EXTRA-LARGE G PROTEINs Interact with E3 Ligases PUB4 and PUB2 and Function in Cytokinin and Developmental Processes[1][OPEN]

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Heterotrimeric GTP-binding proteins (G proteins) composed of Gα, Gβ, and Gγ subunits are conserved signal transduction molecules in animals and plants. In Arabidopsis (*Arabidopsis thaliana*), there are three Gα-like proteins named EXTRA-LARGE G PROTEINs (XLGs) in addition to the canonical Gα protein GPA1. XLGs have been reported to be implicated in multiple pathways, although the underlying mechanisms of their action remain elusive. Here, we report that all three XLGs interact with two closely related plant U-box (PUB) E3 ligases, PUB2 and PUB4. Three XLGs are predominantly localized at the plasma membrane, whereas XLG2 and XLG3 also show nuclear localization. The interactions between PUB2/4 and XLGs suggest that they might function in the same pathways. Indeed, we found that a newly obtained *xlg1/2/3* triple knockout mutant, the *pub4* mutant, and the *pub2/4* double mutant all exhibited defects in cytokinin responses, stamen development, tapetum development, and male fertility. However, the *xlg* single mutants and the *pub2* mutant did not exhibit an obvious defect in these processes, which suggests functional redundancy among the three XLGs and between PUB2 and PUB4. Overexpressing *ARR10* to enhance the cytokinin response in *pub4* or in the *xlg1/2/3* triple mutant partially restored several phenotypes caused by the *pub4* and *xlg1/2/3* mutations. Our findings reveal that the XLGs and PUB2/4 are components in the complex cytokinin signaling networks regulating many developmental and physiological processes.

Heterotrimeric GTP-binding proteins (G proteins) are highly conserved signaling components in eukaryotes. G proteins consist of Gα, Gβ, and Gγ subunits (Hamm, 1998; Oldham and Hamm, 2008). In animals, G proteins are typically regulated by a G-protein-coupled receptor (GPCR). Following recognition of a ligand, GPCR is activated and recruits a G protein. GDP-bound Gα then becomes GTP-bound and separated from Gβγ to activate downstream effectors (Wettschureck and Offermanns, 2005; Li et al., 2007; Oldham and Hamm, 2008).

Arabidopsis (*Arabidopsis thaliana*) contains one canonical Gα (GPA1), one Gβ (AGB1), and three Gγ proteins (AGGs; Urano et al., 2013). In addition, the Arabidopsis genome encodes three EXTRA-LARGE G PROTEINs (XLGs), which have a Gα-like C terminus and an N-terminal region that shares homology among the three XLG proteins but has little similarity to other proteins (Ding et al., 2008). Like canonical Gα proteins, the C-terminal Gα-like domain has the ability to bind and hydrolyze GTP in the presence of Ca^{2+} instead of Mg^{2+} (Heo et al., 2012). All three XLGs were found predominantly localized in the plasma membrane, whereas XLG2 and XLG3 were also found in nuclei (Chakravorty et al., 2015; Maruta et al., 2015). XLGs interact and form complexes with Gβγ and have been reported to share similar function with Gβ and Gγ in many developmental processes and stress responses (Zhu et al., 2009; Chakravorty et al., 2015; Maruta et al., 2015). Like *agb1*, *xlg3* mutants were impaired in the root waving and skewing response and were hypersensitive to ethylene (Pandey et al., 2008). An *xlg* triple mutant, *agb1*, and *agg1/2* also showed increased sensitivity to salt, tunicamycin, and D-Glc and had an increased stomatal density (Chakravorty et al., 2015). XLGs, AGB1, and AGG1/2 are known to be positive regulators of defense responses to several pathogens, and their loss-of-function mutants were compromised in the pathogen-associated molecular pattern-triggered oxidative burst, in disease resistance, or in BIR1-mediated defense

signaling and programmed cell death (Zhu et al., 2009; Delgado-Cerezo et al., 2012; Lee et al., 2013; Liu et al., 2013; Maruta et al., 2015; Liang et al., 2016). XLG2 also interacts with RELATED TO VERNALIZATION1 (RTV1) and is involved in RTV1-mediated flowering time control (Heo et al., 2012).

Ubiquitin-mediated protein degradation is a key regulatory mechanism to modulate protein activities in a wide variety of fundamental cellular processes. In this process, ubiquitin is first activated by the ubiquitin activating enzyme (E1), then transferred to an ubiquitin conjugating enzyme (E2), and finally added to a specific Lys residue of target proteins by ubiquitin ligase (E3). Polyubiquitinated proteins are generally targeted for degradation by the 26S proteasome. Monoubiquitination, however, often regulates protein activities. E3 proteins are highly diversified and are responsible for selecting specific target proteins for ubiquitination. E3 ligases are classified into four categories: HECT, RING-finger, U-box, and PHD-finger. Plant U-box proteins (PUBs) are classified into several subgroups based on the other domains besides the U-box (Yee and Goring, 2009). One subgroup contains Armadillo (ARM) repeats, which are often involved in protein-protein interactions (Hatzfeld, 1999; Samuel et al., 2006). PUB proteins are known to function in diverse biological pathways. *Brassica* ARC1 interacts with an S-locus receptor kinase to regulate self-incompatibility (Stone et al., 2003). Arabidopsis PUB12 and PUB13 ubiquitinate FLAGELLIN-SENSITIVE2 (FLS2) and regulate FLS2-mediated immunity (Lu et al., 2011). Arabidopsis *pub4* mutation causes aberrant expansion and incomplete degeneration of tapetal cells (Wang et al., 2013). The *pub4* mutant also shows increased cell proliferation in both shoot apical meristem and root meristem (Kinoshita et al., 2015a, 2015b). A recent report also shows that PUB4 is involved in individual chloroplast quality control upon oxidative stress (Woodson et al., 2015).

Here, we report the identification of PUB2 and PUB4 as XLG-interacting proteins. A newly generated *xlg1/2/3* triple mutant, the *pub4* single mutant, and the *pub2/4* double mutant were found to exhibit similar morphological phenotypes and were compromised in the cytokinin response. They were also defective in stamen and tapetum development. Overexpression of *ARR10* (a positive regulator of cytokinin signaling) partially restored the phenotypes of *pub4* and the *xlg* triple mutant. Our study indicates that PUB2/4 and XLGs play important roles in multiple developmental and physiological responses and function in the cytokinin responses.

RESULTS

XLGs Interact with PUB4 and PUB2 in Yeast

We performed yeast two-hybrid (Y2H) screen to identify XLG2-interacting proteins. Full-length XLG2 was fused to the GAL4 DNA-binding domain (BD) and used as a bait to screen a normalized Arabidopsis Y2H cDNA library in which each cDNA was fused to the activation domain (AD) sequence. About 5×10^6 clones were screened on the synthetic defined (SD) selection medium ($-$Trp, $-$Leu, $+$X-α-Gal, and $+$aureobasidin A). Positive clones were further grown on the SD medium lacking Trp, Leu, Ade, and His. A total of 37 clones were obtained from the screen. Among them, 12 clones harbored PUB2-encoding sequences and five clones harbored PUB4-encoding sequences. In a similar Y2H screen using the full-length PUB4 as bait, 16 XLG1 clones and seven XLG2 clones were identified among the positive colonies harboring putative PUB4-interacting proteins.

To verify and determine the interactions between XLGs with PUB2 and PUB4 in the Y2H system, all three XLGs (full length) were further tested for their interactions with PUB2 and PUB4. XLG1 and the N terminus of XLG1 were found to cause autoactivation when they were fused to Gal4 DNA-binding domain (Fig. 1A), but the autoactivation could be suppressed by adding 5 mmol 3-amino-1,2,4-triazole (3-AT; Fig. 1B). All of the diploids with AD-PUB2/4 and BD-XLGs could grow on plates with SD/$-$Ade/$-$His/$-$Leu/$-$Trp/X-α-Gal/Aureobasidin A and activate β-galactosidase activity. 3-AT didn't impair growth of these diploids or eliminate β-galactosidase activity (Fig. 1B). These data
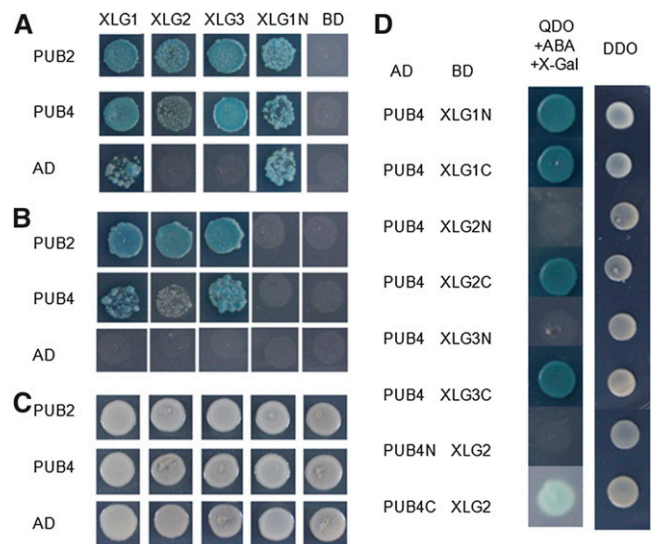

Figure 1. Interactions of XLGs with PUB2/4 in yeast. A, Diploids with BD-XLGs and AD-PUB2/4 were grown on SD plates with SD/$-$Ade/$-$His/$-$Leu/$-$Trp/X-α-Gal (40 μg/mL)/aureobasidin A (200 ng/mL) for 4 d. B, Diploids with BD-XLGs and AD-PUB2/4 were grown on SD plates with SD/$-$Ade/$-$His/$-$Leu/$-$Trp/X-α-Gal (40 μg/mL)/aureobasidin A (200 ng/mL) plus 5 mmol 3-AT for 4 d. C, Diploids with BD-XLGs and AD-PUB2/4 were grown on SD plates with SD/$-$Leu/$-$Trp for 3 d. D, Diploids with full-length/truncated BD-XLGs and full-length/truncated AD-PUB4 were grown on SD plates with SD/$-$Ade/$-$His/$-$Leu/$-$Trp/X-α-Gal (40 μg/mL)/aureobasidin A (125 ng/mL) for 4 d (left) and SD plate with SD/$-$Leu/$-$Trp for 3 d (right). XLG1N, Amino acids 1 to 481 of XLG1; XLG1C, amino acids 482 to 888 of XLG1; XLG2N, amino acids 1 to 460 of XLG2; XLG2C, amino acids 461 to 861 of XLG2; XLG3N, amino acids 1 to 427 of XLG3; XLG3C, amino acids 428 to 839 of XLG3; PUB4N, amino acids 1 to 525 of PUB4; PUB4C, amino acids 526 to 829 of PUB4.

indicate that the three XLGs could interact with both PUB2 and PUB4 in yeast.

To further determine which regions of PUB4 and XLGs interact in yeast, the C terminus containing the ARM-repeat domain (526–829) and N terminus (1–525) of PUB4 were tested for their interactions with XLG2. In addition, the N-terminal regions and the C-terminal Gα domain of all three XLGs were tested for their interaction with PUB4. It was found that the interactions between PUB4 and XLGs all occurred between the C-terminal Gα regions of XLGs and the C-terminal ARM-repeat domains of PUB4 (Fig. 1D; Supplemental Fig. S1A).

XLGs Interact with PUB4 and PUB2 in Plants

We then tested the interactions of PUB2/4 with XLGs in planta. First, the localization of XLGs was determined by transient expression of the XLG proteins fused to GFP in tobacco (*Nicotiana benthamiana*). As shown in Figure 2A, the XLG1-GFP protein was mainly localized to the plasma membrane, while XLG2-GFP and XLG3-GFP were localized to both plasma membrane and nuclei in tobacco (Fig. 2A). The subcellular localization patterns of the XLG proteins are consistent with recent reports (Chakravorty et al., 2015; Maruta et al., 2015). PUB4 was previously reported to be localized in cytoplasm, whereas the interactions between PUB2/4 and XLGs would suggest that PUB2 and PUB4 might also be localized in the plasma membrane. We evaluated the possibility that the previous studies might not yield sufficient resolution to distinguish between the cytosol- and plasma membrane-localized fluorescence signals. We extracted membrane fraction and soluble fraction from PUB4-flag transgenic plants. Although most PUB4 proteins were apparently in the cytosol, some PUB4 proteins could be detected as membrane-associated (Supplemental Fig. S2A). Using the plasma membrane marker PIP2A (Nelson et al., 2007) as the control, it was shown that the PUB2-GFP and PUB4-GFP signals were colocalized with PIP2A at the plasma membrane in
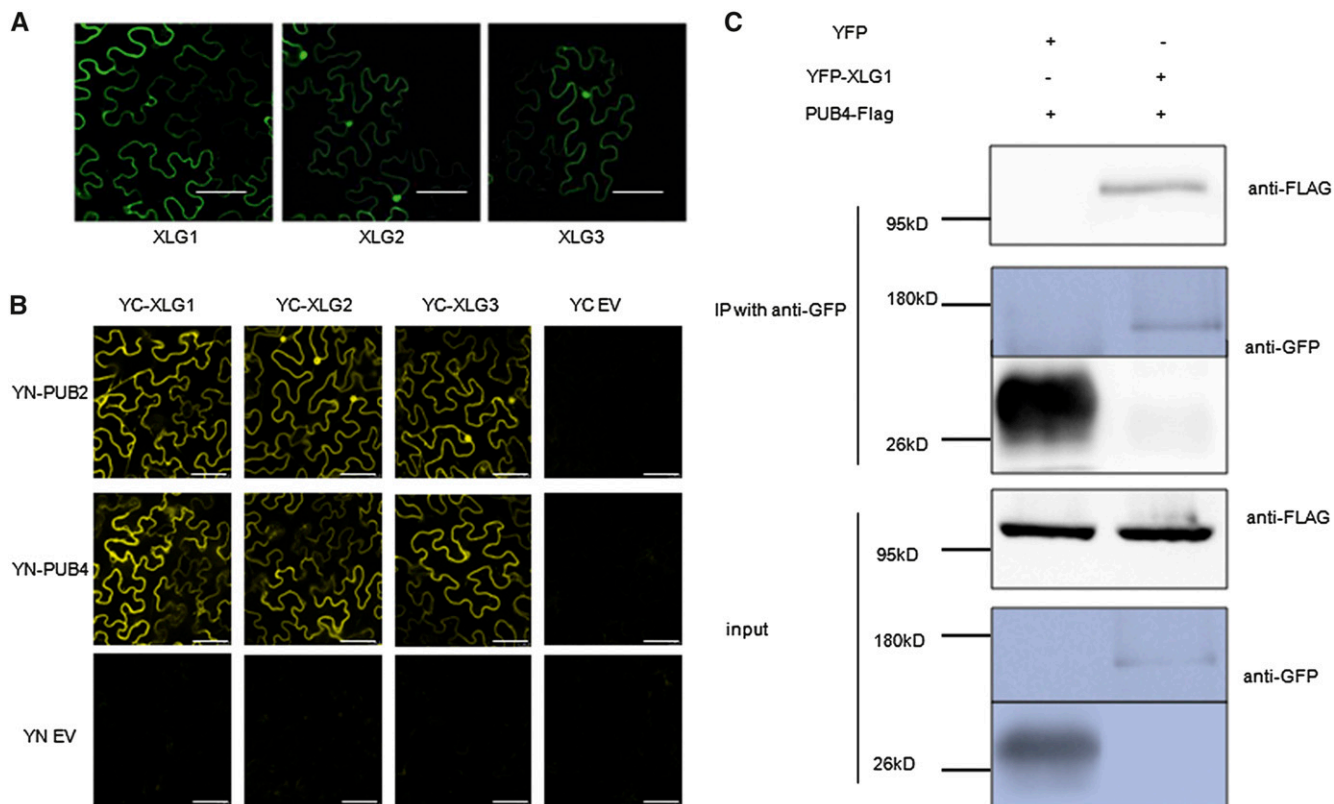

Figure 2. XLGs interact with PUB2/4 in planta. A, The fluorescent signal in cells of tobacco following transient expression of XLG1-GFP (left), XLG2-GFP (middle), and XLG3-GFP (right). Localization of the XLG-GFP fusion proteins was analyzed 48 h after agrobacterial infiltration by confocal laser scanning microscopy. Bars = 80 μm. B, XLGs interact with PUB2/4 in tobacco as revealed by BiFC assay. The PUB2/4 proteins were fused to the N terminus of YFP, and the XLG proteins were fused to the C terminus of YFP. YN and YC refer to the empty vectors with just the N terminus or C terminus of YFP, respectively. The signal was observed 48 h after infiltration. Bars = 61.5 μm. C, Co-IP assay showed that XLG1 interacts with PUB4. Total protein was extracted from transgenic plants expressing both YFP-tagged XLG1 and FLAG-tagged PUB4, and plants expressing both YFP and FLAG-tagged PUB4 were used as a negative control. Proteins were immunoprecipitated with anti-GFP magnetic beads, and the precipitated proteins were detected using a FLAG antibody. PUB4-FLAG could be coimmunoprecipitated with YFP-XLG1 but not with YFP.

tobacco cells (Supplemental Fig. S2B). Similarly, colocalization of PUB4-CFP and YFP-XLG3 was also observed in the tobacco cells (Supplemental Fig. S2C).

Interactions between PUB2/4 and XLGs in planta were examined by bimolecular fluorescence complementation (BiFC) assay. PUB2 and PUB4 were fused to the N terminus of eYFP (YN), and XLGs were fused to the C terminus of eYFP (YC). Each pair of YN-PUB and YC-XLG constructs was delivered into tobacco cells via *Agrobacterium tumefaciens* infiltration. No fluorescence appeared with coinfiltration of empty YN with YC-XLGs or YC with YN-PUB2/4. Reconstituted YFP fluorescence suggested that the interactions between PUB4 and XLGs predominantly occurred in the plasma membrane, whereas PUB2 also interacted with XLG2 and XLG3 in the nuclei (Fig. 2B).

To further confirm the interactions between these two PUBs and XLGs, XLG1 and PUB4 were chosen for coimmunoprecipitation (co-IP) assays using transgenic plants expressing the PUB4-FLAG and YFP-XLG1 fusion proteins. The plants carrying PUB4-FLAG and YFP were used as negative controls. YFP-XLG1 was immunoprecipitated from total leaf protein extract with GFP antibodies, and the co-IPed proteins were then detected using a FLAG antibody (Fig. 2C). PUB4-FLAG was only detected in the precipitate from leaves that expressed both YFP-XLG1 and PUB4-FLAG, but not in that of the negative control, indicating that XLG1 and PUB4 interact in planta.

PUB2 and PUB4 Are Partially Functionally Redundant

The PUB E3 ligase family has more than 60 members in Arabidopsis (Yee and Goring, 2009). Among them, PUB2 shares the highest identity with PUB4. Morphological phenotypes caused by loss-of-function mutation of PUB4 have been reported (Wang et al., 2013) and include slightly smaller rosette sizes and low fertility at

22°C. We obtained a *pub2* mutant (SALK_086656), which contains a T-DNA insertion at the fourth intron and is apparently a knockout allele since we could not detect any *PUB2* transcript by reverse transcription-PCR (Supplemental Fig. S3). The *pub2* mutant did not show any obvious morphological phenotype. The *PUB2* gene appears to be expressed at a lower level than *PUB4* in most tissues according to the public data from Affymetrix Arabidopsis ATH1 Genome Array Data (https://genevisible.com/tissues/AT/Ensembl%20Gene/AT5G67340 and https://genevisible.com/tissues/AT/Ensembl%20Gene/AT2G23140; Supplemental Fig. S4). We also analyzed several sets of microarray and RNA-seq data from EBI-ArrayExpress (http://www.ebi.ac.uk/arrayexpress/). *PUB4* appears expressed at a higher level than *PUB2* in shoot, seedlings, stems, flower buds, pollen, and siliques, while *PUB2* had a much higher expression level in stigma. When treated with either auxin or cytokinin, *PUB4* was found to be expressed at a much higher level than *PUB2* (Zhao et al., 2010).

When PUB2 was ectopically overexpressed under the control of the 35S promoter in the *pub4* mutant, 36 of 43 independent transgenic lines displayed different degrees of fertility restoration (Supplemental Fig. S5). Furthermore, the *pub2 pub4* (*pub2/4*) double mutant is much smaller than *pub4* (Fig. 3, A and B). The above results indicate that PUB2 and PUB4 might have overlapping functions.

XLG1, XLG2, and XLG3 Have Overlapping Functions and Their Triple Mutant Shares Similar Phenotypes to Those of *pub4*

Previous studies revealed that the *xlg* mutants exhibited multiple developmental phenotypes and showed altered responses to both abiotic and biotic

Figure 3. Morphological phenotypes of *pub4*, *pub2/4*, and *xlg1*/2/3*. A, Four-week-old plants of wild type (WT), *pub4*, *pub2/4*, and *xlg1*/2/3* grown in soil under the short-day conditions. Bar = 1 cm. B, Six-week-old plants of wild type, *pub4*, *pub2/4*, and *xlg1*/2/3* grown in soil under the long-day conditions. Bar = 2 cm. C, Nine-day-old seedlings of wild type, *pub4*, *pub2/4*, and *xlg1*/2/3* grown on Murashige and Skoog plates under long-day conditions. Bar = 1 cm. D, Adventitious root numbers of 9-d-old seedlings of wild type, *pub4*, *pub2/4*, and *xlg1*/2/3*. Vertical bars represent mean ± SE ($n >$ 20). Asterisks indicate statistical differences to the wild type ($P < 0.05$).
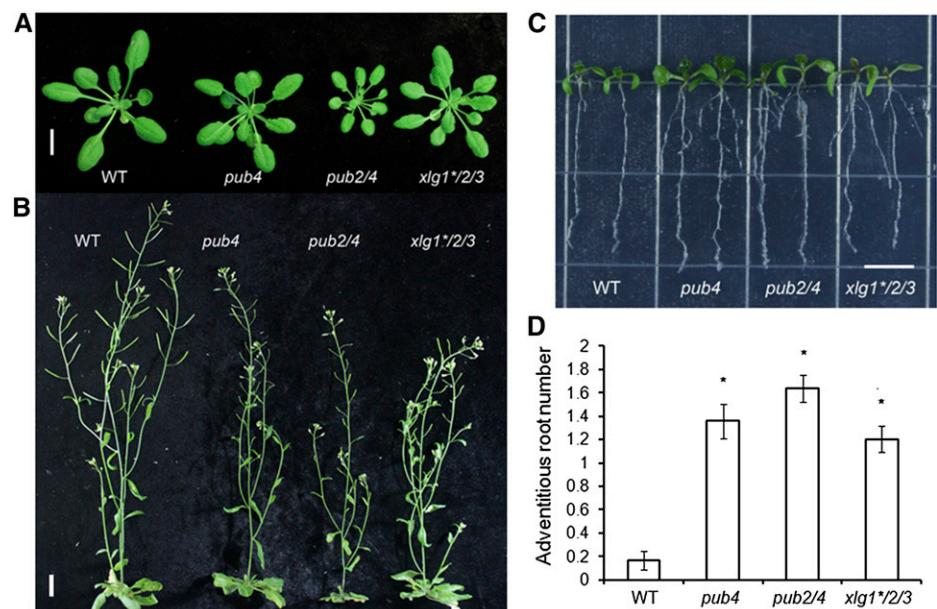
stresses. However, under normal conditions, the *xlg1-1 xlg2-1 xlg3-1* triple mutant in the previous reports lacked an obvious morphological phenotype (Ding et al., 2008; Pandey et al., 2008; Zhu et al., 2009; Chakravorty et al., 2015; Maruta et al., 2015). The *xlg1-1* allele is considered to be a knockdown as *XLG1* expression was still detectable in the mutant, although at a low level (Ding et al., 2008). We obtained another *xlg1* T-DNA insertion allele, *xlg1-2* (SALK_119657), which contains a T-DNA insertion at the fifth intron. *xlg1-2* is apparently a knockout allele since no *XLG1* transcript was detected through RT-PCR (Supplemental Fig. S6). The *xlg1-2* mutant was used in this study. We generated a new triple mutant of *xlg1-2*, *xlg2-1*, and *xlg3-1* (in the Columbia-0 [Col-0] background). The triple mutant is named *xlg1*/2/3* in this report. Although the *xlg* single mutants and double mutants did not show easily discernible morphological phenotypes compared with Col-0 wild type, *xlg1*/2/3* plants, like *pub4*, had a smaller stature (Fig. 3A) when grown in soil and had reduced fertility (Fig. 3B). Many siliques in *xlg1*/2/3* had few seeds (Supplemental Fig. S7). When grown on the Murashige and Skoog medium, young seedlings of those genotypes were slightly bigger compared with the wild type, and they all showed enhanced root systems (Fig. 3, C and D). The primary roots of *pub4* and *pub2/4* but not *xlg1*/2/3* were also longer than the wild type. More lateral and adventitious roots were observed in all three genotypes (Fig. 3, C and D; Supplemental Fig. S8, A and B).

The Cytokinin Response Is Impaired in *pub4*, *pub2/4*, and *xlg1*/2/3*

Some of the *pub4*, *pub2/4*, and *xlg1*/2/3* phenotypes are reminiscent of the mutants defective in the cytokinin response (Hutchison et al., 2006; Riefler et al., 2006). To assess their roles in cytokinin signaling, we treated the *pub* and *xlg* mutants with 0.1 μM 6-benzylaminopurine (BA) in Murashige and Skoog plates for 2 weeks. In the control medium, the fresh weight of *pub4*, *pub2/4*, and *xlg1*/2/3* seedlings was slightly higher than that of wild-type seedlings, likely due to their enhanced root systems (Supplemental Fig. S8, C and D), and the total chlorophyll contents were comparable to the wild type, except a lower chlorophyll level in *pub2/4*. In the presence of exogenous cytokinin, which is known to inhibit seedling growth and development, growth of the wild-type plants was severely arrested and their rosette leaves were pale with a decreased chlorophyll level. In contrast, the *pub4*, *pub2/4*, and *xlg1*/2/3* seedlings were much less sensitive to the BA-caused growth inhibition (Fig. 4A). The *pub4* and *xlg1*/2/3* seedlings had slightly lower fresh weight than the seedlings on the control medium and kept a higher chlorophyll level than the wild type under the BA treatment. The *pub2/4* seedlings were the most insensitive toward cytokinin and only slightly lost their chlorophyll contents under the BA treatment compared with the control
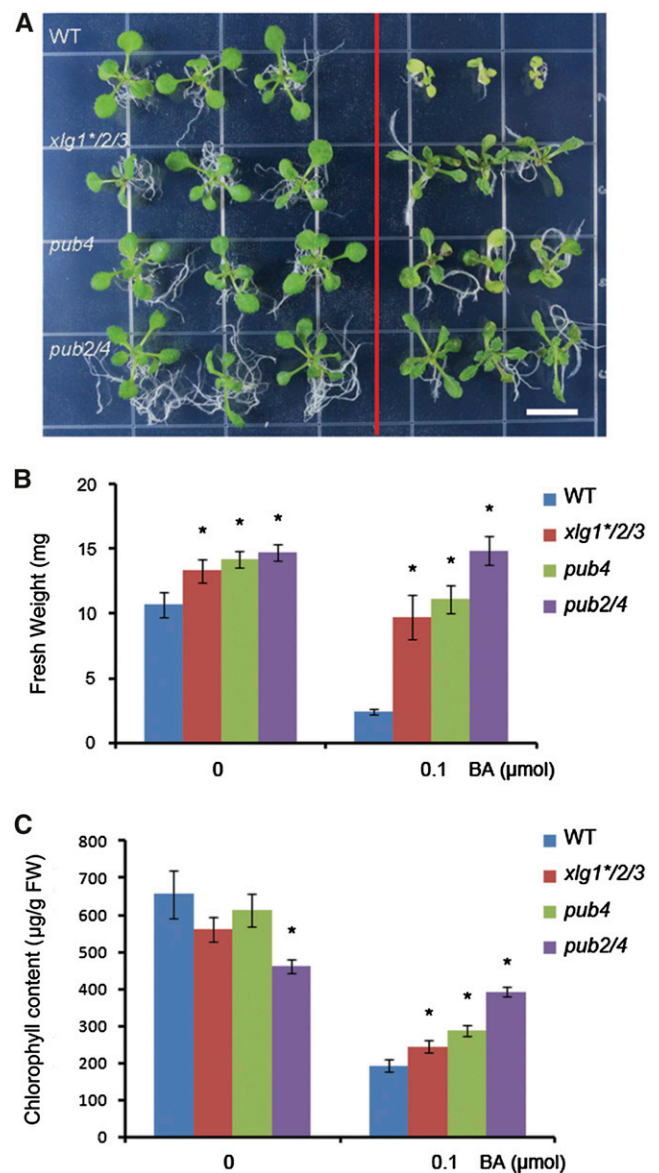

Figure 4. Shoot inhibition by cytokinin in wild type (WT), *pub4, pub2/4*, and *xlg1*/2/3*. A, Fourteen-day-old seedlings grown on the Murashige and Skoog medium without (left) or with 0.1 μmol BA (right). Bar = 1 cm. B, Fresh weight of seedlings of the different genotypes in the medium without and with BA. Fresh weight was determined based on three replicates of 12 14-d-old seedlings for each genotype shown in A. Vertical bars represent mean ± SD. Asterisks indicate statistical differences to the wild type ($P < 0.05$). C, Total chlorophyll contents of seedlings of different genotypes under the conditions shown in A were determined based on three replicates of 12 seedlings each. Vertical bars represent mean ± SD. Asterisks indicate statistical differences to the wild type ($P < 0.05$).

seedlings (Fig. 4). *pub2* and the *xlg* single and double mutants were not significantly different from the wild type in their sensitivity to exogenous BA treatment (Supplemental Fig. S9, A and B). This BA-insensitivity phenotype of *pub4* and *xlg1*/2/3* could be genetically complemented by introduction of *PUB4* into *pub4* or by

introducing *XLG1*, *XLG2*, or *XLG3* into *xlg1*/2/3* (Supplemental Figs. S10 and S11). In addition, another *pub4* mutant, *pub4-3*, displayed a similar phenotype as the *pub4-1* mutant (Supplemental Fig. S9B). These data indicate that the cytokinin-insensitive phenotype is caused by the *xlg* and *pub* mutations.

To further examine the possible involvement of *XLGs* and *PUB2/4* in the cytokinin response, the effect of exogenous cytokinin on hypocotyl elongation inhibition was measured in the wild type and the *xlg* and *pub* mutants. In the dark, obvious hypocotyl elongation inhibition by cytokinin was observed in wild-type seedlings (Fig. 5, A and B). *pub4*, *pub2/4*, and *xlg1*/2/3*

Figure 5. Hypocotyl elongation inhibition of wild type (WT), *pub4*, *pub2/4*, and *xlg1*/2/3* seedlings under the treatment of cytokinin. A, Nine-day-old seedlings of wild type, *pub4*, *pub2/4*, and *xlg1*/2/3* grown on the Murashige and Skoog medium supplemented with DMSO but without BA. Bar = 1 cm. B, Nine-day-old seedlings of wild type, *xlg1*/2/3*, *pub4*, and *pub2/4* grown on the Murashige and Skoog medium with 3 μM BA. Bar = 1 cm. C, Hypocotyl length of wild type, *xlg1*/2/3*, *pub4*, and *pub2/4* under different BA concentrations relative to that grown on DMSO control plates. The hypocotyl elongation of each line with different cytokinin treatment was expressed as a percentage of its DMSO control. Each value represents mean ± SE (n > 10).

were all less sensitive than the wild type to cytokinin, with *pub2/4* the most insensitive among the three genotypes, particularly under the higher cytokinin concentrations (Fig. 5C). We also noticed that all those three mutant seedlings had elongated petioles and open cotyledons in the presence of 3 μM BA (Fig. 5). Again, the hypocotyl elongation phenotype was not significantly different in the *pub2*, *xlg* single, and double mutants from wild type when growing under dark in the presence of cytokinin (Supplemental Fig. S12).

When seedlings were grown in the presence of BA in vertical Murashige and Skoog plates under normal long-day conditions, root sensitivity to cytokinin in *pub4* and *xlg1/*2/3* was not different from that of wild-type plants at low BA concentrations and only slightly less sensitive to 1 μmol BA in those two genotypes (Fig. 6). Root growth of the *pub2/4* mutant, however, showed reduced sensitivity to inhibition by cytokinin at concentrations of 100 nmol and 1 μmol (Fig. 6). These data indicated that those mutations also cause decreased sensitivity to cytokinin for root development, but to a lesser degree compared with the insensitivity to cytokinin for shoot development.

xlg1/2/3* Displays the Similar Tapetum and Stamen Developmental Defects to Those of *pub4*

We observed abnormal numbers of stamens in many flowers of the *pub4*, *pub2/4*, and *xlg1*/2/3* mutants. Normally wild-type plants develop six stamens (four long and two short). However, flowers with four or five stamens frequently appeared in *pub4*, *pub2/4*, and *xlg1*/2/3* mutants (Fig. 7). The missing stamens were always one or two of the short stamens. In *pub4*, approximately 40% of flowers had a reduced stamen number. In *pub2/4* and *xlg1*/2/3*, a majority of flowers lacked one or two stamens (Fig. 7).

It was previously reported that the *pub4* mutant showed aberrant enlargement and incomplete degeneration of the tapetal cells (Wang et al., 2013), leading to failure of pollen release and male sterility. Like *pub4* and *pub2/4*, *xlg1*/2/3* displayed reduced fertility under the normal conditions (Fig. 3B). The reduced fertility was not caused by pollen viability because pollen grains from *xlg1*/2/3* could be stained purple (an indication of viability) using the Alexander staining solution (Supplemental Fig. S13). We then examined whether the sterility of *xlg1*/2/3* is also associated with defect in tapetum development. On dehisced anthers of the wild type, individual pollen grains were visible under light microscopy. However, the pollen grains on the dehisced *xlg1*/2/3* mutant anthers were attached to each other (Fig. 8A). The tapetum development was further examined microscopically. At stage 9, the cells in the wild-type tapetum revealed a thin layer and pollen didn't adhere to each other. However, the tapetum layer in the *xlg1*/2/3* plants was much expanded and the tapetal cells were larger than those in the wild type (Fig. 8B). The pollen grains were also packed
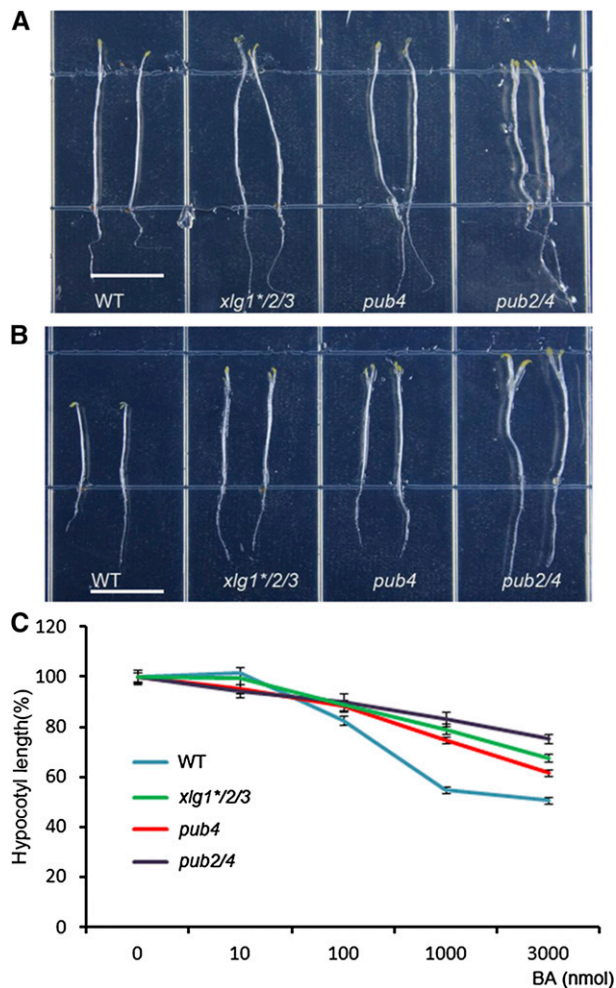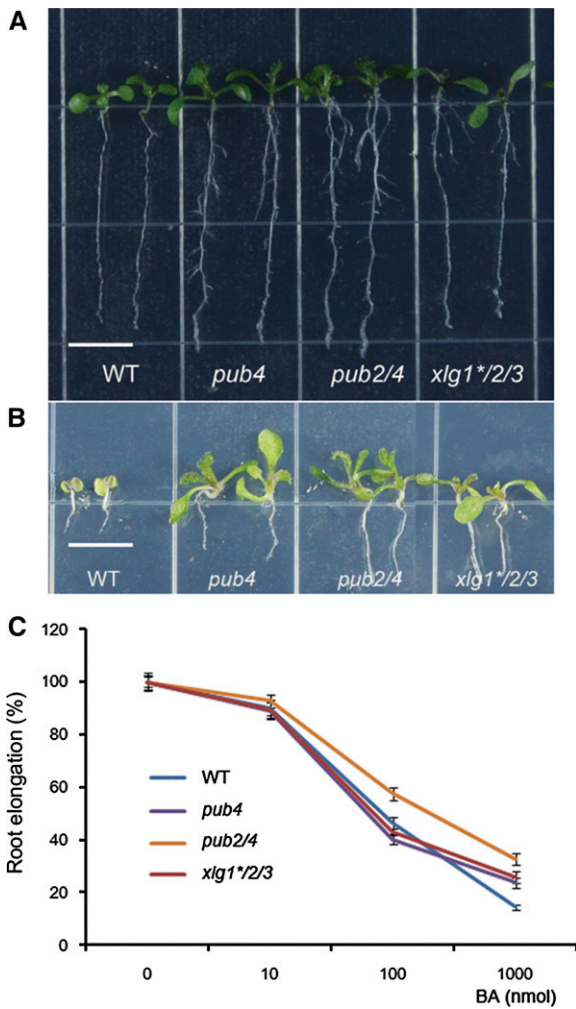
Figure 6. Root elongation inhibition by cytokinin in wild type (WT), *pub4*, *pub2/4*, and *xlg1*/2/3*. A, Nine-day-old seedlings grown on plates without BA. Bar = 1 cm. B, Nine-day-old seedlings grown on Murashige and Skoog plates with 1 μ\mathrm{M} BA. Bar = 1 cm. C, Relative root elongation of seedlings grown on Murashige and Skoog plates with different concentrations of BA relative to seedlings in mock. The root elongation of each line with different cytokinin treatment was expressed as a percentage of its DMSO control. Each value represents mean ± SE ($n \geq 8$).

tightly. The tapetal cells normally start to undergo programmed cell death at stage 10-11 and completely disappear at stage 12-13, leading to release of scattered pollen grains from locules. In the *xlg1*/2/3* mutant, the degeneration of tapetal cells was incomplete, causing pollen grains to adhere to the residual tapetal cells and to fail to be released from the locule walls (Fig. 8B).

ARR10 Overexpression Can Partially Suppress Several Phenotypes Associated with *pub4* and *xlg1*/2/3*

The data above show that *pub4*, *pub2/4*, and *xlg1*/2/3* mutants have reduced cytokinin responses. The type-B response regulators, such as *ARR1*, *ARR10*, and *ARR12*,

are positive regulators of the cytokinin response, and the triple mutation of those three *ARR* genes leads to insensitivity to cytokinin and a dwarf phenotype (Argyros et al., 2008). We overexpressed *ARR1* and *ARR10* in *pub4* under the control of the 35S promoter. *ARR10* overexpression was found to partially restore some phenotypes caused by *pub4*, including the cytokinin response in shoot (Fig. 9A) and pollen release and fertility (Fig. 9, B and C). Overexpression of *ARR10* in *xlg1*/2/3* also partially suppressed, but to a lesser degree, the triple mutant's phenotype of reduced sensitivity to cytokinin (Supplemental Fig. S14). Overexpression of the *ARR1* gene in *pub4* was not found to restore the *pub4* phenotypes.

DISCUSSION

Animals use a large family of plasma membrane GPCRs to perceive various external and internal signals and transduce the signals through a large number of G proteins. Plants have fewer GPCRs, if any, and a much smaller number of G-protein components (Assmann, 2004; Perfus-Barbeoch et al., 2004; Urano et al., 2013; Urano and Jones, 2014), including the single canonical Gα, GPA1. The presence of three XLGs and their known interactions with the multiple Gβγ dimers expand the repertoire of G-protein signaling pathways. Indeed, the XLG mutations have been found to cause various phenotypical changes in developmental and physiological processes (Ding et al., 2008; Pandey et al., 2008; Zhu et al., 2009;
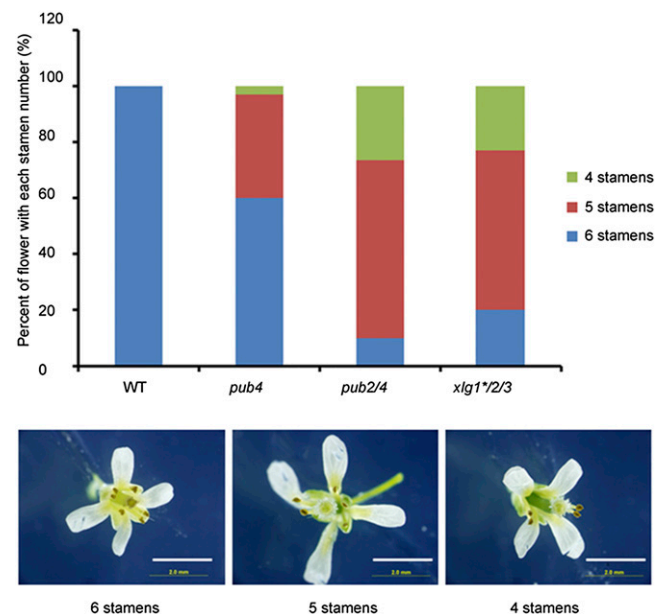

Figure 7. Stamen numbers of wild type (WT), *pub4*, *pub2/4*, and *xlg1*/2/3* mutants. The stamen number was counted from more than 60 flowers for each genotype. Shown below are flowers with normal number (six) of stamens and with reduced numbers of stamens. Bars represent 2 mm.

Figure 8. *xlg1*/2/3* was defective in tapetum development. A, Pollen grains were easily visible on the dehisced anther from the wild type (WT) but not on the anther at the same stages from *xlg1*/2/3*. Bars = 100 μm. B, Wax sections of anthers at different developmental stage in the wild type and *xlg1*/2/3*. The mutant anther showed an abnormally expanded tapetum (T) at stage 10-11. The pollen grains were dispersed in the wild-type anther but adhered to the residual tapetal cells and the anther wall in the mutant. Bars = 20 μm.
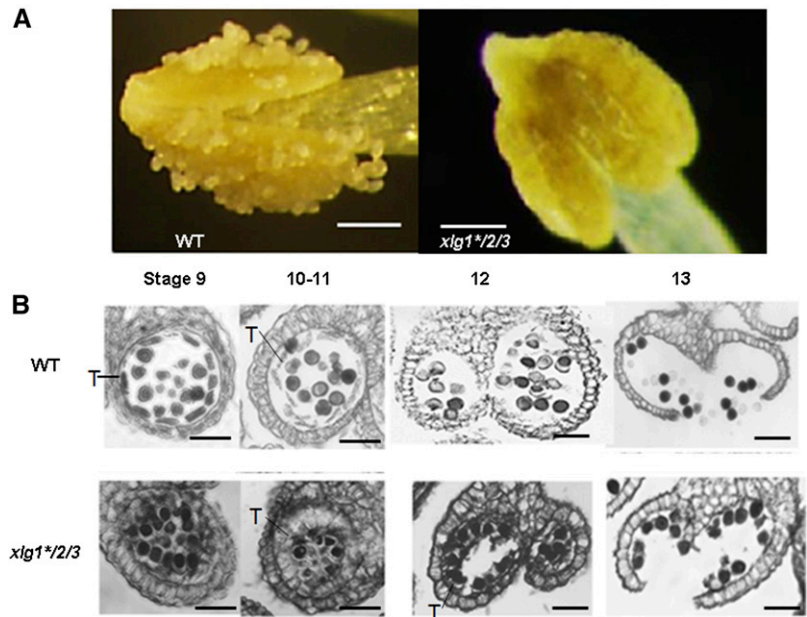
Chakravorty et al., 2015; Maruta et al., 2015; Liang et al., 2016). However, the mechanisms by which the XLGs mediate those pathways remain unclear. The lack of a typical GPCR in plants and other evidences have also raised the possibility that G proteins in plants might function downstream of receptor

kinases, unlike GPCRs in animals (Urano et al., 2013; Nitta et al., 2015).

A previous study indicated that all XLG proteins are localized in the nucleus (Ding et al., 2008). In more recent reports using transient expression and stable transformation, XLG2 and XLG3 were shown to be
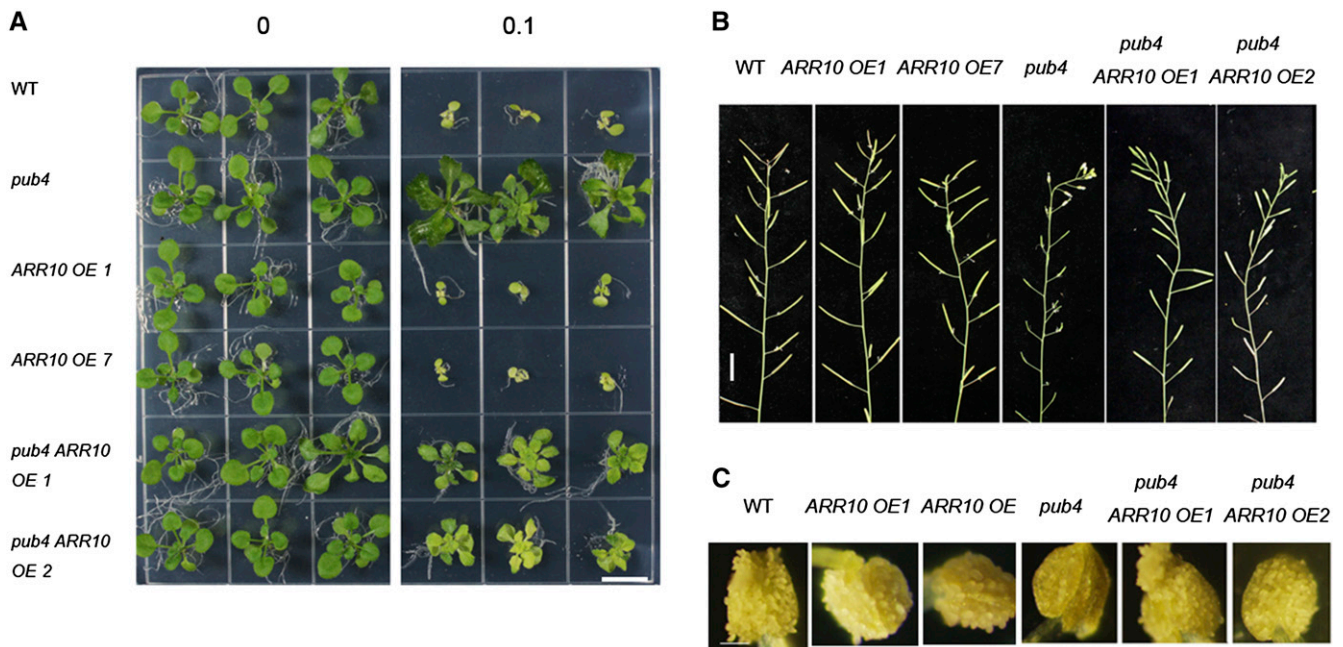
Figure 9. Overexpression of *ARR10* suppressed the *pub4* phenotypes. A, The 14-d-old wild type (WT), two *Pro35:ARR10* lines, *pub4*, and two *Pro-35S:ARR10* lines in the *pub4* background were grown on the Murashige and Skoog plates with DMSO (left) or 0.1 μM BA (right). Bar = 1 cm. B, Phenotypes of 7-week-old wild type, two *Pro35:ARR10* lines, *pub4*, and two *Pro-35S:ARR10* lines in the *pub4* background. Bar = 1 cm. C, Representative anthers of the wild type, two *Pro35:ARR10* lines, *pub4*, and two *Pro-35S:ARR10* lines in the *pub4* background. Individual pollen grains were visible on the anthers from the other genotypes but not from *pub4*. Bar = 100 μm.

localized to both the plasma membrane and nuclei, whereas XLG1 was only associated with the plasma membrane (Chakravorty et al., 2015; Maruta et al., 2015). In this study, we also found plasma membrane localization of all XLGs and additional nuclear location of XLG2 and XLG3. Interactions between XLGs and Gβγ dimers have been reported to occur at the plasma membrane, and overexpression of Gβγ dimers sequestered XLG3 at the plasma membrane (Chakravorty et al., 2015).

The Y2H screen and assay led to the identification of PUB2 and PUB4 as XLGs' putative interacting proteins. BiFC assay indicated that they interacted in planta. The interaction between XLG1 and PUB4 was further confirmed through the in vivo co-IP assay. A previous observation using PUB4-YFP transgenic plants indicated that the PUB4 protein is predominantly localized in the cytosol (Wang et al., 2013); however, this observation cannot rule out the possibility that some of the eYFP signal came from the plasma membrane due to the limited resolution. Additional experimental data in this study further showed that PUB2 and PUB4 are also localized at the plasma membrane. Nuclear localization of PUB4 was reported in another study, and it was suggested that the nuclear localization was likely due to interaction with other proteins (Kinoshita et al., 2015a). Our BiFC data showed that the interaction between XLGs and PUB4 occurred at the plasma membrane, whereas PUB2 was colocalized with XLG2 and XLG3 in both the plasma membrane and the nucleus. It raises the possibility that XLG proteins might sequester PUB4 and PUB2 to the plasma membrane. The tobacco (*Nicotiana tabacum*) PUB4 homolog NtPUB4 is associated with a tobacco chitinase-related receptor-like kinase (NtCHRK1) and localized to the plasma membrane (Kim et al., 2003). Several PUB proteins have been reported to undergo translocation between different cellular compartments, often through their interactions with other proteins (Stone et al., 2003; Samuel et al., 2008; Deb et al., 2014).

The interactions between XLGs and PUB2/PUB4 would suggest that they could function in the same pathways. However, although many phenotypes caused by mutations in these XLG and PUB genes have been described previously, these phenotypes did not suggest that the XLG and PUB proteins are involved in the same biological processes. The *xlg1* allele in the *xlg1/2/3* triple mutant used in the previous studies for characterizing the XLG biological functions carried a T-DNA insertion at the promoter region. This allele is a weak knockdown allele as the mutant still accumulates about 20% *XLG1* transcripts (Ding et al., 2008). In this study, we used another *xlg1* allele (*xlg1-2*), which is likely to be a knockout allele, to generate a new *xlg1/2/3* triple mutant (*xlg1*/2/3*). The new *xlg1*/2/3* mutation indeed causes similar pleiotropic effects on many developmental processes to those caused by *pub4* and *pub2/4*. Those similar phenotypes include male sterility likely caused by aberrant expansion and incomplete degeneration of tapetal cells and a smaller rosette size

that were previously reported for the *pub4* mutant. We also found that both the *xlg* and *pub* mutations lead to insensitivity to cytokinin and reduced numbers of stamens. The multiple similar phenotypes shared by the *xlg* and *pub* mutations, together with their physical interactions, provide convincing evidence that these proteins play roles in the same biological pathways, although the XLG and PUB proteins might also function in other different pathways.

Many PUB proteins have been identified as protein kinase-interacting proteins. In addition to NtPUB4 that interacted with NtCHRK1 in yeast (Kim et al., 2003), the Arabidopsis S-Domain 1 receptor kinases ARK1 and ARK2 phosphorylated PUB9 and PUB13 in vitro (Samuel et al., 2008). PUB13 was also identified as a protein that interacts with BAK1 in the Y2H assay (Lu et al., 2011). BAK1 phosphorylates PUB12 and PUB13, which then form complexes with FLS2 to ubiquitinate FLS2 for degradation, thereby attenuating flagellin-triggered defense signaling (Lu et al., 2011). PUB1 of *Medicago truncatula* interacts with and is phosphorylated by the LYK3 symbiotic receptor and negatively regulates the LKY3 signaling pathway (Mbengue et al., 2010). Our previous study showed that PUB4-FLAG purified from plants appeared to catalyze mono-ubiquitination of itself (Wang et al., 2013), suggesting that it might also catalyze monoubiquitination of its targets. It is unlikely that PUB2/4 target XLGs for polyubiquitination-mediated degradation since the *xlg* triple mutant and *pub2/4* showed very similar phenotypes, instead of opposite phenotypes as would be expected if PUB2/4 negatively regulate XLGs. One possibility is that PUB2/4 could be direct effectors of XLGs and activation of PUB2/4 by XLGs leads to ubiquitination of their target proteins, which remain to be identified. Alternatively, PUB2/4 might activate XLGs by monoubiquitination.

Although knockout of *PUB2* didn't lead to any obvious phenotype, ectopic overexpression of *PUB2* could complement the low fertility phenotype of *pub4*. The *pub2/4* had a smaller size than *pub4*. Besides, *pub2* also enhanced the cytokinin-insensitivity phenotype of *pub4*. All these data indicate that PUB2 and PUB4 have partially redundant functions in multiple developmental processes.

Recently, *pub4* was isolated as a mutant for its reduced sensitivity to root growth inhibition by the CLAVATA3/EMBRYO SURROUNDING REGION-RELATED (CLE) peptides, and the mutation alters cell proliferation in root meristem in a cytokinin-independent manner (Kinoshita et al., 2015a). The cytokinin level in the *pub4* mutant was not changed, but its auxin (indole-3-acetic acid) level was found to be 30% higher than in the wild type (Kinoshita et al., 2015a). We found that the *pub4*, *pub2/4*, and *xlg1*/2/3* mutants all displayed obvious insensitivity to the cytokinin-caused shoot and hypocotyl elongation inhibition. The root elongation inhibition by BA was only slightly reduced in these mutants. The reduced numbers of stamens in the *xlg1*/2/3*, *pub4*, and *pub2/4*

mutants are also reminiscent of the rice (*Oryza sativa*) mutant *lonely guy* (*log*; Kurakawa et al., 2007). *LOG* encodes a cytokinin-activating enzyme and functions in the final step in cytokinin biosynthesis in rice. These results indicate that XLGs and PUB2/4 are positive regulators of the cytokinin signaling pathways. The partial complementation of the *pub4* phenotypes by overexpression of *ARR10*, a positive regulator of the cytokinin response, further supports such a notion. In contrast with some well-studied cytokinin signaling mutants, such as *ahk2/3/4*, *ahp2/3/5*, and *arr1/10/12* that show a severe dwarf phenotype (Nishimura et al., 2004; Hutchison et al., 2006; Riefler et al., 2006; Argyros et al., 2008), the *xlg1*/2/3* and *pub2/4* mutants show a moderate reduction in their sizes, suggesting that XLGs and PUB2/4 might function in more specific developmental and physiological processes. Alternatively, the mild phenotype could be due to functional/pathway redundancy.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

pub4, *xlg2*, *xlg3*, *xlg2/3*, *PUB4-Flag*, and the *pub4* complementation line (*gPUB4*) have been described previously (Ding et al., 2008; Zhu et al., 2009; Wang et al., 2013). *pub2* (SALK_086656) and *xlg1-2* (SALK_119657) were obtained from the Arabidopsis Biological Resource Center at The Ohio State University and crossed to *pub4* or *xlg1-1 xlg2-1 xlg3-1* for generating the *pub2/4* double or *xlg1*/2/3* triple mutant. The mutant alleles are listed in Supplemental Table S1. All lines used in this study are in the Col-0 background, and the primers for genotyping of the mutants are listed in Supplemental Table S2.

Surface-sterilized seeds were plated on the Murashige and Skoog medium with 1% Suc, 0.05% (w/v) MES (pH 5.7), and 0.7% Phytoagar (Sigma-Aldrich). The plates were kept on 4°C for 48 h and transferred to a growth room with either 16 h light/8 h dark (long day) or 9 h light/15 h dark (short day) condition with a light intensity of 100 mol m^{-2} s^{-1}.

Generation of Transgenic Lines

The full-length coding sequences of *XLG1*, *XLG2*, *XLG3*, *PUB4*, *PUB2*, *ARR10*, and the *XLG2* genomic DNA fragment were amplified and cloned into pDONR207 and then transferred into corresponding destination vectors (pEarleyGate 102, pEarleyGate 104, pEarleyGate 201, pMDC83, pEarleyGate 301, modified pEarleyGate 101 without YFP) using LR Clonase II (Invitrogen). The primers used for constructing those constructs are listed in Supplemental Table S2. All the constructs were introduced into *Agrobacterium tumefaciens* strain GV3101, which was then used for transformation of Arabidopsis (*Arabidopsis thaliana*) plants by floral dipping methods (Clough and Bent, 1998).

Y2H Assay

Full-length coding sequences of the *XLG*s, *PUB2/4*, and truncated *XLG*s or *PUB4* were amplified and cloned into pGADT7 or pGBKT7 (Clontech), and the corresponding vectors were introduced into *Saccharomyces cerevisiae* strain Y187 or Y2H Gold yeast cells for mating. The mated strains were grown on the SD medium, the SD medium lacking Trp/Leu, or the SD medium lacking Trp/Leu/His/Ade plus X-α-Gal and aureobasidin A. For library screening, Y2H Gold yeast strains with XLG2-pGBKT7/PUB4-pGBKT7 plasmids were mated to the Y187 Mate and Plate Library (Clontech) and plated on the SD medium lacking Trp/Leu plus X-α-Gal and aureobasidin A plates. The blue clones were picked after 5 d and cultured on the SD medium lacking Trp/Leu and the SD medium lacking Trp/Leu/His/Ade. DNA sequences amplified using T7 and 3AD primers from the positive clones were sequenced and analyzed by BLASTing to TAIR10 transcripts.

BiFC Assay

For BiFC assay, the 35S-YN-pCambia1300 and 35S-YC-pCambia1300 constructs, modified from pSY736 and pSY735 (Bracha-Drori et al., 2004) carrying destination genes, were coexpressed in tobacco (*Nicotiana benthamiana*) cells by agroinfiltration (Chang et al., 2013). Briefly, overnight cultures of agrobacteria were harvested and resuspended to OD$_{600}$ = 0.1 in infiltration buffer containing half-strength Murashige and Skoog liquid medium with 2% (w/v) Suc, 10 mM MES, and 100 μM acetosyringone. Equal volumes of bacterial suspensions were mixed together and infiltrated to 3- to 4-week-old tobacco leaves. Signals were detected 24 to 48 h after infiltration.

Microscopy Analyses

Confocal imaging was carried out with Olympus fluoview 1000 or Leica TCS SP5II confocal microscopes. GFP signal was excited with an argon laser at 488 nm for transient expression of GFP-tagged XLGs. The YFP signal was excited at 514 nm for BiFC assays. CFP was excited at 458 nm, and mCherry was excited at 594 nm. Images were acquired and analyzed with the corresponding software FV10-ASW for Olympus and LAS-AF for Leica.

Co-IP Assay

Leaves from 4-week-old plants were harvested and used for co-IP assays. Proteins were extracted with the buffer containing 50 mM Tris (pH 7.5), 150 mM NaCl, and 0.1% IGEPAL CA-630 and Proteinase Inhibitor Cocktail (Sigma-Aldrich). After a brief sonication, the samples were centrifuged at 12,000g for 15 min at 4°C. The supernatant was incubated with anti-GFP magnetic beads (catalog no. D153-11; MBL) for 2 h to overnight at 4°C with gentle rotation. The beads were then washed four times with PBS. The immunoprecipitated proteins were eluted in the SDS-PAGE loading buffer with heating at 70°C for 10 min. The presence of the corresponding proteins was detected using a FLAG antibody (catalog no. a8592; Sigma-Aldrich) and a GFP antibody (catalog no. 11814460001; Roche), respectively.

Cytokinin Response Assay

For shoot inhibition, Arabidopsis seedlings were grown on horizontal Murashige and Skoog plates containing BA or 0.1% dimethyl sulfoxide (DMSO; as a control). The seedlings were photographed, and the fresh weight and chlorophyll level were determined at day 14 (Argyros et al., 2008). The experiment was repeated twice.

For root elongation assay, Arabidopsis seedlings were grown on vertical Murashige and Skoog plates containing BA or 0.1% DMSO (as a control). The photos were taken and the main root lengths were measured at day 9. The experiment was repeated twice.

For hypocotyl elongation, seedlings of different genotypes were grown on vertical Murashige and Skoog plates in dark. The photos were taken and the hypocotyl lengths were measured at day 9. The experiment was repeated twice.

Accession Numbers

Sequence data from this article can be found in the Arabidopsis Information Resource Web site (www.arabidopsis.org) under the following accession numbers: XLG1 (AT2G23460), XLG2 (AT4G34390), XLG3 (AT1G31930), PUB4 (AT2G23140), PUB2 (AT5G67340), and ARR10 (AT4G31920).

Supplemental Data

The following supplemental materials are available.

Supplemental Figure S1. No autoactivation of BD-XLG1C, XLG2C, XLG3C, and AD-PUB4C was detected.

Supplemental Figure S2. PUB2 and PUB4 were partially localized to the plasma membrane.

Supplemental Figure S3. *pub2-1* is most likely a knockout allele.

Supplemental Figure S4. Relative *PUB4* and *PUB2* expression levels according to Affymetrix Arabidopsis ATH1 Genome Array Data.

Supplemental Figure S5. Overexpression of *PUB2* in *pub4* restored the *pub4* phenotype.

Supplemental Figure S6. *xlg1-2* is most likely a knockout allele.

Supplemental Figure S7. *xlg1*/2/3* produced fewer seeds than the wild type.

Supplemental Figure S8. Primary root length, lateral root numbers, root weight, and root/whole plant fresh weight ratio in seedlings of the wild type, *pub4*, *pub2/4*, and *xlg1*/2/3*.

Supplemental Figure S9. Shoot growth inhibition by cytokinin in the *xlg* and *pub* mutants.

Supplemental Figure S10. Complementation of the *xlg1*/2/3* cytokinin-insensitive shoot growth phenotype by transgenes of *XLG1*, *XLG2*, and *XLG3*.

Supplemental Figure S11. Complementation of the *pub4* cytokinin-insensitive phenotype in shoot growth inhibition by the genomic *PUB4* transgene.

Supplemental Figure S12. Hypocotyl elongation inhibition by cytokinin in *pub2* and *xlg* single and double mutants.

Supplemental Figure S13. The fertility of *xlg1*/2/3* is temperature-dependent.

Supplemental Figure S14. Cytokinin response of the wild type, *xlg1*/2/3*, and two *xlg1*/2/3 Pro-35S:ARR10* lines.

Supplemental Table S1. Mutant alleles used in this article.

Supplemental Table S2. List of primer sequences used in this article.

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