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Qa-SNARE Protein SYP22 Negatively Regulates Brassinosteroid Signaling in the Dark

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Soluble N-ethyl-maleimide sensitive factor attachment adaptor protein receptor (SNARE) domain-containing proteins were mainly involved in vesicle-associated membrane fusion. Genetic screening has revealed the function of SNARE in different aspects of plant biology. Among them, Synthaxin-22 (SYP22) a Qa-SNARE has been reported to have a pleiotropic function in plant development including regulation of leaf waving, shoot gravitropism and flowering time. In this study, we identified a new role of SYP22 in regulation of brassinosteroid (BR) signaling, especially in the dark. SYP22 interacts with BR receptor, brassinosteroid insensitive 1 (BRI1), and overexpression of SYP22 enhanced a weak BRI1 mutant *bri1-5* phenotype. *syp22* mutant exhibits short hypocotyl and it is sensitive to exogenously treated BR while slightly insensitive to BR-biosynthesis inhibitor propiconazole (PCZ) in the dark. Expression levels of BR signaling maker genes *ACS5*, *SAUR15* and *IAA19* were slightly higher, while *BR6OX2*, a BR biosynthesis marker gene, was lower in *syp22* compared to the wild-type. In addition, *syp22* was sensitive to 2,4-D, a synthetic auxin, in the dark. In conclusion, SYP22 is involved in BR- and auxin-mediated hypocotyl growth inhibition in the dark, which might be via interaction with BR and auxin key regulators to alter their internalization in *Arabidopsis*.

Key words: SNARE, trafficking, BR, dark, *Arabidopsis*

INTRODUCTION

Autonomous protein trafficking is one of the major regulatory mechanisms for signal transduction in eukaryotic cells. Membrane trafficking is an intracellular system that can be used to transport proteins; this process includes steps such as budding of transport vesicles in organelles and vesicular fusion to the target membrane. It is well known that SAR/ARF GTPases regulate budding processes, and RAB GTPases and soluble N-ethyl-maleimide sensitive factor attachment protein receptors (SNAREs) are key regulators of membrane attaching and fusion. The SNARE proteins are classified into Qa-, Qb-, Qc-SNARE and R-SNARE groups according to their conserved residues within the SNARE motif (Lipka et al., 2007).

SNAREs are present in the membranes of all subcellular compartments and play a major role in vesicular fusion and membrane biosynthesis. Genome analysis showed that green plants have a larger SNARE family compared with other eukaryotes (Sanderfoot, 2007). Some SNARE proteins are characterized by their function in plants. SYP22/VAM3/SGR3 is a Qa-SNARE that functions in vacuolar and endocytic transport pathways. *syp22* mutants exhibit pleiotropic phenotypes, including wavy leaves, semi-dwarfism, insensitivity to gravity change and late flowering (Ebine et al., 2012; Ohtomo et al., 2005; Shirakawa et al., 2009; Yano et al., 2003). R-SNARE protein VAMP727 forms a complex with SYP22, VTI11 and SYP51. This complex plays a crucial role in vacuolar transport, seed maturation and vacuole biogenesis (Ebine et al., 2008). Negative dominant form of SYP121, another plant Qa-SNARE, suppresses potassium channel KAT1 and water channel PIP25 traffic to the plasma membrane (Besserer et al., 2012; Sutter et al., 2006).

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Additionally, Qc-SNARE protein AtBS14b has been shown to localize in the Golgi apparatus (Lipka et al., 2007). Further, overexpression of AtBS14b altered BR response via interaction with MSBP1 in *Arabidopsis* (Zhu et al., 2014).

Brassinosteroids (BRs) are phytohormones that play a crucial role in plant growth and development, including stem elongation, leaf expansion, vascular differentiation, senescence and stress tolerance (Clouse and Sasse, 1998). BRs are recognized by their receptor Brassinosteroid Insensitve 1 (BRI1) and co-receptor BRI1-Associated receptor Kinase 1 (BAK1), which interacts with BRI1 to enhance BR signaling. Ligand-independent BRI1 trafficking happens between the plasma membrane and the trans-Golgi network/early endosome, followed by degradation in the vacuole (Geldner et al., 2007). VAMP727 and its interacting protein SYP22 were identified to interact with BRI1 (Jones et al., 2014). Previous study also revealed that the hypersensitivity of *bzr1-D* to exogenously supplied BR under dark conditions causes direct activation of BZR1 to *IAA19* and *ARF7* (Zhou et al., 2013). However, the regulatory role of SNARE proteins on BRI1 and BR signaling is still not clear.

Here, we established that *syp22* mutants were sensitive to BR and insensitive to PCZ in the dark. Expression levels of BR signaling marker genes were slightly higher while BR biosynthesis marker gene was slightly lower in *syp22*, indicating that SYP22 negatively regulates BR signaling which may in turn repress BR biosynthesis. In addition, *syp22* was also sensitive to auxin in the dark and had potential to interact with BRI1, PIN5 and other trafficking proteins (GTPase and SNARE). To sum up, these results suggest that SYP22 may regulate intracellular trafficking of the key BR and auxin regulators to modulate hypocotyl growth in the dark.

MATERIALS AND METHODS

PLANT MATERIALS AND GROWTH CONDITIONS

The open reading frame (ORF) regions of *BRI1* (AT4G39400) and *SYP22* (AT5G46860) were recombined into the pABind117 GW binary destination vector and pEarly GW 104 via a Gateway LR reaction, respectively, and *Agrobacterium*-mediated transformation into *N. benthaniama* leaves (Kim et al., 2009). The *YFP-SYP22* construct was transformed into *bri1-5* (WS2 background) for generation of transgenic plants. The primers used to clone *BRI1* and SYP22 ORF regions are listed in Table S1. Surface sterilized seeds were sewed on half-strength Murashige and Skoog (MS) medium with 1.2% agar or MS medium supplied with 2,4-epiBL, 2,4-D or PCZ (Sigma, Saint Louis, MO, USA). After two days of stratification at 4°C, the seedlings were incubated at 22°C in 24-hour low light (~80 μE/m^2) or dark chambers for seven days. The seedling plates were photographed and hypocotyl lengths were measured using ImageJ software. For BL and 2,4-D treatment experiments, seven-day-old seedlings grown in the dark were transferred to media containing 100 nM 2,4-epiBL or 100 nM 2,4-D and the whole seedling were sampled after 3 hours of the treatment.

SPLIT GFP ASSAY IN TOBACCO LEAVES

The N-proximal half of YFP (nYFP) and C-proximal half of CFP (cCFP) sequences were fused to the C-terminal sequences of *BRI1* and N-terminal sequences of *SYP22* in PXNGW and PXCGW vectors, respectively. The primers used to clone *BRI1* and *SYP22* ORF regions were listed in Table S1. For transient expression, all binary constructs were introduced into *A. tumefaciens* strains (GV3101). *Agrobacterium* cells containing split YFP fusion constructs were grown in liquid yeast extract peptone (YEP) medium supplemented with antibiotics (spectinomycin 50 μg/ml and Rifampicin 50 μg/ml). Cultured cells were centrifuged at 4,000 rpm for 5 min at RT, and then the cell pellet was re-suspended in an infiltration buffer (10 mM MES, pH 5.6, 10 mM $MgCl_2$, 200 μM acetosyringone) after removing the supernatant. Cell density was adjusted with infiltration buffer to give an OD_{600} of ~0.5–0.6. Aliquots (0.5 ml) of *Agrobacterium* cells carrying a split GFP fusion constructs were mixed, and then a syringe was used to infiltrate the mixture into the lower surface of *N. benthamiana* leaves. The plants were incubated in a growth chamber for 36 to 48 hours (Kim et al., 2009).

LOCALIZATION OF *BRI1*-GFP AND YFP-SYP22 IN TOBACCO LEAVES

For transient expression of YFP-SYP22 and *BRI1*-GFP fusion proteins, *SYP22* and *BRI1* ORFs were cloned into pEarly-GW 104 and pABind117 GW destination plasmid, respectively, followed by transient expression in *N. benthamiana* leaves by using the *Agrobacterium*-mediated transient expression method (Kim et al., 2009). GFP fluorescence was detected under an Olympus confocal laser-scanning microscope (Fluoview FV 1000, http://www.olympus-global.com/).

RNA EXTRACTION AND QUANTITATIVE RT-PCR ANALYSIS

Total cellular RNA was isolated with TRIzol (Takara, Dalian, China), and subsequently treated with DNase (Promega, Madison, WI, USA) to eliminate genomic DNA contamination. For cDNA synthesis,

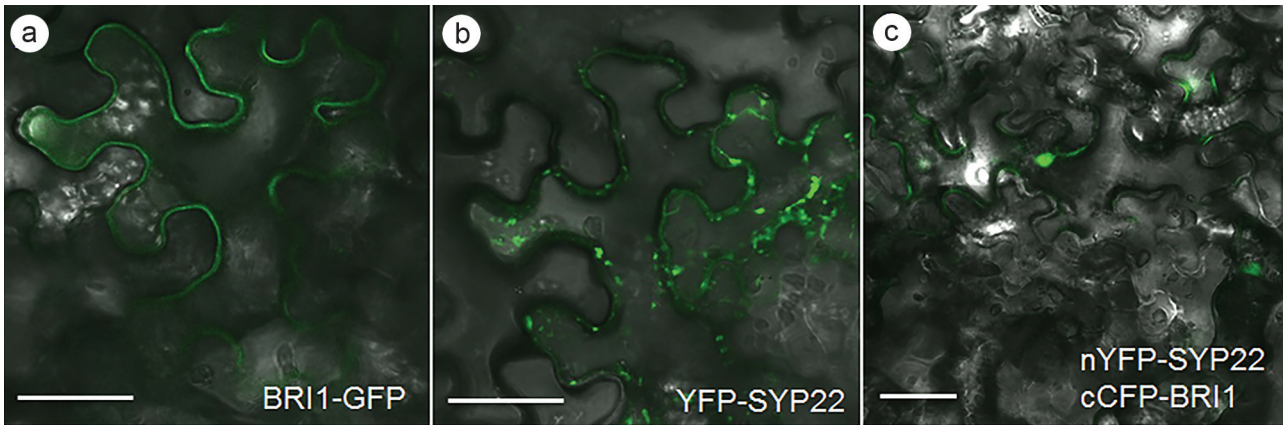

Fig. 1. BRI1 and SYP22 subcellular localization, BRI1 and SYP22 interaction in *N. bethamiana* leaves. BRI1-GFP and YFP-SYP22 proteins were expressed in *N. benthamiana* leaves. (**a** and **b**) Green florescence signals indicated BR1 and SYP22 subcellular localizations and the images were merged with bright field images (DIC). nYFP-SYP22 and cCFP-BRI1 fusion proteins were co-expressed in *N. benthamiana* leaves and YFP signal was observed. (**c**) Green florescence signal indicated the interaction compartments for BRI1 and SY22, and the image was merged with bright field images. Bars = 20 μm.

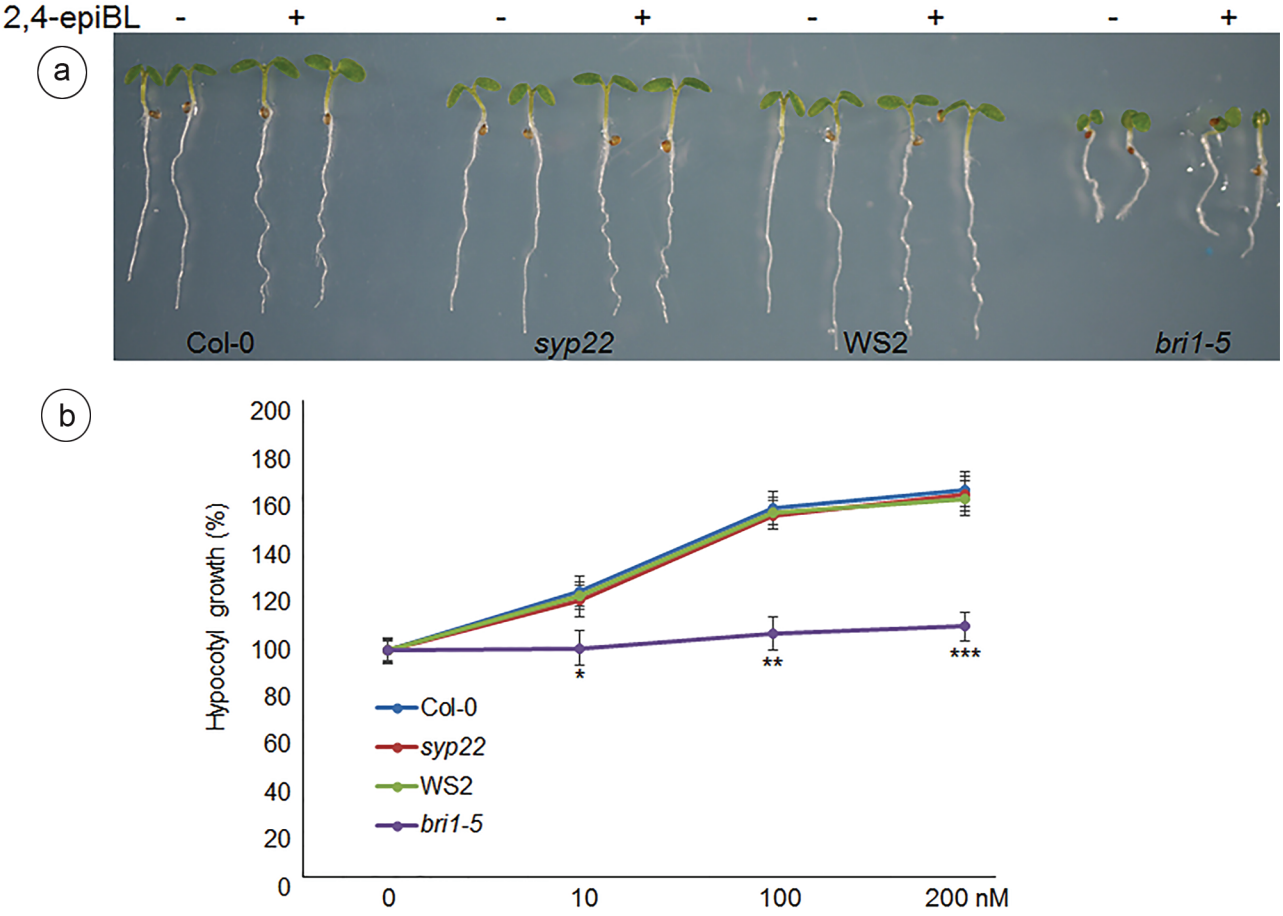

Fig. 2. BL-mediated hypocotyl elongation of *syp22* and *bri1-5* mutants in the light. (**a**) *syp22* and its control Col-0 or *bri1-5* and its control WS2 were grown on half MS medium containing 10 nM 2, 4-epiBL for 5 days. (**b**) Hypocotyl growth patterns of 5-day-old seedlings grown under indicated concentration of 2,4-epiBL were analyzed. Error bars are SE of the means of three independent experiments and more than 10 plants were analyzed each time. Significant differences between WS2 and *bri1-5* were shown (*P<0.05, **P<0.01, ***P<0.001 t test).

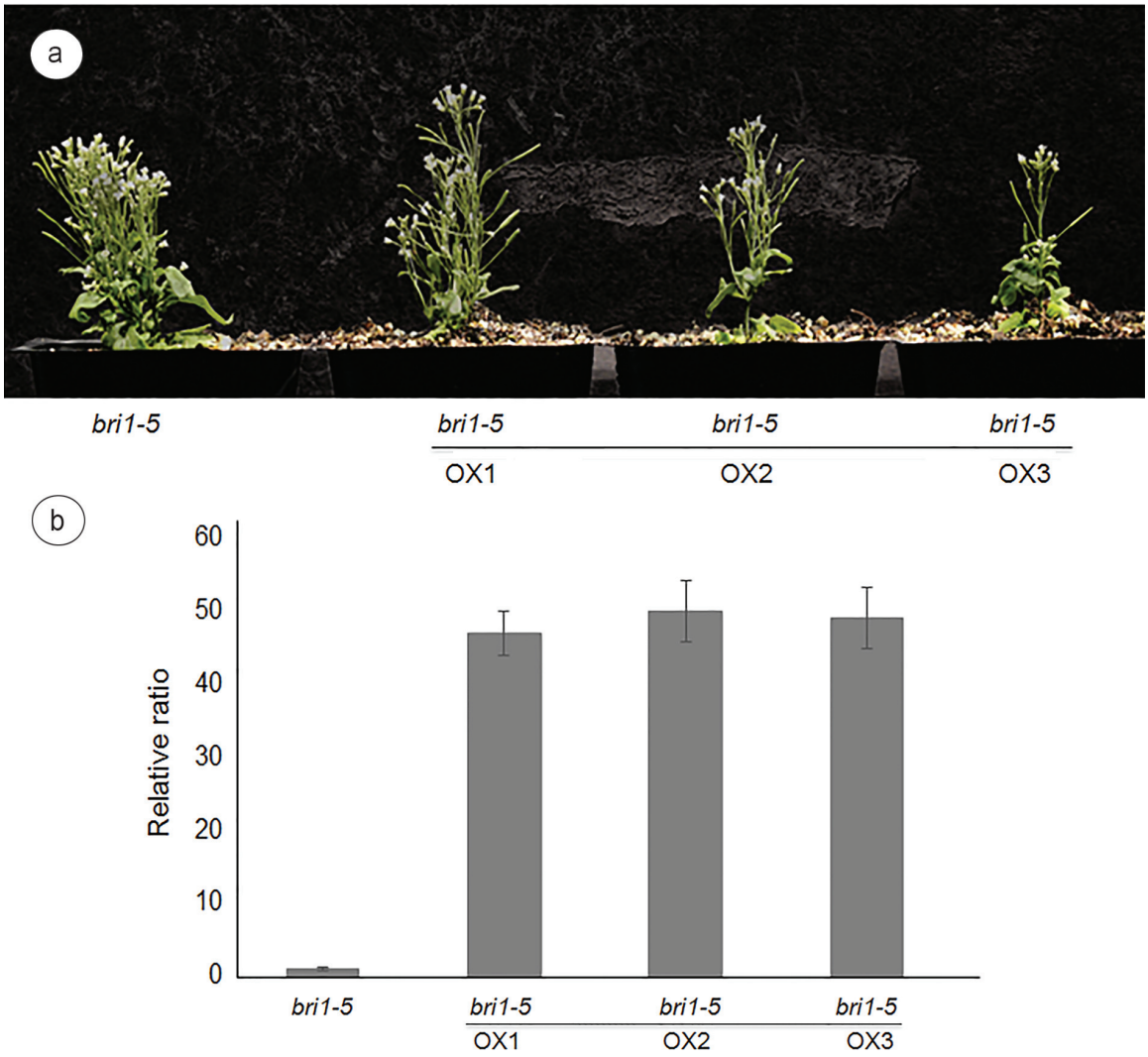

Fig. 3. Phenotypic and transcriptional expressions of *SYP22* in *bri1-5* mutant. (**a**) *bir1-5* and *bri1-5* mutant overexpressing *SYP22* were photographed. (**b**) *SYP22* expression levels from *bri1-5* and *bri1-5* plants overexpressing *SYP22* were analyzed by qRT-PCR. Actin was used as an internal control. OX1, OX2 and OX3 are individual *SYP22* overexpression lines.

GoScriopt™ Reverse Transcription kit was used according to the manufacturer's instruction (Promega, Madison, WI, USA). qRT-PCR analysis used gene specific primers for *BR6OX2*, *ACS5*, *IAA19* and *SAUR15* as described in Oh et al. (2012). *Actin2* was used as control (Chen et al., 2012). All primers used for qRT-PCR were listed in Table S1.

STATISTICAL ANALYSIS

Statistical calculations were performed by prism 5 (GraphPad, San Diego, CA). All data were expressed as mean ± SE. Comparison between two groups was performed by *t* test (*$p<0.05$; **$p<0.01$; ***$p<0.001$).

RESULTS

SYP22 INTERACTS WITH BRI1 AND *syp22* MUTANTS EXHIBIT NORMAL BR RESPONSE IN THE LIGHT

BRI1, a brassinosteroid receptor localized at the plasma membrane and Qa-SNARE protein SYP22, was located in intracellular compartment in tobacco cells (Fig. 1a, b). Interaction between BRI1 and SYP22 was identified via the split GFP assay in

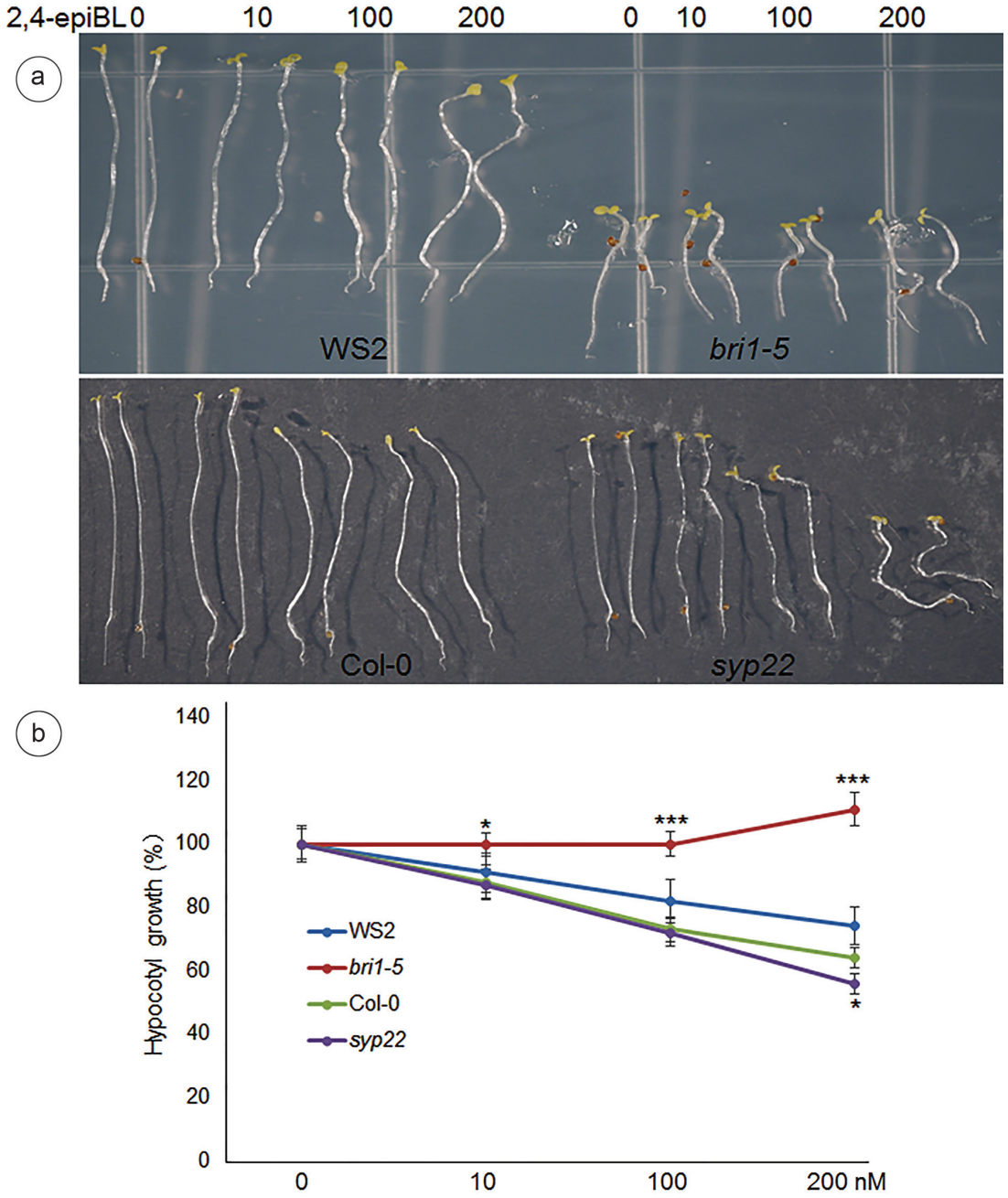

Fig. 4. BL-dependent hypocotyl growth of *bri1-5* and *syp22* mutant plants in the dark. (**a**) *bri1-5* and its control plant WS2 or *syp22* and its control plant Col-o were grown on half MS medium containing indicated concentrations of 2,4-epiBL in the dark. (**b**) Hypocotyl growth patterns of 7-day-old plants shown in (**a**) were measured. Error bars are SE of the means of three independent experiments and more than 10 plants were analyzed each time. Significant differences between WS2 and *bri1-5* or Col-0 and *syp22* were analyzed (*P<0.05, ***P<0.001 t test).

tobacco leaves. Their interaction was mainly observed in the intracellular compartment (Fig. 1c) (Jones et al., 2014). BR-independent BRI1 endocytosis has been known to influence BR signaling, but where *BRI1* activates BR signaling is still ambiguous (Geldner et al., 2007; Irani et al., 2012). Exogenous BR treatment revealed that *syp22* mutant normally responds to BR while *bri1-5*, a weak *BRI1* mutant, is insensitive to BR in the light (Fig. 2). In parallel, SYP22 overexpression plants

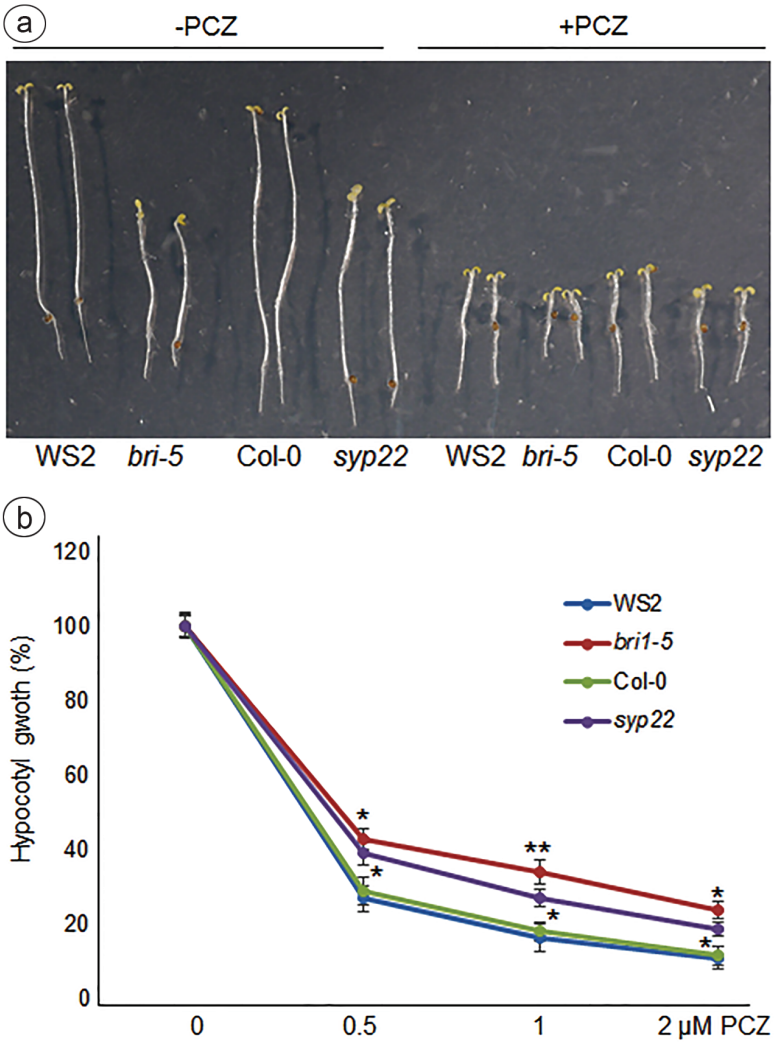

Fig. 5. PCZ-dependent hypocotyl growth of *bri1-5* and *syp22* mutant plants in the dark. (**a**) *bri1-5* and its control plant WS2 or *syp22* and its control plant Col-0 were grown on half MS medium containing 0.5 μM of PCZ in the dark. (**b**) Hypocotyl growth patterns were measured from the plants grown in the half MS medium containing indicated concentrations of PCZ. Error bars are SE of the means of three independent experiments and more than 10 plants were analyzed each time. Significant differences between WS2 and *bri1-5* or Col-0 and *syp22* were analyzed (*P<0.05, **P<0.01 t test).

were generated in WS2 and *bri1-5* backgrounds. SYP22 overexpressors in WS2 background display normal growth and development (data not shown). However, overexpression of *SYP22* in *bri1-5* background showed less shoot branching compared to *bri1-5* mutant (Fig. 3), as well as smaller leaves and shorter petioles (Jones et al., 2014).

syp22 MUTANTS WERE SENSITIVE TO BR WHILE INSENSITIVE TO PCZ IN THE DARK

Brassinosteriods (BRs) are essential hormones in plants, promoting stem and hypocotyl elongation. Furthermore, BR signal is tightly connected with light to regulate photomorphogenesis (Wang et al., 2012). Hypocotyl elongation is sensitive to light conditions, which was used as a character to analyze BR and light response; therefore, *syp22* hypocotyl elongation was analyzed under BR treated conditions in the dark. As shown in Fig. 4a, *syp22* mutant exhibits short hypocotyl compared to Col-0 plants without BR treatment. BR treatment inhibited hypocotyl elongation in the dark along with slight waving. However, *syp22* showed strong hypocotyl waving under high concentration of 2,4-epiBL treatment, while *bri1-5* is insensitive and even slightly elongated upon 2,4-epiBL supply (Fig. 4).

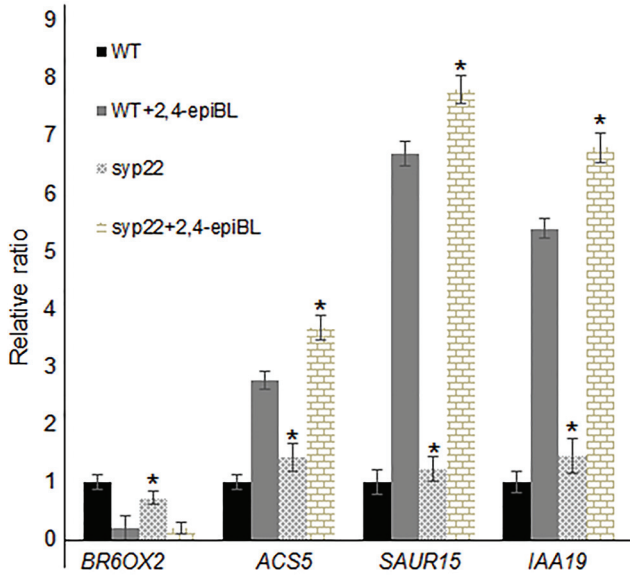

Fig. 6. Expression levels of BR biosynthetic and signaling genes in Col-0 and *syp22* with or without 2,4-epiBL treatment. 7-day-old dark condition grown plants were treated with 100 nM 2,4-epiBL for 3 hours. Expression levels of BR biosynthetic gene (*BR6OX2*) and signaling genes (*ACS5*, *SAUR15* and *IAA19*) were monitored by qRT-PCR. Error bars are SE of the means of three replicates. Significant differences between Col-0 and *syp22* with or without 2,4-epiBL treatment were analyzed (*P<0.05 t test).

In contrast, BR biosynthesis inhibitor PCZ application inhibits hypocotyl elongation. *bri1-5* and *syp22* and their control plants WS2 and Col-0, respectively, were grown on half MS medium containing different concentrations of PCZ. The results showed that *bri1-5* and *syp22* are insensitive to PCZ in the dark (Fig. 5).

BR-MEDIATED MARKER GENE EXPRESSIONS ALTERED IN *syp22* MUTANT

syp22 mutants were sensitive to BR in the dark, therefore, BR-mediated marker gene expressions were further dissected. One-week-old dark condition grown seedlings were treated with 100 nM 2,4-epiBL. qRT-PCR analysis with these samples showed that the expression of *BR6OX2*, a BR biosynthesis enzyme, was dramatically repressed after BR treatment in both Col-0 and *syp22*, but the expression level was lower in *syp22* without BL supply. BR signaling maker genes *ACS5*, *SAUR15* and *IAA19* expressions were obviously induced by 2,4-epiBL treatment and higher in *syp22* than in Col-0 plants with or without BL treatment (Fig. 6), suggesting that SYP22 might regulate BR signaling rather than BR biosynthesis genes.

syp22 MUTANTS ARE SENSITIVE TO AUXIN

Previous study identified BR-mediated hypocotyl inhibition in the dark via activation of auxin signaling (Zhou et al., 2013). Therefore, auxin sensitivity was further analyzed in *syp22* in the dark. 100 nM 2,4-D a synthetic auxin application similar to BR treatment inhibited hypocotyl elongation (Fig. 7a and a). *syp22* exhibited severe waving hypocotyl (Fig. 7a). Further, BR and auxin dependent expression patterns of *SYP22* indicated that *SYP22* is not regulated by two phytohormones in transcriptional level (Fig. 7c). For understanding membrane protein network, a large number of membrane and membrane-associated protein interactions were examined using split ubiquitin yeast two hybrid system (Jones et al., 2014). A Few SYP22 interactors were identified in this assay including PIN5 an auxin efflux carrier, and two trafficking proteins; NPSN11 (a SNARE protein), ROP5/ARAC6 a small GTPase (Fig. 7d). In addition, BRI1 was identified to interact with SYP22 in split GFP test (Fig. 1c), implying a complex regulatory mechanism of hypocotyl elongation by SYP22.

DISCUSSION

syp22 mutants exhibit a short hypocotyl in the dark which is similar to bri1 mutant (Fig. 4). Although *SYP22* expression was not altered by BR (Fig. 7c), SYP22 interacts with *BRI1* in tobacco leaves (Fig. 1c), suggesting an involvement of SYP22 in regulation of BR signaling. *syp22* mutant was sensitive to BR while insensitive to BR biosynthesis inhibitor PCZ in the dark, which is different from the *bri1-5* mutants. *bri1-5* mutant was insensitive to both BR and PCZ treatment (Fig. 4 and 5), suggesting that SYP22 regulates hypocotyl elongation via a complex regulatory circuits including BR signal pathway. In *syp22* plants, *BR6OX2* level was slightly lower, but the expression levels of *ACS5*, *IAA19* and *SAUR15* were slightly higher than in Col-0, indicating a specific regulation of SYP22 on BR signaling, and activation of BR signaling may in turn inhibit biosynthesis in *syp22*. However, *syp22* mutant displayed normal hypocotyl growth and BR response in the light. R-SNARE protein VAMP727 interacts with SYP22 as well as with *BRI1* whereas *syp22/vamp727* double mutant exhibited dwarf phenotype and insensitive response to exogenously treated BL (Ebine et al., 2008; Jones et al., 2014). Complicated connections between BR and light signaling have been reported (Wang et al., 2012). In addition, SNARE proteins normally form a complex, SYP22 forms a complex with VAMP727, VTI11 and SYP51. This complex plays a crucial role in vacuolar transport, seed maturation, and vacuole biogenesis.

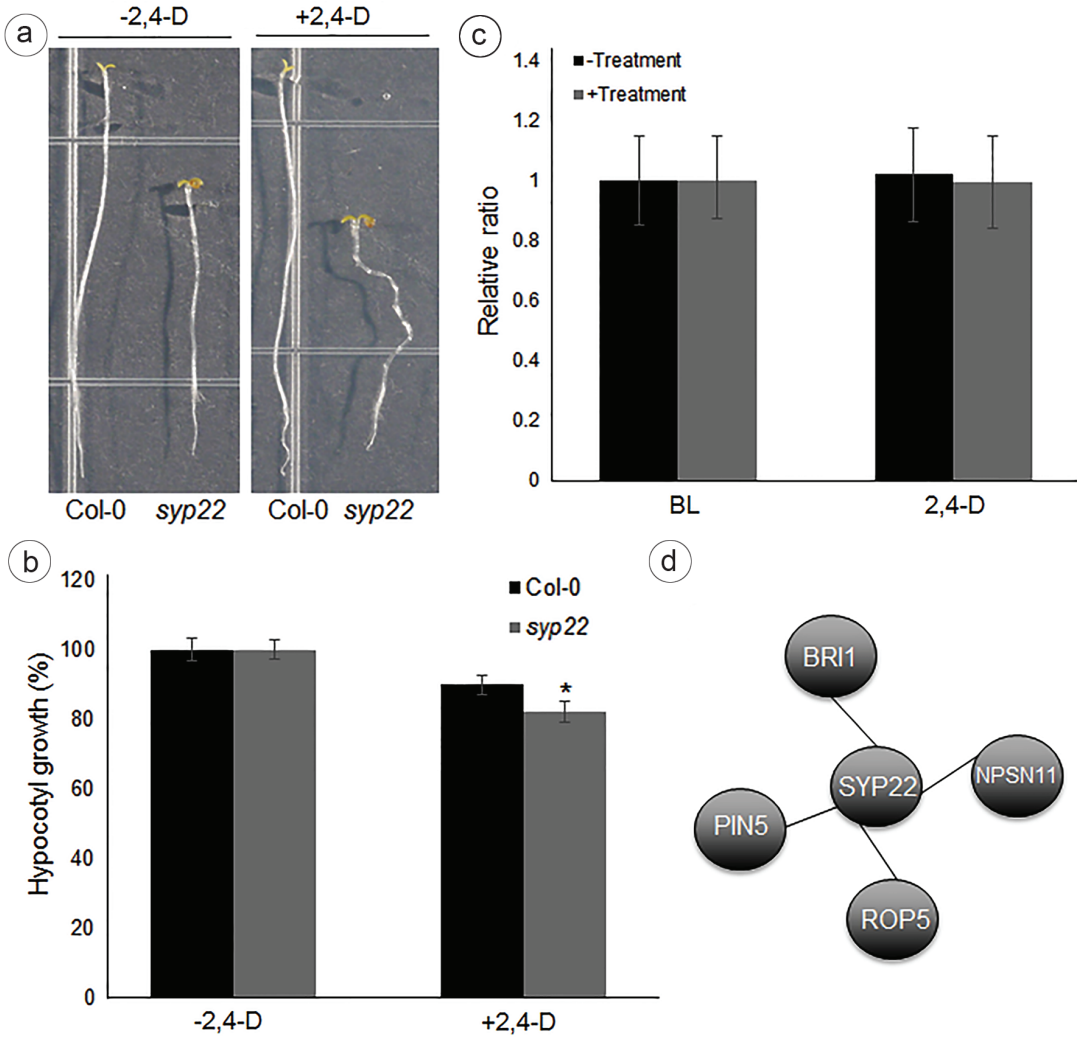

Fig. 7. Auxin-dependent hypocotyl growth of *syp22* and BR- or auxin-mediated expression patterns of SYP22. (**a**) Col-0 and *syp22* plants were grown on half MS medium containing 100 nM 2,4-D for 7 days in the dark. (**b**) Hypocotyl growth patterns of Col-0 and *syp22* plants shown in (a) were measured. The experiments were repeated at least three times and more than 10 plants were analyzed each time. Significant difference between Col-0 and *syp22* after 2,4-D treatment was analyzed (*P<0.05 t test). (**c**) BL and 2,4-D dependent expression levels of SYP22 were analyzed by qRT-PCR. Actin was used as an internal control. (**d**) The putative interactors of SYP22 identified from yeast two hybrid assay were displayed. BRI1: BR receptor; PIN5: auxin efflux carrier; NPSN11: SNARE; ROP5: small GTPase.

VAMP727 was localized at the plasma membrane and endosome while SYP22 localized at the endosome and tonoplast, and missing of both SYP22 and VAMP727 has been known to result in defects of vacuolar protein storage (Ebine et al., 2008). Cell biology study on BRI1 has shown that BRI1 is active in plasma membrane (Irani et al., 2012). These findings imply that *SYP22* mutation may cause less BRI1 degradation in vacuole and recycle more BRI1 to plasma membrane to activate BR signaling. The different SYP22-associated SNARE complex may possibly cause different response of *syp22* mutant to BR in the light and dark. Further genetic and biochemical analyses are required to address the questions.

A *BZR1* dominant mutant *bzr1-D* is hypersensitive to exogenously supplied BR under dark conditions via direct activation of *IAA19* and *ARF7* (Zhou et al., 2013). It indicates that BR-mediated inhibition of hypocotyl elongation causes activation of auxin signaling. Auxin supply to *syp22* mutant resulted in hypocotyl inhibition along with severe waving (Fig. 7a). Membrane associated protein interactome data revealed that SYP22 interacts with one auxin efflux carrier PIN5 in yeast cells (Fig. 7d). BiFC result showed interaction between SYP22 and BRI1, suggesting that SYP22 may control both BR and auxin regulators to modulate hypocotyl elongation. ROP5, a small GTPase, and NPSN11, another

SNARE protein, previously were not identified to be interactors of SYP22 isolated from yeast two hybrid assay. ROP3, ROP6 and ROP9 have been reported to be involved in auxin signaling (Tao et al., 2005; Lin et al., 2012; Nibau et al., 2013). ROP2 has been reported to participate in ABA and BR regulated process (Li et al., 2001). These data suggest that ROP family proteins are involved in diverse aspects of hormonal regulations. SYP22 has been known to interact with different SNARE proteins to make a trafficking complex to differentially regulate plant development. Here, it was established that ROP5 and NPSN11 may play a role in BR and auxin signaling to regulate hypocotyl growth in *Arabidopsis*. Further experiments need to analyze SYP22 regulation in BRI1 and PIN5 trafficking and the role ROP5 and NPSN11 in plant growth and development, especially in hypocotyl elongation.

CONCLUSIONS

SYP22 as a SNARE protein, negatively regulates BR signaling. *syp22* mutants exhibited short hypocotyl and were sensitive to exogenously treated BL while insensitive to PCZ, a BR biosynthesis inhibitor, in the dark. Furthermore, BR biosynthetic gene *BR6OX2* was lower while signaling genes *ACS5*, *SAUR15* and *IAA19* were higher in *syp22* with or without BL treatment. The results of split GFP and mating based split ubiquitin yeast hybrid assays indicate that SYP22 interacts with *BRI1*, PIN5, and two trafficking proteins (NPSN11 and ROP5), suggesting a possible regulatory model by which SYP22 regulates intracellular trafficking of key BR and auxin regulators to modulate hypocotyl elongation in *Arabidopsis*.

AUTHORS CONTRIBUTIONS

TSY, XFZ and Y.H.X designed the research; TSY, XFZ and JHJ performed the research; YHX TSY, XFZ, JHJ and YHX analyzed the data; YHX wrote the paper. All the authors declare that there are no conflicts of interest.

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