Auxin Controls Arabidopsis Adventitious Root Initiation by Regulating Jasmonic Acid Homeostasis

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Vegetative shoot-based propagation of plants, including mass propagation of elite genotypes, is dependent on the development of shoot-borne roots, which are also called adventitious roots. Multiple endogenous and environmental factors control the complex process of adventitious rooting. In the past few years, we have shown that the auxin response factors ARF6 and ARF8, targets of the microRNA miR167, are positive regulators of adventitious rooting, whereas ARF17, a target of miR160, is a negative regulator. We showed that these genes have overlapping expression profiles during adventitious rooting and that they regulate each other's expression at the transcriptional and posttranscriptional levels by modulating the homeostasis of miR160 and miR167. We demonstrate here that this complex network of transcription factors regulates the expression of three auxin-inducible Gretchen Hagen3 (GH3) genes, GH3.3, GH3.5, and GH3.6, encoding acyl-acid-amido synthetases. We show that these three GH3 genes are required for fine-tuning adventitious root initiation in the Arabidopsis thaliana hypocotyl, and we demonstrate that they act by modulating jasmonic acid homeostasis. We propose a model in which adventitious rooting is an adaptive developmental response involving crosstalk between the auxin and jasmonate regulatory pathways.

INTRODUCTION

Adventitious roots arise from aerial organs of the plant either naturally or in response to altered environmental conditions (Geiss et al., 2009; Li et al., 2009). They are required for vegetative propagation of plants and the successful clonal multiplication of elite genotypes of forest, horticultural, and agricultural plant species. The ability to form adventitious roots is a heritable quantitative trait controlled by multiple endogenous and environmental factors (Geiss et al., 2009; Li et al., 2009), among which the plant hormone auxin plays a central role. Auxin is often applied exogenously to promote the development of adventitious roots on stem cuttings of difficult-to-root genotypes. However, exogenous auxin is not always effective, and a better understanding of the molecular mechanisms through which it regulates adventitious rooting is needed in order to improve the rooting of recalcitrant genotypes. So far, only a few genes involved in the regulation of adventitious rooting have been identified. In rice (Oryza sativa), disruption of the auxin-inducible CROWN ROOTLESS1/ADVENTITIOUS ROOTLESS1 gene (CRL1/ARL1), which encodes a member of the plant-specific LATERAL ORGAN BOUNDARY DOMAIN (LBD) protein family, has been shown to prevent the initiation of adventitious crown root primordia (Inukai et al., 2005; Liu et al., 2005). The promoter of the CRL1/ARL1 gene contains specific cis-regulatory elements that interact with the rice transcription factor ARF16, a member of the AUXIN RESPONSE FACTOR (ARF) family (Inukai et al., 2005). ARF16 is the rice ortholog of both ARF7 and ARF19 in Arabidopsis thaliana (Wang et al., 2007). ARF7 and ARF19 regulate lateral root formation in Arabidopsis by activating the expression of LBD16 and LBD29, both of which are phylogenetically closely related to ARL1/CRL1 (Wilmoth et al., 2005; Okushima et al., 2007). ARF transcription factors mediate auxin signaling at the transcriptional level by regulating the expression of auxin-responsive genes (Guilfoyle and Hagen, 2007). The characterization of Arabidopsis argonaute1 (Bohmert et al., 1998) and superroot2 (Delarue et al., 1998; Barlier et al., 2000) single and double mutants allowed us to identify several genes potentially involved in the regulation of adventitious rooting, including one ARF transcription factor, ARF17 (At1g77850), that encodes the target of the microRNA miR160 and three auxin-inducible GH3-like genes, GH3.3 (At2g23170), GH3.5/GH3a/WES1 (At4g27260), and GH3.6/DFL1 (At5g54510; Nakazawa et al., 2001;...

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Tanaka et al., 2002; Sorin et al., 2005, 2006; Park et al., 2007b, 2007a). The first GH3 gene was identified as an early auxin-responsive gene in soybean (Glycine max) (Hagen et al., 1984), and since then, GH3-like genes have been found in many plant species. However, it is only recently that they have been shown to encode enzymes able to conjugate various amino acids to jasmonates, auxins, and benzoates, leading to the activation, inactivation, or degradation of these molecules (Staswick et al., 2005; Chen et al., 2010; Wang et al., 2010; Westfall et al., 2010). We first showed that ARF17 was a negative regulator of adventitious rooting that could potentially integrate auxin and light signaling pathways affecting this process (Sorin et al., 2005). We also showed that ARF17 was a negative regulator of GH3.3, GH3.5, and GH3.6 expression (Sorin et al., 2005), and, in addition, we found that the GH3.3, GH3.5, and GH3.6 protein content was positively correlated with the number of adventitious roots but not with the endogenous auxin content (Sorin et al., 2006). More recently, we demonstrated that adventitious root initiation is controlled in Arabidopsis hypocotyls by a subtle balance between the negative regulator ARF17, which is the target of miR160, and the positive regulators ARF6 (At1g30330) and ARF8 (At5g37020), which are targets of miR167. These three ARFs display overlapping expression domains, interact genetically, and regulate each other’s expression at both the transcriptional and post-transcriptional levels by modulating the availability of the regulatory microRNAs miR160 and miR167 (Gutierrez et al., 2009). In this article, we show that, in contrast to ARF17, ARF6 and ARF8 are positive regulators of the auxin-inducible genes GH3.3, GH3.5, and GH3.6. We also demonstrate that these three GH3 genes have redundant functions and are required for fine-tuning adventitious root initiation in Arabidopsis hypocotyls by modulating jasmonic acid (JA) homeostasis.

RESULTS

The Number of Adventitious Roots in arf Mutants and ARF-Overexpressing Lines Does Not Correlate with Endogenous Auxin Content But Does Correlate with GH3.3, GH3.5, and GH3.6 Transcript Levels

In an attempt to identify the pathway through which the microRNA-targeted genes ARF6, ARF8, and ARF17 regulate adventitious rooting, we selected for evaluation representative mutants and overexpressing lines that we previously characterized as having contrasting adventitious rooting phenotypes (Gutierrez et al., 2009) (Figure 1A). The double mutant arf10-3 arf16-3, which has no phenotype affecting adventitious root growth, was used as a control for the MIR160c-overexpressing line (MIR160c-OX), in which the accumulation of miR160 specifically targets ARF10, ARF16, and ARF17 transcripts for degradation (Wang et al., 2005). In the absence of an arf17 knockout mutant, we used MIR160-OX as an ARF17 downregulated line, as described previously (Gutierrez et al., 2009).

Since auxin is a well-known inducer of adventitious rooting in Arabidopsis (Boerjan et al., 1995; Delarue et al., 1998), it was

![Figure 1](image-url)

**Figure 1.** Variation in the Number of Adventitious Roots in the Hypocotyl of arf Mutants and ARF-Overexpressing Lines Correlates with the Expression Levels of GH3.3, GH3.5, and GH3.6.

(A) Adventitious roots (AR) were counted on seedlings of arf mutant lines and ARF-overexpressing lines. Seedlings were first etiolated in the dark, until their hypocotyls were 6 mm long, and then transferred to the light for 7 d. Data from three independent biological replicates, each of at least 30 seedlings, were pooled and averaged. Error bars indicate se. A one-way analysis of variance combined with the Tukey’s multiple comparison postest indicated that the values marked with asterisks were significantly different from wild-type values (P < 0.01; n > 90). In addition, those marked with hash signs were significantly different from values obtained from single mutants or the ARF17-OX1 line (P < 0.01; n > 90). Col-0, ecotype Columbia; Ws, ecotype Wassilewskijia.

(B) The GH3.3, GH3.5, and GH3.6 transcript abundance was quantified in the hypocotyls of the different arf mutants or overexpressing lines, which had been etiolated and then transferred to the light for 72 h. Gene expression values are relative to expression in the wild type, for which the value is set to 1. Error bars indicate se obtained from three independent biological replicates. A one-way analysis of variance combined with the Dunnett’s comparison postest indicated that the values marked with ns were not significantly different from wild-type values, whereas the others were significantly different (P < 0.05; n = 3).
important to verify whether the increased or reduced number of adventitious roots was due to a higher or lower endogenous content of free indole-3-acetic acid (IAA) in the hypocotyl. Therefore, the level of endogenous free IAA and its major conjugates, IAAsp [N-indole-3-acetyl]-aspartate and IAGlu [N-indole-3-acetyl]-glutamate, was quantified in the hypocotyls of seedlings grown as described previously (Gutierrez et al., 2009). Neither mutations in, nor overexpression of, ARF6, ARF8, or ARF17 significantly altered the endogenous content of free IAA, suggesting that the observed adventitious root phenotype is unlikely to be related to an increase or a reduction in IAA content (see Supplemental Figure 1 online).

To investigate the interactions between GH3.3, GH3.5, and GH3.6, whose expression levels were correlated with the number of adventitious roots (Sorin et al., 2006) and ARF genes, we first analyzed the expression of the three GH3 genes during the early steps of adventitious root formation using transcriptional fusion constructs containing β-glucuronidase (GUS) fused to the respective GH3 promoters. The promGH3.3:GUS, promGH3.5:GUS, and promGH3.6:GUS constructs have overlapping expression patterns with those of promARF6:GUS, promARF8:GUS, and promARF17:GUS and are likewise regulated by light (see Supplemental Figure 2 online) (Gutierrez et al., 2009). Second, we performed real-time quantitative RT-PCR analysis of the relative transcript amounts of the three GH3 genes in the hypocotyls of the various ARF mutant and overexpressing lines (Figure 1B). In the double mutant arf10-3arf16-3, which displayed no difference in adventitious rooting phenotype compared with the wild type, the GH3.3, GH3.5, and GH3.6 transcript levels were not affected. In contrast, the transcript levels of all three genes were reduced in all of the lines showing fewer adventitious roots than the wild type, whereas it was increased in those with a higher number of adventitious roots (Figures 1A and 1B). In agreement with previous data (Sorin et al., 2006), the smaller number of adventitious roots, the lower the amount of GH3.3, GH3.5, and GH3.6 transcripts and vice versa. Since the arf6-3arf8-7 double mutant is sterile, we analyzed a population of progeny from the arf6-3arf8-7 mutant backgrounds, a significant number of which segregated for 25% the level of endogenous free IAA and its major conjugates, IAAsp and IAGlu, was quantified in the hypocotyls of seedlings grown as described previously (Gutierrez et al., 2009). Neither mutations in, nor overexpression of, ARF6, ARF8, or ARF17 significantly altered the endogenous content of free IAA, suggesting that the observed adventitious root phenotype is unlikely to be related to an increase or a reduction in IAA content (see Supplemental Figure 1 online).

In order to assess the potential contributions of the three GH3 genes to the regulation of adventitious rooting, we analyzed adventitious root formation in gh3.3-1, gh3.3-2, gh3.5-1, gh3.5-2, gh3.6-1, and gh3.6-2 single knockout mutant alleles, in gh3.3-1gh3.5-2 and gh3.3-1gh3.6-1 double mutants, and in a gh3.3-1gh3.5-2gh3.6-1 triple mutant grown under conditions described previously (Sorin et al., 2005; Gutierrez et al., 2009) (Figure 2A). We confirmed that none of the single, double, or triple gh3 mutants showed a defect in primary root elongation or secondary root development when grown in our conditions (see Supplemental Figure 4 online).

Interestingly, all single mutants produced significantly more adventitious roots than did the wild type. This was unexpected, given that the results presented above, together with our previous data (Sorin et al., 2006), indicated a positive correlation between the number of adventitious roots and the GH3 transcript or GH3 protein content. This phenotype was not explained by an increased endogenous auxin content since, despite the fact that these three GH3 proteins were shown to conjugate IAA (Staswick et al., 2005), no modification of the endogenous free IAA content was observed in the single mutants (see Supplemental Figure 5 online). In contrast to the single mutants, the gh3.3-1gh3.5-2 and gh3.3-1gh3.6-1 double mutants developed only half the number of adventitious roots compared with the wild type. Also, the gh3.3-1gh3.5-2gh3.6-1 triple mutant produced significantly fewer adventitious roots than the double mutants (Figure 2A), showing the additive effect of the mutations and suggesting a redundant function for GH3.3, GH3.5, and GH3.6 in the regulation of adventitious rooting in Arabidopsis. In this particular context, this result was also contradictory with a role in IAA conjugation and a potential increase of free IAA, since one would expect to see the number of adventitious roots increasing and not decreasing in the double and triple mutants. This did not explain why the single mutants produced more adventitious roots than the wild type. Because the three GH3 genes were strictly coregulated in the different arf mutants and ARF-overexpressing lines, we performed quantitative RT-PCR analysis of the relative transcript amounts of these GH3 genes in the hypocotyl of each single and double gh3 mutant (Figure 2A). Interestingly, the expression of GH3.5 and GH3.6 was upregulated in gh3.3-1 mutant seedlings, whereas that of GH3.3 and GH3.6 was upregulated in gh3.5-2 mutant seedlings, and that of GH3.3 and GH3.5 was upregulated in gh3.6-1 mutant seedlings (Figure 2B). This compensatory effect led to a higher level of total GH3.5+GH3.6, GH3.3+GH3.6, and GH3.3+GH3.5 expression. GH3.3, GH3.5, and GH3.6 belong to a family of 19 genes. Therefore, we checked, in the hypocotyl of the ARF mutant and overexpressing lines, the expression of the other members of the GH3 gene family as well as that of selected auxin-inducible genes shown to be differentially expressed in the ago1-3 versus wild-type hypocotyls (http://urgv.evry.inra.fr/cgi-bin/projects/ CATdb/catdb_index.pl). None of these genes were found to be coregulated with GH3.3, GH3.5, and GH3.6 in the different ARF mutant or overexpressing lines (see Supplemental Figures 3B and 3C online).
transcripts in the single mutants than the total GH3.3+GH3.5+GH3.6 transcript amount in the wild type (Figure 2C), which was likely to explain the increased average number of adventitious roots in the single mutants. The GH3.6 and GH3.5 transcripts also accumulated to higher than wild-type levels in the gh3.3-1gh3.5-2 and gh3.3-1gh3.6-1 double mutants, respectively, but this accumulation was not sufficient to be equivalent to the total GH3.3+GH3.5+GH3.6 transcript amount in the wild type. This likely explains the reduced number of adventitious roots produced by the gh3.3-1gh3.5-2 and gh3.3-1gh3.6-1 double mutants. In conclusion, these results suggest that GH3.3, GH3.5, and GH3.6 redundantly and positively regulate adventitious rooting and that this role is independent from their activity as IAA-amido synthetases.

GH3.3, GH3.5, and GH3.6 Are Likely to Stimulate Adventitious Rooting by Inactivating JA

The results described above suggest that GH3.3, GH3.5, and GH3.6, which are auxin-inducible genes, do not act by regulating auxin levels but might inactivate an inhibitor of adventitious rooting. Among the three proteins, GH3.5 was shown to adenylate salicylic acid (SA) as well as IAA in vitro (Staswick et al., 2005). Therefore, it is possible that, in planta, GH3.3 and GH3.6 proteins could also conjugate SA. Salicylate, therefore, was an obvious candidate to test as a potential target for GH3 proteins. Like SA, JA is a stress-related hormone that can be conjugated by GH3 proteins. The protein product of the gene GH3.11, which is also called JASMONE ACID RESISTANT1 (JAR1), conjugates JA to Ile, producing jasmonoyl-L-isoleucine (JA-Ile), the active signaling molecule recognized by the F-box receptor CORONATINE INSENSITIVE1 (COI1) (Fonseca et al., 2009). In addition, methyl jasmonate was shown to inhibit root growth in Arabidopsis (Staswick et al., 1992), and JA transiently accumulated at the base of Petunia hybrida stem cuttings after mechanical wounding but very rapidly returned to its basal level before adventitious root formation took place (Ahkami et al., 2009). Fattorini et al. (2009) showed that submicromolar amounts of methyl jasmonate promoted adventitious root development from thin cell layers of tobacco (Nicotiana tabacum). This information posttest indicated that the differences observed in the mutants versus the wild type (*), in gh3.6 versus gh3.3 or gh3.5 (#), and in the triple mutant versus the double mutants (##) are significant (P < 0.01; n > 90). Col-0, ecotype Columbia.

(B) The steady state expression levels of GH3.3, GH3.5, and GH3.6 were quantified by quantitative RT-PCR in the hypocotyls of the different gh3 single and double mutants first etiolated in the dark, until their hypocotyls were 6 mm long, and then transferred to the light for 72 h. (C) The relative amount of the total GH3.3+GH3.5+GH3.6 mRNAs in the hypocotyl of single and double gh3 mutants grown as in (B) was quantified by quantitative RT-PCR. For (B) and (C) Gene expression values are relative to the expression in the wild type, for which the value is set to 1. Error bars indicate se obtained from three independent biological replicates. A one-way analysis of variance combined with the Dunnett’s comparison posttest confirmed that the differences between the wild type and the mutants (*) are significant (P < 0.001, n = 3).
twice as much JA compared with the wild type (Figure 3C). Three days after transfer to the light, the JA level was significantly reduced in both wild-type seedlings and the triple mutant. This result indicates that light regulates JA homeostasis. Whether this is due to a downregulation of JA biosynthesis or an activation of JA degradation remains to be determined.

We had previously shown that the early events of adventitious root initiation, such as the expression of the cell cycle gene CYCLOIDEA (CYC) and the CRY1, take place sometime between T0 and 48 h after transfer to the light (Sorin et al., 2005). Therefore, we tested whether GH3.3, GH3.5, and GH3.6 could play a role in the regulation of JA homeostasis by quantifying free JA and the active conjugate JA-Ile in the hypocotyl of wild type and gh3.3-1gh3.5-2gh3.6-1 triple mutant seedlings at T0 and soon after transfer to the light (Figures 3D and 3E). JA content was significantly increased in the triple mutant compared with the wild type, whereas that of JA-Ile was also higher in the triple mutant but was only at a statistically significant level 9 h after transfer to the light. Although low, this increase in endogenous JA-Ile might explain the reduced number of adventitious roots observed in the triple gh3 mutant, since concentration as low as 1 nM exogenously applied JA reduced by 50% the average number of adventitious roots in wild-type seedlings (see Supplemental Figure 6 online).

In conclusion, the accumulation of JA in the triple gh3.3gh3.5gh3.6 mutant suggests that GH3.3, GH3.5, and GH3.6 proteins might conjugate JA to amino acids whose identities have yet to be determined, thereby modulating its level or inactivating it.

GH3.3, GH3.5, and GH3.6 Proteins May Control JA Homeostasis by Conjugating It to Amino Acids

To establish whether GH3.3, GH3.5, and GH3.6 can conjugate JA, end-point assays with the respective glutathione S-transferase (GST) fusion proteins were done, and the products were analyzed by thin layer chromatography. These enzymes conjugate IAA to several amino acids in vitro, but Asp, Met, and Trp are among the better substrates and therefore were tested with JA (Staswick et al., 2005). All three enzymes produce products consistent with JA-Asp, JA-Met, and JA-Trp (Figure 4). In contrast, no detectable product was seen with Ile, in agreement with the fact that relatively little JA-Ile is produced with these enzymes. Our data show that GH3.3, GH3.5, and GH3.6 are able to conjugate JA to amino acids; therefore, the accumulation of JA in the gh3.3-1gh3.5-2gh3.6-1 triple mutant might be explained by a reduction of the conjugation process.

Nevertheless, because an accumulation of JA could also be due to an upregulation of the biosynthesis pathway, we checked the expression of genes encoding the main enzymes involved in JA biosynthesis (see Supplemental Figure 7 online). All genes tested were upregulated in the triple mutant compared with the wild type. JA accumulation in the triple mutant could be due to a combination of increased biosynthesis and reduced conjugation.

Jasmonate Regulates Adventitious Rooting through the COI1 Signaling Pathway

The higher level of JA-Ile that accompanied the increase of JA might activate the COI1 signaling pathway and thereby inhibit
the early stages of adventitious root initiation. To confirm that the JA signaling pathway was upregulated, we analyzed the expression of JAR1, JAZ1, JAZ3, JAZ5, and COI1 genes in the hypcotyl of the wild type and the triple gh3.3-1gh3.5-2gh3.6-1 mutant. JAR1 was upregulated in the hypcotyl of the gh3.3-1gh3.5-2gh3.6-1 mutant compared with wild-type seedlings after they had been transferred to the light for 3 and 9 h (Figure 5A). The relative transcript amounts of the three JA-inducible genes JAZ1, JAZ3, and JAZ5 were upregulated in the hypocotyl of the triple mutant whether it was transferred to the light or not (Figures 5B to 5D), and last, the relative transcript amount of COI1 was shown to the right, migrated with somewhat greater mobility because they were loaded on the plate in organic solvent rather than the enzyme reaction mixture. Free IAA and JA migrated to near the top of the plate.

Figure 4. GH3.3, GH3.5, and GH3.6 Can Conjugate JA to Amino Acids in Vitro.

Thin layer chromatography analysis of JA-amino acid conjugates synthesized by recombinant GST:GH3.3, GST:GH3.5, and GST:GH3.6 proteins. Substrates added to each enzyme reaction mixture are indicated below the image. Concentrations for JA and IAA were 10 and 1 mM, respectively. JA conjugate standards, shown to the right, migrated with somewhat greater mobility because they were loaded on the plate in organic solvent rather than the enzyme reaction mixture.

DISCUSSION

In a previous study, we showed that adventitious rooting on the Arabidopsis hypocotyl is controlled by a complex interaction between three transcription factors from the ARF family, ARF6, ARF8,
and ARF17, and the regulatory microRNAs miR167 and miR160. ARF6 and ARF8 are positive regulators of adventitious rooting, whereas ARF17 is a negative regulator (Gutierrez et al., 2009). In addition, the levels of the proteins encoded by three auxin-responsive genes from the GH3 family (GH3.3, GH3.5, and GH3.6) were positively correlated with the number of adventitious roots, suggesting a role for these genes in the regulation of adventitious rooting (Sorin et al., 2006).

The GH3 family is part of the broader acyl-adenylate/thioester-forming enzyme family, also called the firefly luciferase family (Staswick et al., 2002). In Arabidopsis, there are 19 members that fall into three distinct phylogenetic groups (Staswick et al., 2002, 2005). In group I, GH3.10/DFL2 was described as being involved in red light-specific hypocotyl elongation (Takase et al., 2004), and GH3.11/JAR1 was first shown to be required for the root growth inhibition response to methyl jasmonate (Staswick et al., 1992) and later to catalyze the formation of JA-Ile, the bioactive form of JA (Staswick and Tiryaki, 2004). In addition to GH3.3, GH3.5, and GH3.6/DFL1, group II of the GH3 family comprises five additional members that catalyze the formation of auxin-amino acid conjugates (Staswick et al., 2005). And last, group III is so far the least well characterized. It is composed of nine proteins, one of which (GH3.12/PBS3) has recently been shown to conjugate amino acids to 4-substituted benzoates (Okrent et al., 2009).

We analyzed the relative transcript amount of all GH3 genes and a few additional auxin-responsive genes, and with the exception of GH3.3, GH3.5, and GH3.6/DFL1, it was not correlated with the adventitious root phenotype of the arf mutants and ARF-overexpressing lines. Transcripts were either not detected

**Figure 5.** The COI1 Signaling Pathway Is Required for the Inhibition of Adventitious Rooting by Jasmonate.
ARF6, ARF8, and ARF17 and their regulatory microRNAs interact in a complex network (Gutierrez et al., 2009) and act upstream of GH3.3, GH3.5, and GH3.6. ARF6 and ARF8 are positive regulators of the three GH3s, whereas ARF17 is a negative regulator. GH3.3, GH3.5, and GH3.6 control each other’s expression through a pathway yet to be identified. The three GH3 genes control JA homeostasis. The JA-Ile level in the hypocotyl is determined by the JA level. The level of JA-Ile negatively regulates adventitious rooting through the activation of the COI1 signaling pathway. In conclusion, auxin controls adventitious root initiation through the activation of ARF6 and ARF8 proteins, leading to a down-regulation of the inhibiting COI1 signaling pathway.

in Arabidopsis hypocotyls or their level was either not affected or significantly increased in the different arf mutants or ARF-overexpressing lines. We could then conclude that overall auxin signaling was not affected in the arf mutants and ARF-overexpressing lines and that GH3.3, GH3.5, and GH3.6 are the only GH3 genes out of 19 that are strictly coregulated in the Arabidopsis hypocotyl during adventitious rooting. Furthermore, a decrease or increase in the GH3 transcript level leads to a lower or higher average number of adventitious roots, respectively, confirming the positive correlation we described previously (Sorin et al., 2006).

Exogenous auxin is often used to induce adventitious rooting on stem cuttings of different species (de Klerk et al., 1999; Geiss et al., 2009; Li et al., 2009). In Arabidopsis, mutants overproducing auxin, such as superroot1, superroot2, or yucca1, spontaneously develop numerous adventitious roots on the hypocotyl and, in the case of superroot1, on cuttings of different organs (Boerjan et al., 1995; Delarue et al., 1998; Zhao et al., 2001). Nevertheless, in this work, we show that the variation in the average number of adventitious roots developing on the hypocotyl of representative arf mutants, ARF-overexpressing lines, and the gh3.3, gh3.5, and gh3.6 single and triple mutants does not correlate with a change in the free IAA content. Rather, the data are consistent with our previous results showing that the number of adventitious roots is correlated to the level of GH3.3, GH3.5, and GH3.6 proteins (Sorin et al., 2005, 2006). This was unexpected, given the evidence that these GH3s inactivate IAA by conjugating it to amino acids (Staswick et al., 2005). Consistent with this function, the levels of IAAglu and IAAaspartate were reduced in the single gh3.3-1, gh3.5-2, and gh3.6-1 mutant hypocotyls compared with the wild type. However, this did not increase the endogenous level of free IAA. Surprisingly, in each single mutant, the other two GH3 genes were upregulated, so one might actually have predicted a reduction of free IAA and a reduced number of adventitious roots, which did not occur. Together, these results suggest that these three GH3 proteins are not directly regulating IAA homeostasis in a way that controls adventitious rooting.

The single gh3 mutants developed more adventitious roots than the wild type likely because of the upregulation of the other two remaining GH3 genes, which resulted in a total amount of the three GH3 transcripts that was higher in the single mutants than the total amount in the wild type. In contrast, the double knockout mutants (gh3.3-1gh3.5-2 and gh3.3-1gh3.6-1) developed fewer adventitious roots compared with the wild type, and the gh3.3-1gh3.5-2gh3.6-1 triple mutant developed even fewer than the double mutants. This additive effect indicates that the GH3.3, GH3.5, and GH3.6 genes have redundant functions, but none of them alone can fully compensate for the lack of expression of the other two.

These results indicate that ARF6, ARF8, and ARF17 transcription regulators and their downstream GH3 targets do not control adventitious rooting by modulating free IAA content. Rather, GH3.3, GH3.5, and GH3.6 appear to be promiscuous enzymes that affect the homeostasis of one or more other compounds that negatively regulate adventitious rooting.

Because GH3.5 was described as acting at the crosstalk of auxin homeostasis and SA signaling (Zhang et al., 2007, 2008), we investigated whether SA was an inhibitor of adventitious rooting. The two mutant alleles eds5-1 and eds5-2, which are deficient in SA biosynthesis (Rogers and Ausubel, 1997), produced fewer adventitious roots than the wild type, suggesting that SA was not a negative, but likely a positive, regulator of adventitious rooting. Furthermore, free IAA and SA contents were not altered in the triple gh3 mutant compared with the wild type, either in the dark or 72 h after transfer to the light, although in both genotypes the IAA and SA contents significantly increased after transfer to the light. Thus, the phenotype of the triple gh3 mutant was not due to a modification in the level of free IAA or SA.

In contrast with SA mutants, JA-deficient mutants opr3/dde1 (Sanders et al., 2000; Stintzi and Browse, 2000) and dde2-2 (von Malek et al., 2002) produced more adventitious roots than the wild type, suggesting that jasmonate is an inhibitor of adventitious rooting. Free JA in the hypocotyl of the gh3.3-1gh3.5-2gh3.6-1 triple mutant was twice that in the wild type at T0, and after 3 and 9 h in the light, the percentage of decline in endogenous JA was greater for the triple mutant than for the wild type, but it was still significantly higher than in the wild type at 9 h. Three days after transfer to the light, the amount was markedly decreased in both the wild type and the triple mutant, in agreement with published results showing that JA biosynthesis is regulated by light (Zhai et al., 2007). One way to explain the
higher JA levels in the triple gh3 mutant is if the three GH3 enzymes regulate JA homeostasis by conjugating it to amino acids, leading either to JA catabolism or its unavailability for synthesis of the JA-Ile signal by GH3.11/JAR1. All three enzymes produced JA conjugates in vitro with three of the amino acids tested in this study, but importantly, none synthesized appreciable amounts of JA-Ile. The JA concentration used in the enzyme assays here was 10-fold higher for JA than for IAA, pointing to a role in controlling the level of free JA at least in hypocotyl tissues. In this study, JA-Trp was not detected in the hypocotyl of the wild type or gh3.3-1gh3.5-2gh3.6-1. JA-Trp is an inhibitor of auxin responses, and we previously found low levels in Arabidopsis (Staswick, 2009). In any case, the auxin-inhibiting activity of JA-Trp is not likely responsible for regulating adventitious rooting, because the latter is COI1 dependent whereas JA-Trp activity is COI1 independent. JA-Met and JA-Asp have not been reported because the latter is COI1 dependent whereas JA-Trp activity is COI1 independent. JA-Ile promotes the interaction of the F-box receptor protein COI1 with the JAZ coreceptors, which also function as transcriptional repressors of jasmonate response genes (Thines et al., 2007). JAZ proteins are then ubiquitinated, targeting them for degradation by the 26S proteasome, followed by the activation of associated JA-dependent responses (Chini et al., 2007; Yan et al., 2007; Chung et al., 2008). Further evidence that JA-Ile/COI1 signaling negatively regulates adventitious rooting is the finding that, with the exception of the jasmonate-insensitive jai3 mutant, all of the tested downregulation mutants in this pathway (jar1-12, coi1-16, myc2, and myc2myc3myc4) developed significantly more adventitious roots than the wild type, while the upregulation lines (jar1-1OX and 35S:MYC2) were deficient in root production. The lack of a phenotype in jai3-1 could be explained if this particular JAZ protein (JAZ3) is not involved in adventitious rooting or if other JAZ proteins are redundant for its activity. The JAZ1, JAZ3, and JAZ5 transcripts were elevated in the gh3.3-1gh3.5-2gh3.6-1 triple mutant kept in the dark or transferred to the light, indicating that jasmonate signaling was indeed upregulated in the hypocotyl at this time. The COI1 transcript was also higher in the hypocotyl of the triple mutant 9 h after transfer to the light. The increase in adventitious roots in the double mutants coi1-16arf6-7, coi1-16arf8-7, and coi1-16arf17-OX, in contrast to the reduced rooting in the single arf6-3, arf8-7, and ARF17-OX lines, supports the idea that the COI1-dependent jasmonate signaling pathway acts downstream of the regulatory module composed of three ARFs (ARF6, ARF8, and ARF17) and the three GH3s they regulate (GH3.3, GH3.5, and GH3.6).

In conclusion, our data strongly support the hypothesis that the JA-Ile/COI1 signaling pathway negatively regulates adventitious rooting in Arabidopsis hypocotyls. We propose that auxin stimulates adventitious rooting by inducing GH3.3, GH3.5, and GH3.6 gene expression, via the positive regulators ARF6 and ARF8, leading to an increase in JA conjugation and, as a consequence, a reduction in free JA level. These interactions can be added to our previous model that integrated the interactions between the three ARFs and their regulatory microRNAs (Gutierrez et al., 2009) and are summarized in the model presented in Figure 6. ARF6 and ARF8 have previously been described as positive regulators of JA biosynthesis during flower development (Nagpal et al., 2005; Tabata et al., 2010); here, we show that they are likely to regulate the conjugation of jasmonate to amino acids. Our results highlight a regulatory pathway at the crosstalk of IAA and JA, in which ARF6, ARF8, and ARF17 and their downstream targets GH3.3, GH3.5, and GH3.6 are involved, and demonstrate that JA homeostasis is under auxin control.

METHODS

Plant Material and Growth Conditions

The ARF6-OX, ARF8-OX, and ARF17-OX1 lines have been described previously (Nagpal et al., 2005). Line MIR160c-OX, which overexpresses the gene MIR160c, was described by Wang et al. (2005). The knockout mutants arf8-7 and arf6-3 were described by Gutierrez et al. (2009). The SA-deficient mutant lines eds5-1 and eds5-2 were obtained from Frederick Ausubel. Seeds from the two JA-deficient mutants dde2-2 and opr3 were gifts from Beate Keller and John Browse, respectively. Seeds from coi1-16, jai3-1, myc2, 3SS:MYC2, and the myc2myc3myc4 triple mutant were gifts from Laurens Pauwels.

Insertion lines for the GH3.3, GH3.5, GH3.6, and GH3.11/JAR1 genes were obtained from the Nottingham Arabidopsis Stock Centre. The presence of each insertion was verified using the gene-specific primers listed in Supplemental Table 1 online. The absence or the overexpression of a full-length mRNA was verified for each line by RT-PCR using primers for the coding region (see Supplemental Table 2 online). We identified two gh3.3 mutant alleles, gh3.3-1 (SM_3-39271) and gh3.3-2 (SM_3-17798); two gh3.5 mutant alleles, gh3.5-1 (SALK_014376) and gh3.5-2 (SALK_151766), which was also described by Zhang et al. (2007); two gh3.6 mutant alleles, gh3.6-1 (SALK_133707) and gh3.6-2 (SALK_060813); one gh3.11 mutant allele (SALK_011510) that we called jai1-12 according to Susa and Staswick (2008); and a GH3.11-overexpressing line, JAR1-1OX (SALK_013425).

For generating double mutants with arf6-3, arf8-7, and ARF17-OX1 lines, the coi1-16 allele described by Ellis and Turner (2002) was used. In the F2 population, homozygous arf6-3, arf8-7, and ARF17-OX1 were identified by PCR using the genotyping primers listed in Supplemental Table 1 online. To genotype the coi1-16 point mutation, new derived cleaved-amplified polymorphic sequence primers were designed using the dCAPS Finder 2.0 software (Neff et al., 2002; http://helix.wustl.edu/
dcaps/dcups.html). A mismatch (underlined) was introduced in the F primer to incorporate a restriction site in the PCR product of one allele (see Supplemental Table 1 online). After amplification, the PCR products were digested with XbaI (Fermentas Fast Digest) following the manufacturer's recommendations and separated on a 4% agarose gel. The wild-type allele yielded two fragments of 146 and 19 bp, while col7-16 gave one band of 165 bp.

Adventitious rooting experiments were performed as described previously (Gutierrez et al., 2009).

Expression Profiling of GH3 Genes

A 1.5-kb-long fragment upstream from the start codon of GH3.3, GH3.5, and GH3.6 was amplified from genomic DNA by PCR, cloned using a pENTR/D-TOPO Cloning Kit (Invitrogen), and transferred into the pKFWSF7 binary vector (Karimi et al., 2002) using a Gateway LR Clonase Enzyme Mix (Invitrogen) according to the manufacturer's instructions. Transgenic Arabidopsis thaliana plants expressing the different prorGH3: GUS fusions were produced, and the expression pattern was checked in the T2 progency of 10 to 15 independent transgenic lines. One representative homozygous line for each gene was used for further characterization.

Histochemical assays for GUS expression were performed as described previously (Sorin et al., 2005).

RNA Isolation and cDNA Synthesis

RNAs from arf and gh3 mutants and ARF-overexpressing lines were prepared as described by Gutierrez et al. (2009). Portions (6 μg) of the resulting RNA preparations were treated with DNase using a DNAfree Kit (Ambion), and cDNA was synthesized by reverse transcribing 5 μL of total RNA using SuperScript III reverse transcriptase (Invitrogen) according to the manufacturer's instructions. The reaction was stopped by incubation at 70°C for 10 min; the reaction mixture was then treated with RNaseH (Invitrogen) according to the manufacturer's instructions and diluted by adding 1 mL of distilled water. All cDNA samples were tested by PCR using specific primers flanking an intron sequence to confirm the absence of genomic DNA contamination.

Real-Time RT-PCR Experiments

Transcript levels for Figures 1 and 2 were assessed by quantitative RT-PCR as described by Gutierrez et al. (2009). Transcript levels for Supplemental Figures 2 and 3 online were assessed by quantitative RT-PCR, in assays with triplicate reaction mixtures (final volume, 20 μL) containing 5 μL of cDNA, 0.3 μM of both forward and reverse primers, and 1× FastStart DNA MasterPLUS SYBR Green I (Roche), using a Roche LightCycler, and crossing threshold (CT) values for each sample were acquired with LightCycler software 3.5 (Roche), following an intron sequence to confirm the absence of genomic DNA contamination.

Seventy-two hours after transfer to the light, cotyledons and roots were removed, and an average of 150 hypocotyls (50 to 70 mg) per replicate were harvested, dried on tissue paper, and frozen in liquid nitrogen. Up to nine replicates from three independent experiments were harvested. Samples were extracted, purified, and analyzed by liquid chromatography–multiple reaction monitoring–mass spectrometry as described previously (Kowalczyk and Sandberg, 2001).

IAA and IAA Conjugate Analysis

Seedlings from each genotype were grown in vitro as described above. Seventy-two hours after transfer to the light, cotyledons and roots were removed, and an average of 150 hypocotyls (50 to 70 mg) per replicate were harvested, dried on tissue paper, and frozen in liquid nitrogen. Up to nine replicates from three independent experiments were harvested. Samples were extracted, purified, and analyzed by liquid chromatography–multiple reaction monitoring–mass spectrometry as described previously (Kowalczyk and Sandberg, 2001).

Free IAA, JA, JA-Ile, and SA Quantification

Hypocotyls from the wild type and triple gh3 mutants were collected as described above. JA, JA-Ile, and SA extraction, purification, and quantification were performed according to Bergougnoux et al. (2009), with modifications. To each extract, 20 pmol of [2H4]JA and [2H4]JA-Ile (Olchemim) and 100 pmol of [13C6]IAA (Cambridge Isotope Laboratories) were added as internal standards to check recovery during purification and to validate the quantification. Purified samples were analyzed by the liquid chromatography–tandem mass spectrometry system consisting of an ACQUITY UPLC System (Waters) and a Xevo TQ (Waters) triple quadrupole mass spectrometer. Samples were dissolved in 15% acetonitrile, injected onto the ACQUITY UPLC BEH C18 column (100 × 2.1 mm, 1.7 μm; Waters), and eluted with a linear gradient (0 to 3 min, 15% B; 3 to 10 min, 20% B; 10 to 20 min, 30% B; flow rate of 0.25 mL/min) of 7.5 mM formic acid (A) and acetonitrile (B). Quantification was obtained using a multiple reaction monitoring mode of [M-H]+/[M-H]+ and the appropriate product ion. The multiple reaction monitoring transitions 215.1>59, 326.2>151.1, 141.1>87, and 182.1>136.1 were used for labeling, and 209.1>59, 324.2>151.1, 137.1>93, and 176.1>130.1 were sequences of primers used for all target genes are presented in Supplemental Table 2 online. Due to the high sequence conservation, note that the primers for GH3.13 might also produce an amplicon from GH3.15 transcripts, which was not a problem in our analysis since GH3.15 transcripts are not detectable.

Each amplicon was first sequenced to ensure the specificity of the amplified sequence, and in order to check that the fluorescence signal was derived from the single intended amplicon in the following runs, a melting curve analysis was added to each PCR program.
used for authentic JA, JA-ile, SA, and IAA, respectively. For selective multiple reaction monitoring experiments, optimal conditions were as follows: capillary/cone voltage, 1.0 kV/25 V; source/desolvation gas temperature, 120/550°C; cone/desolvation gas, 70/1000 L/h; collision gas, 0.21 mL/min; low mass/high mass resolution, 2.8/12.5; collision energy, 14 eV; ion energy 1/2, 1.0/0.7 V; entrance/exit, 2.0 V. The limits of detection (signal-to-noise ratio of 1:3) for all analytes were close to 50.0.

**Supplemental Figure 8.** Expression Pattern of promJAZ1:GUS.

**Supplemental Table 1.** Sequences of Primers Used for Genotyping Newly Identified gh3 Mutants and Overexpressing Lines.

**Supplemental Table 2.** Sequences of Primers Used for Quantifying Target Genes by Real-Time RT-PCR.

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**AUTHOR CONTRIBUTIONS**


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