AtSYP71 Is Important for Plant Cell Wall Biosynthesis and Stress Adaptation.

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Research Article

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Abstract

Background: SYP71, the plant-specific Qc-SNARE protein, is reported to regulate vesicle trafficking. SYP71 is localized on the ER, endosome, plasma membrane and cell plate, suggesting its multiple functions. *Lotus* SYP71 is essential for symbiotic nitrogen fixation in nodules. AtSYP71, GmSYP71 and OsSYP71 are implicated in plant resistance to pathogenesis. To date, SYP71 regulatory role on plant development remain unclear.

Results: *AtSYP71*-knockout mutant *atsyp71-4* was lethal at early development stage. Early development of *AtSYP71*-knockdown mutant *atsyp71-2* was delayed, and stress response was also affected. Confocal images revealed that protein secretion was blocked in *atsyp71-2*. Transcriptomic analysis indicated that metabolism, response to environmental stimuli pathways and apoplast components were influenced in *atsyp71-2*. Moreover, the contents of lignin, cellulose and flavonoids as well as cell wall structures were also altered.

Conclusion: Our findings suggested that AtSYP71 is essential for plant development. AtSYP71 probably regulates plant development, metabolism and environmental adaptation by affecting cell wall homeostasis via mediating secretion of materials and regulators required for cell wall biosynthesis and dynamics.

Introduction

Higher plants have developed unique structural characters to deal with environmental changes. Cell wall is an important feature of plant cells which provides cell shapes and strength [1]. Cell wall is a complex extracellular matrix containing cellulose, hemicellulose, pectin polysaccharide, lignin and a small amount of glycoproteins [2]. It plays a variety of essential functions on cell adhesion, cell morphogenesis, material exchange, information transmission, defense and stress tolerance, as well as plant upright growth [3–7]. Plant cell walls are usually divided into two categories: the primary cell walls (PCWs) and secondary cell walls (SCWs) [1].

In *Arabidopsis*, primary cell wall is formed right after cell division and consists of cellulose microfibrils cross-linked by xyloglucan (XyG), and embedded in pectin matrix [8–10]. The secondary cell wall is deposited after cell differentiation and consists of cellulose, hemicellulose and lignin [11]. The content of XyG is the highest in hemicellulose in PCWs and is minor in SCWs [5, 10–12]. XyG turnover is mediated by endotransglucosylases/hydrolases and influences cell elongation, cell wall strength and remodeling [5, 13–16]. In addition, cell wall-specific proteins (e.g. extensins, expansins, hydroxyproline-/glycine-rich proteins and glycoproteins), polysaccharide (e.g. callose) and polyphenols are related to cell wall functions and dynamics [13, 17–21]. Cellulose and hemicellulose are major components in both PCWs and SCWs. Hemicelluloses synthesized at the Golgi apparatus cross link cellulose microfibrils to form a network in cell wall [10, 22–24]. While, pectins are mainly distributed in PCWs, and are involved in regulation of growth and development and response to environment stimuli [18].

Cellulose is synthesized at the plasma membrane (PM) by cellulose synthase (CesA) complexes (CSCs). The CesAs are synthesized in the ER, and assembled into CSCs at the Golgi apparatus [2, 8, 18, 25–28]. PM-targeting and insertion of the CSCs are regulated by various factors, such as PATROL1, CSI1/POM2, CC, STELLO1/2, TRANVIA and the exocyst complex [9, 25, 26, 29–31]. CSC-motility at the PM might be dependent on the PM components, and the CSCs average lifetime is 10-20 min, suggesting a frequent material exchange between intracellular and extracellular spaces [32–34]. The material communication spanned the PM is important for cell wall architecture and remodeling [18, 35, 36]. The cell wall-PM connection via cellulose fibrils and transmembrane proteins is essential for PM-localized protein homeostasis, cell wall biosynthesis and remodeling, as well as signal transduction [37–43].

Lignin is important for cell wall formation [44]. It contributes mechanical strength and rigidity for plants and facilitates water transport through the vascular system. Lignin acts as a physical barrier against biotic and abiotic stress and plays essential roles on plant growth and development [45–50]. As a heteropolymer of monolignols, lignin is synthesized primarily by the General Phenylpropanoid Pathway (GPP), which is the main synthetic pathway for plant secondary metabolites including flavonoids, phenolic acids and stilbenes in addition to monolignols [51].

Monolignols are synthesized in the cytoplasm, or in close to the ER [52, 53], then exported to the cell wall by PM-localized transporters such as ATP binding cassette (ABC) transporters [54–57]. The secreted monolignols could diffuse readily in both PCWs and SCWs for active lignification [52, 58, 59]. The laccases and peroxidases are glycoproteins and essential for lignification [52, 60–62]. Protein glycosylation initiates in the ER lumen, and is completed in the Golgi cisternae [13]. After glycosylation, these oxidative enzymes are exported to the SCW domains for lignification [58]. Lignification initiates while the SCW are still deposited, meaning that laccases and peroxidases are secreted at the same time as hemicelluloses and CSCs do [63–65].

Flavonoids are broadly distributed in plants, which is essential for plant development and defense [66, 67]. Flavonoid compounds are also involved in legume nodulation, auxin transport and male fertility. Moreover, Flavonoids have high application value in nutrition and pharmaceuticals [68]. In plant cells, flavonoids are mainly synthesized in the cytoplasmic face of the endoplasmic reticulum (ER) and then uploaded into the ER lumen [66, 69, 70]. Flavonoids are transported by membrane transporter-mediated transport (MTT) and membrane vesicle-mediated transport (MVT) machineries [67, 68, 70, 71]. Diverse trafficking routes are proposed for flavonoid vacuolar sequestration or secretion to the apoplast [66, 70, 72]. The PM-localized flavonoid transporters are mainly bilitranslocase-like (BTL-like) protein and ATP-binding cassette (ABC) transporters [52, 65, 66, 73]. Both of them are involved in isoflavonoid transport to the apoplast for plant–microbe and symbiotic interactions [66, 70, 74]. SNARE-mediated exocytosis pathways or nondirectional secretion are also hypothesized to mediate in the efflux of flavonoids into the extracellular space [69]. Flavonoids are also proposed a long distance transport, i.e. travel from synthesis sites to other plant organs [65, 66, 75–77], suggesting that flavonoid distribution is an active process possibly mediated by MRP/ABCC-type ABC transporters [67, 77, 78]. PM-localized ABC transporters may transport the tapetosome flavonoids to the pollen surface. This is similar to ABCG10, which secretes

isoflavonoids to the apoplast for plant defense [79]. ABCG1 and ABCG16 are required for suberin and pollen wall extracellular barrier formation [70, 73, 80, 81].

The Golgi apparatus contains many types of glycosyltransferases, substrate transporters and other Golgi-resident proteins important for hemicellulose biosynthesis, assembly and secretion [10, 44, 82]. Moreover, the Golgi apparatus is responsible for modification, sorting and transport of the proteins required for cellulose and lignin biosynthesis at the PM, such as laccases, peroxidases and CSCs [10, 24, 83–85]. Thus, cell wall deposition requires increase of number of Golgi apparatus to serve the needs of an increase in Golgi-residents and cargoes [44, 86, 87]. At the trans-Golgi network (TGN), hemicelluloses and secreted proteins are sorted into transport vesicles and targeted to the PM [88]. The TGN is a sorting hub for both exocytosis and endocytosis [88, 89]. At the TGN, the endocytic vesicles are either re-secreted to the PM or targeted to the vacuoles for degradation [90]. The TGN-PM cycling pathway facilitates membrane components such as lipids and transport machinery being recycled. On the other hand, the cycling and vacuolar degradation pathways coordinately fine regulate homeostasis of the PM-localized proteins, especially under environmental stimuli [11, 18]. The small Cesa compartments (SmaCCs) derived from the Golgi deliver CSCs to the PM during PCW and SCW deposition [33, 91]. SmaCCs are also implicated in CesAs endocytosis and cycling between the TGN and PM [9, 11, 25, 33, 92, 93].

Higher plants have developed a unique vesicle trafficking system to conduct material transport to meet the needs of various cellular activities. Vesicle trafficking carries out intracellular and intercellular material communication and signal transmission [94]. The process of vesicle trafficking includes budding, directional movement, tethering, anchoring and membrane fusion [94–96], and each step is regulated by many factors, such as coatomer, SM, tether, SNARE and Rab proteins [97].

SNARE (soluble N-ethylmaleimide-sensitive factor attachment protein receptor) proteins highly conserved in yeast, mammals and plants usually contain an N-terminal region, a SNARE motif and a transmembrane domain. SNAREs play an important role in fusion of vesicular membrane with the target membrane [98, 99]. According to the conserved amino acids, glutamine or arginine residues, in core of SNARE domain, SNARE proteins are divided into Q-SNAREs (target membrane SNAREs, t-SNAREs) and R-SNAREs (vesicle SNAREs, v-SNAREs) [100]. Q-SNAREs include Qa-SNARE/syntaxin, Qb-SNARE and Qc-SNARE. The three types of Q-SNAREs combine with R-SNARE to form trans-SNARE complex to facilitate membrane fusion [101].

SYP71 is a plant-specific Qc-SNARE [102]. SYP71 is localized on the ER, plasma membrane [103–107], endosome and cell plate [79, 108]. AtSYP71 is proposed to be involved in multiple membrane fusion steps during the secretion process in *Arabidopsis* [109]. Moreover, AtSYP71 coordinates with Qa-SNARE KNOLLE, Qb-SNARE NPSN11 and R-SNAREs VAMP721/722 to mediate membrane fusion in cell plate during cytokinesis [98, 104, 108]. It is reported that *Lotus* LjSYP71 is essential for symbiotic nitrogen fixation in nodules [110]. SYP71 has also been shown to be implicated in plant resistance to biotic stresses. As a component of membrane fusion regulon, SYP71 and partners participate in the defense process in root cells in *Glycine max* [79, 110, 111]. Overexpression of *OsSYP71* enhances rice resistance

to rice blast and tolerance to oxidative stress [108]. In wheat, TaSYP71 regulates resistance to biotrophic obligate fungi *Pst* [112]. *Arabidopsis* SYP71 can be subverted during TuMV infection to facilitate viral pathogenicity by mediating the fusion of the virus-induced 6K2 vesicles with chloroplasts [111]. However, SYP71 effect on cell wall synthesis is elusive.

Here, we report that AtSYP71 regulated secretion pathway. The secretion of the marker, SecGFP, was severely affected in *atsyp71-2* mutant. Knock-down of *AtSYP71* disturbed plant early development. Transcriptomic analysis indicated that metabolism, response to environmental stimuli and stresses pathways and cell wall components were influenced in *atsyp71-2* mutant. Moreover, the contents of lignin, cellulose and flavonoids as well as cell wall structures were also altered. The response of *atsyp71-2* to abiotic stress were abnormal. Our findings suggested that AtSYP71 affected plant development, metabolism and environmental adaptation via regulation on vesicle trafficking.

Results

AtSYP71 is essential for plant growth and development

AtSYP71 gene is composed of nine exons and eight introns (Fig. 1a). To investigate its function, four T-DNA insertion mutants were isolated. In *atsyp71-1*, T-DNA was inserted into 5'-UTR region. In *atsyp71-2* and *atsyp71-3*, T-DNA cassettes were inserted in different sites in the first intron. In *atsyp71-4*, T-DNA was inserted into the seventh intron. RT-qPCR determination indicated that *AtSYP71* expression was downregulated by 30% in *atsyp71-1*, by 70% in *atsyp71-2*, by 40% in *atsyp71-3*, and was depleted in *atsyp71-4* (Fig. 1b). To confirm AtSYP71 protein accumulation in the mutants, we generated anti-AtSYP71 antibodies and performed the western blot analysis. In *atsyp71-1*, AtSYP71 protein accumulation decreased slightly. In *atsyp71-2* and *atsyp71-3*, AtSYP71 protein accumulation decreased dramatically. In *atsyp71-4*, there was no AtSYP71 protein accumulated (Fig. 1c). The root length of seedlings of all the *atsyp71* mutants shortened. The greater the *AtSYP71* expression decreased, the shorter the root length. In *atsyp71-1*, the root length of seven-day-old seedlings was slightly shorter than that of wild type, while the root length of *atsyp71-2* and *atsyp71-3* seedlings were significantly shorter than that of wild type (Fig. 1d, 1f). *atsyp71-4* seeds could germinate, but the roots were not able to elongate, and the cotyledon margin sometimes was incomplete. In most cases, the true leaves could occur, but were very small, and soon turned yellow and vitrified and stop developing, and most of the seedlings died when over seven-day old (Fig. 1e). *atsyp71-1* plants had no obvious growth and development phenotype, whereas bolting of *atsyp71-2* and *atsyp71-3* plants was delayed, and the five-week-old plants were much shorter than those of wild type (Fig. 1g). However, there was no significant difference in the height of *atsyp71* adult (60-day-old) plants (Fig. S1a), suggesting AtSYP71 was essential for morphogenesis and early development. The complemented lines of *atsyp71-2* (*atsyp71-2 com*) and *atsyp71-4* (*atsyp71-4 com*) were generated by introducing *pAtSYP71::GFP-AtSYP71* cassette into *atsyp71-2* or *atsyp71-4* by crossing, respectively. The phenotypes of the complemented lines were restored (Fig. 1c, 1d, 1f, 1g), indicating that *AtSYP71* was the causal gene of the phenotypes of *atsyp71* mutants. The above results suggested that AtSYP71 plays an essential role in regulating plant growth

and development. Since *atsyp71-2* and *atsyp71-3* had similar phenotypes, we used *atsyp71-2* for subsequent analysis.

Secretion was disturbed in *atsyp71-2* mutant

It has been reported that AtSYP71 is localized on the ER, endosome, plasma membrane and cell plate [79, 104, 108, 112–114], suggesting its multiple functions. AtSYP71 associates with Qa-SNARE KNOLLE, Qb-SNARE NPSN11, and R-SNARE VAMP721 or VAMP722 to form a tetrameric endomembrane type SNARE complex to regulate cytokinesis [114]. But the AtSYP71-partners on the ER are not identified yet. Thus, we performed yeast two hybrid (Y2H) analysis and detected the interactions of AtSYP71 with the ER-localized Qa-SNARE AtSYP81 and Qb-SNARE AtSec20, respectively (Fig. 2a). We reported previously that AtSYP81 and AtSec20 interact with ER-localized MAG2 which forms a tethering complex with MIP1, MIP2 and MIP3 [115]. Thus, we also detected interactions between these proteins and found that AtSYP71 interacted with MIP1, MIP2 and MIP3, respectively; AtSYP81 interacted with MAG2, MIP1, MIP2 and MIP3, respectively; and AtSec20 interacted with MAG2, MIP1 and MIP2, respectively (Fig. 2a). To further confirm the interaction between AtSYP71 and these factors, we performed pull down experiment using myc-AtSYP71-overexpressing (AtSYP71/OE) plants followed by Shotgun LC-MS/MS analysis. As expected, we identified MAG2, MIP1 and MIP3 (Fig. 2b). Then, we performed pull down analysis using seven-day-old seedlings of myc-AtSYP71/OE and GFP-AtSYP71/*atsyp71-2* lines. Immunoblot analysis with anti-AtSYP81 antibodies detected AtSYP81 in both of the pull down elution (Fig. 2c). These results suggested that AtSYP71 might combine with AtSYP81 and AtSec20 to form a SNARE complex, and coordinate with the MAG2 tethering complex to mediate Golgi-to-ER vesicle transport.

To clarify effects of *AtSYP71* down-regulation on vesicle trafficking, we analyzed the secretion using the marker SecGFP [116, 117]. Confocal images indicated that in *atsyp71-2* root cells, SecGFP displayed reticular-like structures (Fig. 2e, 2f, yellow arrows) which were not observed in wild type (Fig. 2d). This result suggested that AtSYP71 plays essential role on secretion pathway. Then, we detected accumulation of precursors of seed storage proteins (SSPs) which is considered as a marker for ER-to-vacuolar transport [115, 118–121]. As shown in Fig. S1b, there was no detectable SSP precursors accumulated in *atsyp71-2* seeds, indicating that the ER-to-vacuole pathway was not affected seriously. It is known that when ER export is severely blocked, numerous newly synthesized proteins are trapped inside the ER and induce ER stress [115, 121, 122]. We then determined the expression of *BiP3* which is considered as an ER stress-specific marker. The results indicated that *BiP3* transcriptional level was not significantly altered in *atsyp71-2* (Fig. S1c), suggesting that no severe ER stress was induced, meaning the ER export was not affected seriously. These might because of the complementation from the two AtSYP71 homologues, AtSYP72 and AtSYP73. These results suggested that AtSYP71 probably regulates Golgi-to-ER retrograde pathway timely and spatially.

Transcriptome analysis of *atsyp71-2* mutant

In order to explore the regulatory mechanism of *AtSYP71* on plant growth and development, we performed transcriptome analysis of wild-type and *atsyp71-2* seedlings. The sequencing obtained 34,524

and 33,113 reliable clean reads above 100 bp in wild type and *atsyp71-2*, respectively. After compared with tophat2 software, 87.68% and 86.59% of clean reads can be matched to the reference genome sequence, respectively. Statistical evaluation of sequencing quality value indicated that base Q30% (i.e. the proportion of sequencing error rate less than 0.1%) was 92.37% and 92.16%, respectively (Fig. 3a), illustrating the high quality of transcriptome sequencing and reliable original data for subsequent analysis. Volcano map showed the overall distribution of the differentially expressed genes (DEGs), and totally 165 DEGs were screened. Red dots represented 69 up-regulated DEGs, blue dots represented 96 down regulated DEGs, and green dots represented genes with no significant difference (Fig. 3b).

GO (Gene Ontology) enrichment analysis indicated that the DEGs were mainly concentrated in three aspects: biological process, cellular components and molecular function (Fig. 3c). The biological processes included 26 pathways mainly involved in biotic and abiotic stress, stimuli and defense responses, metabolism and secondary metabolism, and most of DEGs were enriched in stimuli and stress response processes. In cellular components, DEGs were mainly concentrated in cell wall and external encapsulating structure. And in molecular function, DEGs were mainly concentrated in tetrapyrrole binding and heme binding. KEGG (Kyoto Encyclopedia of Genes and Genomes) Enrichment analysis indicated that DEGs were mainly enriched in Biosynthesis of secondary metabolites (20 DEGs), Metabolic pathways (20 DEGs), Glucosinolate Biosynthesis (10 DEGs), 2-Oxocarboxylic acid metabolism (10 DEGs), and Phenylpropanoid biosynthesis (10 DEGs), etc. (Fig. 3d). Above results indicated that down regulation of *AtSYP71* resulted in significant changes in plant stress response, defense, biosynthesis and metabolism.

The expression of cell wall-relating genes were affected significantly in *atsyp71-2*

Go enrichment analysis of the genes in Cellular component (GO:0005575) indicated that DEGs were concentrated in the Extracellular region (GO:0005576), External encapsulating structure (GO:0030312), Cell wall (GO:0005618) and Plant-type cell wall (GO:0009505) (Fig. 4a, colored frames). In these DEGs, we found expression of nine *PRX* family genes (i.e. *PRX4*, *PRX15*, *PRX25*, *PRX37*, *PRX49*, *PRX52*, *PRX53*, *PRX54* and *PRX62*) and one β -D-glucosidase encoding gene, *BGLU25*, were significantly decreased (Fig. 4b). We confirmed the transcriptional levels of these genes by RT-qPCR and the results were consistent with the transcriptomic data (Fig. 4c).

The nine *PRX* genes were in the Extracellular region (GO:0005576) components (Fig. 4A, orange frame). Meanwhile, *PRX15*, *PRX25*, *PRX49*, *PRX52* and *PRX62* were also in the Cell wall (GO:0005618) components belonging to the External encapsulating structure (GO:0030312) (Fig. 4a, red frames); and *PRX25* and *PRX62* were in the Plant-type cell wall (GO:0009505) components (Fig. 4a, apricot yellow frame). *PRX* family proteins which belong to plant specific Type III peroxidases (C₁ Prxs) are involved in auxin metabolism, lignin and suberin biosynthesis, cross-linking of cell wall components, phytoalexin synthesis, and the metabolism of reactive oxygen species (ROS) and reactive nitrogen species (RNS) [123] and are closely related to cell wall dynamics such as cell wall loosening and strengthening [124]. The alteration of expression of the *PRX* genes might affect cell wall biosynthesis.

The β -D-glucosidase BGLU25 functions on Phenylpropanoid biosynthesis pathway which is closely related to the synthesis of lignin, cellulose and flavonoids *etc.* [51]. Moreover, in the Cell wall (GO:0005618) and Extracellular region (GO:0005576) components, expression of some flavonoid and cell wall biosynthesis enzyme-encoding genes were also altered. For example, *LTPG1* (glycosylphosphatidylinositol-anchored lipid protein transfer 1), *LTP2* (lipid transfer protein 2), *LTP5*, *EXPA1* (expansin A1), *EXPA4*, *BBE19/OGOX1* (FAD-binding Berberine Bridge Enzyme 19/oligogalacturonide oxidase 1), *BBE26*, *BBE11*, *XTH20* (xyloglucan endotransglucosylase/hydrolase 20), *XTH22*, *AT4G01700* (Chitinase family protein), *AT5G18470* (Curculin-like lectin family protein), *GSTF6* (glutathione s-transferase 6), *CYP706A1* (cytochrome p450 family 706, subfamily A, polypeptide 1), and *PP2-B13* (PHLOEM PROTEIN 2-B13), were decreased; whereas *BGAL8* (beta-galactosidase 8), *SCPL12* (serine carboxypeptidase-like 12), *JAL22* (jacalin-related lectin 22) and *AT3G16530* (Lectin like protein) were increased in the mutant (Fig. 4d, 4e). Among these genes, *GSTF6*, *CYP706A1*, *BGLU25* and *SCPL12* regulate flavonoid biosynthesis, and *BGAL8*, *EXPAs*, *XTHs*, *LTPs*, *OGOX1*, *ABCG1*, *ATPP2-B13* and *AT4G01700*, participate in cell wall biosynthesis and dynamics. Furthermore, in the Biological process of GO Terms, expression of some transcription factors (TFs) related to cell wall formation and response to stimuli and stress were altered. For example, *WRKY18*, *WRKY46*, *WRKY48*, *WRKY51* and *MYB15* were decreased, whereas *HRS1* was increased (Fig. 4f). MYB15 plays a central role on pathogen-induced Lignification [125], and WRKY18 and WRKY53 coordinate with HAC1 to regulate responses to sugars which are structural components of the cell wall [126, 127]. We confirmed the expression of these TFs by RT-qPCR. Except for *MYB15*, change trends of all other TF genes were consistent with the transcriptomic data (Fig. 4g). Above results suggested that *atsyp71-2* defects probably affected plant development and response to stress via affecting cell wall formation and metabolism.

The cell wall components and structures were affected in *atsyp71-2* mutant

Since expression of many cell wall- and Phenylpropanoid biosynthesis pathway-related genes were altered, we determined the contents of cell wall components and flavonoids in *atsyp71-2* plants. Our results indicated that the contents of lignin, cellulose and flavonoids in *atsyp71-2* roots and stems were significantly lower than those in wild type (Fig. 5).

To figure out effects of decrease of lignin and cellulose on cell wall structure, we observed the caulome. The stem cross sections showed that number of xylem and phloem in *atsyp71-2* was less than that in wild type, and the size of each xylem and phloem was uneven and the distribution was irregular (Fig. 6a). The statistical results indicated that total area of xylem in *atsyp71-2* was significantly less than that in wild type (Fig. 6c). Moreover, structure of xylem in *atsyp71-2* was incomplete, and alignment of the cells was irregular (Fig. 6b, orange dotted frame). Moreover, in epidermis, cortex, phloem and medullary cells in *atsyp71-2*, less inclusions mainly composed of cytoplasm and cellulose were accumulated (ochre arrows) compared with that in wild type. Another remarkable difference was that the degree of lignification of cell walls of interfascicular fiber and pith cells in *atsyp71-2* stem decreased significantly (red and orange arrows). The statistical results indicated that the thickness of cell wall of interfascicular fiber cells was significantly thinner than that of wild type (Fig. 6d). These component and structural

changes of cell wall in *atsyp71-2* might greatly affect the hardness and tenacity of stems and the cell functions, and subsequently affect the plant growth and development.

Stress Response of *atsyp71-2* was altered

Since most of DEGs in transcriptome of *atsyp71-2* mutant were related to environmental stimuli and stress responses, we firstly detected the activities of peroxidases. The activities of superoxide catalase (CAT) and peroxidase (POD) were significantly increased (Fig. 7a, 7b), and the activities of superoxide dismutase (SOD) and ascorbate peroxidase (APX) decreased significantly in *atsyp71-2* (Fig. 7c, 7d). Then, we performed salt, drought and H₂O₂ treatments. Under 120 mM NaCl treatment, root length of *atsyp71-2* only reduced by 5% compared with 34% and 33% decline of that of wild type and *AtSYP71/OE*, respectively (Fig. 7e-7g). Under 75 µM H₂O₂ treatment, root length of *atsyp71-2* only reduced by 9.1% compared with 40.4% and 45.8% decline of that of wild-type and *AtSYP71/OE*, respectively (Fig. 7h-7j). Under 150 mM Mannitol treatment, root length of *atsyp71-2* increased by 8.4% compared with 35.3% and 35.5% decline of that of wild type and *AtSYP71/OE*, respectively, respectively (Fig. 7h-7j). These results indicated that response to stress in *atsyp71-2* altered significantly.

Discussion

AtSYP71 is essential for plant growth and development via regulating secretion of cell wall-related proteins and chemical compounds

That AtSYP71 is a Qc-SNARE with multi-localization on the ER, endosome, PM and cell plate, which are right on the secretion and cycling pathways [79, 104, 108–114], suggests its diverse functions in plant growth and development. AtSYP71 is proposed to be involved in multiple membrane fusion steps during the secretion process in *Arabidopsis* [109]. It is also clarified that AtSYP71 binds with Qa-KNOLLE, Qb-SNARE NPSN11 and R-SNAREs VAMP721 and VAMP722 to form a SNARE complex to regulate cytokinesis [114]. Deficient of AtSYP71 definitely affected AtSYP71-dependent vesicle trafficking and localized organelle functions. The knock-out mutant *atsyp71-4* could germinate, but the roots could not elongate, the leaves were small and the seedlings died soon (Fig. 1C). These phenotypes resembled *syp71^{amiR}*-mutant and *npsn11 syp71^{amiR}*-double mutant which were cytokinesis defective [114]. This might because that depletion of *AtSYP71* seriously disrupted delivery of materials necessary for cell plate formation and the plasma membrane integrity and lead to failure of morphogenesis and subsequent lethality at early development stage. Even the knock-down mutants, *atsyp71-2* and *atsyp71-3* also displayed obvious defects on plant growth and development. On the other hand, Lotus LjSYP71 plays an important role in symbiotic nitrogen fixation, but the *Ljsyp71* mutant grows similarly to wild-type plant when supplied with combined nitrogen. OsSYP71 and TaSYP71 are involved in plant resistance to pathogens, but the mutants didn't show obvious phenotypes under normal growth conditions [108, 114]. These suggested that LjSYP71, OsSYP71 and TaSYP71 are not essential for plant growth and development. This probably because that SYP71 orthologues in different species gain functional division during evolutionary process.

Laccases and peroxidases (i.g. PRXs) required for lignin biosynthesis are glycosylproteins [13, 22, 53]. The ER and the Golgi apparatus are responsible for protein glycosylation. Thus, post transcriptional modification of laccases and peroxidases as well as other enzymes were probably disrupted in *atsyp71* mutants. Secretion, cycling and homeostasis of PM-localized proteins and their motility in the PM depend on the PM components [32–34]. For the above reasons, it is easy to conjecture that secretion, homeostasis of PM and PM-localized proteins were perturbed and subsequently affected expression of many cell wall-related proteins in *atsyp71-2* mutant. For example, *AT4G01700*-encoding protein belongs to Chitinase family which is related to cellulose biosynthesis and cell wall remodeling [128]. The EXPA proteins are responsible for controlling cell wall extension and developmental processes, including cell dissociation and separation [129–132]. LTP1 and LTP2 belong to a family of lipid transfer proteins, and LTP2 plays a role in maintaining the integrity of the cuticle-cell wall interface [133]. LTPG1, a PM-localized lipid transfer protein, regulates cuticular lipid composition [134, 135]. BBE enzymes catalyze the biosynthesis of the secondary metabolites, isoquinoline alkaloids [136]. BBE19/OGOX1, an oligogalacturonide (OG) oxidase, is involved in plant immunity. OGs, a major component of pectin, are a well-known class of damage-associated molecular patterns (DAMPs) that activate immunity and protect plants against microbes [137]. XTH22 is a cell wall-modifying enzyme and is rapidly upregulated in response to environmental stimuli. XTH20, a putative xyloglucan endotransglycosylase/hydrolase expressed primarily in the main and lateral roots, is involved in cell proliferation in incised inflorescence stems [138–140]. PP2-B13, a phloem protein 2-like protein, belongs to F-box-like domain superfamily. PP2 is component of the phloem protein bodies found in sieve elements. PP2 proteins can directly bind with the chitin cell wall and play important roles in defense against pathogens, photoassimilate transport and wound healing [141–143]. BGLU25 belongs to beta-glucosidase family which is involved in cellulose degradation [72, 144].

Beyond that, biosynthesis or/and secretion of some chemicals may also be affected in *atsyp71* mutants. Hemicelluloses are synthesized at the Golgi apparatus and then delivered to the cell wall by transport vesicles [10, 23, 24]. Release of hemicelluloses is controlled by the process of vesicle fusion to the PM which is regulated by SNARE complex including Qc-SNARE AtSYP71. Thus, secretion of hemicelluloses probably was disrupted.

The contents of monolignols and flavonoids synthesized by the Phenylpropanoid biosynthesis pathway decreased in *atsyp71-2* mutant (Fig. 5). A β -D-glucosidase, BGLU25, functioning on Phenylpropanoid biosynthesis pathway, was also changed (Fig. 4). These suggested that Phenylpropanoid biosynthesis pathway was affected. Monolignols, the materials for lignin biosynthesis, are synthesized in the cytoplasm or in close to the ER [52, 53] and then are secreted by PM-localized transporters such as ABC transporters [54–57].

Flavonoids are synthesized on the ER, uploaded into the ER lumen and then transported to the vacuole and the PM by vesicles [66, 69, 70]. The PM-localized ABC transporters mediated flavonoid efflux and long distance transport [52, 65–67, 70, 73–78]. In *atsyp71-2*, expression of ABCG1, an ABC transporter required for suberin secretion [70, 73, 80, 81] was decreased. Although the relationship between ABCG1

and the transport of flavonoids and monolignols is unclear, it cannot be ruled out that the functions of ABCG1 and other transporters changed and affected the transport of flavonoids and monolignols. Another aspect that cannot be ignored is that the biosynthesis of both flavonoids and monolignols is related to the ER. The effect of deficient of AtSYP71 on the ER will certainly influence their biosynthesis. Further study is needed to clarify mechanism underlying AtSYP71 regulation on relating organelle functions, secretion of proteins and chemical compounds as well as homeostasis of PM-localized proteins.

AtSYP71 is closely related to plant stress responses

Abiotic stresses affect plant growth and development [145–147] and induce high level of reactive oxygen species (ROS) production [148–150]. Plants control ROS formation and scavenging through enzymatic and non-enzymatic antioxidative defense systems [151–153]. enzymatic antioxidative systems include superoxide dismutase (SOD), catalase (CAT), peroxidase (POD), ascorbate peroxidase (APX), glutathione reductase (GR), glutathione peroxidase (GPX), glutathione-S- transferase (GST) as well that regulate ROS homeostasis via participate in reduction process of $O_2^{\cdot -}$ to H_2O_2 [153, 154]. CATs potentially directly dismutate H_2O_2 into H_2O and O_2 and is crucial for ROS detoxification under stress [155]. Peroxidases (PODs) breakdown ROS via converting H_2O_2 into H_2O [155, 156]. PODs can further scavenge peroxides induced by SODs [153, 157, 158]. APX has a higher affinity for H_2O_2 than CAT and POD and is thought to play the most essential role in scavenging ROS under stress [153]. SODs provide the first line of defense against the toxic effects of excessive levels of ROS via catalyzing $O_2^{\cdot -}$ to H_2O_2 and O_2 [159]. SODs have diverse forms containing distinct metal ions such as Fe, Mn or Cu/Zn, and are distributed in the apoplast [160], cytosol, chloroplasts, mitochondria and peroxisomes [161]. Respiratory burst oxidase homologues (RBOHs), the plant NADPH oxidases (NOXs), are localized on the plasma membrane and convert oxygen to $O_2^{\cdot -}$ in the apoplast [162]. Under stress conditions, $O_2^{\cdot -}/H_2O_2$ production occurs in cytoplasmic vesicles derived from the plasma membrane or ER [163], and RBOHD is internalized into plasma membrane-derived vesicles [164]. Oxidation also occurs inside the ER lumen to promote protein folding depending on disulfide bonds formation by a FAD-containing ER oxidase (ERO) and protein disulfide isomerases (PDIs) [165]. Apoplastic H_2O_2 influences cell expansion, development and defense by type III peroxidase PRX-mediated cross-linking of cell wall polymers and lignification [166, 167]. In *atsyp71-2*, activities of above four enzymes changed significantly (Fig. 7A-D). In addition to *PRXs*, expression of many redox-related enzymes also altered. For example, Endothelial amine oxidase AOC3, Proline oxidase family FAD-linked oxidoreductase POX1, FAD linked oxidase AT4G20830 and AT5G44400, Multicopper oxidase LAC1 and Copper amine oxidase AT1G31710 (supplemental Table S1). There may be several influencing factors. One is due to defects on vesicle transport. Since the secretion pathway was blocked in *atsyp71-2*, efficiency of the enzyme delivery to destinations might decrease. The second reason could be defects on posttranscriptional modification. For example, expression of UDP-glucosyltransferase, UGT74E2 and AT2G18560 changed (supplemental Table S1). The changes in enzyme activities affected ROS homeostasis and resulted in defects on cellular functions and cell wall structures which leading to weakened plant adaptabilities to environmental stress.

The non-enzymatic components contain ascorbic acids, α -tocopherol, flavonoids, phenolic compounds glutathione, carotenoids and lipids which mitigate oxidative damage by their antioxidant activities through utilization of H_2O_2 [151, 152]. Flavonoids distribute widely in plants and are one of the most bioactive plant secondary metabolites. Flavonoids are usually accumulated in the vacuole and apoplast and have diverse functions in plant development and defense [168, 169]. Many flavonoid biosynthetic genes are induced and result in increase of flavonoids under biotic and abiotic stresses. Flavonoids have antioxidant functions on maintaining the cellular redox balance under environmental stresses. They act as antioxidants depending on their radical reduction potentials and accessibilities [170–180]. In *atsyp71-2*, expression of Phenylpropane synthesis pathway-related genes changed, and the content of flavonoids reduced significantly. This might because of defects on vesicle trafficking which are responsible for flavonoid secretion, and some feedback mechanisms. Changes in the content of other compounds cannot be excluded, since expression of major latex proteins, MLP34 and MLP43, MLP328, MLP329, AT3G26450, AT3G26460 AT4G14060, AT4G23670 and AT4G23680, altered (supplemental Table S1), which could affect latex biosynthesis. Considering of defense function of flavonoids, the decrease of flavonoid contents could be one of the reasons for the change of stress response of *atsyp71-2* mutant.

Besides the enzymatic and non-enzymatic processes, sugars are also antioxidant based on redox balance. Sugars affect gene expression through sugar-specific signaling regulators such as hexokinase (HXK), Snf1-related kinase 1, and INV, which regulate the expression of stress-related genes such SODs, heat shock proteins (HSP) and glutathione-S-transferases (GST) [154, 181]. However, sugars also have dual roles in ROS homeostasis. Excess sugars not only induce increase of cytoplasmic H_2O_2 via metabolic pathways, but also reduce H_2O_2 scavenging through oxidative pentose phosphate (OPP) pathway which activates the antioxidative process [182, 183]. In *atsyp71-2*, expression of S-glucoside biosynthetic process-related genes and some sugar metabolism-related and effector genes altered. For example, Glutathione S-transferases Phi, GSTF6 and GSTF7; HSP proteins MED37C, HSFA6B, HSFA7A and HSP90-1; as well as WRKY18 and WRKY53 which coordinate with HAC1 to regulate responses to sugars [126, 127] (supplemental Table S1). These alterations might severely affect plant adaptation to environmental stimuli. Moreover, in addition to *WRKY* and *MYB* family transcription factors, some other stress response TFs also altered, such as AP2/ERF domain superfamily members, ERF107, ERF018, TINY, AIL6 and RAP2-3; Zinc finger domain-containing BT1, AT5G49665, ZFP3 and RHA4A.

In conclusion, AtSYP71 modulated plant metabolic pathways, development and environmental adaptabilities via regulatory on vesicle trafficking.

Conclusion

The *AtSYP71*-knockout mutant *atsyp71-4* was lethal at early development stage, suggesting that AtSYP71 is essential for plant development. Disturbance of secretion of SecGFP, the secretion marker, suggested that AtSYP71 is required for secretion pathway. Thus, we propose that depletion of AtSYP71 might block the delivery of materials and enzymes required for cell wall biosynthesis and lead to failure of morphogenesis. Transcriptome analysis of the *AtSYP71*-knockdown mutant, *atsyp71-2*, revealed the

disruptions of metabolism, stress response and extracellular components. In addition, contents of lignin, cellulose and flavonoids also altered significantly. These results suggested that AtSYP71 regulates cell wall formation and secondary metabolism. The defects on cell wall structures subsequently affected plant response to abiotic stress. Our findings suggested that AtSYP71 regulates plant development, metabolism and environmental adaptation probably by affecting cell wall homeostasis via mediating secretion of materials and regulators required for cell wall biosynthesis and dynamics.

Materials And Methods

Plant Materials and Growth Conditions

Arabidopsis thaliana ecotype Col-0 was used as wild type. T-DNA mutants were generated from Col-0. *atsyp71-1* (SAIL_813_A05), *atsyp71-2* (SALK_081547), *atsyp71-3* (SALK_201897) and *atsyp71-4* (GABI_367A08) were obtained from the *Arabidopsis* Biological Resource Center (ABRC) at Ohio State University (<https://abrc.osu.edu>). Homozygous plants were isolated by PCR genotyping using the insertion-specific primers listed in Supplementary Table 2. The seeds were surface-sterilized and sown either in soil or on 1/2 Murashige and Skoog medium (PhytoTech) with 1% (w/v) sucrose and 0.8 or 1.2% agar. Plants were grown at 22°C under 16 h: 8 h /light: dark cycles.

Transgenic plants (Col-0) expressing myc (TAP)-tagged *AtSYP71* were generated using a modified pNTAPa vector described by [184].

Abiotic Stress Treatments

For the NaCl, osmotic and oxidative stress treatments, the seeds were sown on 1/2MS medium with 120 mM NaCl, 150 mM mannitol, or 75 mM H₂O₂ and grown vertically. The root length was measured using ImageJ software.

Antibody Preparation

To prepare antibodies against AtSYP71, a polypeptide (Cys-LPARIEAIPDGTAGGPKSTSAWTPSSTTSRPDIKFDS DGRFDDDYFQESN) was synthesized and conjugated to a Carrier protein KLH linked by a N-terminal Cys residue. The peptide–KLH conjugates were injected into two rabbits to generate antibodies. The antibodies were subjected to ProteinA/G purification from the serum and ELISA detection. Polypeptide synthesis and antibody preparation were commissioned to GL Biochem (Shanghai) Ltd.

Immunoblotting

Dodecyl sulfate, sodium salt-Polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblot analysis was performed as described previously [115]. Antibodies were diluted as follows: anti-AtSYP71, 1:2,000; anti-AtSYP81, 1:1,000; anti-12S, 1:20,000; anti-2S3P, 1:5,000 [115]; anti-GFP (B-2:sc-996, Santa Cruz Biotechnology, Inc. Shanghai, China), 1:2,000; anti-myc (9E10:sc-40, Santa Cruz Biotechnology, Inc.

Shanghai, China), 1:2,000; anti-TUA (R0267-1a, Abiocode California, USA), 1: 2,000, respectively. The secondary antibody was against rabbit IgG (ZB2301 and ZSGB-BIO), 1:5000. Immunoreactive signals were detected using a chemiluminescence detection system (LAS-4000 and FYJIFILM).

Confocal Microscopy

Fluorescent images were obtained using a point scanning confocal microscope (Leica TCS SP8). Confocal imaging was preset for GFP (Ex:488 nm, Em:500–550 nm) or for propidium iodide staining (Ex:543 nm, Em: 580–640 nm).

Yeast Two-Hybrid Assay

For the yeast two-hybrid assay, the fragments of *AtSYP71* (cytosolic region), *MIP2* (Sec39 domain) and *MIP3* were amplified using cDNA obtained from seedlings with specific primers and ligated into pEASY-Blunt vector (TransGen, #CB101-01), respectively. After Sanger sequencing confirmation, the fragments were transferred into pGADT7 or pGBKT7 vectors, respectively. *AtSYP81*, *AtSEC20* and *MAG2* constructs were generated in our previous study [115, 121].

The paired constructs were introduced into *Saccharomyces cerevisiae* strain AH109 (Clontech) and selected on SD/-Leu/-Trp medium. The interactions were detected on SD/-Leu/-Trp/-His/-Ade medium.

RNA Extraction and RT-qPCR

Total RNA was extracted as described previously [122] using 9-day-old seedlings grown on 1/2MS medium horizontally. Quantitative reverse transcription-polymerase chain reaction (RT-qPCR) was performed according to the manufacturer's instructions. The specific primers are listed in Supplementary Table 2.

RNA sequencing

Total RNA was extracted from 9-day-old seedlings grown on 1/2MS medium horizontally. Illumina cDNA libraries were constructed with the TruSeq RNA Sample Prep Kits v2 (Illumina, San Diego, CA, USA) following the manufacturer's protocol. Sequencing of the cDNA libraries was performed by pair-end methousing an Illumina HISEQ-x10 with a 150-bp read length and a sequence depth ~20 million uniquely mapped reads.

Sequence trimming, mapping and expression level determination

Reads were trimmed using the CLC Genomics Workbench 6.5.1 (CLC bio, Denmark) with the following parameters: 'quality scores-0.005; trim ambiguous nucleotides-2; remove 5' terminal nucleotides-1; remove 3' terminal nucleotides-1; discard reads below length 25'. Trimmed reads were mapped using the RNA-seq mapping algorithm implemented in the CLC Genomics Workbench to the reference *A. thaliana* genome (TAIR10) allowing only unique mapping (length fraction=1, similarity fraction=0.95). In order to estimate the influence of non-uniquely mapped reads on gene expression we also mapped reads using

the same software and parameters as indicated above, but allowing multiple mapping (up to 10 hits). For each gene, total gene reads (TGR) was determined as the sum of all reads mapped to this gene. To avoid bias due to different library sizes, TGR values were normalized by a size factor as described in Anders and Huber [185].

Identification of DEGs

DEGs were identified using the R package 'DESeq' [185]. A false discovery rate (FDR) of 0.05 and a fold change of 2.0 were chosen as the threshold for significantly differential expression.

GO enrichment analysis

Downregulated and upregulated DEGs were analyzed for GO and other annotation (as key words or protein domain) enrichment using the DAVID gene functional annotation tool <https://david.ncifcrf.gov/>, with an FDR value of 0.05 and a fold change of category representation of 2.0 as the threshold of significance [186, 187].

Enrichment analysis of differential gene KEGG

KEGG enrichment analysis was performed using KOBAS (v2.0). Rich factor, *P*-value and the number of genes indicated the degree of KEGG enrichment. Rich factor means the ratio of enriched DEGs to all genes annotated in the pathway. Therefore, the higher the Rich factor value, the greater the enrichment of the pathway. Parameter setting: Corrected *P*-Value < 0.05.

Determination of lignin

Roots from nine-day-old seedlings or the first segment of stems from 35-day-old plants were harvested and dried at 80 °C to constant weight, then were ground and passed through a 40 mesh sieve. About 5 mg was weighed and put into a 10 mL glass test tube with 1 mL of glacial acetic acid containing 30 % acetyl bromide and 40 uL of perchloric acid, sealed with a sealing film, fully mixed, then incubated in water bath at 80 °C for 40 min, shaking every 10 min. After natural cooling, 1 mL of 2 mol / L NaOH and glacial acetic acid (equal volume) were added and fully mixed. Add 1,960 uL of acetic acid to 40 uL of supernatant and fully mixed. Then, absorbance of the mixture was determined by spectrophotometer at 280 nm wavelength versus the prepared blank.

Determination of cellulose

The roots from –nine-day-old seedlings or the first segment of stems from 35-day-old plants were harvested and incubated overnight in 80% ethanol at 65 °C. Dry materials were ball milled to fine powder. Cellulose content was determined by Anthrone sulfuric acid colorimetry using Cellulose determination kit (CLL-2-Y, Comin, Suzhou Keming Biotechnology Co., Ltd.) according to Xiao et al. (2016). D-Glc (Sigma) was used as a standard for calculation of cellulose content. Three repeats per sample.

Determination of total flavonoids

The flavonoid content was determined by colorimetric assay [188]. A 250 µL of standard solution of rutin at different concentrations or appropriately diluted samples were added to 10 ml volumetric flask containing 1 ml of distillate water, respectively, then, 75 µl of NaNO₂ (5%) was added and mixed thoroughly. After 6 min of incubation, 75 µl of AlCl₃ (10%) was added, fully mixed and incubated for 6 min, then 500 µl of NaOH (1N) was added. Immediately, the solution was diluted by adding 2.5 ml of methanol and mixed thoroughly. Absorbance of the mixture was determined by spectrophotometer at 506 nm wavelength versus the prepared blank. Total flavonoid compounds in plant were indicated as mg rutin equivalents (CE mg/ml). Three repeats per sample.

Analysis of enzyme activities

For determination of activities of ROS-scavenging enzyme, 0.2 g of roots from nine-day-old seedlings were ground in 1.6 ml of precooled 50 mM phosphate-buffered saline (PBS) buffer (pH 7.8). The homogenate was centrifuged at 16,000 g for 20 min at 4°C, and the supernatant was used for the assays. Determination of activities of SOD, POD, CAT and APX were performed according to established protocols [189]. Three biological replicates per sample.

Abbreviations

PCWs: primary cell walls

SCWs: secondary cell walls

PM: plasma membrane

XyG: xyloglucan

CesA: cellulose synthase

CSCs: complexes

GPP: General Phenylpropanoid Pathway

MTT: membrane transporter-mediated transport

MVT: membrane vesicle-mediated transport

ABC: ATP binding cassette

ER: endoplasmic reticulum

BTL-like: bilitranslocase-like protein

SNARE: soluble N-ethylmaleimide-sensitive factor attachment protein receptor

TGN: trans-Golgi network

SmaCCs: small CesA compartments

SSPs: seed storage proteins

DEGs: differentially expressed genes

GO: Gene Ontology

KEGG: Kyoto Encyclopedia of Genes and Genomes

C₃ Prxs: Type III peroxidases

ROS: reactive oxygen species

RNS: and reactive nitrogen species

LTPG1: glycosylphosphatidylinositol-anchored lipid protein transfer 1

LTP2: lipid transfer protein 2

EXPA1: expansin A1

BBE19/OGOX1: FAD-binding Berberine Bridge Enzyme 19/oligogalacturonide oxidase 1

XTH20: xyloglucan endotransglucosylase/hydrolase 20

GSTF6: glutathione s-transferase 6

CYP706A1: cytochrome p450 family 706, subfamily A, polypeptide 1

PP2-B13: PHLOEM PROTEIN 2-B13

BGAL8: beta-galactosidase 8

SCPL12: serine carboxypeptidase-like 12

JAL22: jacalin-related lectin 22

TFs: transcription factors

CAT: catalase

POD: Peroxidase

SOD: Superoxide dismutase

APX: Ascorbate peroxidase

OG: oligogalacturonide

DAMPs: damage-associated molecular patterns

Declarations

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Authors' contributions

LL conceived and supervised the project. LL, XK, and HZ designed the experiments. XK, HZ, XZ and MW generated all material used in this study (cloning, transformations, transgenic plants, and crosses) and figures and tables. XK, HZ, XZ, MW, JZ and ZZ conducted the laboratory experiments. GQ performed the LC-MS/MS analysis. LL and KX drafted the manuscript. XF coordinated the project, and revised the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

All data generated or analysed during this study are included in this published article (and its additional files).

atsyp71-1 (SAIL_813_A05), *atsyp71-2* (SALK_081547), *atsyp71-3* (SALK_201897) and *atsyp71-4* (GABI_367A08) were obtained from the *Arabidopsis* Biological Resource Center (ABRC) at Ohio State University (<https://abrc.osu.edu>). Data sharing not applicable to this article as no datasets were generated or analysed during the current study.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Figures

Figure 1

AtSYP71 gene and T-DNA-tagged mutants. **a**: *AtSYP71* gene structure diagram. Black boxes represent exons, gray lines represent introns, and gray boxes represent untranslated regions (UTRs). The triangles

indicate the T-DNA insertion sites in the mutants. The locations of primers used for RT-qPCR analyses are indicated by arrows. **b**: The relative expression levels of *AtSYP71* in each mutant were detected by RT-qPCR. Three independent experiments per sample, two repeats per experiment. **c**: Western blot was performed to detect accumulation of AtSYP71 or AtSYP71 fusion proteins using anti-myc (for myc-AtSYP71-overexpressing lines), anti-GFP (for GFP-AtSYP71/*atsyp71-2* complementary lines) and anti-AtSYP71 (for endogenous AtSYP71 protein) antibodies. **d**: Seven-day-old seedlings of wild type, *atsyp71* mutants, complementary and overexpression lines. **e**: Five-day-old seedlings of wild type and *atsyp71-4* mutant. Magnified pictures indicated growth defects. **f**: Statistics of root length in panel **d** ($44 \leq n \leq 48$). **g**: Four-week-old plants. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$. Significance was evaluated by Student's *t* test using GraphPad prism 8.0 software (GraphPad, San Diego, USA).

Figure 2

AtSYP71 regulated vesicle trafficking. **a**: Yeast two hybrid analysis of AtSYP71 interactors. Yeast strain AH109 was transformed with the paired constructs as shown. Transformants were streaked onto SD/_Leu/_Trp/_His/_Ade medium. AtSYP81/pGADT7 vs AtSec20/pGBKT7 served as a positive control. Each construct and its corresponding empty vector were used as negative controls. **b**: LC-MS/MS identified AtSYP71-interactors from pull down elution using myc-AtSYP71-overexpressing plants. Hit, peptide_hit_number; score, peptide score. **c**: Immunoblot was performed to detect accumulation of AtSYP81 protein using pull down elution obtained from myc-AtSYP71/OE (left panel) and GFP-AtSYP71/*atsyp71-2* (right panel). **d-f**: Confocal images of SecGFP in Col-0 and *atsyp71-2* background with PI staining.

Figure 3

Transcriptome visualization analysis. **a:** Col-0 and *atsyp71-2* transcription data quality detection. **b:** Volcano plots of differentially expressed genes (DEGs). X axis represents $\log_2$ transformed fold change. Y axis represents $-\log_{10}$ transformed significance. Red points represent up-regulated DEGs. Blue points represent down-regulated DEGs. DEG number are shown in upper right corner. **c:** Gene ontology (GO) analysis of DEGs. GO has three ontologies: molecular biological function, cellular component and biological process. X axis represents DEG number. Y axis represents GO terms. **d:** Top 20 of the DEG enriched functional pathways. X axis represents enrichment factors. Y axis represents pathway names. The color indicates the pvalue (higher: purple, lower: red), the lower pvalue indicates the more significant enrichment. Point size indicates DEG number. The bigger dots indicate the larger number.

Figure 4

Quantitative expression of cell wall-related DEGs. **a**: GO analysis of Cellular component (GO:0005575). DEGs were concentrated in the colored frames. Red frames include more DEGs, yellow frames include less DEGs. **b, d and f**: transcriptomic differential gene expression. **c, e and g**: RT-qPCR validations of panel **b, d and f**. Three independent experiments per sample, two repeats per experiment.

Figure 5

Determination of contents of lignin, cellulose and flavonoids. **a, b and c:** Contents of lignin, cellulose and flavonoids in roots from nine-day-old seedlings. **d, e and f:** Contents of lignin, cellulose and flavonoids of the first segment of stems from 35-day-old plants. *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$; ****, $P<0.0001$. Significance was evaluated by Student's t test using GraphPad prism 8.0 software (GraphPad, San Diego, USA).

Figure 6

Structure of stem cross section. **a**: The paraffin sections of stems with Safranine and Fast Green double staining. **b**: Magnified pictures of part of **a**. **c**: Statistics of total area of xylem. **d**: Statistics of cell wall thickness of inter-fascicular fibre cells. ep, epidermis. co, cortex. if, inter-fascicular fibres. ph, phloem. xy, xylem. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$. Significance was evaluated by Student's t test using GraphPad prism 8.0 software (GraphPad, San Diego, USA).

Figure 7

Plant response to the abiotic stresses. **a-d**: Determination of catalytic activities of CAT (**a**), POD (**b**), APX (**c**) and SOD (**d**) of the roots from nine-day-old seedlings. **e-g**: Seven-day-old seedlings under 120 mM NaCl treatment (**e**) and statistics of root length (**f**) and root length change ratio (**g**). **h-j**: Nine-day-old seedlings under 75 μ M H₂O₂ and 150 mM mannitol treatments (**h**) and statistics of root length (**i**) and root length change ratio (**j**). $39 \leq n \leq 50$. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$. Significance was evaluated by Student's t test using GraphPad prism 8.0 software (GraphPad, San Diego, USA).

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