

Auxin Controls *Arabidopsis* Adventitious Root Initiation by Regulating Jasmonic Acid Homeostasis^W

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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 argonaut1* (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

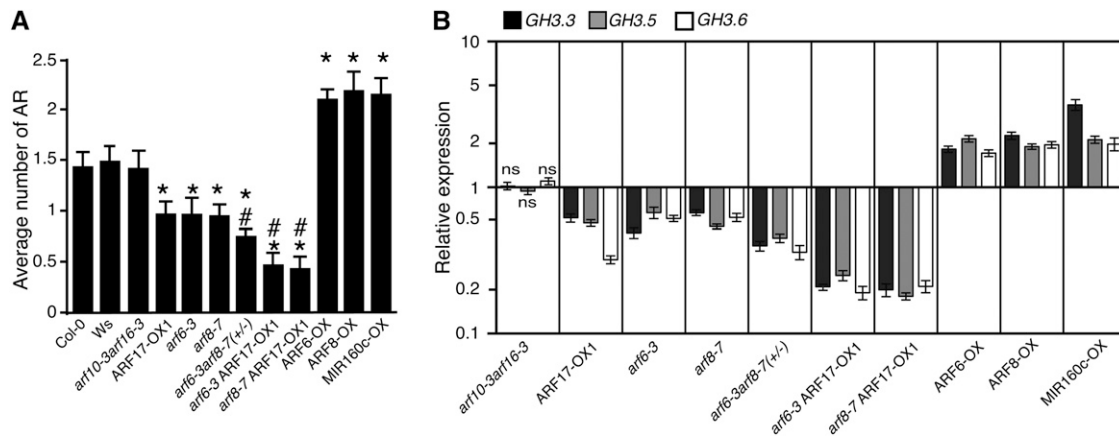


Figure 1. Variation in the Number of Adventitious Roots in the Hypocotyl of *arf* Mutants and *ARF*-Overexpressing Lines Correlates with the Expression Levels of *GH3.3*, *GH3.5*, and *GH3.6*.

(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 \pm SE. A one-way analysis of variance combined with the Tukey's multiple comparison posttest 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 Wassilewskija.

(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 \pm SE obtained from three independent biological replicates. A one-way analysis of variance combined with the Dunnett's comparison posttest 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-ylacetyl)-aspartate] and IAGlu [*N*-(indole-3-ylacetyl)-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 the 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 sesquimutant *arf6-3/arf6-3 arf8-7/ARF8-7* segregating for 25% homozygous double mutants. The average number of adventitious roots was significantly lower than that of the wild type and of the single mutants *arf6-3* and *arf8-7*, indicating an additive effect of the two positive regulators *ARF6* and *ARF8* (Figure 1A). This additive effect was confirmed by the reduced level of the total *GH3.3+GH3.5+GH3.6* relative transcript amount in the sesquimutant compared with that in the single mutants (see Supplemental Figure 3A online). In contrast, the three *GH3* genes were expressed at a higher level in *ARF6*- and *ARF8*-overexpressing lines. The three *GH3* genes were also downregulated in the *ARF17-OX1* line (Figure 1B) (Sorin et al., 2005) and, as expected, upregulated in the *MIR160c-OX* line, in which *ARF17* is downregulated. When *ARF17* was overexpressed in the knockout *arf6-3* and *arf8-7* mutant backgrounds, a significant decrease in the relative amount of transcript of each of the three *GH3* genes was observed compared with the *ARF17*-overexpressing lines or the *arf6-3* and *arf8-7* single mutants (Figure 1B). This confirmed the existence of an additive effect due to the overexpression of the negative regulator *ARF17* in mutants lacking one of the positive regulators (*ARF6* or *ARF8*) of *GH3.3*, *GH3.5*, and *GH3.6* 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).

***GH3.3*, *GH3.5*, and *GH3.6* Positively and Redundantly Regulate Adventitious Rooting**

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*

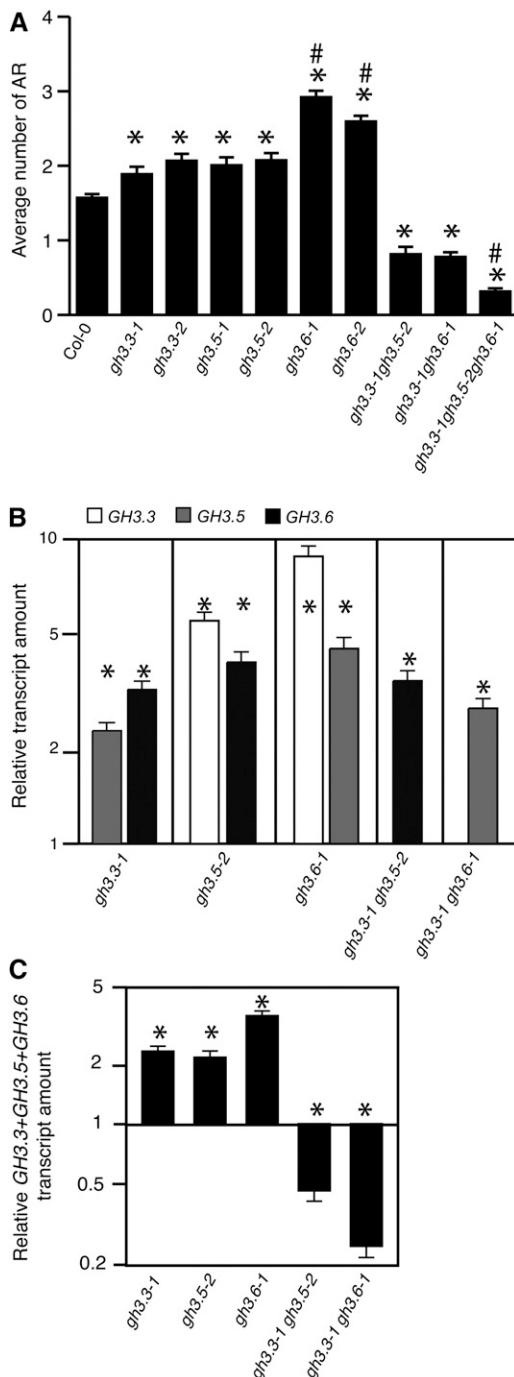


Figure 2. *GH3.3*, *GH3.5*, and *GH3.6* Genes Have Redundant Functions in the Regulation of Adventitious Rooting.

(A) Average number of adventitious roots (AR) in two independent knockout alleles for *gh3.3*, *gh3.5*, and *gh3.6* single mutants, in the double mutants *gh3.3-1gh3.5-2* and *gh3.3-1gh3.6-1*, and the triple mutant *gh3.3-1gh3.5-2gh3.6-1*. 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

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 *JASMONIC 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 ($P < 0.001$, $n = 3$).

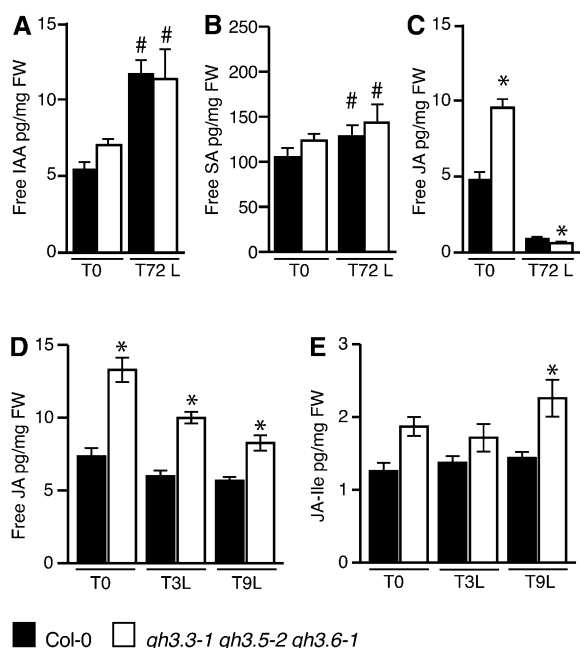


Figure 3. *GH3.3*, *GH3.5*, and *GH3.6* Regulate Adventitious Root Initiation by Modulating JA Homeostasis.

(A) to (C) Endogenous free IAA (A), free SA (B), and free JA (C) contents were measured in the hypocotyl of wild type ecotype Columbia (Col-0) and the *gh3.3-1gh3.5-2gh3.6-1* triple mutant. Seedlings were first etiolated in the dark, until their hypocotyls were 6 mm long (T0), and then transferred to the light for 72 h (T72L). Error bars indicate SD of eight biological replicates. A one-way analysis of variance combined with the Bonferroni posttest indicated that the values indicated by asterisks are significantly different from wild-type Col-0 values and the values indicated by hash signs are significantly different from values observed at T0 ($P < 0.05$; $n = 8$). FW, fresh weight.

(D) and (E) Endogenous free JA (D) and JA-Ile (E) were measured in the hypocotyl of wild type Col-0 and the *gh3.3-1gh3.5-2gh3.6-1* triple mutant, etiolated as in (A) (T0) or transferred to the light for 3 h (T3L) or 9 h (T9L). Error bars indicate SD of nine biological replicates. A one-way analysis of variance combined with the Bonferroni posttest indicated that the values indicated by asterisks are significantly different from wild-type Col-0 values ($P < 0.05$; $n = 9$).

prompted us to also investigate the role of JA in adventitious root formation on *Arabidopsis* hypocotyls.

To verify whether or not the reduced number of adventitious roots observed in the *gh3.3-1gh3.5-2gh3.6-1* triple mutant could be due to a modification of endogenous SA or JA content in the early stages of adventitious root formation, we quantified free IAA, free JA, and free SA in hypocotyls of wild-type seedlings and the *gh3.3-1gh3.5-2gh3.6-1* triple mutant seedlings at time T0 (i.e., etiolated seedlings prior to transfer to the light) and after transfer to the light for 72 h (T72L). No significant differences in the free IAA or free SA content could be observed in the *gh3.3-1gh3.5-2gh3.6-1* triple mutant compared with the wild type at T0 or T72L (Figures 3A and 3B). Both free IAA and free SA contents increased after transfer to the light in both the wild type and the *gh3.3-1gh3.5-2gh3.6-1* triple mutant. In contrast, dark-grown seedlings of the *gh3.3-1gh3.5-2gh3.6-1* triple mutant accumulated

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 *CYCLIN B1*, 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 decreased 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

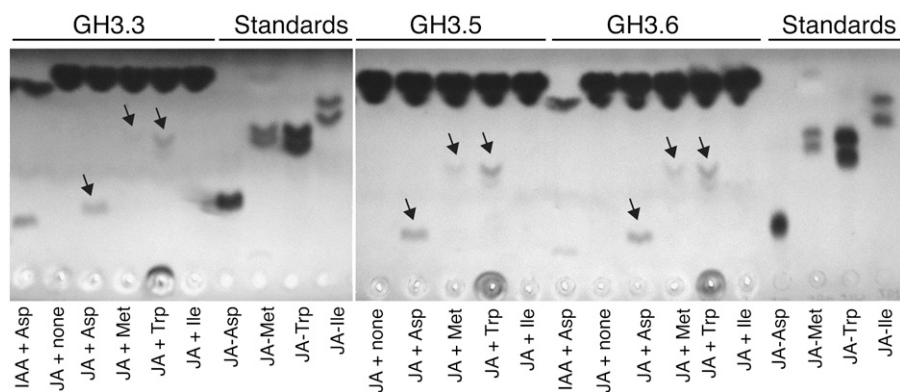


Figure 4. GH3.3, GH3.5, and GH3.6 Can Conjugate JA to Amino Acids in Vitro.

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. Free IAA and JA migrated to near the top of the plate.

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 hypocotyl of the wild type and the triple *gh3.3-1gh3.5-2gh3.6-1* mutant. *JAR1* was upregulated in the hypocotyl 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 upregulated in the triple mutant hypocotyl 9 h after transfer to the light (Figure 5E). We also looked at the expression pattern of *promJAZ1:GUS* in wild-type seedlings grown either in the dark or transferred to the light for 24, 48, or 72 h (see Supplemental Figure 8 online). *promJAZ1:GUS* was expressed in the hypocotyl and root of seedlings either kept in the dark or transferred to the light. In seedlings transferred to the light for 72 h, the expression was restricted to the stele. No staining was observed in the adventitious root primordia (see Supplemental Figure 8C online). This expression pattern overlapped with that of *promARF6:GUS*, *promARF8:GUS*, and *promARF17:GUS*, which we have described previously (Gutierrez et al., 2009).

To further confirm that JA and the COI1 signaling pathways were negatively regulating adventitious root development, we analyzed the phenotypes of several mutants affected in JA biosynthesis and signaling (Figure 5F). We analyzed two mutants altered in JA biosynthesis, *opr3/dde1* (Sanders et al., 2000; Stintzi and Browse, 2000) and *dde2-2* (von Malek et al., 2002). Among the T-DNA insertion lines publicly available, we identified a new knockout mutant allele in the *GH3.11/JAR1* gene that we called *jar1-12* and a *GH3.11/JAR1*-overexpressing line, *JAR1-OX*. We also analyzed the *coi1-16* allele (Ellis and Turner, 2002) altered in the expression of *COI1*, the *jai1/myc2* mutant (Lorenzo et al., 2004) altered in the expression of *MYC2*, a *bHLH* transcription factor that is a direct target of *JAZ* repressors and part of the core signaling module in the jasmonate signaling pathway (Chini et al.,

2007), a *35S:MYC2* line, the triple mutant *myc2myc3myc4* that is as impaired as *coi1-1* in the activation of several, but not all, JA-mediated responses (Fernández-Calvo et al., 2011), and last, the JA-insensitive mutant *jai3-1* altered in the expression of *JAZ3* (Chini et al., 2007). These mutants were characterized under the same conditions used previously (Figure 2A). Both *opr3/dde1* and *dde2-2*, which are affected in JA biosynthesis, developed more adventitious roots than the wild type, confirming a negative effect of JA on adventitious root development (Figure 5F). Interestingly, two SA-deficient mutants, *eds5-1* and *eds5-2* (Rogers and Ausubel, 1997), that we also analyzed produced fewer adventitious roots than did the wild type (Figure 5G), suggesting that SA is possibly a positive regulator of adventitious rooting. The jasmonate-insensitive *jai3-1* mutant did not show any difference in the average number of adventitious roots compared with the wild type, whereas lines in which the JA signaling pathway was upregulated (*JAR1-OX* and *35S:MYC2*) had significantly fewer adventitious roots than the wild type, and those lines with a downregulated JA signaling pathway (*jar1-12*, *coi1-16*, *myc2*, and *myc2myc3myc4*) all had more adventitious roots than the wild type (Figure 5F). Noticeably, *coi1-16* and the *myc2myc3myc4* triple mutant developed the highest average number of adventitious roots, confirming that the *COI1* signaling pathway plays a role in the control of adventitious rooting.

The upregulated JA signaling pathway in the hypocotyl of the *gh3.3-1gh3.5-2gh3.6-1* mutant explains the reduced number of adventitious roots, since the double mutants *arf6-3coi1-16*, *arf8-7coi1-16*, and *coi1-16ARF17-OX* developed more adventitious roots than the wild type, whereas the single *arf* mutants and the *ARF17-OX* line had fewer (Figure 5H). This last result confirms that JA negatively regulates adventitious root initiation through *COI1*, which acts downstream of the *ARF/GH3* module.

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*,

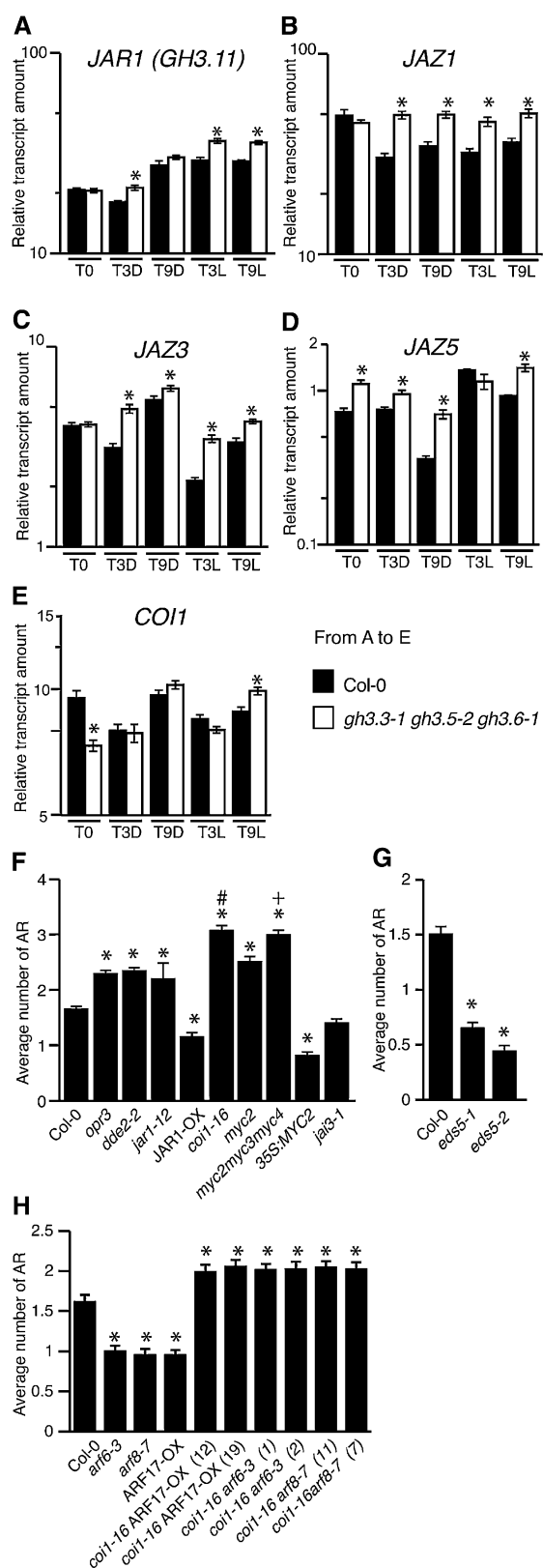


Figure 5. The *COI1* Signaling Pathway Is Required for the Inhibition of Adventitious Rooting by Jasmonate.

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

(A) to (E) Quantification by quantitative RT-PCR of *JAR1 (GH3.11)*, *JAZ1*, *JAZ3*, *JAZ5*, and *COI1* transcripts in hypocotyls of wild-type etiolated seedlings (T0), after an additional 3 or 9 h in the dark (T3D or T9D), and after transfer to the light for 3 or 9 h (T3L or T9L). Values are relative to the expression level of *APT1*, which was used as a reference gene as described in Methods. Error bars indicate SE obtained from three independent biological replicates. A one-way analysis of variance combined with the Tukey's multiple comparison posttest confirmed that the differences between the wild type and the mutants (*) are significant ($P < 0.05$, $n = 3$). Col-0, ecotype Columbia.

(F) and (G) Average number of adventitious roots (AR) in several mutants altered in jasmonate biosynthesis or signaling **(F)** and in the SA-deficient mutant lines *eds5-1* and *eds5-2* **(G)**. 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 test showed that the values indicated by asterisks are significantly different from wild-type values ($P < 0.01$; $n > 90$); the Bonferroni posttest indicates that the value indicated by the plus sign is significantly different from that of the *myc2* mutant and that indicated by the hash mark is significantly different from that of *jar1-12* ($P < 0.01$; $n > 90$). **(H)** Average number of adventitious roots in single *arf6-3* and *arf8-7* mutants and the *ARF17-OX* line and in the corresponding double mutants with *coi1-16*. 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 Dunnett's multiple comparison test showed that the values indicated by asterisks are significantly different from wild-type values ($P < 0.05$; $n > 90$).

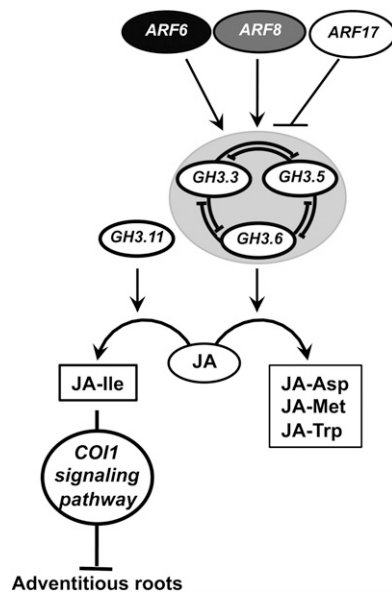


Figure 6. Adventitious Root Initiation: A Proposed Model for a Regulatory Pathway.

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 *GH3*s, 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 *GH3*s inactivate IAA by conjugating it to amino acids (Staswick et al., 2005). Consistent with this function, the levels of IAGlu and IAAsp 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 hypocotyls 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, because previous studies indicated that GH3.3 and GH3.5 were considerably less active on JA (Staswick et al., 2002). Nevertheless, the results suggest that JA could be an alternative substrate for GH3.3, GH3.5, and GH3.6, 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 to occur in *Arabidopsis*, but even if synthesized, they may not accumulate appreciably if they are further metabolized, as is the case for IAAsp.

We also observed that the expression of genes encoding enzymes involved in JA biosynthesis was upregulated in the triple mutant, suggesting that the increase of JA could be due to an upregulation of its biosynthesis. We cannot at present discriminate between these two hypotheses, and indeed, the increase in JA might result from a combination of decreased conjugation and increased biosynthesis in the triple *gh3* mutant. The elevated JA content was accompanied by a small increase in JA-Ile at 9 h after transfer to the light, and this coincided with the upregulation of the *GH3.11/JAR1* transcript. Several JA biosynthesis genes are well established to be transcriptionally upregulated by JA-Ile/COI1 signaling, so reduced conjugation of JA to other amino acids in the triple mutant might free up more JA for JA-Ile synthesis, which in turn could enhance JA production. This is consistent with a model that includes JA-Ile signaling as a negative regulator of adventitious rooting.

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 *jai3-1* 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-OX* 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*, *35S: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 *jar1-12* according to Suza and Staswick (2008); and a *GH3.11*-overexpressing line, *JAR1-OX* (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/dcaps.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 *Xba*I (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 *coi1-16* 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 pKGWFS7 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 *promGH3:GUS* fusions were produced, and the expression pattern was checked in the T2 progeny 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 (5 μ g) of the resulting RNA preparations were treated with DNaseI using a DNAfree Kit (Ambion), and cDNA was synthesized by reverse transcribing 5 μ g of total RNA using SuperScript III reverse transcriptase (Invitrogen) with 2.5 μ g of random hexamers and 500 ng of oligo(dT)₁₂ primer 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 μ L) containing 5 μ L of cDNA, 0.3 μ M of both forward and reverse primers, and 1 \times FastStart DNA Master^{PLUS} SYBR Green I (Roche), using a Roche LightCycler, and crossing threshold (CT) values for each sample were acquired with LightCycler software 3.5 (Roche) using the second derivative maximum method. Transcript levels for Figure 5 and Supplemental Figure 7 online were assessed by quantitative RT-PCR, in assays with triplicate reaction mixtures (final volume, 10 μ L) containing 2 μ L of cDNA, 0.5 μ M of both forward and reverse primers, and 1 \times LightCycler 480 SYBR Green I Master (Roche) on 384-well plates. The plates were filled by an EVO150 robotic liquid handling platform (Tecan), and quantitative PCR was performed with a LightCycler 480 (Roche). The CT values for each sample were acquired with the LightCycler 480 software (Roche) using the second derivative maximum method. All quantifications were repeated with three independent biological replicates.

Steady state levels of uncleaved *ARF* transcripts were quantified using primers spanning the microRNA target site. The following standard protocol was applied for the amplification of each mRNA: 10 min at 95°C, followed by 40 cycles of 10 s at 95°C, 15 s at 60°C (except for *GH3.5*, for which the annealing temperature was 65°C), and 15 s at 72°C. The

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.

Real-Time RT-PCR Data Analysis

APT1 and *TIP41* had previously been validated as the most stably expressed genes among 11 tested in our experimental procedures (Gutierrez et al., 2009) and were used to normalize the real-time RT-PCR data. The normalized expression patterns obtained using both reference genes were similar, so only the data normalized with *APT1* are shown. For Figures 1 and 2 and Supplemental Figure 3 online, CT and PCR efficiency (E) values were used to calculate expression using the formula $E_T^{CT_{WT}-CT_M}/E_R^{CT_{WT}-CT_M}$, where T is the target gene and R is the reference gene, CT is the crossing threshold value, M refers to cDNA from the mutant line, and WT refers to cDNA from the wild type. In these figures, the data for mutants are presented as relative to the wild type, the calibrator. For Figure 5 and Supplemental Figures 2 and 7 online, the expression in the wild type and/or mutants was calculated using the formula $E_R^{CT_{WT}}/E_T^{CT_{WT}}$ [i.e., $(1/E_T^{CT_{WT}})/(1/E_R^{CT_{WT}})$: the normalized relative quantity of template in the original sample], the expression levels of target genes being relative to those of the reference gene. All real-time RT-PCR results presented are means from three independent biological replicates. For each independent biological replicate, the relative transcript amount was calculated as the mean of three technical replicates, using the method for calculation of SE values in relative quantification recommended by Rieu and Powers (2009).

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, SA, and IAA extraction, purification, and quantification were performed according to Bergougnoux et al. (2009), with modifications. To each extract, 20 pmol of [²H₆]JA and [²H₂]JA-Ile (Olchemim) and 100 pmol of [²H₄]SA and [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 \times 2.1 mm, 1.7 μ m; 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>97, 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

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 fmol. The linear range was at least over the 5 orders of magnitude with a correlation coefficient of 0.9989 to 0.9998. For each mutant, three independent experiments were performed, each composed of three biological replicates.

Enzymatic Assays

Enzyme assays were done using GH3-GST fusion proteins produced in *Escherichia coli* as described previously (Staswick et al., 2005). Reactions were for 16 h at 23°C in 50 mM Tris-HCl, pH 8.6, 1 mM MgCl₂, 1 mM ATP, 1 mM DTT, and 2 mM amino acid. Either IAA (1 mM) or JA (10 mM) was included in each reaction. Reactions were analyzed on silica gel 60 F260 plates developed in chloroform:ethyl acetate:formic acid (35:55:10, v/v) and then stained with vanillin reagent (6% vanillin [w/v], 1% sulfuric acid [v/v] in ethanol).

Accession Numbers

Sequence data from this article can be found in the Arabidopsis Genome Initiative or GenBank/EMBL databases under the following accession numbers: *ACX1* (At4g16760) *AIR12* (At3g07390), *AOC2* (At3g25770), *AOS* (At5g42650) *APT1* (At1g27450), *ARF6* (At1g30330), *ARF8* (At5g37020), *ARF17* (At1g77850), *COI1* (At2g39940), *GH3.1* (At2g14960), *GH3.2* (At4g37390), *GH3.3* (At2g23170), *GH3.4* (At1g59500), *GH3.5* (At4g27260), *GH3.6* (At5g54510), *GH3.7* (At1g23160), *GH3.8* (At5g51470), *GH3.9* (At2g47750), *GH3.10* (At4g03400), *GH3.11* (At2g46370), *GH3.12* (At5g13320), *GH3.13* (At5g13350), *GH3.14* (At5g13360), *GH3.15* (At5g13370), *GH3.16* (At5g13380), *GH3.17* (At1g28130), *GH3.18* (At1g48670), *GH3.19* (At1g48660), *GH3.20* (At1g48690), *IAA7* (At3g23050), *IAA17* (At1g04250), *IAA19* (At3g15540), *IAA28* (At5g25890), *JAZ1* (At1g19180), *JAZ3* (At3g17860), *JAZ5* (At1g17380), *KAT2* (At2g33150), *LOX2* (At3g45140), *OPCL1* (At1g20510), *OPR3* (At2g06050), and *TIP41* (At4g34270).

Supplemental Data

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

Supplemental Figure 1. Content of IAA and Its Major Conjugates in Hypocotyls of the Wild Type, *arf* Mutants, and ARF-Overexpressing Lines.

Supplemental Figure 2. Expression Pattern of *GH3.3*, *GH3.5*, and *GH3.6*.

Supplemental Figure 3. Relative Amount of the Sum of *GH3.3*, *GH3.5*, and *GH3.6* mRNAs in the Single *arf6-3* and *arf8-7* Mutants and the *arf6-3arf8-7(+/-)* Sesquimutant.

Supplemental Figure 4. Root Length and Lateral Root Number in *gh3* Single, Double, and Triple Mutants.

Supplemental Figure 5. Content of IAA and Its Major Conjugates in Hypocotyls of the Wild Type and *gh3.3-1*, *gh3.5-1*, and *gh3.6-1* Mutants.

Supplemental Figure 6. Effect of Exogenously Applied JA on Adventitious Rooting.

Supplemental Figure 7. Jasmonate Biosynthesis is Upregulated in the Triple *gh3.3gh3.5gh3.6* Mutants.

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

K.F. and D.I.P. contributed equally to this work. L.G. and G.M. characterized the mutant phenotypes and performed the quantitative PCR analysis. L.G., G.M., and D.I.P. performed the GUS assays. D.I.P. produced the multiple mutants. D.I.P. and M.P. genotyped the mutants. K.F., O.N., and M.K. performed the hormone measurements. P.S. performed the enzymatic assays. H.D. produced the biological material for quantitative PCR analysis and hormone measurements. G.G. produced the *prom:GUS* lines. L.G. and C.B. designed the research and analyzed the data. C.B. conceptualized and supervised the overall project. C.B. wrote the article with inputs from L.G., O.N., M.K., and P.S. P.S. edited the article.

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