mutant plants, several iron-responsive genes are less induced than in the wild type (Li and Schmidt, 2010), indicative of an involvement of UBC13A in processes that utilizes iron. For example, induction of the transcription factor *bHLH38*, an essential regulator of the iron deficiency response, was markedly less pronounced in roots of the *ubc13a* mutant. Notably, under iron-deficient conditions the expression of two genes encoding the iron storage proteins *FER1* and *FER2* was higher in *ubc13a* plants when compared with the wild type, suggesting a higher iron status of the mutant. These results may be interpreted in a sense that more iron

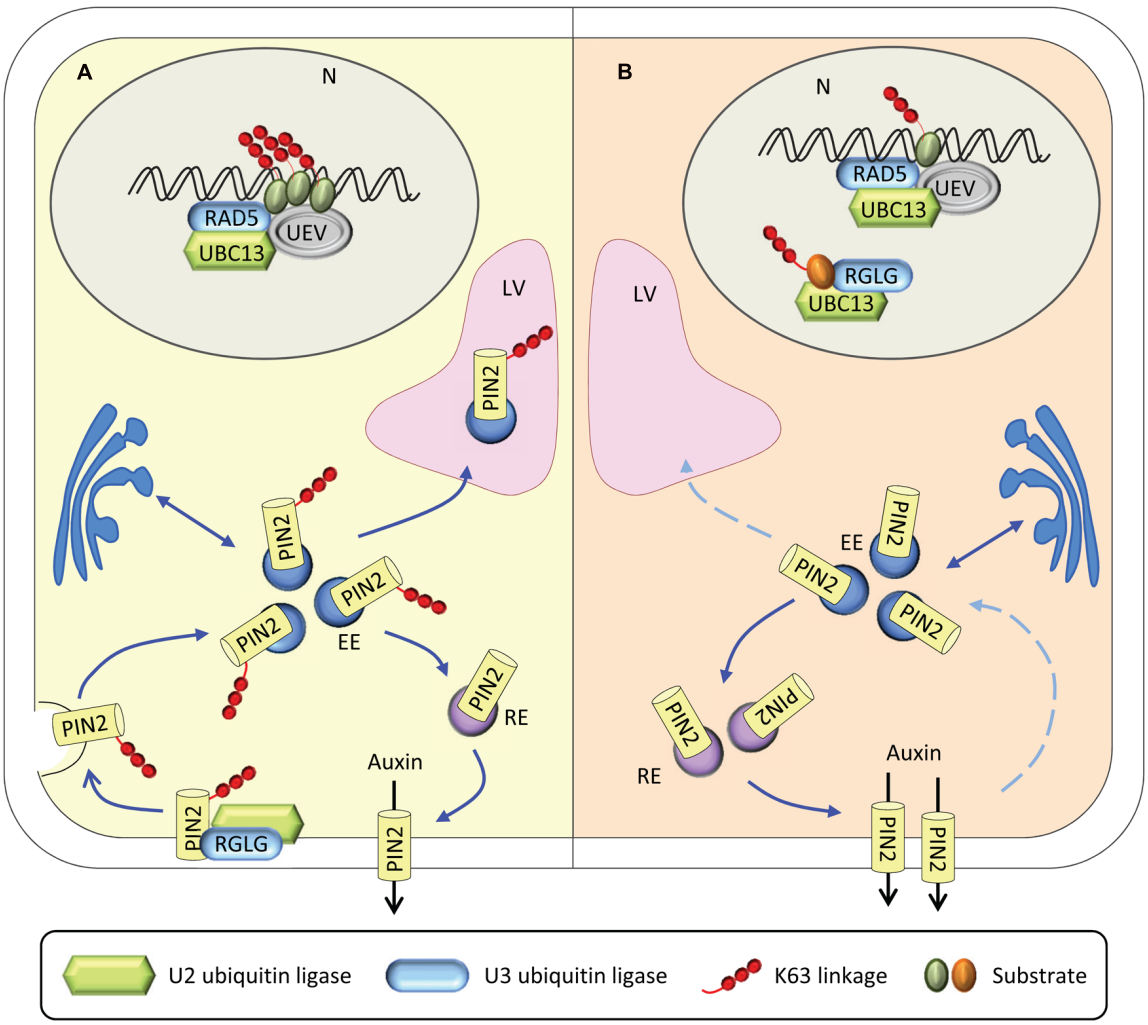

FIGURE 2 | Experimental evidence for processes associated with the formation of K63-linked ubiquitin chains. (A) Under control conditions, RGLG mediates the endocytic turnover of PIN2, thereby regulating the distribution of auxin. UBC13-UEV1-RAD5 is involved in housekeeping DNA damage repair. **(B)** Under iron-deficient conditions, RGLG moves to the nucleus where it interacts with UBC13. UBC13-RGLG1/2 might be associated with DNA repair or with other processes related to genome stability. Loss of PIN2 ubiquitination cause a cessation of endocytotic PIN2 cycling, thereby altering auxin distribution. N, nucleus; LV, lytic vacuole; EE early endosome; RE, recycling endosome.

is available if the UBC13-RGLG pathway is not engaged. Based on the conserved function of UBC13 in DNA damage repair among eukaryotes, it can be assumed that the UBC13-RGLG1/2 complex participates in DNA damage repair.

To test our hypothesis, we germinated seeds from wild-type plants, *rglg1 rglg2*, and *ubc13a ubc13b* double mutants on media containing various concentrations of the DNA-damaging agent methyl methanesulfonate (MMS). While wild-type plants were largely unaffected both under iron-sufficient and iron-deficient conditions, the germination rate of *ubc13a ubc13b* double mutants showed a dramatic decrease in germination rate which was more pronounced under iron-deficient conditions (**Figure 3**). *rglg1 rglg2* mutants showed a less pronounced and iron-independent decrease in seed germination (**Figure 3**). These data are consistent with the assumption that under iron-deficient conditions RGLG1/2 is translocated into the nucleus where it may associate with UBC13 and other E2 proteins, thereby diminishing the efficiency of UBC13 in DNA damage tolerance. UBC13 may have critical, currently unknown functions in DNA metabolism in conjunction with RGLG1/2, probably associated with genome stability (**Figure 2B**).

THE ROLE OF IRON IN DNA SYNTHESIS AND REPAIR

Iron is required for several DNA-related processes. For example, ribonucleotide reductases (RNRs) require iron as an essential cofactor and iron deficiency leads to reduced RNR activity (Cavanaugh et al., 1985; Saletta et al., 2011). RNRs activity is linked to DNA synthesis and tightly regulated in response to iron deficiency in yeast and mammals to assure accurate DNA replication (Furukawa et al., 1992). In yeast, a post-transcriptional regulatory mechanism promotes destabilization of mRNAs involved in non-essential pathways and degradation of such transcripts via

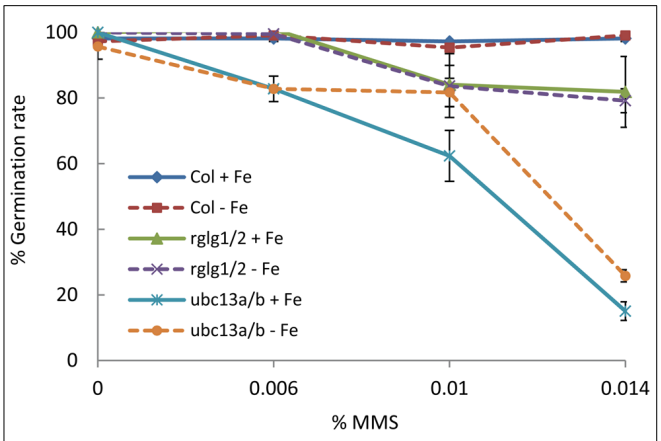

FIGURE 3 | Analysis of DNA damage response during seed germination. Seeds of wild-type plants, *rglg1 rglg2*, and *ubc13a ubc13b* double mutants were placed on either iron-replete or iron-deplete media containing 0, 0.006%, 0.01%, and 0.014% MMS. Data points indicate the percentage of seed germination after 5 days with SD ($n = 36$).

AU-rich element (ARE)-mediated decay (AMD), and supports DNA synthesis and repair via activation of RNR by the Cth2 protein (Martínez-Pastor et al., 2013). Interestingly, many of the down-regulated transcripts are related to the tricarboxylic acid cycle or participate in mitochondrial electron transport, switching respiration to fermentative metabolism (Sanvisens et al., 2011), a response that has also been described for iron-deficient *Arabidopsis* roots (Thimm et al., 2001). Also, storage of iron in the vacuole via CCC1 is inhibited by this pathway, a further parallel to *Arabidopsis* roots in which CCC1-like vacuolar iron transporters are down-regulated in response to iron deficiency (Gollhofer et al., 2011).

In humans, iron deficiency leads to G1/S cell cycle arrest and to the induction of several members of the growth arrest and DNA damage (GADD) gene family members, probably associated with DNA damage resulting from iron deficiency (Saletta et al., 2011). In addition, DNA replication and repair depends on Fe–S clusters (Netz et al., 2010; Pokharel and Campbell, 2012) the synthesis of which is impaired upon iron deficiency (Chen et al., 2004). Defects in Fe–S cluster biogenesis result in genome instability (Veatch et al., 2009). Not surprisingly, mitochondrial dysfunction associated with compromised biogenesis of Fe–S clusters is tightly linked to iron homeostasis (Veatch et al., 2009).

DNA damage applies to the sites of the storage of genetic material, i.e., the nucleus, plastids, and mitochondria. More recently, nucleoli have been associated with a function of sensing DNA damage and as a storage facility for DNA repair proteins (Antoniali et al., 2013). In plants, iron is concentrated in the nuclei, particularly in nuclear substructures that were identified as the nucleoli by Perls/DAB and DAPI co-staining (Roschzttardtz et al., 2011). The nucleolus was described as a central hub for the coordination of stress responses such as DNA repair (Boulon et al., 2010; Antoniali et al., 2013), and for several DNA-repair-related proteins (Gao et al., 2003; Guo et al., 2008). While in plants such information is not available, we speculate that the high iron concentrations in nuclei/nucleoli are important for prioritizing DNA replication and repair when iron becomes limited.

CONCLUSION

In summary, the re-interpretation of the data outlined above hints at a new facet of the iron deficiency response of *Arabidopsis*, which might also be important for other plant species and may thus represent a general aspect in the adaptation of plants to low iron availability. Under iron shortage, DNA-related processes such as replication and repair might be prioritized to secure essential housekeeping functions. While alternative scenarios such as a role of UBC13 in root development that is not linked to DNA repair cannot be ruled out at present, the fact that *ubc13a* mutants appear to have a higher, healthier iron status implies that activation of the DNA damage pathway occurs at the expense of other genes encoding iron-containing proteins. This situation is similar to what has been reported for yeast where iron deficiency limits iron utilization in energy-generating pathways via the action of the RNA-binding protein Cth2 to avoid that essential processes such as DNA synthesis and repair and thus genome integrity are compromised (Martínez-Pastor et al., 2013). While the evidence reported here is indirect and awaits experimental validation, it is tempting to assume that, similar to yeast, post-transcriptional mechanisms that bias the translation of messenger RNAs encoding proteins involved in essential iron-requiring processes such as DNA replication and repair also occur in *Arabidopsis* and other plants. Our initial experiments indicate that both UBC13 and RGLG are involved in DNA repair, but only for *ubc13a ubc13b* mutants iron deficiency severed the effect of MMS. This is consistent with a competition for UBC13 protein in the nucleus due to stress-induced translocation of RGLG1/2 to the nucleus. The role of RGLG1/2 under iron-deficient conditions awaits clarification, but from our results a function for RGLG1/2 in genome stability appears to be plausible. Further experiments addressing the fitness of plants treated with DNA-damaging agents and grown with different iron supply may falsify or validate our hypothesis. The effects associated with adverse environmental condition on genome stability are generally understudied and, despite big steps forward in the past few years (reviewed by Waterworth et al., 2011), not fully understood. From the example outlined here for iron deficiency it might be inferred that mechanisms that are involved in combating the constant assault of environmental stresses on genome stability may be an important component of the plant stress response.

MATERIALS AND METHODS

Arabidopsis thaliana (L.) Heynh Columbia (Col-0) ecotype was used as the wild-type. Seeds of the wild type, *ubc13a* and *ubc13b* mutants were obtained from the Arabidopsis Biological Resource Centre (Ohio State University), *ubc13a ubc13b* double mutants were generated by genetic crossing. The *rglg1 rglg2* mutant lines were provided by Dr. Andreas Bachmeir (University of Vienna). Seeds were surface-sterilized and sown on media as described by Estelle and Somerville (1987), supplemented with 0%, 0.006%,

0.01%, and 0.014% MMS either with or without 40 μM FeEDTA, and stored at 4°C for 2 days. A total of 36 seeds were placed on each plate, three plates were used for each treatment. After 5 days, seed germination was determined and normalized with the rate observed on iron-replete standard without MMS.

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Dynamic regulation and function of histone monoubiquitination in plants

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Polyubiquitin chain deposition on a target protein frequently leads to proteasome-mediated degradation whereas monoubiquitination modifies target protein property and function independent of proteolysis. Histone monoubiquitination occurs in chromatin and is in nowadays recognized as one critical type of epigenetic marks in eukaryotes. While H2A monoubiquitination (H2Aub1) is generally associated with transcription repression mediated by the Polycomb pathway, H2Bub1 is involved in transcription activation. H2Aub1 and H2Bub1 levels are dynamically regulated *via* deposition and removal by specific enzymes. We review knows and unknowns of dynamic regulation of H2Aub1 and H2Bub1 deposition and removal in plants and highlight the underlying crucial functions in gene transcription, cell proliferation/differentiation, and plant growth and development. We also discuss crosstalks existing between H2Aub1 or H2Bub1 and different histone methylations for an ample mechanistic understanding.

Keywords: chromatin, epigenetics, ubiquitin, histone monoubiquitination, transcription regulation, plant development, *Arabidopsis thaliana*

INTRODUCTION

Ubiquitin (Ub) and Ub-like (e.g., SUMO) proteins constitute a family of modifiers that are linked covalently to target proteins. Although ubiquitination (also called ubiquitylation or ubiquitinylation) first came to light in the context of protein destruction, it is now clear that ubiquitination can also carry out proteolysis-independent functions. Ubiquitination can alter biochemical, molecular and/or subcellular localization activities of a target protein. The first ubiquitinated protein to be described was histone H2A in calf thymus, a finding dated more than 36 years ago (Goldknopf et al., 1975; Hunt and Dayhoff, 1977). Yet, only more recently have the underlying mechanisms and regulatory functions of histone ubiquitination begun to emerge (reviewed in Zhang, 2003; Shilatifard, 2006; Weake and Workman, 2008; Braun and Madhani, 2012; Pinder et al., 2013). Histones are highly alkaline proteins, found in the nuclei of eukaryotic cell, which package and order the DNA into structural units named nucleosomes. A nucleosome is composed of roughly 146 bp of DNA wrapping around the histone octamer comprising two molecules each of the four core histones H2A, H2B, H3, and H4 (Luger et al., 1997). Histone monoubiquitination together with other types of posttranslational modifications, e.g., acetylation, methylation, phosphorylation, and SUMOylation, can modulate nucleosome/chromatin structure and DNA accessibility and thus regulate diverse DNA-dependent processes, such as genome replication, repair, and transcription (Zhang, 2003; Shilatifard, 2006; Weake and Workman, 2008; Braun and Madhani, 2012; Pinder et al., 2013).

Ubiquitination occurs *via* conjugation of the C-terminal residue of Ub to the side chain of a lysine (K) residue of the substrate/acceptor protein, a reaction involving three coordinated enzymatic activities (reviewed in Hershko and Ciechanover, 1998). Ub is first activated by an ATP-dependent reaction involving the Ub-activating enzyme E1, then conjugated to the active site cysteine residue of the Ub-conjugating (UBC) enzyme E2, and finally transferred to the target K residue of the substrate protein by the Ub-protein isopeptide ligase E3. Most organisms have only one E1, but dozens of different E2 and hundreds up to thousands of different E3 enzymes, providing the need in coping with effective substrate specificity (Hua and Vierstra, 2011; Braun and Madhani, 2012). Identification and characterization of E3s and some E2s involved in histone ubiquitination had been a key for understanding biological functions of histone ubiquitination in various organisms. Because of its suitability for genomics, genetics, and cellular and molecular biological approaches, *Arabidopsis thaliana* is an ideal model to investigate histone ubiquitination functions. In this review, we focus on this reference plant to expose current progress made on ubiquitination of different types of histones.

H2B MONOUBIQUITINATION IN *Arabidopsis*

GENOME-WIDE DISTRIBUTION OF H2Bub1

Monoubiquitinated H2B (H2Bub1) was first discovered in mouse cells and was estimated to represent about 1–2% of total cellular H2B (West and Bonner, 1980). Later, H2Bub1 was detected widely throughout eukaryotes spanning from yeast to humans and plants (Zhang, 2003; Shilatifard, 2006; Sridhar et al., 2007; Zhang et al., 2007a; Weake and Workman, 2008). The ubiquitination site is mapped to a highly conserved K residue, H2BK123 in budding yeast, H2BK119 in fission yeast, H2BK120 in humans, and H2BK143 in *Arabidopsis*.

Genome-wide analysis revealed that in *Arabidopsis* as in animals H2Bub1 is associated with active genes distributed throughout the genome and marks chromatin regions notably in combination with histone H3 trimethylated on K4 (H3K4me3) and/or with H3K36me3 (Roudier et al., 2011). During early photomorphogenesis, gene upregulation was found to be associated with H2Bub1 enrichment whereas gene downregulation did not show detectable correlation with any H2Bub1 level changes (Bourbousse et al., 2012). In general, H2Bub1 is considered to represent an active chromatin mark broadly involved in genome transcription regulation.

ENZYMES INVOLVED IN REGULATION OF H2Bub1 LEVELS

The budding yeast Rad6 (radiation sensitivity protein 6) was the first factor identified and shown to work as an E2 enzyme involved in catalyzing H2Bub1 formation both *in vitro* and *in vivo* (Robzyk et al., 2000). It contains a highly conserved catalytic UBC domain of approximately 150 amino acids in length with an active-site cysteine for linking Ub. The E3 enzyme working together with Rad6 in catalyzing H2Bub1 formation in budding yeast is Bre1 (Brefeldin-A sensitivity protein 1), which contains a C3HC4-type RING finger domain typical for all E3s (Hwang et al., 2003; Wood et al., 2003). The depletion of either Rad6 or Bre1 eliminates genome-wide H2Bub1 and causes yeast cell growth defects (Robzyk et al., 2000; Hwang et al., 2003; Wood et al., 2003). Human contains at least two homologs of Rad6, namely hHR6A and hHR6B, and two homologs of Bre1, namely RNF20/hBRE1A and RNF40/hBRE1B (Kim et al., 2005; Zhu et al., 2005). In *Arabidopsis*, three homologs of Rad6, namely UBC1, UBC2, and UBC3, were identified and UBC1 and UBC2 but not UBC3 were shown to be redundantly responsible for H2Bub1 formation *in planta* (Cao et al., 2008; Gu et al., 2009; Xu et al., 2009). The two Bre1 homologs HUB1 (HISTONE MONOUBIQUITINATION 1) and HUB2 work non-redundantly, possibly as a hetero-tetramer composed of two copies of HUB1 and two copies of HUB2, in catalyzing H2Bub1 formation in *Arabidopsis* (Fleury et al., 2007; Liu et al., 2007; Cao et al., 2008). H2Bub1 levels are drastically reduced or undetectable in Western blot analysis in the loss-of-function *hub1* and *hub2* single mutants as well as in the *hub1 hub2* and *ubc1 ubc2* double mutants, but are unaffected in the *ubc1*, *ubc2*, and *ubc3* single mutants or in the *ubc1 ubc3* and *ubc2 ubc3* double mutants (Cao et al., 2008; Gu et al., 2009; Xu et al., 2009).

H2Bub1 levels are also regulated by deubiquitination enzymes. Two Ub-specific proteases, Ubp8 and Ubp10, are involved in deubiquitination of H2Bub1 in budding yeast. Strikingly, while Ubp8 acts as a component of the SAGA (Spt-Ada-Gcn5-acetyltransferase) complex specifically in H2Bub1 deubiquitination in transcription activation, Ubp10 functions independently of SAGA and primarily acts in Sir-mediated silencing of telomeric and rDNA regions (reviewed in Weake and Workman, 2008). In human, USP22 acts as Ubp8 ortholog in a SAGA complex in H2Bub1 deubiquitination (Weake and Workman, 2008). In *Arabidopsis*, although a SAGA complex remains uncharacterized so far, the Ub protease UBP26/SUP32 has been shown to deubiquitinate H2Bub1 involved in both heterochromatic silencing (Sridhar et al., 2007) and transcription activation of the *FLC* (*FLOWERING* LOCUS C) gene (Schmitz et al., 2009). More recently, the otubain-like deubiquitinase OTLD1 was reported as implicated in deubiquitination of H2BUb1 and repression of *At5g39160*, a gene of unknown function (Krichevsky et al., 2011).

ROLE OF H2Bub1 IN FLOWERING TIME REGULATION

The timing of flowering is critical for the reproductive success of plants. As compared to wild type, the *hub1* and *hub2* single mutants as well as the *hub1 hub2* and *ubc1 ubc2* double mutants exhibit an early flowering phenotype whereas but the *ubc1*, *ubc2*, and *ubc3* single mutants and the *ubc1 ubc3* and *ubc2 ubc3* double mutants have a normal phenotype (Cao et al., 2008; Gu et al., 2009; Xu et al., 2009). This early flowering phenotype is detectable under both long-day and short-day photoperiod plant growth conditions. Molecular analyses of the mutants indicate that H2Bub1 controls flowering time primarily through transcriptional activation of *FLC* (**Figure 1**). *FLC* encodes a key transcription repressor involved in both the autonomous/developmental and vernalization flowering pathways, and its active transcription is associated with several histone marks, e.g., H3K4me3, H3K36me2/3 and H2Bub1 (reviewed in Berr et al., 2011). In the early flowering mutants *hub1*, *hub2*, *hub1 hub2*, and *ubc1*

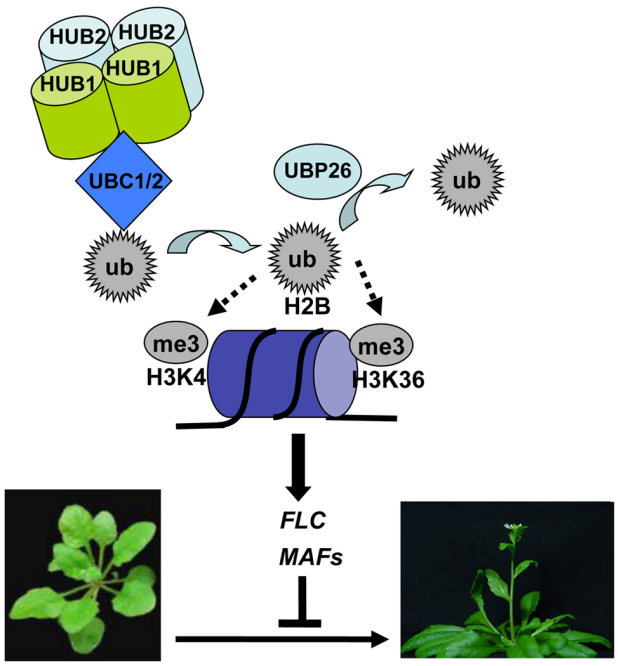

FIGURE 1 | A proposed model for deposition and removal of histone H2B monoubiquitination in transcriptional activation of *FLC* and *MAFs* in flowering time regulation. In this model, HUB1 and HUB2 form a heterotetramer and recruit UBC1 or UBC2 to *FLC*/*MAFs* chromatin, leading to transfer of a ubiquitin (ub) monomer from UBC1 or UBC2 onto H2B. H2Bub1 formation enhances H3K4me3 deposition by methyltransferases, together promoting transcription initiation. UBP26 removes ubiquitin on H2B, favoring H3K36me3 deposition in promoting transcription elongation. Active transcription of *FLC*/*MAFs* represses *Arabidopsis* flowering, a transition from vegetative to reproductive plant development.

ubc2, *FLC* expression levels are reduced and the *FLC* chromatin shows reduced H2Bub1 levels (Cao et al., 2008; Gu et al., 2009). The loss-of-function mutant *ubp26*/*sup32* showed also an early flowering phenotype and reduced *FLC* expression but an elevated level of H2Bub1 in the *FLC* chromatin (Schmitz et al., 2009), indicating that not only H2Bub1 formation but also H2Bub1 removal are necessary for *FLC* transcription. Accompanying H2Bub1 reduction compromised levels of H3K4me3 and to a less extent H3K36me2 were detected at *FLC* in *hub1* and *ubc1 ubc2* (Cao et al., 2008), and reduced level of H3K36me3 but elevated level of H3K27me3 was observed at *FLC* in *ubp26/sup32* (Schmitz et al., 2009). On parallels to the knowledge in yeast, it was proposed that the UBC-HUB-mediated H2Bub1 formation is necessary for H3K4me3 deposition at transcription initiation whereas UBP26/SUP32-mediated H2Bub1 removal is required for H3K36me3 deposition during transcription elongation (Cao et al., 2008; Schmitz et al., 2009). Nonetheless, this hierarchy of histone modifications needs to be cautioned because multiple factors are involved in H3K4me3 and H3K36me2/3 depositions and the SDG8 (SET DOMAIN GROUP 8)-mediated H3K36me2/3 deposition remarkably override H3K4me2/3 deposition in *FLC* transcription (Yao and Shen, 2011; Shafiq et al., 2014). Besides *FLC*, *Arabidopsis* has five *FLC* paralogs, namely *MAF1* (*MADS AFFECTING FLOWERING 1*), *MAF2, MAF3*, *MAF4* and *MAF5*. Some *MAFs* are also downregulated in the early flowering mutants *hub1*, *hub2*, *hub1 hub2*, *ubc1 ubc2,* and *ubp26/sup32* (Cao et al., 2008; Gu et al., 2009; Schmitz et al., 2009; Xu et al., 2009). Thus, H2Bub1 may also regulate flowering time through control of *MAF* gene expression under some plant growth conditions.

H2Bub1 FUNCTION IN OTHER PROCESSES

In addition to flowering, many other processes also involve H2Bub1 as evidenced by studies of the *Arabidopsis hub1* and *hub2* mutants. The *hub* mutants display reduced seed dormancy associated with reduced expression of several dormancy-related genes, including *DOG1* (*DELAY OF GERMINATION 1*), *ATS2* (*ACYLTRANSFERASE 2*), *NCED9* (*NINE-CIS-EPOXYCAROTENOID DIOXYGENASE 9*), *PER1* (*CYSTEINE PEROXIREDOXIN 1*), and *CYP707A2* (Liu et al., 2007). At vegetative growth stages, the *hub* mutants exhibit pale leaf coloration, modified leaf shape, reduced rosette biomass, and inhibited root growth (Fleury et al., 2007). Cell cycle genes, particularly some key regulators of the G2-to-M transition, are downregulated, which could largely explain the plant growth defects of the *hub* mutants (Fleury et al., 2007). A more recent study shows that several circadian clock genes, including *CCA1* (*CIRCADIAN CLOCK ASSOCIATED 1*), *ELF4* (*EARLY FLOWERING 4*) and *TOC1* (*TIMING OF CAB EXPRESSION 1*), are downregulated and their chromatin regions contain lower levels of H2Bub1 in the *hub* mutants, suggesting that H2Bub1 may contribute to the regulation of plant growth fitness to environment through expression modulation of some circadian clock genes (Himanen et al., 2012). It is worth to note that SDG2-mediated H3K4me3 deposition is also required for expression of several circadian clock genes (e.g., *CCA1, TOC1*) and the *hub* mutants exhibit reduced levels of H3K4me3 in chromatin regions of the circadian clock genes (Himanen et al., 2012; Malapeira et al., 2012).

During photomorphogenesis, hundreds of genes show upregulation associated with H2Bub1 enrichment in their chromatin in response to light exposure (Bourbousse et al., 2012). Strikingly, over 50% of these genes gain H2Bub1 enrichment upon the 1 h of illumination, illustrating the highly dynamic nature of H2Bub1 deposition during a likely cell division-independent genome regrogramming process. In contrast to the above discussed cases, in this study the H2Bub1 changes is neither accompanied by any detectable changes of H3K36me3 nor required for H3K4me3 enrichment following six hours of light exposure (Bourbousse et al., 2012). In line with the function of H2Bub1 in gene activation in response to light, the *hub1-3* mutant seedlings are overly light sensitive, exhibiting a photobleaching phenotype (Bourbousse et al., 2012).

The *hub1* mutants also show increased susceptibility to the necrotrophic fungal pathogens *Botrytis cinerea* and *Alternaria brassicicola* (Dhawan et al., 2009). Precise role of H2Bub1 in plant defense against pathogens still remains largely unclear. Structure defects, e.g., thinner cell walls and altered surface cutin and wax compositions, together with impaired induction of some defense genes might have partly contributed to the increased susceptibility to pathogen infection in the *hub* mutant plants (Dhawan et al., 2009; Ménard et al., 2014). It is worthy noting that the *sdg8* mutants impaired in H3K36me3 deposition also display reduced resistance to necrotrophic fungal pathogen infection (Berr et al., 2010, 2012; Palma et al., 2010). It will be interesting to study in future research whether a trans-histone crosstalk between H2Bub1 and H3K36me3 acts on transcription induction in plant response to pathogens.

MECHANISMS OF H2Bub1 IN TRANSCRIPTION REGULATION

So far only limited information is available concerning how H2Bub1 enzymes are recruited to the target chromatin. The evolutionarily conserved PAF1 (Polymerase Associated Factor 1) complex interacts with Pol II (RNA polymerase II) and plays a role as a "platform" for association of enzymes involved in H2bub1, H3K4me3, and H3K36me2/3 deposition, linking histone modifications with active transcription (Shilatifard, 2006; Weake and Workman, 2008; Berr et al., 2011; Braun and Madhani, 2012). A direct interaction between PAF1 complex and Rad6-Bre1 has been detected and shown as required for catalyzing H2Bub1 formation (Xiao et al., 2005). As in yeast and animals, deletion or knockdown of PAF1 components markedly reduces H2Bub1 in *Arabidopsis* (Schmitz et al., 2009). Genetic analysis shows that *HUB2* and *ELF8* encoding a PAF1 subunit act in a same floral-repression pathway in *Arabidopsis* flowering time regulation (Gu et al., 2009). Although physical interaction between UBC-HUB and PAF1 needs future investigation, interactions were observed between UBC and HUB (Cao et al., 2008) and between HUB and MED21 (mediator complex subunit 21), a subunit of the evolutionarily conserved Mediator complex (Dhawan et al., 2009). Mediator complex is associated with both general transcription factors and Pol II and is essential for activator-dependent transcription in all eukaryotes (for a recent review, see Carlsten et al., 2013). Nevertheless, the aforementioned

interactors are generally involved in Pol II transcribed genes and thus cannot fully explain why UBC-HUB targets some but not all active genes. It is reasonable to speculate that UBC-HUB recruitment might also involve some gene-specific yet uncharacterized factors.

The next question is how H2Bub1 affects transcription. In yeast and animals, H2Bub1 can promote transcription elongation by enhancing the recruitment of RNA Pol II and by facilitating nucleosome removal through interplay with FACT (facilitates chromatin transcription), an evolutionarily conserved histone chaperone complex (Pavri et al., 2006; Tanny et al., 2007). FACT acts on displacement of H2A/H2B dimer from a nucleosome core, facilitating transcription elongation on chromatin template. In *Arabidopsis*, FACT genetically interacts with HUB1 and plays critical roles in multiple plant developmental processes (Lolas et al., 2010). Yet its precise interplay with H2Bub1 in transcription regulation needs future investigations.

Alternatively or additionally, H2Bub1 may regulate transcription indirectly through crosstalk with H3K4me3 and H3K36me2/3 (Shilatifard, 2006; Weake and Workman, 2008; Berr et al., 2011; Braun and Madhani, 2012). In line with this idea, lack of H2Bub1 in *Arabidopsis* impairs H3K4me3 and H3K36me2 formation in chromatin at *FLC* and clock genes (Cao et al., 2008; Himanen et al., 2012), and elevated H2Bub1 inhibits H3K36me3 formation in the *FLC* chromatin (Schmitz et al., 2009). Nevertheless, in contrast to the requirement of H2Bub1 for genome-wide H3K4me3 formation in yeast, lack of H2Bub1 in *Arabidopsis* barely affects global H3K4me2/3 and H3K36me2/3 levels, as evidenced by Western blot analysis (Cao et al., 2008; Dhawan et al., 2009; Gu et al., 2009) as well as by ChIP (chromatin immunoprecipitation) analysis of light responsive genes during photomorphogenesis (Bourbousse et al., 2012). It is currently unclear to which extent applies the crosstalk of H2Bub1 with H3K4me2/3 and H3K36me2/3 in *Arabidopsis* gene transcription regulation and what are the molecular mechanisms underlying the crosstalk.

Finally, while H2Bub1 is generally associated with active gene transcription, it can also regulate transcription repression in a chromatin context-dependent manner. The *ubp26/sup32* mutant shows release of transgene and transposon silencing (Sridhar et al., 2007) as well as elevated expression of *PHE1* (*PHERES1*) associated with seed developmental defects (Luo et al., 2008). It has been shown that the silencing release is accompanied by reduction of H3K9me2 and of siRNA-mediated DNA methylation and the *PHE1* expression elevation is associated with a reduced level of H3K27me3. Nevertheless, whether these changes of repressive marks are directly linked with H2Bub1 still need to be investigated.

H2A MONOUBIQUITINATION IN *Arabidopsis*

PRESENCE OF H2Aub1

In contrast to H2Bub1, H2Aub1 has not been found in yeast and has been generally implicated in transcription repression in animal cells (Weake and Workman, 2008; Braun and Madhani, 2012). Albeit its early discovery and high abundance (about 5–15% of the total H2A) in animal cells (Goldknopf et al., 1975; Hunt and Dayhoff, 1977; Zhang, 2003), H2Aub1 function has only more recently begun to be elucidated, thanking to the first identification of the human PRC1 (Polycomb repressive complex 1) component Ring1B (also known as Ring2 and RNF2) as a E3 involved in catalyzing H2Aub1 formation (Wang et al., 2004). In *Arabidopsis*, H2Aub1 was undetectable in a large-scale analysis of histone post-translational modifications by mass spectrometry (Sridhar et al., 2007; Zhang et al., 2007a) and had been thought for a long time to be non-existent (Weake and Workman, 2008). However, five PRC1-like RING-finger proteins, namely AtRING1a, AtRING1b, AtBMI1a, AtBMI1b, and AtBMI1c, have been identified in *Arabidopsis* (Sanchez-Pulido et al., 2008; Xu and Shen, 2008). More recent immunodetection and *in vitro* enzyme activity assays have revealed that these RING-finger proteins are effectively involved in catalyzing H2Aub1 formation in *Arabidopsis* (Bratzel et al., 2010; Li et al., 2011; Yang et al., 2013).

PRC2 AND PRC1 IN H2Aub1 DEPOSITION

Polycomb group (PcG) proteins, first identified in *Drosophila* as repressors of homeotic (*Hox*) genes, are nowadays known to act in multiprotein complexes in transcription repression of a large number of genes in many multicellular organisms including plants (Bemer and Grossniklaus, 2012; Molitor and Shen, 2013; Schwartz and Pirrotta, 2013; Simon and Kingston, 2013). The most intensively studied complexes are PRC1 and PRC2. In *Drosophila*, PRC2 is composed of four core subunits, namely Ez (Enhancer of zeste), Suz12 (Suppressor of zeste 12), Esc (Extra sex combs) and N55 (a 55 kDa WD40 repeat protein), and PRC1 also contains four main subunits, namely Pc (Polycomb), Ph (Polyhomeotic), Psc (Posterior sex combs) and Ring1 (also known as dRing). In mammals, alternate subunit compositions create larger families of related PRC2-type and PRC1-type complexes (Schwartz and Pirrotta, 2013; Simon and Kingston, 2013). Nevertheless, defined biochemical activities of PRC2 and PRC1 are conserved from flies to humans. The classical model proposes a sequential mode of action of the two complexes: PRC2 catalyzes H3K27me3 formation, and PRC1 recognizes the H3K27me3 mark and further mediates downstream H2Aub1 deposition. The PRC1 components, acting as E3 ligases in H2Aub1 formation, are RING-finger proteins: Ring1 in *Drosophila* and Ring1A and Ring1B in human (Braun and Madhani, 2012; Schwartz and Pirrotta, 2013).

In *Arabidopsis*, the four PRC2 core components are highly conserved (**Figure 2**) and encoded by small gene families, and their function in H3K27me3 deposition and transcription repression have been intensively studied (Bemer and Grossniklaus, 2012). In contrast, PRC1 compositions are drastically diverged in plants as compared to animals (Molitor and Shen, 2013). No sequence homologue of Ph could be identified in plants so far. LHP1 (LIKE HETEROCHROMATIN PROTEIN 1), also known as TFL2 (TERMINAL FLOWER 2), binds H3K27me3 and may play a Pc-like function (Turck et al., 2007; Zhang et al., 2007b). This remarkably differs from the distinct roles of HP1 and Pc in animals, where HP1 binds H3K9me3 involved in hetereochromatin formation whereas Pc binds H3K27me3 involved in PRC1-mediated silencing in euchromatin. The best conservations found about PRC1 core components are from RING-finger proteins structured by a RING domain at N-terminus and a Ub-like RAWUL domain at C-terminus (Sanchez-Pulido et al., 2008; Xu and Shen, 2008).

These RING-finger proteins can be classified into two phylogenic groups: the first group comprises *Drosophila* Ring1, human Ring1A and Ring1B, and *Arabidopsis* AtRING1a and AtRING1b; the second group comprises *Drosophila* Psc, human Bmi1, and *Arabidopsis* AtBMI1a, AtBMI1b, and AtBMI1c. Consistent with their sequence conservation, AtRING1a, AtRING1b, AtBMI1a, and AtBMI1b each can ubiquitinate H2A *in vitro*, and loss of function of *AtBMI1a* and *AtBMI1b* causes H2Aub1 reduction *in planta* (Bratzel et al., 2010; Yang et al., 2013).

ROLE OF PRC1-LIKE RING-FINGER PROTEINS IN STEM CELL MAINTENANCE

Plant growth and development largely depend on stem cells located in SAM (shoot apical meristem) and RAM (root apical meristem), whose activities are fine-tuned by multiple families of chromatin factors (Sang et al., 2009; Shen and Xu, 2009). The first uncovered biological role of the *Arabidopsis* PRC1-like RING-finger proteins are on the regulation of SAM activity (Xu and Shen, 2008). While the single loss-of-function mutants *Atring1a* and *Atring1b* have a normal phenotype, the double mutant *Atring1a Atring1b* exhibits enlarged SAM, fasciated stem, and ectopic-meristem formation in cotyledons and leaves. This indicates that *AtRING1a* and *AtRING1b* play a redundant role in stable repression of stem cell activity to allow appropriate lateral organ differentiation. The balances between stem cell maintenance and cell differentiation for organ formation are controlled by specific transcription factors, including KNOX (Class I KNOTTED1-like homeobox) proteins. Strikingly, several *KNOX* genes, e.g., *STM* (*SHOOT-MERISTEMLESS*), *BP* (*BREVIPEDICELLUS*)/*KNAT1*, *KNAT2* and *KNAT6,* are upregulated in *Atring1a Atring1b* (Xu and Shen, 2008). Ectopic expression of *KNOX* genes colocalizes with and precedes ectopic meristem formation. It has been proposed that AtRING1a/b acts as a crucial PRC1 component in conjunction with PRC2 in repression of *KNOX* genes to promote lateral organ formation in the SAM (**Figure 2A**).

ROLE OF PRC1-LIKE RING-FINGER PROTEINS IN EMBRYONIC CELL FATE DETERMINACY

Further characterization of the ectopic meristem structures observed in *Atring1a Atring1b* unravels that these callus structures exhibit embryonic traits (Chen et al., 2010). The *Atbmi1a Atbmi1b* mutant also displays derepression of embryonic traits (Bratzel et al., 2010; Chen et al., 2010). Embryonic callus formation has been observed broadly in somatic tissues of cotyledons, leaves, shoots and roots of the mutant plants. Treatment with an auxin transport inhibitor can inhibit embryonic callus formation in *Atring1a Atring1b*, indicating that a normal auxin gradient is required for somatic embryo formation in the mutant (Chen et al., 2010). Both *Atring1a Atring1b* and *Atbmi1a Atbmi1b* mutants exhibit elevated expression of several key embryonic regulatory genes, including *ABI3* (*ABSCISIC ACID INSENSITIVE 3*), *AGL15* (*AGAMOUS LIKE 15*), *BBM* (*BABYBOOM*), *FUS3* (*FUSCA 3*), *LEC1* (*LEAFY COTYLEDON 1*), and *LEC2* (Bratzel et al., 2010; Chen et al., 2010). It is likely that derepression of these regulatory genes together with *KNOX* has contributed to the ectopic meristem and embryonic callus formation in somatic tissues of the *Atring1a Atring1b* and *Atbmi1a Atbmi1b* mutants (**Figure 2B**). The VAL (VP1/ABI3-LIKE) transcription factors can physically interact with AtBMI1 proteins and the *val1 val2* mutant exhibits comparable phenotype to *Atbmi1a Atbmi1b*, suggesting that VAL and AtBMI1 proteins may form complexes in repression of embryonic regulatory genes during vegetative development (Yang et al., 2013). Notably, loss of VAL or AtBMI1 causes H2Aub1 reduction in chromatin regions at *ABI3*, *BBM*, *FUS3* and *LEC1* but not *STM* (Yang et al., 2013). Future investigation is necessary to clarify whether AtBMI1 and AtRING1 proteins repress *KNOX* transcription *via* H2Aub1 deposition or other independent chromatin remodeling mechanisms.

ROLE OF PRC1-LIKE RING-FINGER PROTEINS IN SEED GERMINATION

Seed germination defines the entry into a new generation of the plant life cycle. It is generally accepted that the process of germination starts with water uptake followed by seed coat rupture and is completed following radicle protrusion (Bentsink and Koornneef, 2008). During the very early phase, the embryonic growth program remains latent and can be reinstated in response to unfavorable environmental cues. With the attainment of photosynthetic competence, the irreversible transition to autotrophic growth is accomplished and embryonic program is stably suppressed. A recent study (Molitor et al., 2014) has identified the *Arabidopsis* PHD-domain H3K4me3-binding AL (ALFIN1-like) proteins as interactors of AtBMI1 and AtRING1 proteins and has demonstrated a crucial function of chromatin state switch in establishment of seed developmental gene repression during seed germination (**Figure 2C**). Loss of AL6 and AL7 as well as loss of AtBMI1a and AtBMI1b retards seed germination and causes transcriptional derepression and a delayed chromatin state switch from H3K4me3 to H3K27me3 enrichment of seed developmental genes, including *ABI3* and *DOG1*. The germination delay phenotype of the *al6 al7* and *Atbmi1a Atbmi1b* mutants is more pronounced under osmotic stress (Molitor et al., 2014), suggesting that AL PHD-PRC1 complexes may participate in regulation of seed germination in response to environmental cues.

ROLE OF PRC1-LIKE RING-FINGER PROTEINS IN OTHER PROCESSES

AtBMI1a and AtBMI1b, also named DRIP1 (DREB2A-INTERACTING PROTEIN 1) and DRIP2, had been reported first as E3 ligases involved in ubiquitination of DREB2A (DEHYDRATION-RESPONSIVE ELEMENT BINDING PROTEIN 2A), a transcription factor controlling water deficit-inducible gene expression (Qin et al., 2008). The *drip1 drip2* mutant shows enhanced expression of water deficit-inducible genes and more tolerance to drought (Qin et al., 2008). Overexpression of *AtBMI1c* accelerates flowering time, which is associated with reduction of *FLC* expression (Li et al., 2011). In addition to SAM maintenance defects and derepression of embryonic traits, the *Atring1a Atring1b* mutant also displays homeotic conversions of floral tissues (Xu and Shen, 2008). Therefore, more precise functions and underlying molecular mechanisms for the PRC1-like RING-finger proteins are still waiting to be uncovered

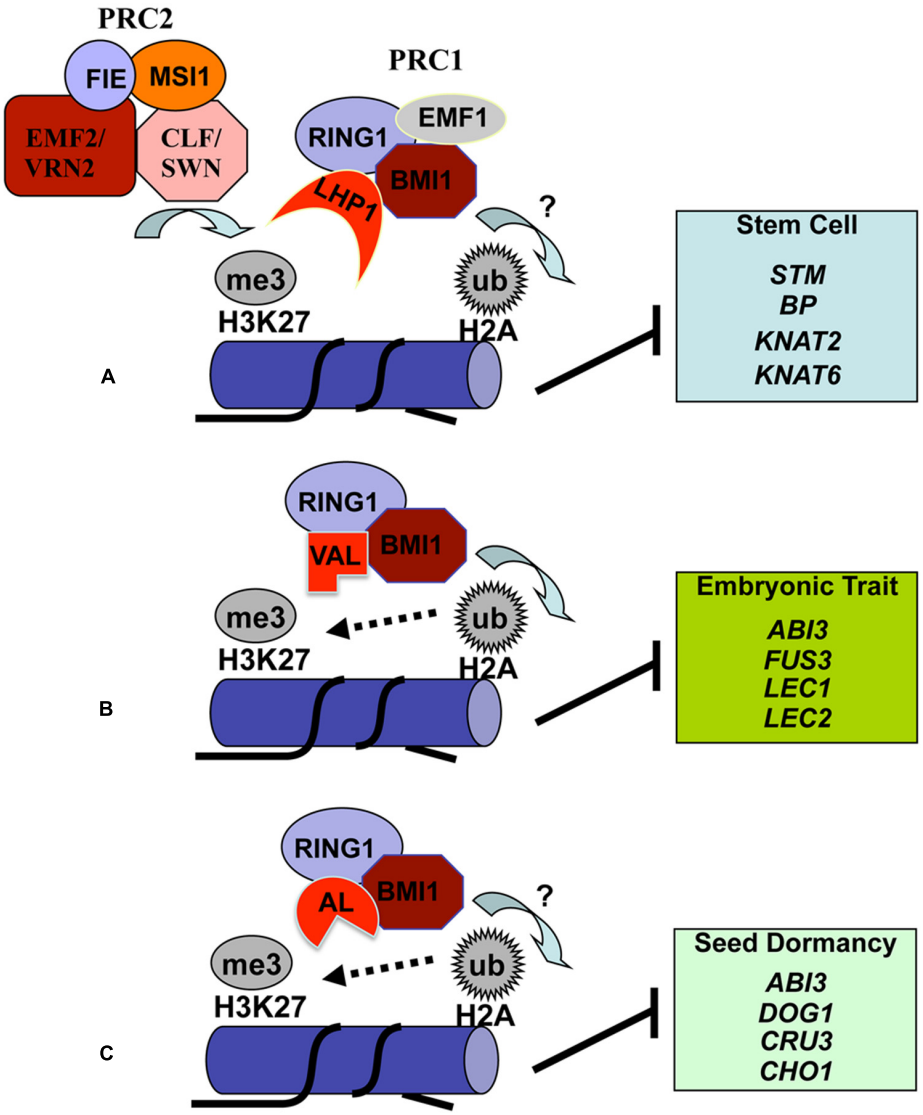

FIGURE 2 | Proposed models for histone H2A monoubiquitination deposition in transcriptional repression of varied target genes. The *Arabidopsis* PRC1-like RING-finger proteins AtRING1a/b (RING1) and AtBMI1a/b/c (BMI1) have the E3 ligase activity in catalyzing H2A monoubiquitination (H2Aub1). Comparable to the classical model of sequential PRC2 then PRC1 action in Polycomb silencing in animal cells, the *Arabidopsis* PRC1-like protein LHP1 binds H3K27me3 pre-deposited by the evolutionarily conserved PRC2 complexes and recruits RING1, BMI1 and possibly also EMF1 through protein–protein interactions **(A)**. This combinatorial action by PRC2 then PRC1 likely plays a broad role in suppression of numerous genes, including the key stem cell regulatory *KNOX* genes that need to be stably repressed during lateral organ development. The transcription factor VAL is involved in recruitment of BMI1 and RING1 in suppression of embryonic trait genes in somatic cells **(B)**. AL proteins bind BMI1 and RING1 and play important roles in suppression of several key seed dormancy regulatory genes to promote germination **(C)**. H3K27me3 deposition at embryonic/seed genes is enhanced by VAL/AL-PRC1 **(B,C)**, unraveling a non-canonical crosstalk between H3K27me3 and H2Aub1. The question marks indicate that H2Aub1 deposition in the specified target gene chromatin still requires future investigation.

during plant development and in plant response to environmental changes.

MECHANISMS OF PRC1-LIKE RING-FINGER PROTEINS IN TRANSCRIPTION REPRESSION

H2Aub1 function in plants is primarily evidenced through investigation of roles of the *Arabidopsis* PRC1-like RING-finger proteins (Xu and Shen, 2008; Bratzel et al., 2010; Chen et al., 2010; Li et al., 2011; Yang et al., 2013). Although these RING-finger proteins act nicely *in vitro* as E3 ligases, their *in vivo* functions in H2Aub1 deposition are still poorly documented. H2Aub1 level in *Arabidopsis* seems very low because large-scale analyses of either the histone-enriched or the Ub-affinity-purified protein preparations fail to detect H2Aub1 (Maor et al., 2007; Sridhar et al., 2007; Zhang et al., 2007a; Manzano et al., 2008; Saracco et al., 2009). H2Aub1 has been detected only by using specific antibodies, and in this case *AtBMI1* genes have been shown to act as positive regulators for H2Aub1 deposition in *Arabidopsis* plants (Bratzel et al.,

2010; Li et al., 2011; Yang et al., 2013). It is unknown whether any deubiquitinases might cause low levels of H2Aub1 in *Arabidopsis*. In animal cells, several deubiquitinases are characterized as specific for H2Aub1 (Weake and Workman, 2008; Simon and Kingston, 2013). Future characterization of *Arabidopsis* H2Aub1 deubiquitinases may provide useful information regarding regulatory mechanisms of H2Aub1 dynamics.

AtRING1 and AtBMI1 proteins physically interact each other and with the H3K27me3-binding protein LHP1 (Xu and Shen, 2008; Bratzel et al., 2010; Chen et al., 2010), providing a possible recruitment mechanism similar to the classical sequential PRC2 then PRC1 silencing pathway in animal cells. However, the *Atring1a Atring1b*, *Atbmi1a Atbmi1b,* or *Atbmi1a Atbmi1b Atbmi1c* mutant exhibits much more severe phenotypic defects than the *lhp1* mutant does, and *lhp1* enhances the *Atring1a Atring1b* mutant defects. It is thus apparent that AtRING1 and AtBMI1 proteins also act independently from LHP1. Recent identification of the transcriptional regulator VAL as AtBMI1-binding protein and of AL as AtRING1 and AtBMI1 interactor provides some novel insight about recruitment mechanisms (Yang et al., 2013; Molitor et al., 2014). It is particular intriguing that loss of AtBMI1 impairs H3K27me3 enrichment at seed developmental genes during seed germination and vegetative growth (Yang et al., 2013; Molitor et al., 2014). It has also been reported that loss of LHP1 impairs H3K27me3 enrichment at flower gene loci in roots (Derkacheva et al., 2013). These recent findings challenge the classic hierarchical paradigm where PRC2-mediated H3K27me3 deposition precedes PRC1 recruitment (**Figure 2**). It is obvious that future investigations are necessary to better understand the composition and function of different PRC1-like complexes in *Arabidopsis*.

CONCLUSIONS AND PERSPECTIVES

Studies over the last few years in the model plant *Arabidopsis* have greatly advanced our knowledge about the roles of H2Aub1 and H2Bub1 in transcription regulation in plant growth and development. In view of additional functions described in animal cells for both H2Aub1 and H2Bub1 in DNA damage repair (Bergink et al., 2006; Marteijn et al., 2009; Chernikova et al., 2010; Ginjala et al., 2011; Moyal et al., 2011; Nakamura et al., 2011), it is anticipated that more roles of H2Aub1 and H2Bub1 in plant response to environmental stresses are waiting to be uncovered. Mutagenesis of enzymes involved in H2Aub1 and H2Bub1 deposition or removal is required to address the question whether these enzymes effectively exert their biological functions *via* H2Aub1 and H2Bub1. Identification and characterization of factors associated with these different enzymes will be essential for understanding molecular mechanisms of their recruitment and function at specific targets within the genome. We need to know whether and how their function is spatially and temporally integrated with plant development. Genome-wide tools need to be further explored to provide a global view of links among enzyme or associated factor binding, H2Aub1/H2Bub1 enrichment, H3 methylation, and Pol II occupation. Crosstalks between H2Aub1 or H2Bub1 and different H3 methylations need to be addressed for chromatin context specificity.

In addition to H2Aub1 and H2Bub1, ubiquitinated H1, H3, and H4 are also found in *Arabidopsis* (Maor et al., 2007; Manzano et al., 2008; Saracco et al., 2009). H3 ubiquitination catalyzed by Rtt101-Mms1 in yeast and by Cul4-DDB1 in human has been recently shown to play an important role in the histone chaperone Asf1-mediated nucleosome assembly (Han et al., 2013). *Arabidopsis* contains a conserved family of CULLINs and CUL4-DDB1 complexes are reported (Shen et al., 2002; Hua and Vierstra, 2011). The Asf1 homologues in *Arabidopsis* are also identified (Zhu et al., 2011). It remains to be investigated whether CUL4-DDB and AtASF1 collaboratively act on nucleosome assembly *via* H3 ubiquitination in epigenetic regulation in *Arabidopsis*.

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E3 ubiquitin-ligases and their target proteins during the regulation of plant innate immunity

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Reversible protein ubiquitination plays a crucial role during the regulation of plant immune signaling. E3 ubiquitin (Ub)-ligase enzymes, which are classified into different families depending on their structural and functional features, confer the specificity of substrate and are the best characterized components of the ubiquitination cascade. E3 Ub-ligases of different families have been shown to be involved in all steps of plant immune responses. Indeed, they have been involved in the first steps of pathogen perception, as they appear to modulate perception of pathogen-associated molecular patterns by pattern-recognition receptors at the plasma membrane and to regulate the accumulation of nucleotide-binding leucine-rich repeat-type intracellular immune receptors. In addition, E3 Ub-ligase proteins are also involved in the regulation of the signaling responses downstream of pathogen perception through targeting vesicle trafficking components or nuclear transcription factors, for instance. Finally, we also discuss the case of microbial effector proteins that are able to target host E3 Ub-ligases, or to act themselves as E3 Ub-ligases, in their attempt to subvert the host proteasome to promote disease.

Keywords: E3 ubiquitin-ligase, microbial effector, plant immunity, 26S proteasome, ubiquitination

INTRODUCTION

Reversible protein conjugation with ubiquitin (Ub), or ubiquitination, is a key regulatory mechanism that controls a variety of cellular processes in eukaryotic cells, including DNA repair, gene transcription, protein activation or receptor trafficking, although the best characterized function of Ub involves selective protein degradation through the 26S proteasome (Vierstra, 2009). Ub becomes covalently attached to lysine residues of intracellular targets *via* an ATP-dependent reaction cascade that involves the sequential action of three enzymes: E1 (Ub-activating), E2 (Ub-conjugating), and E3 (Ub-ligase). The importance of the Ub-related pathway is underlined by the finding that the *Arabidopsis* genome encodes more than 1600 genes (> 6% of the total genome) involved in Ub/26S proteasome system (UPS)-related functions. Most of these genes (> 1400) encode putative E3 Ub-ligases (Mazzucotelli et al., 2006). E3 proteins are classified into four main subfamilies depending on their structural features and mechanism of action: HECT (Homologous to E6-associated protein C-Terminus), RING (Really Interesting New Gene), U-Box and CRL (Cullin-RING Ligases; Vierstra, 2009). HECT proteins form an Ub-E3 intermediate before transfer of Ub to the substrate (Downes et al., 2003). RING and U-box proteins are structurally related single polypeptides that, respectively, use zinc chelation and hydrogen bonds/salt bridges to transfer Ub from the Ub-E2 intermediate to the target (Stone et al., 2005; Yee and Goring, 2009). Ub-ligases containing a RING domain can act independently or as part of a multisubunit CRL complex such as the SCF (Skp1, Cullin, F-box)-type ligase. In this complex, substrate recognition is provided by the F-box protein, whereas the RING protein binds to the E2 (Hua and Vierstra, 2011). By contrast, some RING-type Ub-ligases act independently and determine substrate specificity allowing the interaction between the E2 and the target protein by tethering them in close proximity (Vierstra, 2009).

Plants have developed a multi-layered defense system to ensure their survival in a microbe-rich environment. A first line of pathogen detection is activated after recognition of highly conserved PAMPs/MAMPs (pathogen-/microbe-associated molecular patterns) by specific plant PRRs (pattern-recognition receptors), leading to a form of basal resistance called PTI (PAMP-triggered immunity; Jones and Dangl, 2006). Thriving pathogens evolved to secrete virulence effectors that inactivate crucial PTI regulators thereby counteracting plant defenses. In turn, plants gained the ability to recognize these effectors through resistance (R) proteins, for the most part intracellular NB-LRR (nucleotide-binding-leucine-rich repeat) immune sensors, that lead to a more efficient form of resistance called ETI (effector-triggered immunity; Jones and Dangl, 2006). This specific resistance is frequently associated to development of the hypersensitive response (HR), a form of programmed cell death at the infection site that prevents pathogen spreading (Coll et al., 2011).

In plants, the UPS pathway, and more particularly E3 Ub-ligase proteins, have been shown to be involved in responses to a variety of stimuli (Vierstra, 2009; Robert-Seilaniantz et al., 2011). Since the finding that the SCF complex-interacting protein SGT1 (Suppressor of G2 allele of skp1) is an essential component of *R* gene-triggered disease resistance provided a first connection between the UPS and plant immune signaling (Azevedo et al., 2002), evidence that E3 Ub-ligase proteins act as regulators of plant immunity has increasingly accumulated (Trujillo and Shirasu, 2010; Marino et al., 2012). Indeed, modulation of the expression

of E3 Ub-ligase-encoding genes has been reported following elicitor treatment or inoculation with different pathogens. Moreover, misregulation of E3 Ub-ligase gene expression, using overexpressing, RNA interference (RNAi) and/or mutant lines, results in modulation of plant defense responses following pathogen inoculation [reviewed in (Marino et al., 2012)]. Therefore, it has become increasingly evident that plant E3 Ub-ligase proteins play important roles in the regulation of immune signaling, although the proteins targeted by Ub-ligases are only known in a limited number of cases, and our current knowledge of the involved molecular mechanisms is thus only partial. Here we review positive and negative roles played by E3 Ub-ligases during the regulation of various steps of plant immunity, from pathogen recognition to downstream signaling during both PTI and ETI responses. Due to space limitations, we focus on recent reports about E3 Ub-ligases for which a target protein has been identified during the plant response to bacterial or fungal pathogens, since these particular examples provide insight into the cellular processes involved in regulation of immune signaling. For an overview on UPS-related pathways in response to viral infection we refer the reader to a recent review (Alcaide-Loridan and Jupin, 2012). We also discuss the case of microbial effectors that, to promote disease, either target host E3 Ub-ligases or act as Ub-ligases inside plant cells (**Figure 1**).

PLANT E3 UB-LIGASES INVOLVED IN REGULATION OF PATHOGEN PERCEPTION

Several E3 ligase proteins have been identified as modulators of the first steps of pathogen recognition by plant cells, as they appear to be able to target both PRR and NB-LRR proteins in order to prevent unnecessary activation of defense signaling. In rice (*Oryza sativa*), the RING-type E3 Ub-ligase XB3 interacts with the receptor-like kinase (RLK) protein XA21, which confers resistance to bacterial blight caused by *Xanthomonas oryzae* pv. *oryzae* (*Xoo*; Wang et al., 2006). XB3 has been shown to be required for XA21 accumulation and XA21-mediated resistance to *Xoo*, suggesting that it most likely targets a protein that modulates accumulation of XA21 (Wang et al., 2006). XA21 is able to phosphorylate XB3 but the molecular mechanism underlining activation of defense responses by XB3 remains to be elucidated. Overexpression of members of the XB3 family from rice, *Arabidopsis* and citrus in *Nicotiana benthamiana* induces cell death and this effect is dependent on XB3 catalytic activity, suggesting an evolutionarily conserved role for the XB3 protein family in regulating plant programmed cell death (Huang et al., 2013).

In *Arabidopsis*, the U-box E3 ligases Plant U-Box12 (PUB12) and PUB13 have been involved in attenuation of PTI responses triggered by perception of flagellin, or its active peptide derivative flg22, by the PRRs Flagellin Sensing2 (FLS2) and its co-receptor BAK1. In response to flg22, PUB12 and PUB13 form a BAK1-dependent complex with FLS2 and are able to polyubiquitinate FLS2, but not BAK1 (Lu et al., 2011). BAK1 phosphorylates PUB12 and PUB13 and this phosphorylation is enhanced by flg22 and by the FLS2/BAK1-associated kinase BIK1 (Lu et al., 2011). flg22-dependent signaling is enhanced in *pub12* or *pub13* mutant plants and, in agreement with PUB12 and PUB13 promoting FLS2 degradation, *pub12 pub13* double mutant plants displayed increased resistance to bacterial infection. These data are consistent with the fact that FLS2 undergoes flg22-induced endocytosis and subsequent degradation (Robatzek et al., 2006; Gohre et al., 2008; Beck et al., 2012; Choi et al., 2013). However, whether stabilization of FLS2 in *pub12pub13* plants reflects FLS2 accumulation at the plasma membrane or within an endosomal compartment remains to be determined. A recent report showed that FLS2 degradation occurs in a flg22 time- and dose-dependent manner, which may play a significant role in turning over ligand-occupied FLS2, but the role of PUB12 and PUB13 in this process was not determined in this study (Smith et al., 2013).

Similar to PRR proteins, intracellular NB-LRR immune receptors are also targeted by E3 Ub-ligases, which appear to control R protein accumulation at multiple levels. First, in *Arabidopsis*, the F-box motif E3 Ub-ligase CPR1 interacts with and down-regulates the accumulation of the NB-LRR R proteins Suppressor of *npr1-1* Constitutive1 (SNC1) and Resistant to *P. syringae2* (RPS2), resulting in attenuation of immune signaling (Cheng et al., 2011; Gou et al., 2012). Second, accumulation of SNC1 and RPS4, an additional NB-LRR immune receptor, are also negatively regulated by Suppressor of *rps4-RLD1* (SRFR1), a tetratricopeptide repeat protein (Kim et al., 2010; Li et al., 2010). Since (i) SNC1 levels increased in CPR1-overexpressing plants treated with the proteasome inhibitor MG132 (Gou et al., 2012); (ii) SRFR1 interacts with SGT1; and (iii) increased SNC1 and RPS4 accumulation was also observed in *sgt1* mutant plants (Li et al., 2010), stability of SNC1 and RPS4 is likely regulated by SRFR1 through SGT1 interaction with the SCF complex, revealing an additional molecular mechanism to prevent autoimmunity. Third, mutation of *MOS12* (*modifier of snc1-12*), that encodes an *Arabidopsis* Arg-rich protein homologous to human cyclin L, resulted in altered *SNC1* and *RPS4* splicing patterns and protein levels (Xu et al., 2012). Interestingly, MOS12 interacts with the nuclear U-Box E3 ligases MAC3A and MAC3B, which are required for full R protein-mediated resistance, suggesting that MOS12, MAC3A, and MAC3B contribute to the fine-tuning of *R* gene expression, in a process that appears to be critical for directing appropriate defense outputs (Monaghan et al., 2009; Xu et al., 2012). Finally, a recent report showed an intriguing link between cell cycle regulation and defense signaling (Bao et al., 2013). Omission of the Second Division (OSD1) and its homolog UV-B-Insensitive 4 (UVI4) are two negative regulators of the multisubunit E3 Ub-ligase APC/C (anaphase-promoting complex/cyclosome) that regulates cell cycle progression in *Arabidopsis*. Overexpression of either OSD1 or UVI4 leads to downregulation of APC/C activity, overaccumulation of the APC/C degradation target CYCB1;1, upregulation of several *R* genes, including *SNC1*, and spontaneous cell death and enhanced disease resistance to virulent bacteria (Bao et al., 2013). These data provide further evidence of the intricate control exerted on immune receptor levels in order to regulate defense activation.

PLANT E3 UB-LIGASES INVOLVED IN REGULATION OF DEFENSE-RELATED SIGNALING

Pathogen perception by PRRs stimulates a cascade of signaling events including changes in ion fluxes across the plasma membrane, production of reactive oxygen species (ROS),

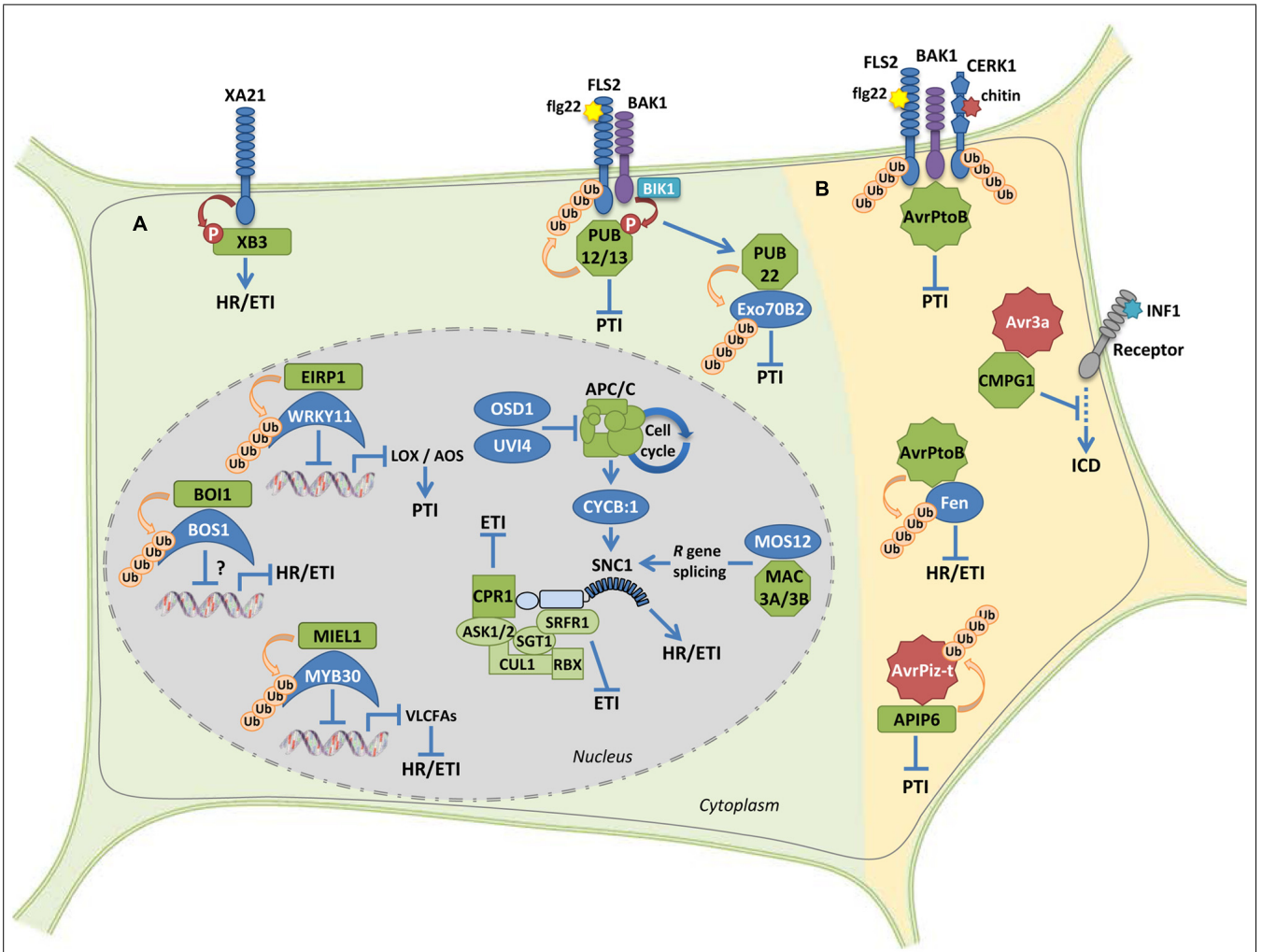

FIGURE 1 | Ub-ligase proteins during the regulation of plant immune signaling. (A) Examples of positive and negative regulatory roles on plant immunity by host E3 Ub-ligases. The RING-type protein XB3 is required for the accumulation of the receptor protein XA21 and promotes downstream HR and defense responses. U-box proteins PUB12 and PUB13 attenuate flg22-mediated PTI signaling. The U-box protein PUB22 targets the exocyst complex subunit Exo70B2, which leads to attenuation of PTI signaling. Accumulation of the intracellular immune receptor SNC1 appears to be controlled by the SCF complex through the interaction with both the F-box CPR1 and the SGT1-interacting protein SRFR1. Modulation of *SNC1* levels by the multisubunit cell cycle-related E3 ligase APC/C or the U-box proteins MAC3A and MAC3B provide additional molecular mechanisms to control immunity. The interaction between the RING-type E3 Ub-ligase EIRP1, and the TF WRK11 results in suppressed transcriptional activation of WRKY11 target genes *AOS* and *LOX1*, thereby enhancing PTI. The RING E3 ligase MIEL1 interacts with the MYB TF MYB30, which results in weakened transcriptional activation of VLCFA-related genes and therefore suppressed HR and defense responses. The RING protein BOI1 interacts with and ubiquitinates the MYB TF BOS1 that is required for pathogen resistance. However, the effect of BOI1 on BOS1 accumulation and transcriptional activation remains unknown. **(B)** Microbial effector proteins acting as or interacting with E3 ligase proteins in the host. The bacterial effector AvrPtoB is able to suppress both PTI and ETI signaling though its respective interaction with FLS2/CERK1 PRRs and the cytoplasmic kinase Fen. The oomycete effector AVR3a suppresses INF1-induced cell death by interacting with and stabilizing the host U-box E3 ligase CMPG1. The fungal effector protein AvrPiz-t targets the RING E3 ligase APIP6, which in turn is able to ubiquitinate AvrPiz-t, resulting in suppressed PTI. RING-, F-box- and U-box-type E3 Ub-ligases are, respectively, represented by rectangles, squares, and octagons. All proteins displaying E3 ligase activity are represented in green. Effectors are represented by stars. See the text for details.

induction of mitogen-activated protein kinases (MAPKs), modulation of host gene transcription and callose deposition at the plant cell wall. Amplitude and duration of these signaling responses must be tightly regulated to ensure an appropriate response. In addition to their role in internalization or degradation of receptors to attenuate downstream signaling, E3 Ub-ligase proteins also regulate the accumulation of plant components involved in defense-related signaling.

Similar to PUB12 and PUB13, PUB22 acts in concert with PUB23 and PUB24 to negatively regulate PTI responses in *Arabidopsis* (Trujillo et al., 2008). Following elicitation with various PAMPs, *pub22/pub23/pub24* triple mutants displayed enhanced early signaling responses, indicating that these three PUB proteins target components involved in defense signaling triggered by different PRRs (Trujillo et al., 2008). Indeed, the exocyst complex subunit Exo70B2 that is involved in vesicle tethering during exocytosis,

has been identified as a cellular target of PUB22 (Stegmann et al., 2012). PUB22 is stabilized in response to flg22 treatment (potentially by inhibition of its autocatalytic ubiquitination activity), leading to Exo70B2 ubiquitination and proteasomal degradation. Exo70B2 is required for both immediate (ROS production, MAPK activation) and later responses (PTI marker gene expression, root growth inhibition) triggered by several PAMPs, indicative of a role in signaling. Indeed, *exo70B2* mutant plants displayed enhanced susceptibility to pathogens (Stegmann et al., 2012). Together, these data suggest a mechanism by which Exo70B2 levels are regulated by quick changes in PUB22 turnover in response to PAMPs and identify a first component of vesicle trafficking required for regulation of plant PTI signaling. In view of these data, it has been proposed that Exo70B2 may contribute to recycling of plasma membrane proteins involved in PAMP-triggered signaling, including NADPH oxidases, ion channels or RLKs such as FLS2. Exo70B2 degradation by PUB22 would thus attenuate the recycling pathway redirecting positive signaling components into the vacuolar degradation pathway and downregulating signaling (Stegmann et al., 2012). As previously discussed, PUB12/PUB13-mediated ubiquitination of FLS2 is expected to modulate its intracellular trafficking (Lu et al., 2011). Since degradation of integral membrane proteins is mediated by the vacuole, signal attenuation is probably simultaneously regulated at various levels of vesicle trafficking.

In Chinese wild grapevine (*Vitis pseudoreticulata*), EIRP1 is an active E3 Ub-ligase whose RING domain is necessary for its activity and also mediates the interaction with the WRKY nuclear transcription factor (TF) VpWRKY11 (Yu et al., 2013). Similar to *EIRP1*, expression of *VpWRKY11* was rapidly induced following fungal infection. VpWRKY11 activated the expression of *AOS* (*Allene Oxide Synthase*) and *LOX2* (*Lipoxygenase2*), two JA-responsive genes that function as negative regulators of basal resistance in *Arabidopsis* (Journot-Catalino et al., 2006). Additionally, in agreement with the observation that co-expression with EIRP1 results in proteasomal degradation of VpWRKY11, *AOS* and *LOX2* expression was, respectively, repressed and induced in EIRP1-overexpressing and RNAi plants. Moreover, EIRP1 overexpression in *Arabidopsis* conferred enhanced resistance to fungal and bacterial pathogens, which correlated with reduced expression of *WRKY11, AOS*, and *LOX2* (Yu et al., 2013). Together, these data identify a RING-type Ub-ligase that plays a positive role in activation of resistance by targeting a TF that acts as a negative regulator of plant defenses.

In contrast, the *Arabidopsis* RING-type Ub-ligase MIEL1 acts as a negative regulator of plant resistance (Marino et al., 2013). Indeed, MIEL1 interacts with the MYB TF MYB30, which activates plant defense responses by up-regulating the expression of genes involved in the production of very long chain fatty acids (VLCFAs; Raffaele et al., 2008). The MYB30-MIEL1 nuclear interaction leads to MYB30 proteasomal degradation, reduced expression of VLCFA-related MYB30 target gene expression and, therefore, attenuation of plant immune responses (Marino et al., 2013). *MIEL1* expression is rapidly repressed in inoculated cells, suggesting that (i) in the absence of the pathogen, MIEL1 may negatively regulate plant defense activation through degradation of MYB30 and that (ii) after pathogen inoculation, repression of *MIEL1* expression may release MYB30 negative regulation, triggering defense (Marino et al., 2013).

BOI1 is an additional nuclear RING-type Ub-ligase that interacts with and ubiquitinates the MYB TF BOS1, which confers resistance to several pathogens in *Arabidopsis* (Mengiste et al., 2003; Luo et al., 2010). This finding suggests that BOS1 may be a target of BOI1. However, no effect of BOI1 on BOS1 transcriptional activity has been reported and both *bos1* mutant and BOI1 RNAi *Arabidopsis* plants (in which the BOS1 protein is expected to accumulate) display enhanced susceptibility to fungal infection (Luo et al., 2010). Therefore, whether BOI1 is able to directly regulate BOS1 protein accumulation remains to be determined.

MANIPULATION OF HOST E3 UB-LIGASE PROTEINS BY MICROBIAL EFFECTORS

The important role played by E3 Ub-ligases during the establishment of plant immune responses to pathogen attack is highlighted by the discovery of microbial effector proteins that evolved the ability to interfere with these host UPS components to promote disease. For example, the AvrPiz-t effector from the rice blast fungus *Magnaporthe oryzae* is translocated into rice cells, where it is able to mediate suppression of PAMP-induced ROS production, inducing susceptibility to *M. oryzae* (Park et al., 2012). AvrPiz-t appears to inhibit the Ub-ligase activity of APIP6, a rice RING-type Ub-ligase that is also able to ubiquitinate AvrPiz-t *in vitro* (Park et al., 2012). Interestingly, AvrPiz-t and APIP6 are both degraded when transiently coexpressed in *N. benthamiana*. Since APIP6 positively regulates flg22-induced ROS generation, induction of defense-related gene expression, and rice resistance to *M. oryzae*, targeting of APIP6 by AvrPiz-t results in suppression of rice PTI responses (Park et al., 2012).

The effector AVR3a from the oomycete *Phytophthora infestans* prevents development of cell death induced by *P. infestans* elicitin INF1. The finding that AVR3a targets and stabilizes the U-box-type Ub-ligase CMPG1 revealed the molecular mechanism behind AVR3a negative regulation of ICD (INF1-triggered Cell Death; Bos et al., 2010). CMPG1 Ub-ligase activity is required for ICD as well as for cell death following elicitor perception at the plasma membrane (Gonzalez-Lamothe et al., 2006; Gilroy et al., 2011). Considering that AVR3a is essential for *P. infestans* virulence, stabilization of CMPG1 by AVR3a suggests that this effector is able to suppress ICD during the biotrophic phase of infection by modifying CMPG1 activity, impeding normal proteasomal degradation of both CMPG1 and its host targets (Bos et al., 2010).

In addition to microbial effectors that are able to target host E3 Ub-ligase proteins, examples of effectors that present E3 Ub-ligase-related domains have also been reported in a diversity of pathogenic microbes including bacteria, fungi, oomycetes, viruses and nematodes (Marino et al., 2012). The best characterized example of microbial E3 ligases is the AvrPtoB effector from *Pseudomonas syringae* that presents a C-terminal domain with remarkable structural homology with RING- and U-box-type Ub-ligases (Janjusevic et al., 2006). AvrPtoB is a modular effector able to suppress PTI signaling. Indeed, AvrPtoB N-terminal

domain is able to interact with the PRRs FLS2 and the chitin receptor CERK1, whereas its C-terminal Ub-ligase domain mediates PRR proteasomal degradation (Gohre et al., 2008; Gimenez-Ibanez et al., 2009). AvrPtoB is additionally able to target the co-receptor protein BAK1 (Shan et al., 2008) and to interfere with MAPK activation downstream of FLS2 (He et al., 2006), although independently of AvrPtoB Ub-ligase activity. Remarkably, AvrPtoB is also able to suppress ETI signaling. Indeed, AvrPtoB interacts with and mediates proteasomal degradation of Fen, a tomato protein kinase that activates plant immunity in response to *P. syringae* carrying Ub-ligase deficient forms of AvrPtoB, as well as cell death responses when overexpressed in *N. benthamiana* (Rosebrock et al., 2007).

CONCLUSIONS AND PERSPECTIVES

Evidence of the involvement of the UPS pathway in the regulation of plant immunity is rapidly mounting. E3 Ub-ligase proteins are the best characterized UPS components playing a role in immune signaling at multiple levels. Nevertheless, based on induction of their expression following elicitation, E1 and E2 enzymes have also been suggested to contribute to plant disease resistance although the molecular mechanisms by which they regulate this process remain poorly characterized (Marino et al., 2012). Interestingly, a recent report provides a first example of the direct involvement of Ub-conjugating proteins in the regulation of plant immunity. In tomato, Fen-interacting protein 3 (Fni3) is a homolog of the *Arabidopsis* E2 enzyme Ubc13. Through interaction with its cofactor *S. lycoperiscum* Uev (Suv), which is an inactive Ub-conjugating enzyme variant, Fni3 catalyzes Lys-63-linked ubiquitination, a non-proteolytic regulatory signal (Mural et al., 2013). Fni3 interacts with Fen but does not affect Fen stability. Fni3 Ub-conjugating activity and interaction with Fen are required for cell death triggered by Fen overexpression in *N. benthamiana* and by several R protein/effector pairs (Mural et al., 2013). Together these results suggest that, in addition to conventional Lys-48-linked ubiquitination that mainly serves as a signal for proteasomal degradation of substrate proteins, other ubiquitination forms are important regulators of plant immune signaling. Consistent with this idea, Lys-63-linked ubiquitination has also been shown to be a target of manipulation by plant pathogens, as the PthA effector from the bacterial pathogen *Xanthomonas axonopodis* pv. *citri* is able to target Ubc13 and Uev in citrus (Domingues et al., 2010).

In conclusion, further characterization of additional UPS components as well as of the distinct fates of ubiquitination targets should contribute to dissecting the complex regulation of plant immune signaling by the UPS.

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The role of ubiquitin and the 26S proteasome in plant abiotic stress signaling

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Ubiquitin is a small, highly conserved, ubiquitously expressed eukaryotic protein with immensely important and diverse regulatory functions. A well-studied function of ubiquitin is its role in selective proteolysis by the ubiquitin-proteasome system (UPS). The UPS has emerged as an integral player in plant response and adaptation to environmental stresses such as drought, salinity, cold and nutrient deprivation. The UPS has also been shown to influence the production and signal transduction of stress-related hormones such as abscisic acid. Understanding UPS function has centered mainly on defining the role of E3 ubiquitin ligases, which are the substrate-recruiting component of the ubiquitination pathway. The recent identification of stress signaling/regulatory proteins that are the subject of ubiquitin-dependent degradation has increased our knowledge of how the UPS facilitates responses to adverse environmental conditions. A brief overview is provided on role of the UPS in modulating protein stability during abiotic stress signaling. E3 ubiquitin ligases for which stress-related substrate proteins have been identified are discussed.

Keywords: abiotic stress, abscisic acid, E3 ubiquitin ligase, 26S proteasome, protein degradation, ubiquitination

INTRODUCTION

The covalent attachment of ubiquitin molecules to selected proteins (referred to as ubiquitination) can influence activity, abundance, trafficking, or localization. The versatility of the ubiquitination pathway lies in the different ways in which ubiquitin molecules can be attached to a selected substrate protein (Komander and Rape, 2012). A single ubiquitin molecule can be attached to one (monoubiquitination) or multiple (multimonoubiquitination) lysine residues within a substrate protein. Another type of modification is the assembly of a chain of ubiquitin molecules (polyubiquitination) on a specific lysine residue within the substrate protein. Ubiquitin contains seven lysine residues each of which can be used to create ubiquitin-ubiquitin linkages, producing structurally diverse polyubiquitin chains (Nakasone et al., 2013). A polyubiquitin chain can be homogeneous using the same lysine residue to build the polymer, or of mixed topology with different lysine residues used to create ubiquitin-ubiquitin linkages. The significance of every type of modification is unknown. However, of the modifications that are understood, each confers a distinct outcome on a specific substrate protein. For example, monoubiquitination, or the attachment of a lysine 63-linked polyubiquitin chain, may serve as a signal for intracellular trafficking or protein activation, respectively (Chen and Sun, 2009). The assembly of a lysine 48-linked polyubiquitin chain is known to signal for the destruction of the modified protein (Thrower et al., 2000).

Ubiquitin-dependent protein degradation involves two distinct and successive steps: the attachment of a polyubiquitin chain consisting of at least four lysine 48-linked ubiquitin molecules to the substrate protein and degradation of the modified protein by the 26S proteasome, a large multi-catalytic protease complex. At the cellular level, the ubiquitin-proteasome system (UPS) is an essential part of regulatory networks that carefully controls the abundance of important enzymes, structural, and regulatory proteins. Plants utilize the UPS to facilitate changes in cellular protein content required for continuous growth, development, and adaptation to their ever changing environment (Stone and Callis, 2007; Vierstra, 2009). In the model research plant *Arabidopsis thaliana* (*At*; *Arabidopsis*), almost 6% of the genome is dedicated to the UPS (Hua and Vierstra, 2011). The majority of these genes encode for ubiquitin ligases (E3s), a central component of the ubiquitination pathway. Recently, E3s have emerged as modulators of plant response to abiotic stresses including drought, cold, salinity, heat, radiation, and nutrient deprivation (Yee and Goring, 2009; Lyzenga and Stone, 2012). Importantly, the action of a single E3 can regulate plant responses to multiple abiotic stresses. The impact of the UPS on abiotic stress tolerance is usually associated with regulating the actions of stress hormones such as abscisic acid (ABA). The significance of the UPS is further exemplified by the finding that multiple ubiquitin ligases are involved in regulating stress hormone signaling. Our understanding of how the UPS facilitate plant responses to various abiotic stresses is aided by recent studies that identified substrates for stress-related E3s. This review provides a brief overview of the role of these E3 ligase-substrates pairings during plant responses to abiotic stresses.

THE UBIQUITIN ENZYMES

Ubiquitination is a multi-step process involving the sequential action of three enzymes: E1 (ubiquitin activating enzyme; UBA), E2 (ubiquitin conjugating enzyme; UBC), and E3 (ubiquitin ligase). The conjugation process begins with the activation of ubiquitin by the E1 followed by transfer of ubiquitin to the E2, forming a thioester linked E2-ubiquitin (E2-Ub) intermediate.

The substrate-recruiting E3 interacts with the E2-Ub allowing for the transfer of ubiquitin to the target (**Figure 1A**). Following the attachment of the initial ubiquitin molecule, the process can be repeated to assemble a polyubiquitin chain (Komander and Rape, 2012). The conjugation process is also reversible. Proteases referred to as deubiquitinating enzymes (DUBs) are able to cleave ubiquitin molecules from modified proteins (Reyes-Turcu et al., 2009). The ubiquitination pathway is hierarchical in that eukaryotic genomes are found to contain one or two E1, 10s of E2 and 100s of E3 encoding genes. For example, the *Arabidopsis* genome is predicted to encode for two E1 isoforms, 37 E2 enzymes and over 1300 E3s or components of E3 complexes (Hatfield et al., 1997; Kraft et al., 2005; Stone et al., 2005; Hua and Vierstra, 2011). The large number of ubiquitin enzymes suggests that many cellular processes are regulated via protein ubiquitination.

The capacity of the ubiquitination pathway to differentially modify numerous proteins is made possible by the abundance and diversity of ubiquitin ligases. The majority of plant E3s are of the homology to E6-associated carboxy-terminus (HECT), U-box, or Really Interesting New Gene (RING) type (**Figure 1A**). The *Arabidopsis* genome is predicted to encode for 7 HECT-type and 64 U-box-type E3s (Downes et al., 2003; Mudgil et al., 2004). Over 470 *Arabidopsis* genes are predicted to encode for RING domain-containing proteins (Stone et al., 2005). Ubiquitin ligases that utilize a RING domain for E2 binding can occur as monomeric E3s or multi-subunit Cullin (CUL) based RING E3 ligases (CRLs; **Figure 1A**). Three types of CRLs have been described in plants, each utilizing a different CUL subunit, CUL1, CUL3a/b, or CUL4 (Hotton and Callis, 2008; Hua and Vierstra, 2011). Each CUL serves as a platform upon which the RING domain-containing (RBX1a/b) and substrate-recruiting sub-units assemble (**Figure 1A**). Substrate-recruiting proteins utilized by plant CRLs belong to either the F-box, Broad complex Tramtrack Bric-a-Brac (BTB), or DDB1 binding WD40 (DWD) families. The F-box family is the largest with over 700 members followed by the DWD and BTB with 85 and 80 members, respectively (Lechner et al., 2006; Gingerich et al., 2007; Lee et al., 2008). The CUL1 based E3s (also referred to as Skp1-Cullin-F-box [SCF]) use the adaptor protein *Arabidopsis* S-Phase kinase-associated protein (ASK) to bind to F-box proteins (Bai et al., 1996; Lechner et al., 2006). CUL4 based E3s are assembled using DNA-damage binding (DDB1) as an adaptor to bind DWD proteins, while CUL3a/b interacts directly with BTB proteins (Gingerich et al., 2007; Lee et al., 2008). The large number of substrate-recruiting subunits and the ability to assemble E3 complexes using one of three CUL proteins makes the CRL group the largest class of ubiquitin ligases.

THE UBIQUITINATION PATHWAY AND ABIOTIC STRESS TOLERANCE

A plants ability to survive abiotic stresses such as salinity, radiation, heavy metals, nutrient deprivation, cold, and drought relies

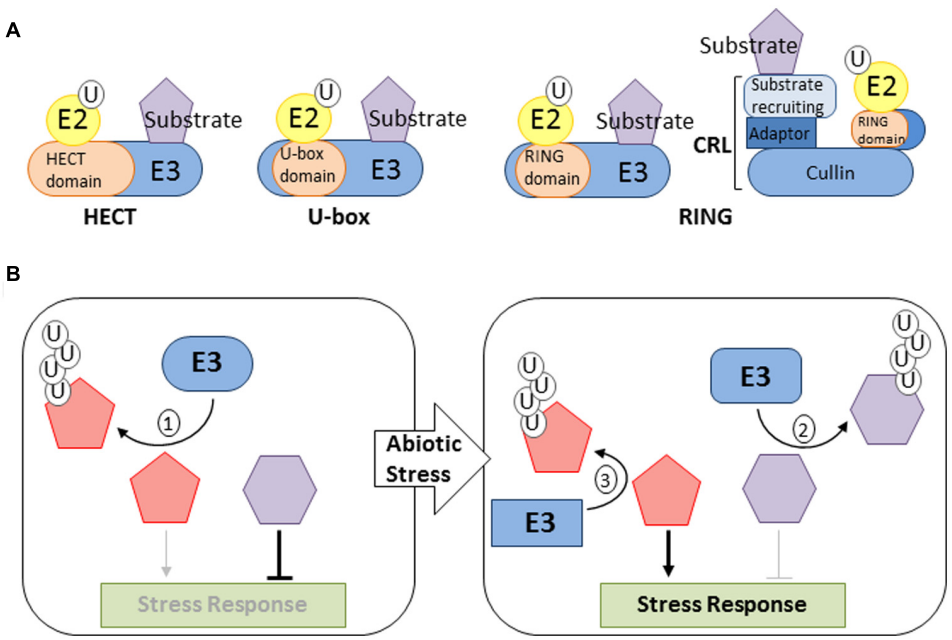

FIGURE 1 | Function of E3 ligases in abiotic stress response. **(A)** Most common type of plant E3s. Ubiquitin ligases are categorized based on the presence of a RING, HECT, or U-box E2-binding domain. RING and U-box domain-containing E3s mediate transfer of ubiquitin (U) directly from the E2-Ub intermediate to the substrate protein. HECT domain-containing E3s form an E3-Ub intermediate prior to the transfer of ubiquitin to the substrate protein. RING domains are found in monomeric E3s and multisubunit CRLs. **(B)** Illustrations of the most common modes of action for E3 ligases in regulating abiotic stress responses. (1) E3 ligases may function as a negative response regulators required to supress stress response pathways by targeting positive regulators for degradation. (2) E3 ligases may promote stress signaling by functioning as positive response regulators that target negative regulators for degradation following stress perception. (3) E3 ligases may also function to attenuate stress signaling by targeting positive regulators for degradation.

heavily on proteomic plasticity. The UPS plays a crucial role in enabling plants to alter their proteome in order to effectively and efficiently perceive and respond to environmental stresses (Smalle et al., 2003; Kurepa et al., 2008). How the UPS functions to facilitate responses to a particular stress depends upon the nature of the substrate protein. For example, ubiquitin-dependent degradation of a positive regulator may serve to supress the response pathway until a stress stimulus is perceived (**Figure 1B**). In this case, ubiquitination of the substrate would cease allowing for accumulation of the regulatory protein and promotion of cellular changes required to acclimate the plant to external conditions. The ubiquitin ligase involved in modifying the regulatory protein would be designated a negative response regulator (Chen and Hellmann, 2013). On the other hand, ubiquitin ligase targeting a negative regulator for degradation in response to a stimulus would enable the activation of signaling pathways required for tolerance of the perceived stress (**Figure 1B**). Many examples of the aforementioned scenario have been reported, some of which are discussed below. Instances of the UPS functioning to attenuate stress signaling have also been described. In these cases, ubiquitin-dependent degradation of a positive regulator occurs following perception of a stress stimulus (**Figure 1B**). Maintenance of a certain level of signal intensity and termination of signal transduction would enable plants to recover and resume normal growth and development once environmental conditions improve.

One of the first indications of the importance of the ubiquitination pathway to abiotic stress tolerance is the finding that *ubiquitin* gene expression is up-regulated in plants exposed to high temperature stress (Genschik et al., 1992; Sun and Callis, 1997). In fact, overexpression of ubiquitin has been shown to increase plant tolerance of salinity and drought conditions (Guo et al., 2008). Since this finding, stress-related roles have been demonstrated for a number of ubiquitin enzymes. Many E2 encoding genes are stress-inducible. Transcript levels of *Glycine max UBC2* (*GmUBC2*; soybean), *Arachis hypogaea UBC2* (*AhUBC2;* peanut) and *Arabidopsis UBC32* (*AtUBC32*) are up-regulated in response to drought and/or salt stress (Zhou et al., 2010; Wan et al., 2011; Cui et al., 2012). Overexpression of *AtUBC32* rendered plants sensitive to salt stress (Cui et al., 2012). Conversely, *AtUBC32* mutant plants are more tolerant of salt stress. Also, transgenic *Arabidopsis* plants overexpressing *Vigna radiata UBC1* (*VrUBC1*; mung bean), *AhUBC2*, or *GmUBC2* were more tolerant of drought stress (Zhou et al., 2010; Wan et al., 2011; Chung et al., 2013). An increasing number of ubiquitin ligases have been shown to be involved in plant responses to various abiotic stresses. A number of excellent review articles provide a detailed listing of many of these E3s (Yee and Goring, 2009; Lee and Kim, 2011; Lyzenga and Stone, 2012; Chen and Hellmann, 2013). This review focuses on examples of E3 ligases for which stress-related substrate proteins have been identified.

Plant response to adverse environmental conditions is a complex and coordinated process involving activation of signaling networks and changes in the expression of hundreds of genes. By modulating the abundance of transcription factors, the UPS may affect the changes in gene expression required to mitigate the potential negative effects of environmental stress. E3 ligases may prohibit transcription activity by targeting the transcription factor for degradation under non-stress conditions. A well-described example is the regulation of dehydration-responsive element binding protein (DREB) 2A by the RING-type E3 ligases DREB2A-interacting protein (DRIP) 1 and DRIP2 (Qin et al., 2008; Morimoto et al., 2013). DREB2A is a transcription factor that regulates the expression of many drought and salt stress-inducible genes (Sakuma et al., 2006a,b). In accordance with UPS regulation, DREB2A only accumulates in transgenic plants treated with proteasome inhibitors (Sakuma et al., 2006a,b; Qin et al., 2008). DRIP1 and DRIP2 are capable of attaching ubiquitin molecules to DREB2A in *in vitro* ubiquitination assays (Qin et al., 2008). Furthermore, DREB2A is stable in *drip1drip2* plants and drought tolerance of the double mutant is further enhanced by overexpression of the transcription factor (Qin et al., 2008). This demonstrates that DREB2A is unstable under non-stress conditions and DRIP1/2 targets the transcription factor for degradation. Exposure to abiotic stresses such as heat and drought stabilize DREB2A and levels of the transcription factor remain elevated during the stress period (Sakuma et al., 2006a; Morimoto et al., 2013). The mechanism underlying the stress-induced stabilization of DREB2A is not known. DRIP1 and DRIP2 localize to and interact with DREB2A within the nucleus (Qin et al., 2008). DREB2A lacking two nuclear localization signals (NLSs) is observed in the cytosol and is more stable compared to the wild type transcription factor (Morimoto et al., 2013). Therefore, under non-stress conditions, DREB2A degradation seems to occur mainly within the nucleus (Qin et al., 2008; Morimoto et al., 2013). A possible mechanism for DREB2A stabilization is stress-induced relocalization of the DRIP1 and DRIP2 to the cytosol. Alternatively, stress-induced ubiquitin-dependent degradation of DRIP1 and DRIP2 may occur within the nucleus. Another example is *Botrytis* Susceptible1 (BOS1), a nuclear-localized R2R3MYB transcription factor that is required for tolerance of drought, salt and oxidative stresses (Mengiste et al., 2003). To demonstrate proteasome-dependent turnover of BOS1, the stability of the transcription factor was assessed in planta using a β-glucuronidase (GUS) reporter system. GUS activity was only detected following treatment with proteasome inhibitors, which indicate inhibition of BOS1 degradation (Luo et al., 2010). Botrytis Susceptible1 Interactor (BOI) is a nuclear-localized RING-type E3 that interacts with BOS1 in plant cells (Luo et al., 2010). BOI is capable of attaching ubiquitin molecules to BOS1 in *in vitro* assays (Luo et al., 2010). Consistent with a role in regulating BOS1 abundance, reduction in *BOI1* expression resulted in reduced tolerance of salt stress (Luo et al., 2010). These results suggest that BOI1 mediate the ubiquitin-dependent turnover of BOS1 under non-stress conditions. Stress-induced stabilization of BOS1 has not been reported.

The UPS involvement in regulating responses to abiotic stresses extends beyond the proteolysis of transcription factors. The RING-type E3 ligases *Arabidopsis* Toxicos EN Levadura (ATL) 6 and ATL31 control the abundance of a 14-3-3 protein required for seedling response to carbon/nitrogen (C/N) stress (Sato et al., 2009, 2011; Maekawa et al., 2012). The ratio between carbon and nitrogen is tightly regulated and changes in availability disrupt early seedling establishment causing post-germinative growth

arrest (Coruzzi and Bush, 2001). Overexpression of 14-3-3χ results in hypersensitivity to C/N stress (Sato et al., 2011). Accordingly, loss of *ATL6* and *ATL31* results in hypersensitivity to C/N stress and overexpression of the 14-3-3χ exaggerates the phenotypes of *atl6atl31* (Sato et al., 2011; Maekawa et al., 2012). Further evidence for ATL6/ATL31-mediated turnover of 14-3-3χ includes ubiquitination of 14-3-3χ by ATL6 and ATL31 during *in vitro* assays and accumulation of 14-3-3χ in *atl6atl31* seedlings (Sato et al., 2011).14-3-3χ protein levels increase in wild type seedlings exposed to C/N stress. Importantly, the C/N stress-induced increase in 14-3-3χ levels does not occur in *atl6atl31* seedlings. This suggests that ATL6/31 mediates the turnover of 14-3-3χ under non-stress conditions and degradation is prohibited during exposure to C/N stress. Another example is *Oryza sativa* drought-induced SINA protein 1 (OsDIS1), a RING-type E3 with high sequence similarity to *Arabidopsis* SINAT5 (Ning et al., 2011). Loss of *OsDIS1* function increased drought tolerance in rice plants. Conversely, transgenic rice plants overexpressing *OsDIS1* displayed reduced drought tolerance. A search for OsDIS1 interacting proteins identified OsNek6, a microtubule-associated serine/threonine protein kinase that belongs to the Never in Mitosis gene A-related kinase family (Vigneault et al., 2007). *Arabidopsis* Nek6 (AtNek6) was previously shown to be involved in microtubule-dependent morphogenesis of epidermal cells (Sakai et al., 2008). However, a positive role for AtNek6 in salt stress response has been reported (Lee et al., 2010; Ning et al., 2011). OsNek6 is degraded by the 26S proteasome and OsDIR1 does contribute to OsNex6 turnover in the absence of stress (Ning et al., 2011). A role for OsNex6 in plant response to drought stress was not reported, however OsDIS1-mediated turnover may function to suppress OsNex6 activity until stress conditions arise.

Ubiquitin-dependent degradation also functions to attenuate stress signaling. An example of this is the RING-type E3 ligase high expression of osmotically responsive gene 1 (HOS1), which mediates the degradation of Inducer of CBF Expression 1 (ICE1), a MYC transcription factor that regulates the expression of cold-responsive genes. HOS1 is capable of catalyzing ICE1 ubiquitination *in vitro* and *in vivo* (Dong et al., 2006). Consistent with a role in mediating ICE1 degradation, overexpression of HOS1 results in reduced expression of cold-responsive genes and increased sensitivity to freezing conditions (Dong et al., 2006). Exposure to cold stress up-regulates *ICE1* expression, however, low temperatures also promote proteasome-dependent degradation of the transcription factor (Chinnusamy et al., 2003; Dong et al., 2006). Turnover of nuclear-localized ICE1 is facilitated by cold-induced relocalization of HOS1 from the cytoplasm to the nucleus (Lee et al., 2001; Dong et al., 2006). The cold-induced HOS1-mediated degradation of ICE1 is suggested to facilitate the transient expression of cold-responsive genes (Chinnusamy et al., 2003; Dong et al., 2006). Another substrate for HOS1 is Constans (CO), a transcription factor that promotes flowering (Putterill et al., 1995; Jung et al., 2012; Lazaro et al., 2012). HOS1 interacts directly with and ubiquitinates CO (Jung et al., 2012; Lazaro et al., 2012). HOS1 regulation of CO abundance provides an explanation for the early flowering phenotype of *hos1* plants (Lee et al., 2001; Lazaro et al., 2012). Similar to the regulation of ICE1, exposure to low temperature promotes HOS1-dependent proteasomal degradation of CO (Jung et al., 2012). HOS1 regulation of CO abundance provides a link between cold stress response and control of flowering.

Another example of the UPS engaging a substrate in response to stress is provided by the RING-type E3 ligases RING domain Ligase 1 (RGLG1) and RGLG2, which regulate the abundance of ethylene response factor 53 (ERF53; Cheng et al., 2012). ERF53 is a drought and salt-responsive AP2/ERF transcription factor (Nakano et al., 2006; Cheng et al., 2012). Loss of both *RGLG1* and *RGLG2* gene function increase drought tolerance, which is consistent with a role for the E3 ligases in regulating ERF53 abundance (Cheng et al., 2012). RGLG1 and RGLG2 interact with and ubiquitinate ERF53 in *in vitro* assays (Cheng et al., 2012). In addition, overexpression of *ERF53* in *rglg1rglg2* plants further enhances drought tolerance of the double mutant and the transcription factor is stable in *rglg1rglg2* plants (Cheng et al., 2012). The RGLG proteins are suggested to be myristoylated and localized predominantly to the plasma membrane, while ERF53 is nuclear localized (Yin et al., 2007; Cheng et al., 2012). Although loss of the predicted myristoylation site disrupts RGLG2 membrane localization, the mutant E3 does not localize to the nucleus (Yin et al., 2007). Whether or not myristoylation regulates E3 ligase activity remains to be seen. However, salt stress does induce the translocation of RGLG2 to the nucleus where it interacts with ERF53 (Cheng et al., 2012). This suggests that RGLG2-mediated degradation of ERF53 occur in response to abiotic stress.

NON-PROTEOLYTIC FUNCTIONS OF UBIQUITIN DURING ABIOTIC STRESS SIGNALING

While the requirement for ubiquitin-dependent protein degradation during response to abiotic stresses is firmly established, the involvement of other types of ubiquitin modification is not well understood. Of interest are the non-proteolytic functions of modifications such as monoubiquitination and lysine-63 linked polyubiquitination. The rice RING-type E3 ligase *Oryza sativa* heat and cold induced 1 (OsHCI1) is involved in tolerance of heat stress (Lim et al., 2013). OsHCI1 is capable of attaching a single ubiquitin molecule to a number of interacting proteins including OsbHLH065, a basic/helix-loop-helix (bHLH) transcription factor. Golgi-localized OsHCI1 translocates to the nucleus of cells exposed to heat shock and nuclear-localized OsbHLH065 in observed in the cytosol when co-expressed with OsHCI1. A role for OsbHLH065 in abiotic stress responses has not been reported. However, it is postulated that OsHCI1-mediated relocalization of nuclear proteins such as OsbHLH065 promotes heat stress tolerance. Monoubiquitination of the boron transporter BOR1 occurs in the presence of high concentrations of boron (Kasai et al., 2011). Boron is an essential nutrient for plant growth and development. Boron deficiency negatively affects yield, and high concentrations are toxic to plants. Plants utilize BOR1 for boron uptake under boron-limiting conditions and overexpression enhances tolerance of boron stress (Takano et al., 2002; Miwa et al., 2006). Boron-induced monoubiquitination of BOR1 is essential for vacuolar sorting and degradation of the transporter (Kasai et al., 2011). RGLG2 interacts with

the E2 enzyme AtUBC35 (also referred to as AtUBC13) and both enzymes can facilitate the formation of lysine-63 linked polyubiquitin chains (Kraft et al., 2005; Yin et al., 2007; Wen et al., 2008). Lysine-63 linked chains have non-proteolytic functions such as endocytosis and protein activation (Chen and Sun, 2009). However, lysine-63 polyubiquitination can also serve as a signal for proteasomal degradation (Saeki et al., 2009). As discussed above, RGLG2's role in abiotic stress response involves targeting the transcription factor ERF53 for proteasomal degradation (Cheng et al., 2012). Of interest is (1) the requirement for RGLG2 generated lysine-63 polyubiquitin chains during stress response and (2) whether RGLG2 modifies ERF53 with the attachment of a lysine-63 or lysine-48 linked polyubiquitin chain. Although the examples are few, the pervasiveness of the ubiquitin modification system suggests that the different types of ubiquitination may regulate aspects of plant responses to abiotic stresses.

UBIQUITIN-DEPENDENT REGULATION OF STRESS HORMONE SIGNALING

Plants utilize hormones to integrate endogenous and exogenous signals. A direct link has been demonstrated between the UPS and the production, perception, signal transduction, and outputs of these hormones. A surprising number of ubiquitin ligases have been shown to control the actions of stress hormones. For example, at least fourteen E3s have been linked to the regulation of ABA synthesis and signaling (**Figure 2**; Lee and Kim, 2011; Liu and Stone, 2011). Abiotic stresses such as drought and salinity increase cellular ABA levels via the induction of ABA biosynthetic genes including *9-cis-epoxycarotenoid dioxygenase 3* (*NCED3)* and *Arabidopsis aldehyde oxidase 3* (*AAO3*; Finkelstein, 2013). The U-box type E3 senescence-associated E3 ubiquitin ligase 1(SAUL1)/plant U-box (AtPUB) 44 negatively regulates ABA biosynthesis by targeting AAO3 for proteasome-dependent degradation (Raab et al., 2009; Salt et al., 2011). Drought tolerance repressor (DOR), a F-box protein that may participate in a CUL1 based RING E3 ligase, is a negative regulator of ABA-mediated responses (Zhang et al., 2008). Drought stressed *dor* plants exhibit enhanced expression of *NCED3* and increased cellular ABA levels. The stress-induced expression of *NCED3* is also enhanced by overexpression of the RING-type E3 XERICO, which is accompanied by increased ABA levels and improved tolerance of drought stress (Ko et al., 2006).

Perception of ABA is mediated by a suite of receptors named pyrabactin resistance 1(PYR1)/PYR1-like (PYL)/Regulatory component of ABA receptor (RCAR; Park et al., 2009; Santiago et al., 2009; **Figure 2**). ABA-bound PYR/PYL/RCAR receptors interact with and inhibit protein phosphatase type 2Cs (PP2Cs), which prohibits the dephosphorylation of sucrose non-fermenting1-related protein kinases (SnRKs; **Figure 2**; Fujii et al., 2009; Ma et al., 2009; Park et al., 2009). The ABA-activated SnRKs are then able to phosphorylate and activate transcription factors and other regulatory proteins involved in facilitating ABA-mediated process required for abiotic stress tolerance (Fujii et al., 2009; Rodrigues et al., 2013). Members of all three SnRK subfamilies, SnRK1, SnRK2, and SnRK3, have been implicated in mediating ABA response/signaling (Fujii et al., 2009; Lyzenga et al., 2013; Rodrigues et al., 2013). PYR/PYL/RCAR, PP2C, and SnRK proteins are considered the core components of the ABA signaling network (Weiner et al., 2010). As shown in **Figure 2**, the UPS regulate the abundance of many of these core components. A search for ubiquitinated proteins in *Arabidopsis* isolated ABA receptor PRY1 and SnRK kinases, SnRK1.1, SnRK2.4, and SnRK2.6/Open Stomata 1 (OST1; Kim et al., 2013). Ubiquitination of the identified targets increased after treatment with proteasome inhibitors, which suggests degradation by the 26S proteasome. De-etiolated 1(DET1)- and DDB1-associated protein 1 (DDA1), which functions as the substrate receptor for a CUL4 based E3 ligase, have been shown to regulate the abundance of ABA receptors PYL4, PYL8, and PYL9 (Irigoyen et al., 2014). ABA prohibits the DDA1-mediated degradation of PYL8 via reducing the ubiquitination of the receptor (Irigoyen et al., 2014). Calcineurin B-like Interacting protein kinase 26 (CIPK26), which belongs to the SnRK3 subfamily, is a positive regulator of ABA signaling (Lyzenga et al., 2013). CIPK26 interacts with two PP2Cs, abscisic acid insensitive (ABI) 1, and ABI2, phosphorylate the ABA-responsive transcription factor ABI5 *in vitro* and seedlings overexpressing CIPK26 are hypersensitive to ABA (Lyzenga et al., 2013). The RING-type E3 ligases, Keep on Going (KEG) interacts with CIPK26 targeting the kinase for degradation by the 26S proteasome.

ABA-mediated responses, such as growth arrest of early seedlings exposed to stress conditions, involve the up or down-regulation of a large number of genes (Seki et al., 2002; Finkelstein, 2013). Changes in ABA-responsive gene expression are mediated by a number of transcription factors including members of the basic leucine zipper (bZIP), AP2/ERF, R2R3, and B3 families (Finkelstein, 2013). The UPS regulates ABA-responsive transcription by modulating the abundance of many of these transcription factors (**Figure 2**). The abundance of the nucleocytoplasmic bZIP transcription factor ABI5 is modulated by KEG (**Figure 2**). ABI5 promote the growth arrest of young seedlings exposed to stress conditions (Lopez-Molina et al., 2001). In the absence of stress, KEG is required to maintain low levels of ABI5 to ensure seedling establishment (Stone et al., 2006; Liu and Stone, 2010). KEG, a *trans*-Golgi network/cytosol-localized E3, ubiquitinates and targets ABI5 for degradation within the cytosol, which would prohibit accumulation of the transcription factor in the nucleus and activation of ABA responses (Gu and Innes, 2011; Liu and Stone, 2013). Elevated levels of ABA promote ABI5 accumulation via increased gene expression and decreased protein turnover. ABA-dependent stabilization of ABI5 protein involves KEG self-ubiquitination and proteasomal degradation (Liu and Stone, 2010). KEG also targets bZIP transcription factors ABRE-binding factors (ABF) 1 and ABF3 for degradation via the 26S proteasome (Chen et al., 2013). Similar to ABI5, ABA prohibits the proteasomal-dependent turnover of ABF1 and ABF3. Compared to other ABA mutants, the phenotype of *keg* seedlings is quite severe and growth arrest occurs in the absence of the hormone. The fact that KEG mediates the degradation of multiple components (CIPK26, ABI5, and ABF1/3) of the ABA signaling pathway helps to explain the lethality of the *KEG* mutation. The abundance of ABI4, an AP2/ERF transcription factor, is also regulated by the 26S proteasome, however the E3 involved is not yet

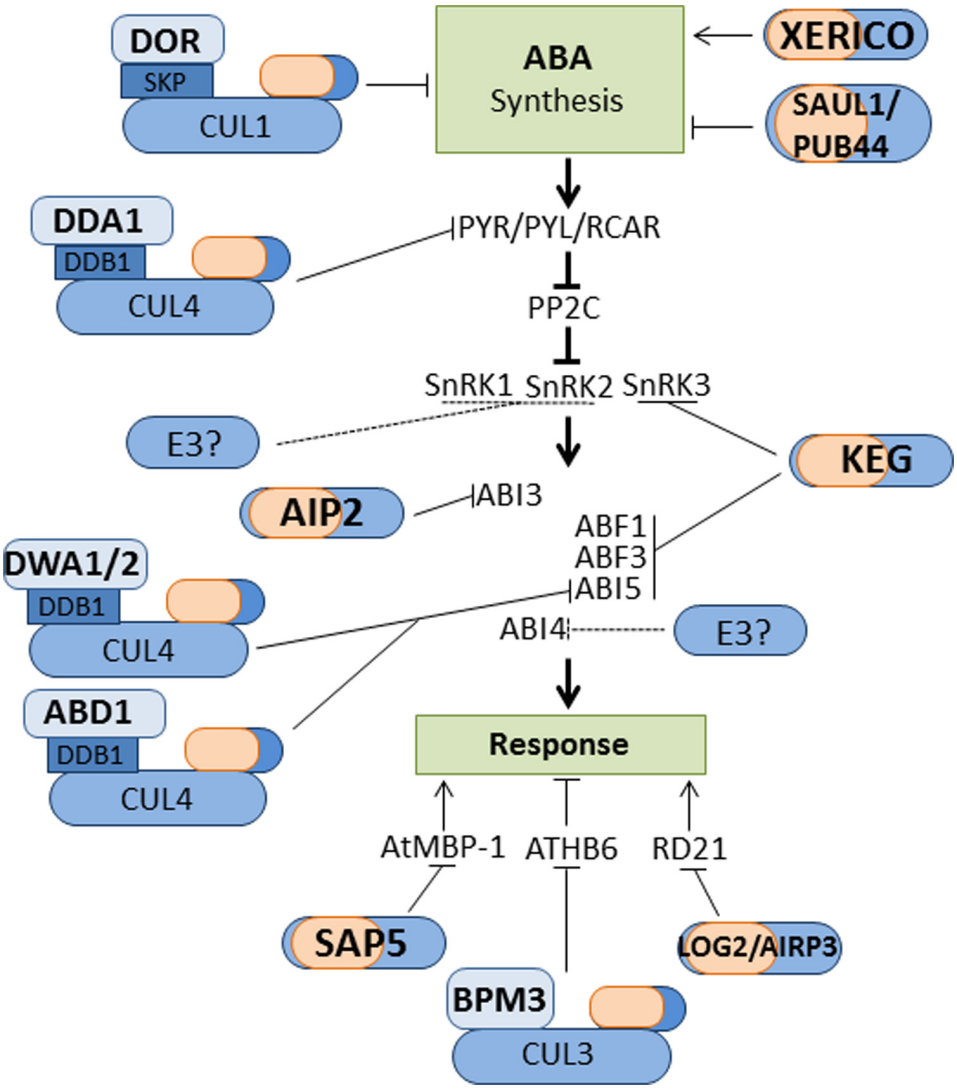

FIGURE 2 | Ubiquitin ligases that regulate ABA signaling. Illustration of E3 ligases that regulate ABA synthesis, signal transduction and response. Not all E3 ligases are shown, mainly those with identified substrates. Question marks and dashed lines denote instances where proteasomal-dependent degradation is reported but the E3 ligase involved is unknown.

identified (Finkelstein et al., 2011). The R2R3-type transcription factor MYB30 negatively regulates ABA signaling (Zheng et al., 2012). MYB30 is targeted for proteasomal degradation by the RING-type E3 MYB30-Interacting E3 Ligase 1 (MIEL1; Marino et al., 2013). MYB30 is multifunctional with additional roles in cell death and pathogen resistance (Marino et al., 2013). MEIL1-mediated degradation of MYB30 suppresses defense signaling in non-infected plants (Marino et al., 2013). Whether or not MIEL1-mediated degradation of MYB30 modulates ABA signaling is yet to be determined.

A monomeric RING-type E3 and two CRLs have been implicated in attenuating ABA signaling. ABI3, a B3 transcription factor, is targeted for proteasomal degradation by the RING-type E3 ABI3-interacting protein 2 (AIP2; Zhang et al., 2005). *aip2-1* accumulate high levels ABI3 compared to wild type and are hypersensitive to ABA. *AIP2* transcript abundance increases in response to ABA application and this correlates with a decrease in ABI3 levels. Thus, ABA promotes the turnover of ABI3, which would assist in suppressing hormone signaling. Nuclear-localized DWD hypersensitive to ABA 1 (DWA1), DWA2, and ABA-hypersensitive DCAF1 (ABD1) negatively regulates ABA signaling by promoting the turnover of ABI5 (Lee et al., 2010; Seo et al., 2014). DWA1, DWA2 and ABD1 proteins function as the substrate-recruiting component of CUL4 based RING E3 ligases (Lee et al., 2010; Seo et al., 2014). ABA treated *dwa1/dwa2* seedlings accumulate higher levels of ABI5 compared to wild type and the double mutants display hypersensitivity to ABA. ABI5 does not accumulate in *dwa1dwa2* in the absence of ABA, which is consistent with the CRL targeting the transcription factor for degradation in the presence of the hormone. Similarly, loss of

ABD1 results in hypersensitivity to ABA, and accumulation of ABI5 following exposure to the hormone (Seo et al., 2014). The BTB protein BMP3, which functions as the substrate-recruiting component of CUL3 based E3 ligase, regulates the abundance of AtHB6, a homeobox-leucine zipper transcription factor. ATHB6 is a negative regulator of ABA response (Himmelbach et al., 2002; Lechner et al., 2011). BMP3 promotes the proteasome-dependent degradation of ATHB6 under non-stress conditions (Lechner et al., 2011). ABA prohibits the turnover of ATHB6. The ABA-induced stabilization of ATHB6 may serve to attenuate ABA responses.

In addition to the above mentioned ubiquitin ligase, many other E3 ligases have been found to be involved in ABA responses. Substrate proteins have been identified for only few of these E3 ligases. Stress Associated Protein 5 (AtSAP5) is an A20/AN1-type zinc finger protein with E3 ligase activity (Kang et al., 2011). AtSAP5 mediate the proteasome-dependent degradation of *Arabidopsis* MBP-1-like protein (AtMBP-1), a positive regulator of ABA responses (Kang et al., 2013). The RING-type E3 ABA-Insensitive RING Protein 3(AtAIRP3)/Loss of GDU2 (LOG2) is a positive regulator of ABA-mediated stress responses (Kim and Kim, 2013). AtAIRP3/LOG2 interacts and ubiquitinates Glutamine Dumper1 (GDU1) and responsive to dehydration 21 (RD21). AtAIRP3/LOG2 ubiquitination of GDU1 is non-proteolytic and regulates the export of amino acids from plant cells (Pratelli et al., 2012). Whereas, AtAIRP3/LOG2 targets RD21 for degradation via the 26S proteasome (Kim and Kim, 2013). RD21 is drought-inducible Cys proteinase (Kim and Kim, 2013). However, it is not known if AtAIRP3/LOG2-mediated degradation of RD21 modulates drought tolerance or ABA responses. ABA-related E3 ligases with no known substrates include U-box-type E3s AtPUB9, AtPUB18, and AtPUB19. Down-regulation of *AtPUB9, AtPUB18*, and *AtPUB19* results in hypersensitivity to ABA, which suggests that the U-box-type E3s are negative regulators of ABA signaling (Samuel et al., 2008; Liu et al., 2011). Interestingly, in the presence of ABA, AtPUB9 is translocated from the nucleus to the plasma membrane. The significance of the ABA-induced relocalization is not known, however, the change in subcellular location may serve to inhibit E3 activity and promote ABA responses. The RING type E3s Salt and Drought Induced RING Finger 1(SDIR1), *Arabidopsis* ABA-insensitive RING protein 1(AtAIRP1), RING-H2 E3 ligase (RHA) 2a, and RHA2b are all positive regulators of ABA-mediated stress responses (Zhang et al., 2007; Bu et al., 2009; Ryu et al., 2010; Li et al., 2011). The identification of substrates for these orphan E3 ligases will shed further light on how the UPS facilities plant responses to and tolerance of adverse environmental conditions.

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A conserved role for the ARC1 E3 ligase in Brassicaceae self-incompatibility

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Ubiquitination plays essential roles in the regulation of many processes in plants including pollen rejection in self-incompatible species. In the Brassicaceae (mustard family), self-incompatibility drives the rejection of self-pollen by preventing pollen hydration following pollen contact with the stigmatic surface. Self-pollen is recognized by a ligand-receptor pair: the pollen S-locus cysteine rich/S-locus protein 11 (SCR/SP11) ligand and the pistil *S* receptor kinase (SRK). Following self-pollen contact, the SCR/SP11 ligand on the pollen surface binds to SRK on the pistil surface, and the SRK-activated signaling pathway is initiated. This pathway includes the armadillo repeat containing 1 (ARC1) protein, a member of the plant U-box (PUB) family of E3 ubiquitin ligases. ARC1 is a functional E3 ligase and is required downstream of SRK for the self-incompatibility response. This mini review highlights our recent progress in establishing ARC1's conserved role in self-pollen rejection in *Brassica* and *Arabidopsis* species and discusses future research directions in this field.

Keywords: ubiquitination, cell signaling, self-incompatibility, *Arabidopsis*, Brassicaceae

INTRODUCTION

Plants have evolved complex signaling networks to survive their sessile existence, and protein ubiquitination underpins many of these systems. One process regulated by ubiquitination is self-incompatibility, which prevents the acceptance of self-pollen by the pistil resulting in increased genetic diversity in a population. E3 ubiquitin ligases have been implicated in two different self-incompatibility systems, the S-Ribonuclease-based self-incompatibility (Solanaceae, Rosaceae, and Plantaginaceae) and the *S* receptor kinase (SRK)-based self-incompatibility (Brassicaceae; reviewed in Hiscock and Allen, 2008; Iwano and Takayama, 2012). This mini review will focus on the role of ubiquitination in the Brassicaceae system that has been well characterized in the *Brassica* and *Arabidopsis* species. Species in the Brassicaceae have dry stigmas, and the pollen grain must receive water for hydration from the stigmatic papilla in order to germinate and grow a pollen tube (Heslop-Harrison and Shivanna, 1977). Therefore, when a pollen grain lands on the stigmatic papilla at the top of the pistil in the flower, the stigmatic papilla can determine if the pollen grain should be accepted or rejected. If a pollen grain is determined to be self-incompatible, the stigmatic papilla will reject it by blocking pollen grain hydration and pollen tube growth. Thus, pollen contact at the stigmatic surface is a major regulatory point for pollination (reviewed in Chapman and Goring, 2010).

THE RECEPTOR-LIGAND PAIR REGULATING SELF-INCOMPATIBILITY IN THE BRASSICACEAE

Initial research in this field was conducted on *Brassica* species (*B. oleracea*, *B. rapa*, *B. napus*) with the identification of two polymorphic loci regulating self-incompatibility. The *Brassica* pollen locus encodes the Cysteine Rich/S-locus Protein 11 (SCR/SP11) protein while the *Brassica* pistil locus encodes the SRK (Schopfer et al., 1999; Cui et al., 2000; Takasaki et al., 2000; Takayama et al., 2000; Silva et al., 2001). Each specific *SCR/SP11-SRK* allele pair comprises a S-haplotype, whereby recognition causes the rejection of self-pollen to prevent inbreeding, and a number of different *Brassica* S-haplotypes has been identified (reviewed in Iwano and Takayama, 2012). Sequences for different S-haplotypes (*SCR/SP11* and *SRK* alleles) have subsequently been identified in other Brassicaceae species including *Arabidopsis lyrata*, *Arabidopsis halleri*, *Arabis alpina*, *Capsella grandiflora*, and a related S-locus region in *Leavenworthia alabamica* (Kusaba et al., 2001; Schierup et al., 2001; Paetsch et al., 2006; Castric et al., 2008; Boggs et al., 2009; Foxe et al., 2009; Guo et al., 2009; Tedder et al., 2011; Chantha et al., 2013). In *Brassica*, when a self-pollen grain contacts a stigmatic papilla, the SCR/SP11 ligand from the pollen coat binds to SRK, and SRK becomes autophosphorylated (Giranton et al., 2000; Kachroo et al., 2001; Takayama et al., 2001; Shimosato et al., 2007). As expected, SRK was found to bind strongest to the corresponding SCR/SP11 ligand, but could also bind weakly to other S-haplotype-encoded SCR/SP11 ligands (Kemp and Doughty, 2007; Naithani et al., 2007; Shimosato et al., 2007). There is one known negative regulator of *Brassica* SRK, Thioredoxin H-like 1 (THL1; Bower et al., 1996; Cabrillac et al., 2001; Haffani et al., 2004). THL1's inhibition is proposed to prevent SRK from auto-activating and signaling before the recognition of SCR/SP11 at the plasma membrane (Giranton et al., 2000; Cabrillac et al., 2001; Ivanov and Gaude, 2009). After binding of SCR/SP11 to SRK, the self-incompatibility signaling cascade is initiated. This rejection is localized to the point of pollen contact, as a single papilla can simultaneously accept a compatible pollen grain and reject a self-incompatible pollen grain (Dickinson, 1995).

REGULATORY PROTEINS ACTING DOWNSTREAM OF SRK

In addition to the role of SCR/SP11 and SRK in mediating initial self-pollen recognition, there are two other proteins that have been identified as positive regulators of the self-incompatibility response in *Brassica*: the M-locus protein kinase (MLPK; Murase et al., 2004; Kakita et al., 2007b) and the E3 ubiquitin ligase, Armadillo (ARM)-repeat containing 1 (ARC1; Gu et al., 1998; Stone et al., 1999, 2003). As well, more recent research from our group has tied the role of Exo70A1, a key component for polarized exocytosis to be negatively regulated by ARC1 in the self-incompatibility response (Samuel et al., 2009; Safavian and Goring, 2013; Indriolo et al., 2014; Safavian et al., Submitted). *B. rapa* MLPK is a Receptor-Like Cytoplasmic Kinase (RLCK) that, through alternate splicing, is localized to the plasma membrane via an N-terminal myristoylation site or an N-terminal hydrophobic region, and both forms can complement *mlpk* mutant stigmatic papillae (Murase et al., 2004; Kakita et al., 2007a). MLPK is proposed to interact with SRK at the plasma membrane, and the SRK-MLPK complex is proposed to phosphorylate downstream signaling proteins (Kakita et al., 2007a,b; Samuel et al., 2008). *A. thaliana* RLCKs that are closely related to MLPK have been identified, but a corresponding role to MLPK in *Arabidopsis* self-incompatibility has not been elucidated yet (Kakita et al., 2007a). So far, the only other known downstream component, ARC1, is a member of the Plant U-box (PUB)/ARM repeat family of E3 ligases (Mudgil et al., 2004; Samuel et al., 2006; Yee and Goring, 2009). While ARC1's role in *B. napus* and *A. lyrata* self-incompatibility has not been disputed (Stone et al., 1999; Indriolo et al., 2012), some debate does exist as to whether ARC1 is required for reconstituting self-incompatibility in *A. thaliana* as discussed below (Indriolo et al., 2014). Part of this will likely turn out to be due to the nature of signaling systems using complex multi-branched pathways; as such, one would expect more signaling proteins to be implicated in the SRK pathway in the future.

Plant U-box-armadillo repeat E3 ligases are involved in a wide variety of plant processes including plant-microbe interactions, abiotic stress responses, hormone responses, and development (Mbengue et al., 2010; Lu et al., 2011; Salt et al., 2011; Liu et al., 2012; Seo et al., 2012; Stegmann et al., 2012; Vogelmann et al., 2012; Wang et al., 2013). Several UND-PUB-ARM E3 ligases have been found to interact with receptor kinases (Samuel et al., 2008; Mbengue et al., 2010; Lu et al., 2011). The conserved U-box domain of $\sim$ 70 residues was originally identified in yeast UFD2 protein and interacts with the E2 conjugating enzyme (Koegl et al., 1999; Hatakeyama and Nakayama, 2003; Andersen et al., 2004; Schulman and Chen, 2005; Wiborg et al., 2008). A subset of the PUB-ARM proteins, including ARC1, contain a conserved U-box N-terminal domain (UND; Mudgil et al., 2004; Samuel et al., 2006). The UND domain is proposed to give specificity for proteins ubiquitinated by ARC1 such as the proposed target, Exo70A1, in the instance of self-incompatibility.

ARC1 IS AN E3 LIGASE INVOLVED IN SELF-INCOMPATIBILITY SIGNALING

Armadillo repeat-containing 1 was originally identified in *B. napus*, through a yeast two-hybrid screen for SRK kinase domain interactors, and ARC1 was found to bind to SRK through its ARM repeat domain (Gu et al., 1998). ARC1 can be phosphorylated by SRK, but is more strongly phosphorylated by MLPK *in vitro* (Gu et al., 1998; Samuel et al., 2008). ARC1 is composed of the three distinct protein domains described above (UND, U-box, ARM repeat domain), and has functional nuclear localization and nuclear export signals. When transiently expressed in tobacco BY2 cells, ARC1 was localized to both the cytoplasm and nucleus, shuttling back and forth between these two compartments (Stone et al., 2003). The function of ARC1's nuclear localization is still unclear, especially as it is expected to be near the plasma membrane for its role in self-incompatibility (**Figure 1**, described in more detail below). When ARC1 was co-expressed with active SRK or MLPK kinase domains, it no longer shuttled to the nucleus suggesting that ARC1 phosphorylation alters its localization and may be important for its function in the self-incompatibility pathway (Stone et al., 2003; Samuel et al., 2008). *B. napus ARC1* displays stigma-specific expression, and the knock-down of *ARC1* expression by antisense suppression resulted in a gained self-pollen acceptance, instead of rejection, indicating a breakdown in the self-incompatible pathway (Gu et al., 1998; Stone et al., 1999). ARC1 was shown to have *in vitro* E3 ligase activity, and the importance of ubiquitination in self-incompatibility came from analyses of ubiquitinated proteins in the stigma. Wild-type *B. napus* stigmas that were pollinated with self-incompatible pollen were shown, by immunoblotting with an anti-ubiquitin antibody, to be enriched in ubiquitinated proteins (Stone et al., 2003; Samuel et al., 2011). In contrast, self-pollinated *ARC1*-antisense-suppressed stigmas had a lower level of ubiquitinated proteins. Therefore, these data suggest that the presence of ARC1 led to ubiquitinated stigma proteins following self-incompatible pollinations. Given that all this work was done in *B. napus*, an outstanding question in the field was whether ARC1's function was conserved in other Brassicaceae species.

ARC1 PLAYS A CONSERVED ROLE IN SELF-INCOMPATIBILITY SIGNALING ACROSS THE BRASSICACEAE

To better understand the Brassicaceae self-incompatibility pathway, one direction of research has examined this trait in *Arabidopsis* species. *A. thaliana* lost the self-incompatibility trait due to mutations in the *SCR*/*SRK* genes while another *Arabidopsis* species, *A. lyrata*, has remained naturally self-incompatible (Kusaba et al., 2001; Schierup et al., 2001). Some *A. thaliana* ecotypes, such as Wei-1 and Old-1, were actually found to carry intact *SRK* genes and exhibited self-incompatibility when pollinated with *A. halleri* pollen expressing the matching *SCR* ligand (Tsuchimatsu et al., 2010). Self-compatible *A. thaliana* has been used as an artificial system for reconstructing self-incompatibility by transforming *A. lyrata SCR* and *SRK* genes into different *A. thaliana* ecotypes (Nasrallah et al., 2004; Boggs et al., 2009; Rea et al., 2010; Tsuchimatsu et al., 2010). Interestingly, transgenic *A. thaliana* expressing *SCR* and *SRK* did not always result in the generation of self-incompatibility. Some transgenic ecotypes such as Sha, Kas-2, and C24 were reported to produce self-incompatible flowers, while other transgenic ecotypes such as Col-0, Mt-0, Nd-0, and No remained self-compatible (Nasrallah et al., 2004; Boggs et al., 2009). However, there is ambiguity

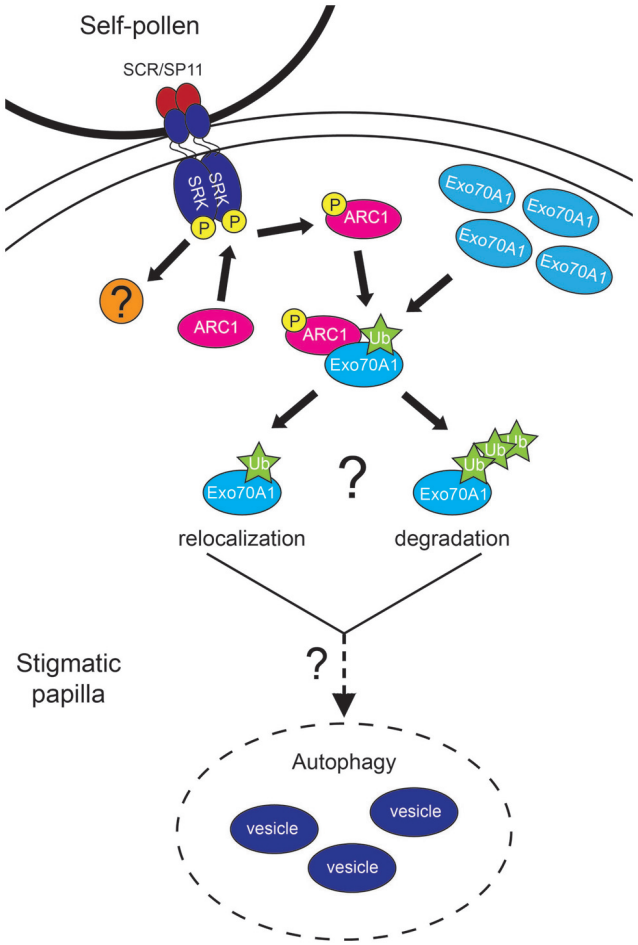

FIGURE 1 | Model for the Self-Incompatibility response in *Arabidopsis*. Upon pollen contact with the stigmatic papilla, the papilla must determine if the pollen grain is self or non-self. For a self-pollen grain, the SCR/SP11 ligand binds to SRK, and SRK will then become phosphorylated. ARC1 interacts with the phosphorylated cytoplasmic kinase domain of SRK and become phosphorylated and activated. ARC1 then seeks its target for ubiquitination, Exo70A1, which is located at the plasma membrane in preparation for exocyst-mediated exocytosis. ARC1, with the help of an E1 activating enzyme and E2 conjugating enzyme, ubiquitinates Exo70A1. The nature of this ubiquitination is not known, as it could be either mono-ubiqitination resulting in Exo70A1 relocalization, or poly ubiquitination leading to degradation of Exo70A1 by the 26S proteosome. Either way, the inhibition of Exo70A1 appears to activate an autophagic response that leads to degradation of the secretory vesicles. There is also another (unknown) factor functioning downstream of SRK in this pathway. How ARC1, this factor, and the ubiquitination of Exo70A1 signals for autophagy is not known. Once the secretory vesicles have been removed, the self-pollen grain will not receive the resources needed for hydration and germination; as a result will be rejected.

in these delineations; for example, transgenic *A. thaliana* Col-0 were sometimes reported as becoming self-incompatible with *SCR* and *SRK* expression (Nasrallah et al., 2002; Kitashiba et al., 2011) and other times reported as remaining self-compatible (Nasrallah et al., 2004; Boggs et al., 2009). The ambiguity appears to stem from the types of assays used to assess self-incompatiblity, and how self-incompatibility is defined; from a very narrow temporal window (Nasrallah et al., 2002) or flower age (Tsuchimatsu et al., 2010) to perhaps a fully self-incompatible flower. The defining purpose of self-incompatibililty is to prevent inbreeding; thus, it would have been clearer if seed set was measured consistently in these studies. Nevertheless, these data suggest that there are some inherent differences between some of the *A. thaliana* ecotypes with regards to the strength of the reconstituted self-incompatibility.

Arabidopsis species diverged 20–40 Mya from *Brassica* species (Franzke et al., 2011), and it has been proposed that *A. thaliana* uses a different signaling pathway downstream of SRK to *Brassica* species (Rea et al., 2010; Kitashiba et al., 2011). However, to date, no candidates have been identified for this proposed pathway. Thus, we were interested in assessing the contributions of ARC1 to self-incompatibility in *Arabidopsis* species. *ARC1* was identified in the *A. lyrata* genome sequence and determined to be deleted in the *A. thaliana* Col-0 and C24 ecotypes (Kitashiba et al., 2011; Indriolo et al., 2012). *ARC1* is expressed in *A. lyrata* stigmas, but also expressed at lower levels in other tissues (Indriolo et al., 2012). Given that some ecotypes such as Wei-1 and Old-1 contained a functional *SRK* gene (Tsuchimatsu et al., 2010), we investigated whether an intact *ARC1* gene was present in any *A. thaliana* ecotypes. Despite screening 357 different *A. thaliana* ecotypes, all ecotypes carried the same deletion resulting in a non-functional *ARC1* gene. Thus, the *ARC1* deletion likely occurred before the different *SRK* and *SCR/SP11* inactivating mutations (since some ecotypes still carried functional *SCR* or *SRK* genes; Indriolo et al., 2012). Further analyses on sequenced genomes from other self-compatible Brassicaceae species revealed that in several self-compatible species, *ARC1* was non-functional due to large deletions or smaller mutations (Indriolo et al., 2012). As well, self-incompatible species carried intact copies of the *ARC1* gene. ARC1's function was then specifically examined in self-incompatible *A. lyrata* to determine if it was necessary for the rejection of self-pollen. *ARC1* expression was knocked down by transforming an *ARC1* RNAi construct into *A. lyrata* resulting in transgenic plants that were no longer able to fully reject self-pollen when self-pollinated. In addition to the observed pollen grain adhesion and pollen tube growth, self-pollinations for transgenic *ARC1* RNAi *A. lyrata* resulted in seed set. In contrast, wild-type self-incompatible *A. lyrata* pollinations resulted in a rejection of all self-pollen and a complete absence of seeds. These results conclusively showed that *ARC1* is required for the complete rejection of self-incompatible pollen in *A. lyrata*, and supported a conserved role for ARC1 downstream of the SRK in both *Brassica* and *Arabidopsis* species (Indriolo et al., 2012). It is important to note though that these results do not preclude the presence of other downstream signaling proteins as only a partial breakdown of self-incompatibility was achieved in both studies (Stone et al., 1999; Indriolo et al., 2012).

Most recently, we have completed the reciprocal part of this research and tested ARC1's role in *A. thaliana* by transforming *A. lyrata ARC1* and *B. napus ARC1* along with the *A. lyrata SCRb* and *SRKb* transgenes (Indriolo et al., 2014). Two ecotypes reported to have differing results when transformed with *A. lyrata SCRb* and

SRKb were selected: Col-0, which remained self-compatible, and Sha, which became self-incompatible (Nasrallah et al., 2004; Boggs et al., 2009). The main question examined was whether transforming *ARC1*, along with *SCRb-SRKb*, into these two ecotypes resulted in a stronger self-incompatibility trait. We observed that transgenic *SCRb-SRKb* Col-0 remained self-compatible as previously reported, but the addition of *ARC1* with *SCRb-SRKb* in Col-0 resulted in clear self-incompatibility. The Sha ecotype was previously reported to display a self-incompatible phenotype with the expression of *SCRb-SRKb* alone, and we did identify some lines that displayed a moderate self-incompatibility trait. However, we found that the addition of *ARC1* resulted in a more robust and stable self-incompatible phenotype with the near complete rejection of self-pollen and little or no seed set (Indriolo et al., 2014). The expression of *ARC1* did not affect the levels of *SCRb* and *SRKb* transcripts, demonstrating that ARC1 functioned at the protein level as a direct component of the self-incompatibility signaling pathway (Indriolo et al., 2014). Interestingly, we observed an approach herkogamy trait in the transgenic *A. thaliana SCRb-SRKb-ARC1* plants, where the stigma was positioned above the anthers to avoid self-pollination. Because of this, manual pollinations were conducted for all the analyses to bypass this additional pollen avoidance trait. Overall, the results of these transgenic experiments clearly showed that the addition of either *A. lyrata ARC1* or *B. napus ARC1* with *SCRb-SRKb* led to stronger self-incompatibility phenotype in both the Col-0 and Sha ecotypes (Indriolo et al., 2014). Furthermore, both *A. lyrata* ARC1 and *B. napus* ARC1 exhibited a matching phenotype; thus despite the 20–40 Mya of divergence between *A. lyrata* and *B. napus*, the ARC1 protein retained a conserved function in self-incompatibility.

EXO70A1, THE TARGET OF ARC1 IN THE SELF-INCOMPATIBILITY PATHWAY

To determine how ARC1 may be driving the rejection of self-pollen, a yeast two-hybrid screen was performed to search for potential targets of ubiquitination by ARC1, and Exo70A1 was identified (Samuel et al., 2009). Further experiments showed that Exo70A1, not only interacted with ARC1, but that it could be ubiquitinated by ARC1 in an *in vitro* assay (Samuel et al., 2009). So how does the ubiquitination of Exo70A1 result in the rejection of self-pollen? Before answering this question, Exo70A1's function in the stigma for compatible pollen responses requires explanation. Exo70A1 has previously been shown to act as a marker for secretory vesicles that are delivered to a target membrane via the exocyst complex (reviewed in He and Guo, 2009; Heider and Munson, 2012; Zárský et al., 2013). In response to compatible pollen, the exocyst complex is proposed to mediate polarized exocytosis by docking secretory vesicles at the stigmatic papillar plasma membrane under the pollen contact point. Observations of RFP:Exo70A1 localized to the plasma membrane in stigmatic papillae of mature flowers supported this hypothesis; that Exo70A1 was localized where it was needed to direct exocyst assembly and delivery of secretory vesicles to facilitate hydration of the compatible pollen grain (Samuel et al., 2009). Both *A. thaliana exo70a1-1* mutant stigmas and transgenic *B. napus* plants with a stigma-expressed *Exo70A1* RNAi knockdown construct were impaired in accepting wild-type compatible pollen validating this prediction. As well, the stigma-specific expression of *RFP:Exo70A1* rescued the stigmatic defect in the *exo70a1-1* mutant (Samuel et al., 2009). Recently, Li et al. (2013) proposed that Exo70A1 has a tissue-specific expression pattern and functions in developing tracheary elements; however, these observations are not consistent with other research published on *A. thaliana* Exo70A1 (reviewed in Zárský et al., 2013). Li et al. (2013) also published that *A. thaliana exo70a1-1* mutant stigmas did not have a defect in accepting compatible pollen. With *A. thaliana* having dry stigmas and previous studies showing that pollen hydration defects can be rescued by high humidity (Preuss et al., 1993; Hülskamp et al., 1995), we tested pollinations under low and high relative humidity and found that this factor may help to explain the discrepancies between these two studies (Safavian et al., Submitted). At low relative humidity, no pollen grain acceptance or seed set was observed on the *exo70a1-1* mutant stigma as we had previously published, but high relative humidity conditions did partially rescue the *exo70a1-1* mutant stigma resulting in some compatible pollen acceptance and seed set (Safavian et al., Submitted). *Exo70A1*'s expression in the stigma was also verified by RT-PCR (Safavian et al., Submitted). The requirement of Exo70A1 in the stigmatic papillae for compatible pollen acceptance makes it an excellent target for ARC1 in the self-incompatibility pathway. If Exo70A1's activity is inhibited through ubiquitination by ARC1, then the required secretory activity would be blocked in the stigmatic papilla at the pollen contact site causing pollen grain rejection (**Figure 1**).

To delve into the idea of secretion being required for compatible pollen acceptance and inhibited for self-incompatible pollen rejection, detailed ultrastructural TEM studies were conducted at different time points following pollinations (Safavian and Goring, 2013; Indriolo et al., 2014). In wild-type compatible pollinations at 10 minutes post-pollination, what appeared to be secretory vesicles were visible at the stigmatic papillar plasma membrane at the point of pollen contact in *A. thaliana* and *A. lyrata* (Safavian and Goring, 2013). Intriguingly, *B. napus* stigmatic papillae appeared to use multivesicular bodies (MVBs) instead for secretion at the point of pollen contact. This switch to MVBs may be connected to the presence of a thicker cell wall and perhaps a need for increased secretion (Safavian and Goring, 2013). This observed secretory activity was disrupted in the *A. thaliana exo70a1-1* mutant stigmas and the transgenic *B. napus Exo70A1* RNAi knockdown stigmas following the application of wild-type compatible pollen (Safavian and Goring, 2013). Thus, these studies indicated that Exo70A1 is necessary for exocytosis in the stigmatic papillae following compatible pollinations for promoting pollen hydration and germination.

Self-incompatible pollinations in *B. napus*, *A. lyrata*, and transgenic *SCRb-SRKb-ARC1 A. thaliana* all displayed signs of disrupted secretory activity (Safavian and Goring, 2013; Indriolo et al., 2014). Originally, we had thought that the outcome of disrupted secretory activity would be an accumulation of secretory vesicles/MVBs in the cytoplasm, and some of this was seen in the transgenic *SCRb-SRKb-ARC1 A. thaliana*. However, in *A. thaliana* and *A. lyrata* stigmatic papillae, autophagy appeared to be induced at 10 min post-self-incompatible pollination. That is, secretory vesicles (and cytoplasm) were being engulfed by autophagosomes and sent to the vacuole for degradation. This

resulted in the presence of autophagic vacuoles and autophagic bodies in the stigmatic papillar vacuoles (Safavian and Goring, 2013; Indriolo et al., 2014). Again, *B. napus* stigmatic papillae showed a different degradation response with MVBs re-directed to the vacuole (Safavian and Goring, 2013). These observations suggest that ARC1 may promote more than just the disruption of Exo70A1-guided secretory activity in self-incompatible pollinations (**Figure 1**). Interestingly in *A. thaliana*, an ecotype specific difference was observed with the addition of *ARC1* with the *SCRb-SRKb* transgenes. The autophagic response was strongest in the transgenic *SCRb-SRKb-ARC1 A. thaliana* Sha ecotype and not as clear in the transgenic *SCRb-SRKb-ARC1 A. thaliana* Col-0 ecotype or the transgenic *SCRb-SRKb A. thaliana* Sha plants lacking *ARC1* (Indriolo et al., 2014). This autophagic response in the transgenic *SCRb-SRKb-ARC1 A. thaliana* Sha ecotype was more similar to that observed in *A. lyrata* self-incompatible pollinations, and was correlated with the strong self-incompatibility response that we observed in *A. thaliana* Sha. Finally, the disruption of vesicle secretion was also observed in the transgenic *SCRb-SRKb A. thaliana* Sha plants lacking *ARC1*, supporting the previous models that there is an additional (unknown) signaling factor(s) functioning downstream of SRK in the cellular response for self-pollen rejection (Rea et al., 2010; Indriolo et al., 2014).

FUTURE RESEARCH DIRECTIONS

The phenotypes arising from the knockdown of *ARC1* in transgenic *ARC1* RNAi *A. lyrata* (Indriolo et al., 2012) and the addition of *ARC1* with *SCRb-SRKb* in transgenic *A. thaliana* (Indriolo et al., 2014) give strong evidence that the *SRK-ARC1* self-incompatibility signaling pathway is conserved between *Arabidopsis* and *Brassica* species. Despite the progress made into elucidating the role of ARC1 in the self-incompatibility pathway, more questions remain on how it functions as an E3 ubiquitin ligase to regulate downstream targets. Future research directions should focus on identifying the *in vivo* protein modifications of ARC1 with Exo70A1 during the self-incompatibility response. If ARC1 ubiquitinates Exo70A1, what is the outcome, redirection of Exo70A1's localization or degradation by the 26S proteasome? Secondly, how is autophagy in *Arabidopsis* or vacuolar targeting of MVBs in *B. napus* induced as part the self-incompatibility response, and how is ARC1 and/or other factors involved in this? Finally, the other unknown signaling factor(s) functioning downstream of SRK needs to be identified and how they function in the cellular response for self-pollen rejection needs to be determined.

AUTHOR CONTRIBUTIONS

Both Emily Indriolo and Daphne R. Goring conceived, designed and wrote this review.

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The NEDD8 modification pathway in plants

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NEDD8, in plants and yeasts also known as RELATED TO UBIQUITIN (RUB), is an evolutionarily conserved 76 amino acid protein highly related to ubiquitin. Like ubiquitin, NEDD8 can be conjugated to and deconjugated from target proteins, but unlike ubiquitin, NEDD8 has not been reported to form chains similar to the different polymeric ubiquitin chains that have a role in a diverse set of cellular processes. NEDD8-modification is best known as a post-translational modification of the cullin subunits of cullin-RING E3 ubiquitin ligases. In this context, structural analyses have revealed that neddylation induces a conformation change of the cullin that brings the ubiquitylation substrates into proximity of the interacting E2 conjugating enzyme. In turn, NEDD8 deconjugation destabilizes the cullin RING ligase complex allowing for the exchange of substrate recognition subunits via the exchange factor CAND1. In plants, components of the neddylation and deneddylation pathway were identified based on mutants with defects in auxin and light responses and the characterization of these mutants has been instrumental for the elucidation of the neddylation pathway. More recently, there has been evidence from animal and plant systems that NEDD8 conjugation may also regulate the behavior or fate of non-cullin substrates in a number of ways. Here, the current knowledge on NEDD8 processing, conjugation and deconjugation is presented, where applicable, in the context of specific signaling pathways from plants.

Keywords: CAND1, COP9 signalosome (CSN), cullin, E3 ubiquitin ligase, F-BOX PROTEIN (FBP), NEDD8, RELATED TO UBIQUITIN (RUB), ubiquitin

NEDD8 IS AN EVOLUTIONARILY CONSERVED REGULATOR

NEDD8 (neural precursor cell expressed, developmentally down-regulated8), in plants and yeasts also known as RELATED TO UBIQUITIN (RUB, hitherto referred to as NEDD8), is a 76 amino acid protein that was originally identified as a highly expressed gene from embryonic mouse brains (Kumar et al., 1993). Amongst all ubiquitin-like modifiers (UBLs), NEDD8 and ubiquitin are most closely related to each other and NEDD8 proteins, like other UBLs, display remarkable sequence conservation across species (Vierstra, 2012). Like ubiquitin, NEDD8 is conjugated to its substrate protein through the formation of an isopeptide bond between its C-terminal glycine and a lysine residue of the target protein (neddylation) but there is no known biological function for free NEDD8.

NEDD8 orthologs can be identified in all eukaryotic species that have sequenced genomes. While NEDD8 is a single gene in humans, mouse, and fruit fly, several copies of *NEDD8* are encoded by the genomes of the plant species *Arabidopsis* (*Arabidopsis thaliana*; Rao-Naik et al., 1998), rice (*Oryza sativa*), *Brachypodium* (*Brachypodium distachyon*), and the moss *Physcomitrella patens* (**Figure 1**). All NEDD8 proteins require proteolytic processing of their C-termini to generate mature NEDD8 with a C-terminal glycine required for NEDD8 conjugation (**Figure 1**). An additional unique feature of plant NEDD8 is the existence of ubiquitin-NEDD8 gene fusions. While gene fusions of ubiquitin to ubiquitin itself or other genes have been reported in other species, *NEDD8* is an unfused gene in animals and yeasts but not in plants. This ubiquitin-NEDD8 fusion structure is found in *Arabidopsis* RUB1 and RUB2 and seems to be conserved among plants, mosses and algae (**Figure 1**; Rao-Naik et al., 1998; Vierstra and Callis, 1999; Shin et al., 2011). In RUB1 and RUB2, a single ubiquitin is fused head-to-tail to the N-terminus of NEDD8 and both ubiquitin-NEDD8 fusions then require post-translational processing to release monomeric ubiquitin and NEDD8 (**Figure 1**). Furthermore, plant genomes contain an unfused monomeric form of NEDD8, *RUB3* in *Arabidopsis*, that can additionally be distinguished from the other *RUB* genes because it lacks an intron that is present at a conserved position in other *RUBs*, e.g., in *Arabidopsis RUB1* and *RUB2* (**Figure 1**). The absence of an intron suggests that this less complex *RUB3* may be more ancient than the intron-containing *RUB1* or *RUB2* or that *RUB3* originated from an mRNA intermediate and a retrotransposition event (Huang et al., 2012).

Similarly to the high sequence conservation observed between human and *Arabidopsis* ubiquitin (96% amino acid sequence identity), also NEDD8 proteins are highly conserved between species (83% identity between human and *Arabidopsis*). This high level conservation is suggestive for an important function of NEDD8 conjugation (neddylation) in eukaryotic cells and a highly conserved neddylation and deneddylation machinery. Indeed, loss-of-*NEDD8* function causes lethality at an early developmental stage in most model organisms and also in plants, with the notable exception of *Saccharomyces cerevisiae* (Lammer et al., 1998; Liakopoulos et al., 1999; Jones and Candido, 2000; Osaka et al., 2000; Tateishi et al., 2001; Ou et al., 2002; Dharmasiri et al., 2003; Maytal-Kivity et al., 2003; Bostick et al., 2004).

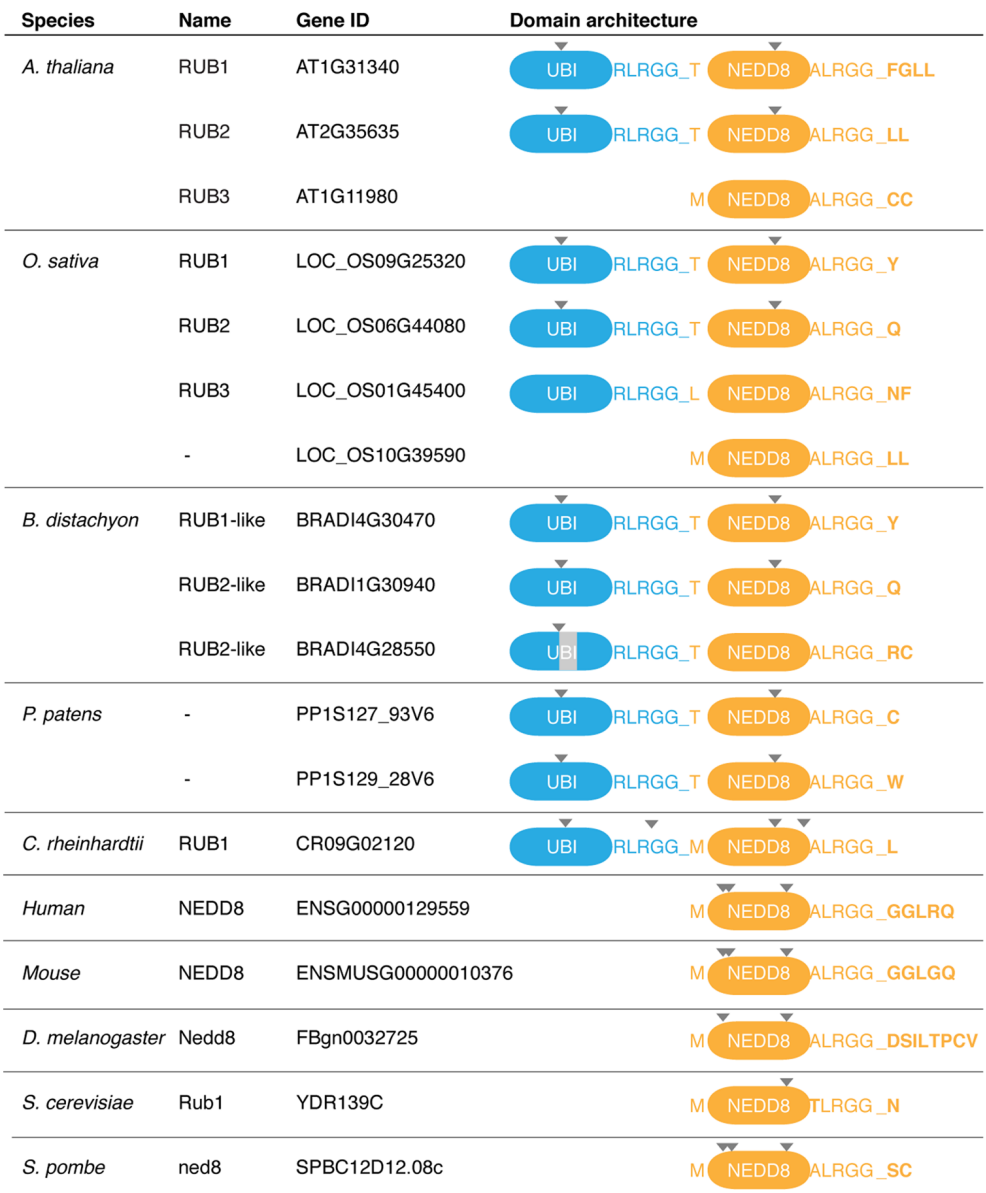

FIGURE 1 | Protein domain organization of NEDD8 proteins from several representative species. Gene identification numbers (Gene IDs) are as listed in www.ensemblgenomes.org. Specifically indicated are the last five amino acids of the respective proteins before the proteolytic cleave sites, the first amino acid of NEDD8 and the proteins' C-terminal amino acids. Proteolytic processing occurs after the C-terminal RGG residues and is indicated by an underscore. Positions of introns in the respective genes are indicated by a triangle. The light gray area in BRADI4G28550 highlights an apparent 22 amino acid deletion in the ubiquitin part of the protein. *A. thaliana* (*Arabidopsis thaliana*), *O. sativa* (*Oryza sativa*, rice), *B. distachyon* (*Brachypodium distachyon*), *P. patens* (*Physcomitrella patens*, moss), *C. reinhardtii* (*Chlamydomonas reinhardtii*, algae), *D. melanogaster* (*Drosophila melanogaster*, fruit fly), *S. cerevisiae* (*Saccharomyces cerevisiae*, baker's yeast), *S. pombe* (*Schizosaccharomyces pombe*, fission yeast).

In *Arabidopsis*, not the single but the combined knockout of the genes *RUB1* and *RUB2* leads to a developmental arrest at the embryonic two-cell stage (Bostick et al., 2004). Thus, *NEDD8* genes and neddylation are essential for growth and development in plants. Plants with reduced *NEDD8* gene expression are dwarfed, partially insensitive to root growth inhibitory concentrations of the plant hormone auxin and also partially defective in auxin-induced lateral root formation (Bostick et al., 2004). As will be outlined below, auxin insensitivity phenotypes are reliable and at the same time the most obvious readouts of neddylation pathway mutants.

NEDD8 PROCESSING

NEDD8 is conjugated to the protein substrates via an isopeptide bond between its C-terminal glycine and a lysine of the target protein (**Figure 2**). NEDD8, like ubiquitin and most UBLs, is expressed as an inactive precursor with a short C-terminal extension that consists of one or several amino acids that need to be

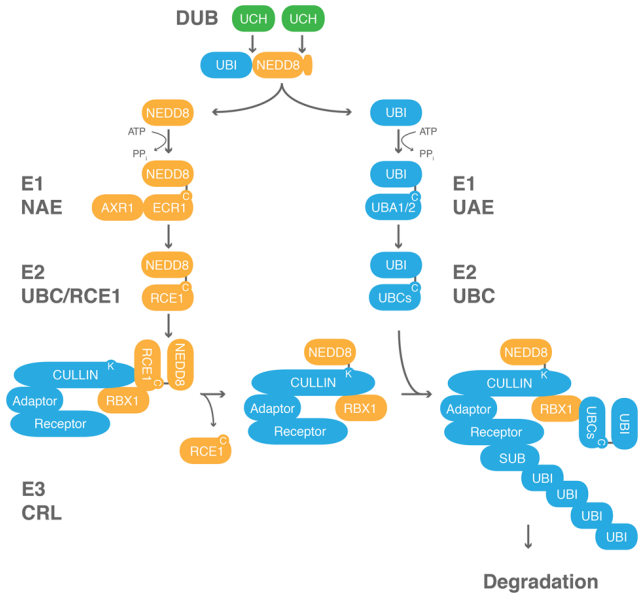

FIGURE 2 | Neddylation and ubiquitin modification are biochemically related processes. Ubiquitin c-terminal hydrolases (UCHs) belong to the family of DUBs that process ubiquitin (UBI)-NEDD8 fusion proteins. UBI and NEDD8 are activated by their conjugation to E1 ubiquitin/NAEs. UBI or NEDD8 from the E1 are then passed via a transthiolation reaction to a protein of the family of E2 ubiquitin-conjugating enzymes, RUB1 CONJUGATING ENZYME1 (RCE1) in *Arabidopsis*. The ubiquitin-charged E2 can then form a complex with an E3 ubiquitin ligase, and ultimately, ubiquitin and NEDD8 are transferred to a lysine residue on the cullin of the E3 and the substrate, respectively. UBI but not NEDD8 can form chains. RBX1 and other proteins not shown here are the proposed NEDD8 ligases. Cullin-RING ligases (CRLs) are the superfamily of E3 ubiquitin ligases that are regulated by neddylation. CRLs are generally composed of a cullin subunit, RBX1, as well as a substrate (SUB) recognition module composed of an adaptor and a substrate receptor protein. C, cysteine; K, lysine.

cleaved off to allow for NEDD8 conjugation (**Figures 1** and **2**; Jentsch and Pyrowolakis, 2000). It has been proposed that the C-terminal extension of ubiquitin, NEDD8, and other UBLs serves to prevent unprocessed proteins to enter into the conjugation pathway but there is, in fact, no experimental evidence supporting this hypothesis (Callis et al., 1995; Rao-Naik et al., 1998). The plant ubiquitin-NEDD8 fusion proteins additionally require removal of the N-terminal ubiquitin by proteolytic cleavage.

NEDD8 processing is carried out by ubiquitin C-terminal hydrolases (UCH) from the family of deubiquitinating enzymes (DUBs). In *S. cerevisiae* and humans, NEDD8 precursor C-terminal processing is facilitated by a dual specificity UCH of the C12 family peptidases, Yuh1 (yeast) or UCHL3 (human, mouse), which also processes the C-terminal extensions of ubiquitin (**Figure 3**; Wada et al., 1998; Johnston et al., 1999; Linghu et al., 2002; Hemelaar et al., 2004; Frickel et al., 2007; Yu et al., 2007). To date, the only isopeptidase known to function exclusively in NEDD8 processing and deconjugation is the C48 family peptidase DEN1/NEDP1/SENP8 from *Drosophila*, and human (**Figure 3**; Gan-Erdene et al., 2003; Mendoza et al., 2003; Wu et al., 2003; Shen et al., 2005; Chan et al., 2008; Shin et al., 2011). However, mouse knockouts of *UCHL3* or *Drosophila* and *Aspergillus* knockouts of *DEN1* are viable although NEDD8 and neddylation are essential in the respective organisms (Kurihara et al., 2000; Chan et al., 2008; Christmann et al., 2013). These findings suggest that mutants of these processing enzymes cannot be fully impaired in NEDD8 processing.

In non-plant species where NEDD8 is expressed as an unfused gene, examining NEDD8 precursor processing *in vivo* is not trivial because NEDD8 has only a short C-terminal extension. Therefore, conjugation of processed NEDD8 to cullins is used as an indirect indication for proper processing. Since cullin neddylation was not impaired in any of the *UCH* gene mutants examined to date, it can be inferred that also NEDD8 processing and conjugation are at least partially functional in these mutants (Kurihara et al., 2000; Chan et al., 2008; Christmann et al., 2013). In the fission yeast *Schizosaccharomyces pombe*, even a Δ*nep1* Δ*nep2* Δ*uch1* Δ*uch2* quadruple mutant lacking the two C48 peptidases orthologous to DEN1 (*NEP1* and *NEP2*) as well as the two C12 peptidases orthologous to yeast Yuh1 (*UCH1* and *UCH2*) shows efficient cullin neddylation (O'Donoghue et al., 2013). Only the additional knockout of the cullin deneddylating enzyme COP9 SIGNALOSOME SUBUNIT5 (CSN5) hinted to a reduced efficiency of NEDD8 precursor processing in this complex mutant because it revealed the absence of cullin1 hyperneddylation that can be observed in the Δ*csn5* deletion strain (O'Donoghue et al., 2013). Taken together, these various findings suggest that there is a functionally redundant family of NEDD8 processing enzymes and that there may be other as yet unknown peptidases that also participate in NEDD8 processing. Furthermore, it is possible that there is no strict specificity among the ubiquitin and NEDD8 processing enzymes *in vivo*.

NEDD8 PROCESSING IN PLANTS

NEDD8 processing hydrolases from plants remain to be identified. In *Arabidopsis*, several peptidases can be classified as C12 and C48 family peptidases based on their homology to UCHs from other species (**Figure 3**). To date, only the C12 peptidases UCH1 and UCH2 have been analyzed at the biological level (Yang et al., 2007). Based on their homology to C12 peptidases, these UCHs would be predicted to have a role in ubiquitin processing. However, although both UCH proteins showed the predicted ubiquitin processing activity *in vitro*, neither the *uch1 uch2* double mutant nor *UCH1* overexpressing lines had apparent changes in the pattern of ubiquitin conjugate formation or in the abundance of free monomeric ubiquitin. At the phenotypic level, mutants or overexpression lines display impaired shoot and flower development and changes in the rate of leaf formation. Altering the abundance of the two *UCH* genes also affects auxin and cytokinin responsiveness. These phenotypes may be explained by defects in selective rather than general protein degradation and this hypothesis is supported by the observation that the degradation of the auxin-labile AUX/IAA AUXIN RESISTANT3 (AXR3) but not that of the light-labile phytochrome A or ELONGATED HYPOCOTYL5 (HY5) proteins appeared to be affected in the *uch* mutants. In summary, these findings suggest that UCH1 and UCH2 may not only act at the level of ubiquitin processing but may also act by selectively regulating the proteasomal degradation of proteins by antagonizing substrate ubiquitylation. Whether UCH1 and UCH2 have a

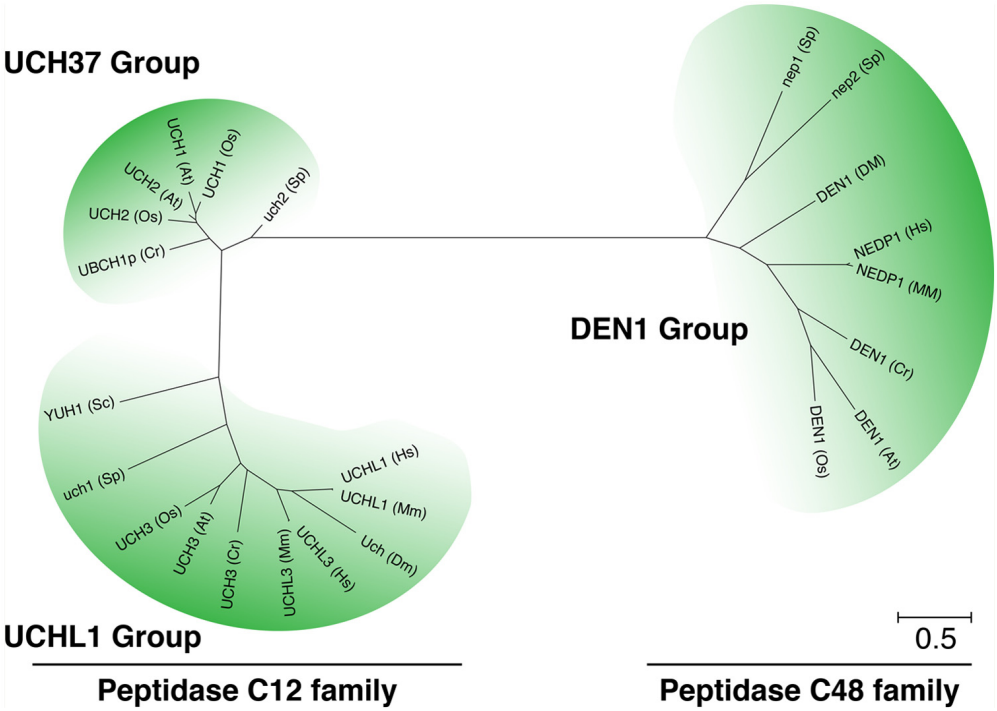

FIGURE 3 | NEDD8 processing is mediated by at least three different classes of peptidases. Unrooted phylogenetic tree of representative members of the UCH and DEN1/NEDP1/SENP8 protein families from *Arabidopsis thaliana* (At), rice (*Oryza sativa*, Os), algae (*Chlamydomonas reinhardtii*, Cr), baker's yeast (*Saccharomyces cerevisiae*, Sc), fission yeast (*Schizosaccharomyces pombe*, Sp), fruit fly (*Drosophila melanogaster*, Dm), mouse (*Mus musculus*, Mm), and human (*Homo sapiens*, Hs). Molecular phylogenetic analysis was performed based on the Maximum Likelihood method and the JTT matrix-based model (Jones et al., 1992) using CLUSTALW algorithm at the EMBL-EBI website (http://www.ebi.ac.uk/Tools/msa/clustalw2/). The tree with the highest log likelihood (−5916.8263) is shown. Initial tree(s) for the heuristic search were obtained automatically by applying the Maximum Parsimony method. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 24 protein sequences. All positions containing gaps and missing data were eliminated. There were a total of 145 positions in the final dataset. Evolutionary analyses were conducted in MEGA5 (Tamura et al., 2011).

role in NEDD8 processing remains to be examined but the comparatively weak morphological phenotype as well as the absence of an apparent cullin neddylation phenotype already suggests that the two UCH proteins may not have a major function in this process.

NEDDYLATION

NEDD8 is conjugated to target proteins in a manner that is highly similar to ubiquitin conjugation (**Figure 2**). NEDD8 is activated by an E1 NEDD8 activating enzyme (NAE) and then passed on to an E2 NEDD8 conjugating enzyme of the ubiquitin-conjugating (UBC) enzyme family from where the protein is ultimately transferred to its substrate protein. The best-studied NEDD8 conjugates are the cullin subunits of cullin-RING-type E3 ubiquitin ligases (CRLs; Hua and Vierstra, 2011). CRLs are a family of evolutionarily conserved E3 ligases that are composed of a core complex, comprised of a cullin subunit and the RING BOX PROTEIN1 (RBX1), as well as a ubiquitylation substrate recognition module. In plants, three different types of CRL complexes can be distinguished based on the identity of the cullin subunits CULLIN1, CULLIN3, or CULLIN4 and the identity of their respective substrate recognition module (Lammer et al., 1998; Ruegger et al., 1998; Dieterle et al., 2005; Figueroa et al., 2005; Gingerich et al., 2005; Bernhardt et al., 2006; Chen et al., 2006). The CRL subunit RBX1 is common to all CRLs and serves to promote NEDD8 conjugation.

Structural analyses of cullin neddylation revealed that NEDD8 conjugation causes a conformational change in subdomains of the cullin and RBX1 subunits (Duda et al., 2008; Boh et al., 2011). Neddylation also eliminates the binding of the exchange factor CULLIN-ASSOCIATED-NEDD8-DISSOCIATED1 (CAND1) and locks the CRL in an active state. Thus, neddylation controls CRL activity by promoting conformational changes that favor substrate ubiquitylation. CRL neddylation can then also lead to the recruitment of additional regulatory factors (den Besten et al., 2012).

THE NEDDYLATION PATHWAY AND AUXIN INSENSITIVITY

As will be discussed in more detail below, loss of cullin neddylation or cullin deneddylation affect CRL function by promoting or, respectively, preventing interactions with the substrate receptor exchange factor CAND1. In the plant and neddylation biology context, the *Arabidopsis* CULLIN1-containing E3 ligase SCF^{TIR1} with the substrate recognition module composed of the F-box protein (FBP) TRANSPORT INHIBITOR RESISTANT1 (TIR1) and its adaptor subunit *ARABIDOPSIS* SKP1 (ASK) is highly

relevant (Ruegger et al., 1998; del Pozo and Estelle, 1999). TIR1, functioning at the same time also as an auxin receptor, binds AUX/IAA transcriptional repressors in an auxin-dependent manner and targets AUX/IAAs for ubiquitylation and degradation by the 26S proteasome (Gray et al., 2001; Tan et al., 2007). AUX/IAAs and auxin-induced AUX/IAA degradation regulate a number of important developmental and morphological processes throughout plant development: *bodenlos* (*bdl*) mutants expressing a stabilized (non-degradable) variant of the AUX/IAA protein bodenlos/iaa14 (bdl/iaa14) are deficient in embryonic root differentiation and are consequently rootless (Hamann et al., 1999; Weijers et al., 2005). *axr3* mutants express the stabilized axr3/iaa17 protein and this mutation allows for root elongation in the presence of root growth-inhibiting auxin concentrations (Gray et al., 2001). The link between auxin insensitivity (auxin resistance), AUX/IAA degradation and CULLIN1 could be established because the CULLIN1 alleles *axr6-1* and *axr6-2* were identified based on their auxin insensitivity (Hobbie et al., 2000; Shen et al., 2002; Hellmann et al., 2003; Quint et al., 2005; Esteve-Bruna et al., 2013). While homozygous *axr6-1* and *axr6-2* loss-of-function mutants arrest development during embryogenesis, the heterozygous mutants display the auxin-insensitive root growth elongation phenotype. Furthermore, double mutants of *axr6-1* or *axr6-2* with other mutants of the auxin and neddylation pathway are defective in root differentiation and thereby mimic the characteristic phenotype of the *bdl* mutant (Hobbie et al., 2000; Hellmann et al., 2003; Quint et al., 2005; Esteve-Bruna et al., 2013).

NEDD8 ACTIVATION

Both, defects in root differentiation as well as auxin-insensitive root elongation have been used extensively as phenotypes for the identification and characterization of *Arabidopsis* neddylation mutants. *auxin resistant1* (*axr1*) is a mutant of the NAE enzyme AXR1 and was identified due to its defects in auxin response that could later be explained by impairment in the degradation of the AUX/IAA protein AXR3 (Lincoln et al., 1990; Leyser et al., 1993; del Pozo et al., 1998). In *Arabidopsis*, *axr1* mutants display an auxin-insensitive root growth phenotype but to fully impair NEDD8 conjugation the function of the AXR1-paralog *AXR1-LIKE* (*AXL*) also needs to be deleted (Dharmasiri et al., 2007). *axr1 axl1* double mutants have a more severe phenotype than *axr1* mutants in that they are defective in embryonic root differentiation and mimic the *bdl* mutant phenotype (Dharmasiri et al., 2007). *Arabidopsis* AXR1 and AXL proteins appear to be equivalent at the biochemical level but interestingly have a differential ability to complement the *axr1* mutant phenotype when expressed from the *AXR1* promoter (Dharmasiri et al., 2007; Hotton et al., 2011). While NEDD8 activation is carried out by a single protein in animals and yeasts, NAE is a heterodimer in plants of AXR1/AXL and E1 C-TERMINAL RELATED1 (ECR1) corresponding to the protein's N- and C-termini, respectively (**Figure 2**; del Pozo et al., 1998; Hotton et al., 2011). An *ecr1-1* mutant was identified in a screen for mutants with differential auxin sensitivity and *axr1/axl* mutants as well as *ecr1* mutants are defective in cullin neddylation (Woodward et al., 2007).

NEDD8 CONJUGATION

RUB1 CONJUGATING ENZYME1 (RCE1) was identified in *Arabidopsis* based on its homology to human UBC12 (del Pozo and Estelle, 1999). An *rce1-1* insertion mutant with significantly reduced *RCE1* expression levels was subsequently isolated and found to be strongly impaired in cullin neddylation (Dharmasiri et al., 2003). *rce1-1* mutants display auxin insensitive root growth phenotypes and fail to differentiate a primary root when combined with *axr1*. Two additional *rce1* alleles were recently found in a suppressor screen of the auxin overproducing *sur2* mutant (Pacurar et al., 2012). Interestingly, both of these *rce1* alleles would be expected to interfere significantly with the biochemical activity of RCE1 since they carry a nonsense and splice site mutation in exon 4, respectively. Analyzing the extent to which RCE1 function is affected in these alleles is certainly interesting because the unexpectedly weak phenotype of these supposedly strong alleles could be considered indicative for the existence of functionally redundant NEDD8 conjugating enzymes.

NEDD8 LIGATION

The CRL core subunit RING BOX1 (RBX1) is one candidate for an E3 NEDD8 ligase (Morimoto et al., 2003). RBX1 is encoded by two genes in *Arabidopsis* and its function as CRL subunit and as NEDD8 ligase was addressed in mutants, antisense and overexpression lines (Gray et al., 2002; Lechner et al., 2002; Schwechheimer et al., 2002). RBX1 interacts with RCE1 and while cullin neddylation is decreased in the absence of *RBX1* it is increased when *RBX1* is overexpressed (Gray et al., 2002).

The protein DEFECTIVE IN CULLIN NEDDYLATION1 (DCN1) has also been described as an E3 NEDD8 ligase (Kurz et al., 2005; Kurz et al., 2008; Meyer-Schaller et al., 2009). Based on yeast studies, it has recently been proposed that DCN1 increases the substrate specificity of RBX1 by directing the RBX1-bound NEDD8-E2 toward the cullin (Scott et al., 2010). Additionally, it was shown that the interaction between DCN1 and UBC12 is regulated by the N-terminal acetylation of the UBC12 E2 enzyme (Scott et al., 2011). The analysis of DCN1-LIKE proteins, of which there are five in humans, has also revealed that at least one member of the protein family, DCNL3, is bound to the plasma membrane (Meyer-Schaller et al., 2009). Studies of a mammalian CULLIN2-containing CRL further revealed that DCN1-LIKE1 can engage in interactions between the cullin and the respective substrate receptor subunit, and more importantly, that this interaction is strengthened when the substrate receptor is loaded with cargo (Heir et al., 2013). At least in this case, DCN1-LIKE1 may function as a sensor for degradation substrate availability and consequently promote neddylation. Thus, DCN1 proteins may contribute to the regulation of E3 ligase activity by targeting E3 ligases to or by activating them in specific subcellular locations.

As yet, DCN1 or DCN1-LIKE proteins have not been analyzed in plants but AT3G12760 is a candidate for a direct DCN1 ortholog from *Arabidopsis*. A second DCN1-LIKE protein, less closely related to DCN1 than AT3G12760, was identified as *anti-auxin resistant3* (*AAR3*) in a screen for mutants that showed resistance to the anti-auxin *p*-chlorophenoxyisobutylic acid. The same screen also identified mutant alleles of *TIR1* and *CULLIN1* and, based on the shared phenotype of these mutants,

AAR3/DNC1-LIKE would qualify as a candidate regulator of NEDD8 ligation and SCF^{TIR1} function (Biswas et al., 2007). Unfortunately, the biochemical function of AAR3/DNC1-LIKE in the context of cullin neddylation has not been examined as yet. There is also one further *Arabidopsis* gene that may encode for an additional DCN1-LIKE protein. Thus, the biochemical and biological functions of *DCN1-LIKE* genes from *Arabidopsis* remain to be investigated.

In yeast, also TFB3, a RING domain subunit of the general transcription factor TFIIH was found to promote neddylation in addition to RBX1 and DCN1 (Rabut et al., 2011). The identification of TFB3 was based on initial observations that CULLIN4 neddylation in yeast was independent from RBX1 and DCN1. The analysis of RING domain protein mutants from yeast then led to the subsequent discovery of Tfb3 as a RING domain protein responsible for the neddylation of CULLIN3 and CULLIN4. A clear homolog of TFB3 is not easily discernable in the plant genomes but the identification of TFB3 from yeast *per se* indicates that it cannot be ruled out that besides RBX1 and DCN1 also other NEDD8 ligases exist in plants.

MLN4924 – A NEDDYLATION INHIBITOR

The importance of the NEDD8-modification pathway in the control of plant development has recently been elucidated in a study with the neddylation inhibitor MLN4924 (Hakenjos et al., 2011). MLN4924 was initially described as an inhibitor of the human NAE E1 enzyme but was subsequently found to also inhibit the NAE E1 subunit ECR1 from predictably all plant species (Soucy et al., 2009; Brownell et al., 2010; Hakenjos et al., 2011). MLN4924 inhibits neddylation in plants and the impairment of CRL function results in the degradation of a number of CRL substrates such as the AUX/IAAs of the auxin pathway, DELLA proteins of the gibberellin pathway and the cell cycle regulator KRP1 (Hakenjos et al., 2011). While the severe phenotypes of strong NEDD8 pathway mutants in *Arabidopsis* and the absence of neddylation mutants in other plant species has as yet hampered studying the role of neddylation in all stages of plant development or in non-*Arabidopsis* species, the availability of MLN4924 now overcomes this limitation (Hakenjos et al., 2011).

NEDDYLATION MUTANTS ARE IMPAIRED IN MANY DIFFERENT CRL FUNCTIONS

As outlined above, AUX/IAA degradation is partially or fully impaired in all mutants of the NEDD8 conjugation pathway and auxin responses are partially or fully blocked in these mutant backgrounds. However, the phenotype of the NEDD8 conjugation mutants is much more complex and not only the consequence of defects in the auxin response pathway. In this regard, it is important to realize that plants predictably have many hundreds of CRLs and that all these CRLs should be impaired in neddylation mutants (Xu et al., 2009). Among these CRLs, SCF^{TIR1} and closely related complexes implicated in auxin responses have a very prominent role because defects in the auxin-regulatory CRLs lead to morphological defects that can easily be examined (Dharmasiri et al., 2005a,b). However, while malfunction of SCF^{TIR1} and closely related complexes is the most visible phenotype of neddylation mutants, all CRL functions should be affected in *axr1* mutants in a manner similar to the defects observed in the auxin pathway. This fact is sometimes overlooked and particularly *axr1* mutants that were amongst the first auxin response mutants to be identified (Lincoln et al., 1990; Leyser et al., 1993) are often being used as auxin pathway-specific mutants. The knowledge about the existence of many other CRL-dependent pathways, also CRL pathways that affect plant growth and morphology clearly argue against using *axr1* mutants or other neddylation mutants as auxin pathway-specific mutations for morphological analyses or genetic interaction studies (Dill et al., 2004; Shen et al., 2007; Stirnberg et al., 2007; Nelson et al., 2011; Waters and Smith, 2013).

CSN PROMOTES CULLIN DENEDDYLATION

NEDD8 can be deconjugated from CRLs through the activity of the COP9 signalosome (CSN; **Figure 4**). CSN is evolutionarily conserved and in most species including plant and mammalian species composed of eight subunits (Chamovitz et al., 1996; Seeger et al., 1998; Wei and Deng, 1998). CSN was originally identified in plants based on mutants that display a constitutively photomorphogenic (cop) phenotype and named following the identification of the causative mutation in the *cop9* mutant (Wei and Deng, 1992; Wei et al., 1994). Similarly to

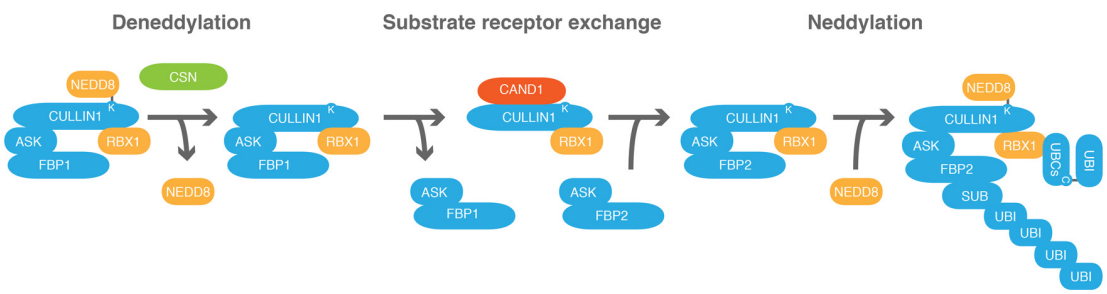

FIGURE 4 | CAND1 regulates the cells CRL repertoire by promoting the exchange of substrate receptor subunits. Schematic representation of the exchange of a hypothetical F-BOX PROTEIN1 (FBP1) of the CRL SCF^{FBP1} against FBP2 following CULLIN1 deneddylation. *ARABIDOPSIS* SKP1 (ASK) proteins are adaptor subunits that link FBPs with CULLIN1. CULLIN1, ASK, RBX1 and FBP form an SCF-type CRL. COP9 SIGNALOSOME (CSN) promotes CULLIN deneddylation. CULLIN-ASSOCIATED-NEDD8-DISSOCIATED1 (CAND1) is an exchange factor that can only associate with deneddylated cullins and promotes substrate receptor exchange. C, cysteine; K, lysine.

light-grown seedlings, *cop* mutants have a short hypocotyl, open cotyledons, and express light-regulated genes when grown in the dark.

CSN REPRESSES PHOTOMORPHOGENESIS IN *ARABIDOPSIS*

In *Arabidopsis*, loss-of-function of the eight CSN subunits results in most cases in the destabilization of the entire CSN complex and in a phenotypically indistinguishable *cop* phenotype, marking constitutive photomorphogenesis as a hallmark phenotype for loss of CSN function (Serino et al., 1999, 2003; Dohmann et al., 2005; Gusmaroli et al., 2007). The *cop* phenotype of *csn* mutants can be explained by their inability to degrade photomorphogenesis regulatory transcription factors such as HY5 in dark-grown seedlings through the activity of the E3 ligase COP1 (Osterlund et al., 2000; Chen et al., 2006; Lau and Deng, 2012). COP1 function is impaired in *csn* mutants leading to a stabilization of the COP1 targets also in the dark. In the wildtype, photomorphogenic development during germination is seemingly controlled by the light-controlled nucleocytoplasmic shuttling of COP1 (von Arnim and Deng, 1994; Osterlund et al., 2000; Pacin et al., 2013). In contrast to the strong photomorphogenesis phenotype of *csn* loss-of-function mutants, mutants with partially impaired CSN function display a number of phenotypes, also including auxin insensitive root elongation (Schwechheimer et al., 2001, 2002; Dohmann et al., 2005, 2008b; Stuttmann et al., 2009; Huang et al., 2013b). This phenotypic similarity was indicative for a connection between CSN, the neddylation pathway, and SCF^{TIR1}-dependent plant growth regulation when knowledge about the biochemical interplay of these components was still unclear (Schwechheimer et al., 2001).

CULLIN DENEDDYLATION IS A FUNCTION OF THE MPN+ DOMAIN SUBUNIT CSN5

COP9 signalosome is closely related to the "lid" of the 26S proteasome. In plants and animals, both protein complexes are composed of six so-called PCI domain subunits and two MPN-domain subunits and they share a set of subunit–subunit interactions within the respective complexes (Glickman et al., 1998; Wei et al., 1998; Fu et al., 2001; Enchev et al., 2010; Kotiguda et al., 2012). The relatedness of the two complexes and their in part shared biochemical function are nicely reflected by the fact that a proteasomal "lid" subunit functionally replaces a "missing" CSN subunit in *Saccharomyces cerevisiae* (Yu et al., 2011). CSN as well as the "lid" have two MPN-domain proteins, which can be further subdivided into an MPN+ domain protein with a catalytically active metalloprotease site, and a catalytically inactive MPN-domain protein that must be derived from the MPN+-domain counterpart (Maytal-Kivity et al., 2002). The MPN+ domain subunits CSN5 and RPN11 confer deneddylation and deubiquitylation activity to the CSN and proteasome "lid" complexes, respectively (Cope et al., 2002; Ambroggio et al., 2004). *csn* mutants from *Arabidopsis* are fully impaired in cullin deneddylation and only traces of, presumably *de novo* synthesized, unneddylated cullin can be detected in *csn* mutants. Interestingly, CSN5 is only functional as a cullin deneddylase when associated with CSN. CSN physically interacts with the cullin and RBX1 subunits of CRLs through its subunits CSN2 and CSN6 and it is thought that these interactions provide CSN with the affinity for its CRL targets (Schwechheimer et al., 2001). Interesting is also the recently identified *csn3-3* allele, which carries a missense mutation in the *CSN3* gene. This mutation strongly impairs auxin responses in the mutant but does neither obviously affect cullin deneddylation nor CSN protein complex integrity (Huang et al., 2013a). Thus, the affected domain of CSN3 may be required for an as yet unknown essential CSN function such as CRL subunit interactions or for the ability of the protein or protein complex to engage in other interactions required for normal auxin responses. This is supported by biochemical analyses combined with structural electron microscopy that suggest that CSN2 and CSN5 interact with the cullin E3 ligase subunit whereas F-box substrate receptors interact with CSN1 and CSN3 (Enchev et al., 2012).

Since *csn* mutants are impaired in the function of presumably hundreds of E3 ligases, it is not surprising that additional physiological defects have been identified in these mutants that can be explained by defects in other CRLs and include defects in SCF^{COI1}-mediated jasmonate signaling (Schwechheimer et al., 2002; Hind et al., 2011), SCF^{SLY1}-mediated gibberellin signaling (Dohmann et al., 2010) as well as defects in cold response (Schwechheimer et al., 2002), cell cycle progression (Dohmann et al., 2008a), and the control of ascorbic acid synthesis (Wang et al., 2013).

CSN REGULATION AND CSN REGULATORS

In view of the large number of CRLs that exist in eukaryotic cells and the importance of CSN-dependent cullin deneddylation for CRL function, it has to be asked how CRL neddylation and CSN-dependent deneddylation are regulated. In this context, it was shown for different CRLs that the availability of ubiquitylation substrate receptor and ubiquitylation substrate promotes CRL formation and cullin neddylation (Bornstein et al., 2006; Emberley et al., 2012). The association of FBP-SKP1 dimers can inhibit CSN function on selected SCF-complexes several fold (Emberley et al., 2012). Thus, in the case of SCF-complexes cullin neddylation and deneddylation are regulated by the presence of FBP-SKP1 dimers and particularly by the presence of a given FBP, assuming that there is no regulation on the level of the SKP1 adaptor subunit, which does not confer substrate specificity to the SCF complexes. CSN is even more strongly inhibited in the presence of degradation substrate and thus degradation substrate and degradation substrate receptor availability negatively regulate CSN activity (Emberley et al., 2012). Furthermore, CSN associates tightly with deneddylated SCF and CSN thereby keeps the CRL complex in a state of low activity after substrate degradation (Emberley et al., 2012). Through this interaction, CSN also prevents cullin neddylation, unless binding of a ubiquitylation substrate triggers its dissociation and allows for cullin neddylation (Enchev et al., 2012).

In addition to the regulation of CSN by CRLs, their subunits and their substrates, there are also other candidate regulators whose function remains to be determined. The 7 kDa protein SMALL ACIDIC PROTEIN1 (SMAP1) is an interesting CSN-interaction partner (Nakasone et al., 2012). *smap1* mutants were identified as *anti-auxin resistant1* (*aar1*) in a mutant screen that also identified mutants of *TIR1*, *CULLIN1*, and *DCN1-LIKE*. Thus the *aar1*/*smap1* phenotype may well be explained by a defect

in the E3 ligase SCF^{TIR1} or its neddylation or deneddylation. Importantly, immunoprecipitates of SMAP1 are very strongly enriched in at least six CSN subunits, indicating that SMAP1 is a CSN interactor and may regulate CSN function. As yet, the analysis of CULLIN neddylation patterns did not reveal any apparent defects in its neddylation or deneddylation but the *aar3*/*smap1* mutant phenotype together with the SMAP1-CSN interaction strongly suggests that SMAP1 is linked to CSN function. Since there is not apparent homolog of SMAP1 outside of the plant kingdom, this function should be plant specific.

The analysis of the CSN-interacting Rig-G protein, a protein related to the *Arabidopsis* protein SPINDLY, provided some insights into how interaction partners could interfere with CSN activity. In the case of Rig-G it is proposed that the protein recruits CSN subunits to the cytoplasm and thereby interferes with CSN assembly in the nucleus (Xu et al., 2013).

CAND1 – A SUBSTRATE RECEPTOR EXCHANGE FACTOR FOR CRLs

Important progress has been achieved in the understanding of the role of neddylation and deneddylation of cullins in the context of CRL assembly and function through analysis of the protein Cullin-associated-Nedd8-dissociated-1 (CAND1). As its name already reveals, CAND1 was identified as an interactor of non-neddylated cullins (Liu et al., 2002; Zheng et al., 2002; Oshikawa et al., 2003). Through a series of elegant experiments from at least three independent laboratories it was recently shown that CAND1 functions as a novel type of exchange factor for CRLs (Pierce et al., 2013; Wu et al., 2013; Zemla et al., 2013). In a highly quantitative and not only therefore remarkable analysis of the diverse protein–protein interactions that can take place between the subunits of SCF-type CRL complexes and CAND1, it could be shown that CAND1 can promote the disassembly of SCF complexes and that FBPs can remove CAND1 from CULLIN1 (Pierce et al., 2013; **Figure 4**). When testing 21 different FBPs it was found that 20 of these could be exchanged using CAND1 as an exchange factor. Thus, in the case of SCF-type CRLs and most likely also in the case of CRLs that are formed with the other cullins, CAND1 can modulate the CRL-complex repertoire of the cell.

CAND1 is unable to interact with neddylated cullins and cullin neddylation stabilizes specific CRLs to prevent substrate receptor exchange (Emberley et al., 2012). Upon cullin deneddylation, CAND1 can become active and modulate the CRL repertoire to optimally match substrate receptor demand. Thus, there must be mechanisms to control CRL deneddylation. Indeed, cullin neddylation and deneddylation are controlled by the presence and absence of degradation substrates or their interactions with substrate receptors (Bornstein et al., 2006; Chew and Hagen, 2007; Emberley et al., 2012). Furthermore, CSN binds preferentially to neddylated CRLs, which may also recruit CSN-associated proteins important for CRL regulation (den Besten et al., 2012). CSN can bind deneddylated cullins but is dissociated in the presence of degradation substrate receptors and degradation substrates (Choo et al., 2011). Consequently, it can be inferred that *csn* as well as *cand1* mutants are deficient in releasing specific substrate receptors. This hypothesis could be experimentally confirmed and it could be shown that substrate receptor activation and substrate degradation are delayed in such mutant backgrounds (Zemla et al., 2013).

CAND1 IN PLANTS

In plants, *cand1* mutants were identified and analyzed in reverse and forward genetic screens. Mutants deficient in CAND1 were isolated as auxin-resistant mutants and the mutant spectrum of *cand1* mutants was recognized as being highly similar to, but also to exceed that of *axr1* mutants (Cheng et al., 2004; Chuang et al., 2004; Feng et al., 2004). Three further *cand1* alleles were identified based on mutants with severe defects in leaf vein patterning (Alonso-Peral et al., 2006). In rice, CAND1 is required for the formation of crown roots and defects in crown root formation are associated with a cessation in the G2/M phase progression in these mutants (Wang et al., 2011). In this context it is interesting to note that also *Arabidopsis csn* mutants are defective in G2/M phase progression (Dohmann et al., 2008a).

Arabidopsis CAND1 also preferentially binds to non-neddylated cullins (Feng et al., 2004). Importantly, two sets of weak *Arabidopsis* mutants exist, the semi-dominant *axr6-1* and *axr6-2* on the one side and the recessive *cul1-6* on the other, carrying missense mutations in almost adjacent positions of CULLIN1. Interestingly, the respective mutant proteins interact differentially with CAND1. While the cul1-6 protein is deficient in CAND1 interaction, axr6-1 and axr6-2 bind more strongly to CAND1 (Feng et al., 2004; Moon et al., 2007). The availability of mutants with weak and strong defects in cullin function, cullin deneddylation, and CAND1 interaction has already permitted to assay the biochemical interactions of the various components at the genetic level (Zhang et al., 2008).

NEDDYLATION SUBSTRATES

EVIDENCE FOR NON-CULLIN NEDDYLATION SUBSTRATES

Despite extensive research, the role and importance of neddylation in cellular processes besides the regulation of CRL activity remains poorly understood. Contrary to the expanding knowledge about ubiquitylated proteins in eukaryotes including plants (Kim et al., 2013) similar studies for NEDD8 have so far not succeeded in consistently identifying non-cullin neddylated proteins (Li et al., 2006; Norman and Shiekhattar, 2006; Jones et al., 2008; Xirodimas, 2008; Bennett et al., 2010; Hakenjos et al., 2011; Hotton et al., 2012). However, there is evidence for the existence of a broad range of neddylated proteins and several non-cullin neddylated proteins have already been identified as summarized in **Table 1**. Loss of function mutants of DEN1/NEDP1/SENP8 from three different species, namely fruit fly, *Schizosaccharomyces pombe* and *Aspergillus nidulans* accumulate neddylated proteins over a broad range of molecular weights. At the same time, these mutants do generally not accumulate neddylated cullins suggesting that DEN1/NEDP1/SENP8 is an important deneddylating enzyme of these non-cullin neddylation substrates (Zhou and Watts, 2005; Chan et al., 2008; Christmann et al., 2013). Also overexpression of NEDD8 leads to the apparent enrichment of many neddylated proteins and this neddylation can be blocked with the inhibitor

Table 1 | Neddylation substrates.

Neddylated protein	Proposed function of neddylation	Species	Reference
E3 ubiquitin ligases			
Cullins, Cul7, and PARC	Increases activity	Eukaryotes	Hori et al. (1999), Sarikas et al. (2011), Calabrese et al. (2011)
Mdm2	Decreases activity	Human	Xirodimas et al. (2004)
Parkin	increases activity	Human	Um et al. (2012), Choo et al. (2012)
BRAP2	–	Human	Takashima et al. (2013)
pVHL	Changes pVHL protein interaction	Human	Stickle et al. (2004), Russell and Ohh (2008)
DIAP1/XIAP	-	Fruit fly/human	Broemer et al. (2010)
DDB1	-	*Arabidopsis*	Hotton et al. (2012)
Transcription factors			
p53	Inhibits transcriptional activity	Human	Xirodimas et al. (2004), Abida et al. (2007)
p73	Inhibits transcriptional activity by sequestering Tap73 to the cytoplasm	Human	Watson et al. (2006)
AICD	Inhibits transcriptional activity	Human	Lee et al. (2008)
E2F1	Inhibits transcriptional activity by blocking protein interaction	Human	Aoki et al. (2012), Loftus et al. (2012)
HIF1α	Stabilizes protein	Human	Ryu et al. (2011)
Transcriptional inhibitors			
BCA3	Activates by promoting protein interaction	Human	Gao et al. (2006)
RCAN1	Stabilizes by inhibiting proteasomal degradation	Human	Noh et al. (2012)
Receptors			
EGFR	Promotes receptor ubiquitylation and ligand induced degradation	Mammals	(Oved et al., 2006)
TβRII	Stabilizes protein	Human	(Zuo et al., 2013)
Kinases			
PINK1	Stabilizes the cytosolic protein form	Human	Choo et al. (2012)
CK1α	–	Human	Huart et al. (2012)
Other			
L11, S14, and other ribosomal proteins	Stabilizes the protein	Human	Xirodimas et al. (2008), Zhang et al. (2012)
SHC	–	Human	Jin et al. (2013)
HUR	Stabilizes the protein	Human	Embade et al. (2012)
Histone H4	Induces complex formation and amplifies Ubi cascade	Human	Ma et al. (2013)
drICE/caspase 7	Reduces catalytic activity	Fruit fly/human	Broemer et al. (2010)
Lag2	–	Yeast	Siergiejuk et al. (2009)
ML3	–	*Arabidopsis*	Hakenjos et al. (2013)

PARK, Parkin-like cytoplasmic protein; Mdm2, murine double minute 2; Parkin, Parkinson juvenile disease protein 2; BRAP2, BRCA1-associated protein; DIAP1, Drosophila inhibitor of apoptosis 1; XIAP, X-linked inhibitor of apoptosis protein; DDB1, damaged DNA binding protein1; VHL, Von Hippel–Lindau disease tumor suppressor; p53, cellular tumor antigen p53; p73, tumor protein p73; AICD, amyloid beta A4 protein; E2F1, transcription factor E2F1; HIF1α, hypoxia-inducible factor 3-alpha; BCA3, breast cancer-associated gene 3; RCAN1, regulator of calcineurin 1; EGFR, epidermal growth factor receptor; TβRII, transforming growth factor-beta receptor type II; PINK1, PTEN-induced putative kinase protein 1; CK1α, casein kinase 1 alpha; L11, ribosomal protein L11; S14, ribosomal protein S14; SHC, Src homology 2 domain-containing-transforming protein C1; HUR, Hu-antigen R; DrICE, Drosophila interleukin-1β-converting enzyme; Lag2, longevity-assurance protein 2; ML3, myeloid differentiation factor-2-related lipid-recognition domain protein 3.

MLN4924 (Hakenjos et al., 2011). Thus, there is evidence that NEDD8-modified proteins other than the cullins can exist but may be low in abundance or only transiently modified under normal conditions.

At the functional level, genetic experiments in *Schizosaccharomyces pombe* showed that the introduction of a specific *cullin1* mutant, which constitutively activates CRLs and therefore renders these CRLs independent from the neddylation machinery, was unable to rescue the phenotype of NEDD8 conjugation mutant *uba3-10* (Girdwood et al., 2011, 2012). This finding may suggest additional biological functions for neddylation that are impaired in *uba3-10* besides the neddylation defect of cullins.

IDENTIFICATION OF NEDDYLATION SUBSTRATES - A DIFFICULT ISSUE

A major problem for the identification of new neddylation substrates is the high sequence similarity between NEDD8 and ubiquitin. Both proteins are sequence identical at their C-termini just downstream of a trypsin cleavage site resulting in an identical di-glycine footprint on a modified protein after trypsin digestion for proteomic analyses (**Figure 1**). Therefore, a di-glycine modification on a lysine of a given peptide cannot unanimously be attributed to either ubiquitin or NEDD8 conjugation.

Ubiquitin and NEDD8 also show a remarkable similarity in their three-dimensional structure and key residues are conserved between the two proteins, most prominently three amino acids (L8, I44, and V70) in the hydrophobic patch that are involved in mediating ubiquitin-protein interactions (Rao-Naik et al., 1998; Whitby et al., 1998; Choi et al., 2009; Girdwood et al., 2011). Thus, a high substrate specificity is required to avoid leakage of ubiquitin or NEDD8 into the respective other modification pathway. Indeed, it has become apparent in the last years that there is a crosstalk between the NEDD8 and ubiquitin conjugation machineries (Hjerpe et al., 2012b; Leidecker et al., 2012; Singh et al., 2012). NEDD8 can be activated by the ubiquitin E1 UBA1 and once activated is conjugated to substrates in a manner similar to ubiquitin (Singh et al., 2012). Inversely, however, NAE specifically activates NEDD8 and does not use ubiquitin as a substrate (Singh et al., 2012). NEDD8 is incorporated in the ubiquitin pathway by UBA1 when the ratio of free NEDD8 to free ubiquitin, which under normal conditions is close to one, shifts toward NEDD8 (Hjerpe et al., 2012a; Leidecker et al., 2012). The leakage of NEDD8 into the ubiquitin pathway leads to the formation of mixed chains with NEDD8 possibly functioning as chain terminator. This mechanism is likely the explanation for the identification of NEDD8 chains in a proteomic study by Jones et al. (2008) using overexpression of a tagged NEDD8 construct. The ability of NEDD8 to form chain linkages is not essential *in vivo* as demonstrated by the viability of a *Schizosaccharomyces pombe* strain carrying a Ned8p mutant construct where all lysine residues that could potentially engage in chain formation were mutated to alanine (Girdwood et al., 2011). Depletion of cellular ubiquitin levels can be caused by knockdown or inhibition of the 26S proteasome but can also have physiological causes such as temperature or oxidative stress (Hjerpe et al., 2012a; Leidecker et al., 2012). While this atypical neddylation has been proposed to act as a stress response to ubiquitin depletion, it is still unclear whether this type of atypical neddylation is biologically relevant *in vivo* (Hjerpe et al., 2012a; Leidecker et al., 2012).

NOVEL NEDDYLATION SUBSTRATES

As outlined above, various studies have led to the identification of novel NEDD8-modified proteins. At the biochemical level, these proteins belong to different protein families including E3 ubiquitin ligases or transcription factors and neddylation has been shown to positively or negatively interfere with their activity (**Table 1**). Neddylation changes the biochemical properties of its target proteins by inducing conformational changes as well as allowing or precluding protein-protein interactions. Apart from its pleiotropic effects on protein degradation through CRL neddylation, neddylation has a prominent role in cell cycle regulation and cellular stress response pathways in human cells and has also been linked to Alzheimer and Parkinson disease (**Table 1**). In plants, the only known non-cullin neddylation substrates are DAMAGED DNA BINDING PROTEIN1 (DDB1) and ML3 from *Arabidopsis*. DDB1 is a subunit of a cullin4 E3 ubiquitin ligase and therefore biochemically close to the cells neddylation machinery (Hotton et al., 2012). ML3 is a protein with intriguing cell biological features that plays a role in pathogen responses (Hakenjos et al., 2013). Interestingly, there is also evidence that ML3 has the ability to bind neddylated proteins in a non-covalent manner. Since the precise biochemical function of ML3 remains to be determined, it is at present unclear what role neddylation has in the control of ML3 function.

Given the very recently acquired understanding of the close interplay between ubiquitylation and neddylation, new and old neddylation targets should undergo close scrutiny to ensure that they are genuine targets for NEDD8 modification (Hjerpe et al., 2012b). A catalog of appropriate characterization criteria has been published (Rabut and Peter, 2008).

CONCLUSION

In this review, we have summarized the current knowledge of the neddylation pathway in eukaryotes with an emphasis on the role of neddylation in plants. While the enzyme pathway for the conjugation and deconjugation of NEDD8 has been elucidated in plants, findings from other eukaryotic model organisms suggest that there are more, unknown players in this pathway that need to be identified to gain a full understanding of the process and its regulation. Particularly the areas of NEDD8 processing, NEDD8 ligases and the identification of non-cullin NEDD8 substrates will require further detailed investigations in the future. Also the presence of ubiquitin-NEDD8 fusion proteins is unique to plants. The analysis of their processing could bear information that may allow understanding how the highly homologous ubiquitin and NEDD8 proteins were derived from each other during evolution.

AUTHOR CONTRIBUTIONS

The authors have contributed in equal parts to the preparation of this manuscript.

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Deubiquitylating enzymes and their emerging role in plant biology

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Ubiquitylation is a reversible post-translational modification that is involved in various cellular pathways and that thereby regulates various aspects of plant biology. For a long time, functional studies of ubiquitylation have focused on the function of ubiquitylating enzymes, especially the E3 ligases, rather than deubiquitylating enzymes (DUBs) or ubiquitin isopeptidases, enzymes that hydrolyze ubiquitin chains. One reason may be the smaller number of DUBs in comparison to E3 ligases, implying the broader substrate specificities of DUBs and the difficulties to identify the direct targets. However, recent studies have revealed that DUBs also actively participate in controlling cellular events and thus play pivotal roles in plant development and growth. DUBs are also essential for processing ubiquitin precursors and are important for recycling ubiquitin molecules from target proteins prior to their degradation and thereby maintaining the free ubiquitin pool in the cell. Here, we will discuss the five different DUB families (USP/UBP, UCH, JAMM, OTU, and MJD) and their known biochemical and physiological roles in plants.

Keywords: deubiquitylation, DUB, USP, UBP, UCH, JAMM, OTU, MJD

INTRODUCTION

Post-translational modification through ubiquitin, or ubiquitylation, plays a key role in many aspects of plant development, growth and environmental- as well as immune responses (reviewed in; Vierstra, 2009, 2012). Ubiquitylation must therefore be strictly controlled and regulated at multiple steps during these processes. The attachment of ubiquitin to the target proteins is carried out by the sequential activities of the ubiquitin activating enzyme (E1), ubiquitin conjugating enzymes (E2s), and ubiquitin ligases (E3s) (reviewed in; Hershko and Ciechanover, 1998). The ubiquitylation status of the substrate proteins is also controlled by the activity of deubiquitylating enzymes (DUBs: also deubiquitinating enzymes or deubiquitinases), hydrolases that remove covalently attached ubiquitin molecules from substrates or hydrolyze the peptide bond between ubiquitin molecules. Notably, whereas the *Arabidopsis* genome encodes more than 1500 E3s (Vierstra, 2012), only around 50 DUBs can be identified. This may owe to the fact that in order to deubiquitylate their targets, DUBs may not need direct interaction with the target proteins themselves but rather interact with the ubiquitin chains and hence, DUBs can deal with a broader range of ubiquitylated target proteins.

DUBs have multiple key roles in the regulation of cellular events. Firstly, they are essential for the activation of ubiquitin molecules after translation. Ubiquitin is translated either as tandem linear ubiquitin repeats or fusion to ribosomal proteins in *Arabidopsis* (Callis et al., 1990, 1995) and has to be processed to single ubiquitin molecules by DUBs in order to be conjugated to their substrates (**Figure 1A**). Secondly, they are responsible for the recycling of the ubiquitin molecules by cleaving them off from the substrates prior their degradation either by the 26S proteasome or by vacuolar proteases (**Figure 1B**). In this way, DUBs contribute to maintain the free ubiquitin pool in the cell. Thirdly, DUBs can also actively regulate cellular processes by influencing the stability of proteins, in that they rescue proteins from degradation by deubiquitylating them before they are recognized by the degradation machinery (**Figure 1C**). Finally, by removing the ubiquitin molecule from its target, DUBs could affect the binding affinity of the target protein to its interactor protein and thereby regulate downstream processes (**Figure 1D**).

In eukaryotes, there are five DUB families that can be classified according to the difference in their catalytic domains [Reviewed in (Komander et al., 2009; Reyes-Turcu et al., 2009)]: the ubiquitin-specific proteases (UBPs or USPs), the ubiquitin C-terminal hydrolases (UCHs), the ovarian tumor proteases (OTUs), the Machado–Joseph domain (MJD)- or Josephine domain proteases and the JAB1/MPN/MOV34 (JAMM) proteases. All DUBs are cysteine proteases, except DUBs of the JAMM family, which are zinc metalloproteases that require a coordinated Zinc ion in their active sites. Some of the DUBs display also hydrolysis activity toward other ubiquitin-like proteins, like Nedd8/RUB (Kumar et al., 1993; Callis et al., 1995; Hochstrasser, 1996), SUMO (Matunis et al., 1996), or ISG15/UCRP (Loeb and Haas, 1992), suggesting a complex regulatory mechanism surrounding ubiquitin- and ubiquitin-like modifications.

In most of the cases, interaction of DUBs with their target proteins is mediated outside of the catalytic domain by scaffold proteins or adaptor proteins whereas structural characteristics of the catalytic domain mediate the specificity toward certain ubiquitin linkages (reviewed in; Komander et al., 2009). The structure of the catalytic domains also determines whether the DUB cleaves ubiquitin chains from the distal or the proximal end. Only a few cases were reported in which the DUB were shown to interact directly with its ubiquitylated substrate protein (reviewed in; Reyes-Turcu et al., 2009). In addition,

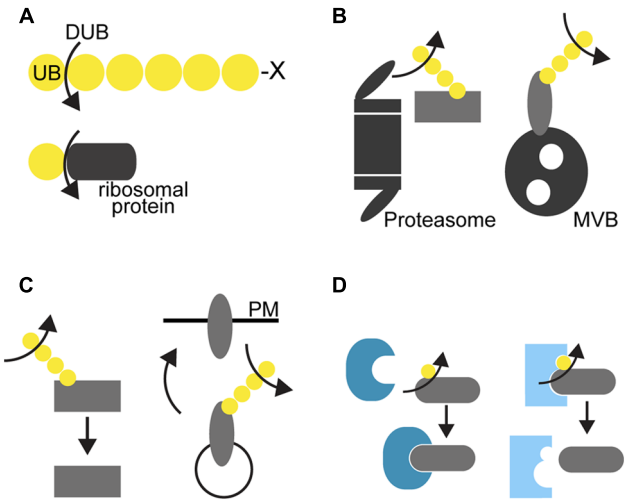

FIGURE 1 | Cellular function of DUBs. (A) Ubiquitin is translated as tandem ubiquitin repeats with several amino acid extension (depicted as X) at the C-terminus or as fusion to ribosomal proteins in plants. DUBs process the peptide bond between ubiquitin and its fusion protein to produce ubiquitin monomers that can be then conjugated to its substrate proteins. **(B)** DUBs can remove ubiquitin chains from its target proteins and recycle ubiquitin molecules prior to degradation by the 26S proteasome (left) or before the sequestration into the intraluminal vesicles of the multivesicular body (right). Deubiquitylation can start at the distal end as shown here or at the proximal end or in the interior of polyubiquitin chains. **(C)** Removal of the ubiquitin chains by DUBs can inhibit their recognition by the degradation machinery and thus rescues them from degradation regardless whether the protein is a cytosolic proteasomal substrate (left) or a membrane cargo (right). **(D)** Ubiquitylation can serve as an interaction signal for the modified protein. By removing the ubiquitin moiety, DUBs could change the binding affinity of its target protein to another protein, either by enabling (left) or by disabling the binding of the unmodified protein to its interacting protein.

DUB activity can be regulated at the transcriptional or post-translational level in that the DUBs themselves can be phosphorylated, SUMOylated or ubiquitylated (reviewed in; Huang and Cochran, 2013). Thus, it is difficult to associate a specific DUB family to one cellular process, rather, the biochemical and physiological function of each DUB has to be examined individually.

As summarized in the following sections, accumulating evidence indicate an important role of DUBs not only in yeast and mammals but also in various aspects of plant biology. However, in contrast to target protein regulation by the ubiquitylation machinery, understanding of the molecular mechanisms of cellular and physiological functions of DUBs in plants has just started.

DUB FUNCTION IN PLANTS

UBIQUITIN-BINDING PROTEINS

The UBPs form the largest subfamily of cysteine protease DUBs in *Arabidopsis* with 27 members that can be classified in 14 subfamilies based on their domain organization (Yan et al., 2000; **Figure 2A**). Most of the UBPs have additionally to their catalytic domain further domains that enable them to interact with different proteins, allowing UBPs to be involved in a broad range of biological processes. However, to date, the molecular functions of UBPs are far from being well resolved in plants, UBP26 being the only member among this family for which the target protein, Histone H2B, is identified (Sridhar et al., 2007).

UBP1 and UBP2 are close homologs to each other and are unique to plants (Yan et al., 2000). They are both active DUBs that can hydrolyze K48-linked ubiquitin chains *in vitro*. T-DNA insertion mutants *ubp1* and *ubp2* are phenotypically indistinguishable from wild type plants under normal conditions or standard stress conditions that were tested for known proteasomal mutants. However, when grown in the presence of the Arg analog canavanine, mutants show stunted growth, shorter roots and display chlorotic leaves, indicating that UBP1 and UBP2 are necessary for resistance to canavanine.

UBP3 and UBP4 are highly homologous to each other (Doelling et al., 2007). *ubp3* and *ubp4* single mutants do not show obvious phenotypes whereas the double mutant *ubp3ubp4* shows lethality, indicating redundant functions between UBP3 and UBP4. UBP3 and UBP4 are probably required for pollen transmission since *ubp3ubp4* is defective in gametogenesis and shows also pollen germination defects.

UBP12 and UBP13 show activity toward K48-linked diubiquitin (Ewan et al., 2011). The *ubp12ubp13* double mutant, but not the single mutants, showed lethality, indicating redundant functions between *UBP12* and *UBP13*. *UBP12* and *UBP13* were identified as genes that were up-regulated in response to *Pst* DC3000 infection (Brazma et al., 2003). Accordingly, RNAi line that has reduced levels of both *UBP12* and *UBP13* shows increased disease resistant upon *P. syringae* infection. These results indicated that both UBP12 and UBP13 act as negative regulators in *Arabidopsis* immune response.

Arabidopsis UBP14 is a functional homolog of the yeast Ubp14p and is a ubiquitously expressed DUB that cleaves K48-linked chains and Ub-X-βgal, but not UBQ1 (Doelling et al., 2001). The *ubp14* mutant arrested growth during embryo development. The arrested embryos accumulated high amount of ubiquitylated proteins, indicating that UBP14 is an essential DUB, required for proper embryogenesis. Interestingly, UBP14 was also identified as the causative gene of an EMS mutant *phosphate deficiency root hair defective 1* (*per1*) which is defective in Pi deficiency-induced root hair formation (Li et al., 2010). *per1* shows reduced levels of UBP14/PER1 protein, and failed to respond to Pi starvation by increasing the frequency and length of root hairs, implicating UBP14/PER1 function also in the adaptation to changes in phosphate/nutrient availability in the environment.

UBP15 can cleave peptide bonds between tandem ubiquitin and localizes both to the cytosol and the nucleus (Liu et al., 2008). *UBP15* is mainly expressed in leaves, which is in accordance to its proposed function in defining leaf pattern and shape of the leaf margin by controlling cell proliferation. Many genes including cell cycle or flowering genes are misregulated in the *ubp15* mutant, which may be the cause for the developmental defects observed in this mutant. Further genetic analysis has suggested that UBP15 and UBP16 might function redundantly. The *ubp19* mutant was described as embyo-lethal, but no further analysis is yet performed.

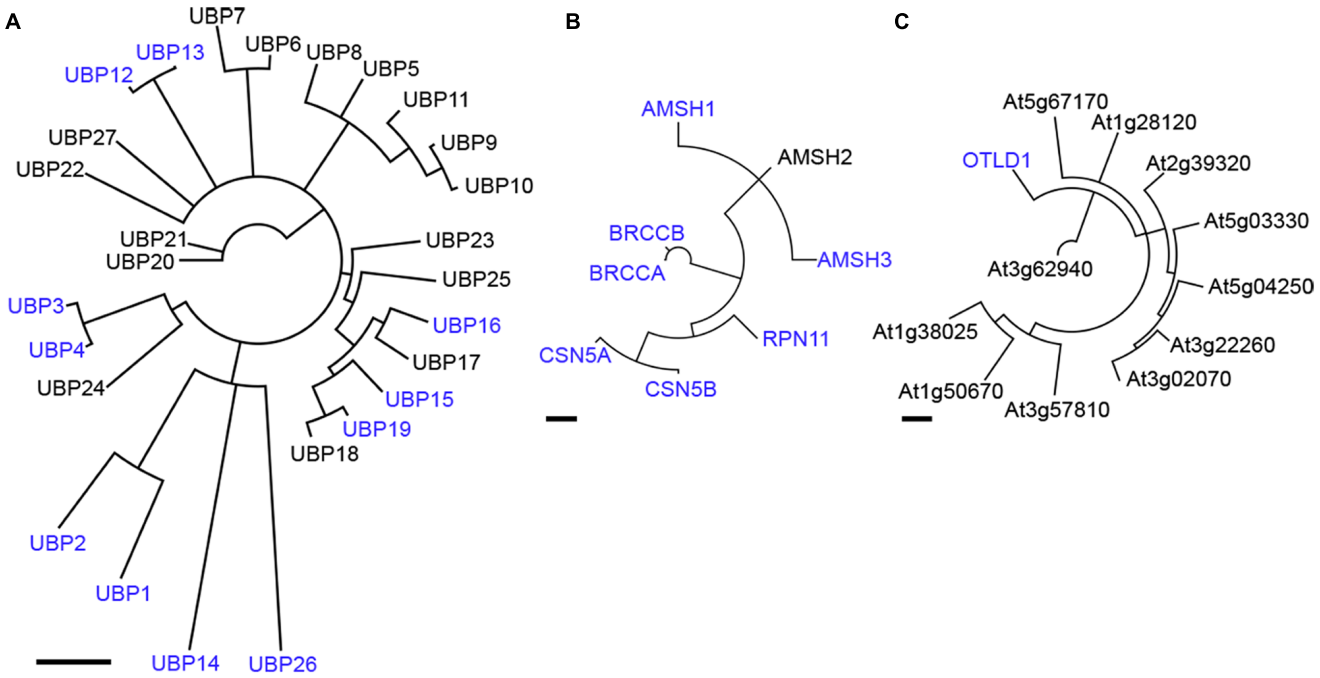

FIGURE 2 | Phylogenetic analyses of *Arabidopsis* UBP-, JAMM-, and OTU-domain proteinases. A Neighbor-Joining consensus tree based on amino acid sequences surrounding the catalytic domain for *Arabidopsis* UBP- **(A)**, JAMM- **(B)** and OTU- **(C)** domain proteins is shown. Scale bars indicate 0.5 aa substitutions per site. DUBs mentioned in the text are highlighted in blue. Note that CSN5A and CSN5B shown in **(B)** are deneddylating- and not deubiquitylating enzymes.

UBP26/SUP32 was first identified in a suppressor screen of *ros1-1*, a mutant with enhanced gene silencing (Sridhar et al., 2007). UBP26 can deubiquitylate monoubiquitylated histone H2B *in vitro*, and the *ubp26-1* mutant accumulates ubiquitylated histone H2B. Further experiments suggested that histone H2B deubiquitylation by UBP26 is important for heterochromatic histone H3 methylation and DNA methylation and hence, for proper gene silencing. Further studies have identified the MADS-box gene *PHERES1* to be probably under this regulation. A T-DNA insertion line of *UBP26* arrested growth at the embryo stage, probably due to the misregulation of *PHERES1* that is normally under the strict regulation of genomic imprinting (Luo et al., 2008).

The *ubp26-1* mutant also shows misregulation of the *FLOWERING LOCUS C* (*FLC*) gene, which leads to an early flowering phenotype of the mutant (Schmitz et al., 2009). Expression of *FLC* is decreased in the *ubp26-1* mutant and ubiquitylated H2B was observed to accumulate in the *FLC* chromatin. Deubiquitylation of H2B by UBP26 probably keeps the levels of H3K27me3 low, thereby allowing activation of *FLC* gene expression. UBP26, together with OTLD1 that is mentioned below, are examples in which DUBs play an active regulatory function that is not directly associated with protein degradation.

UBIQUITIN C-TERMINAL HYDROLASES

Deubiquitylating enzymes of this family contain a UCH domain, first identified in the yeast Uch1p, which has a structural feature distinct from other DUBs. Mutational studies based on human UCH proteins have revealed a size-filtering mechanism that allows UCH proteins to hydrolyze small ubiquitin adducts more efficiently than ubiquitin chains or large ubiquitin fusion proteins (Popp et al., 2009). For this specificity, UCH proteins are thought to be mainly involved in ubiquitin recycling rather than regulating substrate proteins through deubiquitylation, though several mammalian studies also indicate regulatory roles for UCH family DUBs.

In *Arabidopsis*, three UCH domain proteins were identified and characterized (Yang et al., 2007). *Arabidopsis* UCH1 and UCH2 contain a related C-terminal extension of 100 aa that is missing in UCH3. UCH2 was shown to be able to cleave peptide and/or isopeptide bonds bound to ubiquitin and showed activity toward K48 chains *in vitro*. UCH1 and UCH2 are expressed ubiquitously and GFP-fusion proteins of UCH1 and UCH2 are localized to the nucleus like the 26S proteasome, however, stable association with the proteasome could not be demonstrated. Both UCH1 overexpressing plants as well as a *uch1uch2* double mutant show a number of developmental phenotypes including altered sensitivity to auxin and cytokinins. Moreover, auxin signaling mutants *axr1-3* and *axr2* show both synergy with UCH1 overexpressing lines and in accordance with this, stability of AUX/IAA proteins were found to be specifically modified in the UCH1 overexpressor and *uch1-1uch2-1* double mutant, indicating the involvement of UCH proteins in the auxin signaling pathway.

JAMM DOMAIN PROTEINS

The JAMM domain DUBs are zinc metalloproteases that contain a catalytic MPN+ domain (Maytal-Kivity et al., 2002). The MPN+ domain coordinates two zinc ions that activate a water molecule to attack the ubiquitin isopeptide bond. One member of the family, human AMSH-Like protease (AMSH-LP), was the first DUB that was co-crystalized with diubiquitin and structural studies provided insightful information regarding the K63-specificity of this DUB (Sato et al., 2008). Eight JAMM domain proteins are present in *Arabidopsis* (**Figure 2B**), most of them being associated with key regulatory roles. CSN5, which is encoded by two homologous genes *CSN5A* and *CSN5B* in *Arabidopsis*, is a JAMM domain protease and the catalytic subunit of the COP9 signalosome that specifically hydrolyzes the ubiquitin-like molecule Nedd8/RUB, rather than ubiquitin (Chamovitz et al., 1996; Cope et al., 2002).

RPN11 was first identified as a subunit of the 26S proteasome regulatory particle in yeast (Glickman et al., 1998) and was subsequently shown to possess deubiquitylating activity (Verma et al., 2002). *Arabidopsis* has one homolog of RPN11, which was shown to be part of the purified *Arabidopsis* 26S proteasome (Book et al., 2010). RPN11 function is primarily required for the deubiquitylation of proteasomal substrates prior to degradation and recycling of ubiquitin molecules.

In contrast to RPN11 and CSN5, AMSH3 is not a stable subunit of a multi protein complex (Isono et al., 2010). It is an essential DUB in *Arabidopsis*, since the *amsh3* null mutants show seedling lethality and a number of intracellular trafficking defects, implicating its function in this pathway. AMSH1, an AMSH3 homolog, and AMSH3 both interact with ESCRT-III subunits (Katsiarimpa et al., 2011, 2013) and are probably involved in the deubiquitylation of plasma membrane cargos at the multivesicular body. Furthermore, mutants of both *amsh1* and ESCRT-III show defects in autophagic degradation, indicating that the ESCRT-III- and AMSH-dependent trafficking pathway is also contributing to the regulation of autophagy (Katsiarimpa et al., 2013).

Arabidopsis BRCC36A and BRCC36B are homologs of mammalian BRCC36, a DUB that was shown to interact with a protein complex containing BRCA1 (Dong et al., 2003) and is recruited to the site of DNA damage (Inui et al., 2011). Though *Arabidopsis brcc36* mutants are viable and phenotypically indistinguishable from wild-type plants, the *brcc36a* mutant shows defects in intra- and inter-chromosomal homologous recombination as well as in DNA crosslink repair (Block-Schmidt et al., 2011). BRCC36 was also shown to be epistatic to BRCA1, indicating its involvement in BRCA1 regulation, probably as part of a multi protein complex including BRCC36 and BRCA1, as proposed in other organisms.

OVARIAN TUMOR PROTEASES

Ovarian tumor proteases are cysteine protease DUBs that contain the OTU-domain, which was first identified in the product of the drosophila *ovarian tumor* gene and is found in virus, bacteria and eukaryotic organisms (Kumar et al., 1993). A recent study using structural and enzymatic analyses of OTU proteases have revealed the mechanism of ubiquitin linkage specificity of human OTU DUBs (Mevissen et al., 2013). The *Arabidopsis* genome contains 12 OTU domain-containing genes (**Figure 2C**) most of which are uncharacterized yet. OTUs are involved in a variety of cellular processes in yeast and mammals but in plants so far only one OTU-protein, OTLD1, was characterized in relation to a specific biological process.

OTLD1 is an otubain-like DUB that was found in a yeast two-hybrid screen using the histone demethylase KDM1C as bait (Krichevsky et al., 2011). Thus OTLD1, like UBP26 mentioned above, is implicated in histone deubiquitylation. OTLD1 was shown to bind to histones and possess DUB activity specifically toward ubiquitylated H2B but not toward H2A. In both a KDM1C mutant *swp1-1* and an *otld1* T-DNA insertion mutant, gene derepression was observed, indicating that KDM1C and OTLD1 function together to repress gene expression via histone deubiquitylation.

MACHADO–JOSEPH DOMAIN

MJD DUBs are named after the chronic degenerative Machado–Joseph disease. In MJD patients, a cysteine proteinase DUB called Ataxin 3 is modified in its poly Q tract, which probably causes alteration in its structure and interaction with other proteins (reviewed in; Costa Mdo and Paulson, 2012). Ataxin 3 contains the catalytic DUB domain named Josephine-domain and is implicated in proteasome-dependent protein quality control. *In silico* search in the *Arabidopsis* genome database shows three Josephine domain-containing proteins (AT1G07300, AT2G29640, and AT3G54130), the function of which has yet to be elucidated.

FUTURE RESEARCH ON PLANT DUBs

Although many lines of evidence suggest that not only ubiquitylating enzymes but also DUBs can actively regulate substrate fate, elucidation of the molecular function of individual DUBs in plants has just begun. Studies in the past decade, mainly conducted using yeast and mammalian models, have shown important house keeping- as well as diverse regulatory functions of DUBs in different pathways.

DUBs like the yeast Doa4p (Dupre and Haguenauer-Tsapis, 2001), human and plant AMSH proteins (McCullough et al., 2004; Isono et al., 2010; Katsiarimpa et al., 2011) and human USP8/USPY (Mizuno et al., 2006; Row et al., 2006) were shown to be involved in the regulation of cargo endocytosis and stability. However, it is still an open question whether ubiquitylated endocytosis cargos are direct targets of these DUBs.

DUBs regulate their substrates not only by determining their proteolytic fate. For example, as it was also shown in plants, histone H2A or histone H2B ubiquitylation status is controlled by multiple DUBs (Joo et al., 2007; Sridhar et al., 2007; Zhu et al., 2007; Nakagawa et al., 2008; Schmitz et al., 2009). The ubiquitylation status of histones affects their methylation status and thus controls gene expression in the corresponding chromatin region. In TGFß signaling, two human DUBs, USP9x, and USP15, were shown to control monoubiquitylination of their substrates Smad4 and R-SMADs, respectively. The ubiquitylation status of these proteins affects their DNA-binding capacity and hence downstream gene activation (Dupont et al., 2009; Inui et al., 2011). It is an

intriguing future topic whether plant DUBs are also part of the conserved or plant-specific signaling cascades.

As these examples show, in addition to the understanding of the spatio-temporal regulation of DUBs themselves, the identification of DUB substrates is crucial for the elucidation of individual DUB function. Since structural studies indicate that in most of the studied cases the interaction of DUBs with the ubiquitin chain, but not with their specific target proteins, is the prerequisite for deubiquitylation, the identification of *bona fide* DUB targets is not an easy task. With the advance in quantitative proteomics coupled with the use of suitable mutants and biochemical tools, it is to be expected that we will get a better insight into plant DUB targets in the near future. Further studies should reveal the sophisticated balancing mechanisms of ubiquitylation and deubiquitylation by which substrate fate and thus important intracellular and physiological processes in plants are regulated.

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Plasma membrane-association of SAUL1-type plant U-box armadillo repeat proteins is conserved in land plants

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Post-translational protein modification plays a pivotal role in the regulation and specific turnover of proteins. One of these important modifications is the ubiquitination of target proteins, which can occur at distinct cellular compartments. At the plasma membrane, ubiquitination regulates the internalization and thus trafficking of membrane proteins such as receptors and channels. The Arabidopsis plant U-box (PUB) armadillo repeat (PUB-ARM) ubiquitin ligase SAUL1 (SENESCENCE-ASSOCIATED UBIQUITIN LIGASE1) is part of the ubiquitination machinery at the plasma membrane. In contrast to most other PUB-ARM proteins, SAUL1 carries additional C-terminal ARM repeats responsible for plasma membrane-association. Here, we demonstrated that the C-terminal ARM repeat domain is also essential and sufficient to mediate plasma membrane-association of the closest Arabidopis paralog AtPUB43. We investigated targeting of PUB-ARM ubiquitin ligases of different plant species to find out whether plasma membrane-association of SAUL1-type PUB-ARM proteins is conserved. Phylogenetic analysis identified orthologs of SAUL1 in these plant species. Intracellular localization of transiently expressed GFP fusion proteins revealed that indeed plasma membrane-association due to additional C-terminal ARM repeats represents a conserved feature of SAUL1-type proteins. Analyses of transgenic Arabidopsis plants overexpressing N-terminally masked or truncated proteins revealed that interfering with the function of SAUL1-type proteins resulted in severe growth defects. Our results suggest an ancient origin of ubiquitination at the plasma membrane in the evolution of land plants.

Keywords: SAUL1, U-box, ARM repeat, armadillo, plasma membrane, ubiquitin

INTRODUCTION

Post-translational modifications of proteins regulate their function and abundance. Ubiquitination of target proteins, in which the C-terminus of ubiquitin is covalently bound to other proteins and attached to itself in growing ubiquitin chains, modifies protein function in case of mono-ubiquitination and triggers degradation of poly-ubiquitinated proteins via the 26S proteasome. E3 ubiquitin ligases are crucial for attachment of ubiquitin to target proteins that are specifically recognized. This specific ubiquitination of proteins via E3 ubiquitin ligases requires the preceding activation of ubiquitin by E1 ubiquitin-activating enzymes and the subsequent transfer to E2 ubiquitin-conjugating enzymes.

Different types of E3 ubiquitin ligases have been classified (Vierstra, 2009). Among these, plant U-box (PUB) proteins represent the most recently identified type of E3 ubiquitin ligases. In Arabidopsis, the group of PUB proteins comprises 64 members (Azevedo et al., 2001; Wiborg et al., 2008). These PUB proteins contain the highly conserved U-box that is required for E2 binding (Pringa et al., 2001). In addition, most of the members contain armadillo (ARM) repeats, which likely constitute interfaces for protein-protein interactions, and are thus named PUB-ARM proteins. They support various functions during plant growth and development such as light and hormone signaling, self-incompatibility, cell death and senescence, as well as stress and pathogen response. Up to now, the latter responses involve the largest number of PUB-ARM proteins indicating a substantial function of this type of E3 ubiquitin ligases for plant survival following stress or pathogen attack (Raab et al., 2009; Yee and Goring, 2009; Mbengue et al., 2010; Bergler and Hoth, 2011; Liu et al., 2011; Park et al., 2011; Salt et al., 2011; Li et al., 2012; Vogelmann et al., 2012).

Specific functions of PUB-ARM proteins depend on their intracellular targeting that is likely defined by the presence of ARM repeat domains. Generally, these domains have been implicated in the localization of eukaryotic ARM repeat proteins to the nucleus, to the cytoplasm, or to polymerized actin (Aberle et al., 1994; Hulsken et al., 1994; Graham et al., 2000; Coates et al., 2006; Yang et al., 2007). For PUB-ARM proteins, localization in nucleus and cytoplasm as well as association with the endoplasmic reticulum (ER) and with the plasma membrane has been demonstrated (Amador et al., 2001; Stone et al., 2003; Cho et al., 2008; Samuel et al., 2008; Drechsel et al.,

2011). Plasma membrane-association of the Arabidopsis SAUL1 (SENESCENCE ASSOCIATED UBIQUITIN LIGASE 1) protein depends on an elongated C-terminus with additional ARM repeat domains that are unique to SAUL1 and its two paralogs AtPUB42 and AtPUB43. Whereas deletion of these additional ARM repeat domains resulted in the loss of plasma membrane-association, these C-terminal ARM repeats were sufficient to mediate plasma membrane-association of other cytoplasmic proteins (Drechsel et al., 2011).

Here, we were aiming to identify PUB-ARM proteins with elongated C-terminus carrying additional ARM repeats in other plant species and to test whether the presence of these C-terminal ARM repeat domains also mediates plasma membrane-association of these orthologs of the Arabidopsis SAUL1 protein.

MATERIALS AND METHODS

CLONING OF DNA CONSTRUCTS

For PCR amplification of DNA fragments, cDNAs were isolated from the aerial parts of young *Arabidopsis thaliana* seedlings grown on soil at long day-conditions (16 h light/8 h dark) at 22°C for 2 weeks, leaves of *Populus trichocarpa* plants grown on soil at long day-conditions (16 h light/8 h dark) for several months, *Physcomitrella patens* grown in sterile culture, and from *Oryza sativa* ssp. *Japonica* cv. *Nipponbare* leaf material from seedlings grown on soil in long day-conditions (16 h light/8 h dark) at 26°C for 4 weeks. Total RNA was isolated with Trizol reagent (Invitrogen, Karlsruhe, Germany). RT-PCRs were performed with the High Capacity RNA-to-cDNA Master Mix (Invitrogen). For generation of fusion proteins between full-length or truncated PUB-ARM proteins and GFP, the respective open reading frames were amplified by PCR from cDNA using the primer pairs listed in Supporting **Table S1**. The reverse primers harbored a wobble base to generate PCR fragments with or without a stop codon. The amplified fragments were cloned into pENTR/D-TOPO (Invitrogen, Karlsruhe, Germany), verified by sequencing, and recombined into destination vectors pMDC43 (Curtis and Grossniklaus, 2003) for GFP fusion to the N-terminus and pK7FWG2.0 (Karimi et al., 2002) for fusion of GFP to the C-terminus.

PROTOPLAST ISOLATION, TRANSFORMATION, AND CONFOCAL ANALYSIS

Protoplasts were isolated from fully expanded leaves of 3–4 week-old Arabidopsis plants grown on soil. Leaves were roughened using sandpaper, transferred to protoplasting buffer (500 mM sorbitol, 1 mM $CaCl_2$, 0.03% pectolyase Y23, 0.75% cellulose YC and 10 mM MES-KOH, pH 5.6–6.0), and incubated in the dark at 22°C for 1.5 h with gentle agitation (60–75 rpm). Protoplasts were separated from undigested material by filtration through a 50 µm nylon mesh and sedimented by centrifugation for 8 min at 100 × g. The pellet was resuspended in MaMg buffer (400 mM sorbitol, 15 mM $MgCl_2$, 5 mM MES-KOH, pH 5.6). Protoplast transformation was essentially performed as previously described (Abel and Theologis, 1994). Transformed protoplasts were transferred into small petri dishes and incubated for 24 h in the dark at 22°C prior to analysis by confocal laser scanning microscopy as described previously (Drechsel et al., 2011).

TRANSIENT TRANSFORMATION OF TOBACCO LEAVES

For transient transformation of *Nicotiana benthamiana* leaves, *Agrobacterium tumefaciens* strain C58C1 (Deblaere et al., 1985) harboring the respective DNA construct was grown at 29°C in LB supplemented with 50 µg ml^{-1} kanamycin to the stationary phase. Bacteria were sedimented by centrifugation at 5000 g for 15 min at room temperature and resuspended in infiltration buffer (10 mM $MgCl_2$, 10 mM MES, KOH pH5.7). Cells were infiltrated into the abaxial air spaces of 2–4-week-old *N. benthamiana* plants. GFP fluorescence was monitored by confocal laser scanning microscopy 24–48 h past infiltration as described previously (Drechsel et al., 2011).

PHYLOGENETIC ANALYSIS

A multiple sequence alignment of 150 PUB-ARM proteins from Arabidopsis (*A. thaliana*), rice (*Oryza sativa*), poplar (*Populus trichocarpa*) and moss (*Physcomitrella patens*) was generated employing MAFFT 6 (Katoh et al., 2005) with the E-INS-i routine and the BLOSUM 45 matrix at http://mafft.cbrc.jp/alignment/server/. In the first phylogenetic analysis, the full alignment (2683 amino acid positions) was used, in a second approach a reduced alignment without any gap position (210 amino acids positions) was employed. Bayesian phylogenetic analyses were performed with MrBayes 3.1.2 (Huelsenbeck and Ronquist, 2001). We assumed the WAG model of amino acid evolution (Whelan and Goldman, 2001) with gamma distribution of substitution rates. Metropolis-coupled Markov chain Monte Carlo (MCMCMC) sampling was performed with one cold and three heated chains. Two independent runs were performed in parallel for 5 million generations each. Starting trees were random and the trees were sampled every 1000th generation. Posterior probabilities were estimated on the final 4000 trees (burnin = 1000).

RESULTS

PLASMA MEMBRANE-ASSOCIATION OF PUB43 DEPENDS ON ITS C-TERMINAL ARM REPEAT DOMAIN

Plasma membrane-association was observed for the two SAUL1 paralogs AtPUB42 and AtPUB43, but not for any other member of the Arabidopsis PUB-ARM protein family (Drechsel et al., 2011). For SAUL1 it has been demonstrated that this specific localization depends on the additional ARM repeat domain in the C-terminus that is unique to SAUL1, AtPUB42, and AtPUB43. The domain structure of AtPUB43 is schematically depicted in **Figure 1A**. To test whether the C-terminal ARM repeat domain is also essential for plasma membrane-association of AtPUB43, we analyzed the localization of fusion proteins between GFP and truncated AtPUB43 proteins by confocal laser scanning microscopy on transformed Arabidopsis protoplasts. As for SAUL1, deletion of the N-terminal part of AtPUB43 in AtPUB43ΔARM$_{1-6}$-GFP proteins did not result in the loss of plasma membrane-association (**Figure 1B**). This deletion protein, however, was not equally distributed in the plasma membrane, but occurred in large patches (**Figures 1B,C**). A similar pattern has been observed for SAUL1 proteins with truncated and/or masked N-terminus previously (Drechsel et al., 2011). In a next step, either ARM repeats 7–12 or 10–12 were deleted in AtPUB43. Both proteins, AtPUB43ΔARM$_{7-12}$-GFP

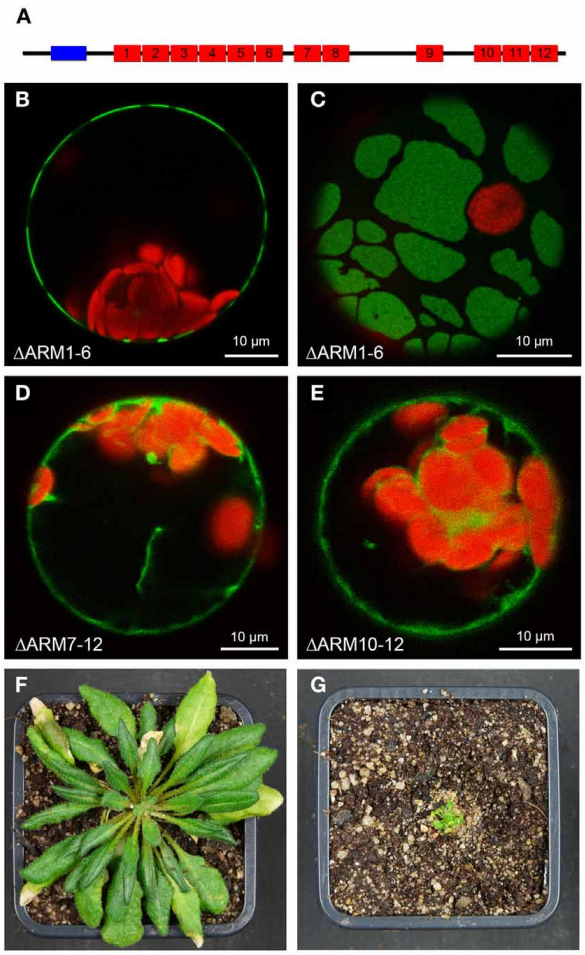

FIGURE 1 | Involvement of C-terminal ARM repeats in plasma membrane-association of AtPUB43. (A) Schematic representation of the AtPUB43 protein domain structure. U-box and ARM repeats are depicted in blue and red, respectively. **(B)** Confocal analysis of fluorescence of an Arabidopsis leaf protoplast expressing AtPUB43ΔARM$_{1-6}$-GFP fusion proteins. GFP fluorescence depicted in green was only detected at the plasma membrane. Chlorophyll auto-fluorescence within the chloroplasts is depicted in red. **(C)** Confocal analysis of fluorescence of an Arabidopsis leaf protoplast expressing AtPUB43ΔARM$_{1-6}$-GFP fusion proteins. The representative top-view showed that GFP signals and thus the AtPUB43ΔARM$_{1-6}$-GFP fusion proteins were localized in membrane patches. **(D)** Confocal analysis of fluorescence of an Arabidopsis leaf protoplast expressing AtPUB43ΔARM$_{7-12}$-GFP fusion proteins. GFP fluorescence depicted in green was detected in the cytosol. Chlorophyll auto-fluorescence within the chloroplasts is depicted in red. **(E)** Confocal analysis of fluorescence of an Arabidopsis leaf protoplast expressing AtPUB43ΔARM$_{10-12}$-GFP fusion proteins. GFP fluorescence depicted in green was detected in the cytosol. Chlorophyll auto-fluorescence within the chloroplasts is depicted in red. **(F)** Growth phenotype of wildtype plants grown for 12 weeks in short-day conditions. Photon flux density was 80–100 µmol m^{-2} s^{-1}. **(G)** Growth phenotype of CaMV35S::YFP-SAUL1 plants grown for 12 weeks in short-day conditions. Photon flux density was 80-100 µmol m^{-2} s^{-1}.

and AtPUB43ΔARM$_{10-12}$-GFP, were localized to the cytoplasm and not to the plasma membrane (**Figures 1D,E**). These data indicated that indeed the C-terminal ARM repeat domain is essential and sufficient for plasma membrane-association of AtPUB43 and that this is a general feature of SAUL1-type E3 ubiquitin ligases. The analysis of transgenic Arabidopsis plants overexpressing GFP-AtPUB43ΔARM$_{1-6}$ showed that the C-terminus by itself had no effect on plant growth and development (**Figure S1**). To test whether masking the N-terminus of SAUL1-type proteins, which eventually leads to patchy distribution of the protein at the plasma membrane (Drechsel et al., 2011 and **Figure 2E**), may affect growth and development, we analyzed CaMV35S::YFP-SAUL1 plants. Indeed, overexpression of YFP-SAUL1 fusion proteins resulted in a severe growth defect when compared to growth of wildtype plants (**Figures 1F,G**).

SAUL1-TYPE PUB-ARM PROTEINS FROM RICE ARE ASSOCIATED TO THE PLASMA MEMBRANE

Recently, the PUB-ARM protein family has been described in rice (Zeng et al., 2008). Five members of this protein family, namely OsPUB21, OsPUB22, OsPUB23, OsPUB24, and OsPUB25 possess an elongated C-terminus and carry an additional C-terminal ARM repeat domain like their Arabidopsis orthologs. We investigated whether the rice SAUL1 orthologs were also associated to the plasma membrane. For that purpose, GFP-OsPUB23 fusion proteins were expressed in Arabidopsis protoplasts and their fluorescence analyzed by confocal laser scanning microscopy. GFP fluorescence indicated plasma membrane-association of GFP-OsPUB23 (**Figure 2A**). To confirm this localization, GFP-OsPUB23 was co-expressed with AtINT4-RFP. The AtINT4 inositol transport protein was localized to the plasma membrane as described previously (**Figure 2B**, c.f. Schneider et al., 2006). Merging green and red fluorescence of GFP-OsPUB23 and AtINT4-RFP, respectively, clearly showed that OsPUB23 is associated to the plasma membrane (**Figures 2C,D**). When isolating the cDNA clone of *OsPUB21*, we not only found the long form *OsPUB21.1*, but also recovered the truncated *OsPUB21.2* missing the additional C-terminal ARM repeat domain. Whereas GFP-OsPUB21.1 was also associated to the plasma membrane (**Figure 2E**), GFP-OsPUB21.2 was not localized to the plasma membrane but to the cytoplasm (**Figure 2F**). Masking the N-terminus of Os-PUB21.1 by GFP again resulted in patchy distribution at the plasma membrane (**Figure 2E**). Indeed, rice SAUL1-type PUB-ARM proteins were associated to the plasma membrane and this localization was dependent on the elongated C-terminus carrying the additional ARM repeat domain.

IDENTIFICATION AND PHYLOGENETIC ANALYSES OF PUB-ARM PROTEINS FROM *POPULUS TRICHOCARPA* AND *PHYSCOMITRELLA PATENS*

To test whether SAUL1-type PUB-ARM proteins with additional C-terminal ARM-repeats exist in other plants and in mosses, we were aiming to identify PUB-ARM proteins in *Populus trichocarpa* and in the moss *Physcomitrella patens*. For that purpose, we used the protein sequences of SAUL1, AtPUB42, and AtPUB43 including their elongated C-terminus to BLAST search the respective genomes on the phytozome website (www.phytozome.net). When using this approach to identify PUB-ARM proteins in rice, we could re-identify all PUB-ARM proteins that were recently published by Zeng et al. (2008). We therefore decided to follow this approach on *P. trichocarpa* and *P. patens*. In *P. trichocarpa*

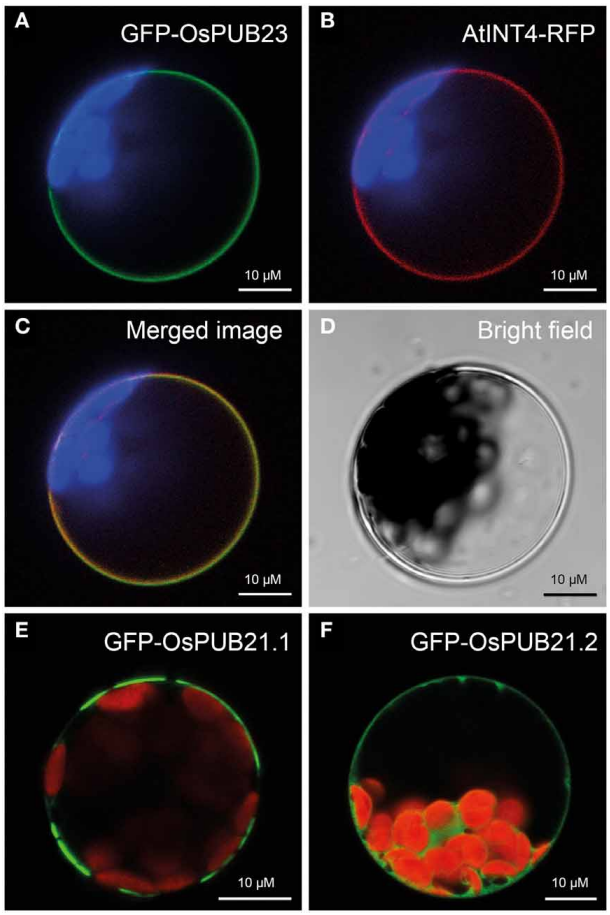

FIGURE 2 | Localization of rice SAUL-type PUB-ARM proteins at the plasma membrane. (A) Localization of GFP-OsPUB23 fusion proteins at the plasma membrane of Arabidopsis protoplasts. Confocal laser scanning microscopy detected fluorescence of GFP-OsPUB23 proteins depicted in green at the plasma membrane of protoplasts co-expressing GFP-OsPUB23 and AtINT4-RFP. Chlorophyll auto-fluorescence within the chloroplasts is depicted in blue. **(B)** Localization of AtINT4-RFP fusion proteins at the plasma membrane. Confocal laser scanning microscopy detected fluorescence of AtINT4-RFP proteins depicted in red at the plasma membrane of protoplasts co-expressing GFP-OsPUB23 and AtINT4-RFP. Chlorophyll auto-fluorescence within the chloroplasts is depicted in blue. **(C)** Merged image of **(A,B)**. Yellow signals derived from the overlap of green and red fluorescences from **(A,B)**. **(D)** Bright filed image of the protoplast analyzed in **(A–C)**. **(E)** Localization of GFP-OsPUB21.1 fusion proteins at the plasma membrane. Confocal laser scanning microscopy detected fluorescence of GFP-OsPUB21.1 proteins depicted in green at the plasma membrane of transformed protoplasts. Chlorophyll auto-fluorescence within the chloroplasts is depicted in red. **(F)** Localization of GFP-OsPUB21.2 fusion proteins at the plasma membrane. Confocal laser scanning microscopy detected fluorescence of GFP-OsPUB21.1 proteins depicted in green in the cytosol of transformed Arabidopsis protoplasts. Chlorophyll auto-fluorescence within the chloroplasts is depicted in red.

and *P. patens*, 50 and 31 PUB-ARM proteins were identified, respectively.

Two multiple sequence alignments were applied for Bayesian phylogenetic analyses. The first alignment covered 2683 amino acid positions and included the full-length proteins. In the second alignment all gaps were removed, resulting in 210 positions. Both alignments gave very similar trees, which recovered the classes I–IV of PUB-ARM proteins, as defined by Zeng et al. (2008) (**Figure 3A**; Supporting **Figures S2**, **S3**). SAUL1 and SAUL-like paralogs of Arabidopsis and rice are members of class IV. SAUL1 (AtPUB44) and AtPUB43 are closely related and form a common branch with three PUB-ARM proteins from rice (OsPUB23–25) and two from poplar (Pt0002s00910 and Pt0005s27480) (**Figure 3B**). AtPUB42 is on a different branch within class IV and is related to rice OsPUB21 and 22, as well as two proteins of poplar (Pt0008s11210 and Pt0010s14630). Six proteins of the bryophyte *P. patens* are related to the clade of SAUL1-like proteins of the vascular plants. In addition, five proteins from *P. trichocarpa* and five from *P. patens* form a distinct clade within class IV, which is more distantly related to SAUL1. SAUL1 orthologs within the class IV proteins displayed sequences identities of more than 40%. Comparison of SAUL1 with class I–III PUB-ARM proteins revealed low identity scores of less than 26%. In all cases and in contrast to all other PUB-ARM proteins, SAUL1-type PUB-ARM proteins consist of a clearly higher number of amino acids due to their specific domain organization, namely their elongated C-terminus that contains additional ARM repeat domains. Based on these criteria, additional BLAST searches identified putative SAUL1-type PUB-ARM proteins in all land plants listed on the phytozome website, suggesting conservation in land plants (not shown).

SAUL1-TYPE PUB-ARM PROTEINS FROM *POPULUS TRICHOCARPA* AND *PHYSCOMITRELLA* PATENS ARE ASSOCIATED WITH THE PLASMA MEMBRANE

Two PUB-ARM proteins from each organism, one SAUL1-type and one without the additional C-terminal ARM repeat domain, were selected to investigate conservation of SAUL1-type plasma membrane-association in *Populus trichocarpa* and *Physcomitrella patens*. Confocal laser scanning microscopy on transformed Arabidopsis leaf cell protoplasts and tobacco leaf epidermal cells analyzed localization of GFP fusion proteins. The poplar SAUL1-type PUB-ARM protein Pt0005s27480 (POPTR_0005s27480) localized to the plasma membrane as indicated by GFP fluorescence of GFP-Pt0005s27480 (**Figure 4A**) or Pt0005s27480-GFP (not shown) fusion proteins. In contrast, the same approach showed that the poplar PUB-ARM protein Pt0004s02840 (POPTR_0004s02840) that is lacking the C-terminal ARM repeat domain was localized to cytoplasm and nucleus (**Figure 4B** and not shown). The same results were obtained in *Physcomitrella patens*. Whereas fusion proteins with the SAUL1-type PUB-ARM protein Pp1s3_414V6 were associated with the plasma membrane, the Pp1s67_203V6 protein that also does not contain the additional ARM repeat domain localized to cytoplasm and nucleus (**Figures 5A,B**). To confirm localization of Pt0005s27480 and Pp1s3_414V6 at the plasma membrane, both GFP fusion proteins were co-expressed with the plasma membrane sugar transport protein UmSrt1 from the phytopathogenic fungus *Ustilago maydis* fused to RFP (**Figures 4C**, **5C**). The UmSrt1 transport protein was localized to the plasma membrane as described previously (c.f. Drechsel et al., 2011). Merging green and red fluorescence of GFP-Pt0005s27480

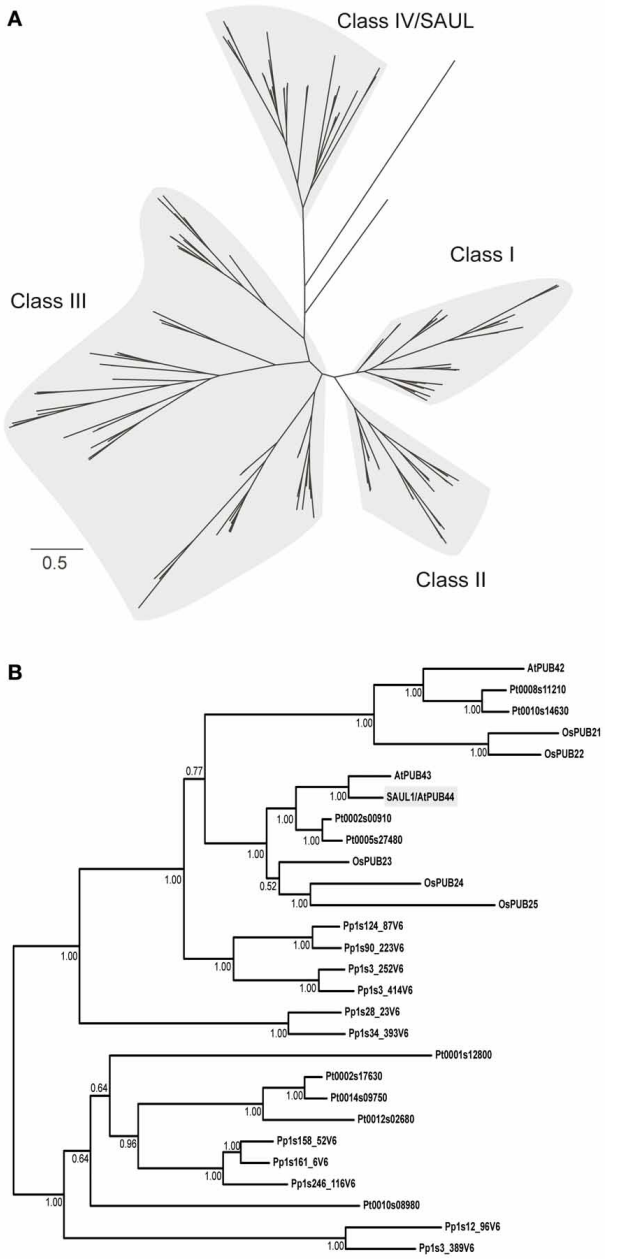

FIGURE 3 | Phylogenetic relationship of PUB-ARM proteins of Arabidopsis, rice, poplar and moss. (A) Simplified Bayesian phylogenetic tree depicting the relationship among class I–IV PUB-ARM proteins, as defined by Zeng et al. (2008). The bar represents 0.5 PAM distance. The tree derived from the analysis of the 2683 amino acid alignment; the full tree in Supporting **Figure S2**. **(B)** Subtree of **(A)** depicting the relationship among class IV proteins, which include SAUL1-type proteins. Arabidopsis SAUL1 (AtPUB44) is highlighted in gray; the numbers at the nodes are Bayesian posterior probabilities.

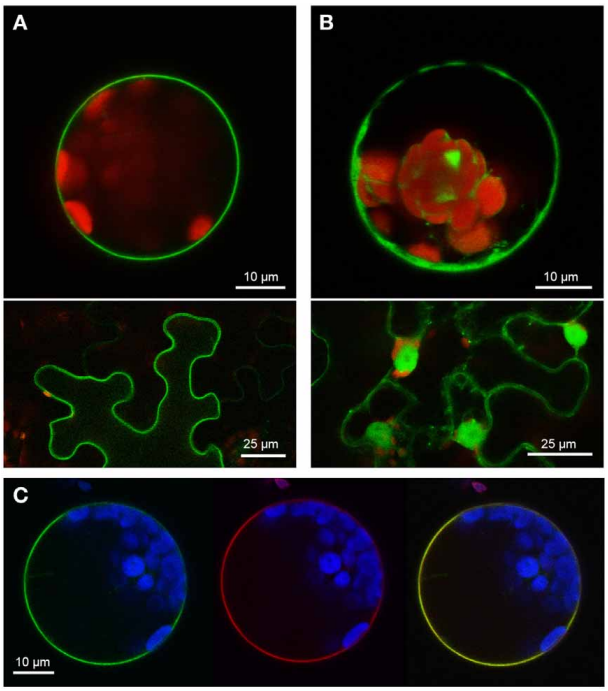

FIGURE 4 | Localization of SAUL-type PUB-ARM proteins from *Populus trichocarpa* at the plasma membrane. (A) Confocal analysis of fluorescence of Arabidopsis leaf protoplasts (top panel) and tobacco leaf epidermal cells (bottom panel) expressing GFP-Pt0005s27480 fusion proteins. GFP fluorescence depicted in green was detected at the plasma membrane in both cell types. Chlorophyll auto-fluorescence within the chloroplasts is depicted in red. **(B)** Confocal analysis of fluorescence of Arabidopsis leaf protoplasts (top panel) and tobacco leaf epidermal cells (bottom panel) expressing GFP-Pt0004s02840 fusion proteins. GFP fluorescence depicted in green was detected in cytosol and nucleus in both cell types. Chlorophyll auto-fluorescence within the chloroplasts is depicted in red. **(C)** Localization of GFP-Pt0005s27480 fusion proteins at the plasma membrane of Arabidopsis protoplasts. Confocal laser scanning microscopy detected fluorescence of GFP-Pt0005s27480 proteins depicted in green (left) and RFP-UmSrt1 (middle) proteins depicted in red at the plasma membrane of protoplasts co-expressing GFP-Pt0005s27480 and RFP-UmSrt1. Merging both images resulted in yellow signals (right) derived from the overlap of green (GFP-Pt0005s27480) and red (RFP-UmSrt1) fluorescences. Chlorophyll auto-fluorescence within the chloroplasts is depicted in blue.

and RFP-UmSrt1 (**Figure 4C**) or GFP-Pp1s3_414V6 and RFP-UmSrt1 (**Figure 5C**) resulted in yellow signals and clearly showed that both SAUL1-type proteins are associated to the plasma membrane.

DISCUSSION

Plant growth and development depend on specific degradation of proteins or on modification of protein functions following the attachment of ubiquitin. PUB armadillo repeat (PUB-ARM) proteins represent one class of E3 ubiquitin ligases that function in ubiquitination of target proteins. Whereas the majority of Arabidopsis PUB-ARM proteins were localized to the cytoplasm and/or nucleus, SAUL1 and its paralogs AtPUB42 and AtPUB43 were associated with the plasma membrane (Drechsel et al., 2011). This unique association with the plasma membrane is due to their exceptional protein structure. Alike all other PUB-ARM proteins, SAUL1-type proteins contain the U-box at the N-terminus important for E2 binding and a neighboring set of ARM repeats that is likely crucial for target binding. However,

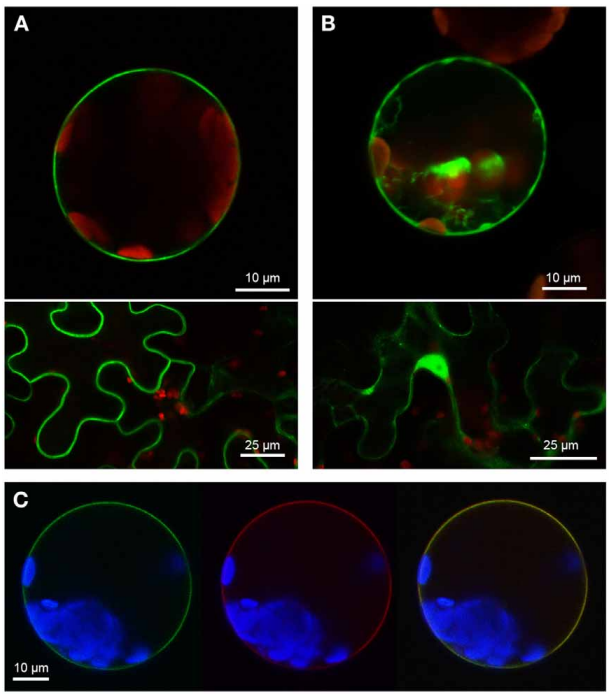

FIGURE 5 | Localization of SAUL-type PUB-ARM proteins from *Physcomitrella patens* at the plasma membrane. (A) Confocal analysis of fluorescence of Arabidopsis leaf protoplasts (top panel) and tobacco leaf epidermal cells (bottom panel) expressing GFP-Pp1s3_414V6 fusion proteins. GFP fluorescence depicted in green was detected at the plasma membrane in both cell types. Chlorophyll auto-fluorescence within the chloroplasts is depicted in red. **(B)** Confocal analysis of fluorescence of Arabidopsis leaf protoplasts (top panel) and tobacco leaf epidermal cells (bottom panel) expressing GFP-Pp1s67_203V6 fusion proteins. GFP fluorescence depicted in green was detected in cytosol and nucleus in both cell types. Chlorophyll auto-fluorescence within the chloroplasts is depicted in red. **(C)** Localization of GFP-Pp1s3_414V6 fusion proteins at the plasma membrane of Arabidopsis protoplasts. Confocal laser scanning microscopy detected fluorescence of GFP-Pp1s3_414V6 proteins depicted in green (left) and RFP-UmSrt1 (middle) proteins depicted in red at the plasma membrane of protoplasts co-expressing GFP-Pp1s3_414V6 and RFP-UmSrt1. Merging both images resulted in yellow signals (right) derived from the overlap of green (GFP-Pp1s3_414V6) and red (RFP-UmSrt1) fluorescences. Chlorophyll auto-fluorescence within the chloroplasts is depicted in blue.

in addition to these domains SAUL1-type PUB-ARM proteins are equipped with an additional set of ARM repeats at the elongated C-terminus. This domain has been shown to be essential and sufficient for plasma membrane-association of Arabidopsis SAUL1-type PUB-ARM proteins (**Figure 1**, Drechsel et al., 2011).

Here, we showed that rice SAUL1 orthologs, which have been identified by Zeng et al. (2008), also localized to the plasma membrane, and that the additional ARM repeat domain at the C-terminus is essential for this localization (**Figure 2**). To further prove that additional ARM repeats in an elongated C-terminus and thus plasma membrane-association are conserved features of SAUL1-type PUB-ARM proteins, we identified PUB-ARM proteins in *P. trichocarpa* and *P. patens*. In both plant species our search identified a group of SAUL1-type proteins, and we could demonstrate that these were associated to the plasma membrane indicating that this was a conserved function (**Figures 4**, **5**). Our phylogenetic analyses identified SAUL1-type proteins as members of class IV PUB-ARM proteins, as defined by Zeng et al. (2008) (**Figure 3**). The basic topology of the tree of PUB-ARM proteins followed that outlined by Zeng et al. (2008) and recovered the classes I, II, and IV identified in rice. Only class III was found paraphyletic in our analyses, comprising of in fact three distinct clades. In addition to Arabidopsis and rice, SAUL1-like proteins were also found in poplar and moss, suggesting an ancient origin of this type of ubiquitin ligase early in the evolution of land plants. There is a second group of proteins within class IV that is related to the SAUL1-like proteins, which has no orthologs in Arabidopsis and rice (**Figure 3B**). It remains to be demonstrated whether these proteins are also associated with the membrane and have functions similar to SAUL1.

To unravel and understand the function of PUB-ARM proteins, their target proteins have to be identified. To this end, we could show that interfering with the function of SAUl1-type proteins by masking the N-terminus with YFP resulted in severe growth defects in CaMV35S::YFP-SAUL1 plants (**Figure 1**). The N-terminal YFP may lead to conformational changes in SAUL1 and thus prevent correct binding to the respective E2 enzyme through the U-box that is contained in the SAUL1 N-terminus (Aravind and Koonin, 2000; Pringa et al., 2001). The future identification of SAUL1 target proteins will show whether masking the N-terminus may affect target recognition.

For some cytosolic/nuclear PUB-ARM proteins, the substrates have been identified. In the proteasome, subunits of the 19S regulatory particle represent targets of CaPUB1 and AtPUB22 (Cho et al., 2006, 2008). Degradation of these subunits results in reduced levels of functional 26S proteasomes, which may affect environmental stress responses. In rice, the PUB-ARM E3 ligase SPL11 (SPOTTED LEAF11) monoubiquitinates and thus negatively regulates SPIN1 (SPL11-interacting protein1), a member of the STAR (Signal Transduction and Activation of RNA) protein family. SPIN1 functions as a negative regulator of flowering in rice (Vega-Sanchez et al., 2008). The Arabidopsis PUB-ARM proteins AtPUB12 and AtPUB13 poly-ubiquitinate FLS2 (PRR FLAGELLIN-SENSING 2), thereby promote its degradation, and have a function in flagellin-induced immune responses (Lu et al., 2011). Recently, it was demonstrated that AtPUB22 mediated degradation of components of the exocyst complex thus affecting PAMP-triggered signaling (Stegmann et al., 2012). In contrast to the described examples, target proteins of plasma membrane-associated PUB-ARM proteins have not been identified yet.

A possible function of ubiquitination at the plasma membrane mediated by SAUL1-type PUB armadillo repeat E3 ubiquitin ligases could be the internalization of membrane proteins and thus the regulation of plasma membrane protein composition. The requirement of ubiquitin as a signal for internalization has primarily been demonstrated in yeast and mammals (Rotin et al., 2000; Hicke and Dunn, 2003). In plants, endocytosis can also remove transporters (PIN1) and receptors (BRI1, FLS2) from the plasma membrane (Geldner et al., 2001; Russinova et al., 2004; Robatzek et al., 2006). Generally, endosomal removal cannot only lead to receptor inactivation and down-regulation of

signaling, but may also stimulate signaling in case of accumulation of activated receptors in endosomes. Ubiquitination is involved in endocytosis of the Arabidopsis flagellin receptor FLS2 (FLAGELLIN-SENSING2) that results in its degradation (Gohre et al., 2008). Recently, it has been shown that flagellin induces association of FLS2 with the PUB-ARM proteins AtPUB12 and AtPUB13, which direct ubiquitination and turnover of FLS2 (Lu et al., 2011). The down-regulation of the plasma membrane transport proteins IRT1 (IRON-REGULATED TRANSPORTER1) and BOR1 (BORON TRANSPORTER1) also requires ubiquitination (Barberon et al., 2011; Kasai et al., 2011). Endocytosis and changes in protein stability were also induced by artificial mono-ubiquitination of the PM ATPase PMA and PIN2 (Herberth et al., 2012; Leitner et al., 2012). In addition, these and other studies link ubiquitination to the endosomal sorting complex required for transport (ESCRT) (Raiborg and Stenmark, 2009; Furlan et al., 2012; Scheuring et al., 2012). We hypothesize that plasma membrane-associated SAUL1-type E3 ubiquitin ligases may be responsible for mono-ubiquitination of membrane proteins to induce their endocytosis. Alternatively, they could be involved in processing plasma membrane-anchored regulatory proteins via poly-ubiquitination (Hoppe et al., 2001). Future identification of targets of SAUL1-type E3 ubiquitin ligases will help to unravel their molecular function at the plasma membrane.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://www.frontiersin.org/journal/10.3389/fpls.2014.00037/abstract

Figure S1 | Growth and development of Arabidopsis plants overexpressing GFP-AtPUB43ΔARM$_{1-6}$ fusion proteins.

Figure S2 | Phylogeny of PUB-ARM proteins of Arabidopsis, rice, poplar, and moss, as recovered by Bayesian analysis of the alignment covering 150 Proteins and 2683 amino acid positions.

Figure S3 | Phylogeny of PUB-ARM proteins of Arabidopsis, rice, poplar, and moss, as recovered by Bayesian analysis of the alignment covering 150 Proteins and 210 amino acid positions.

Table S1 | Primer pairs for amplification the respective open reading frames by PCR.

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SCF^{SLF}-mediated cytosolic degradation of S-RNase is required for cross-pollen compatibility in S-RNase-based self-incompatibility in *Petunia hybrida*

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Many flowering plants adopt self-incompatibility (SI) to maintain their genetic diversity. In species of Solanaceae, Plantaginaceae, and Rosaceae, SI is genetically controlled by a single *S*-locus with multiple haplotypes. The *S*-locus has been shown to encode S-RNases expressed in pistil and multiple SLF (*S*-locus F-box) proteins in pollen controlling the female and male specificity of SI, respectively. S-RNases appear to function as a cytotoxin to reject self-pollen. In addition, SLFs have been shown to form SCF (SKP1/Cullin1/F-box) complexes to serve as putative E3 ubiquitin ligase to interact with S-RNases. Previously, two different mechanisms, the S-RNase degradation and the S-RNase compartmentalization, have been proposed as the restriction mechanisms of S-RNase cytotoxicity allowing compatible pollination. In this study, we have provided several lines of evidence in support of the S-RNase degradation mechanism by a combination of cellular, biochemical and molecular biology approaches. First, both immunogold labeling and subcellular fractionation assays showed that two key pollen SI factors, PhS_{3L}-SLF1 and PhSSK1 (SLF-interacting SKP1-like1) from *Petunia hybrida*, a Solanaceous species, are co-localized in cytosols of both pollen grains and tubes. Second, PhS_{3L}-RNases are mainly detected in the cytosols of both self and non-self-pollen tubes after pollination. Third, we found that PhS-RNases selectively interact with PhSLFs by yeast two-hybrid and co-immunoprecipitation assays. Fourth, S-RNases are specifically degraded in compatible pollen tubes by non-self SLF action. Taken together, our results demonstrate that SCF^{SLF}-mediated non-self S-RNase degradation occurs in the cytosol of pollen tube through the ubiquitin/26S proteasome system serving as the major mechanism to neutralize S-RNase cytotoxicity during compatible pollination in *P. hybrida*.

Keywords: self-incompatibility, self-pollen incompatibility, cross-pollen compatibility, S-RNase localization, SCF^{SLF}, ubiquitin/26S proteasome system

INTRODUCTION

Self-incompatibility (SI) is a mating strategy that allows individual of a species to discriminate self (genetically related) from non-self (genetically unrelated), and thus accept cross-pollen whereas reject self-one. These outcomes are referred to as cross-pollen compatibility (CPC) and self-pollen incompatibility (SPI), respectively. In many species of flowering plants, such discrimination is genetically controlled by a single *S*-locus with multiple haplotypes, self-pollen rejection occurs when the pollen *S* haplotype matches either of the two pistil *S* haplotypes and the *S*-locus has been shown to encode at least two components from both the pollen and pistil sides, controlling the male and female expression of SI specificity, respectively (De Nettancourt, 2001; Takayama and Isogai, 2005; Franklin-Tong, 2008; Zhang et al., 2009). In Solanaceae, Plantaginaceae and Rosaceae, the pistil *S* encodes a T2 family ribonuclease, named S-RNase (McClure et al., 1989; Clark et al., 1990; Lee et al., 1994; Murfett et al., 1994; Sassa et al., 1996; Xue et al., 1996), whereas the pollen *S* has been shown to encode multiple paralogous F-box proteins called the *S* Locus F-box (SLF) in Solanaceae and Plantaginaceae (Lai et al., 2002; Zhou et al., 2003; Sijacic et al., 2004; Qiao et al., 2004a; Kubo et al., 2010; Williams et al., 2014) and SLF (Entani et al., 2003) or *S*-haplotype-specific F-box protein (SFB) in Rosaceae (Ushijima et al., 2003; Yamane et al., 2003; Sassa et al., 2007). In addition, other factors, not encoded by the *S*-locus, have been found in pistil side and are involved at different stages of the SI reaction. So far, three essential pistil-modifier factors, 120K (120 kDa glycoprotein), HT-B protein and NaStEP (*Nicotiana alata* stigma expressed protein), have been identified in *Nicotiana* species (McClure et al., 1999; Hancock et al., 2005; Jimenez-Durán et al., 2013). 120K is a style-specific glycoprotein that is taken up by pollen tubes during pollination (Lind et al., 1996; Schultz et al., 1997). Suppression of 120K expression by RNAi prevents *S*-specific pollen rejection (Hancock et al.,

2005). Immunolocalization shows that HT-B is also taken up by pollen tubes during pollination. Loss-of-function assays showed that HT-B is essential for pollen rejection (McClure et al., 1999; Kondo et al., 2002; O'Brien et al., 2002; Sassa and Hirano, 2006; Puerta et al., 2009). NaStEP is a Kunitz-type proteinase inhibitors and a positive regulator of HT-B stability in *Nicotiana alata* pollen tubes (Busot et al., 2008; Jimenez-Durán et al., 2013).

S-RNase is a pistil-specific glycoprotein and initially synthesized in transmitting cells of the style and then secreted into extracellular matrix of the transmitting tract tissue (Cornish et al., 1987; Anderson et al., 1989). S-RNase is very abundant and mainly found in the transmitting track of a mature style where the growth of self-pollen tube is arrested after pollination (Cornish et al., 1987; Xue et al., 1996). It is proposed that S-RNase likely functions as a cytotoxic ribonuclease to degrade RNA by gaining access to self-pollen tube whose growth is thus arrested, but non-self-pollen tube growth is unaffected (McClure et al., 1990; Luu et al., 2000; Liu et al., 2009). The S-RNase is necessary for the pistil to recognize and reject self-pollen (Huang et al., 1994; Lee et al., 1994; Murfett et al., 1994). Furthermore, the S-RNase alone determines the pistil specificity of SI (Karunanandaa et al., 1994).

The first pollen *S* determinant, *AhSLF-S$_2$*, was identified as an F-box gene in *Antirrhinum hispanicum*, a member of Plantaginaceae (Lai et al., 2002). Most F-box proteins usually serve as a component of an SCF (SKP1/Cullin1/F-box) ubiquitin ligase complex that often results in its target substrate polyubiquitination and degradation (Smalle and Vierstra, 2004). Thus, the finding of AhSLF-S$_2$ provided a clue to the biochemical mechanism of SI. It was proposed that its potential function is involved in the ubiquitin/26S proteasome system (UPS) (Lai et al., 2002). The UPS pathway uses ubiquitin-activating enzyme E1, ubiquitin-conjugating enzyme E2 and ubiquitin ligase E3 to catalyze substrate polyubiquitination for degradation by the UPS pathway (Bai et al., 1996). SCF complex is a class of multisubunit E3 ligase involved in recognition and polyubiquitination of specific target proteins for degradation by the UPS pathway (Cardozo and Pagano, 2004; Willems et al., 2004). For Cullin1, as a scaffold protein, its N-and C-terminal domains interact with SKP1 and Rbx1, respectively. The F-box domain of F-box proteins interacts with SKP1 and a separate domain with their specific substrates (Zheng et al., 2002). Importantly, Qiao et al. (2004a) have shown that this C-terminal region of AhSLF$_2$ interacts with both its cognate self and non-self S-RNases. In addition, SSK1 (SLF-interacting SKP1-like 1) proteins have been shown to be a novel class of pollen-specific SKP1-like proteins that bridge Cullin1 and SLF proteins to form an SCF^{SLF} complex in *A. hispanicum* (Plantaginaceae), *Petunia* (Solanaceae) as well as in both *Prunus* and *Pyrus* (Rosaceae) (Huang et al., 2006; Zhao et al., 2010; Matsumoto et al., 2012; Xu et al., 2013; Entani et al., 2014; Li et al., 2014; Yuan et al., 2014). Taken together, these results showed that both SLF and SSK1 are components of an SCF complex. In addition, Cullin1 has been shown to be involved in both SI and UI (unilateral incompatibility) in Solanum (Li and Chetelat, 2010, 2013). Thus, an S-RNase degradation model has been proposed to explain the biochemical mechanism of S-RNase-based SI. The model posits that non-self S-RNases are degraded via the UPS pathway mediated by SCF^{SLF} complex in cross pollen tubes so that S-RNase cytotoxicity is restricted, whereas self S-RNase is somehow able to escape degradation to exert its cytotoxicity to pollen tubes (Qiao et al., 2004a; Hua and Kao, 2006). By contrast, the S-RNase compartmentalization model also has been proposed for the S-RNase restriction mechanism (Goldraij et al., 2006; McClure, 2009; McClure et al., 2011). This model posits that the majority of S-RNases are sequestered in vacuoles of pollen tube with a minority entering the cytosols to be recognized by SLF. Sequestered S-RNases are thus spatially separated from cellular RNAs. Self-recognition is hypothesized to release S-RNases from vacuoles and subsequently to inhibit self-pollen tube growth, whereas cross recognition would stabilize vacuoles to continue to sequester S-RNases. Therefore, it remains unclear how the cytotoxic effect of S-RNase is specifically restricted in compatible pollination.

To address this issue, in this study, we determined the subcellular location of two key pollen SI factors, PhS$_{3L}$-SLF1 and PhSSK1, as well as of the pistil factor PhS$_{3L}$-RNase in pollen tubes after pollination in *P. hybrida*. Our results revealed that PhS$_{3L}$-SLF1 and PhSSK1 are localized in cytosols of pollen grains and tubes where the majority of S-RNases are also located in the cytosols after pollination. In addition, we found that PhSLFs interact with PhS-RNases. Furthermore, we found that non-self S-RNases are degraded through the SCF^{SLF}-mediated UPS pathway in compatible pollen tubes. Taken together, our results demonstrate that the SCF^{SLF}-mediated cytosolic degradation of S-RNase serves as the major mechanism to restrict its cytotoxicity to cross-pollen tubes in *P. hybrida*, providing an important insight into the biochemical and cellular mechanisms of S-RNase-based SI.

RESULTS

IDENTIFICATION OF THE POLLEN-*S* GENES OF *P. HYBRIDA*

To identify the pollen-*S* genes in *P. hybrida*, we first cloned four *PhSLF* alleles by a homology-based method from self-incompatible *S$_1$S$_1$*, *S$_3$S$_3$*, *S$_{3L}$S$_{3L}$*, and *S$_v$S$_v$* homozygous plants as described (Clark et al., 1990; Robbins et al., 2000; Qiao et al., 2004b). PhS$_1$-SLF1 (GenBank accession number GQ121443.1), PhS$_3$A-SLF1 (GenBank accession number AY639403.1), PhS$_{3L}$-SLF1 (GenBank accession number GQ121445.1) and PhS$_v$-SLF (GenBank accession number GQ121446.1) were found to belong to Type-1 SLFs (Supplementary Figures S1A,B) based on the classification by Kubo et al. (2010). We then isolated a promoter fragment derived from a 2120 bp sequence upstream of a pollen-specific gene *PhS$_3$A-SLF1*. A binary vector *pBI101* containing the *PhS$_3$A-SLF1* promoter fragment fused with a downstream GUS reporter gene (Supplementary Figure S2A) was introduced into self-incompatible lines of *S$_1$S$_v$* and *S$_3$S$_{3L}$* haplotypes, respectively. GUS activity analysis of the transgenic plants and wild-type revealed that this putative promoter fragment was sufficient to drive the GUS expression specifically in the anther (Supplementary Figure S2B), resulted from its expression in the pollen grains (Supplementary Figure S2C). To determine whether the *PhSLF* alleles encode the pollen *S*-determinants, we generated transgenic plants by introducing three alleles, *PhS$_1$-SLF1*, *PhS$_{3L}$-SLF1*, and *PhS$_v$-SLF1* driven by the *PhS$_3$A-SLF1* promoter into different self-incompatible lines of *S* haplotypes, respectively. Competitive interaction, where expression of two

heteroallelic pollen-*S* genes in the same pollen grain causes breakdown of pollen self-incompatibility, has been used to test the validity of the pollen-*S* genes (Lewis, 1947; Sijacic et al., 2004; Qiao et al., 2004b). The *PhS$_{3L}$-SLF1* transgene caused breakdown of self-incompatibility in S_3S_{3L} but not $S_{3L}S_v$ haplotypes (Supplementary Figures S3A,B, Supplementary Tables S1,S2), indicating that it functions as the pollen-*S* product against non-self S$_3$-RNase. Consistent with this result, all progeny from self-pollination of the primary S_3S_{3L}/*PhS$_{3L}$-SLF1* plants inherited *S$_3$-RNase* and the transgene, with an S_3S_3 to S_3S_{3L} segregation ratio of ca.1:1 as predicted (Supplementary Figure S4A, Supplementary Table S2). We also examined the inheritance of the *PhS$_{3L}$-SLF1* transgene and progeny *S*-genotypes from pollination of wild-type S_3S_{3L} plants with the pollen from the S_3S_{3L}/*PhS$_{3L}$-SLF1*-3 transgenic plants. All progeny also inherited *S$_3$-RNase* and the transgene, with an S_3S_3 to S_3S_{3L} segregation ratio of ca.1:1 as predicted (Supplementary Figure S4A). Furthermore, the S_1S_v/*PhS$_{3L}$-SLF1::FLAG* plants also caused self-incompatibility breakdown (Supplementary Figure S3C, Supplementary Table S3). All progeny from self-pollination of the primary S_1S_v/*PhS$_{3L}$-SLF1::FLAG* plants inherited *S$_1$-RNase* and the transgene, with an S_1S_1 to S_1S_v segregation ratio of ca.1:1 as predicted (Supplementary Figure S4B, Supplementary Table S3). Nevertheless, the *PhS$_1$-SLF1* transgene did not show a competitive interaction in the S_1S_v transgenic plants (Supplementary Figure S3D, Supplementary Table S4), and the *PhS$_v$-SLF1* transgene did not show a competitive interaction in the $S_{3L}S_v$ transgenic plants either (Supplementary Figure S3E, Supplementary Table S5), showing that the *PhS$_1$-SLF1* and *PhS$_v$-SLF1* do not function as the pollen-*S* at least against S$_v$-RNase and S$_{3L}$-RNase, respectively. Taken together, these results showed that the *PhS$_{3L}$-SLF1* and *PhS$_{3L}$-SLF1::FLAG* act as the functional pollen-*S* against *S$_3$-RNase* and *S$_1$-RNase*, respectively.

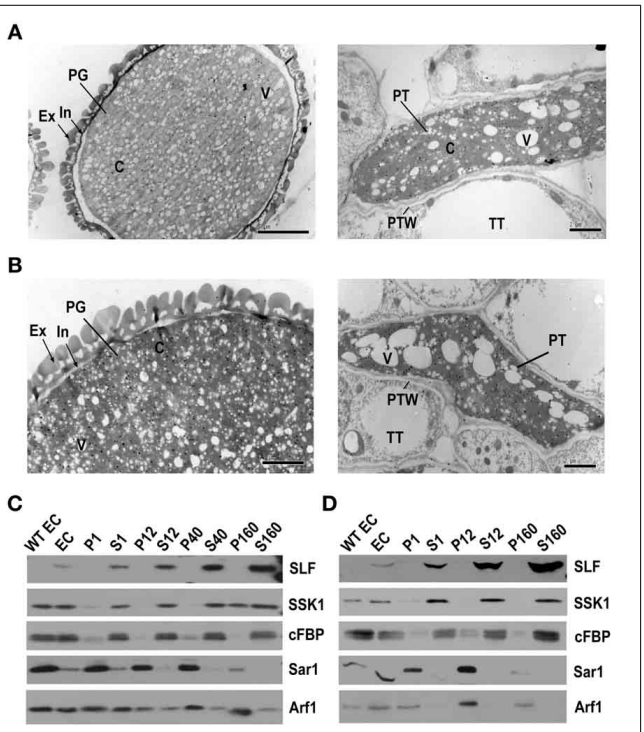

FIGURE 1 | PhS$_{3L}$-SLF1 and PhSSK1 are both located in the cytosol of pollen grains and pollen tubes. (A) Immunogold labeling of PhS$_{3L}$-SLF1::FLAG in pollen grain (left) and pollen tube (right). **(B)** Immunogold labeling of PhSSK1 in pollen grain (left) and pollen tube (right). PG, pollen grain; Ex, exine; In, intine (arrow); C, cytosol; V, vacuole; PT, pollen tube; PTW, pollen tube wall; TT, transmitting tract tissue. **(C)** Western blot detection of PhS$_{3L}$-SLF1::FLAG (SLF) and PhSSK1 (SSK1) in subcellular fractions of pollen grains. **(D)** Western blot detection of PhS$_{3L}$-SLF1::FLAG (SLF) and PhSSK1 (SSK1) in subcellular fractions of *in vitro* germinated pollen tubes. WT EC and EC denote entire cell homogenates from wild-type and the transgenic pollen grains or pollen tubes, respectively. Pellet fractions (P1, P12, P40, and P160) and supernatant fractions (S1, S12, S40, and S160) are derived from differential centrifugation at 1000, 12,000, 40,000, and 160,000 g, respectively. cFBP, Sar1, and Arf1 are marker antibodies for cytosol, endoplasmic reticulum (ER) and Golgi, respectively.

PHS$_{3L}$-SLF1 AND PhSSK1 BOTH LOCALIZE TO THE CYTOSOLS OF POLLEN GRAINS AND POLLEN TUBES

To examine the localization of the pollen SI factors PhS$_{3L}$-SLF1 and PhSSK1, we performed immunogold labeling and subcellular fractionation experiments. For immunogold labeling experiment of PhS$_{3L}$-SLF1, we used FLAG antibody to detect its expression in the mature pollen grains and pollinated pollen tubes of the S_1S_v/*PhS$_{3L}$-SLF1::FLAG* transgenic plants exhibiting the competitive interaction described above. The FLAG gold particles corresponding to PhS$_{3L}$-SLF1::FLAG are distributed in the cytosols of both pollen grains and pollen tubes rather than associated with any discernible organelle (**Figure 1A**). This finding is similar to that found for AhSLF-S$_2$ in *A. hispanicum* (Wang and Xue, 2005). As controls, the FLAG antibody did not label any signal in both pollen grain, pollen tube and pollinated style of wild-type (Supplementary Figure S5A), confirming the specificity of FLAG antibody.

To examine the subcellular location of PhSSK1, *P. hybrida* SLF-interacting SKP1-like1, an adaptor for an SCF^{SLF} complex, we used PhSSK1 antibody to detect its expression in the mature pollen grains and pollinated pollen tubes of wild-type plants. First, to examine the specificity of the PhSSK1 antibody, we extracted proteins from different tissues for western blot analysis and found strong signals at approximately 30 kDa in the pollen grains and pollen tube extracts, and no signal was found with the protein extracts from other organs, including leaf and pistil, indicating that the antibody is specific for its target protein. In addition, western blotting analysis using rabbit pre-immune serum for PhSSK1 antibody showed no apparent signal in the protein extracts from all of these organs examined (Supplementary Figure S5B). In addition, we further performed immunogold labeling using rabbit pre-immune serum as a negative control and the pre-immune serum did not label any signal in wild-type pollen grain and pollen tube as well as pollinated style (Supplementary Figure S5C). Thus, the PhSSK1 antibody has strong target specificity and we performed immunogold labeling using the antibody to detect the subcellular location of PhSSK1. The immunogold labeling results showed that the PhSSK1 gold particles exhibited a similar distribution to the PhS$_{3L}$-SLF1::FLAG in the cytosols of both pollen grains and tubes (**Figure 1B**). Taken together, these results

indicated that the pollen SI factors PhS$_{3L}$-SLF1 and PhSSK1 are both localized in the cytosols of the pollen grains and pollinated pollen tubes.

Nevertheless, because it is hard to clearly distinguish the endomembrane system from the cytosols in our immunogold labeled sections, we further carried out subcellular fractionation experiments. The PhS$_{3L}$-SLF1::FLAG (SLF) was always co-purified with the supernatant fractions and significantly enriched in the S160 fraction, representing the cytosolic fraction (Cherry, 1974; Quail, 1979; Arrese et al., 2001; Agrawal et al., 2011; Rangel et al., 2013) of pollen grains (**Figure 1C**) and pollen tubes (**Figure 1D**), consistent with the immunogold labeling results. PhS$_{3L}$-SLF1::FLAG specific signal detected in pollen grains and pollen tubes of transgenic plant, but not in wild-type pollen grains and pollen tubes (**Figures 1C,D**), indicating that FLAG antibody is specific for its target protein. In addition, subcellular fractionation experiment further showed a similar location for PhSSK1 (SSK1) (**Figures 1C,D**). Interestingly, SSK1 showed a dynamic localization seen in P160 fractions of the pollen grains (**Figure 1C**), similar to the Golgi marker Arf1 observed previously (Pertl et al., 2009) though it was not clear how this occurs. Taken together, these results showed that the pollen factors PhS$_{3L}$-SLF1 and PhSSK1 are located in the cytosols of the pollen grains and pollen tubes.

S-RNases ARE PREDOMINANTLY LOCALIZED IN THE CYTOSOLS OF BOTH COMPATIBLE AND INCOMPATIBLE POLLEN TUBES

Previous studies have shown that S-RNases appear to have different locations. Luu et al. found that they are located in the cytosols of pollen tubes in *Solanum chacoense* by immunocytochemical labeling (Luu et al., 2000). Whereas Goldraij et al. (2006) showed that they are largely compartmentalized in vacuoles in *Nicotiana* by immunolocalization. To examine the localization of PhS$_{3L}$-RNase (GenBank accession number AJ271065.1), we performed both immunogold labeling and subcellular fractionation experiments. To examine the specificity of PhS-RNase antibody, proteins from different tissues were extracted and detected. After western blotting, strong signals were only found in the $S_{3L}S_{3L}$ and S_3S_3 style extracts, but no signal was found in pollen grains, pollen tubes, leaves, S_1S_1 and S_vS_v styles. As a negative control, western blotting analysis using rabbit pre-immune serum for PhS-RNase antibody detected, no apparent signal in all of the organs examined (Supplementary Figure S5D). We further performed the immunogold labeling using the PhS-RNase antibody and rabbit pre-immune serum as negative controls. PhS-RNase antibody did not label any signal in pollen tube without style extract treatment (Supplementary Figure S5E) and the pre-immune serum also did not label any signal in pollinated pistil (Supplementary Figure S5F). To further confirm the PhS-RNase antibody specificity, total proteins of $S_{3L}S_{3L}$ styles were isolated and analyzed by two-dimensional polyacrylamide gel and western blot. A strong signal was observed at approximately 30 kDa and pH 9.0 (Supplementary Figure S6A). Then the spot was excised from the gel and identified by liquid chromatography/tandem mass spectrometry (LC-MS/MS). In the MASCOT search, a total of 204 peptides matched the sequence of PhS$_{3L}$-RNase in total 900 peptides identified by the LC-MS/MS spectra, and matched peptides have a coverage 43% sequence of PhS$_{3L}$-RNase (Supplementary Figures S6A,B). The other peptides were identified from the 30 kDa protein spot did not match PhS$_{3L}$-RNase (Supplementary Table S6). In addition, other unidentified minor fragments were judged to be derived from contaminated and/or degraded proteins. Therefore, our proteomic data indicated that the 30 kDa protein represents PhS$_{3L}$-RNase. Taken together, these results demonstrated that the PhS-RNase antibody specifically detects its target protein.

For immunogold labeling experiment of PhS$_{3L}$-RNase, we used the PhS-RNase antibody to detect its expression in compatible (CPC) and incompatible (SPI) pollinated styles. Both CPC and SPI styles at 6, 12, and 24 h post-pollination were stained with aniline blue and monitored by a fluorescence microscope (Supplementary Figure S7), showing that the growth of incompatible pollen tubes is arrested around the upper one-third of the style and the compatible pollen tubes have reached the bottom of the style 24 h post-pollinations as described previously (Singh et al., 1992). The CPC and SPI styles of 6 h post-pollination were used to observe the stylar regions containing the apical tips of pollen tubes, where their elongations occurred. The subcellular localization of PhS$_{3L}$-RNase was observed for the transverse sections of the CPC and SPI stigmas at 6 h post-pollination, the upper one-third of CPC and SPI styles at 12 h post-pollination, the bottom of styles at 24 h post compatible pollination and the upper one-third of styles at 24 h post incompatible pollination, respectively. The PhS$_{3L}$-RNase gold particles were found to be evenly distributed in the cytosols of both CPC and SPI pollen tubes (relatively close to the pollen tube tip) growing in styles at 6, 12, and 24 h post-pollination, respectively, but not found in vacuoles and any other discernible organelles (**Figures 2A,B**). In particular, the gold particles were not found in vacuoles when significant cytosol vacuolization occurred in pollen tubes growing in style around 24 h post compatible pollination. It is noteworthy that there are still some gold particles in the pollen tubes within the style around 24 h post compatible pollination (**Figure 2A**). In addition, a distal zone from the pollen tube tip (upper one-third) of CPC after 24 h also were observed by immunogold labeling, and this result showed that the PhS$_{3L}$-RNase gold particles also are found to be evenly distributed in the cytosols but not found in vacuoles of 24 h post compatible pollination (**Figure 2C**). A region without pollen tubes showing PhS$_{3L}$-RNase accumulation occurred mainly in extracellular matrix (ECM) of a mature pistil as a positive control (**Figure 2D**). Taken together, these results indicated that PhS$_{3L}$-RNases are predominantly localized in the cytosols of the pollen tubes in styles of early, middle, and late stages after both CPC and SPI pollinations.

To further examine the subcellular localization of PhS$_{3L}$-RNase, we carried out a similar immunogold labeling experiment using an *in vitro* pollen germination system. In order to test the validity of *in vitro* pollen germination system in *P. hybrida*, we carried out the following experiments. Often, compatible pollen tubes grow about 2 cm in pistil at 24 h post-pollination in *P. hybaida*. Pollen tubes also can grow about 2 cm in *in vitro* pollen germination medium after 24 h (Supplementary Figure S8A), indicating that pollen tube growth *in vitro* system

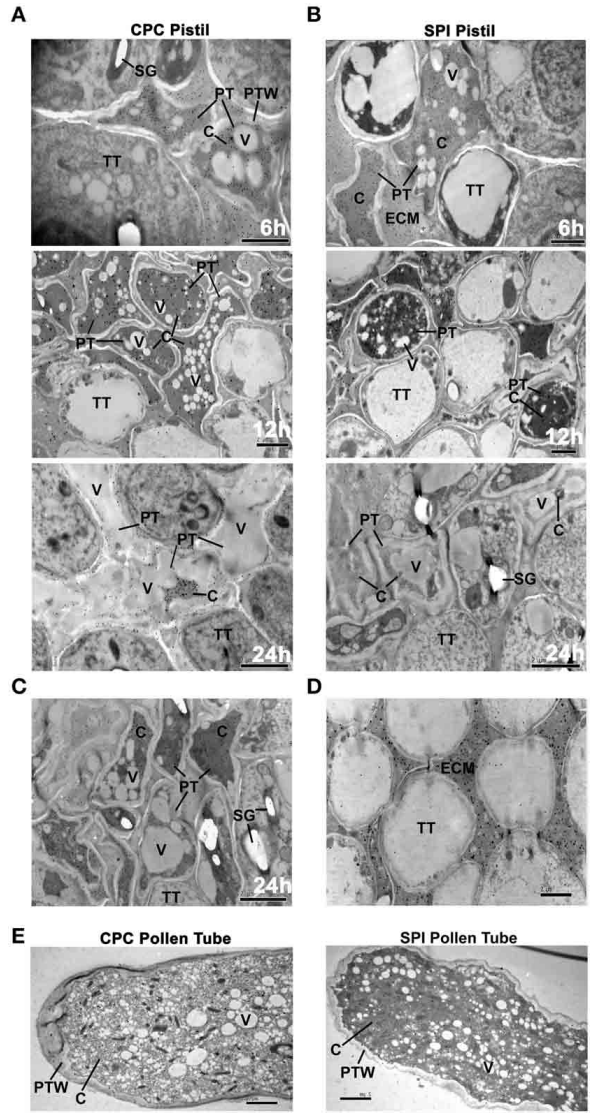

FIGURE 2 | S-RNases localize to the cytosol of both compatible and incompatible pollen tubes. (A) Immunogold labeling of PhS_{3L}-RNase in cross-sectioned pistils of 6, 12, and 24 h post compatible pollination (CPC), respectively. A zone that is relatively close to the pollen tube tip was observed. **(B)** Immunogold labeling of PhS_{3L}-RNase in cross-sectioned pistils after 6, 12, and 24 h post incompatible pollination (SPI), respectively. A zone that is relatively close to the pollen tube tip was observed. **(C)** Immunogold labeling of PhS_{3L}-RNase in cross-sectioned pistils after 24 h post compatible pollination. A zone that is relatively close to the upper one-third pollen tube was observed. **(D)** A region without pollen tube showing PhS_{3L}-RNase accumulation mainly in ECM of a mature pistil as positive control. **(E)** Immunogold labeling of PhS_{3L}-RNase in *in vitro* germinated pollen tube of CPC (S_v) and SPI (S_{3L}), respectively (longitudinal-section). PT, pollen tube; PTW, pollen tube wall; TT, transmitting tract tissue; C, cytosol; V, vacuole; SG, starch granule; ECM, extracellular matrix.

is largely similar to that *in vivo* pollination reaction. In addition, the S_v and S_{3L} pollen tubes were continuously challenged with $S_{3L}S_{3L}$ style extracts to mimic the CPC and SPI pollinations, respectively, both CPC and SPI pollen tube growth was inhibited compared to controls, but the SPI pollen tubes showed a more significant growth inhibition compared with CPC pollen tubes (Supplementary Figures S8B,C, Supplementary Table S7). Nevertheless, the control pollen tubes without stylar extracts showed much better growth compared with both CPC and SPI ones, suggesting that the extracts contained a strong inhibitory activity of pollen tube growth. Further work is needed to address this issue. The results showed that *in vitro* (5 h) compatible pollen tubes are about 0.02 cm, and incompatible about 0.017 cm (Supplementary Figures S8B,C), also similar to that of compatible and incompatible tubes (6 h) *in vivo* (Supplementary Figure S7). Taken together, these results showed that the *in vitro* pollen germination system in *P. hybrida* (Supplementary Figure S8) are similar to that by Qiao et al. (2004a) in *Antirrhinum* and Meng et al. (2014) in apple. The *in vitro* pollen germination system can exclude a high background interference of S-RNases in style. Although the style extracts *in vitro* appeared to lose their activity after long time incubation, this system can mimic the CPC and SPI reaction for at least first several hours of the reaction. Using the *in vitro* pollen germination system, we further examined the subcellular localization of PhS_{3L}-RNase in pollen tubes, and the immunogold labeling results showed that the PhS_{3L}-RNase gold particles were found to be distributed in the cytosols of both CPC and SPI pollen tubes (**Figure 2E**). Taken together, these results showed that PhS_{3L}-RNases are localized in the cytosols of the CPC and SPI pollen tubes in the *in vitro* pollen germination system, similar to that found in *in vivo* (**Figures 2A,B**).

For detection of smaller membranous structures such as endosomes and prevacuolar compartment (PVC), immunogold labeling is not feasible because of the size of commercial colloidal gold is 5–20 nm, mostly 10 nm for transmission electron microscope (TEM), which is beyond the thickness of biomembrane. In addition, it is clearly seen that immunogold particles are not present in the vacuoles, starch granules and mitochondria structures from the images of S-RNases immunogold labeling in pollen tubes (**Figures 2A–C**). Both vacuoles and starch granules are electron-luculent in TEM pictures. The edge of vacuoles is clearer and smooth in pollen tube. However, the starch granules are more dim and irregular. To exclude the possibility of endosome, endoplasmic reticulum (ER), Golgi and PVC localization of S-RNase, we carried out subcellular fractionation experiments using the pulse challenged pollen tubes of the *in vitro* pollen germination system. Both S_v (CPC) and S_{3L} (SPI) pollen tubes were treated with $S_{3L}S_{3L}$ style extracts as described above. The western blot results showed that the overwhelming majority of PhS_{3L}-RNase was always co-purified with the supernatant fractions and significantly enriched in the S160 fractions of both CPC and SPI pollen tubes (Supplementary Figure S9A), representing the cytosolic fractions (Cherry, 1974; Quail, 1979; Arrese et al., 2001; Agrawal et al., 2011; Rangel et al., 2013), indicating that PhS-RNases have a predominant cytosolic location but a minor amount associated with a microsome-like fraction. Taken together, these results demonstrated that PhS_{3L}-RNases are predominantly localized in the cytosols of the CPC and SPI pollen tubes, similar to that found in *S. chacoense* (Luu et al., 2000).

PhSLF INTERACTS WITH PhS-RNase IN BOTH YEAST AND POLLEN TUBES

Although our results have shown that PhS_{3L}-RNase, PhS_{3L}-SLF1, and PhSSK1 are all localized to the cytosol of pollen tubes, it remained unclear whether an interaction between PhSLFs and PhS-RNases occurs. To examine the physical interaction between PhSLFs and PhS-RNases, we used a yeast two-hybrid assay. The C-terminal coding sequences of *PhS_{3L}-SLF1*, *PhS_1-SLF1*, and *PhS_v-SLF1* (after removal of F-box domain) were introduced into *pGBKT7* vector and expressed as a fusion to GAL4 DNA binding domain (BD), whereas the full-length coding sequences of *PhS_{3L}-RNase*, *PhS_3-RNase* (GenBank accession number U07363.1) and *PhS_v-RNase* (GenBank accession number AJ271062.1) were introduced into *pGADT7* vector and expressed as a fusion to transcriptional activating domain (AD). Yeast cells co-transformed with the *PhS_{3L}-SLF1-C* and the *PhS_3-RNase*, *PhS_{3L}-RNase* or *PhS_v-RNase* constructs grew well on both Leu-/Trp- and Leu-/Trp-/His-/Ade-media, showing that a physical interaction had occurred between PhS_{3L}-SLF1-C and *PhS_3-RNase*, *PhS_{3L}-RNase* or *PhS_v-RNase* in yeast. In addition, the C-terminal region of PhS_v-SLF1 only interacts with PhS_v-RNase, rather than the PhS_3-RNase and PhS_{3L}-RNase. We did not detect interactions between the C-terminal region of PhS_1-SLF1 and PhS_3-RNase, PhS_{3L}-RNase or PhS_v-RNase. Yeast transformed with the negative control plasmids *AD::PhS-RNases* and *pGBKT7* or *BD::PhSLFs* and *pGADT7* did not grow (**Figure 3A**). Furthermore, the β-galactosidase reporter activity was detected and appeared to be positive in yeast cells co-transformed with

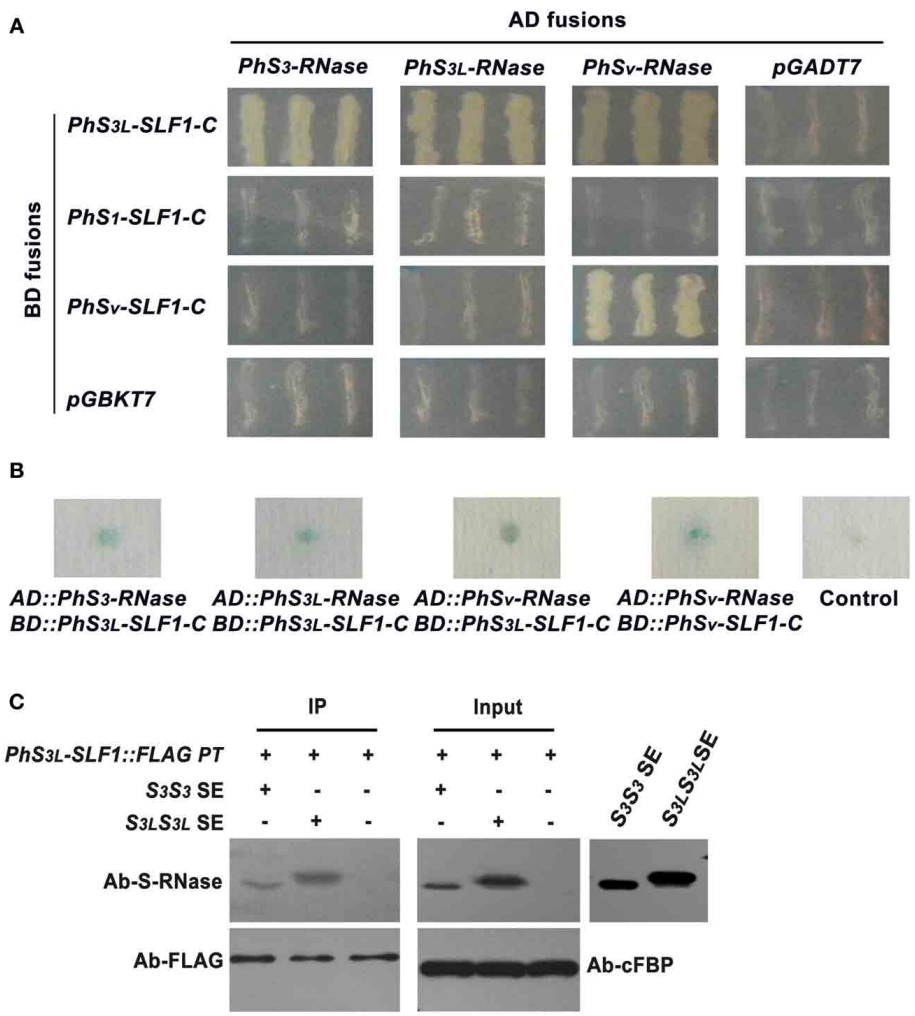

FIGURE 3 | PhSLFs selectively interact with PhS-RNases. (A) Yeast two-hybrid assays between PhSLFs and PhS-RNases. Cells of yeast strain AH109 containing various combinations of bait (BD fusions) and prey (AD fusions) were tested for their growth on selective medium SD/-Ade-His-Leu-Trp. The empty vectors *pGBKT7* and *pGADT7* were negative controls. **(B)** The β-galactosidase activity assay was used to further test the interaction of PhSLFs and PhS-RNases. The combination of empty *pGBKT7* and *pGADT7* was used as a negative control. **(C)** Co-immunoprecipitation assays between PhS_{3L}-SLF1::FLAG and PhS_3-RNase or PhS_{3L}-RNase using *in vitro* pollen germination system. Untreated pollen tubes were used as negative control (CK). Samples from SPI (treated with S_3S_3 SE), CPC (treated with $S_{3L}S_{3L}$SE) and CK pollen tubes were immunoprecipitated by FLAG antibody and detected by PhS-RNase antibody and FLAG antibody, respectively. Challenged but not immmunoprecipitated SPI, CPC, and CK pollen tube samples were loaded as input. cFBP was detected as input loading control. PT, pollen tube; SE, style extract.

BD::PhSLFs-C and *AD::PhS-RNases* (**Figure 3B**). Taken together, these results show that PhSLFs selectively interact with PhS-RNases in yeast cells.

To confirm the interactions between the PhSLF and PhS-RNase, we performed a co-immunoprecipitation experiment. To examine the co-immunoprecipitation specificity of FLAG antibody, *in vitro* germinated *S_1/PhS$_{3L}$-SLF1::FLAG* pollen tubes from selfed progeny of the primary *S_1S_v/PhS$_{3L}$-SLF1::FLAG* plants described above were continuously treated with *$S_{3L}S_{3L}$* style extracts, whereas wild-type *S_1* and *S_{3L}* pollen tubes were continuously challenged with *$S_{3L}S_{3L}$* style extracts were used as negative controls, respectively. Equal amounts samples were immunoprecipitated by FLAG antibody and detected by PhS-RNase antibody. Western blot results showed that PhS$_{3L}$-RNase detected in the sample of *PhS$_{3L}$-SLF1::FLAG* pollen tubes, but no apparent signal was found in wild-type *S_1* and *S_{3L}* pollen tubes (Supplementary Figure S10). Thus, the FLAG antibody has a strong target specificity and we thus performed co-immunoprecipitation experiment using the antibody to detect the interactions between PhS$_{3L}$-SLF1 and PhS$_3$-RNase or PhS$_{3L}$-RNase. *In vitro* germinated *S_1/PhS$_{3L}$-SLF1::FLAG* pollen tubes were continuously treated with *S_3S_3* and *$S_{3L}S_{3L}$* style extracts to mimic CPC and SPI responses, respectively. Untreated pollen tubes were used as negative control. Equal amounts of the treated and untreated pollen tube samples were immunoprecipitated by FLAG antibody and detected by both PhS-RNase and FLAG antibodies, respectively. Western blot results showed that the specific proteins, similar to that detected in the wild-type *S_3S_3* and *$S_{3L}S_{3L}$* style extracts, were also detected in the treated pollen tubes by the PhS-RNase antibody, but not detected when using the untreated pollen tubes (**Figure 3C**), showing that PhS$_3$-RNase and PhS$_{3L}$-RNase were specifically precipitated by the anti-FLAG antibody in both CPC and SPI responses. Taken together, these results showed that the PhS$_{3L}$-SLF1 interacts with PhS$_3$-RNase and PhS$_{3L}$-RNase.

PhS$_{3L}$-RNases ARE POLYUBIQUITINATED IN CROSS-COMPATIBLE POLLEN TUBES

Previously, AhS-RNases have been shown to be polyubiquitinated in *A. hispanicum in vivo* (Qiao et al., 2004a). To examine whether PhS-RNases were polyubiquitinated in compatible pollination *in vivo*, *$S_{3L}S_{3L}$* styles were pollinated with *S_{3L}* (SPI) and *S_v* (CPC) pollen, respectively. Because S-RNase is highly abundant in the style (Roalson and McCubbin, 2003), it is difficult to detect any difference of ubiquitinated S-RNase between compatible and incompatible pollen tubes within the pollinated styles due to a high background. To overcome the potential problems, the CPC and SPI pollen tubes were dissected out from styles of 13, 16, and 20 h post-pollination for lysis and protein extraction. The dissected pollen tube samples are enriched for pollen tubes (Supplementary Figure S11). Ubiquitination was examined by co-immunoprecipitation using PhS-RNase antibody. Western blot signals of polyubiquitinated PhS$_{3L}$-RNases were specifically detected in the CPC pollen tube samples after 16 and 20 h post-pollination by both PhS-RNase and ubiquitin antibodies, but little in the SPI samples (**Figure 4A**). Thus, these results indicated that PhS$_{3L}$-RNases are polyubiquitinated in compatible pollen tubes in *P. hybrida in vivo*.

To confirm this finding, we examined PhS$_{3L}$-RNase polyubiquitination using the *in vitro* pollen germination system as described. Both *S_{3L}* (SPI) and *S_v* (CPC) pollen tubes were challenged with *$S_{3L}S_{3L}$* style extracts in conjunction with or without MG132 (a peptide aldehyde effectively blocking the proteolytic activity of the 26S proteasome complex) treatment, respectively. The challenged *S_v* and *S_{3L}* pollen tube samples were immunoprecipitated using PhS-RNase antibody and detected by both PhS-RNase and ubiquitin antibodies, respectively. Polyubiquitinated PhS$_{3L}$-RNase was predominantly detected in the CPC pollen tube samples, but not in the SPI samples (**Figure 4B**). In addition, polyubiquitinated PhS$_{3L}$-RNases were accumulated to a higher level after MG132 treatment in the CPC samples but also detected in the SPI samples to some extent (**Figure 4B**), indicating that polyubiquitinated PhS$_{3L}$-RNases are degraded in the CPC pollen tubes. Style extracts and immunoprecipitation of the untreated

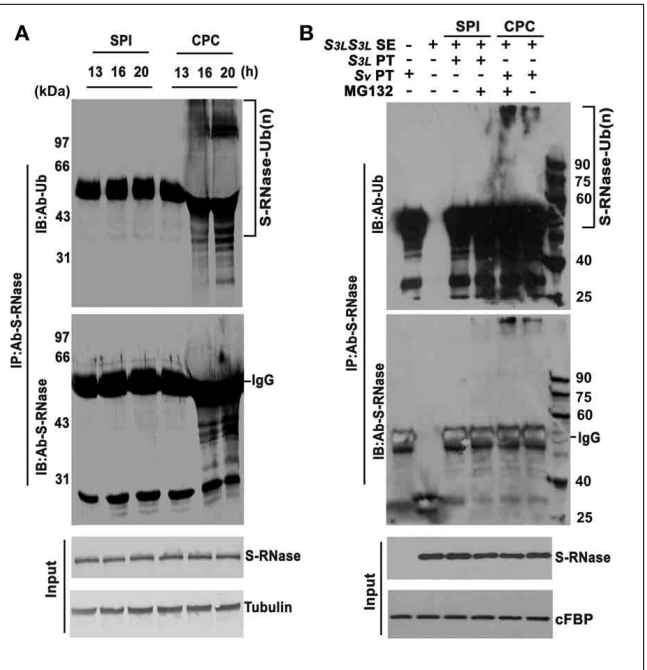

FIGURE 4 | S-RNases are polyubiquitinated in compatible pollen tubes. **(A)** PhS-RNases are polyubiquitinated in pollen tubes of compatible pollination. Pollen tubes were dissected from styles after 13, 16, and 20 h post-pollination. Samples extracted and immunoprecipitated by PhS-RNase antibody and detected by ubiquitin (Ub) antibody and PhS-RNase antibody. PhS-RNase and tubulin were detected as input and loading control. **(B)** PhS-RNases are polyubiquitinated in CPC response in *in vitro* pollen germination system. *In vitro* germinated *S_{3L}* (SPI) and *S_v* (CPC) pollen tubes challenged by *$S_{3L}S_{3L}$* style extract were subjected to MG132 treatment (+) or not (–). Samples of SPI, CPC, and CK were immunoprecipitated by PhS-RNase antibody and detected by ubiquitin (Ub) antibody and PhS-RNase antibody, respectively. Style extracts and immunoprecipitation of the untreated pollen tubes samples were used as negative controls. Challenged but not immmunoprecipitated pollen tube samples were loaded as input. PhS$_{3L}$-RNase and cFBP were detected as input and loading control. S-RNase-Ub (n) denotes polyubiquitinated S-RNase. IgG indicates heavy chain. Molecular weights in kilodalton (kDa) are shown on right or left side of the blots.

pollen tube samples did not show any signal as negative controls (**Figure 4B**). Taken together, these results showed that PhS_{3L}-RNases are indeed polyubiquitinated in the CPC pollen tubes in the *in vitro* germination system, similar to that found *in vivo* (**Figure 4A**). In general, proteins with long polyubiquitin chains (at least four ubiquitin monomers) are recognized and degraded by the UPS (Thrower et al., 2000; Weissman, 2001). Thus, the increase of polyubiquitinated PhS_{3L}-RNases after MG132 treatment suggested that the UPS pathway is involved in S-RNase degradation of the CPC pollen tubes.

S-RNase DEGRADATION BY THE UPS PATHWAY IN THE CROSS-COMPATIBLE POLLEN TUBES IS MEDIATED VIA SCF^{SLF}

The subcellular fractionation experiments showed that PhS_{3L}-RNases were significantly reduced in the cytosol of the compatible pollen tubes, but remain essentially unchanged in SPI responses (Supplementary Figures S9A,B). Moreover, PhS_{3L}-RNases were polyubiquitinated and their polyubiquitination levels increased after MG132 treatment in cross-compatible pollen tubes (**Figures 4A,B**). Thus, our results showed that PhS_{3L}-RNases are degraded in the cross pollen tubes by the UPS pathway. Because of a high background expression level of PhS_{3L}-RNase in style, it would be hard to directly examine whether PhS_{3L}-RNases are degraded in the compatible pollen tubes *in vivo*. Instead, we carried out a pulse chase experiment using the *in vitro* germinated pollen tubes as described above. Both S_{3L} (SPI) and S_v (CPC) pollen tubes were challenged with $S_{3L}S_{3L}$ style extracts and then rinsed and allowed to grow in fresh media in conjunction with or without MG132 treatment, respectively. Equal amounts of the SPI and CPC pollen tube samples were loaded and detected by PhS-RNase antibody. Western blot showed that PhS_{3L}-RNase level was significantly decreased in CPC but still remained essentially unchanged in SPI response (**Figure 5A**), confirming that PhS_{3L}-RNases are degraded in the CPC pollen tubes. In addition, MG132 treatment partially hindered the degradation of PhS_{3L}-RNases in CPC but not in SPI response (**Figure 5A**), supporting the previous finding that PhS_{3L}-RNase degradation occurs by the UPS pathway in cross-compatible pollen tubes.

To validate this finding, we further performed the following experiment also using the *in vitro* germinated pollen tubes. Both S_{3L} (SPI) and S_v (CPC) pollen tubes were continuously treated with $S_{3L}S_{3L}$ style extracts, respectively. Equal amounts of the SPI and CPC pollen tube samples were loaded and detected by PhS-RNase antibody. Western blot showed that PhS_{3L}-RNases were preferentially accumulated in incompatible pollen tubes, whereas PhS_{3L}-RNase were not accumulated in compatible tubes when treated continuously with the style extracts (**Figure 5B**), showing that PhS_{3L}-RNase is continuously degraded in cross-compatible pollen tubes.

To examine whether PhS_{3L}-RNase degradation is mediated by SLF, we used the S_3S_{3L}/PhS_{3L}-*SLF1* plants exhibiting breakdown of self-incompatibility described above (Supplementary Figures S3B, S4A and Supplementary Table S2) and carried out the following experiments. We first identified the S_3S_3/PhS_{3L}-*SLF1* plants from the T_1 progeny of S_3S_{3L}/PhS_{3L}-*SLF1* and collected the pollen grains to perform the pulse chase experiment as described

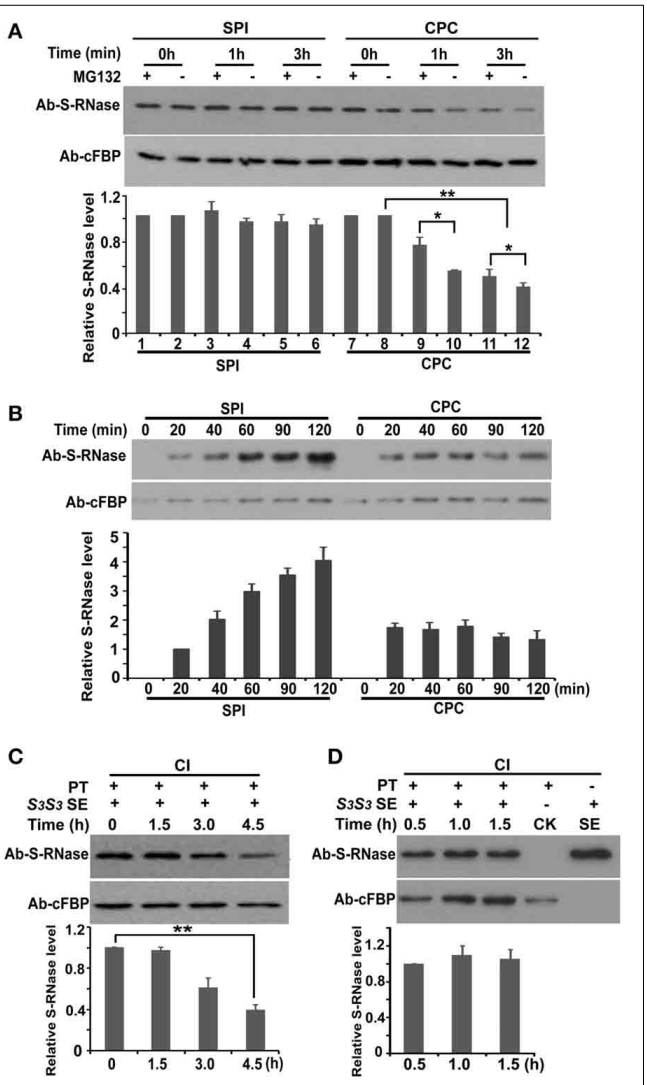

FIGURE 5 | SCF^{SLF} directly mediates non-self S-RNase degradation by the 26S proteasome pathway. (A) Time course analysis of PhS_{3L}-RNase levels in pulse challenged SPI (S_{3L}) and CPC (S_v) pollen tubes in conjunction with (+) or without (−) MG132 treatment. **(B)** Time course analysis of PhS_{3L}-RNase accumulations in continuously challenged SPI (S_{3L}) and CPC (S_v) pollen tubes. *In vitro* germinated S_{3L} and S_v pollen tubes were continuously treated with $S_{3L}S_{3L}$ style extract. PhS_{3L}-RNase levels in pollen tubes were monitored by western blot analysis. cFBP was detected as loading control. **(C)** Time course analysis of PhS_3-RNase levels in pulse challenged pollen tubes with stylar extracts. The pollen tubes were derived from the transgenic plants exhibiting competitive interaction (CI) between the transgene PhS_{3L}-*SLF1* and S_3 haplotype. PhS_3-RNase was detected by western blot to monitor its dynamics in S_3/PhS_{3L}-*SLF1* pollen tubes. **(D)** Time course analysis of PhS_3-RNase levels in continuously challenged pollen tubes with stylar extracts. The transgenic S_3/PhS_{3L}-*SLF1* pollen tubes were continuously treated with S_3S_3 style extract and monitored for S_3-RNase level through the time course. CK indicates pollen tubes before treatment. cFBP was detected as loading control. PT, S_3/PhS_{3L}-*SLF1* pollen tube; SE, style extract. Column charts in both **(A–D)** show quantitative S-RNases levels determined by Quantity One software using three replicates. *T* tests were done between the designated lanes. Single and double asterisks denote significant and extremely significant difference, respectively.

above. The *S_3/PhS$_{3L}$-SLF1* pollen tubes were pulse challenged with S_3S_3 style extracts and then rinsed and left to grow in fresh media to monitor PhS$_3$-RNase levels. Western blot analysis showed that PhS$_3$-RNase levels in the *S_3/PhS$_{3L}$-SLF1* pollen tubes after being challenged with S_3S_3 style extract were found to be gradually reduced (**Figure 5C**), similar to that in compatible pollen tubes (**Figure 5A**), showing that the degradation of PhS$_3$-RNase is mediated by PhS$_{3L}$-SLF1 action.

To validate this finding, we performed the following experiments. The *S_3/PhS$_{3L}$-SLF1* pollen tubes were continuously treated with S_3S_3 style extracts. Equal amounts of the samples were loaded and detected by PhS-RNase antibody. Western blot analysis showed that PhS$_3$-RNase was not accumulated in the continuously challenged *S_3/PhS$_{3L}$-SLF1* pollen tubes (**Figure 5D**), similar to that in compatible pollen tubes (**Figure 5B**), showing that S$_3$-RNase is degraded continuously in the *S_3/PhS$_{3L}$-SLF1* pollen tubes. Taken together, these results suggested that S-RNase degradation in compatible pollen tubes is mediated by action of non-self SCF^{SLF}.

DISCUSSION

Self and non-self-recognition of the pistil and pollen factors is one of the most intriguing processes of self-incompatibility. The pistil *S* determinant S-RNase activity has been shown to serve as cytotoxins to inhibit the pollen tube growth (McClure et al., 1990; Liu et al., 2009). Thus, compatible pollen tube must take an effective mechanism to restrict the S-RNase activity. Recently, two S-RNase restriction mechanisms, the S-RNase degradation or the S-RNase compartmentalization have been proposed to explain S-RNase fate during SI responses. In this study, we have provided several lines of evidence to support the S-RNase degradation mechanism. First, both immunogold labeling and subcellular fractionation showed that the two key pollen SI factors of *P. hybrida*, *PhS$_{3L}$-SLF1* and PhSSK1, localize to the cytosols of both pollen grains and tubes and that the majority of S-RNase also has a similar localization after pollination, consistent with their joint roles in the pollen tubes. Second, both yeast two-hybrid and co-immunoprecipitation results showed PhSLFs and PhS-RNases directly interacts with each other, indicating that their recognition occurs in the cytosol where the initial self and non-self-signaling for the downstream discriminative responses could occur. Third, polyubiquitinated PhS-RNases were predominantly detected in the CPC pollen tubes both *in vivo* and *in vitro* by immunoprecipitation assay and their levels increased after MG132 treatment, consistent with the UPS pathway is involved in the polyubiquitinated S-RNase degradation (Zhang et al., 2009). Finally, we showed that the S-RNase degradation is mediated by action of non-self SCF^{SLF}. Thus, our findings show that S-RNase degradation serves as the major mechanism for restricting S-RNase cytotoxicity in compatible pollen tubes. This conclusion is consistent with the most of the previous results (Takayama and Isogai, 2005; Zhang et al., 2009; Iwano and Takayama, 2012).

On the basis of our results and other recent findings, we propose a model for S-RNase-based self-incompatibility (**Figure 6A**). In this model, S-RNases are taken up into the cytosols of both CPC and SPI pollen tubes. In the cytosol, a repertoire of SLFs and S-RNases interact to trigger self or non-self-discriminative

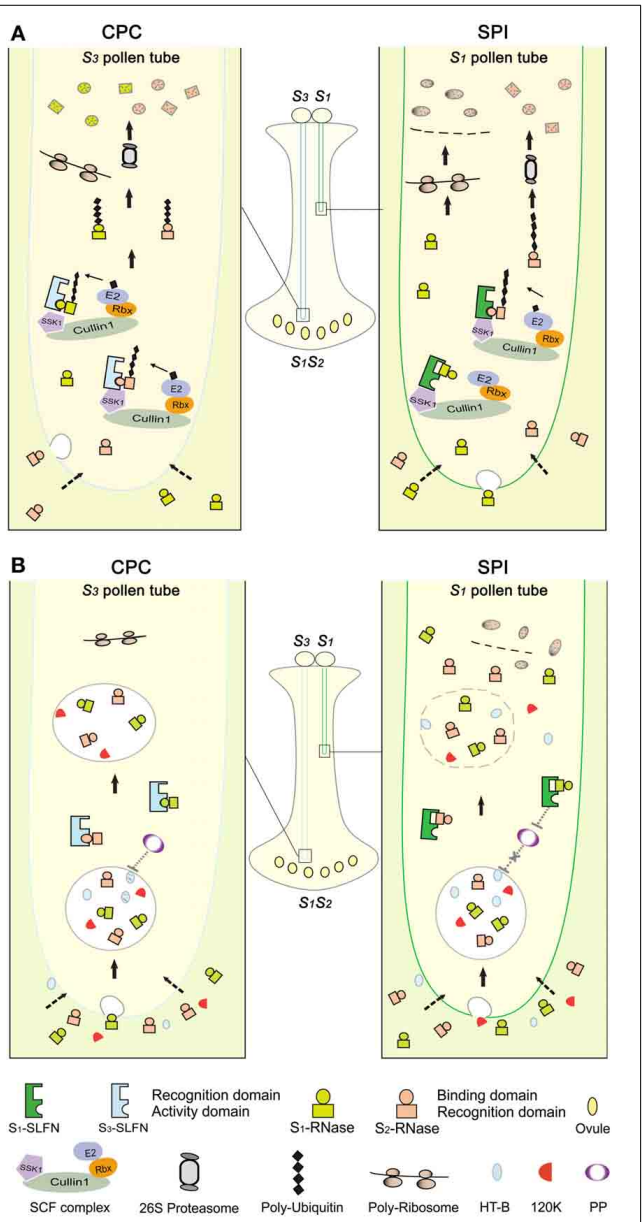

FIGURE 6 | Models of S-RNase-based self-incompatibility. (A) S-RNase degradation model in *Petunia*. In CPC response, cross S_3 pollen lands on the stigma, germinates and grows into an S_1S_2 style. Both S$_1$- and S$_2$-RNases enter the S_3 pollen tube by endocytosis or an unknown pathway (dotted arrows). Subsequently, the hypothetical binding domains of S$_1$- and S$_2$-RNases interact with a hypothetical activity domain of the pollen *S* (i.e., SLF in *Solanaceae* and *Plantaginaceae*) in the cytosol of the pollen tube. Then, one or more SLF (SLFN) in S_3 pollen tube form functional SCF$^{S3\text{-}SLFN}$ complexes to tag S$_1$- and S$_2$-RNases with a polyubiquitin chain, which are subsequently degraded by the 26S proteasome. Thus, ribosomal RNAs escape from degradation by S-RNases in cross-pollen tubes, resulting in normal pollen tube growth. In SPI, during which self S_1 pollen lands on the S_1S_2 style, both S$_1$- and S$_2$-RNases enter the S_1 pollen tube. Similar to CPC response, non-self S$_2$-RNases bind to a hypothetical activity domain of the pollen *S* in the cytosol of the pollen tube. Then, one or more SLF (SLFN) in S_1 pollen tube form SCF$^{S1\text{-}SLFN}$ complexes to tag S$_2$-RNase with a polyubiquitin chain, resulting in its degradation by the 26S proteasome. In contrast, the recognition domain of self S$_1$-RNase binds to a hypothetical

(Continued)

FIGURE 6 | Continued
recognition domain of SLF resulting in the formation of a non-functional SCF$^{S1\text{-}SLFN}$ complex, thus self S-RNase escapes degradation and acts as a cytotoxin to inhibit the pollen tube growth. **(B)** S-RNase compartmentalization model in *Nicotiana*. S-RNases, 120K and HT-B all enter the pollen tubes. In CPC response, HT-B degradation possibly occurs by a hypothetical pollen protein (PP), thus S-RNase is sorted to a vacuolar compartment. In SPI, the interaction between S-RNase and SLF has been postulated to stabilize HT-B, perhaps by inhibiting the PP action. Subsequently, the vacuolar compartment breaks down, releasing S-RNase and acts as a cytotoxin to inhibit the pollen tube growth.

responses, respectively. Two hypothetical separate domains for SLF (recognition and activity domain) and S-RNase (recognition and binding domain) presumably mediate self and non-self-interaction between them as previously suggested (Kao and McCubbin, 1996). All SLF members in a given pollen tube form a spectrum of SCF^{SLF} complexes. In compatible pollen tubes, all SCF^{SLF} complexes could mediate the polyubiquitination of non-self S-RNases, which are destined to degradation via the UPS pathway. Whereas in incompatible pollen tubes, though non-self S-RNase is targeted for degradation similar to that in CPC, self S-RNase somehow escapes degradation by a yet unknown specific interaction between SLF and S-RNase, leaving it intact to exert a cytotoxic effect on self-pollen tubes and resulting in their growth inhibition.

Similar to previous models (Kao and Tsukamoto, 2004; Zhang et al., 2009; Chen et al., 2010), our model also predicts that S-RNases interacts with its self or non-self SLFs through different domains. But, it is not known where those domains are positioned in both S-RNases and SLFs. Our preliminary results suggest that the PhSLFs can selectively interact with "self" and "non-self" PhS-RNases in yeast system, and future studies will need to confirm these results *in vivo*. In addition, the "self" interaction between SLFs and S-RNases also have been detected in *Petunia inflata* (Solanaceae), *A. hispanicum* (Plantaginaceae) and apple (Rosaceae) (Qiao et al., 2004a; Hua and Kao, 2006; Yuan et al., 2014). Nevertheless, the interaction affinity analysis between PiS-RNase and PiSLF has shown that PiSLF interacts with non-self PiS-RNases more strongly than with self S-RNase and vice versa for SLFs in *Petunia inflata* (Hua et al., 2007). Recently, Kubo et al. found that a repertoire of *SLF* genes constituting the pollen *S* in a given *S*-haplotype and collectively control the pollen specificity against non-self S-RNases. They predicted that each type of SLF interacts with a subset of non-self S-RNases in a species and all SLF are required to detoxify all non-self S-RNases (Kubo et al., 2010). Thus, it is likely that a complicated relationship occurs among S-RNases and SLFs. In pollen tubes of a given *S*-haplotype, all of SLF proteins recognize and interact with self S-RNase but must through different domains from that with all non-self S-RNases. These differential interactions between S-RNase and self/non-self SLF might serve as an initial differential signaling mechanism for the downstream compatible and incompatible responses but further studies are needed to determine if this is the case *in vivo*.

The interactions between SLFs with non-self S-RNases have been shown to result in S-RNases polyubiquitination which is mediated by SCF^{SLF} complexes in our study, but it is not clear which lysine site(s) are ubiquitinated and whether monoubiquitination of S-RNases also occur. Other recent studies also showed that SCF^{SLF} complexes poly-ubiquitinate non-self S-RNases, resulting in their degradation *in vitro* (Entani et al., 2014; Yuan et al., 2014). Interestingly, some gold particles of PhS$_{3L}$-RNase were also detected in the cytosol of the compatible pollen tubes even 24 h post pollination (**Figure 2A**), indicating that not all S-RNases are degraded in compatible pollen tubes. Recent studies also have shown that SI response requires the presence of a minimum level of S-RNase in the style, an amount referred to as a threshold level. A threshold value of S-RNases in the style is required for pollen rejection or acceptance (Qin et al., 2006; Soulard et al., 2014). Thus, in our results, S-RNase is likely degraded to a threshold value, but not all S-RNase would be degraded in cross pollen tubes. In addition, Qin et al. (2005) showed that replacing only one lysine residue in the C4 region (that is conserved in solanaceous S-RNases) with arginine did not affect SI function of S$_{11}$-RNase in *S. chacoense*, indicating that this lysine is not involved in the S-RNase ubiquitination and degradation. In addition, F-box proteins themselves are known to be ubiquitinated and regulated by the UPS in mammals, yeast and plants (Galan and Peter, 1999; Wirbelauer et al., 2000; Stuttmann et al., 2009). Recently, Chen et al. (2012) found that a novel conserved ubiquitin-binding domain structure in the C-terminal regions of both PhS$_{3L}$-SLF1 and PhS$_1$-SLF1. Therefore, identification of potential ubiquitination sites and types of both S-RNases and SLFs is required to address those questions.

The S-RNase degradation model (**Figure 6A**) depicts of the S-RNase restriction mechanisms in compatible pollen tube, incompatible pollination, and competitive interaction. However, it is not entirely clear how the incompatible reaction occurs in pollen tubes. Recent studies have shown that S-RNases depolymerize actin cytoskeleton, trigger mitochondrial alteration and DNA degradation response in incompatible pollen tube, indicating that programmed cell death may occur in incompatible reaction of pears (*Pyrus pyrifolia*) (Liu et al., 2007; Wang et al., 2009; Wang and Zhang, 2011). In addition, studies have shown that HT-B protein, stylar 120 kDa glycoprotein and NaStEP are required for pollen rejection in *Nicotiana* but not for specificity (McClure et al., 1999; Hancock et al., 2005; Jimenez-Durán et al., 2013). Although these pistil factors have been identified, the underlying mechanisms for their action are currently unknown.

The S-RNase compartmentalization mechanism (**Figure 6B**) has been proposed for the S-RNase restriction mechanism during CPC responses in *Nicotiana*, but it remains unclear whether it functions in parallel, in sequence with the degradation mechanism. The two models make different presumptions about the interaction between S-RNases and SLFs. In the S-RNase degradation model, non-self S-RNase is degraded as a direct result of their interaction, indicating that SLFs are essential factors, consistent with genetic and molecular evidence of competitive interaction in both Solanaceae and Plantaginaceae (Golz et al., 1999; Xue et al., 2009; Sun and Kao, 2013). In the compartmentalization model, S-RNases are sequestered into a vacuole by a non-*S*-specific mechanism and an interaction between S-RNases and SLFs interferes with this process. In compatible pollinations,

pollen overcomes rejection by degrading HT-B and compartmentalizing S-RNase, indicating that HT-B is essential factor. However, it is not clear how the S-RNase and SLF interaction controls HT-B degradation and vacuole membrane breakdown. Our results also appear to suggest that different mechanisms of the pollen reception are operating between *Nicotiana* and *Petunia*. It would be interesting to examine these potential differences by a comparative analysis using additional Solanaceaous species. Nevertheless, pollen tubes might possess a multilayered resistance mechanism against S-RNase where most non-self S-RNases are degraded to detoxify its activity but a small amount is compartmentalized and/or distributed in cytosol. In fact, our subcellular fractionation results showed that a minute amount of S-RNases are associated with a microsom-like fraction (**Figure S9A**). What role, in any, of the microsome-associated S-RNase plays in CPC and SPI responses remains to be determined.

In conclusion, our results showed that S-RNases are degraded in the cytosol of pollen tubes through the SCF^{SLF}-mediated UPS pathway during the CPC response in *P. hybrida*. However, further studies including a detailed analysis of both S-RNase and SLF ubiquitinations and their types are urgently needed to address the biochemical mechanism of the SPI response and a potential link between the cytosolic and microsome-associated S-RNases during SPI and CPC responses.

MATERIALS AND METHODS

PLANT MATERIALS AND TRANSFORMATION

Wild-type self-incompatible homozygous *P. hybrida* lines ($S_{3L}S_{3L}$, S_vS_v, S_1S_1, and S_3S_3) have been previously described (Clark et al., 1990; Robbins et al., 2000; Qiao et al., 2004b). Heterozygous S_3S_{3L}, $S_{3L}S_v$, and S_1S_v were derived from crosses of related SI lines. Ti plasmid constructs were separately electroporated into *Agrobacterium tumefaciens* strain LBA4404 (Invitrogen), and transgenic plants were generated by Agrobacterium-mediated leaf disk transformation (Lee et al., 1994; Qiao et al., 2004b).

POLYMERASE CHAIN REACTION (PCR)

The genomic DNA or cDNA were amplified by PCR using rTaq or ExTaq DNA Polymerase (Takara). Genotyping assays were carried out with genomic DNA. PCR primers are shown in Supplementary Table S8. They were used at a final concentration of 5 μM in 20 μl reactions. The PCR condition was 95°C for 5 min for hot start, then 35 cycles of the following: 95°C for 30 s, annealing at 52–58°C (about 3–5°C below the Tm of the primers used) for 30 s, extension at 72°C for 90 s, and finally incubated at 72°C for 10 min.

POLLINATION

All of the pollinations were performed using open flowers. In the process of cross-pollination, anthers were removed from the flower of the plant serving as female recipient before dehiscence to prevent self-pollination. Pollinated flowers were covered with paper bags. Pollinated styles used in immunogold labeling, aniline blue staining and ubiquitination assay were performed as follows: self-pollinations were performed with wild-type $S_{3L}S_{3L}$ plants and cross-pollinations with pollen from S_vS_v plants on $S_{3L}S_{3L}$ plants.

IN VITRO GERMINATION OF POLLEN TUBES

Mature pollen grains were suspended and incubated in liquid pollen germination medium (PGM, 20 mM MES, 15% PEG4000, 2% sucrose, 0.07% $Ca(NO_3)_2{\cdot}4H_2O$, 0.02% $MgSO_4{\cdot}7H_2O$, 0.01% KNO_3, 0.01% H_3BO_3, pH 6.0) at 25°C in the dark. For the treatment of pollen tubes, pollen tubes cultured for about 1.5 h were collected by centrifugation at 1000 g for 1 min and then suspended in fresh PGM with style lysates. For fractionation, immunogold labeling, co-immunoprecipitation, ubiquitination and S-RNase degradation assays, pollen tube samples were collected by centrifugation as above and then rinsed twice with fresh PGM to removal the S-RNases in PGM. Style lysates were prepared by grinding pistils to a fine powder and protein were extracted with PGM.

IMMUNOGOLD LABELING AND ELECTRON MICROSCOPY

Pollen grains, treated pollen tubes or pollinated styles (at least 2 or 3 replicates of each sample) were chemically fixed overnight at 25°C in 2.5% glutaraldehyde and 1.5% paraformaldehyde in 0.1 M PBS (pH 7.4). Samples fixed were rinsed several times with 0.1 M PBS and dehydrated sequentially with 30, 50, 70, 80, 90, 95, and 100% ethanol. Dehydrated samples were embedded in LR-White Resin (Sigma-Aldrich, USA). The embedded samples were sectioned into 90 nm sections with ultramicrotome (Leica EM UC6, German). The ultra-thin sections were placed on Formvar-coated nickel grids. Sections were blocked with PBS-Glycine buffer (PBS with 50 mM glycine) followed by PBGT buffer (PBS with 0.1% galtel, 1.0% BSA, 0.1% Tween 20) and incubated at 4°C overnight with primary antibody (1:2000 dilution of monoclonal anti-FLAG antibody for PhS_{3L}-SLF1 labeling and 1:3000 dilution of polyclonal antibodies for PhSSK1 and PhS-RNase labeling, respectively). Polyclonal antibodies to PhSSK1 and PhS-RNase have been previously described (Zhao et al., 2010). Primary antibody labeled sections were washed with PBGT buffer 6 times and subjected to labeling with secondary antibody conjugated to 20 nm diameter colloidal gold particles at 1:20 dilution in PBGT buffer for 90 min at room temperature. After sequential washes with PBGT buffer, PBS-Glycine buffer (PBS with 50 mM glycine) and deionized water, twice for each wash, labeled sections were subjected to Danscher's enhancer solution at 25°C for 25 min to enlarge gold particles. Sections were stained with 2% uranyl acetate for 15 min and observed by using a HITACHI H-7500 transmission electron microscope (Danscher, 1981; Holgate et al., 1983).

SUBCELLULAR FRACTIONATION AND IMMUNODETECTION

Pollen grains or *in vitro* germinated pollen tubes were homogenized with FastPrep-24 system (MP Biomedicals, USA) in homogenization buffer (HB) containing 330 mM sucrose, 150 mM KCl, 50 mM Tris-MES, pH 7.5, with additives of 1 mM EDTA, 1 mM PMSF, 1 mM DTT, and protease inhibitor cocktail. The fractionation procedure was performed on ice or at 4°C. The homogenate was subjected to differential centrifugation of three or four sequential steps with centrifuge force at 1000, 12,000, 40,000 g (omitted in three-step ones) and 160,000 g, respectively. The resulting pellets were suspended with HB buffer while the supernatant fraction was subjected to next step of centrifugation,

before which an aliquot was stored for detection. Protein samples were quantified by the Bradford method. Equal amounts of protein samples were applied to 12% SDS-PAGE gels and transferred to PVDF membranes (GE Healthcare, USA) for western blot detection. The amounts of proteins were calculated from immuno signal determined by Quantity One software (Bio-Rad, USA). Antibodies used are as below, monoclonal antibody to the FLAG tag is a commercial product (Sigma-Aldrich, USA), polyclonal antibodies to organelle markers are all purchased (Agrisera, Sweden).

ISOLATION AND IDENTIFICATION OF PhS_{3L}-RNase BY LC-MS/MS

$S_{3L}S_{3L}$ styles were extracted by trichloroacetic acid-acetone precipitation method. Proteins were dissolved from the dried precipitate using lysis-buffer (7 M urea, 2 M thiourea, 4% CHAPS, 65 mM DTT, 0.5% IPG buffer 3–10, 0.002% bromophenol blue.). Protein concentration was determined by the Bradford method. About 500 μg protein sample was loaded onto an IPG strip holder for a 24 cm, pH 3–10 non-linear gradient IPG strip (Bio-Rad) for the isoelectric focusing. For the SDS-PAGE, the equilibrated IPG gel strips were placed onto 12% gel. One gel was transferred to PVDF membranes for western blot detection. The other gel was visualized by Coomassie Brilliant Blue staining. The target protein was excised from the gel and digested with trypsin. Then the digested peptides identified by LC-MS/MS by Beijing protein innovation co., Ltd. All of the peptide fragments of the proteins were identified by the MASCOT server in-house search engine and compared against a merged database consisting of the Solanceae database and containing PhS_{3L}-RNase. MASCOT search parameters were set as follows: threshold of the ions score cutoff, 0.05; peptide tolerance, 10 ppm; MS/MS tolerance, 0.2 Da; and peptide charge, 1+, 2+ or 3+. The search was also set to allow one missed cleavage by trypsin, carboxymethylation modification of Cys residues, and variable oxidation of Met residues.

ANILINE BLUE STAINING OF POLLEN TUBES WITHIN STYLE

Pollinated styles were chemically fixed in ethanol: glacial acetic acid (3:1) solution 24 h or more at 25°C. Then, rinsing three times in water, pistils were transferred to 8 N sodium hydroxide solution for 8 h to clear and soften the tissue. After rinsing in water, the softened pistils were soaked in a 0.1% solution of water-soluble aniline blue dye dissolved in 0.1 N K_3PO_4 for 8–24 h in the dark at room temperature. The pollen tubes in the styles are smeared or are observed whole under a conventional or dissecting microscope by direct illumination with ultraviolet light of Olympus BX53 fluorescence microscope. Observations are made in a darkened room.

YEAST TWO-HYBRID ASSAYS

The full-length *PhS-RNases* were cloned into *pGADT7* (Clontech, CA, USA), respectively, to produce fusion proteins with the GAL4 activation domain. The C-terminal of *PhSLF*s(removing N-terminal approximately 60 amino acid long F-box motif) was introduced into *pGBKT7* (Clontech, CA, USA), to form recombinants with the GAL4 DNA binding domain. The various combinations of BD and AD vectors were co-transformed into yeast strain AH109 and grown on SD/-Leu-Trp medium at 30°C for 3 days. The clones were subsequently grown on SD/-Ade-His-Leu-Trp medium at 30°C for 7 days to test interaction. The Combinations of *AD::PhS-RNases* with empty BD are used for detecting PhS-RNases self-activation. The combinations of *BD::PhSLF* with empty AD were carried out to evaluate PhSLFs self-activation in yeast two-hybrid assays. The combinations of empty AD with empty BD indicate negative controls. For β-galactosidase assay, yeast clones grown for 48 h at 30°C were transferred onto filter paper, and the clones were lysed in liquid nitrogen, and then 5 mL Z buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, and 1 mM $MgSO_4$, pH 7.0) containing β-mercaptoethanol was added and incubated at 30°C (for <8 h) and checked periodically for the appearance of blue color.

CO-IMMUNOPRECIPITATION

Mature pollen grains from transgenic plants of S_1/PhS_{3L}-*SLF1::FLAG* were suspended and incubated in liquid pollen germination medium described above. Total style proteins were extracted from S_3S_3 and $S_{3L}S_{3L}$ homozygous plants and co-incubated with germinated pollen tubes, respectively. For immunoprecipitation, anti-FLAG affinity gel (Sigma-Aldrich) was mixed with pollen tube extracts and co-incubated for 4 h at 4°C on a rotary shaker. After the affinity gel was washed with PBS buffer to remove all of the non-specific proteins. The protein being eluted from the resin can be monitored by measuring the absorbance of the eluant at 280 nm and further washed until the absorbance difference of the wash solution coming off the column is less than 0.05 vs. a wash solution blank as described procedure in ANTI-FLAG® M2 affinity gel (Sigma-Aldrich). Bound proteins were eluted with 0.1 M glycine HCl, pH 3.5. After equilibration of the eluted protein with 0.5 M Tris-HCl, pH 7.4, with 1.5 M NaCl, 2× SDS-PAGE sample loading buffer (125 mM Tris-HCl, pH 6.8, with 4% SDS, 20% glycerol, and 0.004% bromphenol blue) was added to each sample and control. The samples were denatured at 100°C for 5 min and then equal amount of protein samples were subjected to 12% SDS-PAGE gels and then transferred to PVDF membranes for western blot detection. Membranes were incubated with primary antibodies (1:3000 dilution of polyclonal antibody for PhS-RNase and 1:2000 dilution of monoclonal anti-FLAG antibody), and then incubated with anti-rabbit or anti-mouse IgG conjugated to horseradish peroxidase followed by chemiluminescence detection and then exposed to film.

UBIQUITINATION ASSAY

For the ubiquitination assay of *in vivo*, pollen tubes were dissected from pollinated styles. Pollinated styles were placed in extraction buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10 mM $MgCl_2$, 1 mM EDTA, 1 mM PMSF, 0.1% NP-40, 10% glycerin and 1×protease inhibitor cocktail), then pollen tubes were carefully pulled out through a small slit in the style as described by Goldraij et al. (2006) in stereomicroscope and were frozen in liquid nitrogen. For the ubiquitination assay of *in vitro* pollen germination system, S_{3L} and S_v pollen tubes were continuously treated 1.5 h with $S_{3L}S_{3L}$ style extract in conjunction with or without MG132 (40 μM), respectively, and then lysed in extraction buffer. Protein concentration was determined by the Bradford method. Protein extracts were incubated with primary antibody

(polyclonal antibody for PhS-RNase) at 4°C for 4 h on a rotary shaker. Protein G agarose beads (IP50 Kit, Sigma-Aldrich, USA) were added and samples co-incubated for a further 2 h at 4°C on a rotary shaker. Samples were washed using IP buffer provided in the Kit. The protein/bead mixture was denatured at 100°C for 5 min and then equal amounts of protein samples were loading on 12% SDS-PAGE gels and then transferred to PVDF membranes for western blot detection. Membranes were incubated with primary antibodies (1:3000 dilution of polyclonal antibody for PhS-RNase and 1:2000 dilution of polyclonal antibody for ubiquitin), and then incubated with anti-rabbit IgG conjugated to horseradish peroxidase followed by chemiluminescence detection and then exposed to film.

IN VITRO S-RNase DEGRADATION ASSAYS

For pulse challenged assay, *in vitro* germinated pollen tubes were co-incubated with style extracts for 1 h and then rinsed with style extracts and allowed pollen tubes to grow in fresh media to monitor S-RNase dynamics in pollen tubes. For continuously challenged assay, *in vitro* germinated pollen tubes were co-incubated with style extracts at different time points, and then lysed in extraction buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10 mM $MgCl_2$, 1 mM EDTA, 1 mM PMSF, 0.1% NP-40, 10% glycerin and 1 × protease inhibitor cocktail). Protein concentration was determined by the Bradford method. Equal amounts of protein samples were loaded on 12% SDS-PAGE gels and then transferred to PVDF membranes for western blot detection. Membranes were incubated with primary antibodies (1:3000 dilution of polyclonal antibody for PhS-RNase), and then incubated with anti-rabbit IgG conjugated to horseradish peroxidase followed by chemiluminescence detection and then exposed to film.

PHYLOGENETIC ANALYSIS

Analysis of *SLF* genes based on deduced amino acid sequences were carried out using a neighbor-joining method with 1000 bootstrap replicates using MEGA version 5.0 (Tamura et al., 2011). *PhS_1-SLF1*, *PhS_3A-SLF1*, *PhS_{3L}-SLF1*, and *PhS_v-SLF1* were cloned in this study; S_{17} alleles are from *P. axillaris* (Tsukamoto et al., 2005) and all others from *P. hybrida* (Kubo et al., 2010).

AUTHOR CONTRIBUTIONS

Yongbiao Xue conceived and designed the experiments. Wei Liu, Jiangbo Fan, Junhui Li, and Yanzhai Song performed the experiments. Qun Li and Yu'e Zhang provided technical support. Wei Liu and Yongbiao Xue were principally responsible for drafting the final manuscript. All authors provided intellectual content and contributed to manuscript revisions.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://www.frontiersin.org/journal/10.3389/fgene.2014.00228/abstract

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Distinct phylogenetic relationships and biochemical properties of Arabidopsis ovarian tumor-related deubiquitinases support their functional differentiation

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The reverse reaction of ubiquitylation is catalyzed by different classes of deubiquitylation enzymes (DUBs), including ovarian tumor domain (OTU)-containing DUBs; experiments using *Homo sapiens* proteins have demonstrated that OTU DUBs modulate various cellular processes. With the exception of OTLD1, plant OTU DUBs have not been characterized. We identified 12 *Arabidopsis thaliana OTU* loci and analyzed 11 of the encoded proteins *in vitro* to determine their preferences for the ubiquitin (UB) chains of M1, K48, and K63 linkages as well as the UB-/RUB-/SUMO-GST fusions. The *A. thaliana* OTU DUBs were shown to be cysteine proteases and classified into four groups with distinct linkage preferences: OTU1 (M1 = K48 > K63), OTU3/4/7/10 (K63 > K48 > M1), OTU2/9 (K48 = K63), and OTU5/11/12/OTLD1 (inactive). Five active OTU DUBs (OTU3/4/7/9/10) also cleaved RUB fusion. OTU1/3/4 cleaved M1 UB chains, suggesting a possible role for M1 chains in plant cellular signaling. The different substrate specificities of the various *A. thaliana* OTU DUBs indicate the involvement of distinct structural elements; for example, the OTU1 oxyanion residue D89 is essential for cleaving isopeptide bond-linked chains but dispensable for M1 chains. UB-binding activities were detected only for OTU2 and OTLD1, with distinct linkage preferences. These differences in biochemical properties support the involvement of *A. thaliana* OTU DUBs in different functions. Moreover, based on the established phylogenetic tree, plant- and *H. sapiens*-specific clades exist, which suggests that the proteins within these clades have taxa-specific functions. We also detected five OTU clades that are conserved across species, which suggests that the orthologs in different species within each clade are involved in conserved cellular processes, such as ERAD and DNA damage responses. However, different linkage preferences have been detected among potential cross-species OTU orthologs, indicating functional and mechanistic differentiation.

Keywords: Arabidopsis, deubiquitylation, OTU, ubiquitin, DUB

INTRODUCTION

By modulating the stability, activity, interaction, or subcellular localization of critical regulatory and mechanistic components, the covalent attachment and removal of ubiquitin (UB), i.e., ubiquitylation and deubiquitylation, are essential mechanistic and regulatory elements of numerous cellular processes, such as chromatin silencing, transcriptional activation, mRNA splicing and export, cell division, DNA damage response, intracellular trafficking, and signal transduction (Hershko and Ciechanover, 1998; Komander et al., 2009; Reyes-Turcu et al., 2009).

The close connection between reversible ubiquitylation and almost all aspects of cellular processes and organismic functions is primarily due to the many components involved, which include substrates, conjugation enzymes, deconjugation enzymes, and diverse signals derived from the small but highly conserved UB proteins and their cognate binding partners, which decipher the signals (Smalle and Vierstra, 2004; Fu et al., 2010). The numerous protein-interacting interfaces of UB and attachment as a monomer or polymers with distinct linkages enables UB assembly on substrates to yield diverse signals (Komander and Rape, 2012). The attachment of monomeric UB and UB chains through eight different linkages, including the linear M1, K6, K11, K27, K29, K33, K48, and K63 linkages, produces different topoisomers with distinct functional roles. While K48-linked UB chains target modified proteins for proteasomal degradation (Chau et al., 1989), K63-linked chains are critical for signaling complex assembly, endocytosis, and DNA damage responses (Chen and Sun, 2009). K11- and K29-linked chains are alternative proteasomal degradation signals for cell-cycle regulators and UB fusion degradation pathway substrates, respectively (Johnson et al., 1995; Jin et al., 2008). More recently, linear UB chains and typical K63 linkages have emerged as important signals for NF-κB activation pathways in mammals (Rieser et al., 2013). The cellular functions of the other atypical chains remain elusive (Chen and Sun, 2009; Kulathu and Komander, 2012).

While conjugation enzymes, including activation enzymes (E1s) and, in particular, the UB carriers (E2s) and ligases (E3s), are essential for UB signal assembly on substrates, the reverse

reaction, which is catalyzed by a diverse set of deubiquitylation enzymes (DUBs), is equally important for modulating the formation and disassembly of the diverse ubiquitin signals. DUBs have been extensively studied in budding yeast (*Saccharomyces cerevisiae*) and mammals for their roles in various cellular and organismic processes and their biochemical properties, including structural elements, catalytic mechanisms, UB binding, and substrate specificities (Reyes-Turcu et al., 2009). Similar to those from other eukaryotic species, Arabidopsis (*Arabidopsis thaliana*) DUBs are grouped into five classes and comprise the second most abundant enzymatic component of the UB system (Komander et al., 2009). Four of these classes are cysteine proteases, including 3 UB C-terminal hydrolases (UCH) (Yang et al., 2007), 27 UB-specific processing proteases (UBP/USP) (Yan et al., 2000; Liu et al., 2008), 12 *ovarian tumor*(OTU)-related proteases (this study), and 2 Josephin/Machado-Joseph disease proteases (MJD) (At3g54130 and At2g29640). The other class, belonging to the zinc-dependent metalloproteases, comprises at least 6 JAB1/MPN/Mov34 (JAMN) domain-containing proteases, including AMSH1-3, the proteasome subunit RPN11, and BRCC36A/B (Yang et al., 2004; Isono et al., 2010; Block-Schmidt et al., 2011).

Based on phenotype analyses of T-DNA insertion mutants, *A. thaliana* DUBs of various classes play important roles in plant growth and development. However, the specific cellular processes and mechanistic components involved have not been determined for most of the plant DUBs described. Among the UCH DUBs, UCH1 and UCH2 are likely involved in auxin signaling and are critical for shoot architecture and leaf morphology (Yang et al., 2007). The best characterized plant DUBs are the UBPs, including UBP1 and UBP2, which are required for the turnover of aberrant proteins (Yan et al., 2000); UBP3 and UBP4, which are essential for male gametophyte development (Doelling et al., 2007); UBP12 and UBP13, which play as negative regulators in immune response (Ewan et al., 2011); UBP14 and UBP19, which are important for embryogenesis (Doelling et al., 2001; Liu et al., 2008); and UBP15, which is critical for vegetative and reproductive growth (Liu et al., 2008). UBP26 is involved in both transcriptional suppression and activation. It is required for seed development through modulation of the repressive histone mark H3K27me3 on the Polycomb group complex-targeted gene *PHERES1* (Luo et al., 2008). UBP26 is also required for heterochromatic silencing of transgenes and transposons by affecting the methylation of DNA and histone H3 at various lysines (Sridhar et al., 2007). By contrast, UBP26 is required for transcriptional activation of *FLOWERING LOCUS C (FLC)* to suppress flowering (Schmitz et al., 2009). In the *ubp26* mutant, ubH2B accumulates globally and at the *FLC* locus. Moreover, the activating histone mark H3K36me3 and the repressive mark H3K27me3 were decreased and increased, respectively, at the *FLC* locus, resulting in transcriptional suppression. Among the JAMN DUBs, AMSH1 and AMSH3 are likely involved in deubiquitylating endocytosed plasma membrane cargos by interacting with the ESCRT-III subunits (Katsiarimpa et al., 2011, 2013). BRCC36A and BRCC36B are homologs of mammalian BRCC36, a component of a protein complex containing BRCA1, and are likely involved in intra- and inter-chromosomal homologous recombination (Block-Schmidt et al., 2011). With the exception of OTLD1, the functional and biochemical properties of the *A. thaliana* OTU DUBs have not been characterized. OTLD1 may function in a histone-modifying repressor complex harboring the histone lysine demethylase KDM1C to suppress specific gene expression through histone deubiquitylation and demethylation (Krichevsky et al., 2011).

The OTU domain was initially identified in the *Drosophila melanogaster OTU* gene product (Steinhauer et al., 1989) and subsequently observed in protein sequences from various eukaryotes, including animals and plants, viruses, and a single bacterium *Chlamydia pneumonia* (Makarova et al., 2000). The *Homo sapiens* OTU domain-containing proteins OTUB1 and OTUB2 were subsequently identified as novel DUBs in experiments employing UB derivatives with thiol-reactive C-terminal groups (Borodovsky et al., 2002). Subsequent extensive biochemical, structural, and functional analyses have primarily been conducted with *H. sapiens* OTU DUBs; these studies have provided extensive information on their biochemical properties and important regulatory roles in signaling cascades.

The phylogenetic relationships between OTU DUBs from different species have not been reported. A phylogenetic tree established for *H. sapiens* OTU proteins identified four major clades: the OTUB clade (OTUB1 and OTUB2), the OTUD clade (OTUD1, OTUD2/YOD1, OTUD3, OTUD4, OTUD5/DUBA, OTUD6A, OTUD6B, and ALG13), the A20-like clade (A20/TNFAIP3, Cezanne/OTUD7B, Cezanne2/OTUD7A, TRABID/ZRANB1, and VCPIP1/VCIP135), and the OTULIN clade (OTULIN) (Mevissen et al., 2013). Members of the A20 and OTULIN clades are the most extensively studied *H. sapiens* OTU DUBs. A20, OTUD7B, and OTULIN generally play a negative regulatory role in various NFκB signaling pathways (Boone et al., 2004; Wertz et al., 2004; Enesa et al., 2008; Hitotsumatsu et al., 2008; Turer et al., 2008; Hymowitz and Wertz, 2010; Fiil et al., 2013; Hu et al., 2013; Keusekotten et al., 2013; Rivkin et al., 2013). The OTUB clade members, particularly OTUB1, have also been extensively characterized. OTUB1 is involved in immune responses (Soares et al., 2004), estrogen receptor-mediated transcription (Stanišiæ et al., 2009), and the DNA damage response (Nakada et al., 2010). The other, less-examined *H. sapiens* OTU DUBs and their potential orthologs from other species also play important roles in various cellular processes and signaling pathways. OTUD5/DUBA regulates interferon signaling (Kayagaki et al., 2007). Whereas VCIP1 is involved in CDC48-mediated Golgi membrane fusion (Wang et al., 2004), *H. sapiens* YOD1 and its possible yeast ortholog Otu1 are involved in p97/CDC48-mediated ERAD (Rumpf and Jentsch, 2006; Ernst et al., 2009).

In contrast to the characterized USPs, which have promiscuous linkage preferences (Faesen et al., 2011), the *H. sapiens* OTU DUBs have more strict linkage specificities (Mevissen et al., 2013). The distinct linkage specificities associated with OTU DUBs could be exploited in restriction analyses to determine the linkage types of the ubiquitin chains conjugated on endogenous substrates (Fiil et al., 2013; Hospenthal et al., 2013; Mevissen et al., 2013), to purify ubiquitylated substrates with specific linkage types, and to assemble UB chains with specific linkage(s) (Bremm et al., 2010). Such restriction applications are essential

tools for ubiquitylation biology. Extensive biochemical and structural analyses have revealed multiple mechanisms for determining the linkage specificities of OTU DUBs, including the presence of additional UB binding domain(s), ubiquitylation sites, S1' and S2 substrate sites on the OTU core, and substrate-assisted catalysis (Wang et al., 2009; Juang et al., 2012; Licchesi et al., 2012; Wiener et al., 2012; Keusekotten et al., 2013; Mevissen et al., 2013). However, to precisely manipulate the linkage specificities of particular OTU DUBs, more extensive biochemical and structural analyses are required to determine the exact molecular bases underlying the selectivity for UB dimers and UB polymers of specific linkages.

Given the potential importance of *A. thaliana* OTU DUBs in plant growth and development, we identified 12 *A. thaliana* loci encoding OTU DUBs. We characterized the biochemical properties (i.e., substrate binding and specificities) of 11 *A. thaliana OTU* loci-encoded proteins with a complete OTU domain. The *A. thaliana* OTU DUBs were classified into four groups with distinct substrate binding and specificities. Based on the phylogenetic tree established using OTU sequences from different species, conserved and plant-specific OTU DUBs were identified. Distinct substrate specificities were observed among possible cross-species orthologs within the same phylogenetic clades, suggesting potential mechanistic and functional differentiation. The distinct biochemical properties and phylogenetic relationships of *A. thaliana* OTU DUBs support their functional differences. Based on the phylogenetic tree, we discuss the possible unique and conserved functional roles of *A. thaliana* OTU DUBs.

RESULTS

A. THALIANA HAS AN OTU-DUB FAMILY WITH 12 PHYLOGENETICALLY DISTINCT MEMBERS

To characterize *A. thaliana* OTU-DUBs, 12 loci, *OTU1-5, OTLD1, and OTU7-12*, encoding OTU domain-containing proteins were identified through database searches using the OTU domain sequences for *H. sapiens* OTUB1 (NP_060140) and OTUB2 (NP_075601) as well as *S. cerevisiae* Otu1 (P43558) as the initial queries (**Table 1**). The exon-intron organization and coding sequences of these *A. thaliana* loci were determined through a sequence comparison with the corresponding PCR-amplified full-length cDNAs as well as available cDNAs and ESTs from the TAIR database (http://www.arabidopsis.org/) (**Figure S1**). Based on the splicing products detected, a single isoform exists for the *OTU1-3*, *OTU9-10*, and *OTU12* loci; two isoforms exist for the *OTU5*, *OTLD1*, *OTU7*, and *OTU11* loci (referenced with *a* and *b* extensions); and five isoforms exist for *OTU4* (*OTU4a-e*) (**Table 1**; **Figure S1**). With the exception of *OTU4b* and *OTU4d-e*, for which the encoded proteins were predicted to have lost or disrupted OTU domains due to frame shifts from alternative splicing, the remaining *OTU* loci encode potential OTU domain-containing DUBs. However, *OTU8* is likely a pseudogene because corresponding multiple isolated cDNAs were consistently derived from alternative splicing using a different 3′ junction of the second annotated intron (61 bp upstream of the predicted junction), which generated a frame-shift and downstream start codon located on the third annotated exon, resulting in an N-terminally truncated OTU domain. Accordingly, a search of the GENEVESTIGATOR microarray databases (https://www.genevestigator.com/gv/plant.jsp) revealed extremely low expression levels for *OTU8* transcripts and moderate to high expression levels for *OTU1-2*, *OTU4-5, OTLD1*, *OTU9*, and *OTU11-12* transcripts across various primary and cultured tissues from different organ sources (**Figure S2**). Microarray probes are not available for *OTU3*, *OTU7*, and *OTU10*. In general, constitutive expression of the eleven active *OTU* loci in various tissues, including roots, inflorescence stems, rosette and cauline leaves, flowers, and siliques, was detected by RT-PCR (**Figure S3**).

To examine the phylogenetic relationships between the 12 potential *A. thaliana* OTU-DUBs, a phylogenetic tree (**Figure 1**) was generated based on the aligned peptide sequences from the OTU domains derived from the *A. thaliana*, *Oryza sativa*, *H. sapiens*, and *S. cerevisiae* OTU proteins. With the exception of

Table 1 | *A. thaliana* loci encoding OTU-DUBs.

Gene	Locus	Peptide length (aa)/MW (kDa)	Domains identified (coordinates)[a]	Full-length cDNA accession[b]
OTU1	At2g28120	306/34.4	Peptidase_C65 (41–294) OTU (87–289)	JQ013442
OTU2	At1g50670	208/23.4	OTU (11–123)	JQ013443
OTU3	At2g38025	234/26.3	OTU (82–230)	JQ013444
OTU4a	At3g57810	317/35.8	OTU (174–300)	JQ013445
OTU4b		217/24.2	OTU* (174–216)	BX824976
OTU4c		274/30.8	OTU (131–257)	JQ013446
OTU4d		195/21.8	OTU* (131–191)	JQ013447
OTU4e		75/8.3	ND	JQ013448
OTU5a	At3g62940	332/37.4	OTU (187–321) Coiled coil (19–86)	JQ013449
OTU5b		316/35.7	OTU (172–306) Coiled coil (4–71)	JQ013450
OTLD1a	At2g27350	506/55.3	OTU (222–333) UBA-like (453–491)	JQ013451
OTLD1b		505/55.3	OTU (222–333) UBA-like (452–490)	AY058065
OTU7a	At5g67170	375/41.5	OTU (43–155) SEC-C (313–333)	JQ013452
OTU7b		231/26.9	OTU (43–155)	JQ013453
OTU8	At2g39320	154/18.3	OTU* (1–62) Coiled coil (85–150)	JQ013454
OTU9	At5g04250	345/39.2	OTU (210–322)	JQ013455
OTU10	At5g03330	356/41.7	OTU (219–331)	JQ013456
OTU11a	At3g22260	245/28.2	OTU (107–219)	JQ013457
OTU11b		240/27.7	OTU (107–219)	JQ013458
OTU12	At3g02070	219/25.6	OTU (85–197)	JQ013459

[a] *The proteins encoded by OTU4b, OTU4d, OTU4e, and OTU8 had disrupted (OTU*) or lost (ND) OTU domains due to frame shifts caused by alternative splicing.*

[b] *With the exception of OTU4b and OTLD1b, for which full-length cDNAs are available in the TAIR database, the full-length cDNAs for the various His- or His/GST-tagged wild-type recombinant constructs were isolated from various cDNA libraries by PCR (**Table S1**).*

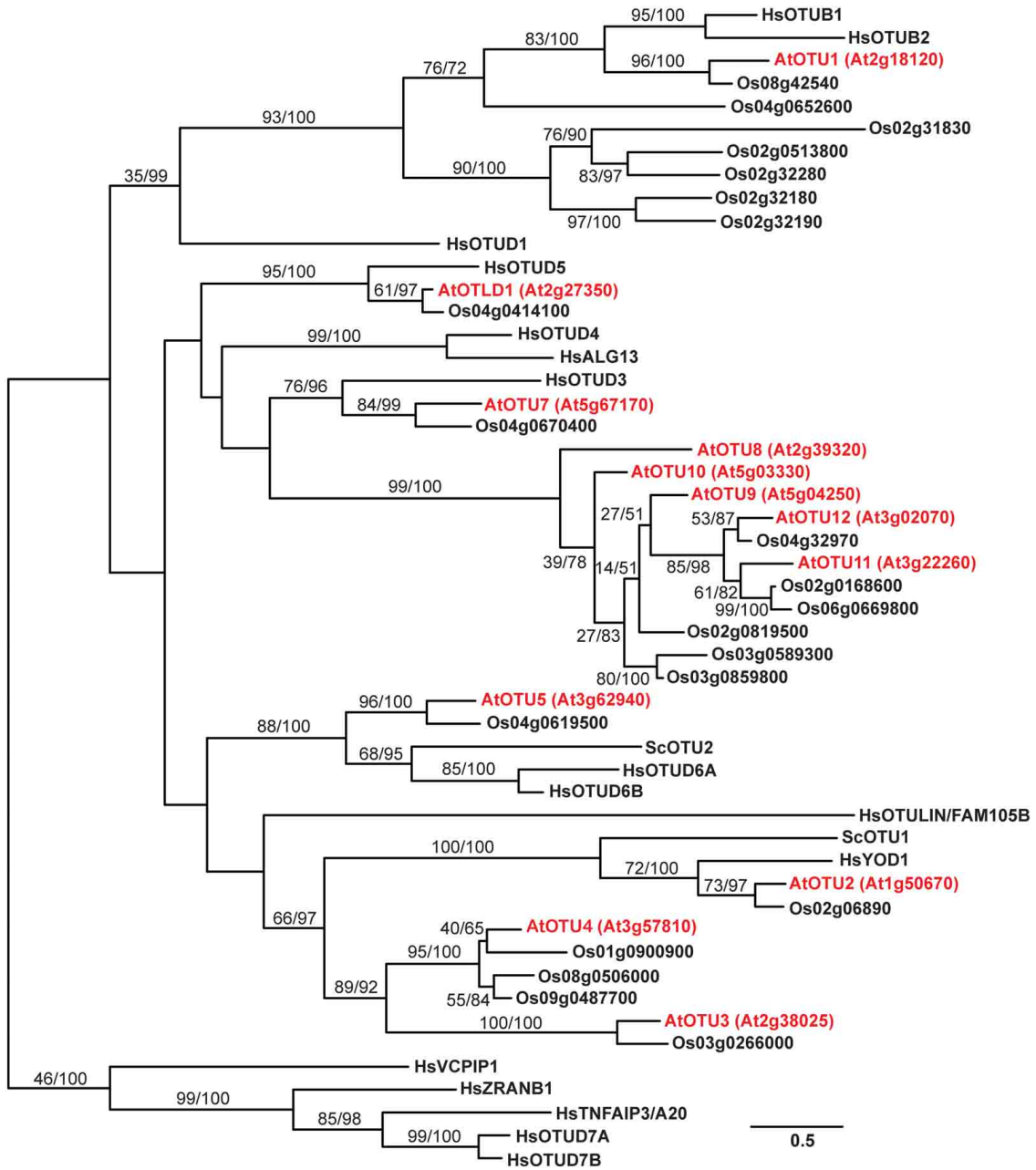

FIGURE 1 | A phylogenetic tree for the OTU proteins from *A. thaliana, O. sativa, S. cerevisiae,* and *H. sapiens*. The phylogenetic tree was generated using aligned OTU domain sequences from *A. thaliana, O. sativa, H. sapiens,* and *S. cerevisiae*. The branch labels separated by a slash indicate bootstrap support and Bayesian posterior probability. The branch length is based on the maximum likelihood results, and the scale represents the branch length that is proportional to the number of amino acid substitutions. The sequences from *A. thaliana*, *O. sativa*, *H. sapiens*, and *S. cerevisiae* are identified by the prefixes At, Os, Hs, and Sc, respectively, as abbreviations of their binomial names. The *A. thaliana* OTUs are marked in red with corresponding locus numbers in parentheses. The accession numbers for the analyzed sequences are listed in the Materials and Methods.

OTU8-12, which are in the same clade, each of the remaining *A. thaliana* OTU proteins belongs to a distinct clade in the predicted phylogenetic tree. A single potential *O. sativa* ortholog is associated with each of the *A. thaliana* OTU2-3, OTU5, OTLD1, and OTU7 clades. However, multiple potential *O. sativa* orthologs are associated with the clades containing *A. thaliana* OTU1, OTU4, and OTU9. The closer phylogenetic relationship among the *A. thaliana* and *O. sativa* OTU orthologs within the same clades compared with OTU proteins from the same species in different clades indicates that the orthologs within a clade evolved before the divergence of dicot and monocot species. Among the *A. thaliana* and *O. sativa* orthologs in the same clade, the nearly identical exon/intron organization of their corresponding loci further supports their close phylogenetic

relationship (**Figure S1**). Five clades harbor OTU proteins derived from *A. thaliana*, *O. sativa*, and *H. sapiens*; the two clades containing *A. thaliana* OTU2 and OTU5 also include *S. cerevisiae* Otu1 and Otu2, respectively. The five clades conserved across species likely evolved before their common ancestors diverged. *A. thaliana* OTU1, OTU2, OTU5, OTLD1, and OTU7 belong to the same clades as *H. sapiens* OTUB1/OTUB2, *H. sapiens* YOD1/*S. cerevisiae* Otu1, *H. sapiens* OTUD6A/OTUD6B/*S. cerevisiae* Otu2, *H. sapiens* OTUD5, and *H. sapiens* OTUD3 respectively, which suggests that these *A. thaliana* OTU proteins have distinct, evolutionarily conserved functions. No plant orthologs are associated with the four *H. sapiens*-specific clades, which include OTUD1; OTUD4/ALG13; the arbitrary outgroup clade harboring VCPIP1, ZRANB1, TFNAIP3/A20, OTUD7A, and OTUD7B; and the OTULIN/FAM105B clade. Similarly, three plant-specific clades harboring *A. thaliana* OTU3, OTU4, and OTU8-12 with one or multiple *O. sativa* homologs are present.

A. THALIANA OTU-DUBs EXHIBIT DISTINCT SUBSTRATE SPECIFICITIES

The lengths of the encoded *A. thaliana* OTU proteins range from 208 to 506 amino acids, with predicted molecular weights of ∼23.4–55.3 kDa (**Table 1**). Each protein includes an OTU domain (PFAM accession number: PF02338) with a length of 113–203 amino acids. Similar to its *H. sapiens* counterparts OTUB1 and OTUB2, *A. thaliana* OTU1 includes a larger Peptidase_C65 domain (PF10275) that covers the OTU domain. The OTU domain is located at the center of OTLD1; the C-terminal region of OTU1, OTU3-5, and OTU9-12; and the N-terminal region of OTU2 and OTU7. All of the *A. thaliana* OTU domains detected include the conserved catalytic Cys-His-Asp/Asn triad, similar to known active OTU-DUBs such as *H. sapiens* OTUB1 (**Figure S4**). In addition to the OTU domain, a few known domains were identified that are associated with the *A. thaliana* OTU proteins, including a coiled-coil domain in OTU5a (19–86) and OTU5b (4–71), a SCOP UBA-like domain (d1fya) in OTLD1a (453–491) and OTLD1b (452–490), and a potential nucleic acid-binding SEC-C motif (PF02810) in OTU7a (313–333) (**Table 1**).

The distinct phylogenetic relationships of the *A. thaliana* OTU proteins strongly suggest functional differentiation. To further explore this possibility, we characterized the differences in their biochemical properties, including deubiquitylation substrate specificities and binding activities against K48- and K63-linked UB chains. Therefore, we purified recombinant wild-type, site-specific, and deletion variants of the *A. thaliana* OTU proteins, which were expressed in *Escherichia coli* as His-tagged or His- and glutathione *S*-transferase (His/GST)-double tagged proteins (**Table S1**). The deubiquitylation substrate specificities of the *A. thaliana* OTU proteins OTU1-3, OTU4c-d, OTU5b, OTLD1a, OTU7a, OTU9-10, OTU11a, and OTU12 were examined (**Table 2**). With the exception of OTU4c and OTU10, which were examined using the His/GST-tagged versions due to low yields of their His-tagged forms, the His-tagged versions were analyzed. His/GST-tagged OTU1 was also analyzed and compared with the His-tagged form to examine whether fusion of the large GST tag affected catalytic activity. We expected that the His-tagged OTU4d would be catalytically defective due to a truncated OTU domain. His-tagged *H. sapiens* OTUB1 was analyzed as a control and for comparison. Other OTU isoforms were not analyzed for various reasons, such as low yields of both tagged recombinant proteins (OTU4a), small sequence variation from the analyzed isoforms (OTU5a, OTLD1b and OTU11b), a large C-terminal truncation (OTU7b), and lost or disrupted OTU domains (OTU4b, OTU4e, and OTU8).

The substrate specificities of the potential *A. thaliana* OTU DUBs were examined by determining their *in vitro* cleavage activities on isopeptide bond-linked UB tetramers with K48- and K63-linkages; peptide bond-linked linear UB dimers, trimers, and tetramers; and peptide bond-linked UB-, RUB-, and SUMO-GST fusion proteins. As summarized in **Table 2**, seven of the 11 examined *A. thaliana* OTU proteins exhibited deubiquitylation activities with distinct substrate specificities. The selectivity of the potential *A. thaliana* OTU DUBs for K48- and K63-linked UB chains was first determined (**Table 2**; **Figure 2**). Both His- and His/GST-tagged OTU1, similar to its *H. sapiens* counterpart OTUB1, displayed a clear preference for K48-linked UB chains. However, unlike OTUB1, which did not exhibit detectable activity for K63-linked chains, *A. thaliana* OTU1 displayed moderate activity with this chain linkage. By contrast, while OTU4c displayed a slight cleavage preference for K63-linked UB chains, OTU3, OTU7a, and OTU10 displayed stronger cleavage activities for K63-linked UB chains. However, OTU2 and OTU9 exhibited approximately similar cleavage activities for both linkage types. The remaining *A. thaliana* OTU proteins examined, OTU5b, OTLD1a, OTU11a, and OTU12, did not exhibit detectable *in vitro* cleavage activity for either chain linkage or other tested substrates, which suggests these proteins are cryptic *in vitro* or have specificity for other, unexamined chain linkages. As predicted, the OTU4d with a disrupted OTU domain displayed no significant cleavage activity against either chain linkage.

Among the seven *A. thaliana* OTU proteins active against isopeptide bond-linked UB chains, OTU1, OTU3, and OTU4c also exhibited cleavage activities against peptide bond-linked linear UB polymers. Both His- and His/GST-tagged OTU1 proteins exhibited strong cleavage activities with all tested linear UB polymers, and OTU3 and OTU4 exhibited weak to moderate cleavage activities with linear UB trimers and tetramers but not dimers (**Table 2**; **Figure 3**; **Figure S5**). Moreover, whereas *A. thaliana* OTU1 exhibited similarly stronger catalytic activities for peptide bond- and K48 isopeptide bond-linked UB tetramers than for K63-linked UB tetramers, OTU3 and OTU4c had stronger catalytic activities for isopeptide bond-linked UB tetramers of both linkages than peptide bond-linked linear UB tetramers (**Figure 3**). Interestingly, in contrast to the strong catalytic activities detected with *A. thaliana* OTU1, the *H. sapiens* OTUB1 was inactive with peptide bond-linked linear UB chains (**Table 2**; **Figure S5**). With the exception of OTU1 and OTU2, the active *A. thaliana* OTU proteins also displayed cleavage activities against peptide bond-linked UB- and RUB-GST fusion proteins but not the SUMO-GST fusion protein (**Table 2**; **Figure 4**; **Figure S6**). A cleavage

Table 2 | Distinct biochemical properties of *A. thaliana* OTU-containing deubiquitinases.

Name[a]	Tag	Ub4		Linear chain			Ub/Ubl-GST fusion			Chain[b]
		K48	K63	Ub2	Ub3	Ub4	Ub	Rub	Sumo	
OTU1	His	+++[c]	+	+++	+++	+++	–	–	–	–
OTU1	GST	+++	+	+++	+++	+++	–	–	–	–
OTU2	His	+	+	–	–	–	–	–	–	K48 ≈ K63
OTU3	His	++	+++	–	+	+	++	+	–	–
OTU4c	GST	+++	+++	–	++	++	++	+	–	–
OTU4d	His	–	–	–	–	–	–	–	–	–
OTU5b	His	–	–	–	–	–	–	–	–	–
OTLD1a	His	–	–	–	–	–	–	–	–	K48 > K63
OTU7a	His	+	++	–	–	–	++	+	–	–
OTU9	His	++	++	–	–	–	++	+	–	–
OTU10	GST	++	+++	–	–	–	++	+	–	–
OTU11a	His	–	–	–	–	–	–	–	–	–
OTU12	His	–	–	–	–	–	–	–	–	–
HsOTUB1	His	+++	–	–	–	–	–	–	–	ND

[a] *OTU4d contains a disrupted OTU domain.*

[b] *UB chain binding was analyzed by GST pull-down assays using GST-fused proteins; –, absence of binding; ND, not determined.*

[c] *The approximate deubiquitylation activities are quantitatively designated by +++, ++, +, and – for strong, moderate, weak, and absent activity, respectively.*

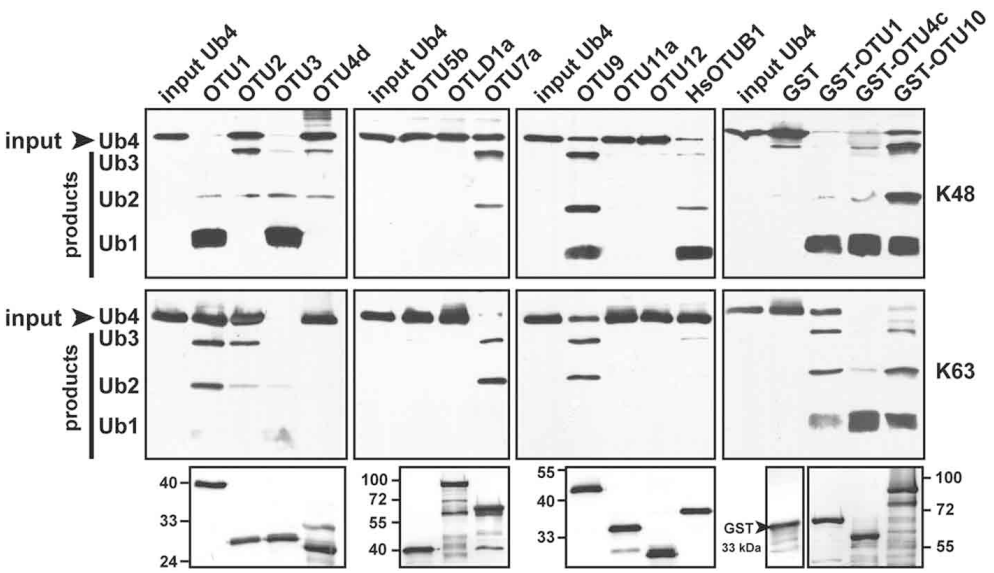

FIGURE 2 | The *A. thaliana* OTU-containing proteins display distinct cleavage preferences for K48- and K63-linked UB tetramers. *A. thaliana* OTU proteins cleavage activities for K48- and K63-linked UB tetramers (Ub4) were analyzed using purified His- (OTU1-3, OTU4d, OTU5b, OTLD1a, OTU7a, OTU9, OTU11a, and OTU12) or His/GST-tagged (OTU1, OTU4c, and OTU10) forms. The His-tagged *H. sapiens* otubain 1 (HsOTUB1) was analyzed for comparison. GST was included as a negative control for His/GST-tagged OTU proteins. A substrate incubated without enzymes was used as a negative input control (input Ub4). The inputs and their cleavage products are labeled on the left as trimers (Ub3), dimers (Ub2), and monomers (Ub1) and were visualized by immunoblotting using antisera against *H. sapien*s UB (α-UB). Duplicate input OTU proteins were visualized through immunoblotting using antisera against the His-tag to ensure approximately equivalent enzyme input levels (bottom panels). The molecular weight markers are labeled on the left or right.

preference for UB-GST over RUB-GST was evident (**Table 2**; **Figure 4**).

We examined the cleavage activities for isopeptide-bond linked ubiquitin tetramers with K48 and K63 linkages; OTU1, OTU3, OTU4c, OTU7a, OTU9, and OTU10 generally displayed optimum activity at neutral pH (**Figure S7**). However, for K63-linked chain cleavage, OTU9 and OTU10 exhibited optimum activity at slightly alkaline pH values. These neutral and slightly alkaline pH optima suggest that the active *A. thaliana* OTU DUBs examined are likely not vacuolar proteases.

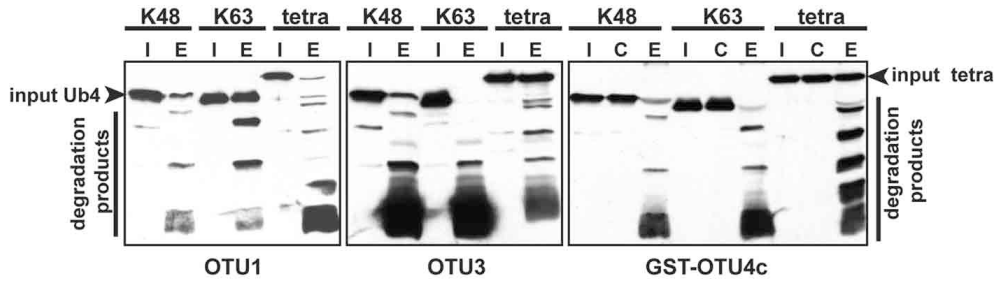

FIGURE 3 | The relative cleavage activities of *A. thaliana* OTU1, OTU3, and OTU4c against linear (tetra) and K48- or K63-linked UB tetramers. The cleavage activities were analyzed using His-tagged OTU1 and OTU3 and His/GST-tagged OTU4c (E). GST was included as a negative control for GST-OTU4c (C). A substrate incubated without enzymes was used as a negative input control (I). The inputs and their cleavage products are labeled on the left or right; the degradation products were visualized by immunoblotting with α-UB.

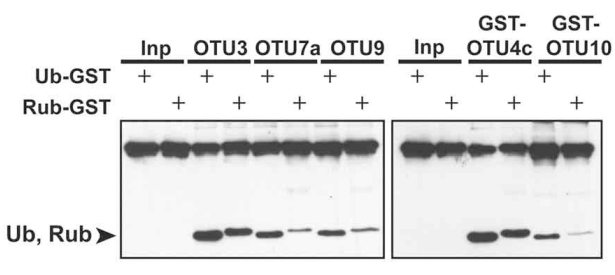

FIGURE 4 | The relative cleavage activities of *A. thaliana* OTU3, OTU4c, OTU7a, OTU9, and OTU10 for UB- and RUB-fused GST. The cleavage activities were analyzed using His-tagged OTU3, OTU7a, and OTU9 as well as His/GST-tagged OTU4c and OTU10. The substrates incubated without enzymes were used as negative input controls (Inp). The specific substrate used is indicated by "+." The cleavage products Ub and Rub, which are labeled on the left, and the inputs were visualized by immunoblotting with α-HA.

A. THALIANA OTU-DUBs ARE CYSTEINE PROTEASES WITH A CONSERVED CATALYTIC TRIAD

The presence of a conserved catalytic triad (**Figure S4**) suggests that the *A. thaliana* OTU DUBs are cysteine proteases similar to those characterized from *H. sapiens* and *S. cerevisiae*. As tested using the His-tagged OTU1, the cleavage activities for K48- and K63-linked UB tetramers were inhibited by UB aldehyde and N-ethylmaleimide but not by the metalloprotease inhibitors 1,10-phenanthroline and EDTA or the serine protease inhibitor phenylmethylsulfonyl fluoride (**Figure 5**). Furthermore, site-specific mutations of the conserved catalytic triad in OTU1, OTU4c, and OTU7a or of a conserved residue that is potentially critical for stabilizing the oxyanion reaction intermediate in OTU1 (D89) completely abolished cleavage activities for K48- and K63-linked UB tetramers (**Figures 6A–C**; OTU1 variants C92S, D89E, and H288R, OTU4c-C136S, and OTU7a-C48S). The same mutations also generally abolished the linear UB polymer cleavage activities of OTU1 and OTU4c (**Figures 6D,E**) and the UB- and RUB-GST fusion protein cleavage activities of OTU4c (**Figure 6F**). Interestingly, although cleavage of isopeptide bond-linked UB tetramers was abolished, OTU1-D89E still cleaved linear UB polymers to a similar extent as the wild-type protein (**Figure 6D**).

A. thaliana OTU1 D89 and corresponding Asp residues in other OTU DUBs, including *H. sapiens* OTUB1 and OTUB2, were initially predicted to be involved in catalytic triad formation by polarizing active-site histidine (Balakirev et al., 2003). Instead, crystal structure analyses revealed that either an Asp or Asn (D290 of OTU1) located one residue away in the C-terminal direction from the active-site histidine is involved in catalytic triad formation through hydrogen bonds to orient the active-site histidine (Nanao et al., 2004; Edelmann et al., 2009). However, the backbone amides of the originally predicted Asp residues of OTUB1, OTUB2, A20, and yeast Otu1 (corresponding to OTU1 D89) are critical for stabilizing the oxyanion reaction intermediate (Nanao et al., 2004; Komander and Barford, 2008; Edelmann et al., 2009).

The substrate selectivity of various *A. thaliana* OTU DUBs may be an intrinsic property of their respective OTU domains. An N-terminal 43-residue deletion in OTU4c did not modify its catalytic activities with the examined isopeptide and peptide bond-linked substrates (**Figures 6B,E,F**; OTU4c-NΔ). Similarly, the cleavage of K48- and K63-linked tetra ubiquitin chains by N-terminal deletion variants of OTU9 and OTU10 (**Table S1**, OTU9-NΔ1-100, OTU9-NΔ1-172, OTU10-NΔ1-125, and OTU10-NΔ1-173) containing intact OTU domains was comparable to that catalyzed by the corresponding wild-type proteins (data not shown).

A. THALIANA OTU2 AND OTLD1 BIND UB CHAINS WITH DISTINCT LINKAGE PREFERENCES

DUBs including OTUs often harbor UB-binding domains (Komander et al., 2009). An appendage of the UB-binding domain(s) may restrict or broaden the linkage specificities, as has been observed for UIM, ZnF, and an ankyrin repeat domain in *H. sapiens* OTUD1, OTUD2, and TRABID, respectively (Licchesi et al., 2012; Mevissen et al., 2013). Additional UB-binding domain(s), such as the multiple NZFs of TRABID, are involved in targeting to substrate sites and enhance cleavage activity with longer UB chains (Licchesi et al., 2012). Furthermore, the different C-terminal linkage-specific UB chain-binding motifs (ZnF1-7) in *H. sapiens* A20 are crucial for its recruitment by distinct signaling proteins and complexes (Bosanac et al., 2010; Skaug et al., 2011; Tokunaga et al., 2012; Verhelst et al., 2012; Lu et al., 2013).

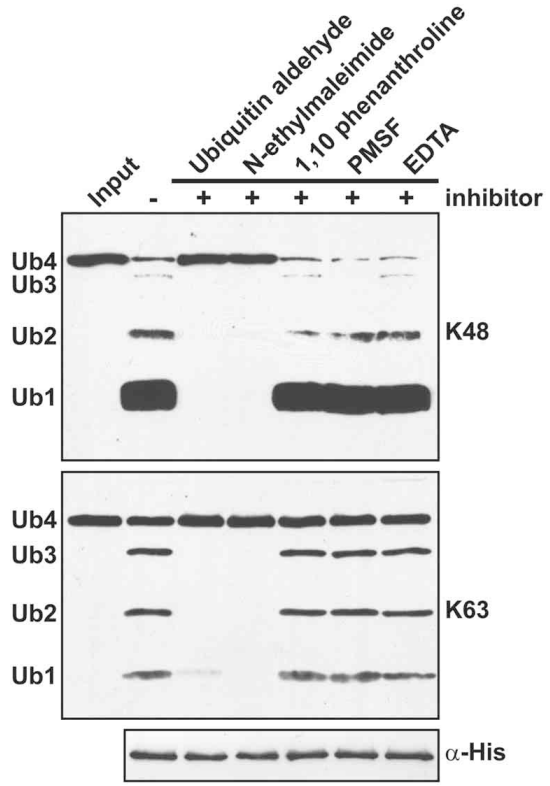

FIGURE 5 | *A. thaliana* OTU1 is catalytically inhibited by cysteine protease inhibitors. The effects of different protease inhibitors on the cleavage activity of His-tagged *A. thaliana* OTU1 (300 nM) for K48- and K63-linked UB tetramers (250 ng) were examined by comparing the resulting cleavage products after pre-incubation (5 min at 4°C) and the catalytic reaction (1 h at 37°C) in the absence (−) and presence (+) of 0.5 μM UB aldehyde, 0.5 μM N-ethylmaleimide, 0.5 μM 1,10-phenanthroline, 1 mM phenylmethylsulfonyl fluoride (PMSF), or 1 mM EDTA. The input substrate incubated without OTU1 was used as a negative control (Input). The input Ub4 and cleavage products Ub3, Ub2, and Ub1, labeled on the left, were detected by immunoblotting with α-UB. Duplicate samples were examined using antisera against the His-tag to confirm that OTU1 was input at equivalent levels (α-His).

Because binding preferences for UB chains with different linkages may contribute to substrate selectivity or intracellular targeting, we examined the association between the *A. thaliana* OTU proteins and K48- and K63-linked UB chains *in vitro* through pull-down assays using the GST-fusion proteins (**Table S1**). OTU1-3, OTU4c, OTU5b, OTLD1a, OTU7a, OTU9-10, OTU11a, and OTU12 were examined. However, binding of K48- and K63-linked UB chains was detected only for OTU2 and OTLD1a (**Table 2**; **Figures 7A,B**). OTU2 exhibited approximately equivalent binding affinities for K48- and K63-linked UB chains, while OTLD1a displayed a clear binding preference for K48-linked UB chains.

OTU2 AND OTLD1 HARBOR NOVEL UB-BINDING DOMAINS

We delineated the critical domains or residues involved using GST-fused deletion and/or site-specific variants (**Figures 8A**, **9A**; **Table S1**). Binding assays using OTU2 terminal truncations indicated that both the N- (1–99) and C-terminal regions (179–208) contain structural elements that are critical for binding K48- and K63-linked UB chains. The association with K48- and K63-linked UB chains was abolished for all OTU2 terminal deletion mutants (**Figures 8A,B**; CΔ1, CΔ2, and NΔ1). A PROSITE Zinc_Finger_C2H2 motif (http://prosite.expasy.org/PS00028) containing four well-spaced Cys and His residues was identified at the OTU2 C-terminus (179–201). Individual mutants of the Cys and His residues in this domain still bound to the UB chains with affinities nearly equivalent to that of the wild-type protein (**Figures 8A,B**; C179S, C182S, H195R, and H201R). However, the double mutant C179S/H195R exhibited completely abolished (K48-linked chain) or severely affected (K63-linked chain) binding. These results also support a critical role for the OTU2 C-terminus in UB-chain association. The predicted zinc finger motif likely does not exist because any single mutation of the predicted residues involved in zinc chelation would have disrupted the zinc finger and C-terminal structure. Because the OTU2 N-terminal region containing the OTU domain is critical for chain binding, OTU2 was treated with NEM to determine if the catalytic cysteine is essential. Association with both UB chain types was abolished when OTU2 was pre-incubated with NEM, suggesting a critical role for the catalytic cysteine in chain binding (**Figure 8B**; OTU2-NEM). However, the catalytic cysteine (C16) is actually not essential for UB chain binding because the site-specific mutant bound UB chains as well as the wild-type protein (**Figure 8B**; C16S). The deleterious effect of NEM on OTU2 UB chain binding indicates that other Cys residues are likely critical. We examined the roles of two other available Cys residues (C63 and C99) in the N-terminal half of OTU2 in UB chain association. Association with both chain types was almost completely abolished in OTU2 variants with single and double substitutions of these Cys residues (**Figures 8A,B**; C63S, C99S, and C63S/99S). Most known UB-binding domains and motifs, such as UIM and UBA, are formed from small and restricted UB-binding protein regions, and thus the involvement of scattered critical residues/domains in UB chain association by OTU2 is unique and suggests that a novel structural domain formed from a broader sequence region is likely involved in UB binding.

Although OTLD1a contains a UBA-like domain at the C-terminus, a C-terminal-truncated variant in which the UBA-like domain was removed exhibited K48-linked chain binding activity equivalent to that of the wild-type protein (**Figures 9A,B**; CΔ1). Based on additional experiments with various terminal deletion variants, the UB chain binding region is likely positioned between residues 211–410; N-terminal deletion variants truncated before residue 211 and C-terminal deletion variants truncated after 410 bound K48-linked chains (**Figures 9A,B**; NΔ1-2 and CΔ1-4). An internal fragment (211–444) encompassing OTU domain (OTUf) also bound K48-linked chains.

DISCUSSION

We characterized *A. thaliana* OTU domain-containing DUBs; based on extensive studies from other species, these proteins likely function as critical mechanistic elements with important cellular and organismic processes. The distinct phylogenetic relationships

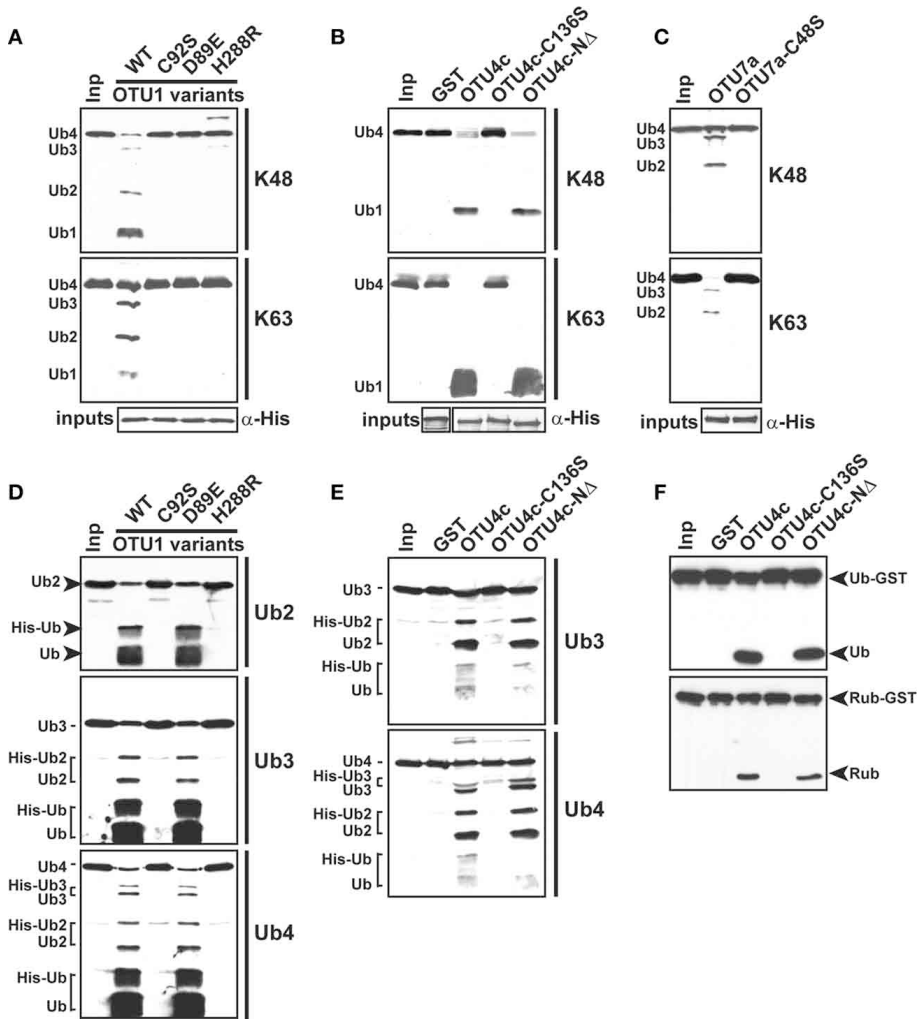

FIGURE 6 | The conserved catalytic triad is required for the deubiquitylation activities of *A. thaliana* OTU1, OTU4c, and OTU7a. **(A)–(E)** The catalytic roles of the conserved D89 in OTU1, the conserved catalytic triad in OTU1, OTU4c, and OTU7a, and the 43 N-terminal residues in OTU4c for cleavage of K48- and K63-linked UB tetramers **(A)–(C)**, peptide bond-linked linear UB dimers (Ub2), trimers (Ub3), and tetramers (Ub4) **(D,E)**, and peptide bond-linked UB- and RUB-GST fusions **(F)** were examined by comparing the cleavage activities of the site-specific or deletion mutants, which included the OTU1 variants C92S, D89E, and H288R; OTU4c-C136S; OTU4c-NΔ; and OTU7a-C48S, with those of the corresponding wild-type proteins. Substrates incubated without enzyme were used as negative input controls (Inp). The inputs and their cleavage products are labeled on the left or right and were visualized by immunoblotting with α-UB **(A)–(E)** or α-HA **(F)**. Duplicate input OTU variants were detected by immunoblotting with antisera made against His-tag to confirm equivalent enzyme input levels (α-His). The His- (OTU1 and OTU7a variants) or His/GST-tagged (OTU4c variants) forms were used. GST was included as a negative control for the GST-fused OTU4c variants.

and biochemical properties observed suggest that the *A. thaliana* OTU-DUBs identified are likely involved in different functions.

Similar to OTU DUBs characterized from other species, the *A. thaliana* OTU DUBs are cysteine proteases; as determined with OTU1, OTU4, and OTU7 by the importance of the conserved catalytic triad and an oxyanion residue and through specific inactivation with cysteine protease inhibitors. Based on *in vitro* DUB assays, the *A. thaliana* OTU DUBs have distinct substrate preferences. Because ubiquitin dimers with all eight linkages have become available more recently (El Oualid et al., 2010; Virdee et al., 2010; Hospenthal et al., 2013) and most long atypical chains, such as K6, K27, K33, and K29 are not available, we analyzed only the linear (M1-linked), K48-linked, and K63-linked UB chains. Seven of the 11 *A. thaliana* OTU domain-containing proteins exhibited deubiquitylation activities with distinct substrate preferences. We categorize the *A. thaliana* OTU DUBs into four groups based on substrate preferences (**Table 2**; **Figures 2–4**). The first group consists of a single member, OTU1, which exhibited equivalently stronger preferences for linear and K48-linked UB chains than that for K63-linked chains. The second group includes OTU3, OTU4, OTU7, and OTU10, which exhibited slightly or clearly stronger preferences for K63-linked chains than K48-linked and linear chains. OTU3 and OTU4 had weak to moderate cleavage activities for linear UB tetramers and trimers,

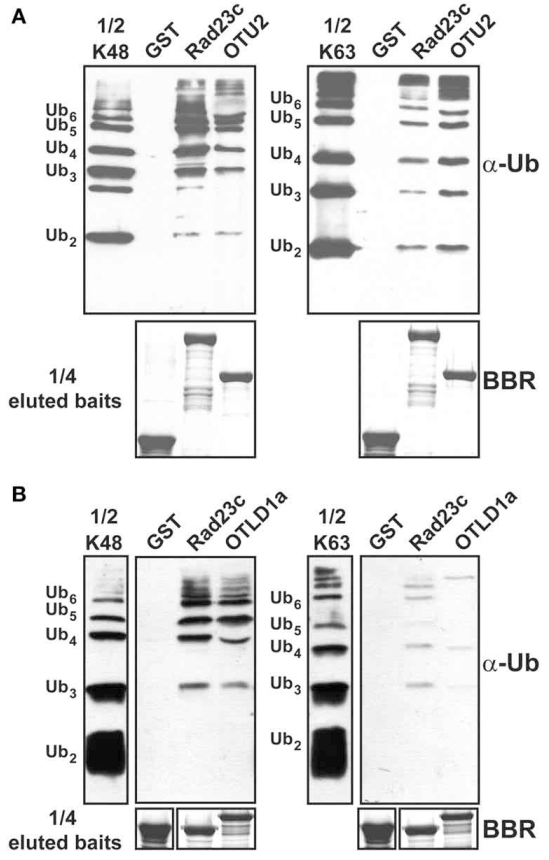

FIGURE 7 | OTU2 and OTLD1 associate with isopeptide bond-linked UB chains and have distinct preferences for different linkage types. (A) OTU2 exhibited equivalent binding affinities for K48- and K63-linked UB chains. **(B)** OTLD1a prefers to bind K48- over K63-linked UB chains. Five micrograms of K48- or K63-linked UB chains (2–6 units, labeled on the left) were subjected to pull down by GST-fused OTU2 **(A)**, OTLD1a **(B)**, and negative (GST) and positive (AtRAD23c) control proteins. One microgram of the input chains (1/2 K48 or K63) and two-fifths of the eluted pulled-down products were analyzed by immunoblotting with α-Ub. One-tenth of the eluted pulled-down products (1/4 eluted baits) were visualized by Brilliant Blue R staining (BBR) to confirm equally immobilized baits.

and OTU7 and OTU10 were inactive toward the linear UB chains. The third group contains OTU2 and OTU9, which exhibited nearly equivalent preferences for K48- and K63-linked UB chains and no cleavage activity toward linear UB chains. The fourth cryptic group includes OTU5, OTLD1, OTU11, and OTU12, which were inactive against all substrates examined *in vitro*.

The absence of *in vitro* cleavage activity for OTU5, OTLD1, OTU11, and OTU12 did not completely rule out their potential function as DUBs *in vivo* because only a limited number of substrates were tested. A cryptic or auto-inhibited conformation has been associated with DUBs of various classes, including OTUs, that have been examined *in vitro* (Nanao et al., 2004; Messick et al., 2008). Moreover, catalytic activation of a specific cryptic enzyme may require association with additional factor(s) or assembly into protein complex(es). DUBs of various classes, including OTUs, often function in complexes (Sowa et al., 2009). The cryptic enzyme can also be activated by post-translational modifications such as phosphorylation, as has been observed for *H. sapiens* OTUD5 (the possible *A. thaliana* OTLD1 ortholog), for which phosphorylation is critically involved in substrate binding (Huang et al., 2012). The conserved OTU5 catalytic cysteine is irreplaceable for rescuing mutant phenotypes, which supports the notion that its deubiquitylation activity is functionally relevant and that it is active *in vivo* (Radjacommare and Fu, unpublished results). Thus, it is worth examining the cryptic OTU DUBs for their *in vitro* cleavage activities toward ubiquitin chains with other linkages that have recently become available and for their importance of the catalytic sites *in vivo*. It is also worth identifying the interacting proteins/substrates for the cryptic group of *A. thaliana* OTU proteins. However, some of the inactive *A. thaliana* OTU DUBs may be involved in non-catalytic functions, as demonstrated for OTUB1 and A20 (Nakada et al., 2010; Skaug et al., 2011; Juang et al., 2012).

Although we examined cleavage activities with only three UB linkages, we compared the linkage preferences of the *A. thaliana* OTU DUBs with potential orthologs in other species, particularly *H. sapiens*. Differences on linkage preference have been clearly detected among the potential orthologs from different species, suggesting functional and/or mechanistic differentiation. A strict linkage preference is generally associated with OTU DUBs in *H. sapiens*-specific clades as determined using UB dimers of all eight linkages (Mevissen et al., 2013). OTUD1, OTUD4, OTUD7A/OTUD7B, and OTULIN have strict preferences for K63-, K48-, K11-, and M1-linked UB chains, respectively. However, the other three *H. sapiens*-specific OTUs have cryptic (ALG13), dual- (A20 and VCPIP1, K11/K48), or multiple (TRABID/ZRANB1, K29 = K33 > K63) linkage preferences. Among OTU DUBs in the five conserved phylogenetic clades, whereas both *H. sapiens* and *Caenorhabditis elegans* OTUB1 orthologs have strict specificity for K48 linkages (Wang et al., 2009; Mevissen et al., 2013), *A. thaliana* OTU1 has equally strong preferences for linear and K48-linked chains over K63-linked chains. The other *H. sapiens* OTUB1 homolog, OTUB2, also has distinct substrate preferences; it cleaves K63-linked chains with greater activity than K48- and K11-linked chains. In addition, although the orthologs from *H. sapiens* (YOD1/OTUD2), *S. cerevisiae* (Otu1), and *C. elegans* (Otu1) have generally similar multiple cleavage activities (K29 = K33 = K27 > K11 > K48), *A. thaliana* OTU2 has equivalently moderate activities for K48- and K63-linked UB chains. Moreover, whereas the potential *H. sapiens* ortholog (OTUD3) has dual cleavage preferences for K6- and K11-linked chains, *A. thaliana* OTU7 has greater cleavage activity for K63-linked chains than K48-linked chains. By contrast, two potential orthologs, *A. thaliana* OTU5 and *H. sapiens* OTUD6B, from the same phylogenetic clade are both inactive for each linkage examined. However, a slightly more divergent *H. sapiens* ortholog, OTUD6A, has cleavage activity for multiple linkages (K29 = K33 = K27 > K11 > K63) (Mevissen et al., 2013). Because *H. sapiens* OTUD5/DUBA is activated by phosphorylation with dual preferences (K6 = K11) (Huang et al., 2012; Mevissen et al., 2013), potential activation through phosphorylation and the linkage specificity of the potential *A. thaliana* ortholog OTLD1, which is inactive in our DUB assay, must be further investigated. However, for an extensive cross-species

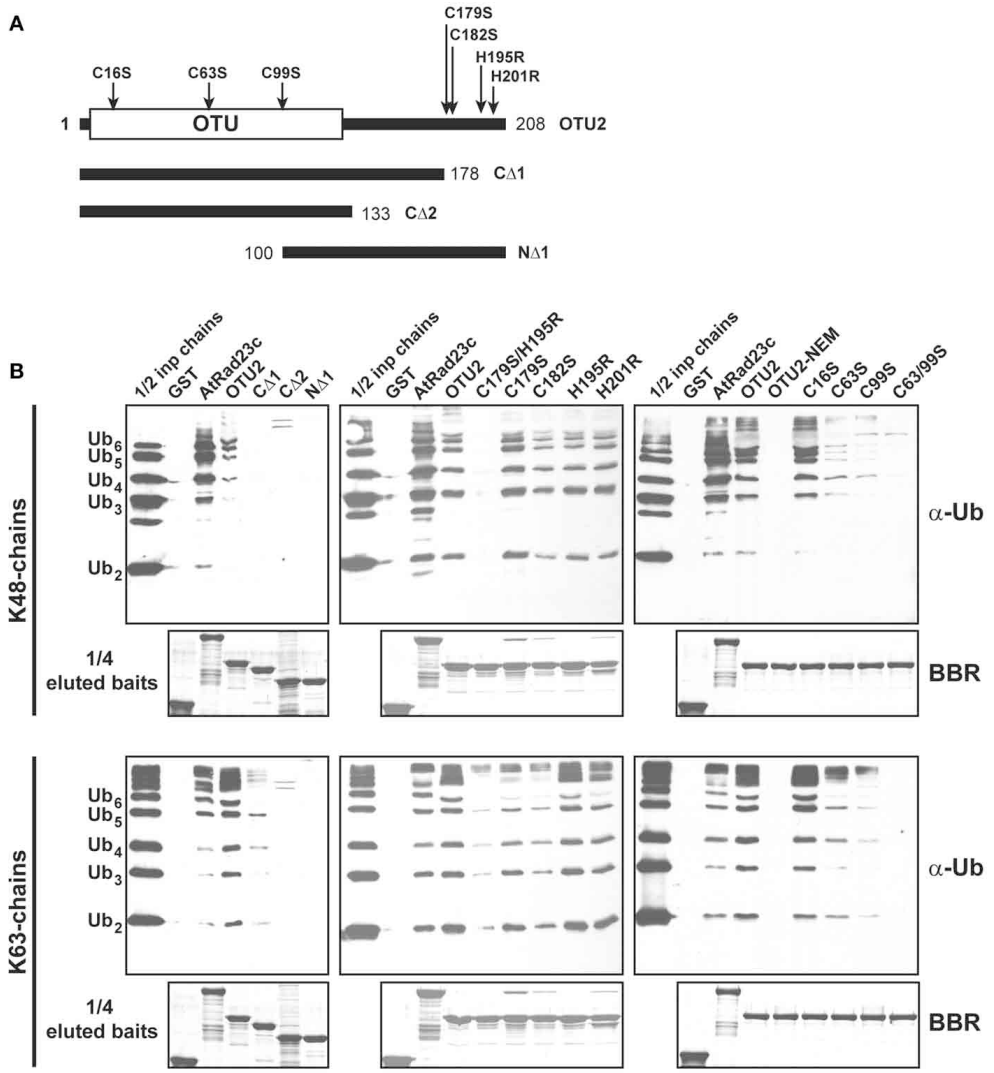

FIGURE 8 | Residues located at both the OTU2 N- and C-termini are critical for ubiquitin chain binding. (A) Schematic diagrams of the site-specific and deletion variants of OTU2 that were used to delineate the domains and residues involved in UB binding. The site-specific mutations and OTU domain are indicated. The numbers indicate the coordinates for the OTU2 variant termini. **(B)** GST pull-down products. Five micrograms of K48- or K63-linked UB chains (2–6 units, labeled on the left) were subjected to pull down by GST-fused OTU2 variants and negative and positive control proteins (GST and AtRAD23c, respectively). One microgram of the input chains (1/2 inp chains) and two-fifths of the eluted pulled-down products were analyzed by immunoblotting using antisera against human UB (α-Ub). One-tenth of the eluted pulled-down products (1/4 eluted baits) were visualized by Brilliant Blue R staining (BBR) to confirm equivalently immobilized baits. Wild-type OTU2 pre-incubated with 0.5 μM *N*-ethylmaleimide was analyzed to examine the involvement of catalytic and non-catalytic cysteine residues (OTU2-NEM).

comparison of linkage specificities among OTU DUBs, more extensive *in vitro* assays with UB dimers or longer UB chains with all linkages are necessary.

With the exception of OTU1, which has equivalent peptidase and isopeptidase activities and exhibits similarly strong cleavage activities for linear and K48-linked ubiquitin chains, the active *A. thaliana* OTU DUBs are generally isopeptidases. However, although OTU3 and OTU4 have stronger preferences for K63 isopeptide bond-linked chains, both OTU DUBs also have weak to moderate activities for linear ubiquitin chains. In contrast to *A. thaliana*, among 16 *H. sapiens* OTU DUBs with a complete catalytic triad, only the recently discovered OTULIN has strict cleavage activity for linear M1-linked UB chains. Modulation of linear ubiquitin chain assembly and disassembly has emerged as an important regulatory mechanism for signal transduction in mammals (Iwai, 2011; Rieser et al., 2013). The observed cleavage activities for linear chains by *A. thaliana* OTU DUBs, particularly OTU1, suggest a potential functional relevance of linear UB chains in plants. However, linear chain assembly components of the E3 ligase LUBAC are restricted to vertebrates and select invertebrate lineages and are not found in plants (Keusekotten et al., 2013).

In contrast to human OTU DUBs, which are all inactive against NEDD8 (RUB)-based peptide substrates (Edelmann et al., 2009;

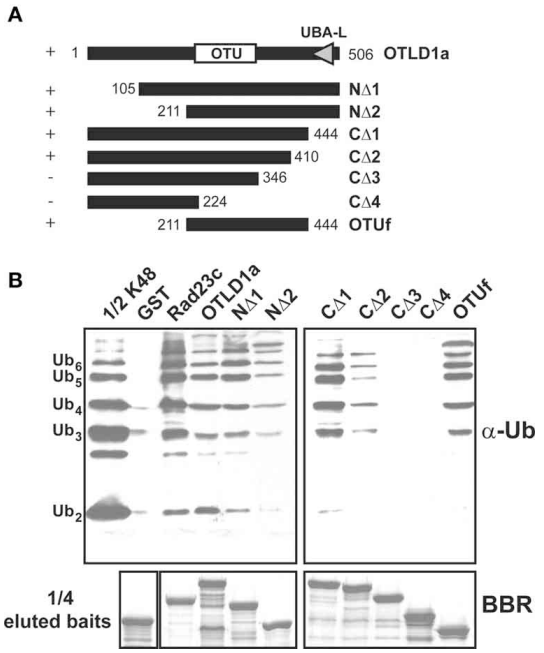

FIGURE 9 | The OTLD1a region spanning 211–410 but not the UBA domain is critical for UB chain binding. (A) Schematic diagrams show the OTLD1a deletion variants that were used to delineate the domain involved in UB chain binding. The OTU and UBA-like (UBA-L) domains are indicated. Positive and negative binding activity against K48-linked UB chains are indicated by + and − on the left. The numbers indicate the OTLD1a variant termini coordinates. **(B)** Pull-down products of the GST-fused OTLD1a variants. Five micrograms of K48-linked UB chains (2–6 units, labeled on the left) were subject to pull down by GST-fused OTLD1a variants and negative and positive control proteins (GST and AtRAD23c, respectively). One microgram of the input chains (1/2 K48) and two-fifths of the eluted pulled-down products were analyzed by immunoblotting with α-Ub. One-tenth of the eluted pulled-down products (1/4 eluted baits) were visualized by Brilliant Blue R staining (BBR) to confirm equivalently immobilized baits.

Mevissen et al., 2013), five of the seven active *A. thaliana* OTU DUBs (OTU3-4, OTU7, OTU9, and OTU10 but not OTU1 and OTU2) have cleavage activities for UB- and RUB-GST fusion proteins, with a clear preference for UB-GST. Although RUB-GST is an artificial substrate, the activities detected for this substrate indicate that *A. thaliana* OTU DUBs may play a role in modulating RUB conjugation, which is important for regulating CULLIN-based E3 ligase activities (Stratmann and Gusmaroli, 2012).

The distinct substrate preferences of various *A. thaliana* OTU DUBs suggest structural differences in the substrate recognition mechanisms. The strict preferences of OTU1 and OTU2 for peptide- and/or isopeptide bond-linked UB chains but not UB- and RUB-GST fusion proteins suggest that contacts with both distal and proximal UBs are critical for cleavage. Similarly, *H. sapiens* OTUB1 does not cleave UB-GFP (Balakirev et al., 2003). The importance of proximal UB binding has been demonstrated with *H. sapiens* OTULIN, which is inactive against active-site probes such as UB propargylamide and UB-AMC (Keusekotten et al., 2013; Rivkin et al., 2013). The remaining *A. thaliana* OTU DUBs can cleave UB- and RUB-GST fusion proteins, which suggests that the leaving group (either UB or GST) has little influence on catalysis. However, the clear linkage preferences among these OTU DUBs still favor the structural differences surrounding the isopeptide linkage at the proximal UB (S1′) binding sites. The importance of S1′ binding sites for linkage specificity has been established for *H. sapiens* OTUB1, OTULIN, and OTUD1-3 (Wang et al., 2009; Juang et al., 2012; Wiener et al., 2012; Keusekotten et al., 2013; Mevissen et al., 2013). Using *H. sapiens* OTUD1-3, conserved S1′ binding sites have been observed between orthologs from different species, but these binding sites diverged significantly between different OTUD families (Mevissen et al., 2013).

It is generally believed that linear and K63-linked UB chains have similar structural conformations (Trempe, 2011; Komander and Rape, 2012). However, *A. thaliana* OTU1 can discriminate between these linkages, and it selectively cleaves linear and K48-linked chains over K63-linked chains, indicating structural differences between linear and K63-linked UB chains. The presence of structural differences between linear and K63-linked chains is also supported by the different cleavage activities of OTU3-4, OTU7, and OTU10 toward these linkages. The structural elements that are critical for OTU1 recognition are likely similar in linear and K48-linked UB chains but diverge in K63-linked chains. Alternatively, *A. thaliana* OTU1 may utilize distinct recognition elements in the cleavage of linear and K48-linked UB chains. Consistent with the latter notion, the critical OTU1 residues involved in catalyzing isopeptide bond-linked or peptide bond-linked UB chains are not identical; the Asp residue (D89) is critical for cleaving isopeptide bond-linked chains but is dispensable for cleaving linear chains (**Figure 6**), which indicates a role for D89 in stabilizing an oxyanion reaction intermediate that is relevant when cleaving isopeptide bond-linked chains but not linear chains. Different OTU1 residue(s) are likely used to stabilize the oxyanion reaction intermediate for linear chain cleavage.

Among the eleven *A. thaliana* OTU DUBs examined, we found that OTU2 and OTLD1 bind UB chains with distinct linkage preferences. Although a ZnF-like motif and UBA-like domain were identified in OTU2 and OTLD1, respectively, the ZnF-like structure likely does not exist in OTU2, and OTLD1 UBA is dispensable for UB-binding, suggesting the involvement of novel domains. Consistent with our results, neither a bound zinc ion nor the ZnF domain was detected in the yeast ortholog Otu1 in crystal structural analyses (Messick et al., 2008). In addition, UB binding to the predicted ZnF-like domain region of the potential *H. sapiens* ortholog YOD1/OTUD1 was not detected in nuclear magnetic resonance experiments (Mevissen et al., 2013). Interestingly, both the N- and C-terminal domains/residues of OTU2 are critical for binding to UB chains, which suggests that the UB-binding structure is formed from a broad region of the protein. However, the functions of the UB binding activities in *A. thaliana* OTU2 and OTLD1 must be further investigated.

Although the UB binding assays were conducted at conditions (4°C and absence of DTT) unfavorable for UB cleavage, the detection of UB chain binding activities could be compromised with OTU DUBs (OTU1, OTU3, OTU4, OTU7, OTU9,

and OTU10) that are active with the tested linkage types; because the tested K48- and K63-linked chains could be cleaved during the prolonged pull-down assays. It is thus necessary to further examine UB chain binding preferences for those active OTU DUBs using inactive variants and additional linkage types. Moreover, the functional relevance of any detected UB binding activities needs to be further examined.

Based on the phylogenetic tree established in this study, both *H. sapiens*- and plant-specific clades exist, which suggests that these OTU DUBs are involved in taxa-specific functions. The three plant-specific clades include the clade harboring *A. thaliana* OTU8-12, which also harbors six *O. sativa* homologs; the clade harboring *A. thaliana* OTU3/*O. sativa* Os03g0266000; and the clade harboring *A. thaliana* OTU4/*O. sativa* Os01g0900900/Os08g0506000/Os09g0487700. The functions of the plant-specific OTU DUBs are unknown. Notably, a large plant-specific clade harbors *A. thaliana* OTU8-12 and 6 *O. sativa* orthologs, suggesting a functional importance of this clade. The *H. sapiens*-specific clades include OTUD1, OTUD4/ALG13, OTULIN (FAM105B), and VCPIP1/ZRANB1 (TRABID)/TNFAIP3 (A20)/OTUD7A/OTUD7B; the latter two clades generally have important regulatory functions in NFκB signaling (Lee et al., 2000; Evans et al., 2001, 2003; Wertz et al., 2004; Keusekotten et al., 2013). The human-specific VCPIP1 is involved in fundamental p97-mediated reassembly of mitotic organelles (Uchiyama et al., 2002; Wang et al., 2004), which suggests that alternative plant DUB(s) in plant-specific OTU or another class are likely involved in similar processes.

Five clades conserved across species harbor potential orthologs from *A. thaliana*, *O. sativa*, and *H. sapiens*. Two of these conserved clades also harbor potential orthologs from *S. cerevisiae*. Specifically, *A. thaliana* OTU1, OTU2, OTU5, OTLD1, and OTU7 as well as their corresponding *O. sativa* orthologs belong to the clades harboring *H. sapiens* OTUB1/OTUB2, *H. sapiens* YOD1/*S. cerevisiae* Otu1, *H. sapiens* OTUD6A/OTUD6B/*S. cerevisiae* Otu2, *H. sapiens* OTUD5 (DUBA), and *H. sapiens* OTUD3, respectively. Consistent with the relationships in the phylogenetic tree, nearly identical gene structures were observed among the *A. thaliana* and *O. sativa* orthologs. Moreover, the potential *A. thaliana*, *H. sapiens*, and *S. cerevisiae* orthologs from the conserved clades have similar domain organization and high sequence identities/similarities over extended regions (**Figure S8**).

The potential cross-species orthologs in each of the five cross-species conserved OTU DUB clades are likely devoted to similar cellular functions. Because *S. cerevisiae* Otu1 and *H. sapiens* YOD1 are involved in ERAD (Rumpf and Jentsch, 2006; Ernst et al., 2009), the potential *A. thaliana* ortholog OTU2 may also be involved in ERAD. However, we observed distinct linkage specificities between *A. thaliana* OTU2 and *H. sapiens*, *S. cerevisiae*, and *C. elegans* orthologs, as described above. In addition, whereas both *H. sapiens* YOD1/OTUD2 and *S. cerevisiae* Otu1 interact with p97/Cdc48 through an N-terminal UBL or UBX domain, the *A. thaliana* OTU2 has a relatively shorter N-terminus and does not interact with CDC48 (**Figure S8** and data not shown). These results suggest potential mechanistic differentiation, such as a requirement for additional factor(s) in *A. thaliana* OTU2 recruitment by CDC48.

The human K48-specific OTUB1 plays an important role in DNA damage responses by directly inhibiting UBC13 (UBE2N) activity (Nakada et al., 2010); a non-catalytic function that utilizes K48-linkage recognition is critical for this inhibition (Juang et al., 2012; Wiener et al., 2012). Similar non-catalytic inhibition of processes mediated by the UBE2D and UBE2E families by OTUB1 is also likely (Nakada et al., 2010; Juang et al., 2012; Sun et al., 2012; Wiener et al., 2012). Because potential *A. thaliana* orthologs in the UBC13 and UBE2D-E families (http://www.arabidopsis.org/) exist, it would be interesting to examine whether a similar E2-inhibition function is associated with *A. thaliana* OTU1 and whether it is also involved in DNA damage responses. However, given its strong and moderate cleavage activities for linear and K63-linked chains, respectively, in addition to the K48 linkage catalysis and recognition, *A. thaliana* OTU1 may also be involved in other functions, such as modulating linear chain-mediated signaling.

Because only *A. thaliana* OTLD1 has been examined, the plant OTU DUBs contribute little to the functional implications of cross-species conserved OTU DUBs. However, *A. thaliana* OTLD1 (the potential *H. sapiens* OTUD5/DUBA ortholog) likely acts in a histone-modifying repressor complex that harbors histone lysine demethylase KDM1C to suppress specific gene expression through histone deubiquitylation and demethylation (Krichevsky et al., 2011). Interestingly, *A. thaliana* OTU5 (the potential *H. sapiens* OTUD6A/6B ortholog) has been observed in nuclear protein complex(es) and is involved in modulating histone marks on major flowering repressors to regulate flowering time (Radjacommare and Fu, unpublished results). These results suggest that OTU DUBs may be involved in chromatin modification-mediated transcriptional regulation in addition to their predominant roles in cellular signaling. It would be interesting to examine whether the corresponding *S. cerevisiae* and/or *H. sapiens* orthologs also have similar functions and whether these orthologs are associated with evolutionarily and functionally conserved protein complexes.

MATERIALS AND METHODS

BIOINFORMATICS

The *A. thaliana* and *O. sativa OTU* loci were identified through reiterative searches of the TAIR (http://www.arabidopsis.org/index.jsp) and NCBI (http://blast.ncbi.nlm.nih.gov/) databases using the *H. sapiens* OTUB1 and OTUB2 and *S. cerevisiae* Otu1 sequences as the initial queries. The protein domains were identified using SMART from the ExPASy server (http://expasy.org/tools/). Routine DNA and protein sequence analyses were performed using GCG Version 11.1.3-UNIX (Accelrys Inc.).

To generate the phylogenetic tree, the OTU domain sequences were aligned using MUSCLE with default settings (Edgar, 2004). The resulting alignment was used to infer the phylogeny using maximum likelihood and Bayesian methods. We used the PhyML program for the maximum likelihood method (Guindon and Gascuel, 2003). To estimate the level of support for each internal branch, we generated 1000 non-parametric bootstrap samples of the alignment using the SEQBOOT program from the PHYLIP package (Felsenstein, 1989), and we repeated the phylogenetic inference as described above.

For the Bayesian approach, we used the program MrBayes (Ronquist and Huelsenbeck, 2003; Altekar et al., 2004) to infer the posterior probability distributions. The accession numbers for the OTU-containing sequences from *O. sativa*, *H. sapiens*, and *S. cerevisiae* with abbreviated binominal name prefixes are as follows: AK120577 (Os01g0900900), EAY84610 (Os02g 06890), BAD38558 (Os02g31830), BAD26139 (Os02g32180), EEC73285 (Os02g32190), BAD26147 (Os02g32280), AK067291 (Os02g0168600), NP_001046946 (Os02g0513800), AK119352 (Os02g0819500), NP_001049654 (Os03g0266000), AK072986 (Os03g0589300), AK071971 (Os03g0859800), AK240901 (Os04g32970), AK101471 (Os04g0414100), AK066247 (Os04g0 619500), AK107489 (Os04g0652600), AK103099 (Os04g0 670400), AK073551 (Os06g0669800), EEE69074 (Os08g42540), NP_001062186 (Os08g0506000), NP_001063527 (Os09g0 487700), Q9NP73 (HsALG13), NP_060140 (HsOTUB1), NP_075601 (HsOTUB2), NP_001138845 (HsOTUD1), Q5T2D3 (HsOTUD3), EAX05048 (HsOTUD4), NP_060072 (HsOTUD5), Q7L8S5 (HsOTUD6A), AAH29760 (HsOTUD6B), NP_570971 (HsOTUD7A), NP_064590 (HsOTUD7B), Q96BN8 (HsOTULIN/FAM105B), NP_006281 (HsTNFAIP3/A20), NP_079330 (HsVCPIP1), NP_061036 (HsYOD1), CAB64449 (HsZRANB1), P43558 (ScOTU1), and P38747 (ScOTU2).

RECOMBINANT PROTEIN PURIFICATION

To express the various His- and His/GST-tagged OTU proteins, the corresponding full-length coding regions were PCR-amplified using PfuTurbo (Agilent Technologies) and cloned in-frame into pET28 or pET42, respectively (EMD Millipore). The clones, specific primers designed to add appropriate restriction sites for cloning, template cDNA libraries, and vectors are listed (**Table S1**). To express the site-specific OTU variants, mutagenesis was performed using PfuTurbo in accordance with the manufacturer's instructions (Agilent Technologies); the specific primers are listed (**Table S1**). The sequences for the expression constructs were verified by DNA sequence analysis using an ABI PRISM 3700 DNA Analyzer (Life Technologies). Recombinant protein expression in *E. coli* BL21 (DE3) (Novagen) and purification has been previously described (Fatimababy et al., 2010).

IN VITRO DEUBIQUITYLATION ASSAY

The *in vitro* deubiquitylation assay was performed as previously described (Balakirev et al., 2003). The purified recombinant OTU proteins and their variants were incubated at a concentration of 300 nM each with the different substrates in reaction buffer (150 mM NaCl, 0.5 mM DTT, and 20 mM Tris-HCl, pH 8) at 37°C for 1 h. To determine the optimal pH, the cleavage reactions were performed in buffers containing 150 mM NaCl and 0.5 mM DDT at different pH values (20 mM acetate buffer at pH 4.5, 20 mM phosphate buffer at pH 6.5, 20 mM Tris-HCl buffer for pH 7.5–8.5, and 20 mM 3-cyclohexylamino-1-propanesulfonic acid for pH 9.5–10.5). The hydrolysis assay was performed at 37°C for 20 min. The reactions were stopped by boiling the samples for 5 min in 2× SDS-PAGE sample buffer. The inputs and cleavage products were separated by SDS-PAGE and detected by immunoblotting with rabbit polyclonal antisera raised against *H. sapiens* UB (sc-9133) or mouse monoclonal antisera raised against the influenza hemagglutinin (HA) epitope (sc-7392) (Santa Cruz Biotechnology). The input protein concentrations were determined using the protein assay reagent (Bio-Rad Laboratories). The substrates were used in 250 ng quantities for the K48- and K63-tetraubiquitin chains (Boston Biochem); 100, 200, and 250 ng for the purified recombinant linear UB dimers, trimers, and tetramers, respectively; and 250 ng for the purified recombinant HA-tagged UB, RUB, and SUMO fusion proteins.

UB CHAIN BINDING

The GST pull-down assay and detection of pulled-down products by immunoblotting has been described previously (Fatimababy et al., 2010; Lin et al., 2011). The Lys48- and Lys63-linked UB chains (Ub2-7) were purchased from Boston Biochem.

ACCESSION NUMBERS

The sequence data from this article can be found in the Arabidopsis Genome Initiative or GenBank/EMBL databases under the accession numbers listed in **Table 1**.

AUTHOR CONTRIBUTIONS

Hongyong Fu and Ramalingam Radjacommare designed the research; Ramalingam Radjacommare and Raju Usharani performed the research; Hongyong Fu, Ramalingam Radjacommare, and Chih-Horng Kuo analyzed the data; and Hongyong Fu wrote the article.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/journal/10.3389/fpls.2014.00084//abstract

Figure S1 | Comparison of the exon and intron organizations of the *A. thaliana* and *O. sativa OTU*-containing loci in each phylogenetic clade.

Figure S2 | Expression of various *A. thaliana OTU* loci across a wide variety of tissues was detected using GENEVESTIGATOR.

Figure S3 | Relatively constitutive expression of various *A. thaliana OTU* loci was observed by RT-PCR analyses.

Figure S4 | The protein sequence alignment of the OTU domains from *A. thaliana* OTU1-5, OTLD1, OTU7-12, and *H. sapiens* OTUB1.

Figure S5 | *A. thaliana* OTU1, OTU3, and OTU4c display cleavage activities for peptide bond-linked UB polymers.

Figure S6 | *A. thaliana* OTU3, OTU4c, OTU7a, and OTU9-10 cleave UB- and RUB- but not SUMO-GST fusion proteins.

Figure S7 | Optimal cleavage activities of various *A. thaliana* OTU proteins for K48- and K63-linked ubiquitin tetramer were observed at neutral pH.

Figure S8 | Potential orthologs from different species within the conserved phylogenetic clades have similar domain organizations and extended sequence similarities.

Table S1 | *E. coli* intermediate and final constructs used to express *A. thaliana* OTU-deubiquitylation proteins and their test substrates.

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