



# Recent advances on the posttranslational modifications of EXTs and their roles in plant cell walls

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The genetic set up and the enzymes that define the O-glycosylation sites and transfer the activated sugars to cell wall glycoprotein Extensins (EXTs) have remained unknown for a long time. We are now beginning to see the emerging components of the molecular machinery that assembles these complex O-glycoproteins on the plant cell wall. Genes conferring the posttranslational modifications, i.e., proline hydroxylation and subsequent O-glycosylation, of the EXTs have been recently identified. In this review we summarize the enzymes that define the O-glycosylation sites on the O-glycoproteins, i.e., the prolyl 4-hydroxylases (P4Hs), the glycosyltransferases that transfer arabinose units (named arabinosyltransferases, AraTs), and the one responsible for transferring a single galactose (galactosyltransferase, GalT) on the protein EXT backbones. We discuss the effects of posttranslational modifications on the structure and function of extensins in plant cell walls.

**Keywords:** plant cell wall, O-glycosylation, proline hydroxylation, O-glycoproteins, extensins, cell expansion

## INTRODUCTION

The major components of primary plant cell walls include a complex composite of networks of cellulose microfibrils, branched xyloglucans, and a diverse pectin matrix (Somerville et al., 2004; Cosgrove, 2005). In addition, a structural role has been clearly assigned to the hydroxyproline-rich O-glycoprotein extensins (EXTs) in building and maintaining the growing cell wall (Lampport and Northcote, 1960; Hall and Cannon, 2002; Held et al., 2004; Cannon et al., 2008; Lampport et al., 2011; Velasquez et al., 2011a,b). EXTs are plant cell wall O-glycoproteins that belong to the hydroxyproline-rich glycoproteins (HRGPs) superfamily with a modular highly repetitive sequence that includes the O-glycosylated Ser-(Hyp)<sub>3-4</sub> repeats and some of the crosslinking motifs Val-Tyr-Lys, Val-Tyr-Lys-Tyr-Lys, Tyr-Tyr-Tyr-Lys, or Tyr-X-Tyr, all of a hydrophobic nature (Figure 1A; Fry, 1982; Brady et al., 1998; Held et al., 2004; Cannon et al., 2008). EXTs can also occur in the context of natural, chimeric glycosylated polypeptides, e.g., EXT-arabinogalactan proteins (Lind et al., 1994), leucine-rich repeat (LRR) EXT proteins (Rubinstein et al., 1995), or lectin-EXTs (Kieliszewski and Lampport, 1994). Here in this review, we will highlight the major breakthroughs of the posttranslational modifications of EXTs and how these modifications affect the function of EXTs within the primary plant cell wall.

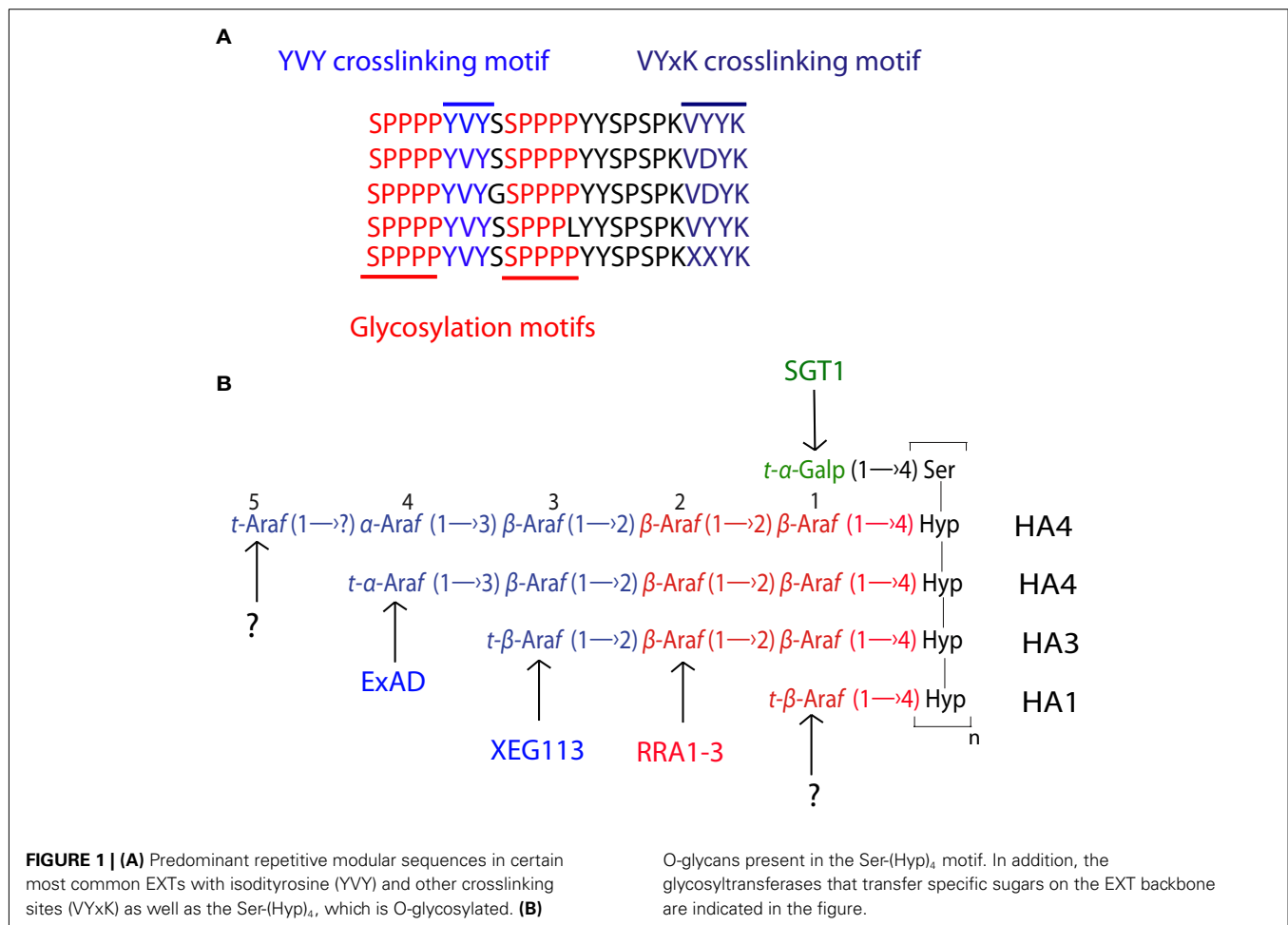
Although the protein sequences that define the O-glycosylation site on HRGPs are partially known (Kieliszewski and Lampport, 1994; Shpak et al., 1999, 2001), the glycosyltransferases that transfer the activated sugars to the protein backbone (arabinose and galactose) have remained a mystery for decades. Now we are beginning to perceive the emerging picture of the molecular machinery that assembles sugars into these complex O-glycoproteins

which comprises an important part of the cell wall. Most of the genes involved in the posttranslational modification of EXTs that includes proline hydroxylation and subsequent O-glycosylation are now being identified (Egelund et al., 2007; Cannon et al., 2008; Gille et al., 2009; Velasquez et al., 2011a), increasing our understanding of plant cell wall EXTs synthesis and their roles in cell expansion during growth.

While several aspects of EXTs have been reviewed recently (Kieliszewski et al., 2010; Showalter et al., 2010; Lampport et al., 2011), the present review focuses specifically on the enzymes that define the O-glycosylation sites, namely the prolyl 4-hydroxylases (P4Hs) and the glycosyltransferases that transfer arabinosyl units (named arabinosyltransferases, AraTs) or a single galactosyl residue (galactosyltransferase, GalT) to the protein EXT backbones. Finally, the structure and functions of the O-glycoprotein EXTs are discussed in the context of cell wall expansion during growth.

## STEPS TO BUILD MATURE, FUNCTIONAL EXTs

Structural O-glycoproteins EXTs that are secreted into plant cell walls are first shaped by several posttranslational modifications that include signal peptide processing in the ER, hydroxylation of peptidyl-proline residues into hydroxyproline (Hyp) in the ER-Golgi apparatus (Lampport and Northcote, 1960; Lampport, 1967; Yuasa et al., 2005; Velasquez et al., 2011a), O-glycosylation on Hyp and Ser by the action of several glycosyltransferases, mostly in the Golgi apparatus, although early steps could be taking place in the ER (Figure 1B; Shimma et al., 2011; Velasquez et al., 2011a), and finally, covalent intra- and inter-molecular crosslinking at the plant cell wall level (Fry, 1982; Held et al., 2004). All these



posttranslational modifications of EXTs are crucial for the function of EXTs in the plant cell wall during cell development and growth (Hall and Cannon, 2002; Cannon et al., 2008; Velasquez et al., 2011a,b).

Two P4H families, the collagen P4Hs (C-P4Hs) and the hypoxia-inducible transcription factor (HIF)-associated P4Hs, are responsible for the hydroxylation of collagens and HIF, respectively (Myllyharju, 2003, 2008). The vertebrate C-P4Hs are  $\alpha_2\beta_2$  tetramers, in which the  $\alpha$ -subunits possess the enzymatic activity and the  $\beta$ -subunits are identical to the enzyme disulfide isomerase. The tetrameric assembly is required for stability and full activity. In contrast, plant P4Hs and also HIF-P4Hs are monomers (Hieta and Myllyharju, 2002; Myllyharju, 2003; Koski et al., 2007, 2009). Plant P4Hs are membrane-bound enzymes that catalyze the hydroxylation of peptidyl-proline to yield hydroxyproline by introducing an oxygen atom between the hydrogen and carbon atoms of the peptidyl-proline adding merely 16 atomic mass units (Lampport, 1963). From four possible isomers of hydroxyproline, the most common isomer in nature is (2*S*,4*R*)-4-hydroxyproline, herein after referred to as Hyp (*trans*-4-hydroxyproline) in reference to the *trans*-relationship between the -COOH and -OH substituents on the pyrrolidine ring (Hieta and Myllyharju, 2002; Myllyharju, 2003). In addition, the *cis*-hydroxyproline (2*S*,4*S*)-4-hydroxyproline is reported to be present in the toxic cyclic peptides

from *Amanita* mushrooms (e.g., alpha-amanitin and phalloidin; Alexopoulos et al., 1996). The P4Hs belong to a family of 2-oxoglutarate-dependent dioxygenases that require 2-oxoglutarate and O<sub>2</sub> as co-substrates, Fe<sup>2+</sup> as a cofactor and ascorbate to prevent the rapid inactivation by self-oxidation (Hieta and Myllyharju, 2002; Koski et al., 2007, 2009). From a mechanistic point of view,  $\alpha$ -ketoglutarate is oxidatively decarboxylated to produce succinate and CO<sub>2</sub>. The conversion of Pro to Hyp in the secretory pathway provides reactive hydroxyl groups for further modification such as O-glycosylation. In addition, the product of this reaction, poly-(4-hydroxyproline), has an even greater tendency than polyproline to adopt a helical polyproline II-type conformation (Lampport, 1977, 1980; Van Holst and Varner, 1984; Valentin et al., 2010).

*In vitro* and *in vivo* characterization of plant P4Hs has been carried out in *Arabidopsis thaliana* (Hieta and Myllyharju, 2002; Tiainen et al., 2005; Vlad et al., 2007; Asif et al., 2009; Velasquez et al., 2011a), *Nicotiana tabacum* (Yuasa et al., 2005), *Dianthus caryophyllus* (Vlad et al., 2010), and the green alga *Chlamydomonas reinhardtii* (Keskihaio et al., 2007). Only the activity of AtP4H1 and AtP4H2, out of the 13 P4Hs encoded in the *Arabidopsis* genome, have been fully characterized *in vitro*, and only CrP4H1 has been crystallized, without and with the (Ser-Pro)<sub>5</sub> peptide as a substrate (Koski et al., 2007, 2009). Both AtP4Hs hydroxylate proline-rich peptides of both extreme types: arabinogalactan proteins (AGPs)

and EXTs. However, AtP4H5 seems to be more specific as yeast two-hybrid assays and molecular modeling strongly suggest its preference for polyproline II peptide instead of the AGP-type substrate (Velasquez et al., 2011a). Moreover, three AtP4H T-DNA insertional mutants (*Atp4h2,5,13*) exhibited a clear short root hair phenotype and reduced root Hyp levels as well. Remarkably, overexpression of these P4Hs displayed extra long root hairs (Velasquez et al., 2011a,b) and much higher Hyp contents in roots when compared to WT Col-0 (Estevez et al., unpublished results). Interestingly, heterologous peptides that mimic collagen (X-Pro-Gly)<sub>n</sub> are also hydroxylated by some plant P4Hs, though inefficiently (Tanaka et al., 1981; Kaska et al., 1988).

Based on multiple pieces of evidence, it became clear that proline hydroxylation of HRGPs, and specifically EXTs, are essential for polarized cell expansion in root hairs. Therefore, it is tempting to imagine that homologous P4Hs and HRGPs will also be relevant in cell expansion in other rapidly expanding cell types such as pollen tubes or dark-grown hypocotyls. On the other hand, suppression of the gene encoding 1 out of 10 *Chlamydomonas reinhardtii* prolyl 4-hydroxylases (CrP4H1), which hydroxylates algal HRGP sequences, led to a defective cell wall (Keskiäho et al., 2007), suggesting a major unique structural role of certain HRGPs as targets of CrP4H1 in this unicellular green algae. In two other studies, a direct role of P4Hs in hypoxia stress and during different stages of plant growth and development is speculated, suggesting that P4Hs could be acting as putative oxygen sensors (Vlad et al., 2007; Asif et al., 2009). Nevertheless, further studies are needed to confirm this hypothesis.

The extent and type of O-glycosylation in HRGPs, and specifically in EXTs, can be predicted by the “Hyp contiguity” hypothesis, according to which O-glycosylation correlates with the primary sequence of the protein where location and context of Hyp residues are crucial (Kieliszewski and Lampport, 1994; Kieliszewski, 2001; Shpak et al., 1999, 2001). Generally, wherever Hyp residues are contiguous in the amino acid sequence, arabinosylation is predominant in EXTs, whereas arabinogalactans are rather added to clustered, non-contiguous Hyp residues in AGPs. However, it is worth mentioning that isolated Hyp units were also arabinogalactosylated in some cases (Zhao et al., 2002). Another clear exception to this hypothesis is exemplified by the small CLE-like peptides (e.g., Tob/Tom-HypSys, PSY1, CLV3, and CLE2) where single proline units are modified with  $\beta$ -linked-L-arabinofuranosides with identical linkages/stereochemistry of the innermost three arabinose units found in the EXTs (Ryan and Pearce, 2003; Ito et al., 2006; Kondo et al., 2006; Ohyama et al., 2009; Matsuzaki et al., 2010), suggesting that similar P4Hs and glycosyltransferases participate in their posttranslational modifications. It is important to note that Hyp O-glycosylation has not been found in animal cells, which makes HRGPs unique in their structure and functions for plant cells.

EXT O-glycosylation in the Ser-(Hyp)<sub>3-4</sub> repeats involves a series of glycosyltransferases that add a single galactose unit to serine and 4–5 arabinose units, in a linear fashion, to Hyp. The minimal set of activities necessary for synthesis of the entire glycomodule in EXTs (Figure 1B) may comprise: one Ser- $\alpha$ -D-galactosyltransferase acting exclusively on serine, one Hyp- $\beta$ -L-arabinofuranosyltransferase, two  $\beta$ -(1  $\rightarrow$  2)-L-arabinofuranosyl

transferases, one  $\alpha$ -(1  $\rightarrow$  3)-L-arabinofuranosyltransferase and a last arabinosyltransferase adding the fifth arabinofuranose with a yet unknown regio-chemistry (Shimma et al., 2011; Velasquez et al., 2011a). Three glycosyltransferases involved in extensins maturation have been recently reported after characterizing EXT O-glycosylation in T-DNA mutant lines of the corresponding genes in *Arabidopsis* (Egelund et al., 2007; Gille et al., 2009; Shimma et al., 2011; Velasquez et al., 2011a). The arabinosyltransferase that would add the second  $\beta$ -(1  $\rightarrow$  2)-arabinosyl unit in the Hyp-O-arabinosides is the proposed Reduced Residual Arabinose 3 (RRA3). The one likely to transfer the third  $\beta$ -(1  $\rightarrow$  2)-arabinose was named as XyloEndoGlucanase (XEG113), both belonging to the GT77 Carbohydrate Active EnZymes (CAZY) family (Egelund et al., 2007; Gille et al., 2009; Velasquez et al., 2011a). Finally, the AraT that would transfer the fourth  $\alpha$ -(1  $\rightarrow$  3)-L-arabinose moiety was identified very recently as Extensin Arabinose Deficient transferase (ExAD) within the GT47 family (Petersen et al., unpublished results). In addition, the galactosyltransferase that adds galactose on serine in the Ser-(Hyp)<sub>3-4</sub> repeats was recently reported as SGT1 (Shimma et al., 2011). To the present day, the arabinosyltransferase that adds the first and the last (fifth) arabinose remains unknown (Figure 1B). Remarkably, all the AraTs T-DNA mutants identified so far with deficient O-arabinosylation in EXT backbones displayed a clear root hair phenotype, suggesting that O-glycosylation on EXT is crucial on EXT function during cell expansion at least in this cell type and also in dark-grown hypocotyls for the mutant *xeg113-2* (Gille et al., 2009; Velasquez et al., 2011a,b). The evolutionary conservation from unicellular green algae to vascular plants of the Ser-(Hyp)<sub>3-4</sub> glycomodule implies an essential function of O-glycosylation in EXT shape and biological role.

## IMPACT OF HYDROXYLATION AND O-GLYCOSYLATION ON EXT FUNCTION

It has been suggested that the O-glycans increase HRGP solubility, resistance to proteolytic degradation and thermal stability (Kieliszewski et al., 1989, 2010; Ferris et al., 2001; Shpak et al., 2001; Lampport et al., 2011). The structural and functional consequences of O-glycosylation in polyproline EXTs remained unclear although some evidence suggested that this posttranslational modification could provide conformational stability to these macromolecules (Lampport, 1980; Van Holst and Varner, 1984; Stafstrom and Staehelin, 1986). To understand the biological meaning of the lack of hydroxylation/O-glycosylation on EXTs, possible conformational changes induced by proline hydroxylation and subsequent O-arabinosylation on the minimal EXT repeat Ser(Pro)<sub>4</sub> and Ser(Hyp)<sub>4</sub> were tested by molecular dynamics modeling. In fact, O-glycosylation clearly appears to stabilize the helical conformation of the EXT minimal peptide whereas incomplete hydroxylation and O-glycosylation favors its flexible and disorganized conformation (Velasquez et al., 2011a) as it was previously suggested by circular dichroism on both short and repetitive EXT synthetic peptides and native EXTs (Van Holst and Varner, 1984; Shpak et al., 2001). In another recent study, it was found that O-glycosylation of Hyp residues causes a dramatic increase in the thermal stability of the polyproline type-II (PP-II) helix, which may be explained by a network of inter-glycan and glycan-peptide hydrogen bonds

(Owens et al., 2010). Analysis of the  $\beta$ -O-glycosylated polyproline model peptides indicates that Hyp O-glycosylation leads to a significant increase in conformational stability (Owens et al., 2010; Velasquez et al., 2011a). Since contiguous O-glycosylation seems to bring about a rise in conformational stability, similar increases in the conformational stability of EXTs are likely to occur. Based on this, we theorize that EXTs with incomplete hydroxylation/O-glycosylation have an enormous negative impact on EXTs overall shape, affecting EXTs interactions with the surrounding environment, including those with other EXTs in the network or with other modifying enzymes such as EXT-specific peroxidases (PER<sub>ext</sub>; Velasquez et al., 2011a). The latter belong to the type-III apoplasmic peroxidases that form a large multigene family present in all land plants (Passardi et al., 2004).

Based on the *in vitro* activity of several peroxidases (Everdeen et al., 1988; Brownleader et al., 1995; Schnabelrauch et al., 1996; Magliano and Casal, 1998; Jackson et al., 2001; Price et al., 2003), it is proposed that the *in vivo* crosslinking of EXTs might involve multiple PER<sub>ext</sub>, although their molecular roles and specific targets remain unknown. Some of these enzymes could catalyze the polymerization of EXT monomers via di-isodityrosine and pulcherosine, a tetramer and trimer of tyrosine, respectively (Fry, 1982; Brady et al., 1996, 1998) to form a covalent cross-linked network at the plant cell wall level (Cannon et al., 2008). For example, tomato PER<sub>ext</sub> exhibited a strong activity onto diverse types of extensins (Schnabelrauch et al., 1996). Moreover, PER<sub>ext</sub> activity might be modulated *in vivo*, as shown by a study that used a natural inhibitor of extensin crosslinking isolated from a cell wall compartment containing extensins and peroxidases (Brownleader and Dey, 1993; Brownleader et al., 2000). The high number of genes encoding type-III- peroxidases, in addition to the biochemical variety of their substrates, is still one of the main obstacles for the assigning of their specific functions *in vivo*. However, in *Arabidopsis*, by means of co-expression analysis of well known root hair cell wall-associated genes, a few putative EXT peroxidases such as PER13 and PER73 were identified, suggesting that at least some of them are responsible for the crosslinking of EXTs specifically in the root hair cell walls (Velasquez et al., 2011a).

## EXTENSIN FUNCTION

The known roles of extensins in cell wall assembly, cell shape and growth raise the question about the function for each individual extensin molecule (Hall and Cannon, 2002; Cannon et al., 2008; Velasquez et al., 2011a). Recently, it was reported that EXTs can form, at least *in vitro*, a tridimensional covalent network through Tyr linkages mediated by EXT peroxidases between individual EXT molecules and also via self-recognition and alignment of hydrophilic O-glycosylated Ser-(Hyp)<sub>3-4</sub> repeats and hydrophobic peptide-crosslinking modules (Cannon et al., 2008). Thus, the ordered EXT monomers assembly in the plant cell wall would involve a zipper-like endwise association via crosslinking at the ends of the molecules (Kieliszewski et al., 2010; Lampport et al., 2011). It is also proposed that the oligomeric extensin interacts with pectins by a simple acid-base reaction forming a supramolecular ionic structure in the nascent cell wall, which would serve

as a template for further cell wall deposition (Cannon et al., 2008; Kieliszewski et al., 2010). This hypothesis was recently confirmed *in vitro* by using a layer-by-layer method to construct a multilayer thin film used to investigate the interactions between extensin and pectin. Multilayer formation was successfully produced, thereby evidencing the occurrence of interactions between EXTs and pectin (Valentin et al., 2010). In addition, covalent EXT-pectin crosslinks were also suggested (Qi et al., 1995; Nuñez et al., 2009).

Although the *Arabidopsis* genome encodes up to 63 putative extensins of which 20 would be defined as classical extensins, where most of them share both motifs for O-glycosylation and Try-mediated crosslinks (Figure 1A), 12 are shorter extensins and 31 are extensin-like chimeras and hybrid-extensins that could contain other protein domains (Cannon et al., 2008; Showalter et al., 2010; Lampport et al., 2011), only one extensin mutant, *root-, shoot-, hypocotyl-defective (rsh) ext3*, has been shown to have a nearly lethal phenotype (Hall and Cannon, 2002; Cannon et al., 2008). However, only two other single *ext* mutants, such as the *leucine-rich repeat extensins 1 and 2 (lrx1 and lrx2)* with aberrant root hairs (Baumberger et al., 2001, 2003a,b; Ringli, 2010) and the *proline-rich extensin-like receptor kinase perk13* displaying longer root hairs (Humphrey et al., 2007), have been reported, thus suggesting a low degree of EXT genetic redundancy. In support of this notion, a number of root hair-relevant extensins have been found taking advantage of network analysis of co-expression with other cell wall genes known to be important for root hair growth, like expansins EXP7 and EXP18 (Won et al., 2009), proline-rich proteins PRP1 and PRP3 (Fowler et al., 1999; Bernhardt and Tierney, 2000) and leucine-rich extensin protein LRX1 (Baumberger et al., 2003a,b). Consistently, the same EXTs were identified by functional genomics on root hair mutants (Baumberger et al., 2003a,b; Jones et al., 2006; Deal and Henikoff, 2010). Through these two approaches, it was possible to identify several EXTs that seem to be important for root hair growth. This was later confirmed when T-DNA homozygous mutant lines (*ext6, ext7, ext10*, etc.) of these EXTs turned out to exhibit drastically shorter root hairs (Velasquez et al., 2011a,b). All these EXT-related mutants highlight an essential role of each individual EXT type on cell expansion and growth, specifically in root hairs. This suggests that location and function of each individual EXT type within the network at the cell wall are relevant to the whole EXT network assembly.

Finally, from an evolutionary perspective, highly organized but non-covalent EXT-like cell wall networks are ancient, as they occur in several unicellular and multicellular green algae (Sumper and Hallmann, 1998; Ferris et al., 2001; Bollig et al., 2007; Lee et al., 2007; Sørensen et al., 2011). In agreement, type-III peroxidase genes seem to be absent in green algae genomes (Passardi et al., 2004). Hence, a covalently cross-linked EXT network seems to have been a secondary development in evolution that led to strong plant cell walls, enabling terrestrial colonization, and turgor-driven growth in land plants.

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