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Auxin-Callose-Mediated Plasmodesmal Gating Is Essential for Tropic Auxin Gradient Formation and Signaling

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SUMMARY

In plants, auxin functions as a master controller of development, pattern formation, morphogenesis, and tropic responses. A sophisticated transport system has evolved to allow the establishment of precise spatiotemporal auxin gradients that regulate specific developmental programs. A critical unresolved question relates to how these gradients can be maintained in the presence of open plasmodesmata that allow for symplasmic exchange of essential nutrients and signaling macromolecules. Here we addressed this conundrum using genetic, physiological, and cell biological approaches and identified the operation of an auxin-GSL8 feedback circuit that regulates the level of plasmodesmal-localized callose in order to locally downregulate symplasmic permeability during hypocotyl tropic response. This system likely involves a plasmodesmal switch that would prevent the dissipation of a forming gradient by auxin diffusion through the symplasm. This regulatory system may represent a mechanism by which auxin could also regulate symplasmic delivery of a wide range of signaling agents.

INTRODUCTION

A prominent issue in the biology of multicellular organisms is how non-cell-autonomous signaling molecules cross the boundary that is conventionally defined as the cell membrane. Auxin, a quintessential hormone involved in controlling plant development, pattern formation, morphogenesis, and tropic responses, requires coordinated polarized transport to establish the requisite precise spatial and temporal gradients (Brunoud et al., 2012; Leyser, 2005; Sabatini et al., 1999; Vanneste and Friml, 2009; Zhao et al., 2010). The genes underlying the formation of such local auxin gradients, including those that mediate the polarized transport of this hormone, are now well characterized and provide a general principle for hormone action (Bennett et al., 1996; Christensen et al., 2000; Jürgens and Geldner, 2007; Michniewicz et al., 2007; Noh et al., 2003; Sorefan et al., 2009; Weijers and Friml, 2009; Wiśniewska et al., 2006).

In *Arabidopsis thaliana*, a specific positioning of efflux carriers, including PIN-FORMED (PIN) and P-glycoproteins, allows for the export of auxin from the cytoplasm of donor cells into the cell wall (Murphy et al., 2002; Wiśniewska et al., 2006). Transfer of this released auxin into the adjacent receiver cells is accomplished by active H^+/IAA^- symport mediated by AUX1/LAX family members (Bennett et al., 1996; Kleine-Vehn and Friml, 2008; Yang et al., 2006). This combination of processes, termed polar auxin transport (PAT), provides distinctive patterns of auxin distribution in the embryo, shoot and root apical meristems, apical hook, and sites of lateral root initiation, as well as during phototropic and gravitropic responses (Band et al., 2012; Brunoud et al., 2012; Vanneste and Friml, 2009). Interdicting the efflux transporters causes abnormal/dispersed patterns of auxin distribution in these various domains, with a concomitant alteration in developmental programming (Cheng et al., 2006; Ljung et al., 2005; Zhao et al., 2001; Benková et al., 2003; Christie et al., 2011; Friml et al., 2002a, 2002b, 2003).

Modeling studies have shown that the efficacy of the PAT system is quite sensitive to the degree to which auxin can move within tissues by diffusion (Bayer et al., 2009; Jönsson et al., 2006; Smith et al., 2006). Hence, regulation of the boundaries for passive auxin movement appears to be crucial, in terms of establishing/maintaining an auxin gradient. These modeling studies confined their attention to passive diffusion across the plasma membrane. Interestingly, another potential pathway for auxin diffusion would be through plasmodesmata (PD) (Lucas and Lee, 2004). These plant-specific intercellular organelles establish a cytoplasmic continuum between neighboring cells ($10^3 \sim 10^5$ PD/cell), termed the symplasmic pathway (Robards, 1975), which functions in the delivery of nutrients, such as sucrose and amino acids, essential for supporting the high levels of metabolism taking place within developing tissues. Furthermore, PD also facilitate the cell-to-cell transport of proteins and RNA, some of which play essential

roles in controlling developmental events within the same cellular domains covered by auxin gradients (Kim et al., 2005c; Lucas et al., 1995, 2009; Schlereth et al., 2010; Xu and Jackson, 2010).

Because auxin is an organic acid of a size (~200 Da) similar to that of sucrose and, given that sucrose moves by diffusion through PD, this raises the question as to how an auxin gradient could be established and maintained in the presence of open PD. One possibility is that control over PD permeability would be tightly coordinated with the auxin signaling network, in order to produce and maintain auxin gradients when the need arises for developmental reprogramming.

A number of cellular components involved in regulating PD permeability have been identified (Benitez-Alfonso et al., 2009, 2013; Kobayashi et al., 2007; Lee et al., 2011; Simpson et al., 2009; Stonebloom et al., 2009; Vatén et al., 2011), and callose, a β-1,3-glucan, appears to be a key factor (Lucas et al., 2009). Studies of GLUCAN SYNTHASE LIKE 8 (GSL8), an enzyme involved in callose synthesis, and β-1,3-glucanase, the enzyme involved in callose turnover, have established that PD callose deposition can regulate trafficking through PD of transcription factors and viral movement proteins (Chen and Kim, 2009; Guseman et al., 2010; Levy et al., 2007). Further evidence for the role of callose in regulating PD permeability was also provided by studies of the *gfp arrested trafficking* (*gat*) mutant (Benitez-Alfonso et al., 2009). The regulation of PD callose deposition and cell-to-cell connectivity is also critical in determining the pattern of lateral root formation (Benitez-Alfonso et al., 2013).

In this study, we used seedling phototropic responses to explore the relationship between PD permeability and PAT. We demonstrate the presence of an auxin-PD-callose feedback circuit that downregulates symplasmic permeability in order to permit the formation of an asymmetric auxin gradient essential for these plant tropic responses. Importantly, we show that reduced PD callose, and the concomitant increase in PD permeability, allows for PD-mediated auxin diffusion, thereby preventing the establishment of the auxin gradient and, hence, resulting in seedling hypocotyls being unresponsive to light. Our studies reveal that development of a spatial auxin gradient requires de novo synthesis of PD-localized callose, regulated by an auxin-mediated plasmodesmal switch. This regulatory system may represent a mechanism by which auxin could regulate symplasmic delivery of a wide range of signaling agents.

RESULTS

Hypocotyl Tropic Responses Require GSL8-Derived PD Callose Deposition

To test the hypothesis that regulation of PD callose deposition is essential for the establishment of a localized auxin gradient, a screen was conducted for *Arabidopsis* mutants lacking PD callose. The *Arabidopsis* genome contains 12 genes encoding putative callose synthases (GSL1–12) (Chen et al., 2009). Among the 11 *gsl* mutants tested, with the exception of the gametophytic lethal mutant *gsl10*, only *gsl8/calS10/massue/chorus* lacked aniline blue-based PD callose staining (Figure 1A). This finding is consistent with GSL8 being the major enzyme involved in PD callose deposition (Guseman et al., 2010).

Because *gsl8* is a cytokinesis-defective mutant that showed abnormal embryogenesis and seedling lethality (Chen et al., 2009; Thiele et al., 2009), transgenic plants expressing a double-stranded (*ds*)*GSL8* RNAi construct, under the control of the dexamethasone (dex)-induction system (Aoyama and Chua, 1997), were employed to knock down the *GSL8* transcript level. These lines phenocopied the *gsl8* mutant with dwarf phenotype and abnormal hook opening and had specifically reduced *GSL8* transcript levels (Chen et al., 2009) (Figure 1B; Figures S1A–S1C available online). Real-time RT-PCR assays further confirmed that a 6 hr dex treatment caused a 90% reduction in *GSL8* transcript level compared to wild-type and dex (−) plants (Figure 1C). Interestingly, this reduction in *GSL8* transcripts was accompanied by significant increases in transcripts of other family members, most notably *GSL5* and *GSL7* (Figure 1B). Importantly, as aniline blue signal was barely detectable in dex-treated *dsGSL8 RNAi* (hereafter referred to as *dsGSL8*+dex) seedlings (Figure 1A), it would appear that the upregulated *GSL* genes do not contribute to PD callose deposition. Taken together, these experiments establish that PD callose, produced by the action of GSL8, is downregulated in these *dsGSL8*+dex plants.

To explore the involvement of PD callose in auxin gradient formation, we chose the hypocotyl as a model system, as here an asymmetric auxin distribution is a prerequisite for the phototropic and gravitropic responses that mainly depend on cell elongation rather than division. Both wild-type and uninduced control *dsGSL8 RNAi* (−dex) (hereafter referred to as *dsGSL8*−dex) seedlings displayed normal tropisms (Figures 1D and 1E). However, *dsGSL8*+dex seedlings were unresponsive to either light (Figure 1D) or gravity (Figure 1E). The growth reduction of the *dsGSL8*+dex hypocotyl might contribute to the observed reduced tropic responses. This possibility was unlikely based on studies obtained using younger seedlings (*dsGSL8*±dex). Here we observed similar growth characteristics, but distinctive phototropic and gravitropic differences (Figures 1D and 1E; Figure S1D). This suggested that the nonphototropic response of the *dsGSL8*+dex seedlings was not caused by a defect in elongation per se, but rather from an inability to undergo differential elongation between the shaded and illuminated sides of the hypocotyl. These findings are significant, as dex-inducible *GAL4:GR* control seedlings (Figure S1E) and all other tested *gsl* mutants (Figure S1F) exhibited normal phototropic responses.

An asymmetric distribution of callose was observed in phototropically responding *dsGSL8*−dex hypocotyls (Figures 1F–1H and 1L). In contrast, no such asymmetric deposition was detected in *dsGSL8*+dex nonresponsive hypocotyls (Figures 1I–1L). To confirm whether the nonphototropic nature of these *dsGSL8*+dex hypocotyls was associated with a reduction in PD callose, an anticallose antibody was used to visualize callose associated with PD (Figure S1G). Our immunogold studies clearly demonstrated that, in comparison to the wild-type control (Figure 1M), the level of PD callose was greatly reduced in *dsGSL8*+dex hypocotyls (Figures 1N and 1P; Figure S1G). Equivalent results were obtained with hypocotyls from the *gsl8* mutant (Figures 1O and 1P; Figure S1G), thereby indicating the effectiveness of the dex-induction system in reducing the level of GSL8 and, thus, PD callose. However, as some residual PD-associated callose was still detected in the *gsl8* and