Strigolactones Suppress Adventitious Rooting in Arabidopsis and Pea[1][C][W][OA]

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Adventitious root formation is essential for the propagation of many commercially important plant species and involves the formation of roots from nonroot tissues such as stems or leaves. Here, we demonstrate that the plant hormone strigolactone suppresses adventitious root formation in Arabidopsis (*Arabidopsis thaliana*) and pea (*Pisum sativum*). Strigolactone-deficient and response mutants of both species have enhanced adventitious rooting. *CYCLIN B1* expression, an early marker for the initiation of adventitious root primordia in Arabidopsis, is enhanced in *more axillary growth2* (*max2*), a strigolactone response mutant, suggesting that strigolactones restrain the number of adventitious roots by inhibiting the very first formative divisions of the founder cells. Strigolactones and cytokinins appear to act independently to suppress adventitious rooting, as cytokinin mutants are strigolactone responsive and strigolactone mutants are cytokinin responsive. In contrast, the interaction between the strigolactone and auxin signaling pathways in regulating adventitious rooting appears to be more complex. Strigolactone can at least partially revert the stimulatory effect of auxin on adventitious rooting, and auxin can further increase the number of adventitious roots in *max* mutants. We present a model depicting the interaction of strigolactones, cytokinins, and auxin in regulating adventitious root formation.

Adventitious root formation is the process of root initiation from a nonroot tissue, and as an energetically expensive process, it is tightly regulated to prevent the loss of valuable plant resources on unnecessary root formation. The ability to induce adventitious root formation in cuttings is central to plant industries worldwide. However, many plant species are recalcitrant to clonal propagation due to difficulties in inducing adventitious roots, and this poses significant limitations on the forestry and horticulture industries. Although clonal propagation has been used for centuries, we have only recently begun to understand adventitious root formation at the genetic, molecular, and biochemical levels (Gutierrez et al., 2009; Liao et al., 2010; Negi et al., 2010).

There are two main developmental pathways that can lead to adventitious root formation. Direct root formation involves roots forming from stem tissues such as the pericycle in the hypocotyl of Arabidopsis (*Arabidopsis thaliana*; Goldfarb et al., 1998) and is similar to lateral root development. Indirect root formation involves the production of callus tissue prior to adventitious root development and is different from lateral root formation because the earliest events include divisions from cambium and cambium-associated cells, while lateral root formation occurs specifically from the pericycle. This type of adventitious rooting is most commonly found where roots form from stem cuttings (Goldfarb et al., 1998).

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Several plant hormones control adventitious root formation (Blakesley et al., 1991). The most well studied of these is auxin, which promotes both adventitious and lateral root formation (Cooper, 1936; Boerjan et al., 1995; Delarue et al., 1998; Zhao et al., 2001; Negi et al., 2010). The auxin-overproducing mutants such as *superroot1* (*sur1*; Boerjan et al., 1995), *sur2* (Delarue et al., 1998), and *yucca* (Zhao et al., 2001) produce more adventitious and lateral roots than the wild type. Previous studies suggest that auxin regulates multiple stages of adventitious and lateral root formation (for review, see Blakesley et al., 1991). Auxin is required prior to, during, and after the early cell divisions that lead either to roots or callus (Smith and Thorpe, 1975; Diaz-Sala et al., 1996; Greenwood et al., 2001). Although it has been known that auxin is essential for adventitious root formation, little research has focused on the regulation of adventitious root formation by auxin at the molecular level. Differences in expression profiles between emerging adventitious and lateral roots suggest that each root type is regulated by a unique set of signaling pathways (Gutierrez et al., 2009). Despite the clear requirement of auxin for adventitious root formation, previous studies have demonstrated that there are no consistent differences in auxin transport, metabolism, or free indole-3-acetic acid (IAA) between easy-to-root and hard-to-root cuttings (Diaz-Sala et al., 1996; Krisantini et al., 2006). Although it is possible that auxin response could account for some of the differences between adventitious root phenotypes, it may also reflect the importance of other signaling pathways in the regulation of adventitious root induction.

In addition to auxin, cytokinins are important regulators of adventitious root formation (Debnath, 2008; Konieczny et al., 2009). Taking a shoot cutting results in a decrease in endogenous cytokinin compared with intact plants (Bollmark et al., 1988). It is plausible that this lowered cytokinin level may form part of the signal to the cutting that new roots are needed. This idea is supported experimentally, because treatment with exogenous cytokinins results in strong suppression of adventitious root formation (Bollmark and Eliasson, 1986; De Klerk et al., 2001). Consistent with these results, the cytokinin receptor mutant *arabidopsis histidine kinase4* (*ahk4*) and plants overexpressing either *CYTOKININ OXIDASE1* (*CKX1*) or *CKX2*, resulting in higher breakdown of cytokinin, display enhanced adventitious root production (Werner et al., 2003; Lohar et al., 2004; Kuroha et al., 2006, Riefler et al., 2006). Similarly, fewer adventitious roots are formed on cuttings of petunia (*Petunia hybrida*) or tomato (*Solanum lycopersicum*) that have enhanced cytokinin synthesis due to the overexpression of an *ISOPENTENYLTRANSFERASE* gene (Groot et al., 1995; Clark et al., 2004). Collectively, these results demonstrate a role for cytokinin in negatively regulating adventitious root formation.

Although it is well established that auxin and cytokinin are important for lateral root and adventitious root development, their mechanisms of action have yet to be fully elucidated, and it can be expected that other signaling pathways play important roles. For example, considering the concerted action of auxin, cytokinin, and strigolactones in the regulation of shoot branching (for review, see Ongaro and Leyser, 2008; Dun et al., 2009; Waldie et al., 2010), it is possible that the hormone strigolactone may play an intermediary role downstream of auxin because auxin regulates the activity of strigolactone biosynthesis genes and thus might affect strigolactone levels (Brewer et al., 2009). Strigolactone signaling mutants have also been shown to have moderately enhanced lateral root formation (Kapulnik et al., 2011; Ruyter-Spira et al., 2011).

Strigolactones are a novel class of plant hormones that were originally discovered for their promotion of mycorrhizal association (Akiyama et al., 2005) and parasitic weed seed germination (Matusova et al., 2005), but more recently they have been found to negatively regulate bud outgrowth (Gomez-Roldan et al., 2008; Umehara et al., 2008). Orthologous strigolactone signaling and synthesis genes have been found in all higher plant species examined, most notably Arabidopsis, pea (*Pisum sativum*), petunia, tomato, rice (*Oryza sativa*), and *Chrysanthemum* species, in which mutations in these genes result in increased bud outgrowth phenotypes (Drummond et al., 2009; Beveridge and Kyozuka, 2010; Liang et al., 2010). The enhanced branching in strigolactone-deficient mutants is reversed by addition of the synthetic strigolactone GR24. Similarly, strigolactone response mutants also have an enhanced branching phenotype, but they are unable to respond to GR24, supporting the role of affected factors in the perception of strigolactones (Gomez-Roldan et al., 2008; Umehara et al., 2008).

While shoot branching has been well characterized in mutants impaired in strigolactone signaling, nothing has been reported about their adventitious root phenotypes. Here, we provide evidence that strigolactones suppress the earliest stages of adventitious root formation.

RESULTS AND DISCUSSION

Strigolactones Suppress Adventitious Root Formation in Arabidopsis Hypocotyls

It has been demonstrated previously that adventitious roots can be induced on Arabidopsis hypocotyls by etiolating seedlings for several days (Gutierrez et al., 2009; Sorin et al., 2005, 2006). To assess whether strigolactones have a role in regulating adventitious root formation, Arabidopsis mutants with defects in strigolactone synthesis (*more axillary growth1* [*max1*], *max3*, and *max4*) or response (*max2*) were grown in the adventitious root induction assay using 4 d of etiolation followed by transfer to the light. All four mutants showed a significant increase in adventitious root formation compared with wild-type controls (Fig. 1A). The *max1*, *max3*, and *max4* mutants produced 1.5- to 3-fold more adventitious roots than controls. The *max2* mutant displayed the highest number of

adventitious roots, producing approximately 5-fold more roots than the control ($P < 0.05$; Fig. 1A). The increase in adventitious root formation in strigolactone mutants has been demonstrated in four independent experiments for Arabidopsis and across two genetic backgrounds (Columbia [Col-0] and Wassilewskija [Ws-4]; Supplemental Fig. S1). These results indicated a role for strigolactones in regulating adventitious rooting in Arabidopsis.

To determine if strigolactones can restore the adventitious rooting phenotype of the strigolactone synthesis mutants, the synthetic strigolactone GR24 was added exogenously to the wild-type and strigolactone mutants of Arabidopsis. Treatment of wild-type plants resulted in the suppression of adventitious root formation from an average of 0.9 adventitious roots per plant to zero roots per plant (Fig. 1A). Strigolactone treatment of the strigolactone synthesis mutants *max1*, *max3*, and *max4* repressed adventitious rooting close to the levels observed in GR24-treated wild-type plants. In contrast, the strigolactone response mutant, *max2*, did not display a significant reduction in adventitious rooting in response to GR24 treatment.

Hormones generally function in a dose-dependent manner, so to provide further support for a role of strigolactones in the regulation of adventitious root formation, we treated the wild type and the synthesis mutant (*max4*) with a range of concentrations of GR24. The suppression of adventitious root formation was indeed dose dependent (Fig. 1B), and in both the wild type and *max4*, inhibition was observed when a concentration of 100 nM GR24 or higher was applied. These results support the role of strigolactones as a plant hormone negatively regulating adventitious root formation.

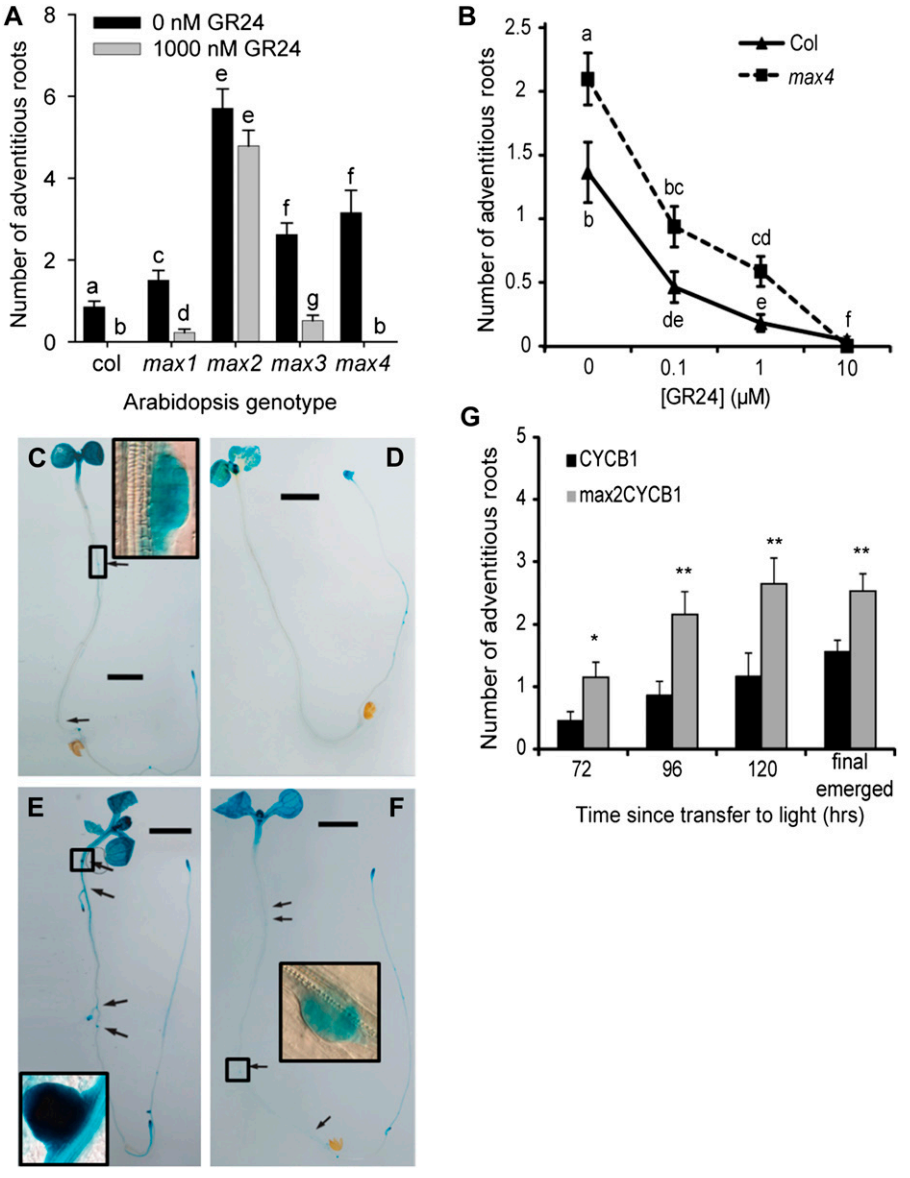

Figure 1. Strigolactones inhibit adventitious root formation in pea cuttings and intact Arabidopsis hypocotyls. A, Strigolactones inhibit adventitious root formation in Arabidopsis. Synthesis mutants of Arabidopsis Col-0 ecotype are *max1-1*, *max3-11*, and *max4-1*, while the response mutant is *max2-1*. $n > 60$. B, GR24 (synthetic strigolactone) inhibits adventitious root formation in a dose-dependent manner. Col is the Col-0 wild type, and *max4-1* is the strigolactone production mutant. C to F, *CYCB1*:*GUS* wild type (C), *CYCB1*:*GUS* wild type with GR24 (D), *max2*-*CYCB1*:*GUS* control (E), and *max2*-*CYCB1*:*GUS* with 1,000 nM GR24 (F). Arrows point to adventitious roots or primordia, and boxes show the hypocotyl/root boundary. Bars = 2 mm. G, Graphical representation of the number of primordia or roots present on *CYCB1*:*GUS* plants at increasing times since plates were transferred to the light. Means across replicates are presented with SE. * $P < 0.05$, ** $P < 0.01$ (Student's *t* test). In A, B, and G, plants were scored after 10 d of light (15 d after germination). In all panels, means are presented ± SE. Different letters represent means that are significantly different at $P < 0.05$ (Student's *t* test). [See online article for color version of this figure.]

Together, the results in this section demonstrate that adventitious rooting is negatively regulated by strigolactone via the MAX2 response pathway. This finding widens the known physiological functions of strigolactones and also highlights similarities between branching and adventitious root development. Recently, two studies have demonstrated that low concentrations of strigolactones also inhibit lateral root initiation in a *max2*-dependent manner (Kapulnik et al., 2011, Ruyter-Spira et al., 2011). These papers support the role of strigolactones in root developmental processes.

Strigolactones Block the First Visible Stages of Adventitious Root Formation in Arabidopsis Hypocotyls

The first visible stage of adventitious root formation in Arabidopsis hypocotyls occurs when founder cells (i.e. pericycle cells adjacent to the xylem poles) become activated and divide to form the root primordium (Boerjan et al., 1995). To determine whether strigolactones influence the initiation of cell divisions or only act later, we used the *CYCLIN B1* (*CYCB1*):*GUS* reporter to visualize the first divisions of the founder cells (Fig. 1, C–F; Himanen et al., 2002). The number of regions expressing *CYCB1*:*GUS* 120 h after transfer to the light (Fig. 1G) was correlated with the number of adventitious roots that form from the hypocotyls under the same conditions (Fig. 1G, final emerged). Sites of enhanced reporter gene activity (Fig. 1, C–F) were observed in the hypocotyls of wild-type plants as early as 72 h after transfer from dark to light (Fig. 1G). The number of sites with enhanced *CYCB1*:*GUS* activity continued to increase after 96 and 120 h in the light, but no increase was observed after 7 d in the light (Fig. 1G, final emerged). The number of sites with high reporter gene activity observed in *max2 CYCB1*:*GUS* (Fig. 1, E and G) plants was significantly higher than in the controls at all time points examined and showed a similar trend over time. In addition, GR24 treatment of wild-type plants (Fig. 1, D and G) reduced the number of sites with marker activity, while the number of sites in *max2* was unaffected by GR24 treatment (Fig. 1, F and G). Consequently, similar to the effects on the number of adventitious roots (Fig. 1A), the abolition of the strigolactone response and the supply of exogenous strigolactone to wild-type plants caused changes in the expression of a marker for an early stage of lateral root formation. Therefore, these results support a role for strigolactones in suppressing development at or before the first divisions of the founder cells required for adventitious root formation in Arabidopsis.

MAX2 Expression in the Xylem of Arabidopsis Hypocotyls Is Sufficient for Repressing Adventitious Root Formation

To investigate where strigolactone signaling is important for controlling adventitious root formation at the cellular level, we examined the adventitious rooting of transgenic plants that specifically express *MAX2* in different tissues in the *max2* mutant (Agusti et al., 2011). When *MAX2* was expressed under the control of a xylem-specific promoter *NAC SECONDARY WALL THICKENING PROMOTING FACTOR3* (*NST3*; Mitsuda et al., 2007), the level of adventitious root formation was restored to wild-type levels (Fig. 2A). When *MAX2* was expressed under the control of phloem-specific (Fig. 2A, *ALTERED PHLOEM DEVELOPMENT* [*APL*]; Bonke et al., 2003), endodermis-specific (Fig. 2B, *SCARECROW* [*SCR*]; Wysocka-Diller et al., 2000), or procambium-specific (Fig. 2B, *WUSCHEL RELATED HOMEOBOX4* [*WOX4*]; Hirakawa et al., 2010) promoters, the adventitious rooting was intermediate between *max2* control

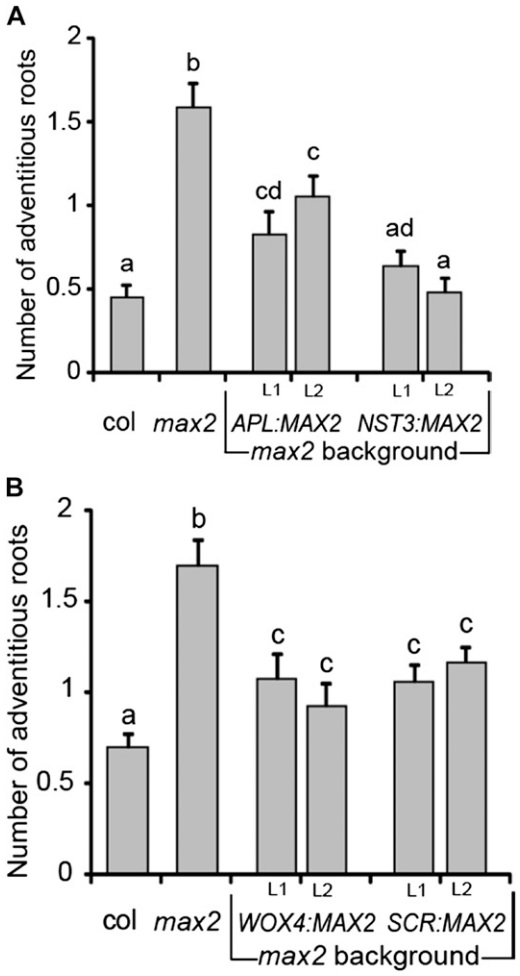

Figure 2. Expression of *MAX2* in the xylem is sufficient to restore adventitious rooting. Constitutive expression of *MAX2* in a *max2* background is shown in the phloem (*APL*:*MAX2*) or in the xylem (*NST3*:*MAX2*; A) and in the procambium (*WOX4*:*MAX2*) or in the starch sheath (*SCR*:*MAX2*; B). Controls are wild-type Col-0 and the *max2* mutant. Adventitious rooting was done in two different lines (L1 and L2) in each of *APL*:*MAX2*, *NST3*:*MAX2*, *WOX4*:*MAX2*, and *SCR*:*MAX2* promoter constructs. Plants were scored after 10 d of light (15 d after germination). Means are presented ± SE. Different letters represent means that are significantly different at $P < 0.05$ (Student's *t* test).

and wild-type plants (Fig. 2). This suggests that the expression of *MAX2* in xylem-associated cells is most important for strigolactone regulation of adventitious rooting. This is not unexpected, given that *MAX2* is expressed in a range of cell types and tissues, including cambium- and xylem-associated cells (Shen et al., 2007; Stirnberg et al., 2007), and that adventitious roots of Arabidopsis hypocotyls arise in pericycle cells that are aligned with the xylem poles (Boerjan et al., 1995). Unfortunately, *MAX2* could not be expressed specifically in the pericycle of the hypocotyl, due to the lack of suitable enhancer trap lines; however, our results suggest that strigolactone signaling in the xylem is sufficient to regulate pericycle-derived adventitious rooting. The non-cell-autonomous repression of adventitious rooting in the pericycle by *MAX2* expression in the other nonxylem tissues allows for the possibility that strigolactones may regulate a short-range signal that can move from these tissues into the pericycle. Root hair development is also regulated by *MAX2*-dependent strigolactone signaling and likely involves non-cell-autonomous signaling, because root hairs are formed from epidermal cells, whereas, as mentioned above, *MAX2* is expressed primarily in the vasculature (H. Koltai, personal communication; Kapulnik et al., 2011).

MAX4 and *MAX3* Expression in Arabidopsis Hypocotyls

Because etiolation and deetiolation are important for adventitious rooting in the hypocotyl, we measured the expression of *MAX4* and *MAX3* in hypocotyls at different times after transfer to the light. In three biological replicates, we found that expression of both *MAX4* and *MAX3* was induced by light over time ($P < 0.01$ and $P < 0.05$ for *MAX4* and *MAX3*, respectively). No increase in *MAX4* and *MAX3* expression could be observed in dark-grown plants ($P = 0.629$ and $P = 0.531$; Fig. 3). *MAX2* expression has been analyzed in the same tissue samples, and no change in expression pattern was observed (data not shown). In terms of adventitious root regulation, it could be that, in the dark or immediately after transfer to the light, cells in the hypocotyl become competent to induce roots. We anticipate that the increased strigolactone biosynthesis may then prevent further roots from forming. In tomato, light also stimulates the expression of the strigolactone biosynthesis gene *SlCCD7* in the roots, which also corresponded to higher strigolactone levels (Koltai et al., 2011). In that study, however, the authors did not analyze expression in tissues other than the roots; therefore, it is possible that the hypocotyl of tomato also may respond to light.

Previous studies have shown that the hypocotyl could be a potent source of strigolactones, as inserting wild-type hypocotyl segments between strigolactone biosynthetic mutant scion and rootstock can revert mutant scions almost to the wild type (Napoli, 1996; Booker et al., 2004). However, the production of strigolactones in these segments may be up-regulated through feedback and may not under normal conditions produce high quantities of strigolactones. Our results on the expression of strigolactone biosynthesis genes support the suggestion that the hypocotyl, which is the site for adventitious root initiation, may also be a site of strigolactone production. These findings also emphasize that, in Arabidopsis, the root is not the only location with strigolactone production.

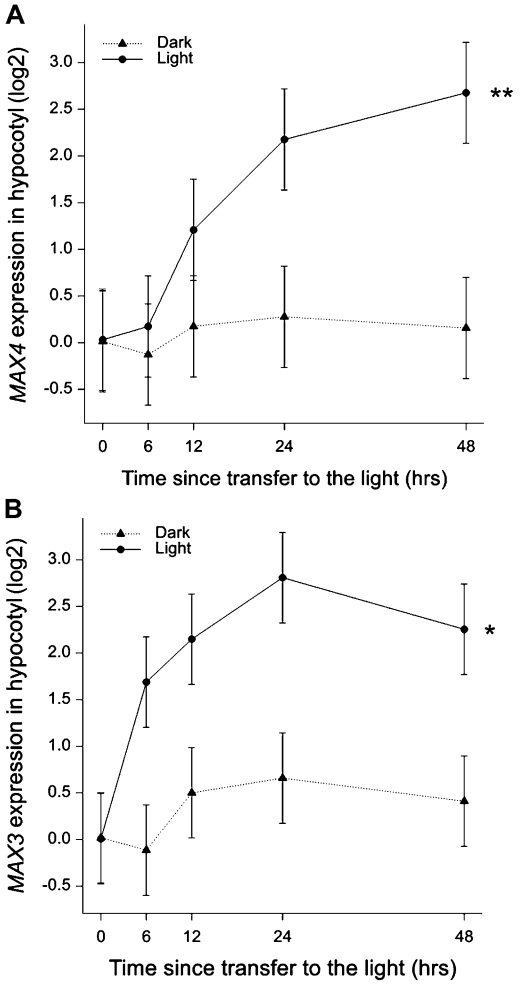

Figure 3. *MAX4* and *MAX3* expression is light induced in the hypocotyl. Relative expression of *MAX4* (A) and *MAX3* (B) is shown in dark-grown hypocotyls that were transferred to light 5 d after germination (circles) or not transferred to light (triangles). Data and error bars represent means of three biological replicates ± SE ($n = 3$). Asterisks indicate statistically significant increases in expression over time (** $P < 0.01$, * $P < 0.05$).

Strigolactones Suppress Adventitious Root Formation Independently of Cytokinins

Cytokinins are well-known suppressors of adventitious root formation; therefore, it is plausible that strigolactones could act through cytokinins to suppress adventitious root formation or vice versa. We have previously shown that pea and Arabidopsis strigolactone

mutants have decreased cytokinin levels in the xylem (Beveridge et al., 1997a, 1997b; Foo et al., 2007), which could potentially explain the increased adventitious rooting observed in these mutants. Therefore, we explored the interdependency between strigolactones and cytokinin in regulating adventitious root formation.

First, we examined the effect of cytokinin treatment on the strigolactone synthesis and response mutants. Cytokinin treatment of wild-type plants resulted in the expected suppression of adventitious root formation, reducing the mean number of roots per plant from 0.9 to zero ($P < 0.05$; Fig. 4A). Cytokinin treatment of the strigolactone mutants *max1*, *max2*, *max3*, and *max4* repressed adventitious rooting to close to wild-type levels. Each mutant displayed a significant reduction in adventitious root number to a similar extent as observed in cytokinin-treated wild-type plants. These results indicate that cytokinins do not suppress adventitious rooting by acting solely through the regulation of strigolactone levels or strigolactone signaling. Because the cytokinin responsiveness is not impaired in the strigolactone mutants, we have raised the possibility that the low-xylem cytokinins observed in strigolactone mutants (Beveridge et al., 1997a, 1997b; Foo et al., 2007) are actually the reason for the enhanced adventitious root formation.

If this assumption were true, then mutants affected in cytokinin biosynthesis or perception should display reduced strigolactone responsiveness. Arabidopsis mutants with reduced cytokinin synthesis (*isopentenyltransferase1* [*ipt1*] *ipt5 ipt7*) or reduced cytokinin perception (*ahk3 ahk4*) displayed increased (3-fold and 1.5-fold, respectively) adventitious root production ($P < 0.05$) relative to wild-type controls (Fig. 4B). Strigolactone treatment of *ipt1 ipt5 ipt7* and *ahk3 ahk4* mutants resulted in a substantial reduction in adventitious roots (87% and 96%, respectively; $P < 0.05$; Fig. 4B). These results, together with the results from cytokinin treatment (Fig. 4A), suggest that strigolactones and cytokinins act independently in adventitious rooting.

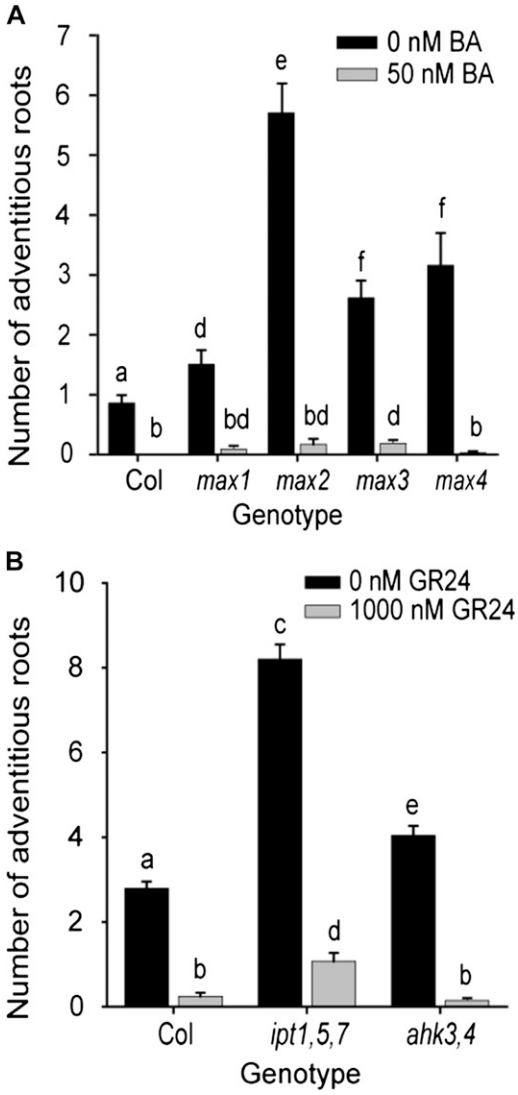

Figure 4. Cytokinin mutants are strigolactone responsive and strigolactone mutants are cytokinin responsive. Mutants were grown on plates containing control and 50 nM benzylaminopurine (BA; A) or 1,000 nM GR24 (B) medium. A, *max1-1*, *max3-11*, and *max4-1* are synthesis mutants, and *max2-1* is a response mutant. $n > 40$. B, *ipt1 ipt5 ipt7* triple mutants produce less cytokinin than the Col-0 wild type, while the *ahk3 ahk4* double mutant is defective in cytokinin reception. In both panels, plants were scored 10 d after transfer to the light (15 d after germination). Means are presented ± SE, and different letters represent means that are significantly different ($P < 0.05$) using Student's *t* test.

Auxin Can Promote Adventitious Root Formation Independently of Strigolactones

The importance of auxin in promoting adventitious rooting has been well established (for review, see Blakesley et al., 1991), and the application of auxin to the base of cuttings is routinely used for plant propagation (Ritchie, 1991). Auxin has been shown to promote *MAX3/CCD7* and *MAX4/CCD8* gene expression (Foo et al., 2005; Hayward et al., 2009), whereas our data support an inhibitory role for strigolactones in adventitious root formation (Fig. 1), suggesting that auxin does not act on adventitious root formation by promoting the transcription of *MAX3* and *MAX4*. To tease apart how strigolactones and auxin may interact, we first examined the strigolactone responsiveness of the auxin-overproducing line *35S:YUCCA1* (Zhao et al., 2001). The *35S:YUCCA1* line produced an increased number of adventitious roots compared with the wild type, consistent with the ability of auxin to promote adventitious root formation (Fig. 5A). When treated with 1,000 nM GR24 in the medium, wild-type plants displayed an 88% reduction in adventitious root number after 10 d of light ($P < 0.05$), whereas, under the same conditions, strigolactone treatment resulted in a 50% reduction in root production in the *35S:YUCCA1* mutant. When auxin content was manipulated by exogenous treatment of 100 nM IAA (Fig. 5B), the number of adventitious roots formed in wild-type

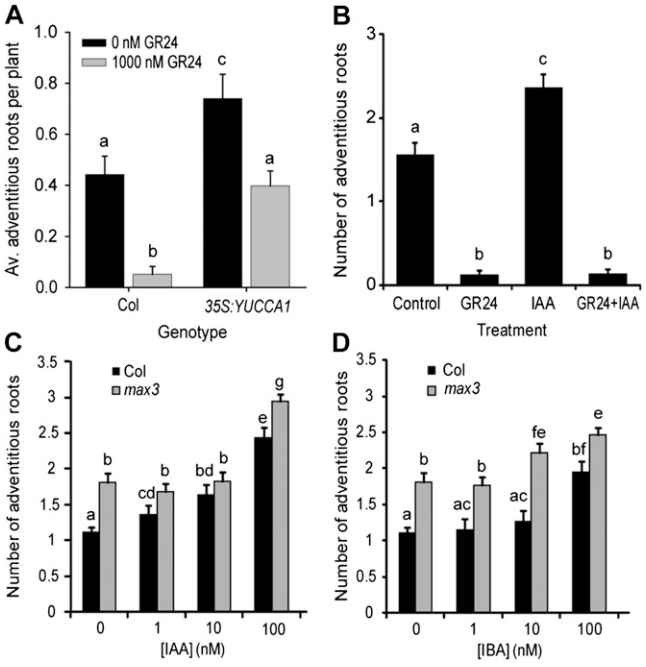

Figure 5. GR24 (synthetic strigolactone) suppresses the number of adventitious roots formed in plants with high auxin, and *max3-11* produces more adventitious roots in response to increasing concentrations of IBA and IAA. A, *35S:YUCCA1* is the auxin-overproducing line, and Col is the Col-0 wild type. $n > 60$. B, Wild-type plants were treated with 100 nM IAA and 1,000 nM GR24 together and separately. C, The synthesis mutant (*max3-11*) and the Col-0 wild type respond to IAA. D, Both genotypes also respond to IBA. $n > 120$ (20 seedlings per plate and six plates per treatment). All plants were scored after 10 d. Means are presented ± SE. Different letters represent means that are significantly different (Student's *t* test, $P < 0.05$).

Arabidopsis was enhanced, whereas strigolactone treatment resulted in a reduction in root number (Fig. 5B). Treatment of wild-type plants with both 100 nM IAA and 1,000 nM GR24 reduced adventitious root formation, such that the number of roots formed was not significantly different from that in wild-type plants treated with strigolactone alone. The difference in the effect of *YUCCA1* overexpression and exogenous auxin application is likely to be due to the constitutive/ubiquitous increase in auxin production in the *35S:YUCCA1* line, including in the rooting zone, in comparison with the exogenous auxin treatment. Further experiments investigating different hormone concentrations and examining auxin localization and transport would be required to determine exactly how these hormones interact. However, our studies indicate that strigolactones can cause inhibitory effects, even in the presence of elevated auxin content.

To test whether auxin signaling can act downstream or independently of strigolactones, we examined the effect of auxin treatment on adventitious rooting in the Arabidopsis *max3* mutant (deficient in the synthesis of strigolactones). For this experiment, we used two types of auxins, IAA and indole-3-butyric acid (IBA), as both of these auxins have been reported previously to induce adventitious roots (Blakesley et al., 1991). Both the wild type and *max3* produced more adventitious roots when treated with either IAA or IBA (Fig. 5, C and D). Wild-type plants showed increasing numbers of adventitious roots with increasing IAA concentration, displaying 2.2-fold more roots 15 d after germination when exposed to 100 nM IAA. At this concentration of IAA, the *max3* mutant also displayed a significant (1.6-fold) increase in root number. These results are not consistent with a role of IAA acting through strigolactones to regulate adventitious rooting. It is worth noting that wild-type plants responded to all concentrations of IAA (1–100 nM), whereas only *max3* displayed an increase in the number of adventitious roots when supplied with 100 nM IAA. However, when supplied with IBA, the *max3* mutant responded to 10 nM IBA, whereas only wild-type plants displayed an increase in adventitious root production at 100 nM IBA. These exogenous hormone studies show that auxin can promote adventitious rooting even in the absence of strigolactone and that strigolactone can suppress adventitious rooting even in the presence of high auxin content (Fig. 5). These findings indicate that the auxin and strigolactone pathways are largely independent, although, as discussed below, this is probably an oversimplification.

Next, we examined the genetic relationship of the *AUXIN RESISTANT1* (*AXR1*) and *MAX* pathways. To do this, we compared adventitious root formation in the wild-type, *axr1* (an auxin response mutant), *max*, and *axr1-max* double mutants. Consistent with previous results (Fig. 1), the *max* mutants produced more adventitious roots than the wild type. In contrast, *axr1* mutants produced almost no adventitious roots (Fig. 6). All four of the *axr1 max* double mutant lines tested formed no roots, similar to the *axr1* mutant alone, supporting a model in which the *MAX* genes act on an *AXR1*-dependent pathway or where there is an absolute requirement for *AXR1* in adventitious rooting. Although *AXR1* may not be specific to auxin signaling, the phenotype of the *axr1 max* double mutants and the reduced response to strigolactone in *35S:YUCCA1* plants suggest that auxin and/or strigolactone may

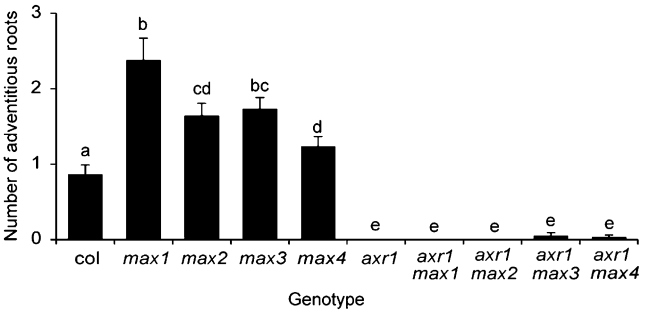

Figure 6. *axr1* and *axr1 max* produce no adventitious roots. $n > 60$. Means are presented ± SE. Different letters indicate significantly different means at $P < 0.05$ (Student's *t* test).

be involved in some kind of cross talk, as discussed below. Alternatively, because auxin signaling is required to respond to the auxin buildup prior to founder cell initiation, it is possible that *AXR1* function is required prior to strigolactones in the early stages of adventitious root formation.

In support of a more direct cross talk between strigolactone and auxin, it has recently been suggested that strigolactones reduce the amount of auxin moving basipetally (Bennett et al., 2006; Brewer et al., 2009; Crawford et al., 2010; Ruyter-Spira et al., 2011). Given that auxin transport can inhibit adventitious root formation in cuttings (Liu and Reid, 1992; Ford et al., 2001; Marks et al., 2002) and if strigolactones reduce the amount of auxin reaching the rooting zone of Arabidopsis hypocotyls, then it is not surprising that adventitious root formation would be reduced under wild-type strigolactone production. A similar explanation has been proposed for the strigolactone inhibition of lateral root development (Kapulnik et al., 2011; Ruyter-Spira et al., 2011). The expression of *MAX2* under various vascular-associated promoters led to the suppression of adventitious rooting in the *max2* background and demonstrated the likely transport of a signal downstream of MAX2. Auxin is a potential candidate for this signal, although it is unclear how

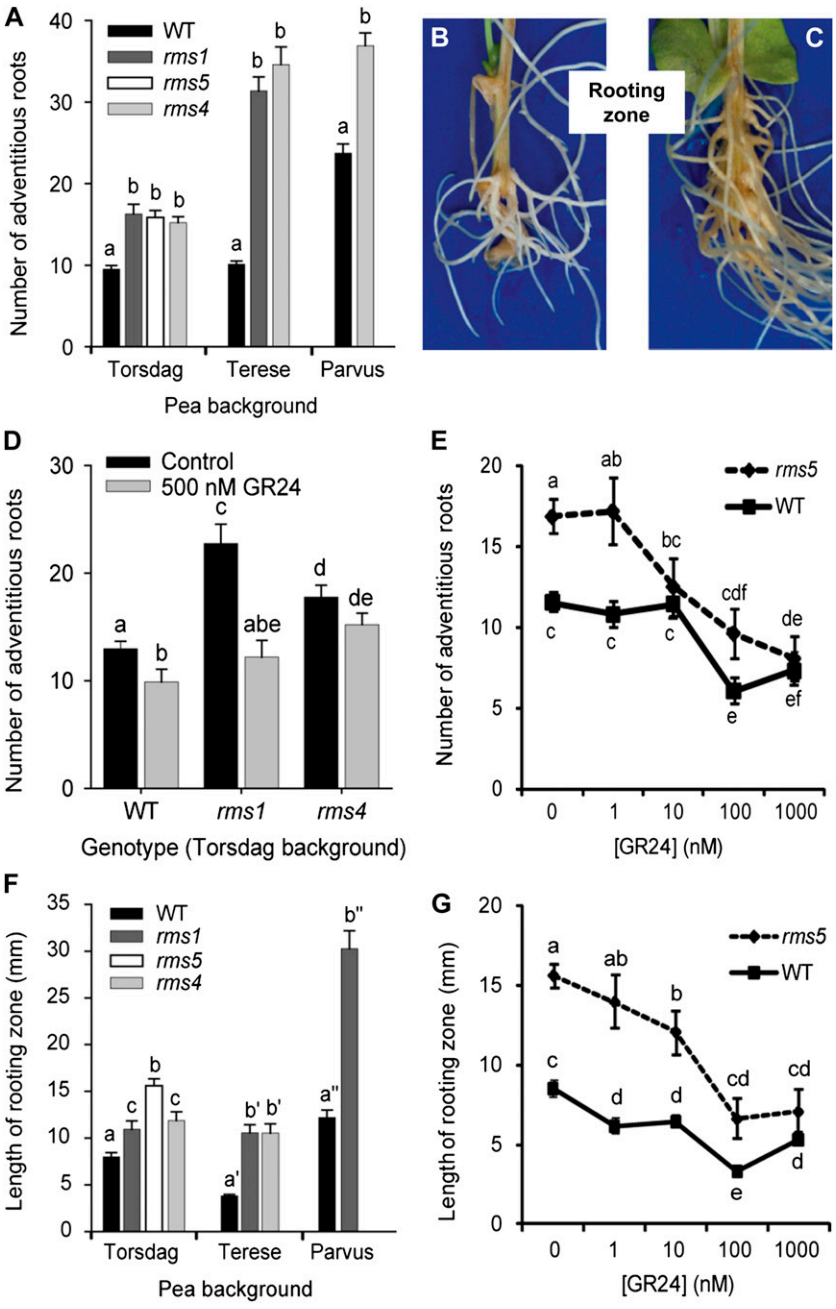

Figure 7. Strigolactones inhibit adventitious root formation in pea cuttings and intact Arabidopsis hypocotyls. A, Adventitious root phenotypes of strigolactone mutants across three pea backgrounds (Torsdag, Térèse, and Parvus). Mutants of *rms* in the Térèse background and *rms5* and *rms4* in Parvus were not available (missing columns). The number of plants (*n*) in each bar is greater than 30 in each background. WT is the wild type; *rms1-2* (Torsdag and Parvus), *rms1-10* (Térèse), and *rms5-3* (Torsdag) are synthesis mutants; *rms4-1* (Torsdag) and *rms4-3* (Térèse) are response mutants. B and C, Photographs of the bases of pea cuttings from the wild type (B) and *rms5* (C) showing the difference in numbers of roots and the rooting zones. D, GR24 (synthetic strigolactone) inhibits adventitious rooting in the wild type and the synthesis mutant (*rms1*) but not in the response mutant (*rms4*). Treatment was 500 nM GR24 for the first 48 h after cutting. $n = 15$. E, GR24 inhibits adventitious root formation in a dose-dependent manner. Wild-type and *rms5-3* cuttings were treated with 0, 1, 10, 100, or 1,000 nM GR24 for the first 48 h after cutting. $n = 15$. F, Strigolactone mutants produce adventitious roots from a larger region of stem (rooting zone). The number of plants (*n*) in each bar is greater than 30 in each background. G, The rooting zone decreased after treatment with 0, 1, 10, 100, or 1,000 nM GR24. *rms5-3* is the synthesis mutant. $n = 15$. In all panels, the plants were scored 21 d after cutting. Means are presented ± SE. Different letters represent means that are significantly different ($P < 0.05$) by Student's *t* test. [See online article for color version of this figure.]

MAX2-dependent strigolactone signaling in the phloem, which is distant from the xylem poles, could be as equally effective as in other vascular-associated cells if the downstream signal was auxin itself.

Strigolactones Also Regulate Adventitious Rooting in Pea

To observe whether strigolactones regulate adventitious rooting in a different species, shoot cuttings of wild-type pea and strigolactone synthesis (*ramosus1* [*rms1*]/*ccd8* and *rms5/ccd7*) and response (*rms4*) mutants were taken, and the cut ends were placed in water to allow adventitious roots to form. We observed an increase in adventitious root formation in strigolactone mutants in nine independent experiments across three genetic backgrounds for pea, and this is consistent with a role for strigolactones in negatively regulating adventitious rooting. There was a 1.5- to 3-fold enhancement of adventitious rooting in the mutants compared with the wild type (Fig. 7, A–C). Furthermore, exogenous application of strigolactone (GR24) resulted in a reduction of adventitious rooting in the strigolactone synthesis mutant *rms1* ($P < 0.05$), to levels similar to those observed in wild-type plants, but caused no significant change in the strigolactone response mutant *rms4* (Fig. 7D). While the synthesis mutants showed a 41% reduction in response to GR24, treatment of wild-type cuttings with 500 nM GR24 for 48 h resulted in a 23% reduction in adventitious root production (Fig. 7D). Similar to Arabidopsis, the response to exogenous strigolactone was dose dependent in both the wild type and *rms5* (Fig. 7E). In *rms5*, this was visible using concentrations of 1 nM GR24 and higher, while the wild type responded to concentrations of 100 nM and higher. The highest concentration of GR24 reduced the average number of roots produced from 11 to seven in the wild type and from 17 to eight in *rms5* (Fig. 7E). These results support the role of strigolactones as a plant hormone regulating adventitious root formation across divergent species. The onset of adventitious root formation in Arabidopsis hypocotyls and pea stems occurs via different processes, through the induction of formative divisions of initial cells in the hypocotyl pericycle in Arabidopsis after light induction and via dedifferentiation of cells in the stem of pea without light induction. Therefore, the similar phenotypes and responses to strigolactones in both hypocotyls and stems demonstrate a general role for strigolactones in restricting organogenesis.

Strigolactones Reduce the Size of the Adventitious Rooting Zone in Pea

Alterations in adventitious root numbers may result in changes in the density of adventitious rooting, the length of the stem from which initials can form (rooting zone), or a combination of both. In three genetic backgrounds of pea, the length of the rooting zone was significantly longer when strigolactone production or response was interrupted (Fig. 7F). When the synthetic strigolactone GR24 was applied exogenously to a strigolactone synthesis mutant or the wild type, the rooting zone decreased in a dose-dependent manner (Fig. 7G). GR24 at 1,000 nM was sufficient to reduce the size of the rooting zone in *rms5* to that of wild-type controls (Fig. 7G). The increase in length of the rooting zone in the strigolactone mutants does not correlate with internode and, presumably, cell length, as the pea strigolactone mutants have shorter internodes than wild-type plants. These results suggest that strigolactones regulate the size of the rooting zone in cuttings of pea.

In Arabidopsis, adventitious roots formed along the entire hypocotyl rather than forming specifically in a region directly above the root-shoot junction, so measurements of the rooting zone were not meaningful. In contrast to light-grown Arabidopsis seedlings, there was no significant difference in hypocotyl length of etiolated *max* mutant seedlings.

The density of adventitious roots responded less predictably in strigolactone mutants (Supplemental Fig. S2A) and to strigolactone treatments in pea (Supplemental Fig. S2B), presumably due to differences in genetic background. From this, it appears that strigolactones have less effect on the density of adventitious roots but instead regulate the region in which the adventitious roots can form.

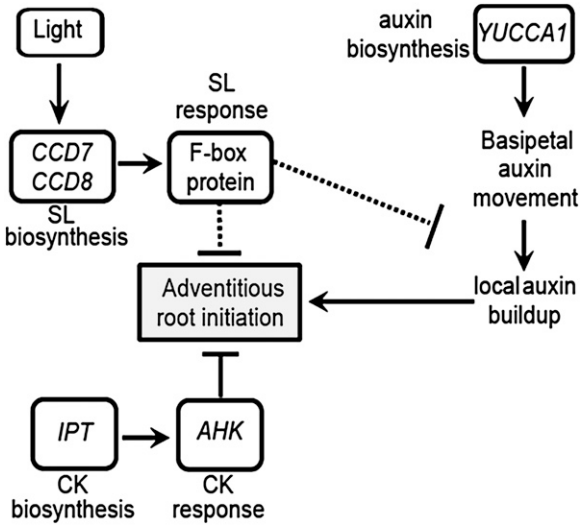

Figure 8. Simplified model of the regulation of adventitious root formation in pea stem cuttings and Arabidopsis intact hypocotyls. Arrows represent promotion and flat-ended lines represent repression. CCD7 and CCD8 are light responsive and are required for the synthesis of strigolactone (SL). Local response to strigolactone requires the RMS4/MAX2 F-box protein. Local signaling and response to strigolactone suppress adventitious root formation. The dashed lines represent possible ways that strigolactone could regulate adventitious rooting (i.e. directly or via regulation of the amount of local auxin levels). Solid lines represent interactions demonstrated in this study. In a cutting, the root-derived strigolactone message is disconnected, thus removing the repression of adventitious root formation.

CONCLUSION

We have developed a simplified model (Fig. 8) to explain the role of strigolactones in controlling adventitious root formation based on our findings. We have demonstrated that strigolactone signaling, via the xylem expression of *MAX2*, acts to inhibit adventitious root initiation (Fig. 2). Furthermore, this regulation is light dependent in the hypocotyl (Fig. 3).

We have also demonstrated that strigolactone and cytokinin act independently to inhibit adventitious root formation (Fig. 4). Similarly, auxin signaling is necessary for adventitious root formation (Fig. 6), and it is possible that strigolactones negatively regulate auxin levels in the pericycle, thereby reducing adventitious root initiation (Fig. 5).

In summary, the research presented here has demonstrated a new function for strigolactones in the suppression of adventitious root initiation. We have tested and presented a model describing how strigolactones may interact with cytokinin and auxin to control adventitious root formation. With further study, the manipulation of strigolactone signaling through strigolactone inhibitors may become a useful tool for improving the cutting propagation of commercially important species.

MATERIALS AND METHODS

Arabidopsis Growth and Adventitious Root Formation

Unless otherwise stated, Arabidopsis (*Arabidopsis thaliana*) plants were grown by first surface sterilizing the seeds for 1 min in 70% ethanol and then for 20 min in 30% bleach with 0.1% Triton X-100. Seeds were sown on square agar plates containing 10 g L^{-1} phytagel (Sigma-Aldrich; http://www.sigmaaldrich.com), 5 g L^{-1} Suc, 1.5 g L^{-1} Murashige and Skoog salts (Phytotechnology Laboratories; http://www.phytotechlab.com), and 0.5 g L^{-1} MES and then stratified in the dark at 4°C for 3 d. Arabidopsis seedlings were grown in 16-h/8-h day/night conditions at 22°C.

Induction and measurement of adventitious roots in intact Arabidopsis plants were performed as described previously (Sorin et al., 2005) with minor modifications. Briefly, wild-type Col-0 and Ws-4 as well as mutant homozygous seed lines *max1* (*At2g26170*), *max2* (*At2g42620*), *max3* (*At2g44990*), *max4* (*At4g32810*), *max2/MAX2:APL*, *max2/MAX2:NST3*, *max2/MAX2:SCR*, *max2/MAX2:WOX4*, *ipt1 ipt5 ipt7*, *ahk3 ahk4*, and *35S:YUCCA1* were placed in the dark in a controlled growth cabinet at 22°C for 5 d. The new *max4-9* allele is the FLAG204D03 line (provided by INRA) in the Ws-4 ecotype. The plates were then exposed to light (16-h days) at 22°C for 7 d. The number of adventitious roots on each seedling was then counted. Adventitious roots were only counted above the root-shoot junction (and not including any roots that formed at the junction). For Arabidopsis experiments using GR24, 1,000 nM was applied. IAA and IBA were dissolved in ethanol and diluted to treatment concentrations of 0, 1, 10, and 100 nM. Phytohormone treatments were added to the agar medium at the desired concentration prior to pouring the plates.

Cloning and Transgenic Lines

As described by Agusti et al. (2011), to generate the *APL:MAX2* (*pSH1*) construct, the *MAX2* open reading frame was amplified by PCR and cloned into a vector containing the *APL* promoter (Sehr et al., 2010), using *Nco*I/*Pci*I and *Pst*I restriction sites. For the generation of *NST3:MAX2* (*pSH2*), we amplified a 3,028-bp genomic fragment upstream of the *NST3* start codon using the primers *NST3for3* and *NST3rev6* and a 523-bp genomic fragment downstream of the *NST3* stop codon using the primers *NST3for7* and *NST3rev3*. The fragments were cloned into the *Kpn*I and *Not*I restriction sites of *pGreen0229* (Hellens et al., 2000). The *MAX2* open reading frame was cloned into the *Nco*I/*Pci*I and *Bam*HI restriction sites generated between both promoter fragments. To produce the *SCR:MAX2* (*pSH3*) construct, a 2,517-bp genomic fragment upstream of the SCR start codon and a 554-bp genomic fragment downstream of the *SCR* stop codon were amplified using the primer pairs *SCRprom3/SCR_Prom_R* and *SCR_Prom3′_F/SCR_Prom3′_R*, respectively. The fragments were cloned into *pGreen0229*. The *MAX2* open reading frame was cloned into the *Bam*HI and *Xma*I restriction sites generated between both promoter fragments. To generate the *WOX4:MAX2* (*pSH5*) construct, we amplified a 2,943-bp genomic fragment upstream of the *WOX4* start codon using the primers *WOX4for1* and *WOX4rev1* and a 646-bp genomic fragment downstream of the *WOX4* stop codon using the primers *WOX4for9* and *WOX4rev2*. The fragments were cloned into *pGreen0229*, and the *MAX2* open reading frame was cloned into the *Nco*I/*Pci*I and *Bam*HI restriction sites generated between both promoter fragments. All lines were homozygous, and all constructs were sequenced; after plant transformation into *max2-1* mutants, single-copy lines were identified by Southern analyses, and representative lines were used for further investigations. All primers mentioned in this section are listed by Agusti et al. (2011).

Histology

GUS staining was performed as described by Vanneste et al. (2005), and stained seedlings were mounted in 90% lactic acid (Acros Organics). The slides were analyzed using differential interference contrast microscopy (Olympus BX51).

Gene Expression

Plants were grown and etiolated as described above on square agar plates to induce adventitious rooting. After etiolation, half of the plates were transferred to the light and the other half remained in the dark. The cotyledons, hypocotyls, and roots were harvested at 0, 6, 12, 24, and 48 h after transfer to light together with cotyledons, hypocotyls, and roots of plants that were not transferred to the light. For each time, hypocotyls from around 100 plants were used. Tissues were harvested for three biological repeats and stored at −80°C. Total RNA was extracted with the RNeasy Plant Mini Kit (Qiagen) according to the manufacturer's protocol. Genomic DNA was removed by DNase treatment, and the RNA samples were purified through NH_4Ac (5 M) precipitation. Samples were quality controlled and quantified with a Nano-Drop Spectrophotometer (Isogen). One microgram of RNA was reverse transcribed into cDNA with the iScript cDNA synthesis kit (Bio-Rad) and subsequently diluted 25 times. Real-time quantitative reverse transcription-PCR was done on a LightCycler 480 (Roche Diagnostics) with SYBR Green for detection, in triplicate on a 384-multiwell plate, in a total volume of 5 μL and a cDNA fraction of 10% or in a total volume of 10 μL and a cDNA fraction of 20%, for detection of *MAX4* and *MAX3* expression, respectively. Cycle threshold values were obtained with the accompanying software and analyzed with the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). The obtained values were normalized against those of CASEIN KINASE II, which was used as an internal standard. Averages were made of the three biological repeats at each time point after transfer to the light, and statistical analysis of *MAX4* and *MAX3* expression profiles was done by one-way ANOVA, using GenStat version 13 software (Payne, 2010).

Pea Seedling Growth/Germination

Seeds of pea (*Pisum sativum*) were planted in tubes (45 × 45 × 75 mm) filled with premium blend potting mix (7:2:1 pine bark:peat blend:sand) and watered every second day for 2 weeks under 16-h daylength with day/night temperatures of 23°C/18°C. The dwarf Térèse seeds were germinated in the dark, and emerged seedlings were given 2 d of dark, 1 d of light, 2 d of dark, and then 10 d of light to encourage basal internode elongation.

Pea Cutting Conditions

Seedlings with five to six leaves expanded (including scale leaves) were cut above the second scale leaf, and the bases were placed in 20 mL of treatment solution. All cuttings were kept within a transparent enclosure under the conditions above; bases were kept in dark enclosures. Treatment solutions were supplied at the time of cutting and were reapplied at 24 h. After 21 d, the number of adventitious roots (those emerging directly from the stem) were counted, and the length of the rooting zone (distance from the base to the

uppermost root) and the density (number of roots per millimeter of stem) were measured. GR24 (www.chiralix.com) was dissolved in acetone, and then treatments were made up in water. For the dose-response experiments, concentrations of 0, 1, 10, 100, and 1,000 nM GR24 were used. For other pea experiments using GR24, 500 nM was applied. IAA and IBA were dissolved in ethanol, diluted to treatment concentrations, and applied to the base of the cuttings for 48 h.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Number of adventitious roots in the Ws-4 background of Arabidopsis.

Supplemental Figure S2. The rooting zone is suppressed by strigolactones, while the density of adventitious roots is not controlled by strigolactones.

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