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Structural and Functional Characterization of Arabidopsis GSK3-like Kinase AtSK12

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Plant GSK3-like kinases are key regulators that modulate a broad range of physiological processes such as cell growth, stomatal and flower development, responses for abiotic and biotic stress, and carbohydrate metabolism. Arabidopsis Shaggy/GSK3-like kinases (AtSK) consist of ten members that are classified into four subfamilies (I~IV). Only one of these Arabidopsis GSK3s, BIN2 (also named AtSK21), has been characterized by biochemical and genetic studies. BIN2 acts as a negative regulator in brassinosteroid (BR) signaling that controls cell growth and differentiation. Recent studies suggest that at least seven AtSKs are involved in BR signaling. However, specificities for the substrates and the functional differences of each member of the family remain to be determined. Here we report structural characteristics and distinct function of AtSK12 compared with BIN2. AtSK12 has a longer N-terminal extension, which is absent in BIN2. Transgenic plants overexpressing the AtSK12 mutant carrying deletion of N-terminal region display more severe dwarf phenotypes than those of the wild-type AtSK12. Microscopic analysis reveals that N-terminal-deleted AtSK12 accumulates in the nucleus. This implies that structural difference in the N-terminal region of AtSK members contributes to their subcellular localization. In contrast to BIN2, overexpression of AtSK12 does not cause a stomatal cluster. Furthermore, we show that YODA MAPKKK, which controls stomatal development, interacts with BIN2 but not with AtSK12. Our results suggest that AtSK12 mediates BR-regulated cell growth but not stomatal development while BIN2 regulates both processes. Our study provides evidence that different GSK3 members can have overlapping but non-identical functions.

INTRODUCTION

Shaggy/Glycogen synthase kinase 3 (GSK3) is a serine/threonine kinase that is highly conserved in all eukaryotes. Mammal GSK3s function as key regulators in a wide range of cellular processes including metabolic homeostasis, embryo development, neuronal growth and differentiation (Hur and Zhou, 2010; Kaidanovich-Beilin and Woodgett, 2011; Sutherland, 2011). In contrast to the two isoforms of GSK3s (GSK3α and GSK3β) in mammals, plants contain divergent GSK3-like kinases in their genomes (Yoo et al., 2006). In Arabidopsis, there are ten GSK3-like kinases. So far, Arabidopsis Shaggy/GSK3-like kinases (AtSK) appear to have diverse functions in the regulation of growth, responses for abiotic and biotic stress, flower and stomatal development (Gudesblat et al., 2012; Jonak and Hirt, 2002; Saidi et al., 2012). Of these, the molecular action of plant GSK3-like kinases was best understood in brassinosteroid (BR) signaling (Choe et al., 2002; He et al., 2002; Kim et al., 2009; 2012; Li and Nam, 2002; Peng et al., 2008; Perez-Perez et al., 2002; Rozhon et al., 2010).

BR is a steroid hormone that regulates a wide range of physiological responses and developmental processes of plants. BR-deficient and BR-insensitive mutants show pleiotropic defects including extreme dwarf, male sterility, dark-green and curled leaves, and abnormal skotomophogenesis, suggesting that BR plays essential roles in plant growth and development (Choe, 2004; Kim and Wang, 2010; Wang et al., 2012). Since the *bin2-1* mutant carrying E263K mutation was isolated as a BR-insensitive mutant (Choe et al., 2002; Li and Nam, 2002; Perez-Perez et al., 2002), genetic and biochemical analyses have illustrated that BIN2, a GSK3-like kinase, negatively regulates BR-responsive transcription factors, BZR1 and BZR2, leading to the inhibition of plant growth when BR levels are low. BIN2 phosphorylation of BZR1/2 causes the inhibition of BZR1/2 activity through multiple mechanisms such as cytoplasmic retention mediated by 14-3-3 protein, loss of DNA binding activity, and degradation by 26S proteasome (Gampala et al., 2007; He et al., 2002; Ryu et al., 2007; Vert and Chory, 2006; Zhao et al., 2002). Of ten GSK3-like kinases in Arabidopsis, recent studies suggest that seven members including BIN2 are involved in BR signaling (Kim et al., 2009; Rozhon et al., 2010). In particular, AtSK12 was shown to interact with and phosphorylate BZR1, like BIN2. Overexpression of AtSK12 in Arabidopsis causes a typical dwarf phenotype shown in BR-insensitive mutant. Fur-

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thermore, AtSK12 protein level was regulated by brassinolide (a most active BR) and BSU1 phosphatase, suggesting that AtSK12 also acts as a negative regulator in BR signal transduction pathway (Kim et al., 2009). Although many AtSKs appear to be involved in BR signaling, their specificities for substrates and interacting proteins in BR signaling are poorly understood.

Mammal GSK3 activity is inhibited by N-terminal Ser phosphorylation (Ser9 of GSK3β, Ser21 of GSK3α) and facilitated by Tyr phosphorylation (Tyr 216 of GSK3β, Tyr 279 of GSK3α) (Cohen and Goedert, 2004). Phosphorylated Ser 9/21 of GSK3β/α competitively binds to the primed substrate-binding pocket of GSK3, leading to the inhibition of GSK3 activity (Doble and Woodgett, 2003). However, plant GSK3-like kinases do not share the homology with N-terminal phosphorylation motif of mammal GSK3s (Jonak and Hirt, 2002). The functional role of N-terminal region of plant GSK3-like kinase remains unknown. Instead, it was shown that dephosphorylation of a Tyr residue equivalent to Tyr216/279 of human GSK3β/α is a key mechanism to inhibit plant GSK3-like kinase activity. BSU1 phosphatase inhibits BIN2 through direct dephosphorylation Tyr200 of BIN2 (Kim et al., 2009; 2011).

Recently, it was demonstrated that BIN2 integrates BR signaling and stomatal development in Arabidopsis (Gudesblat et al., 2012; Kim et al., 2012; Khan et al., 2013). Stomata formed by a pair of guard cells are essential for gas exchange and evaporation of water during photosynthesis and respiration throughout plant life. These specialized epidermal cells are established from protodermal cells through sequential asymmetric and symmetric cell divisions (Bergmann and Sack, 2007). In Arabidopsis, MPK3/6 activated by sequential phosphorylation through YODA MAPKKK and MKK4/5 negatively regulates stomatal development by direct phosphorylation of a bHLH transcription factor, SPEECHLESS, which initiates cell fate transition (Lampard et al., 2008). Previously, we showed that the *bin2-1* gain-of-function mutant and transgenic plant overexpressing BIN2 generate abnormal stomatal cluster, resulting in high ratio of stomata/pavement cells in epidermis of abaxial leaves. It was further demonstrated that BIN2 interacts with and inactivates YODA MAPKKK, leading to the acti-vation of SPEECHLESS. Bikinin, a specific inhibitor of GSK3-like kinases, strongly suppressed the stomatal cluster in both wild-type and the *bin2-1* gain-of-function mutant, indicating that BR-regulated GSK3-like kinases mediate not only cell growth but also stomatal development (Kim et al., 2012). However, func- tional relevance of other AtSKs in stomatal development remains unknown.

In this study, we further analyzed structure and function of AtSK12 belonging to subfamily I, in comparison with animal GSK3 and BIN2. Consistently, transgenic plants overexpressing AtSK12 lacking N-terminal region showed more severe dwarf phenotypes than those of full length AtSK12. The truncated AtSK12 without N-terminal region accumulates in the nucleus, indicating that nuclear localization of AtSK members is affected by their N-terminal sequence variation. Furthermore, our phenotypic and biochemical analysis strongly suggest that AtSK12 regulates cell growth through BZR1/BZR2 interaction, but not stomatal development or YODA MAPKKK activity.

MATERIALS AND METHODS

Plant materials

Arabidopsis thaliana Columbia-0 (Col-0) was used as the ecotype background of all transgenic plants. The Arabidopsis and tobacco (*Nicotiana benthamiana*) plants were grown in greenhouse under 16 h light/8 h dark cycles.

Plasmids

The coding sequences of wild-type or deleted form of AtSK12 were cloned into pENTR/SD/D-TOPO vectors (Invitrogen, USA). Mutations were generated by site-directed mutagenesis (Stratagene, USA). Each entry clones were subcloned into gateway compatible pDEST15 (GST), pEarleyGate 101 or 104 vectors by GateWay LR reactions. The sequences of oligos used in cloning are described in Supplementary Table 1.

Yeast two hybrid assays

Each cDNA was cloned into gateway compatible pXDGATcy86 (DNA binding domain) or the pGADT7 vector (activation domain). Combinations of BD- and AD-fusion plasmids were transformed into the yeast cells, and interaction was tested on Synthetic Dropout (SD) or SD-Histidine containing 0 to 5 mM 3-amino-1, 2, 4-triazole. All reagents related to yeast culture were purchased from Clontech (USA).

In vitro kinase assays

Induction and purification of proteins expressed from *E. coli* was performed as described previously (Tang et al., 2008). 0.5 μg of GST-AtSK12 or GST-Ndel-AtSK12 was incubated with 2 μg of MBP-BZR1 in the kinase buffer (20 mM Tris, pH 7.5, 1 mM $MgCl_2$, 100 mM NaCl and 1 mM DTT) containing 100 μM ATP and 10 μCi ^{32}P-γ -ATP at 30°C for 3 h. Kinase activity of ATSK12 and Ndel-AtSK12 was analyzed by SDS-PAGE followed by autoradiography.

Transgenic plants, transient expression, and microscopy

Agrobacterium strains GV3101 carrying various binary vector constructs were transformed into Arabidopsis by using the floral dipping method. Agrobacterium-mediated transient expression in tobacco was performed as described previously (Gampala et al., 2007). Fluorescence of YFP was analyzed by SP5 confocal microscope (Leica Microsystems, Germany).

Quantification of fluorescent signal

The ratio between nuclear and cytoplasmic fluorescent signals of YFP-tagged AtSK12 variants was quantified as previously described (Gampala et al., 2007). Briefly, a fixed size (18 pixels) was drawn from representative areas within the nucleus, cytoplasm, and background of each cell, and then integrated densities were measured. Five repeat measurements were performed for 10 independent cells expressing YFP-AtSK12 variants. Each value obtained from nucleus or cytoplasm was subtracted by average of background values and then nuclear to cytoplasmic ratio was calculated. All images were analyzed by ImageJ.

Stomatal observation

The 10 cotyledons of 8-dpg seedlings of each genotype were cleared in ethanol:acetic acid and dipped in chloral hydrate solution (Water:Chloralhydrate:Glycerol = 3:8:1, v/w/v) followed by mounting on slides. For quantification, 2-3 images at 400X magnification were captured per abaxial cotyledon using DIC microscopy (Nikon, Eclipse 80i). Statistical analysis was performed by Sigmaplot software (SystatSoftware Inc., USA).

RESULTS

Characteristics of protein sequence of Arabidopsis GSK3-like kinases

Two isoforms (GSK3α and GSK3β) of mammal GSK3s share almost identical kinase domain, but differ mainly in their N-

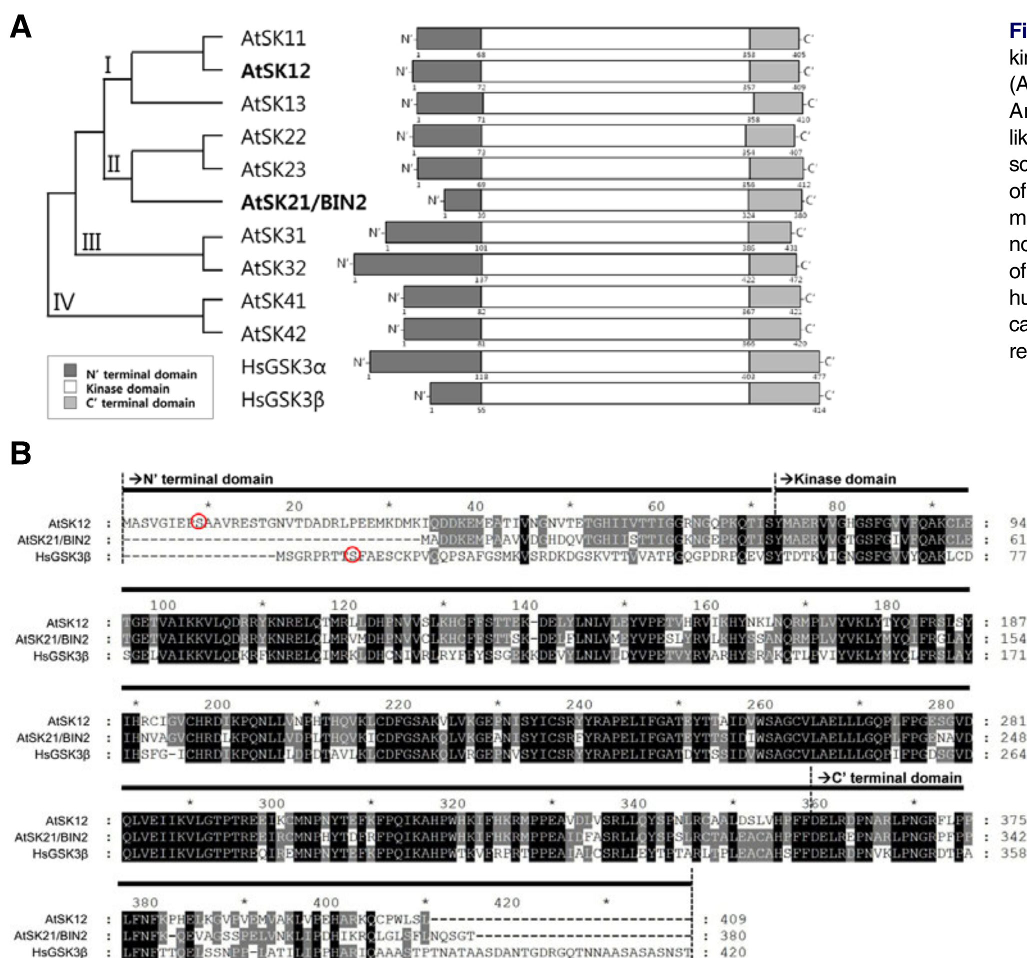

Fig. 1. Arabidopsis GSK3-like kinases and their structures. (A) Phylogenetic tree of the Arabidopsis GSK3/Shaggy-like kinases (AtSKs) (left) and schematic domain structure of AtSKs compared with human GSK3s (Right). (B) Amino acid sequence alignment of AtSK12, AtSK21/BIN2, and human GSK3β. Circle indicates Ser 9 in N-terminal region of GSKs.

terminal domains. AtSK members also have highly conserved kinase domains and variable N-terminal regions (Fig. 1A). However, it is not known whether N-terminal domains of AtSKs have any functional role or not. Because BIN2 has a very short length of N-terminal region compared with mammal GSK3s and other nine AtSKs (Figs. 1A and 1B), we selected AtSK12 belonging to subfamily I as a model to address functional role of N-terminal region of AtSKs. AtSK12 contains longer N-terminal extension (31 amino acids) than BIN2.

AtSK12 binding to BR signaling components in yeast cells

To test whether AtSK12 has a functional difference compared with BIN2 in BR signaling, we first examined the interaction of AtSK12 to BR signaling components. In the yeast two-hybrid assays, AtSK12 interacted with BZR1 but not with BRI1 or BIN2 (Supplementary Fig. 1). When substrate specificities between AtSK12 and BIN2 were compared by using BZR1 and BZR2 as well-known substrates, both AtSK12 and BIN2 interacted with BZR1 and BZR2 without any preference in their binding (Fig. 2A). Next, we created N-terminal 31 amino acid deletion (Ndel), C-terminal 29 amino acid deletion (Cdel), or E297K (corresponding to the bin2-1 gain-of-function mutation) mutation on AtSK12, and compared their binding specificity with BZR1. Deletion of N-terminal region or E297K mutation in AtSK12 did not affect BZR1 binding activity in the yeast cells. Interestingly, C-terminal deletion abolished the AtSK12 binding to BZR1, suggesting that C-terminal region composed of 29 amino acids is responsible for BZR1 binding (Fig. 2B).

Phenotype of transgenic plants overexpressing N-terminal-deleted AtSK12

To further investigate the functional role of N-terminal region of AtSK12 *in vivo*, we generated transgenic Arabidopsis plants overexpressing wild-type AtSK12 or mutant AtSK12 (Ndel-AtSK12 or E297K-AtSK12). As shown in Fig. 3, T1 transgenic plants displayed a wide range of phenotypes from weak to extreme dwarf, reminiscent of plants overexpressing BIN2 or *bin2-1* (E263K-BIN2) (Li and Nam, 2002). The percentage of transgenic plants showing strong phenotype was significantly higher in the T1 population transformed with E297K-AtSK12 than that of the wild-type AtSK12 (Fig. 3). This result indicates that 297th glutamic acid of AtSK12 has same functional role with 263rd glutamic acid of BIN2. Importantly, N-terminal deletion of AtSK12 significantly increased the percentage of T1 population showing a strong phenotype compared with that of wild-type AtSK12 (Fig. 3). When mRNA and protein levels of transgene were compared in individual T1 plants overexpressing wild-type or Ndel-AtSK12, strong phenotype of Ndel-AtSK12 transgenic plants was not due to higher level of mRNA or protein (Supplementary Fig. 2). This result strongly suggests that

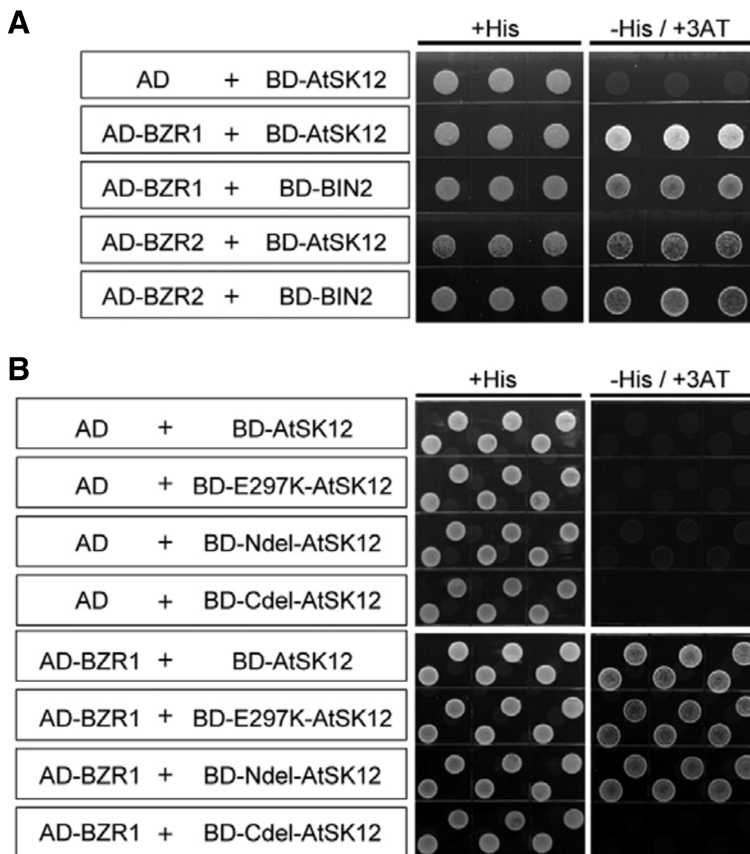

Fig. 2. Interaction between AtSK12 and BZR1 or BZR2 in yeast two-hybrid assays. (A) Both AtSK12 and BIN2 interact with BZR1 as well as BZR2. (B) The effect of mutation on AtSK12 was assayed by BZR1 interaction in yeast cells. Various AtSKs (wild-type, N-terminal 31 amino acids deletion, C-terminal 29 amino acids deletion) were fused to DNA binding domain (BD) while BZR1 or BZR2 was fused to activation domain (AD). Yeast growth on -His indicates interaction. 3-Amino-1,2,4-triazole (3-AT) was added as a competitive inhibitor of HIS3 enzyme.

N-terminal region of AtSK12 negatively regulates AtSK12 action in plant cells.

Nuclear accumulation of N-terminal-deleted AtSK12

Although N-terminal amino acid sequences of AtSK12 do not share homology with that of the human GSK3β, the N-terminal region of AtSK12, but not BIN2, contains Ser residue at 9th position, which is a critical phosphorylation site in regulation of the human GSK3β (Fig. 1B). To examine possible role of Ser9 phosphorylation in AtSK12, we generated S9A mutation on AtSK12 or E297K-AtSK12, and transformed it into wild-type Arabidopsis. However, S9A mutation did not affect phenotypic strength in T1 transgenic plants compared with its control, confirming that a loss of Ser9 in AtSK12 is not related to strong activity of Ndel-AtSK12 (Supplementary Fig. 3), and Ser9 of AtSK12 does not contribute to the regulation of AtSK12 activity, unlike the human GSK3β.

To understand how overexpression of AtSK12 possessing N-terminal deletion caused stronger phenotype than that of wild-type AtSK12 in Arabidopsis, we compared kinase activity between wild-type and Ndel-AtSK12 for BZR1 substrate. However, kinase activity phosphorylating BZR1 was very slightly decreased by deletion of N-terminal domain of AtSK12 in *in vitro* kinase assay (Supplementary Fig. 4). This result suggests that strong phenotype caused by Ndel-AtSK12 in transgenic Arabidopsis is not due to a change of catalytic activity.

It was previously reported that nuclear localization of BIN2 is crucial for BZR2 regulation. Transgenic plants overexpressing BIN2 tagged with nuclear localization signal show much stronger dwarf phenotype than those of wild-type BIN2 (Vert and Chory, 2006). This prompted us to investigate subcellular localization of AtSK12. When subcellular localization of yellow fluorescent protein (YFP)-fused AtSK12, Ndel-AtSK12, and E297K-AtSK12 was observed by transient expression in a tobacco leaf epidermis, the fluorescent signal of wild-type AtSK12 was detected both in nucleus and cytoplasm similar to BIN2 (Fig. 4). While YFP-E297K-AtSK12 showed slightly increased nuclear/cytoplasmic ratio compared with wild-type AtSK12, N-terminal deletion greatly increased nuclear/cytoplasmic ratio of AtSK12 (Fig. 4). Moreover, nuclear/cytoplasmic ratio of Ndel-AtSK12 was also significantly increased in transgenic Arabidopsis (Supplementary Fig. 5). This observation strongly suggests that N-terminal region of AtSK12 negatively regulates nuclear localization of the protein. Similar to BIN2, enhanced nuclear localization of AtSK12 will more effectively inhibit BR-responsive transcription factors BZR1/2.

AtSK12 regulates cell growth but not stomatal development

Recently, we demonstrated that BIN2 functions as a key regulator in stomatal development of Arabidopsis leaves (Kim et al., 2012). Plants overexpressing the BIN2 and *bin2-1* mutants showed abnormal stomatal clusters and an increased number of stomata in the abaxial leaf epidermis. To further compare the function of AtSK12 and BIN2, we investigated the functional involvement of AtSK12 in stomatal development. Surprisingly, unlike BIN2 overexpression plants, transgenic plants overexpressing wild-type AtSK12 or mutant AtSK12 showed very subtle phenotypes in stomatal development compared with BIN2

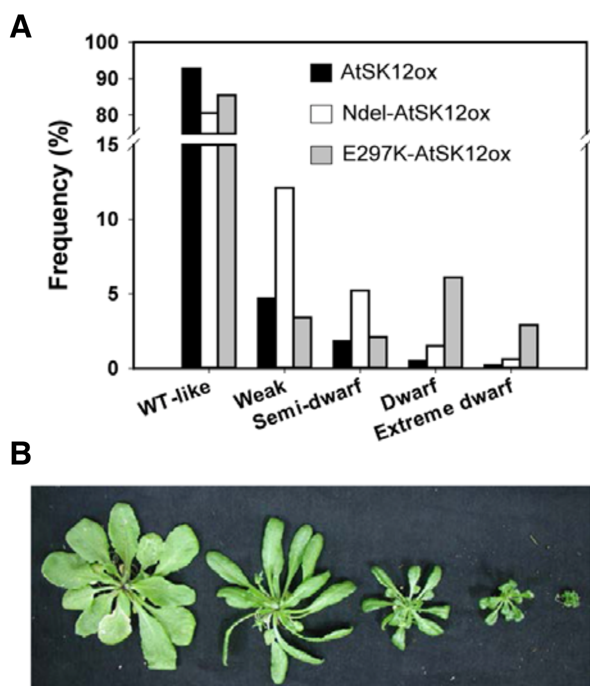

Fig. 3. Phenotypes of transgenic Arabidopsis plants overexpressing WT AtSK12, Ndel-AtSK12, or E297K-AtSK12. (A) Phenotypic frequency of each T1 transgenic plants. A total of 1009, 1166, and 724 T1 transgenic plants were counted, respectively. (B) Various phenotypes of T1 transgenic plants overexpressing Ndel-AtSK12. Phenotype categories indicated in (A) was classified based on these phenotypes.

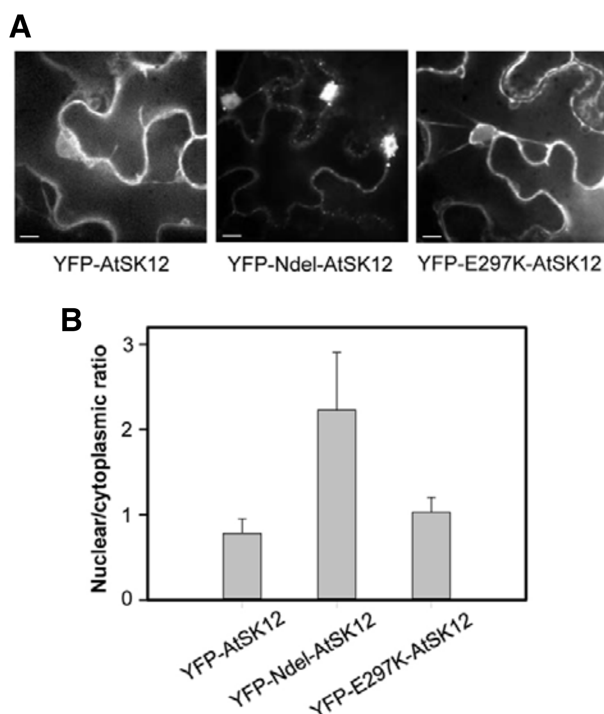

Fig. 4. Subcellular localization of YFP-tagged WT, Ndel-AtSK12, and E297K-AtSK12. (A) The indicated constructs were transformed into tobacco leaf epidermal cells. (B) Quantification of nuclear/cytoplasmic ratio. Scale bars, 10 um.

overexpression plants. Stomatal index was similar to wild-type plant and stomatal clusters were rarely observed (Fig. 5). This result indicates that AtSK12 regulates cell growth, but not stomatal cell development, while BIN2 modulates both.

To further address functional difference of Arabidopsis GSK3-like kinases, we cloned ten GSK3-like kinases and examined interaction with YODA MAPKKK in yeast cells. Consistent to stomatal phenotype, three GSK3-like kinases belonging to subfamily II including BIN2 strongly interacted with YODA MAPKKK while other GSK3-like kinases including AtSK12 showed very weak or no interaction with YODA MAPKKK (Fig. 6). Thus, of seven GSK3-like kinases regulating cell growth through BZR1 interaction, AtSK21, 22, and 23 mainly interact with YODA, which controls stomatal development.

DISCUSSION

Functional roles of N-terminal and C-terminal regions of Arabidopsis GSK3-like kinase

In this study, we analyzed the relationship between structure and function of Arabidopsis GSK3-like kinases. While the deletion of C-terminal of AtSK12 abolished AtSK12 interaction to BZR1 in yeast cells, interaction strength of Ndel-AtSK12 to BZR1 was not altered compared with full-length of AtSK12. Our genetic studies indicated that AtSK12 is more active when N-terminal 31 amino acids are deleted. When Ndel-AtSK12 was overexpressed in wild-type plants, the frequency of the dwarf mutant phenotype is significantly increased than that of full-length AtSK12, indicating that N-terminal region of AtSK12 has an inhibitory role in action of AtSK12 (Fig. 3). N-terminal region of mammal GSK3s also appears to have an inhibitory function in regulating GSK3 activities. Several kinases such as Akt, Protein kinase A, and ribosomal S6 kinase phosphorylate Ser 9/21 of GSK3β/α. Then, phosphorylated Ser 9/21 residue of GSK3β/α competes with primed phosphate of substrate in substrate binding pocket, resulting in reduction of GSK3 phosphorylation of substrate (Kaidanovich-Beilin and Woodgett, 2011). However, Arabidopsis GSK3-like kinases have variable N-terminal domains, which do not contain Ser 9/21 phosphorylation motif conserved in mammals. Nevertheless, N-terminal region of AtSK12 possesses a Ser residue at 9th position of protein sequence. However, the S9A mutation on AtSK12 did not change phenotypic frequency of transgenic T1 population, indicating that Ser 9 phosphorylation of AtSK12 is not responsible for regulation of AtSK12 activity (Supplementary Fig. 3).

Our microscopic analysis indicates that YFP-Ndel-AtSK12 accumulates more in the nucleus than YFP-AtSK12 and YFP-E297K-AtSK12. It was reported that nuclear localization of BIN2 increases the BIN2 suppression of BZR1/2, leading to the inhibition of plant growth (Vert and Chory, 2006). Our results suggest that N-terminal region of AtSK12 contributes to modulate nuclear localization of AtSK12 in plant cells. We could not detect dominant nuclear localization of E297K-AtSK12 compared with Ndel-AtSK12 although transgenic plants overexpressing E297K-AtSK12 showed more severe phenotypes. It seems that a strong phenotype of E297K-AtSK12 is mainly due to escaping BSU1 regulation (Kim et al., 2009). It remains unknown how subcellular localization of AtSKs is regulated. AtSKs may interact with specific protein responsible for nuclear locali

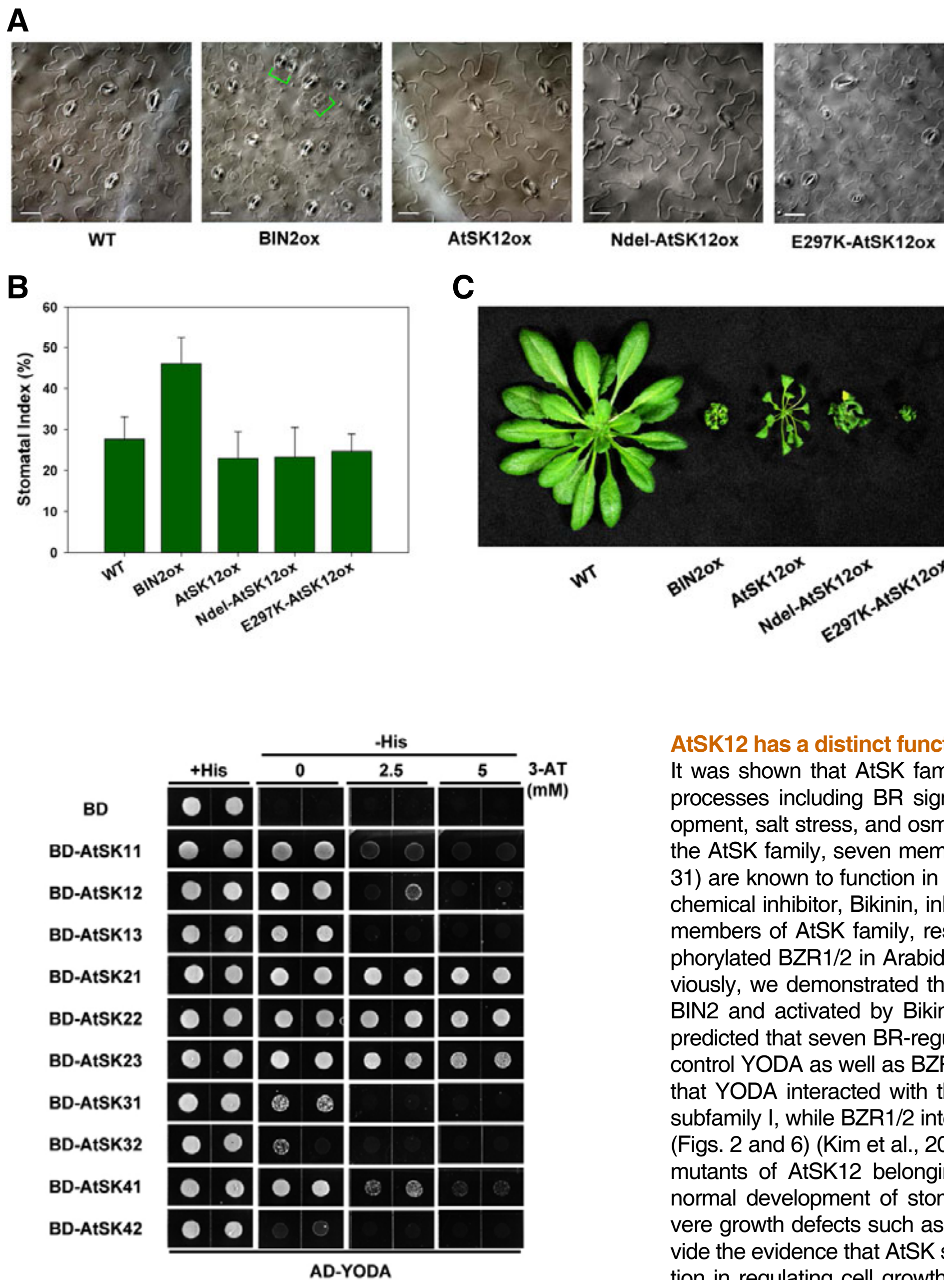

Fig. 5. Stomatal and growth phenotypes of transgenic plants overexpressing BIN2 or AtSK12 variants. (A) Differential interference contrast (DIC) microscopy images of abaxial leaf epidermis of Arabidopsis overexpressing BIN2, WT AtSK12, Ndel-AtSK12, and E297K-AtSK12. Brackets indicate stomatal cluster. Scale bar indicates 50 um. (B) Quantification of stomatal cells of plants shown in (A). Error bars indicate standard deviation. (C) Growth phenotype of 4-week-old transgenic plants overexpressing BIN2 or AtSK12 variants used in stomatal observation. AtSK12ox, Ndel-AtSK12ox, and E297K-AtSK12ox plant represent phenotype of semi-dwarf, dwarf, and extreme dwarf categorized in Fig 3, respectively.

Fig. 6. YODA interacts with AtSKs belonging to subfamily II. BD-fused ten AtSKs were transformed into yeast cells expressing AD-fused YODA. Yeast growth on medium containing high concentration of 3-AT indicates high affinity binding between YODA and AtSKs.

zation or they can be anchored by fatty acid acylation. We found that N-terminal region of AtSK12 contains putative myristoylation site (Gly 5). However, G5A mutation did not change localization of AtSK12, suggesting that AtSK12 is not anchored by fatty acid acylation (Supplementary Fig. 6). Taken together, our studies provide the evidence that subcellular localization of AtSK12 is regulated by N-terminal sequence of AtSK12. Thus, it seems that regulation mechanism mediated by N-terminal region of GSK3s is different in mammals and Arabidopsis.

AtSK12 has a distinct function compared with BIN2

It was shown that AtSK family mediates various physiological processes including BR signaling, stomatal and flower development, salt stress, and osmotic stress. Of the ten members of the AtSK family, seven members (AtSK11,12,13,21,22,23, and 31) are known to function in BR signaling (Saidi et al., 2012). A chemical inhibitor, Bikinin, inhibits the kinase activities of seven members of AtSK family, resulting in accumulation of dephosphorylated BZR1/2 in Arabidopsis (De Rybel et al., 2009). Previously, we demonstrated that YODA MAPKKK is inhibited by BIN2 and activated by Bikinin treatment. Thus, it was simply predicted that seven BR-regulated AtSK family members might control YODA as well as BZR1/2. However, our results showed that YODA interacted with the AtSK subfamily II, but not with subfamily I, while BZR1/2 interacted with both subfamily I and II (Figs. 2 and 6) (Kim et al., 2009). Consistently, gain-of-func-tion mutants of AtSK12 belonging to subfamily I showed almost normal development of stomatal cell, although they have severe growth defects such as a dwarfism (Fig. 5). Our data provide the evidence that AtSK subfamily I and II have similar function in regulating cell growth, but distinct roles in stomatal cell differentiation. Our study suggests that different AtSK members may have overlapping as well as specific functions.

Each member of the AtSK family shares highly conserved kinase domain. Given that amino acid sequences in N- and C-terminal region of AtSK subfamily I and II are highly variable, it seems that each terminal region flanking kinase domain may be responsible for determination of substrate specificity. Indeed, our data support that C-terminal region of AtSK12 is essential for BZR1 binding. Comparison of different substrate binding using mutation in C-terminal region will expand our understanding about relationship between structure and function of plant GSK3s proteins.

Note: Supplementary information is available on the Molecules and Cells website (www.molcells.org).

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