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RESEARCH ARTICLES

Auxin and Light Control of Adventitious Rooting in Arabidopsis Require ARGONAUTE1[W]

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Adventitious rooting is a quantitative genetic trait regulated by both environmental and endogenous factors. To better understand the physiological and molecular basis of adventitious rooting, we took advantage of two classes of *Arabidopsis thaliana* mutants altered in adventitious root formation: the *superroot* mutants, which spontaneously make adventitious roots, and the *argonaute1* (*ago1*) mutants, which unlike *superroot* are barely able to form adventitious roots. The defect in adventitious rooting observed in *ago1* correlated with light hypersensitivity and the deregulation of auxin homeostasis specifically in the apical part of the seedlings. In particular, a clear reduction in endogenous levels of free indoleacetic acid (IAA) and IAA conjugates was shown. This was correlated with a downregulation of the expression of several auxin-inducible *GH3* genes in the hypocotyl of the *ago1-3* mutant. We also found that the *Auxin Response Factor17* (*ARF17*) gene, a potential repressor of auxin-inducible genes, was overexpressed in *ago1-3* hypocotyls. The characterization of an *ARF17*-overexpressing line showed that it produced fewer adventitious roots than the wild type and retained a lower expression of *GH3* genes. Thus, we suggest that *ARF17* negatively regulates adventitious root formation in *ago1* mutants by repressing *GH3* genes and therefore perturbing auxin homeostasis in a light-dependent manner. These results suggest that *ARF17* could be a major regulator of adventitious rooting in Arabidopsis.

INTRODUCTION

Adventitious root formation is a complex process that is affected by multiple endogenous factors, including phytohormones, and environmental factors, such as wounding and light. The molecular mechanisms by which adventitious root formation is regulated are still poorly understood. Auxin plays a central role (Blakesley, 1994) and may interact with other endogenous factors or environmental stimuli, such as light. It was shown that auxin and light act antagonistically on the development of adventitious roots in *Eucalyptus saligna* and *E. globulus* (Fett-Neto et al., 2001). Recently, Niemi et al. (2005) showed that light sources with different spectra could affect adventitious root and mycorrhyza formation in Scots pine (*Pinus sylvestris*) in vitro. *Arabidopsis thaliana* serves as a valuable model system for dissecting the molecular mechanisms involved in the control of adventitious root initiation by diverse environmental signals. For example, King and Stimart (1998) have shown that several ecotypes of *A. thaliana* differ in their capacity to produce adventitious roots on the hypocotyl in response to auxin and that low and high rooting responses might be controlled by several genes acting independently in an additive-dominant manner. More recently, Konishi and Sugiyama (2003) identified temperature-sensitive mutants of Arabidopsis altered in adventitious rooting.

To gain further insight into the interaction between light and auxin in the regulation of adventitious rooting, we took advantage of two classes of mutants, *superroot1* (*sur1*) and *sur2* and *argonaute1* (*ago1*), that we described previously (Boerjan et al., 1995; Delarue, 1996; Bohmert et al., 1998; Delarue et al., 1998; Camus, 1999). *sur1* and *sur2* are auxin overproducers that spontaneously develop adventitious roots on the hypocotyl as a consequence of increased endogenous auxin levels (Boerjan et al., 1995; Delarue et al., 1998). Although SUR2 is primarily involved in indole glucosinolate production, SUR1 is apparently required for the production of all glucosinolates in Arabidopsis (Barlier et al., 2000; Bak et al., 2001; Mikkelsen et al., 2004).

The *ago1* mutant was first identified as a leaf developmental mutant (Bohmert et al., 1998). *AGO1* is the founding member of a gene family that is conserved among eukaryotes (Bohmert et al., 1998), the members of which play a crucial role in the regulation of posttranscriptional gene silencing and related

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mechanisms (Fagard et al., 2000; Hammond et al., 2001; Carmell et al., 2002; Morel et al., 2002). AGO proteins are also called PPD proteins, because they all retain the conserved PAZ and PIWI domains (Cerutti et al., 2000). These proteins have been shown in both *Drosophila melanogaster* and human cells to be core components of the RNA-induced silencing complex, which targets mRNA for degradation using a microRNA (miRNA) as a guide (Hutvagner and Zamore, 2002; Ishizuka et al., 2002). Recently, it was shown that the role of the *AGO1* gene in the miRNA pathway and its own regulation by this particular pathway are crucial for plant development (Vaucheret et al., 2004). Kidner and Martienssen (2004) reported that the leaf polarity defect observed in *ago1* could be explained by an abnormal distribution of miRNAs targeting *PHABULOSA* and *PHAVOLUTA* transcription factors, which are known to control leaf polarity in plants. It was also shown that the steady state levels of several transcription factor targets of miRNAs were increased in rosette leaves of strong and weak alleles of *ago1* (Vaucheret et al., 2004).

During our further characterization of the phenotype conferred by *ago1*, we discovered that, unlike the root, the apical part of *ago1* mutants displayed resistance to auxin-mediated hypocotyl elongation and a defect in adventitious root formation in response to auxin. This prompted us to investigate a potential interaction between light and auxin in the regulation of adventitious rooting using an allelic series of *ago* alleles and *ago sur* double mutants. The data presented here demonstrate that the defect in adventitious root formation in *ago1* mutants correlates with an alteration of auxin homeostasis and a hypersensitivity to light. We show that the mRNA of *Auxin Response Factor17* (*ARF17*) accumulates in the hypocotyls of *ago1* and demonstrate that deregulation of ARF17 expression and, as a consequence, *GH3* gene expression at least in part explain the adventitious root phenotype of *ago1* mutants. Thus, we conclude that *AGO1*, through its action on the regulation of *ARF17* expression, regulates genes involved at the cross talk between auxin and light signaling during adventitious root development.

RESULTS

The experiments described in this article were done using one null and four hypomorphic *ago1* mutants. The strong phenotype of *ago1-3* has been described previously (Bohmert et al., 1998; Camus, 1999). In this study, we confirmed by protein gel blot analysis and sequencing that *ago1-3* is a null allele (see Supplemental Table 1 and Supplemental Figure 1A online). The four new hypomorphic mutants (*ago1-32* to *ago1-35*) were identified in a screen for mutants displaying a phenotype similar to that of other previously described hypomorphic allele mutants (Morel et al., 2002). *ago1-32* shows a weak phenotype similar to that of *ago1-26*, whereas *ago1-33*, *ago1-34*, and *ago1-35* are similar to *ago1-27* (Morel et al., 2002). These mutants can grow in soil and, except for *ago1-32*, are fertile.

The Apical Part of *ago1* Seedlings Is Specifically Impaired in Auxin Response

When wild-type seedlings were germinated on media containing increasing concentrations of picloram, an auxin-type herbicide (Hansen and Grossmann, 2000), their hypocotyls showed a maximum size at a concentration of 5 μM (Figure 1A). Unlike the wild type, *ago1-3* plants showed no elongation of the hypocotyl when grown under the same conditions (Figure 1A). This defect of hypocotyl elongation in response to auxin was confirmed with the four weak allele mutants (Figures 1B and 2A). However, the root growth of the *ago1-3* null mutant was inhibited normally on media containing different concentrations of either picloram (Figure 1C) or the auxins naphthylacetic acid (1-NAA) or indoleacetic acid (IAA) (data not shown). To investigate whether *ago1* hypocotyl elongation was resistant to the increased endogenous content of auxin, double mutants between the different *ago1* alleles and the *sur2* auxin overproducer were produced. The *sur2-1 ago1-3*(hyb) double mutant is in a hybrid genetic background between Columbia (Col-0) and Wassilewskija (Ws) because it comes from a cross between a homozygote *sur2-1* plant in the Ws ecotype and a heterozygote for the *ago1-3* mutation in the Col-0 background. This hybrid genetic background will be referred to as (hyb) in the rest of the article. *sur2 ago1* root had the same length as the *sur2* root and was shorter than roots of the wild type or single *ago1* mutants (Figure 1D) and therefore responded normally to increased endogenous levels of auxin. Conversely, the double mutant hypocotyl remained short (Figures 1E and 2B). The apical part of 8-d-old light-grown *sur2-1 ago1-3*(hyb) double mutants contained twice as much free IAA than did the wild type (C. Sorin, K. Ljung, J.D. Bussell, G. Sandberg, and C. Bellini, unpublished data), indicating that the *ago1-3* mutation indeed induced resistance to increased levels of endogenous auxin that in the wild type were shown to induce elongation of the hypocotyl (Gray et al., 1998). On the contrary, when grown at high auxin concentrations, which inhibit wild-type hypocotyl elongation, the *ago1-3* mutant was as sensitive as the wild type and showed reduction of hypocotyl elongation (Figures 2C and 2D). We also examined whether the *ago1* hypocotyl could elongate under other growth conditions. Both the null and the weak allele mutants were grown on medium containing concentrations of gibberellic acid that promote hypocotyl elongation in the wild type. All *ago1* alleles analyzed responded to gibberellic acid and elongated in the same proportion as the wild type (see Supplemental Figure 2 online). When grown in the dark, plants representing the weak alleles showed no mutant phenotype (see Figure 8). In the dark, the null allele *ago1-3* was shorter than the wild type but was almost 10 times longer than in the light. The growth rate of *ago1-3* followed that of the wild type for the first 3 d after germination, but then it decreased (Figure 1F).

To test whether, similar to the other auxin-resistant mutants (Pickett et al., 1990; Wilson et al., 1990; Timpte et al., 1995; Leyser et al., 1996), *ago1-3* showed cross-resistance to cytokinin or ethylene, the mutant was grown on a medium containing either cytokinin or 1-aminocyclopropane-1-carboxylic acid, an ethylene precursor. Like the wild type, *ago1-3* responded normally to both hormones (see Supplemental Figure 3 online). The double mutant between *ago1-3* and the cytokinin overproducer *amp1* (Chaudhury et al., 1993) showed a clear additive effect of both mutations (see Supplemental Figures 3B and 3C online).

Based on all of these results, we conclude that *ago1* represents a new class of auxin-resistant mutants in which the hypocotyl is specifically resistant to auxin-mediated elongation.

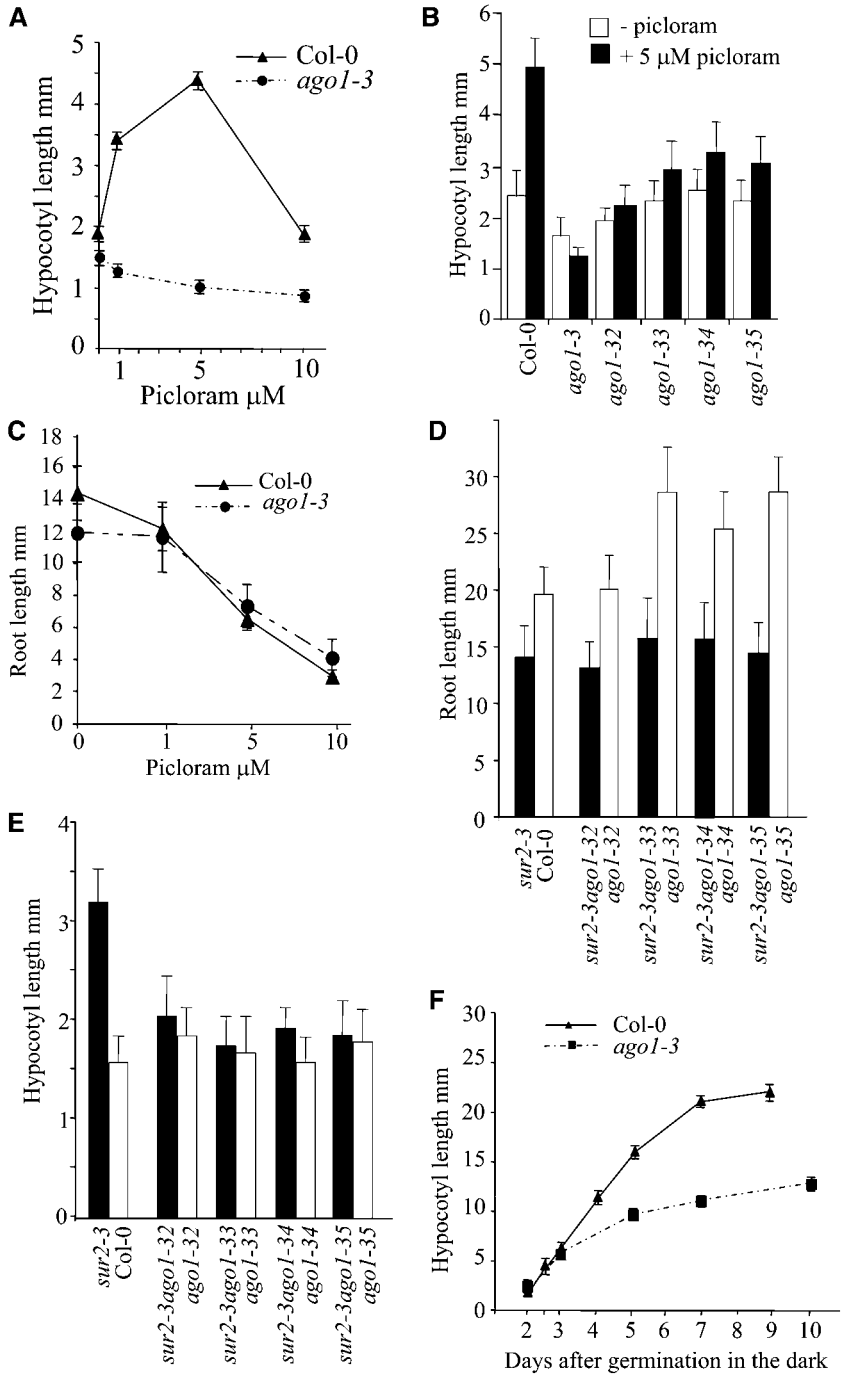

Figure 1. The Apical Part but Not the Root of *ago1* Mutants Is Resistant to Auxin.

(A) Hypocotyl length of wild-type and *ago1-3* siblings grown in vitro on increasing concentrations of picloram, 8 d after germination in the light.
(B) Hypocotyl length of wild-type and different *ago1* mutants grown in vitro in the presence or absence of 5 µM picloram, 8 d after germination in the light.
(C) Root length of wild-type and *ago1-3* siblings grown on increasing concentrations of picloram, 8 d after germination in the light.
(D) and **(E)** Root **(D)** and hypocotyl **(E)** length of Col-0 seedlings and siblings from a plant homozygous for the *sur2-3* mutation and heterozygous for the different weak alleles of *ago1*, 8 d after germination in the light.
(F) Hypocotyl length of wild-type and *ago1-3* siblings grown in vitro in the dark. The hypocotyl was measured at different time points.
Error bars indicate SD.

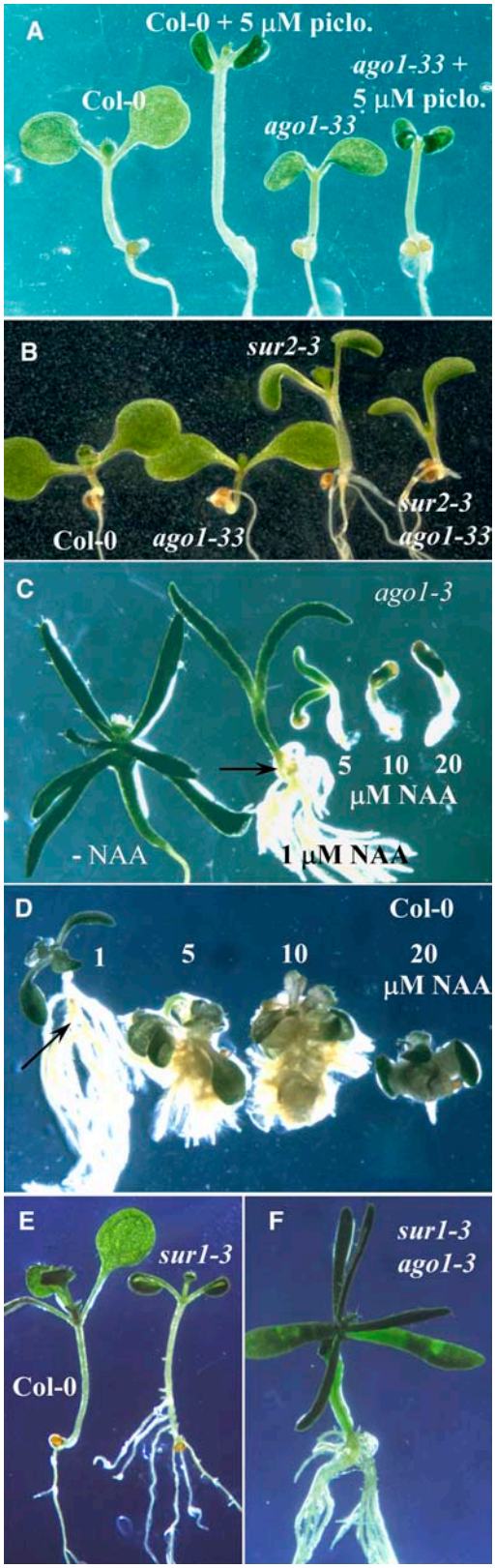

Figure 2. Auxin Resistance in the Apical Part Is Associated with a Defect in Adventitious Root Formation.

(A) Eight-day-old wild-type and *ago1-33* siblings germinated and grown in the light in the presence or absence of 5 μM picloram.
(B) Wild-type and *ago1-33* siblings (left) and *sur2-3* and *ago1-33 sur2-3* siblings (right). Seedlings were germinated and grown in the light for 8 d.
(C) and **(D)** Three-week-old *ago1-3* **(C)** and wild-type **(D)** siblings grown in the light on media without or with increasing concentrations of 1-NAA. Arrows indicate the hypocotyl/root junction.
(E) Wild-type Col-0 (left) and *sur1-3* (right) seedlings grown for 15 d in vitro.
(F) One-month-old *sur1-3 ago1-3* double mutant.

ago1-3 Is Altered in Adventitious Root Formation but Not in Lateral Root Development

Because only the apical part of *ago1* seedlings was resistant to auxin, we analyzed their capacity to produce adventitious roots either in response to exogenous auxin or in the auxin overproducer *sur1* or *sur2* background. When germinated and grown in the light in the presence of auxin, *ago1-3* seedlings, unlike wild-type seedlings, were unable to develop adventitious roots on the hypocotyl (Figures 2C and 2D). Double mutants between *ago1-3* and the auxin overproducer *sur1-3* were also unable to produce adventitious roots from the hypocotyl (Figures 2E and 2F). We previously showed that adventitious roots in the hypocotyl initiate from the pericycle cells adjacent to the xylem poles, similar to lateral roots (Boerjan et al., 1995). Therefore, we checked whether *ago1-3* mutant roots were able to initiate and develop lateral roots in response to exogenous auxin. We used the *CYCB1*:*uidA* promoter fusion as a reporter gene to monitor lateral root formation. Indeed, *CYCB1* is one of the earliest genes expressed in the pericycle cells that will develop into a lateral root (Beeckman et al., 2001). As shown in Figures 3A to 3D, *ago1-3* was able to initiate and develop lateral roots in response to 1 μM 1-NAA in a similar way to the wild type. These results indicate that, although adventitious and lateral roots develop from pericycle cells, *ago1* is specifically impaired in adventitious root formation.

When Primarily Etiolated, *ago1-3* Seedlings Can Develop a Few Adventitious Roots in Response to Auxin

Because wild-type hypocotyl can spontaneously develop adventitious roots when it has been etiolated in the dark, and also because *sur1* and *sur2* have a longer hypocotyl than wild-type seedlings, we wondered whether the defect in adventitious rooting from *ago1* hypocotyls was linked to its defect in elongation in the light in response to auxin. Mutant and wild-type seedlings were grown in the dark for different periods of time, and hypocotyl length was measured before they were transferred to the light for 1 week. After being etiolated for 2.5 d in the dark, both wild-type and mutant plants had an average hypocotyl length of 5 mm (Figure 1F), and most of wild-type seedlings developed at least one adventitious root when transferred to the light for 1 week (Figure 4A). The proportion of mutant seedlings able to develop at least one adventitious root remained extremely low irrespective of hypocotyl size (Figure 4A). This result suggested that a defect in hypocotyl elongation was not sufficient to explain the defect in adventitious rooting. Therefore,

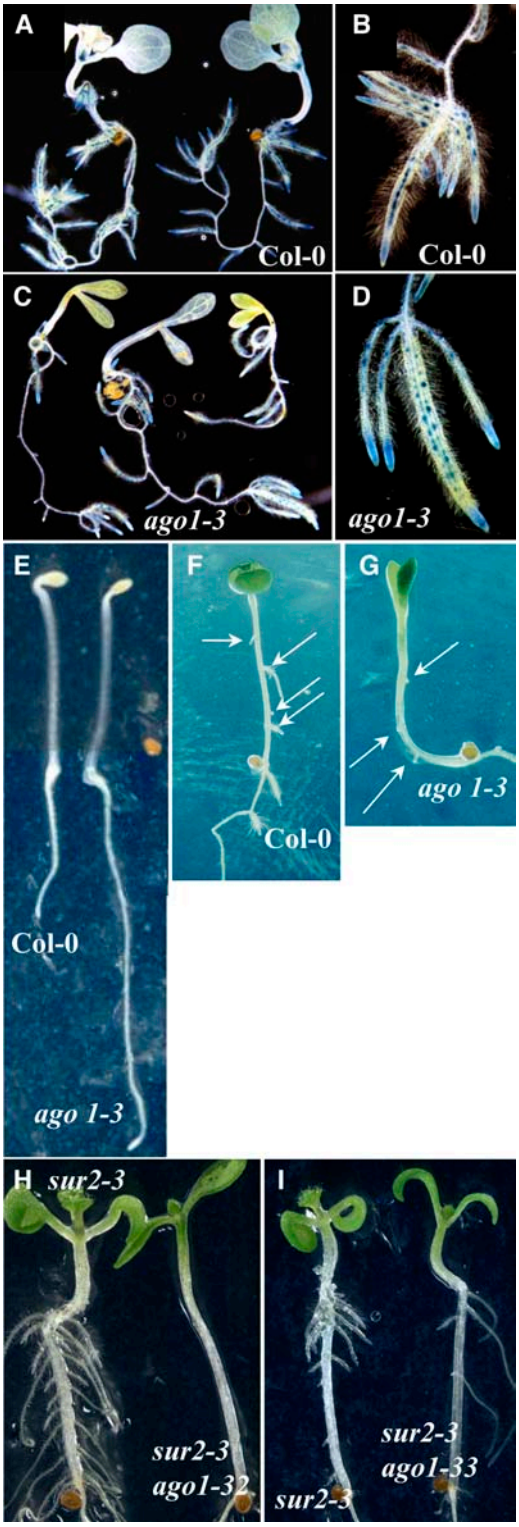

Figure 3. Adventitious Root Initiation, but Not Lateral Root Initiation, Is Affected in *ago1-3*.

(A) to **(D)** Siblings from a heterozygous *ago1-3* plant expressing the *CycB1*:*uidA* marker gene were germinated and grown in the light for 5 d on a medium without auxin and then transferred to a medium containing 1 μM NAA for 6 d.

(A) and **(B)** Wild type.

(C) and **(D)** *ago1-3*.

(E) Siblings from a heterozygous *ago1-3* parent plant were germinated and grown in the dark for 2.5 d before transfer to the light.

(F) Col-0 could make up to four adventitious roots after etiolation and transfer to the light for 1 week on a medium containing 1 μM 1-NAA. Arrows indicate adventitious roots.

(G) *ago1-3* siblings could very rarely develop up to three adventitious roots in the same conditions as in **(F)**. Arrows indicate adventitious roots.

(H) and **(I)** *sur2-3* and *sur2-3 ago1* etiolated siblings after 1 week in the light. *ago1-32* is the intermediate allele mutant **(H)**, and *ago1-33* is one of the weakest alleles **(I)**.

we tested the capacity of the *ago1-3* mutant to develop adventitious roots in response to exogenous auxin after hypocotyl elongation in the dark. Mutant and wild-type siblings were etiolated for 2.5 d before transfer to the light (Figure 3E). They were then transferred onto a medium without auxin or containing 1 μM 1-NAA. Addition of auxin significantly increased the proportion of *ago1-3* seedlings developing one or more adventitious roots (Figures 3F, 3G, 4B, and 4C). Figures 3F and 3G illustrate the fact that wild-type and *ago1-3* seedlings could sometime make up to four and three adventitious roots, respectively, when transferred onto auxin-containing medium after etiolation. Nevertheless, this remained a rare event, as *ago1-3* showed an average of one adventitious root and the wild type in the same conditions had three adventitious roots (Figure 4C). However, weak allele mutants etiolated for 2.5 d before transfer to the light on a medium containing 1 μM 1-NAA produced almost as many adventitious roots as the wild type (data not shown).

Adventitious roots were also scored in etiolated seedlings of the different *sur2 ago1* double mutants at 7 d after transfer to the light. The *sur2-1 ago1-3*(hyb) mutant developed as many adventitious roots as the wild type in the absence of auxin but never more (Figure 4D). Double mutants with the weak alleles developed more adventitious roots but never as many as the single *sur2* mutant in the same conditions (Figures 3H, 3I, and 4D).

The *sur2-1 ago1-3*(hyb) Double Mutant Can Initiate Adventitious Roots in the Dark

To check whether the results described above were linked to a defect in the initiation of adventitious roots or to a blockage of the subsequent development of initiated adventitious roots, we monitored the expression of the *CycB1*:*uidA* marker gene in the *sur2-1 ago1-3*(hyb) double mutant. Seedlings were etiolated in the dark to obtain 5-mm hypocotyls before transfer to the light for 1 to 7 d. Figures 5A and 5C show that *sur2-1*(hyb) did not express the reporter gene at 2.5 d after germination in the dark in the hypocotyl but clearly showed adventitious root initiation at 1 d after transfer to the light (Figure 5C), which regularly increased with time (Figures 5B and 5C). Unexpectedly, the *sur2-1 ago1-3*(hyb) double mutant already expressed the reporter gene in hypocotyl pericycle cells before being transferred to the light (Figures 5A and 5C). In contrast with *sur2-1*(hyb), though, no more primordia were initiated on the hypocotyl even several days

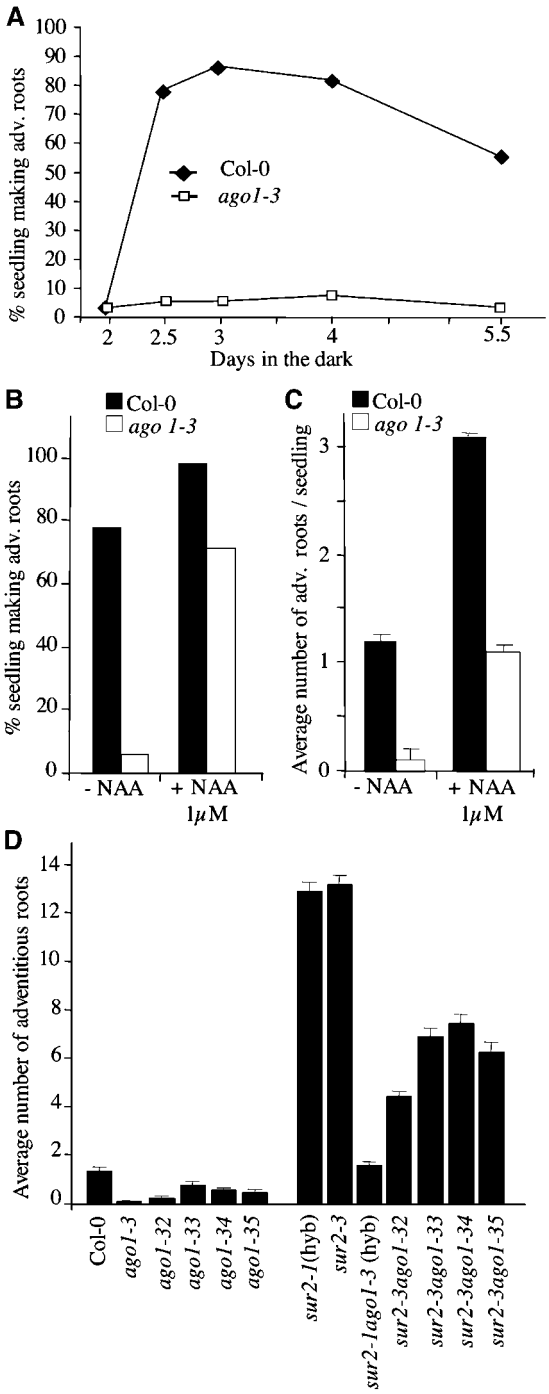

Figure 4. Auxin Stimulates Adventitious Roots on Etiolated *ago1* Seedlings but Never as in the Wild Type.

Emergent adventitious roots were scored 7 d after transfer to the light. At least 40 seedlings were used for each data point. This was repeated on three independent biological replicates.

(A) Siblings from a heterozygous *ago1-3* mother plant were germinated and grown in the dark for different times, then transferred to the light. The proportion of seedlings forming one or more adventitious roots was determined after 1 week in the light.

(B) Proportion of wild-type and mutant seedlings forming one or more adventitious roots after transfer to the light for 1 week on a medium with or without 1-NAA.

(C) Average number of adventitious roots formed on wild-type or mutant seedlings in the absence or presence of 1-NAA. Error bars indicate SE.

(D) Average number of adventitious roots formed on Col-0, *sur2*, the five *ago1* mutants, and the five *sur2 ago1* double mutants after etiolation and transfer to the light for 1 week. Error bars indicate SE.

after transfer to the light (Figures 5B and 5C). These results indicate that light is required for the induction of adventitious root initiation and development in *sur2-1*(hyb) mutants but has an inhibitory effect in *ago1-3 sur2-1*(hyb) double mutants. Because adventitious roots in *ago1-3 sur2-1*(hyb) seedlings were initiated in the dark and stopped after transfer to the light, the *ago1-3* mutation may alter light regulatory pathways.

Auxin Homeostasis Is Altered in the Apical Part of *ago1-3* Seedlings but Not in the Root

The fact that *sur2 ago1* double mutants developed fewer adventitious roots than *sur2* mutants under the same growth conditions suggested that the *ago1* mutation could affect endogenous auxin levels. Therefore, we determined the endogenous content of free IAA in the different genotypes. Seedlings of the wild type, *ago1-3*, *sur2-1*(hyb), and *sur2-1 ago1-3*(hyb) were grown in the dark for 2.5 d and then transferred to the light for 24, 48, or 72 h. Figure 6A shows that *ago1-3* entire seedlings had a slightly lower free IAA content than the wild type either in the dark or after transfer to the light ($P < 0.05$). A more pronounced effect of the *ago1-3* mutation could be observed in the auxin-overproducing *sur2* background. The double mutant *sur2-1 ago1-3*(hyb) had a reduction in free IAA compared with the single *sur2-1*(hyb) mutant ($P < 0.01$), although the level remained higher than in the wild type ($P < 0.01$). Interestingly, when IAA was quantified in the root only, no differences between *sur2-1*(hyb) and *sur2-1 ago1-3*(hyb) were detected (Figure 6B). In the dark, the *ago1-3* root had the same auxin content as the wild-type root, and no clear differences were observed after transfer to the light (Figure 6B). On the contrary, when free IAA was quantified in the apical part of the seedlings (hypocotyl plus cotyledons), the endogenous content in the *ago1-3* mutant was similar to the wild-type level in the dark, but it decreased after transfer to the light ($P < 0.01$). This effect was even more striking in the *sur2-1*(hyb) background. The free IAA content clearly increased in the apical part of *sur2-1*(hyb) after transfer to the light ($P < 0.05$) but decreased in that of the *sur2-1 ago1-3*(hyb) double mutant ($P < 0.05$) (Figure 6C).

The IAA biosynthesis rate was measured in the apical part of the different genotypes at 72 h after transfer to the light by monitoring the incorporation of deuterium via de novo synthesis of IAA, as described previously (Ljung et al., 2001). Although the biosynthesis rate was low at that stage of development, incorporation of deuterium could be measured, indicating that some IAA was synthesized. The rate of synthesis was slightly lower but significantly different in the *ago1-3* mutant compared with the wild type ($P < 0.05$); nevertheless, no difference was detected between the auxin overproducer *sur2-1* mutant and the *sur2-1 ago1-3*(hyb) double mutant (Figure 7A). Similar results

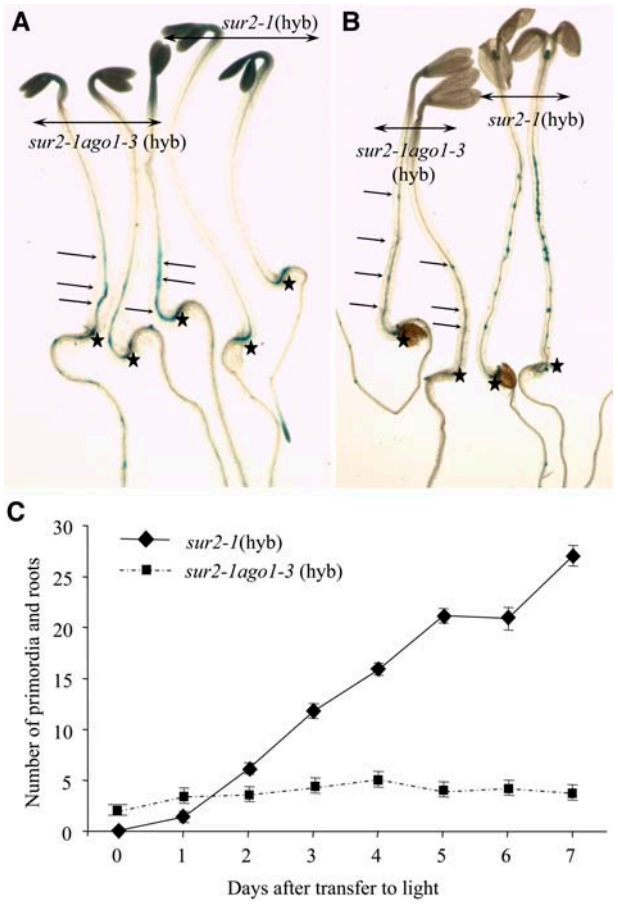

Figure 5. *sur2 ago1* Double Mutants Initiate Adventitious Roots in the Dark.

(A) and **(B)** *CycB1*:*uidA* expression in etiolated siblings of *sur2-1* and *sur2-1 ago1-3*(hyb), 2.5 d after germination in the dark **(A)** and after transfer to the light for 3 d **(B)**. Arrows indicate GUS staining in the *sur2-1 ago1-3*(hyb) hypocotyl. Stars indicate the junction between the hypocotyl and the root. **(C)** *CycB1*:*uidA* expression in etiolated siblings of *sur2-1* and *sur2-1 ago1-3*(hyb) was monitored in seedlings germinated and grown in the dark for 2.5 d and transferred to light for 1 to 7 d. This allowed identification and scoring of the initiation of very early primordia as well as older emerging roots. Error bars indicate SE.

were obtained with entire seedlings (data not shown). By contrast, incorporation of deuterium was almost not detectable in the root of both genotypes (data not shown), indicating that, at that stage of development, the auxin was synthesized mainly in the apical part. These results indicate that the *AGO1* gene might be required for the regulation of auxin biosynthesis. Nevertheless, the decrease of auxin content in *sur2-1 ago1-3*(hyb) cannot be explained by a lower biosynthesis rate compared with *sur2-1*, suggesting that *ago1-3* may be altered in other pathways that regulate auxin homeostasis. Thus, we measured the endogenous contents of several IAA metabolites in the different genotypes and showed that the levels of the amide conjugates IAAsp ($P < 0.01$), IAAla ($P < 0.05$), and IAAglu ($P < 0.05$) were lower in *ago1-3* and *ago1-3 sur2-1*(hyb) compared with Col-0 and *sur2-1*(hyb), respectively (Figures 7C to 7E), suggesting an inhibition of the IAA conjugation pathway in the *ago1-3* background. The level of oxoindole-3-acetic acid was not statistically different at that stage of development between *ago1-3* and Col-0 seedlings or in the *ago1-3 sur2-1*(hyb) double mutant compared with *sur2-1*(hyb) siblings (Figure 7B). Nevertheless, because the decrease of IAA content in *sur2-1 ago1-3*(hyb) compared with *sur2-1*(hyb) cannot be explained by a reduction of IAA biosynthesis or increased conjugation, further analyses are needed to check for a potential activation of the catabolic pathway in *ago1-3*. These results suggest that *AGO1* influences the overall regulation of auxin homeostasis in the apical part of Arabidopsis seedlings.

ago1 Is Hyperresponsive to Light

Although the *ago1-3* mutant did not show a characteristic deetiolated phenotype (short hypocotyl, open and expanded cotyledons), some aspects of its phenotype in the dark, such as the shorter hypocotyl and longer primary root (Figure 3E), suggest a defect in light perception or in light regulatory pathways. Indeed, *ago1-3* had a root that was almost three times longer than the wild-type root for the same hypocotyl size at 2.5 d after germination in the dark (Figure 3B). This root length was equivalent to that of *ago1-3* or wild-type roots when the seedlings were germinated and grown in the light for 7 d (data not shown). The different *ago1* alleles were grown under different monochromatic light conditions. They were grown under low-fluence constant red light (cR) (9 $\mu E \cdot m^{-2} \cdot s^{-1}$), low-fluence constant blue light (cB) (4 $\mu E \cdot m^{-2} \cdot s^{-1}$), or very low-fluence constant far-red light (cFR) (0.25 $\mu E \cdot m^{-2} \cdot s^{-1}$) for 7 d. In these conditions, the wild type was shorter than when it was grown in the dark, but its hypocotyl still elongated. The *ago1-3* mutant did not elongate at all in any light conditions except in the dark (Figure 8A), suggesting a general upregulation of light regulatory pathways. The weak allele mutants were all shorter than the wild type in cR, cB, and cFR (Figure 8A). The weakest allele mutants displayed similar elongation of the hypocotyl in cB but showed more heterogeneity in cR and cFR. Three of the hypomorphic mutants, *ago1-32*, *ago1-33*, and *ago1-34*, were almost as short as the null mutant *ago1-3* in very low-fluence cFR. These results suggested that *ago1* mutants were hypersensitive to light. That four of them were considerably shorter in cFR suggested a deregulation in the phytochrome A (PHYA)–dependent pathway. To test this hypothesis, we generated double mutants with *phyA*. As shown in Figure 8B, we observed a clear epistasy of the *phyA* mutation on *ago1-3*, *ago1-33*, and *ago1-35* in cFR. These results strongly suggest that at least the light regulatory pathways mediated by PHYA are upregulated in *ago1*.

Expression of *GH3* Genes Is Downregulated in *ago1-3*

We have analyzed the expression of several auxin-inducible genes in the *ago1-3* mutant. Using RNA gel blot experiments, we have checked the expression of six auxin-inducible *Aux/IAA* genes (*IAA1*, *IAA4*, *IAA7*, *IAA14*, *IAA17*, and *IAA19*) (Abel et al., 1995; Rouse et al., 1998; Nagpal et al., 2000; Fukaki et al., 2002; Tatematsu et al., 2004). None of the six genes was upregulated in *ago1-3* compared with the wild type in the absence of auxin (data

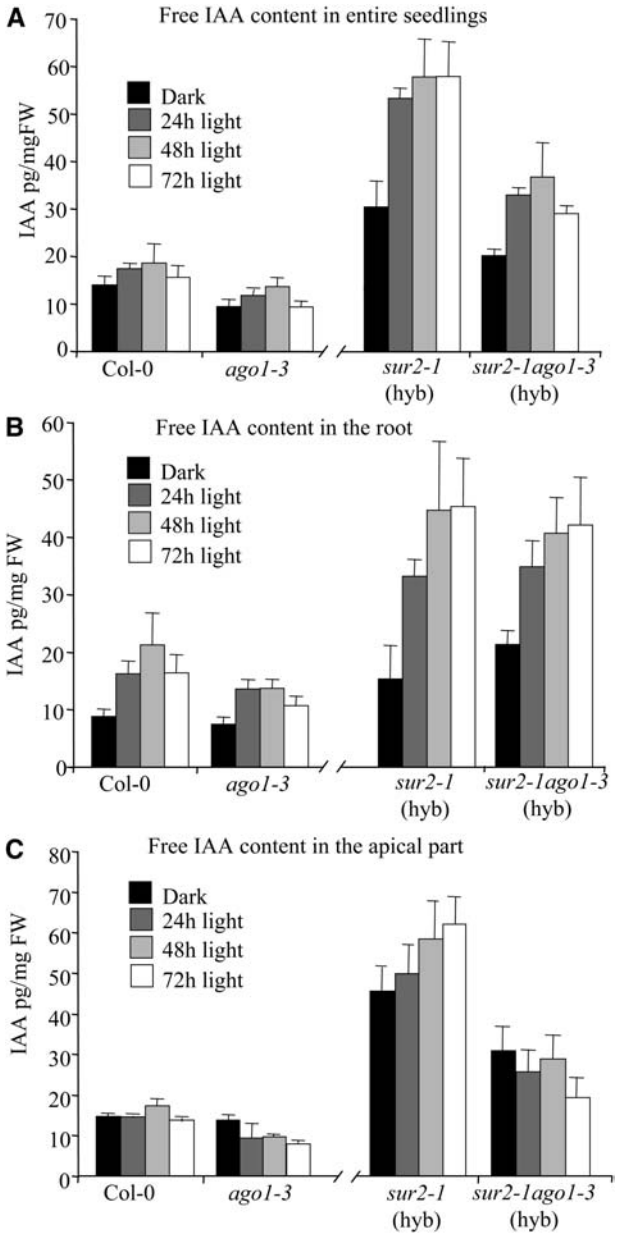

Figure 6. Free IAA Content in Col-0, *ago1-3*, *sur2-1*(hyb), and *ago1-3 sur2-1*(hyb) Seedlings.

The endogenous free IAA level was measured in seedlings germinated and grown for 2.5 d in the dark, then transferred to the light for 24, 48, or 72 h. FW, fresh weight.

(A) Entire seedlings. Free IAA content was lower in *ago1-3* than in the wild type ($P < 0.5$). The *sur2-1 ago1-3*(hyb) double mutant contained significantly more auxin than the wild type ($P < 0.01$) but less than *sur2-1*(hyb) ($P < 0.01$).

(B) Root. The auxin content increased in all the genotypes after transfer to the light. No significant difference between *ago1-3* and Col-0 or *sur2-1*(hyb) and *sur2-1 ago1-3*(hyb) could be detected.

(C) Apical part (cotyledons plus hypocotyl). The auxin content decreased significantly in *ago1-3* seedlings after transfer to the light ($P < 0.01$) and was significantly lower than that in the wild type after 72 h in the light ($P < 0.01$). The auxin content increased significantly in *sur2-1*(hyb) after transfer to the light ($P < 0.05$) but decreased in *sur2-1 ago1-3*(hyb) ($P < 0.05$).

Three biological replicates were used for each data point. Error bars indicate SD. A *t* test was performed according to http://graphpad.com/quickcalcs/ttest1.cfm.

not shown). All of them were induced by auxin, although the induction in *ago1-3* was weaker in the case of *IAA7* and *IAA14* (data not shown).

The expression patterns of four auxin-inducible reporter genes were also analyzed. *IAA2*:*uidA*-, *DR5*:*uidA*-, *SAUR-AC*:*uidA*-, and *GH3*:*uidA*-expressing lines were crossed with heterozygous *ago1-3* plants. Wild-type and mutant plants were grown for 1 week on medium without auxin or containing 1 μM 1-NAA. Upon analysis of histochemical β-glucuronidase (GUS) staining, we observed the same expression pattern of *DR5*:*uidA*, *IAA2*:*uidA*, or *SAUR-AC*:*uidA* as well as the same response to auxin treatment between wild-type and *ago1-3* plants. By contrast, *GH3*:*uidA* expression was induced in the root and in the hypocotyl of the wild type when grown in the presence of 1 μM 1-NAA but never in the hypocotyl of *ago1-3* mutants, which, however, showed a normal induction in the root (Figures 9A and 9B). In the absence of auxin, light-grown wild-type and *ago1-3* seedlings showed similar patterns of expression in the root at the points of lateral root initiation. Nevertheless, in contrast with the wild type, the apical meristem was rarely stained in 1-week-old, light-grown *ago1-3* seedlings (Figures 9A and 9B). Because these differences between the wild type and *ago1-3* were observed in light-grown seedlings, we checked the conditions used for adventitious root induction. Seedlings were first etiolated for 2.5 d and then transferred to long-day conditions for 2 d. After 44 h, they were transferred in liquid culture medium in the presence or absence of 10 μM 1-NAA and kept in the same growth conditions for 4 h, then stained overnight for GUS expression. In the absence of auxin, *GH3*:*uidA* was expressed in all hypocotyls of wild-type seedlings, whereas in *ago1-3*, expression was restricted to the bottom third of the hypocotyl. The roots of nontreated wild-type or *ago1-3* seedlings mainly expressed *GH3*:*uidA* at the site of lateral root initiation. As described previously, the *ago1-3* root is longer than the wild-type root and initiates several lateral roots in the dark, which could explain the greater GUS expression in roots of etiolated *ago1-3* compared with the wild type. When seedlings where treated with 10 μM 1-NAA, *GH3*:*uidA* was induced all along the root of both wild-type and *ago1-3* siblings (Figures 9C and 9D). *GH3*:*uidA* expression was also stronger in hypocotyls of both the wild type and *ago1-3* after auxin treatment (Figures 9C and 9D). Nevertheless, expression of the reporter gene still was not induced in the upper part of *ago1-3* hypocotyls (Figure 9D). These results suggest that the regulation of *GH3-like* gene expression could be altered in *ago1-3*.

The mRNA of the Auxin Response Factor *ARF17* Accumulates in the Hypocotyl of *ago1-3*

It was recently shown that *ago1* mutants accumulate several miRNA targets, including two auxin response factors, *ARF8* and *ARF17*, in rosette leaves (Vaucheret et al., 2004). Five *ARF* genes

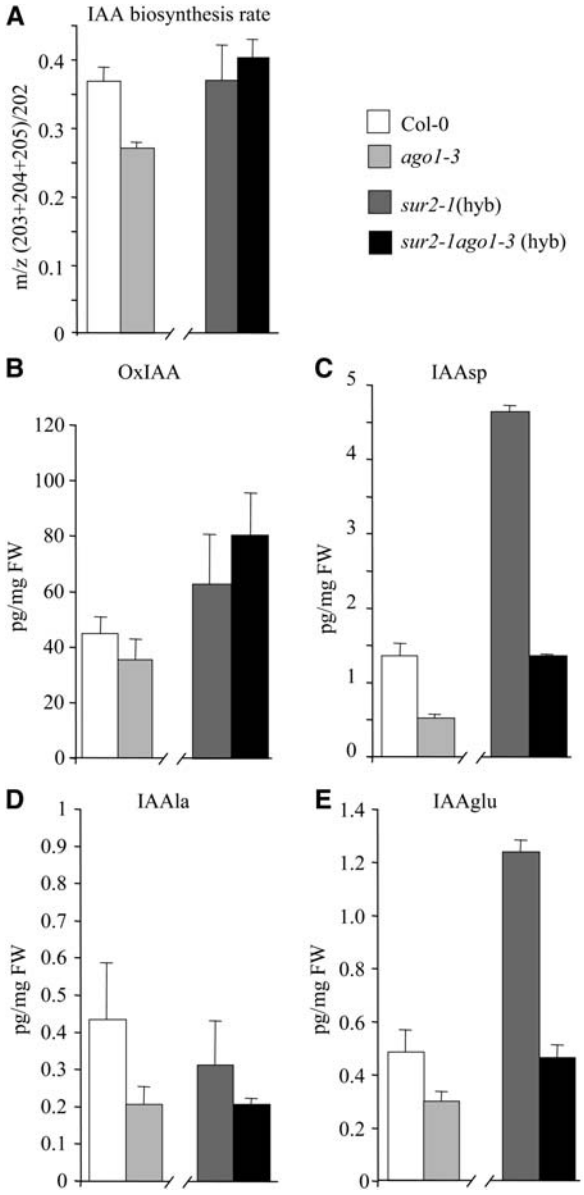

Figure 7. IAA Biosynthesis Rate and Level of IAA Metabolites in Col-0, *ago1-3*, *sur2-1*(hyb), and *ago1-3 sur2-1*(hyb) Seedlings.

(A) IAA biosynthesis rate in the apical part of seedlings (hypocotyl plus cotyledons), 72 h after transfer to the light. Four replicates were used for each data point. Error bars indicate SD.

(B) to **(E)** Quantification of IAA metabolites. Measurements were performed on entire seedlings grown for 2.5 d in the dark for oxoindole-3-acetic acid **(B)**, *N*-(indole-3-acetyl)-Asp **(C)**, *N*-(indole-3-acetyl)-Ala **(D)**, and *N*-(indole-3-acetyl)-Glu **(E)**. Four replicates were used for each data point. Error bars indicate SD. FW, fresh weight.

were identified as potential targets for miRNAs: *ARF6*, *ARF8*, *ARF10*, *ARF16*, and *ARF17* (Rhoades et al., 2002). To address whether any of these genes could be involved in AGO-modified auxin responses during adventitious root formation, we analyzed their expression in wild-type and *ago1-3* hypocotyls that had been etiolated for 2.5 d in the dark and transferred to the light for 48 h. A significant sevenfold increase in the steady state level of *ARF17* mRNA was detected in the hypocotyl of the *ago1-3* mutant (Figure 10A). Moreover, the *ARF10* mRNA showed a twofold increase, and no significant difference was detected for *ARF6*, *ARF8*, or *ARF16*. Additionally, no difference in expression was detected for either *ARF7*/*NPH4* or *ATHB2*, analyzed as transcription factors putatively not targeted by miRNAs and because they were shown previously to act in the cross talk between light and auxin signaling pathways (Stowe-Evans et al., 2001; Morelli and Ruberti, 2002).

ARF17 Represses the Expression of *GH3* Genes and Negatively Regulates Adventitious Root Formation

ARFs bind auxin response elements present in the promoter of auxin-inducible genes such as *Aux*/*IAA*, *SAUR*, and *GH3* and either repress or activate their transcription (Tiwari et al., 2003). Although repression activity has not been demonstrated for *ARF17*, its sequence is more closely related to the repressor group of *ARF*s (Tiwari et al., 2003). Therefore, we suggest that the overexpression of *ARF17* in *ago1-3* could negatively regulate the expression of several *GH3*-related genes and in this way repress adventitious root formation. To test this hypothesis, we characterized a SALK line containing a T-DNA insertion in the promoter region of the *ARF17* gene. Initial RT-PCR on the line SALK 062511 indicated the presence of an *ARF17* transcript in T-DNA homozygotes. The molecular characterization of the insertion indicated the presence of two T-DNAs as inverted repeats ~200 bp 5′ of the coding sequence (CDS) and ~100 bp upstream of the start of transcription (Figure 10B). The pROK vector used in the generation of SALK lines (Alonso et al., 2003) carries a *Cauliflower mosaic virus* 35S promoter oriented toward the left border, which was likely to induce the expression of *ARF17*. Real-time PCR was performed as described above and indicated that the line was in fact an overexpresser. The abundance of *ARF17* transcript was increased 7 times with respect to wild-type siblings in 12-d-old entire seedlings (data not shown) and ~13 times in hypocotyls etiolated for 2.5 d followed by 2 d in the light (Figure 10C). Importantly, because the primers bound the miRNA target sequence in *ARF17*, the excess transcript is not immediately degraded through miRNA processes and is able to accumulate. As this line has retained kanamycin resistance (see http://signal.salk.edu/tdna_FAQs.html), a single T-DNA insertion locus was confirmed by screening the segregation of selfed progeny derived from heterozygous plants on kanamycin-supplemented medium (data not shown). Homozygote *ARF17 OX* plants did not show any obvious phenotypic difference compared with wild-type siblings: they grew normally in soil and were fully fertile (data not shown). To verify the hypothesis that ARF17 could repress *GH3* expression, we analyzed the transcript abundance for *GH3-3*, *GH3-5* (*AtGH3a*), and *GH3-6* (*DFL1*) in hypocotyls etiolated for 2.5 d followed by 2 d in the light. All three genes were significantly repressed in *ARF17 OX* (Figure 10D). Because the expression of these three genes was correlated to the adventitious root number (C. Sorin, L. Negroni, T. Balliau, H. Corti, M.P. Jacquemot, M. Daventure, G. Sandberg, M. Zivy, and C. Bellini, unpublished

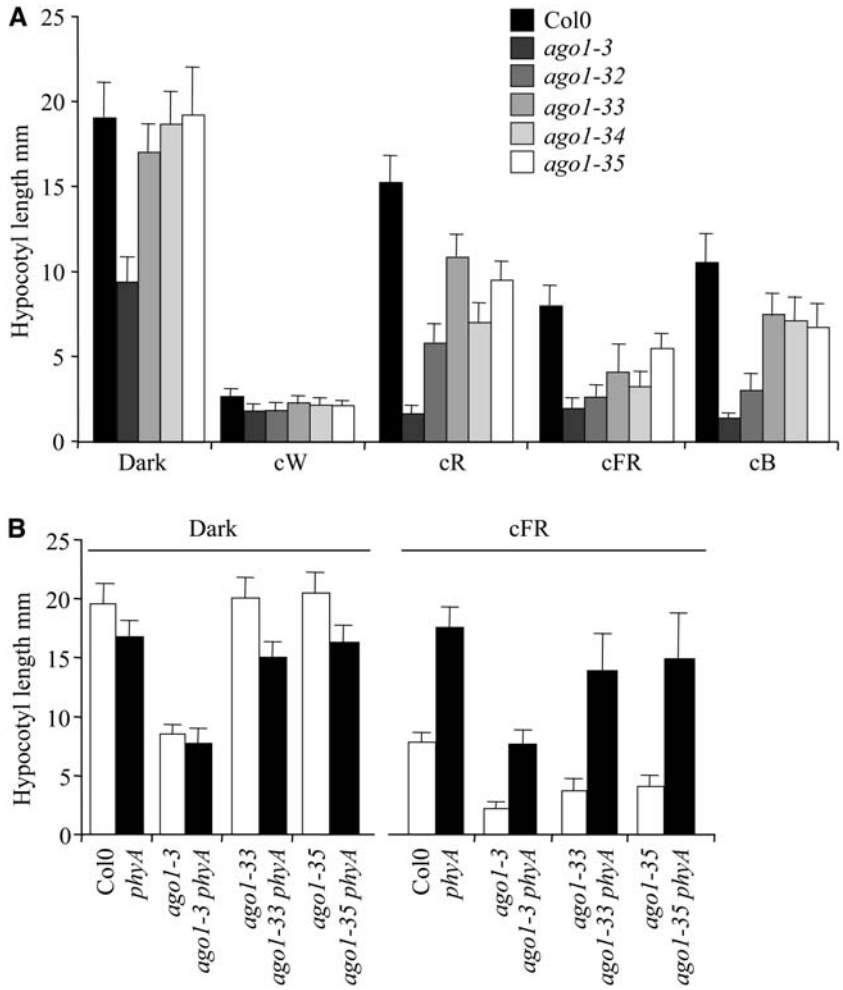

Figure 8. *ago1* Is Hyperresponsive to Light.

(A) Hypocotyl length of the different *ago1* mutants grown in various light conditions. Hypocotyl length was measured on wild-type and mutant siblings from the different alleles grown in vitro under different light conditions for 8 d. cW, constant white light (150 $\mu E \cdot m^{-2} \cdot s^{-1}$); cR, constant red light (9 $\mu E \cdot m^{-2} \cdot s^{-1}$); cFR, constant far-red light (0.25 $\mu E \cdot m^{-2} \cdot s^{-1}$); cB, constant blue light (3.7 $\mu E \cdot m^{-2} \cdot s^{-1}$). Error bars indicate SD.

(B) *phyA* is epistatic to *ago1* in far-red light. Hypocotyl length of Col-0, *ago1-3*, *phyA*, *phyA ago1-3*, *phyA ago1-33*, and *phyA ago1-35*, 8 d after germination either in the dark or in cFR. Error bars indicate SD.

data), we checked whether *ARF17 OX* was altered in the development of adventitious roots. Indeed, although the adult plant did not have an obvious phenotype, *ARF17 OX* produced fewer adventitious roots than did wild-type siblings after etiolation and transfer to the light for 1 week (Figures 10E and 10F). These results suggest that *ARF17* could negatively regulate adventitious root formation by repressing *GH3* gene expression.

DISCUSSION

ago1 Mutants Represent a New Class of Auxin-Resistant Mutants Altered in Their Capacity to Develop Adventitious Roots

We have demonstrated that the null and weak *ago1* allele mutants analyzed in this study are specifically resistant to auxin in the apical part of the seedling. Indeed, they were not able to elongate in the presence of picloram, which is known to stimulate hypocotyl elongation in the wild type (Delarue et al., 1998). Nevertheless, *ago1* was able to respond to auxin concentrations that inhibit hypocotyl elongation. The root responded normally to all of the auxins tested, suggesting that the auxin resistance was restricted to the apical part. Because the hypomorphic mutants display the same auxin resistance in the hypocotyl as the null mutant, this cannot be attributed to the pleiotropic developmental phenotype of the null mutant. Therefore, *ago1* differs from other auxin-resistant mutants described to date, such as *axr1*, *aux1*, *axr2*, *axr3*, and *axr4*, which were selected for the resistance of the root to inhibitory concentrations of auxin (Estelle and Somerville, 1987; Pickett et al., 1990; Wilson et al., 1990; Hobbie and Estelle, 1995; Leyser et al., 1996). These auxin-resistant mutants, except *axr4*, also display cross-resistance to other

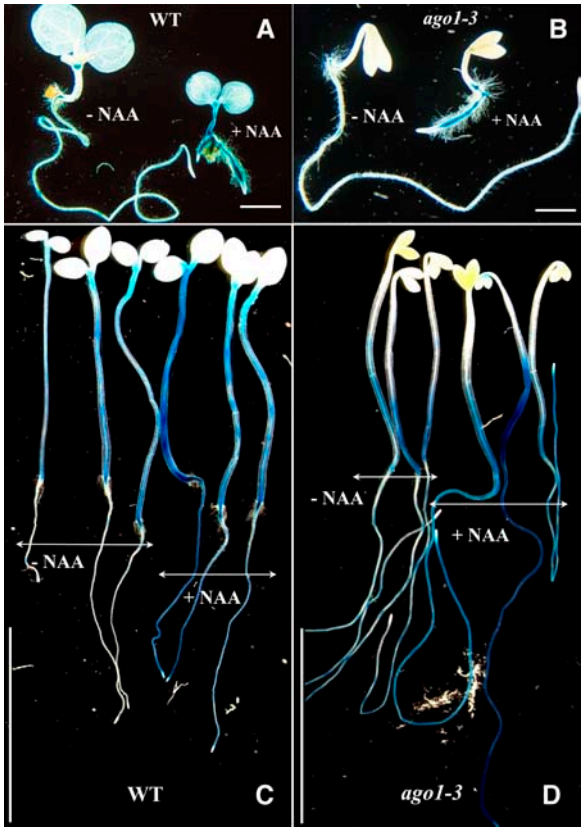

Figure 9. Expression of the *GH3*:*uidA* Reporter Gene in *ago1-3* Seedlings.

(A) and **(B)** *GH3*:*uidA* expression in wild-type **(A)** and *ago1-3* **(B)** seedlings geminated and grown in the light for 8 d in the absence or presence of 1 μM 1-NAA. Bars = 2 mm.

(C) and **(D)** *GH3*:*uidA* expression in wild-type **(C)** and *ago1-3* **(D)** seedlings that were etiolated for 2.5 d in the dark before transfer to the light for 44 h. Seedlings were then transferred to liquid culture medium without or with 10 μM 1-NAA for 4 h in the same growth conditions. GUS staining was overnight. From left to right in each panel: three nontreated seedling and three seedlings treated with NAA. Bars = 6 mm.

hormones, such as cytokinin and ethylene. This was not the case for *ago1* mutants, which responded normally to exogenous cytokinin, ethylene, and gibberellic acid. Although *ago1-3* has an altered developmental phenotype, it is still able to behave like the wild type in various growth conditions. The defect in hypocotyl elongation in the presence of picloram is not attributable to a general problem of elongation, because the *ago1* mutants were able to elongate in the light when grown on medium containing gibberellic acid or in the dark. Together, our results show that the apical part of *ago1* is specifically resistant to auxin-mediated hypocotyl elongation.

This auxin resistance is also characterized by a reduced number of adventitious roots in the hypocotyl in response to auxin. Unlike the hypocotyl, the roots of the *ago1-3* null mutant were still able to normally initiate lateral roots in response to exogenous auxin, as shown by the normal induction and expression of the *CycB1*:*uidA* reporter gene in the roots of mutant seedlings. These results strongly support the hypothesis that different regulatory pathways control lateral root and adventitious root initiation, although both root types initiate from pericycle cells. A mutation in *AGO1* clearly uncouples these pathways.

We further demonstrated that the defect in adventitious rooting is not related to defective hypocotyl elongation, because *ago1-3*, unlike the wild type, was barely able to produce adventitious roots irrespective of the size of its hypocotyl. However, when seedlings were first etiolated and then transferred to the light on medium containing 1 μM 1-NAA, most of the *ago1-3* seedlings could make at least one adventitious root, but the average number of adventitious roots always remained lower than in the wild type in the presence of auxin. This was also observed in the *sur2 ago1* double mutant, indicating that *ago1* plants were able to initiate adventitious roots under certain conditions. This means that the pericycle cells in the hypocotyl were still able to reenter the cell cycle in an *ago1* background and that the inhibition occurred at a different level. Indeed, when the expression of the *CycB1*:*uidA* reporter gene was monitored in *sur2-1*(hyb) and *sur2-1 ago1-3*(hyb), we observed that, unlike *sur2-1*, the double mutant had already initiated adventitious roots at 2.5 d after germination in the dark. Nevertheless, the number of adventitious roots did not increase after transfer to the light, unlike in *sur2-1* seedlings. These results could be explained by two hypotheses. (1) The *ago1-3* mutation modifies the endogenous auxin content in the double mutant *sur2-1 ago1-3*(hyb), leading to a reduction or a blockage of adventitious rooting. This, however, would not explain the initiation of adventitious roots in the *sur2-1 ago1-3*(hyb) hypocotyl in the dark. (2) The *ago1-3* mutation could modify light regulatory pathways in such a way that it affects auxin homeostasis and has an inhibitory effect on adventitious root initiation and development in *ago1* or *sur2 ago1*. To further test the latter hypothesis, we analyzed the behavior of *ago1* mutants in different light conditions and measured the endogenous content of IAA and IAA metabolites in the different genotypes.

Light Regulatory Pathways Are Upregulated in *ago* Mutants

Although the hypomorphic mutants did not show any significant phenotype in the dark, the null allele mutant *ago1-3* had a shorter hypocotyl and a longer root than did the wild type. This observation supported the hypothesis of a potential deregulation of light perception and signaling, which was further tested by growing *ago1* mutants under different light conditions (white, cR, cB, and cFR light). Under all conditions tested, the null allele mutant *ago1-3* remained as short as in white light, and all of the weak alleles displayed a significant inhibition of hypocotyl growth compared with the wild type under low fluences of cR, cB, and cFR ($P < 0.01$). The different weak allele mutants did not show exactly the same behavior. Thus, it should be possible to classify the different *ago1* hypomorphic alleles according to their behavior in different light conditions and possibly to identify alleles more specifically affecting one or the other pathway. If such alleles could be identified, they could be used in global approaches, such as transcriptome analysis, to identify candidate genes potentially involved in the different light regulatory

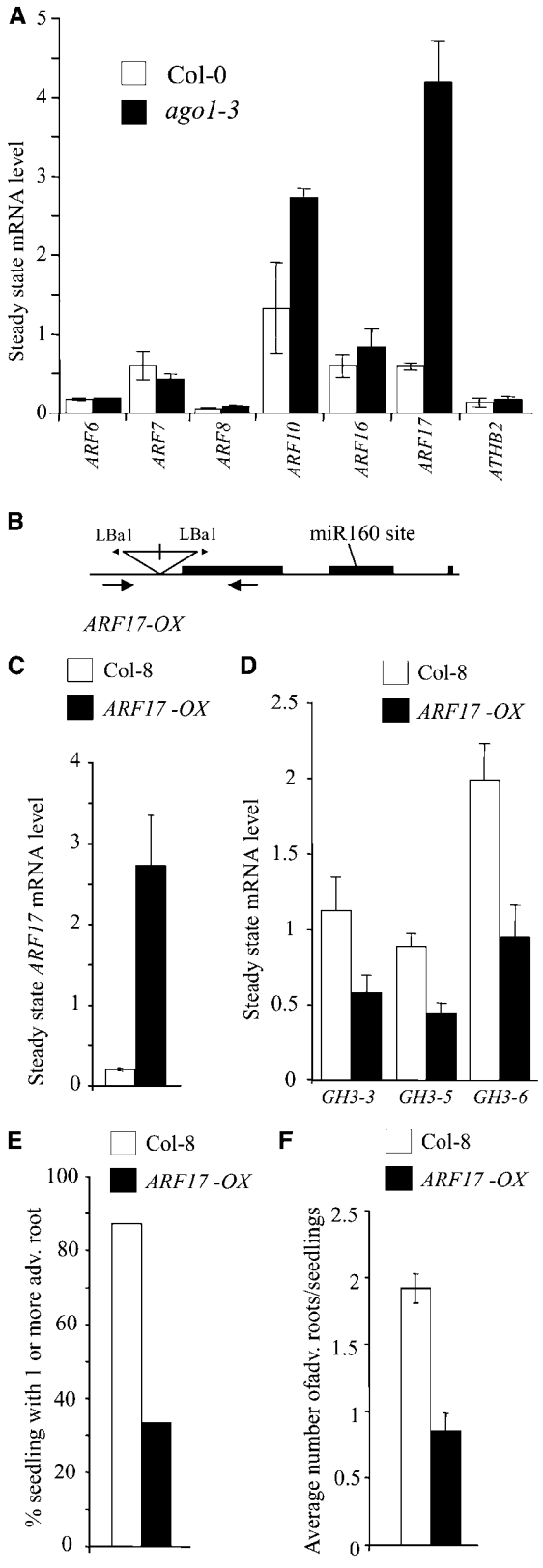

Figure 10. The Auxin Response Factor *ARF17* Represses *GH3* Genes and Adventitious Root Formation.

pathways. Three of the hypomorphic mutants were almost as short as the null allele mutant *ago1-3* in extremely low-fluence cFR, suggesting that a deregulation of the *PHYA*-dependent pathway might account for part of the phenotype of these *ago1* mutants. Analysis of the double mutants with *phyA* showed a *phyA* epistasy on *ago1*, confirming that the *PHYA*-dependent signaling pathways were upregulated in *ago1* mutants. Double mutants with other mutants affected in light perception or signaling are currently being analyzed to determine the relative contribution of the different pathways to the regulation of adventitious rooting.

ago1-3 Disrupts Auxin Homeostasis in the Apical Part of the Seedlings

Measurements of the endogenous level of free IAA showed that it was lower in *ago1-3* and *ago1-3 sur2-1*(hyb) entire seedlings than in their respective controls Col-0 and *sur2-1*(hyb). Interestingly, free IAA content was shown to decrease in the apical part of *ago1-3* and *ago1-3 sur2-1*(hyb) after transfer to the light, unlike in the root, where the auxin content was the same as in the respective controls Col-0 and *sur2-1*(hyb). In *ago1-3*, this could be explained by a lower auxin biosynthesis rate compared with that in the wild type. This result indicated that the *AGO1* gene might regulate genes involved in the regulation of auxin

(A) Total RNAs were extracted from hypocotyls of Col-0 and *ago1-3* siblings etiolated in the dark for 2.5 d and then transferred for 48 h into the light. The indicated mRNAs were quantified by real-time quantitative PCR using primers surrounding putative miRNA cleavage sites. *ATHB2* and *ARF7* were used as controls not targeted by miRNAs. Expression for each gene was normalized to that of *ACTIN2*. Error bars indicate SE of two independent biological replicates.

(B) Scheme of the *ARF17 OX* T-DNA insertion line SALK 062511. Black boxes represent CDS, lines represent introns, untranslated regions, or promoters. The positions of the inverted repeat T-DNA insertion and left border primers (Lba1) are indicated. Approximate positions of the genomic primers used in genotyping are indicated with arrows below the transcript.

(C) Relative abundance of *ARF17* transcript in *ARF17 OX* and Col-8 hypocotyls etiolated for 2.5 d and exposed to light for 2 d. Expression for the gene was normalized to that of *ACTIN2*. Quantification was made by real-time quantitative PCR using primers surrounding the putative miRNA cleavage site. Error bars indicate SE of two independent biological replicates.

(D) Relative abundance of *GH3-3*, *GH3-5*, and *GH3-6* transcripts in *ARF17 OX* and Col-8 hypocotyls etiolated for 2.5 d and exposed to light for 2 d. Quantification was performed using semiquantitative RT-PCR as described in Methods. Expression for the genes was normalized to that of *18S* rRNA. Error bars indicate SE of three independent RT-PCR replicates ($P < 0.01$). These experiments were repeated on two independent biological replicates.

(E) Proportion of wild-type and *ARF17 OX* siblings developing one or more adventitious roots after etiolation and transfer to the light for 1 week.

(F) Average number of adventitious roots formed on *ARF17 OX* and Col-8 after etiolation and transfer to the light for 1 week. Error bars indicate SE ($P < 0.01$; $n > 30$). Observations were done on three independent biological replicates.

biosynthesis. Nevertheless, the effect of *ago1-3* on the auxin biosynthesis rate could not be detected in an auxin overproducer background, and no differences in auxin biosynthesis were detected in *ago1-3 sur2-1*(hyb) compared with *sur2-1*(hyb). Therefore, the lower level of free IAA in the apical part of the *ago1-3 sur2-1*(hyb) double mutant in the dark and its decrease after transfer of the seedlings to the light cannot be explained only by the defect in biosynthesis. This suggested that *AGO1* has a more general role in the regulation of auxin homeostasis and might also be needed, directly or indirectly, for the regulation of auxin conjugation and/or catabolism. Indeed, the level of IAA conjugates was reduced in *ago1-3* and *ago1-3 sur2-1*(hyb), indicating that IAA conjugation is downregulated in *ago1-3*. No significant increase in the level of oxoindole-3-acetic acid (one of the primary catabolites) could be detected in *ago1-3 sur2-1*(hyb) compared with *sur2-1*(hyb), suggesting that auxin catabolism might not be affected, although results observed with *ago1-3 sur2-1*(hyb) suggest that further analysis is required. Together, these results allow for the conclusion that *AGO1* regulates, directly or indirectly, genes that control different aspects of auxin homeostasis in Arabidopsis.

ARF17 and *GH3* Might Control Adventitious Rooting by Modulating IAA Homeostasis in a Light-Dependent Manner

Expression of auxin-inducible genes was analyzed in the *ago1-3* mutant. No significant difference between the wild type and *ago1-3* was detected, except for the *GH3*:*uidA* fusion. Unlike in the wild type, this reporter gene was not induced in the *ago1-3* mutant hypocotyls when the seedlings were grown in the light in the presence of auxin. Nevertheless, when seedlings were first etiolated before transfer to the light, expression of *GH3*:*uidA* was detected in both wild-type and *ago1-3* hypocotyls. However, in *ago1-3*, the expression pattern was different from that in the wild type and restricted to the bottom part of the hypocotyl. Auxin treatment increased the expression of *GH3*:*uidA* in both wild-type and *ago1-3* siblings, but the expression pattern was not modified in the *ago1-3* hypocotyl. This indicated that exogenous NAA could not induce *GH3*:*uidA* expression in the upper part of *ago1-3* hypocotyl. These results suggested a potential deregulation of the expression of endogenous *GH3* genes in *ago1-3*. This hypothesis was confirmed by results that we recently obtained by analyzing, through two-dimensional gel electrophoresis, the protein profile of the mutant hypocotyl (C. Sorin, L. Negroni, T. Balliau, H. Corti, M.P. Jacquemot, M. Daventure, G. Sandberg, M. Zivy, and C. Bellini, unpublished data). The expression of three GH3 proteins, GH3-3 (Staswick et al., 2002), GH3-5 (AtGH3a) (Tanaka et al., 2002), and GH3-6 (DFL1) (Nakazawa et al., 2001), was positively correlated with adventitious root formation, and they accumulated in the hypocotyls of *sur2-1* but not *ago1-3 sur2-1*(hyb) (C. Sorin, L. Negroni, T. Balliau, H. Corti, M.P. Jacquemot, M. Daventure, G. Sandberg, M. Zivy, and C. Bellini, unpublished data). Because the endogenous auxin level in *sur2-1 ago1-3*(hyb) was at least twice the wild-type level, the downregulation of *GH3* genes in an *ago1* background is not strictly related to the auxin content. Interestingly, it has been shown that *GH3*-related proteins can adenylate, in vitro, several phytohormones, such as jasmonate, IAA, and salicylic acid (Staswick et al., 2002). *GH3-3*, *GH3-5*, and *GH3-6* genes are induced by auxin (Hagen and Guilfoyle, 2002), and the encoded proteins belong to subgroup II of GH3 proteins that were reported to adenylate IAA in vitro (Staswick et al., 2002). Staswick et al. (2005) reported that six recombinant GH3 proteins, including the three listed above, could produce, in vitro, auxin conjugates with several amino acids and that a *DFL1*-overexpressing line contained an increased level of IAA-Asp. Based on these observations, we conclude that the reduction in auxin conjugates in an *ago1-3* background is probably attributable to the reduced expression of *GH3* genes. Some *GH3* genes are regulated by both light and auxin (Hsieh et al., 2000; Nakazawa et al., 2001; Tanaka et al., 2002; Takase et al., 2003), suggesting that they could act at the cross talk of auxin and light signaling pathways. Based on our results and those described in the literature, we propose that an abnormal regulation of several *GH3*-related genes in *ago1* mutants alters auxin homeostasis in a light-dependent manner. Here, we also show that among five *ARF*s that are targeted for degradation by a miRNA, only *ARF17* mRNA accumulates to a high level in the hypocotyl of *ago1-3*. *ARF17* belongs to the auxin response factor family that is represented by 23 members in Arabidopsis (Hagen and Guilfoyle, 2002). Because ARF17 lacks the characteristic protein–protein interaction domains present in the majority of ARFs and that are necessary for the interaction with other ARFs and Aux/IAA proteins (Kim et al., 1997; Ulmasov et al., 1999; Guilfoyle and Hagen, 2001), it is unlikely that ARF17 interacts either with other ARFs or with Aux/IAA proteins. ARFs bind auxin response elements present in the promoter of auxin-inducible genes such as *Aux/IAA*, *SAUR*, and *GH3* and either repress or activate their transcription (Tiwari et al., 2003). Although repression activity has not been demonstrated for *ARF17*, its sequence is more closely related to the repressor group of *ARF*s (Tiwari et al., 2003). Therefore, the overexpression of ARF17 in the *ago1-3* mutant could negatively regulate the expression of several *GH3*-related genes. This was confirmed by the characterization of an *ARF17*-overexpressing line, which showed a clear reduction in the expression of *GH3-3*, *GH3-5* (*AtGH3a*), and *GH3-6* (*DFL1*). *ARF17 OX* also produced significantly fewer adventitious roots than did the wild type, confirming the correlation between adventitious rooting and the expression level of *GH3* genes observed in our proteomic experiments (C. Sorin, L. Negroni, T. Balliau, H. Corti, M.P. Jacquemot, M. Daventure, G. Sandberg, M. Zivy, and C. Bellini, unpublished data). Therefore, we conclude that the overexpression of *ARF17* in *ago1* is likely to be at least partially responsible for the defect in adventitious root formation.

Although at a lower level than *ARF17*, *ARF10* also accumulates in *ago1-3*. *ARF10* is also a potential repressor of auxin-inducible genes, and it is phylogenetically related to *ARF17* and *ARF16* (Okushima et al., 2005). Thus, it will be interesting to analyze whether its accumulation also contributes to the auxin-related phenotype conferred by *ago1-3* and what could be the relative contribution of ARF10 and ARF17 in the control of adventitious root development.

Interestingly, it was shown recently that *ARF8*, which belongs to the activator group of *ARF*s, could positively regulate the

expression of three *GH3* genes, including *GH3-5* and *GH3-6*, and in this way possibly modulate auxin homeostasis (Tian et al., 2004). Although *ARF8* is targeted by a miRNA, its expression is not modified in the hypocotyl of *ago1-3*. We suggest that *ARF17* is likely to compete with *ARF8* in the regulation of *GH3* gene expression.

We have demonstrated that the defect in adventitious rooting of *ago1* mutants correlates with a perturbation of auxin homeostasis in the apical part of the seedling and the upregulation of light signaling pathways. Because *ago1-3* is affected in biosynthesis and conjugation and potentially in degradation processes, we conclude that *AGO1* is required for the proper regulation of auxin homeostasis in the apical part of the plant. We have also shown that expression of several *GH3* genes is likely to be repressed by the auxin response factor *ARF17* in the hypocotyl of the *ago1-3*, where the defect in auxin homeostasis was observed. Therefore, we suggest that *ARF17* and *GH3* could be involved in the control of adventitious root formation. Because several *GH3* genes were shown to act in the cross talk between light and auxin signaling pathways, we suggest that *ARF17* could be a major regulator of adventitious root formation by repressing *GH3* genes and therefore modulating auxin homeostasis in a light-dependent manner.

METHODS

Plant Material and Growth Conditions

The origins of the different *Arabidopsis thaliana ago1* mutants are described in the supplemental data online. *sur1-3*, *sur2-1*, and *sur2-3* mutants have been described (Boerjan et al., 1995; Delarue et al., 1998; Barlier et al., 2000). A SALK insertion line (ecotype Col-8) with a T-DNA insertion in the promoter region of the *ARF17* gene was obtained from the Nottingham Arabidopsis Stock Centre (reference number SALK 062511). Seeds from the *phyA-211* mutant were obtained through the Nottingham Arabidopsis Stock Centre (reference number N6223). The *GH3*:*uidA* construct, which contains the promoter of the soybean *GH3* gene (Hagen et al., 1991), was a gift from T. Guilfoyle (University of Missouri, Columbia, MO). The production of Arabidopsis transgenic lines was described by Delarue (1996). The *SAUR-AC1*:*uidA*- (Gil and Green, 1996), *IAA2*:*uidA*-, and *DR5*:*uidA*-expressing lines were provided by P. Gil (Michigan University, East Lansing, MI), A. Marchant (UPSC, Sweden), and B. Scheres (Utrecht University, The Netherlands), respectively. The *CycB1*:*uidA*-expressing line was provided by D. Inzé (VIB, Gent, Belgium). Heterozygous plants segregating the *ago1-3* mutation were crossed with the different transgenic lines. Wild-type and *ago1-3* seedlings expressing the marker genes were selected in F2 progeny.

Double mutants were obtained by crossing either heterozygous plants for *ago1-3* or *ago1-32* alleles or homozygous *ago1-33*, *ago1-34*, or *ago1-35* plants with heterozygous *sur1-3* (Col-0) or homozygous *sur2-1* (Ws), *sur2-3* (Col-0), or *phyA-211* (Col-0). Double mutants were selected in the F3 generation. Because *ago1-3* and *sur2-1* are in two different ecotypes, the double mutant *ago1-3 sur2-1*, the background of which is a mix of Ws and Col-0, was always compared with its siblings, which we called *sur2-1*(hyb) to avoid confusion.

For seed production and crosses, the plants were grown in a greenhouse. Seeds were sown on soil, and seedlings were transferred into individual pots 10 d after germination. Plants were grown under 16 h of light with 10 to 15°C night temperature and 20 to 25°C day temperature.

For in vitro culture, seedlings were grown as described previously (Santoni et al., 1994). The conditions in the controlled-environment chambers were as follows: 150 $\mu E \cdot m^{-2} \cdot s^{-1}$ irradiance provided by BIOLUX fluorescent tubes, 16 h of light, 60% relative humidity, 15°C night temperature, and 20°C day temperature. 1-NAA was dissolved in ethanol. Picloram was dissolved in DMSO.

For dark growth conditions, Petri dishes were wrapped with four layers of aluminum foil and placed vertically.

For experiments in different light qualities, the plates were placed under continuous irradiance at a constant temperature of 20°C. Blue light (460 nm, 3.7 $\mu E \cdot m^{-2} \cdot s^{-1}$) was provided by TLD tubes 36W/18 (Philips, Montpellier, France) filtered by a Plexiglas layer (blauw number 627; Rohm and Haas, Philadelphia, PA). Red light (660 nm, 9 $\mu E \cdot m^{-2} \cdot s^{-1}$) was provided by light-emitting diodes (NLS01 number 9600; Nijssen, Utrecht, The Netherlands). Far-red light (700 to 760 nm, 0.25 $\mu E \cdot m^{-2} \cdot s^{-1}$) was provided by True-Lite fluorescent tubes (Bio-Elektrik, Leinburg, Germany) filtered by a layer of Plexiglas (Rohm and Haas).

Histochemical GUS Assays

Histochemical assays for GUS expression were performed as described previously (Mollier et al., 1995).

Hypocotyl and Root Measurements

Hypocotyl and root measurements were performed as described by Gendreau et al. (1997). At least 30 seedlings were used for each data point. All measurements were done on two independent biological replicates. Error bars in the figures indicate SD.

Scoring of Adventitious Roots

Seedlings were etiolated in the dark for 2.5 d and then transferred to the light. Emergent adventitious roots were scored at 7 d after transfer to the light. At least 40 seedlings were used for each data point. This was repeated on three independent biological replicates.

Adventitious root primordia and emerging adventitious roots were scored in *sur2-1 ago1-3*(hyb) and *sur2-1* expressing the *CycB1*:*uidA* reporter gene. *GUS* staining was performed before counting blue dots (very early primordia) and emerging roots on the hypocotyls using a stereomicroscope. For each data point, 30 seedlings were analyzed. The experiment was repeated three times for time point 0 (i.e., seedlings grown in the dark only).

Molecular Characterization of the *arf17* Insertion Line

The line SALK 062511 (Alonso et al., 2003) carries a T-DNA insertion ~200 bp 5′ of the CDS and ~100 bp upstream of the start of transcription. The flanking sequence tag for this line (http://signal.salk.edu/cgi-bin/tdnaexpress) runs upstream into the promoter. PCRs of genomic DNA performed using the SALK T-DNA primer Lba1 (http://signal.salk.edu/tdnaprimers.html) in combination with the ARF17 genomic primers ARF17-1S-LF (5′-CGAGGGATAAGCACAAAAACATGA-3′) and ARF17-1S-RR (5′-CCGTTGTTAGCAAGTGACGCC-3′) indicated the presence of two T-DNAs as inverted repeats. Sequencing across the left border toward ARF17-1S-RR confirmed the insertion point and the presence of the inverted repeat (Figure 10B). The pROK vector used to generate SALK lines (Alonso et al., 2003) carries the *Cauliflower mosaic virus* 35S promoter oriented toward the left border. The presence of the intact 35S promoter was confirmed by sequencing.

PCR Experiments

Hypocotyls were collected from mutant and wild-type siblings segregating from heterozygous parents that had been grown in the dark for 2.5 d, then in the light for 2 d. Two independent biological replicate experiments

were performed. RNA was extracted from approximately 50 mg of hypocotyls using the RNAqueous kit (Bio-Rad, Hercules, CA). cDNAs were made using the iScript kit (Bio-Rad).

Real-time RT-PCR template quantification was performed using a Bio-Rad iCycler with iQ SYBR Green Supermix (Bio-Rad). This followed a two-step protocol: 95°C for 3 min, followed by 50 cycles of denaturation at 95°C for 30 s and annealing/extension at 60°C for 45 s. Melt curves were derived after each amplification by increasing the temperature in 0.5°C increments from 55 to 95°C. Reactions were performed in triplicate for each sample. Acceptable standard curves were those for which $1 \geq E \geq 0.85$ and $r^2 \geq 0.985$, where E is the PCR efficiency and r^2 is the correlation coefficient obtained with the standard curve.

Primers for At3g18780/*ACTIN2*, At5g37020/*ARF8*, and At1g77850/*ARF17* are given by Vazquez et al. (2004). Other primers were as follows: At1g30330/*ARF6* (5′-TGCGAAGCGAGCTTGCTC-3′ and 5′-GCTCACAAACTCCGGCCAAG-3′); At5g20730/*ARF7* (5′-AGAACTCAATCTTTTGGTGTC-3′ and 5′-CGTTTTTGCACCTTTGTATAAG-3′); At2g28350/*ARF10* (5′-ACAATGGCGGTGGCGAGTC-3′ and 5′-GATGGTGATCCGAAGAGTTGTTGAG-3′); At4g30080/*ARF16* (5′-AAGCCCGTTAAGCTCTGTTC-3′ and 5′-GGAGGAGGAGGTGGTCTATTC-3′); and At4g16780/*ATHB2* (5′-ACATGAGCCCACCCACTAC-3′ and 5′-GAAGAGCGTCAAAAGTCAAGC-3′). Quantification of each gene was normalized to *ACTIN2*.

Semiquantitative RT-PCR was performed using as internal standard the 18S rRNA primers/competimers (Ambion, Austin, TX). PCR amplification was performed using Taq DNA polymerase (New England Biolabs, Beverly, MA). For each gene-specific primer pair, the optimum annealing temperature and linear amplification range were predetermined using 0.012 μg of RNA per 10 μL of PCR assay. The sequences of the gene-specific primers used for RT-PCR were as follows: At2g23170/*GH3-3* (5′-AAGTTTGTGCGGAGGAAGAA-3′ and 5′-AAAGCGGGCTGAAGTGTGT-3′); At4g27260/*GH3-5* (5′-AATGCCAACAATCGAAGAGG-3′ and 5′-CTTGCACTCAAATTCCACGA-3′); and At5g54510/*GH3-6* (5′-CCTATGCTGGGCTTTACAGG-3′ and 5′-ACCAGGGGACCATTTAGGAC-3′). The PCR products were resolved on a 1.5% agarose gel and stained with ethidium bromide. The PCR products were then quantified by scanning densitometry. A *t* test was performed according to http://graphpad.com/quickcalcs/ttest1.cfm.

Analysis of Endogenous IAA Content

Seedlings were grown in the dark for 2.5 d and then transferred to the light for 24, 48, or 72 h. Entire seedlings, the apical part (cotyledons plus hypocotyl), or roots from several seedlings were pooled to obtain an average of 15 mg of fresh weight.

Samples were extracted, purified, and analyzed by gas chromatography–selected reaction monitoring–mass spectrometry as described previously (Edlund et al., 1995). Calculation of the isotopic dilution factors was based on the addition of 50 pg [$^{13}C_6$]IAA/mg tissue. A *t* test was performed according to http://graphpad.com/quickcalcs/ttest1.cfm.

Quantitative Analysis of IAA Conjugates and Catabolites

Seedlings were grown in the dark for 2.5 d. Entire seedlings were pooled to obtain 30 to 70 mg of fresh weight. Samples were extracted, purified, and analyzed by liquid chromatography–multiple reaction monitoring–mass spectrometry as described previously (Kowalczyk and Sandberg, 2001). A *t* test was performed according to http://graphpad.com/quickcalcs/ttest1.cfm.

Deuterium-Feeding Experiments

Seedlings were grown in the dark for 2.5 d and then transferred to the light for 48 h. Then, entire seedlings, apical parts, or roots were transferred to liquid culture medium (Santoni et al., 1994) with or without 30% deuterated water for 20 h. The samples were then weighed and frozen in liquid nitrogen. The extraction and purification were performed as described previously (Ljung et al., 2001). Calculation of isotopic dilution was based on the addition of 50 pg [$^{13}C_6$]IAA/mg tissue. Analysis by gas chromatography–high-resolution mass spectrometry was done at a resolution of at least 10,000, and isotopomers of the base peaks of methylated and trimethylsilylated IAA were measured with mass-to-charge ratios of 202.105, 203.112, 204.118, 205.124, and 208.125. Corrections were incorporated for the contribution of natural isotopic abundances to mass-to-charge ratios of 203 to 205. The incorporation of deuterium into the IAA molecule was then calculated. Corrections for background were made by analyzing the samples from the control plants grown without deuterated water. A *t* test was performed according to http://graphpad.com/quickcalcs/ttest1.cfm.

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NOTE ADDED IN PROOF

The word hybrid is misused in this article. A hybrid is an F1 product. The double mutant *sur2-1 ago1-3* is indeed in a recombinant genotype between Col-0 and Ws. Hybrid should be understood here as recombinant genotype.