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A novel semi-dominant mutation in *brassinosteroid signaling kinase1* increases stomatal density

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Stomata play a pivotal role in balancing CO₂ uptake for photosynthesis and water loss via transpiration. Thus, appropriate regulation of stomatal movement and its formation are crucial for plant growth and survival. Red and blue light induce phosphorylation of the C-terminal residue of the plasma membrane (PM) H⁺-ATPase, threonine, in guard cells, generating the driving force for stomatal opening. While significant progress has been made in understanding the regulatory mechanism of PM H⁺-ATPase in guard cells, the regulatory components for the phosphorylation of PM H⁺-ATPase have not been fully elucidated. Recently, we established a new immunohistochemical technique for detecting guard-cell PM H⁺-ATPase phosphorylation using leaves, which was expected to facilitate investigations with a single leaf. In this study, we applied the technique to genetic screening experiment to explore novel regulators for the phosphorylation of PM H⁺-ATPase in guard cells, as well as stomatal development. We successfully performed phenotyping using a single leaf. During the experiment, we identified a mutant exhibiting high stomatal density, *jozetsu* (*jzt*), named after a Japanese word meaning 'talkative'. We found that a novel semi-dominant mutation in BRASSINOSTEROID SIGNALING KINASE1 (BSK1) is responsible for the phenotype in *jzt* mutant. The present results demonstrate that the new immunohistochemical technique has a wide range of applications, and the novel mutation would provide genetic tool to expand our understanding of plant development mediated by brassinosteroid signaling.

KEYWORDS

Arabidopsis thaliana, brassinosteroid signaling, whole-mount immunohistochemistry, InsituPro, genetic screening, next-generation sequencing

Introduction

Stomata consist of pairs of guard cells on the plant epidermis and function to balance CO₂ uptake for photosynthesis and water loss via transpiration. Stomatal movement (opening and closing) is regulated in response to various environmental and endogenous signals, including light (blue and red light)/darkness, CO₂, humidity, the phytohormone abscisic acid (ABA), and drought stress (Roelfsema et al., 2012; Inoue and Kinoshita, 2017; Jezek and Blatt, 2017; Lawson and Matthews, 2020). Stomatal development is also influenced by environment and endogenous stimuli, such as light, CO₂, phytohormones (ABA, auxin, and brassinosteroids), temperature, and water/osmotic stress (Qi and Torii, 2018; Han et al., 2021).

Light-induced stomatal opening is driven by the activation of the plasma membrane (PM) H⁺-ATPase in guard cells, which generates an electrochemical gradient across the PM to promote ion influx and a consequent turgor increase through osmotic water uptake by the cell (Shimazaki et al., 2007; Inoue and Kinoshita, 2017; Fuglsang and Palmgren, 2021; Palmgren, 2023). The activation of PM H⁺-ATPase is mediated by the phosphorylation of its C-terminal penultimate residue, threonine (Thr), in guard cells (Kinoshita and Shimazaki, 1999, 2002). Previous studies have identified regulators for blue light-induced phosphorylation of PM H⁺-ATPase in guard cells (Kinoshita et al., 2011; Takemiya et al., 2013a, 2013b; Hayashi et al., 2017). Although a PM-localized protein kinase insensitive to a potent protein kinase inhibitor, K-252a, is suggested to phosphorylate the penultimate Thr in PM H⁺-ATPase (Hayash et al., 2010), it has not been identified. Moreover, photosynthesis-dependent phosphorylation and clade D type2C protein phosphatase-mediated dephosphorylation of PM H⁺-ATPase (Ando and Kinoshita, 2018; Wong et al., 2021; Akiyama et al., 2022; Ando et al., 2022) raise a hypothesis that unidentified regulators are involved in the above processes in guard cells. Note that PM H⁺-ATPase trafficking in guard cells regulated by environmental stress greatly affects its activity and stomatal movement (Xue et al., 2018; Xia et al., 2019; Baena et al., 2024).

Stomatal development is a sequential process of cell division and differentiation. In *Arabidopsis thaliana* (Arabidopsis), stomatal development begins with a subset of protodermal cells called meristemoid mother cells, which undergo several asymmetric cell divisions to generate meristemoid cells surrounded by stomatal lineage ground cells. Subsequently, the meristemoid cells differentiate into guard mother cells (GMCs). Finally, GMCs undergo a symmetrical division, resulting in the formation of a pair of guard cells (Qi and Torii, 2018; Han et al., 2021). These processes are regulated by three basic helix-loop-helix (bHLH) transcription factors: SPEACHLESS (SPCH; MacAlister et al., 2007), MUTE (Pillitteri et al., 2007), and FAMA (Ohashi-Ito and Bergmann, 2006), as well as bHLH-leucine zipper proteins SCREAME(SCR)/INDUCER OF CBF EXPRESSION1 and SCR2 (Kanaoka et al., 2008). The stability of the stomatal bHLH proteins is regulated by the mitogen-activated protein kinase (MAPK) cascade, which includes YODA (YDA), MKK4/5/7/9, and MPK3/6 (Bergmann et al., 2004). The SPCH/SCR heterodimer is responsible for the initiation and proliferation of the stomatal lineage through asymmetric divisions. The MUTE/SCR dimer halts the asymmetric division mediated by

SPCH/SCR and induces the differentiation of meristemoids into GMCs. The FAMA/SCR complex facilitates the last symmetric division and restricts further cell division in guard cells.

A phytohormone brassinosteroid (BR) coordinates plant growth and development (Zhu et al., 2013). PM-localized leucine rich repeat-receptor kinase (LRR-RK) BRASSINOSTEROID INSENSITIVE1 (BRI1) functions as a BR receptor (Li and Chory, 1997; Kinoshita et al., 2005). BR binding activates BRI1 kinase activity, involving the recruitment of the coreceptor kinase BRI1-ASSOCIATED RECEPTOR KINASE1 (BAK1; Li et al., 2002; Nam and Li, 2002), dissociation from inhibitory proteins (Wang and Chory, 2006), and transphosphorylation between BRI1 and BAK1 (Wang et al., 2008). Activated BRI1 phosphorylates substrate proteins, including BRASSINOSTEROID SIGNALING KINASE1 (BSK1; Tang et al., 2008). The phosphorylated BSK1 induces BRI1-SUPPRESSOR1-mediated inactivation of GSK3-like kinase BRASSINOSTEROID INSENSITIVE2 (BIN2) to regulate downstream transcriptional regulation by BRASSINAZOLE RESISTANT1 (BZR1; He et al., 2002; Wang et al., 2002; Yin et al., 2002; Kim et al., 2011). In Arabidopsis leaves, BR has been shown to negatively regulate stomatal development. *bri1* and dominant *bin2* mutations result in a clustered stomata phenotype, whereas a mutation in *BZR1* does not affect stomatal development (Kim et al., 2012). Additionally, BIN2 has been shown to inhibit YDA activity (Kim et al., 2012). Thus, BIN2 is considered to regulate the MAPK cascade to control downstream stomatal transcription factors (Qi and Torii, 2018).

BSK1 is one of the members of receptor-like cytoplasmic kinase sub-family (RLCK-XII), which is consisted of 12 members in Arabidopsis (BSK1–BSK12; Tang et al., 2008). BSK proteins consist of the N-terminal kinase domain and the C-terminal tetratricopeptide repeats. BSK proteins do not contain a transmembrane region; however, myristoylation is suggested to enable their association with the PM, which may be required for their functions (Tang et al., 2008). The BSK family proteins exhibit functional redundancy, and genetic analysis of single mutants failed to show clear morphological defects, except for *bsk3*, which exhibits sensitivity to the BR biosynthesis inhibitor and insensitivity to brassinolide (Tang et al., 2008). Sreeramulu et al. (2013) indicated that knock-out of at least three BSK genes, including *bsk3*, is required for the morphological defects and altered response to exogenous brassinolide.

We previously established an immunohistochemical detection method for the guard-cell PM H⁺-ATPase and its phosphorylation status of the penultimate threonine. In this method, leaves instead of isolated epidermis or guard cells, were used as materials (Ando and Kinoshita, 2018, 2019). The use of isolated epidermis or guard cells often limits the applicability of conventional methods. Therefore, the new method employing leaves was designed to overcome these restrictions and have a broader range of applications. To demonstrate this, we carried out a genetic screening experiment as a model study. Genetic screening generally involves handling a single leaf, often from a dwarf plant, making it challenging to obtain a sufficient amount of epidermal tissue. Utilizing a commercial liquid-handling robot, we successfully performed simultaneous sample preparation and phenotyping for multiple samples. During the

experiment, we identified a mutant with high stomatal density. Further analysis revealed that the mutation responsible for the phenotype is a novel semi-dominant allele of BSK1. Our mutant serves as a genetic tool to elucidate the function of BSK1 protein in plant development.

Materials and methods

Plant materials and growth conditions

Arabidopsis thaliana was used as experimental material. Ethyl methanesulphonate (EMS)-mutagenesis using *phot2-1* was carried out previously (Lightner and Caspar, 1998), and M₂ population was used for screening. Columbia-0 (Col-0) was used as control plant for *bsk1* (SAIL_140_C04) and DNA extraction. The T-DNA insertion was confirmed by genomic PCR using primer sets shown in Supplementary Table S1. Landsberg *erecta* (*Ler*) was used for a map-based cloning experiment. Plants were grown on soil under fluorescent lamps in the growth room. Photon flux densities, day length, temperature, and relative humidity was approximately 50 $\mu\text{mol m}^{-2} \text{s}^{-1}$, 16-h, 20–24°C, and 40–60%, respectively.

Immunohistochemical screening for stomatal traits

Previously developed immunohistochemical technique using leaves (Ando and Kinoshita, 2018) was employed as screening tool wherein phosphorylation of the penultimate residue of PM H⁺-ATPase (Thr) in guard cells was visualized. Leaves were collected from each M₂ plants, then they were illuminated with red light (150 $\mu\text{mol m}^{-2} \text{s}^{-1}$) and blue light (50 $\mu\text{mol m}^{-2} \text{s}^{-1}$) simultaneously for 15 min. Illuminated leaves were fixed and attached to a microscope slide as described previously. Liquid handling including tissue permeabilization, blocking, antibody application, and washing the material steps was automated by Insitu Pro VSi (Intavis). Observed phenotype was confirmed using the remained leaves from the same M₂ plants if possible, and validated in M₃ plants.

Stomatal density and index

For measurement of stomatal density and index, leaves were fixed and cleared according to the previous study (Kang et al., 2009). Six images per leaf were obtained, then the density and index were calculated on each image. Arithmetic mean of the six images was calculated as a representative value for the corresponding leaf. Data represent arithmetic means of the representative values obtained from at least three leaves with standard deviations.

Next-generation sequencing

F₂ plants were obtained by crossing EMS mutant line and its progenitor and those exhibiting mutant phenotype were selected

and DNA was extracted from them in a bulk with the High pure PCR template preparation kit (Roche) according to the manufacturer's instructions. The bulk population genomic DNA was subjected to whole-genome sequencing and analyzed by Mitsucal for mapping and identification of a mutation as described previously (Suzuki et al., 2018). Reanalysis of the mutation by sanger sequencing was performed using a primer set shown in Supplementary Table S1.

Construction of plasmid vectors and transformation of plants

Genomic fragment of *BSK1* was amplified by nested PCR from Col-0 using primer sets shown in Supplementary Table S1. The fragment including –1,650 bp to +3,742 bp of the start codon was fused to pCAMBIA1300 digested with EcoRI using In-Fusion HD Cloning kit (Clontech). *Agrobacterium tumefaciens* (GV3101) was transformed with the construct, then used for generation of the transgenic plants.

Statistical analyses

Statistical comparison of means was conducted using R software (R Core Team, 2023). Student's *t* test or Dunnett's test using *multcomp* package (Hothorn et al., 2008) was carried out for two independent means or multiple means with single control, respectively. Segregation ratio was analyzed by chi-square goodness of fit test. *P* < 0.05 was considered statistically significant.

Results

Genetic screening based on the immunohistochemical visualization of guard-cell PM H⁺-ATPase

Previously, we developed a novel immunohistochemical technique for visualizing PM H⁺-ATPase and the phosphorylation of its penultimate residue, Thr, in guard cells using whole leaves (Ando and Kinoshita, 2018). Conventional techniques, such as the isolation of guard cell protoplasts (Ueno et al., 2005), require isolation of a sufficient amount of epidermis before the experiments, limiting their application based on plant amount, size, or both. To assess the versatility of the new immunohistochemical technique, we applied it to genetic screening, where plants must be analyzed individually, and thus only single, and sometimes small, leaves are available for the experiment. Utilizing a liquid-handling robot, we successfully semi-automated the process of immunohistochemical visualization of guard-cell PM H⁺-ATPase to prepare many specimens simultaneously. We screened the M₂ population of EMS-mutagenized *phot2-1* (*phot2*-EMS; about 8,700 plants) and Col-0 (Col-EMS; about 3,200 plants) based on the phosphorylation status of guard-cell PM H⁺-ATPase in leaves illuminated with red (150 $\mu\text{mol m}^{-2} \text{s}^{-1}$) and blue light (50 $\mu\text{mol m}^{-2} \text{s}^{-1}$) for 15 min (Figure 1A).

During the experiment, we identified a plant that exhibited increased stomatal density, which was named *jozetsu* (*jzt*) after a Japanese word meaning ‘talkative’ (Figure 1B). The successful isolation of plants with stomatal defects demonstrates that our immunohistochemical technique using a leaf works even when the plant material is limited.

Characterization of *jzt* plant

To identify the responsible mutation in *jzt* plant, we conducted further investigations on *jzt* and characterized this mutant. Initially, we examined whether *jzt* exhibits morphological defects other than the stomatal density. As shown in Figure 2A, we observed that *jzt* plants displayed a dwarf phenotype compared to its progenitor *phot2*, suggesting that the putative responsible mutation is involved

not only in the stomatal development but also in general plant development. Next, we analyzed the stomatal index and density to quantitatively assess the *jzt* phenotype. In *phot2* leaves, the stomatal index was approximately 20% on average, whereas *jzt* leaves exhibited a 1.5-fold higher stomatal index (Figure 2B). Stomatal density in *phot2* and *jzt* was around 150 and 480 stomata mm^{-2} , respectively. These results indicate that *jzt* may have a defect in guard-cell differentiation during the stomatal development.

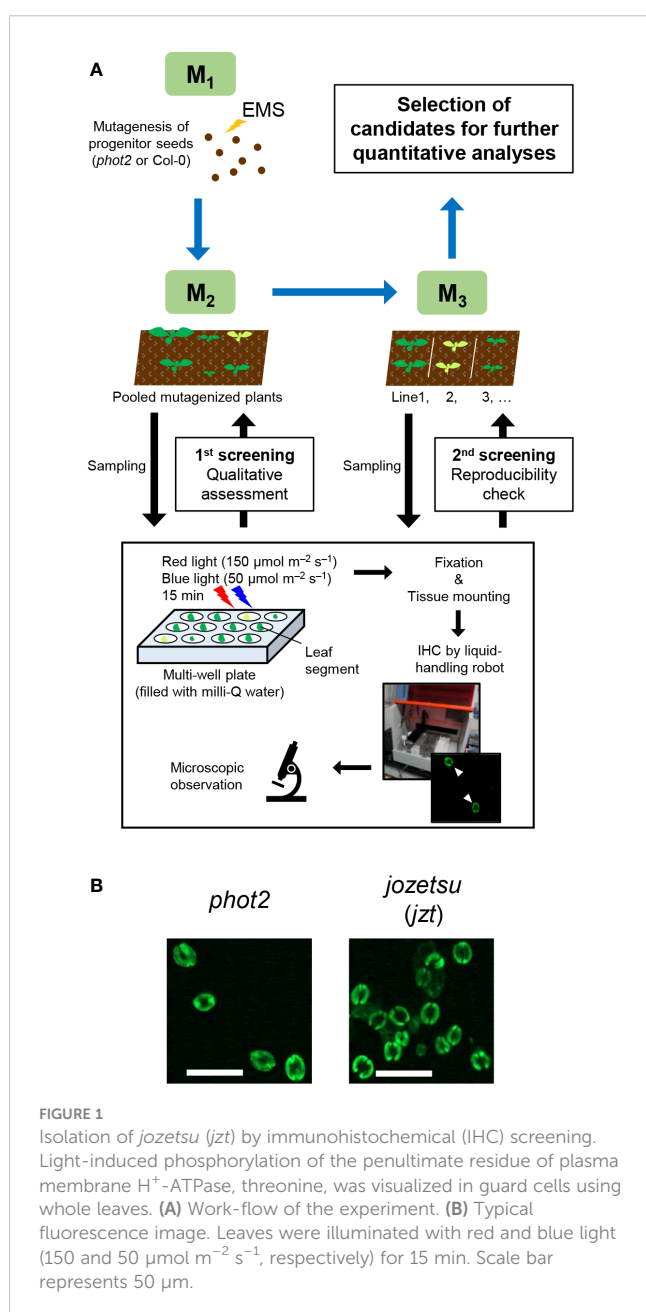
Identification of the mutation responsible for *jzt* phenotypes

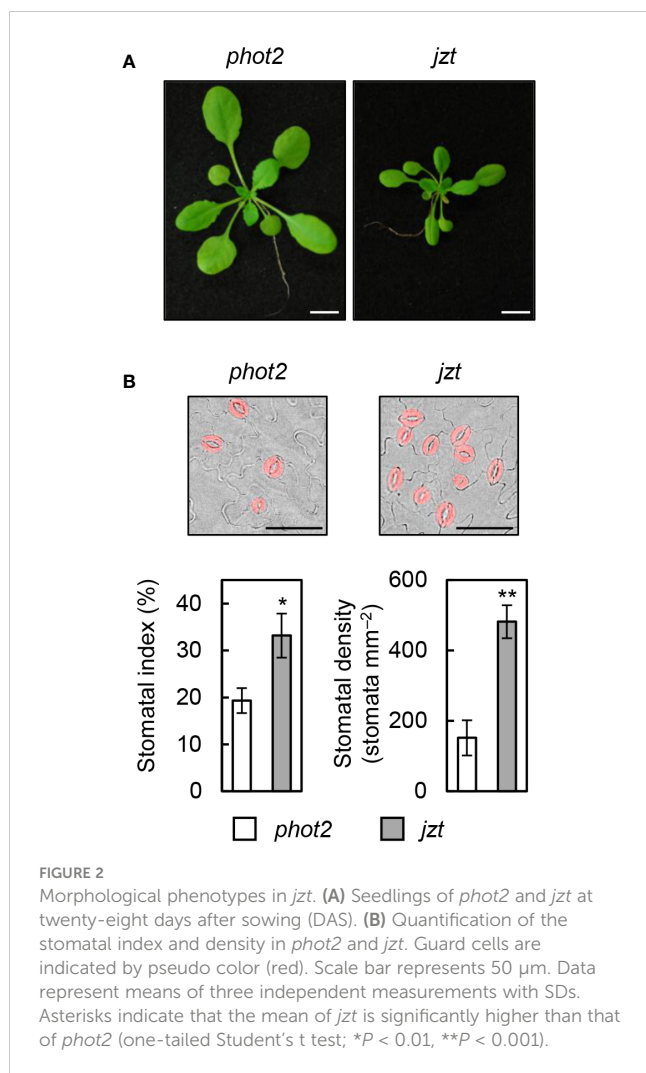
We crossed *jzt* with *Ler* or *phot2* and conducted map-based cloning or Next-generation sequencing, respectively, to identify the mutation responsible for the *jzt* phenotypes. Map-based cloning revealed that the responsible mutation is located between 16.6 and 16.9 Mb of the chromosome 4 (Figure 3A). Through this, we identified single nucleotide substitutions that cause missense mutations in two genes: At4g35150 and At4g35230 encoding O-methyltransferase family protein and BSK1, respectively (Figures 3B, C; Supplementary Figures S1A, B). Since BSK1 is a signaling complex for BR and BR has been shown to be a negative regulator of stomatal development in leaves (Tang et al., 2008; Kim et al., 2012). Public microarray data indicated that *BSK1* transcripts are detectable throughout the entire plant, including leaves, whereas those of *At4g35150* were indicated to express only in the developing embryo (Supplementary Figure S1C). These results strongly suggested that the responsible mutation is the substitution (G2262 to A) in *BSK1*, causing an amino acid change (Glu395 to Lys) at the linker region between the kinase domain and the tetratricopeptide repeat (TPR; Figure 3C).

To validate the above results, we transformed *jzt* with a wild-type genomic *BSK1* fragment, including its putative promoter region, and investigated whether the transgene complements the phenotypes in *jzt*. Although the dwarf phenotype was not fully restored in the transgenic lines (Figure 4A), they exhibited reduced stomatal index and density compared to *jzt* (Figures 4B–D). Therefore, the defect in stomatal development in *jzt* is most likely to be caused by the novel mutation in *BSK1*.

The novel mutation in *BSK1* exhibits semi-dominant features

BSKs exhibit functional redundancy, and only the *bsk3-1* mutant shows insensitivity to exogenous BR treatment (Tang et al., 2008; Sreeramulu et al., 2013). A recent study indicated that a double knock-out of *BSK1* and its homolog *BSK2* is required to cause the defects in stomatal development similar to those observed in *jzt* (Neu et al., 2019). Consistent with these studies, we could not observe the high stomatal density phenotype in the T-DNA insertion *bsk1* mutant (Supplementary Figure S2). These results suggest that the novel mutation in *BSK1* identified in this study does not simply result in the functional loss of *BSK1* protein. Then, we hypothesized that the mutation is a dominant allele rather than a recessive loss-of-function allele. To test this, we reanalyzed the F_2 population obtained by crossing



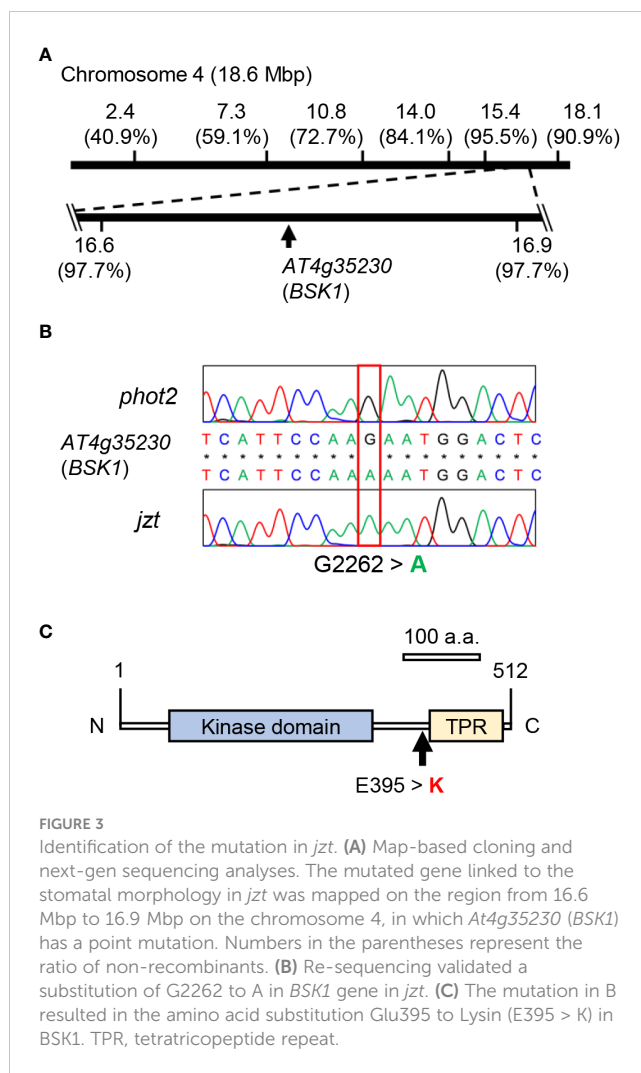


jzt and *phot2* to quantify the stomatal phenotype in each plant in the population. We confirmed that a quarter of the F_2 plants exhibited an extremely high stomatal density like *jzt* (> 400 stomata mm^{-2}). Another quarter of the population showed *phot2*-like phenotype, where the stomatal density was below 150 stomata mm^{-2} . Interestingly, we found that about half of the F_2 plants showed partially increased stomatal density (150–230 stomata mm^{-2}). The segregation ratio of “*phot2*-like”: “intermediate”: “*jzt*-like” was fitted to the well-known 1: 2: 1 autosomal semi-dominant mode of inheritance (Figure 5). These results indicate that the novel mutation observed in BSK1 (hereafter referred to *bsk1-4D*) is a semi-dominant allele and thus capable of causing morphological defects by itself.

Discussion

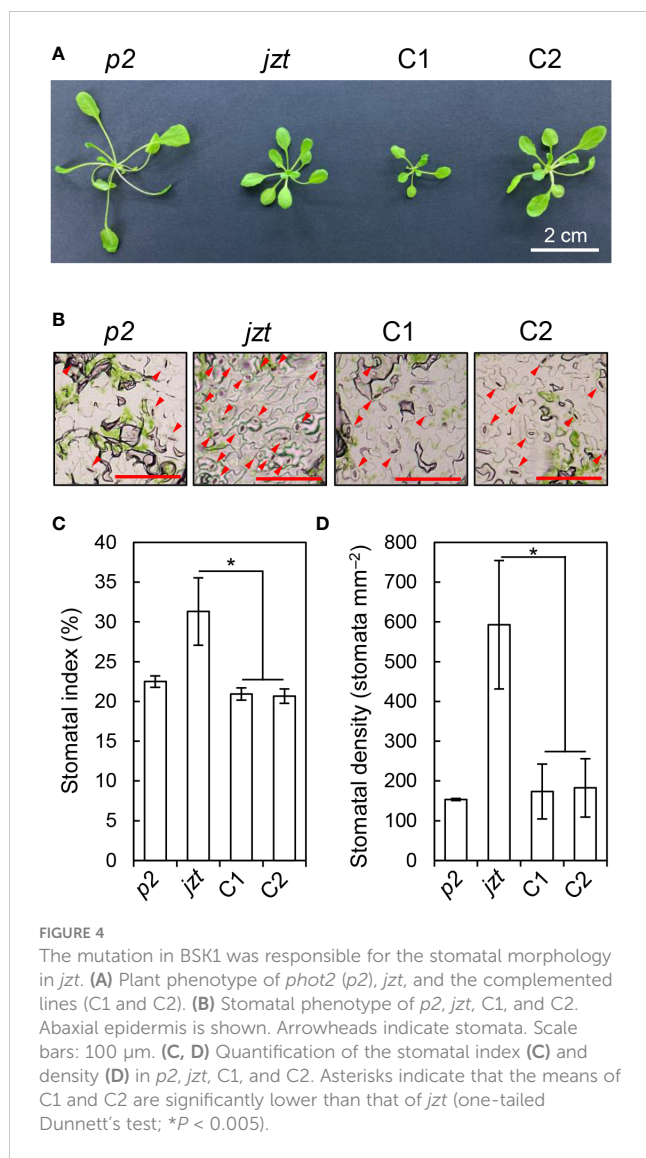
Application of the immunohistochemical detection of PM H^+ -ATPase in leaves

The genetic screening conducted in this study is characterized by the direct observation of stomata. Although similar experiments can be found in literature studying guard-cell differentiation (Pillitteri



et al., 2007), most previous studies on stomatal physiology have examined phenotypes that indirectly represent plant transpiration through stomata. For example, changes in leaf surface temperature or leaf weight serves as an index for water loss through transpiration (Merlot et al., 2002; Hashimoto et al., 2006; Negi et al., 2008; Tsuzuki et al., 2011; Takemiya et al., 2013a; Tomiyama et al., 2014; Yamauchi et al., 2016). The use of these phenotypes in genetic screening enables the simultaneous handling of multiple samples. On the other hand, immunohistochemical experiment appears to be unsuitable for handling many samples at once, as it involved various treatments with chemical solution as well as washing materials. Recently, however, commercial liquid-handling robots have enabled the automation of immunohistochemistry or *in situ* hybridization experiments (Friml et al., 2003; Matsuzaki et al., 2010). Here, we also demonstrated that the immunohistochemical detection of guard-cell PM H^+ -ATPase can be semi-automated by the robot. Genetic screening, taking advantage of the liquid-handling robot, is ongoing to isolate mutants that exhibit defects in the light-induced phosphorylation of guard-cell PM H^+ -ATPase, which will be reported in a future paper.

Previous techniques for detecting guard-cell PM H^+ -ATPase relied on the isolation of epidermal tissues containing guard cells,



and thus, the application is restricted by the availability of epidermis from the plants (Ueno et al., 2005; Hayashi et al., 2011). In this context, the immunohistochemical technique using leaves was expected to enable us to conduct experiments such as genetic screening, where we have to handle individual, and sometimes dwarf, leaves (Ando and Kinoshita, 2018). Successful phenotyping by the immunohistochemistry using a single leaf even in dwarf plants like *jzt/bsk1-4D*, demonstrates that our technique has a broad range of application. Thus, it would enable various genetic investigations of guard-cell PM H⁺-ATPase in plants with small and/or few leaves like early-flowering mutants (Kinoshita et al., 2011).

Novel dominant mutation in BSK1

In this study, we identified a novel semi-dominant mutation in *BSK1* that causes morphological defects, including the overproduction of stomata. Incomplete complementation of the

dwarf phenotype in *jzt/bsk1-4D* by genomic BSK1 may support that the mutation is a semi-dominant allele (Figure 4A). The BSK family genes exhibit functional redundancy, and simultaneous knock-out of BSK genes is required to induce the morphological defects (Sreeramulu et al., 2013; Neu et al., 2019). BSK1 is considered one of the substrates of BRI1 in BR signaling (Tang et al., 2008). Crosstalk exists between leucine-rich-repeat receptor-like kinases (LRR RLKs), including BRI1, which regulates the plant growth, development, and innate immunity (Zhu et al., 2013). Previously, the *bsk1-1* mutation was identified as a suppressor mutation for the powdery mildew resistance phenotype in *ENHANCED DISEASE RESISTANCE2*, and BSK1 was shown to associate with FLAGELLIN SENSING2 (FLS2), another LRR RLK that functions in the immune response (Shi et al., 2013). In contrast to the mutation in *jzt/bsk1-4D*, *bsk1-1* is a recessive allele that causes a missense mutation in the TPR domain of BSK1 (Shi et al., 2013). TPR was originally identified in yeast and has been considered to be involved in protein-protein interaction (Hirano et al., 1990; Sikorski et al., 1990; Blatch and Lässle, 1999). As *bsk1-1* does not affect the BSK1-FLS2 interaction itself, a functional defect other than the protein interaction may be caused by the *bsk1-1* mutation (Shi et al., 2013). *jzt/bsk1-4D* is located in the C-terminal region of the linker between the kinase domain and TPR (Figure 3C). Previous reports and the present results imply that the C-terminal region of BSK1, including Glu395 might function in the regulation of or interaction with protein(s) that regulates guard-cell differentiation. The putative target of BSK1 may include other BSK family proteins. Sreeramulu et al. (2013) indicated that loss-of-function of BSK1 restores the insensitivity to 24-epibrassinolide in *bsk3,4,6,7* mutant, suggesting an antagonistic interaction between BSK1 and other BSK family proteins. It would be interesting to investigate the stomatal phenotype in higher-order *bsk* mutants, including *jzt/bsk1-4D* mutation. The *jzt/bsk1-4D* mutation identified in this study would be a beneficial tool to uncover the protein function of BSK1 in the plant development.

Perspective

As the phosphorylation of guard-cell PM H⁺-ATPase was detected in *jzt/bsk1-4D* (Figure 1B), BSK1 may not be necessarily required for light-induced phosphorylation of PM H⁺-ATPase in guard cells. Given that BR signaling has pleiotropic functions (Zhu et al., 2013); however, BSK1 might function in other stomatal response, i.e. immunity (Zeng et al., 2010). Stomatal closure is a part of the immune response to restrict bacterial invasion, whereas the pathogenic bacteria have a mechanism for stomatal reopening to achieve their infection (Melotto et al., 2006). Bacterial invasion also induces a systemic reduction of stomatal density in new leaves emerged after the inoculation (Dutton et al., 2019). Previous studies have revealed a significant role of PM H⁺-ATPase in the plant-pathogen interaction including stomatal movement, in which RPM1-interacting protein 4 (RIN4) function as PM H⁺-ATPase activator for stomatal reopening (Liu et al., 2009; Elmore and

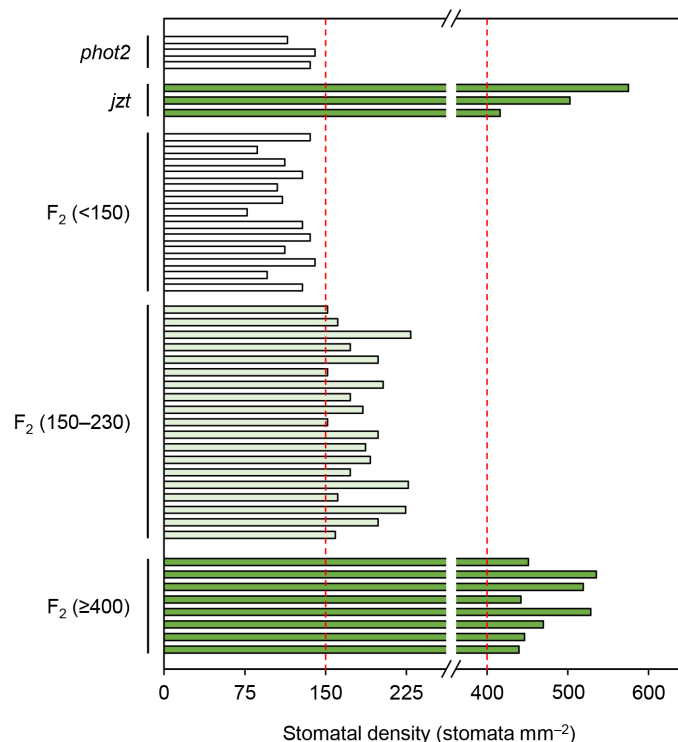


FIGURE 5

The substitution in *BSK1* in *jzt* was semi-dominant. Stomatal density of the F_2 population ($n = 40$ plants) obtained from the crossing of *phot2* and *jzt* were analyzed. Three phenotypes were observed: *phot2*-like (stomatal density < 150 stomata mm^{-2}), *jzt*-like (≥ 400 stomata mm^{-2}), and intermediate ($150 - 230$ stomata mm^{-2}). The segregation ratio was 13: 19: 8, which was fitted to the 1: 2: 1 segregation (chi-squared test, $\chi^2 = 1.35$, $P = 0.51$).

Coaker, 2011). RIN4 is a putative phosphorylation target of FLS2, which is likely to be regulated by BSK1 as described above (Shi et al., 2013; Ray et al., 2019). It is noteworthy that BR induces the phosphorylation of PM H^+ -ATPase in the hypocotyl of etiolated seedlings (Minami et al., 2019). These results imply a functional connection between BSK1 and the stomatal immune response. The *jzt/bsk1-4D* mutant would also be a useful genetic resource to investigate this hypothesis.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding authors.

Author contributions

EA: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing. KT: Investigation, Writing – original draft. TS: Investigation, Writing – original draft, Methodology,

Software. TK: Writing – original draft, Investigation, Methodology, Software, Conceptualization, Data curation, Formal analysis, Funding acquisition, Project administration, Resources, Supervision, Validation, Visualization, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fpls.2024.1377352/full#supplementary-material>

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