Proteomic Analysis of Different Mutant Genotypes of Arabidopsis Led to the Identification of 11 Proteins Correlating with Adventitious Root Development[1][W]

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A lack of competence to form adventitious roots by cuttings or explants in vitro occurs routinely and is an obstacle for the clonal propagation and rapid fixation of elite genotypes. Adventitious rooting is known to be a quantitative genetic trait. We performed a proteomic analysis of Arabidopsis (*Arabidopsis thaliana*) mutants affected in their ability to develop adventitious roots in order to identify associated molecular markers that could be used to select genotypes for their rooting ability and/or to get further insight into the molecular mechanisms controlling adventitious rooting. Comparison of two-dimensional gel electrophoresis protein profiles resulted in the identification of 11 proteins whose abundance could be either positively or negatively correlated with endogenous auxin content, the number of adventitious root primordia, and/or the number of mature adventitious roots. One protein was negatively correlated only to the number of root primordia and two were negatively correlated to the number of mature adventitious roots. Two putative chaperone proteins were positively correlated only to the number of primordia, and, interestingly, three auxin-inducible GH3-like proteins were positively correlated with the number of mature adventitious roots. The others were correlated with more than one parameter. The 11 proteins are predicted to be involved in different biological processes, including the regulation of auxin homeostasis and light-associated metabolic pathways. The results identify regulatory pathways associated with adventitious root formation and represent valuable markers that might be used for the future identification of genotypes with better rooting abilities.

In dicotyledonous plants, adventitious roots can be defined as roots that develop from organs such as leaves and stems under unusual circumstances. Adventitious root formation is a key step in vegetative propagation of woody or horticultural species, and problems associated with rooting of cuttings frequently result in significant economic losses (De Klerk et al., 1999). It is a complex process known to be affected by factors such as phytohormone levels, phenolic compounds, light, or wounding. One of the major endogenous factors known to play a role in adventitious rooting is the hormone auxin. Auxins have been shown to be effective inducers of adventitious roots in many woody species (Selby et al., 1992; Díaz-Sala et al., 1996; Goldfarb et al., 1998; De Klerk et al., 1999). Light is one of the major environmental factors known to influence adventitious root development, and light and auxin act antagonistically in adventitious root development in *Eucalyptus saligna* and *Eucalyptus globulus* (Fett-Neto et al., 2001). Niemi et al. (2005) recently showed that light quality could affect adventitious root and mycorrhiza formation in Scots pine (*Pinus sylvestris*) in vitro.

While the physiology of adventitious root formation is reasonably well known, the genetic and molecular mechanisms involved are still poorly understood. The few genetic and molecular studies of adventitious rooting that have been undertaken have shown that it is a heritable character, and quantitative trait loci analyses of adventitious rooting in trees (Han et al., 1994; Marques et al., 1999) and in Arabidopsis (*Arabidopsis thaliana*; King and Stimart, 1998) showed that it

[1] This work was supported by the Institut National de la Recherche Agronomique (C.B. and M.Z.) and the Swedish Foundation for Strategic Research (C.B.). C.S. received a Ph.D. fellowship from the Ministère de la Recherche et de l'Enseignement Supérieur and from the Swedish Foundation for Strategic Research.

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[W] The online version of this article contains Web-only data.

Article, publication date, and citation information can be found at www.plantphysiol.org/cgi/doi/10.1104/pp.105.067868.

is a quantitative genetic trait as expected for a character controlled by multiple factors. Recently, Brinker et al. (2004) investigated gene expression during adventitious root formation in *Pinus contorta* hypocotyls pulse treated with auxin and harvested at different developmental time points of root development. More than 200 genes were differentially expressed during the different phases analyzed. These genes were related to auxin transport, photosynthesis, cell replication, or cell wall synthesis (Brinker et al., 2004). In Arabidopsis, nine temperature-sensitive mutants have been isolated that are altered in different stages of adventitious root formation from hypocotyl explants (Konishi and Sugiyama, 2003). One of the mutations has been identified. It is in the *MOR1/GEM1* gene that encodes a microtubule-associated protein, suggesting that microtubule organization is important during root meristem initiation. Most model systems have compared explants treated with exogenous auxin with non-auxin-treated material, making it difficult to distinguish events specifically associated with adventitious rooting from those resulting from exogenous auxin application. We took advantage of two classes of Arabidopsis mutants affected in adventitious root formation for identifying molecular markers associated with the initiation and developmental phases of adventitious rooting. The *superroot1* (*sur1*) and *superroot2* (*sur2*) mutants are auxin overproducers that spontaneously develop adventitious roots on the hypocotyl (Boerjan et al., 1995; Delarue et al., 1998). The *superroot* genes have been identified, and both were shown to encode enzymes of the indole-glucosinolate pathway (Bak et al., 2001; Mikkelsen et al., 2004). *SUR2* encodes CYP83B1 (Barlier et al., 2000), which converts indole-3-acetaldoxime in 1-aci-nitro-2 indolyl-ethane, and *SUR1* encodes the C-S lyase that acts one step later, catalyzing the conversion of *S*-alkylthiohydroximate to thiohydroximate (Bak et al., 2001; Mikkelsen et al., 2004). The blockage of either of these steps results in the redirection of indole-3-acetaldoxime toward indole-3-acetic acid (IAA) biosynthesis (Bak et al., 2001; Mikkelsen et al., 2004).

The *argonaute1* (*ago1*) mutant was first characterized as a leaf developmental mutant (Bohmert et al., 1998). We showed recently that both the null allele *ago1-3* and hypomorphic alleles were altered in their ability to produce adventitious roots (Sorin et al., 2005). *AGO1* is the founding member of a gene family that is conserved among eukaryotes (Bohmert et al., 1998) and has been shown to be involved in the regulation of posttranscriptional gene silencing (Fagard et al., 2000; Morel et al., 2002). More recently, the *AGO1* gene was shown to play a crucial role in the regulation of gene expression through the microRNA (miRNA) pathway, where it is potentially required for miRNA-directed mRNA cleavage (Vaucheret et al., 2004). *ago1* has a pleiotropic developmental phenotype, some aspects of which can be explained by the deregulation of transcription factors targeted by miRNAs (Jones-Rhoades and Bartel, 2004). We recently showed that the defect in adventitious rooting in *ago1* mutants can at least partially be explained by the accumulation of the auxin response factor *ARF17* (Sorin et al., 2005), whose expression is regulated by the miRNA *mir160* (Jones-Rhoades and Bartel, 2004; Mallory et al., 2005). A transgenic line overexpressing *ARF17* was shown to produce less adventitious roots than the wild type, suggesting that *ARF17* could be a major regulator of adventitious rooting. To identify other genes involved in adventitious root development, we coupled genetic and physiological analysis with a proteomic characterization of different single and double mutants.

This approach has already been successfully used for the characterization of Arabidopsis developmental mutants (Santoni et al., 1994, 1997) and the identification of proteins correlated to a developmental process. The first characterization of two-dimensional (2-D) protein patterns of Arabidopsis mutants led to the identification of proteins positively correlated with hypocotyl elongation, one of which was actin (Santoni et al., 1994). Biochemical distance indices between Arabidopsis mutants could be estimated from 2-D electrophoresis data and correlated with phenotypic and physiological analysis of the mutants (Santoni et al., 1997). More recently, proteome analysis has become a major approach for functional characterization of plants (for review, see Canovas et al., 2004). It has proven useful for the identification of proteins associated with a range of developmental or physiological processes (de Vienne et al., 1999; Plomion et al., 2000; Bae et al., 2003; Gallardo et al., 2003; Mang et al., 2004; Schiltz et al., 2004; Vincent et al., 2005).

In this article, we describe the analysis and comparison of 2-D protein profiles of hypocotyls of *ago1-3*, *sur1-3*, *sur2-1*, and *sur2-1ago1-3* Arabidopsis mutants during initiation of adventitious roots. This showed that, although *AGO1* is involved in the regulation of gene expression through the miRNA pathway, its mutation does not induce more variations in hypocotyls than a mutation in *SUR1* or *SUR2*. We also showed significant differences between the *sur1* and *sur2* protein profiles, although the two genes act in the same biosynthesis pathway. Finally, we identified 11 proteins, including three auxin-inducible GH3-like proteins, whose content correlated either positively or negatively with early and/or late phases of adventitious root development.

RESULTS

Analysis of 2-D Protein Patterns of the Different Genotypes

This work describes proteomic variations between the *ago1-3*, *sur1-3*, and *sur2-1* Arabidopsis mutants and the *sur2-1ago1-3* double mutant, with particular reference to the early developmental events associated with adventitious root initiation in the hypocotyl. All four genotypes behave differently in terms of adventitious root development. The auxin overproducers *sur1-3*

and *sur2-1* spontaneously develop adventitious roots on the hypocotyl either in the light (Boerjan et al., 1995; Delarue et al., 1998) or when etiolated prior to transfer to the light (Sorin et al., 2005). In contrast, the *ago1-3* mutant and the *sur2-1ago1-3* double mutant were unable or barely able to produce adventitious roots when grown directly in the light or after being etiolated prior to transfer to the light (Sorin et al., 2005).

Seeds were germinated in the dark and young seedlings were etiolated until they were about 5 mm and then transferred to the light for 48 h, as described previously (Sorin et al., 2005). Although wild-type, *ago1*, and *superroot* mutants have very different adult phenotypes, they are phenotypically almost identical to each other at this developmental stage, that is, before any adventitious root primordia have emerged from the hypocotyls of wild-type or *superroot* mutants (Sorin et al., 2005). Microscopic observation of cleared hypocotyls showed that, in all genotypes, adventitious roots initiated from pericycle cells opposite to xylem poles, as described previously (Boerjan et al., 1995; Delarue et al., 1998; Takahashi et al., 2003; Sorin et al., 2005). Nevertheless, a clear difference in the number of very early root primordia could be observed between the genotypes as shown by the early differential expression of the *CyclinB1*:*uidA* reporter gene that was used to monitor adventitious root initiation in *sur2-1* mutants and *sur2-1ago1-3* double mutants (Sorin et al., 2005). This developmental stage was therefore suitable for protein profiling. Soluble proteins were prepared from dissected hypocotyls and subjected to 2-D PAGE (Fig. 1) as described in ''Materials and Methods.'' The proteomic data were then analyzed for correlation of the abundance of individual proteins with the number of adventitious roots and/or the endogenous auxin content among the different genotypes. In this way, genes whose expression was correlated to adventitious root formation could be identified.

We first estimated the number of proteins that varied significantly between genotypes (Table I). After spot detection, 2-D gels were aligned and matched to a reference gel created by Progenesis software, where 1,147 reproducible spots were identified. Out of a total of 1,147 spots analyzed, a similar percentage was significantly affected by the *sur1-3* or the *sur2-1* mutation, 6.6% in *sur1-3* (Columbia [Col]-0) and 7.4% in *sur2-1* (Wassilewskija [Ws]), compared to their corresponding wild types, Col-0 and Ws, respectively.

As the *sur1-3* and *sur2-1* mutants are in two different genetic backgrounds (Col-0 and Ws, respectively), spots displaying significant ecotype variation were removed prior to comparison of the 2-D profiles (Table I). Nineteen spots were shown to be variable in both *sur1-3* and *sur2-1*, which represents 32% and 31% of the variable spots in *sur1-3* and *sur2-1*, respectively. Analysis of the *ago1-3* (Col-0) versus wild-type (Col-0) 2-D profiles showed that 7.1% of the spots were affected by the mutation (Table I). Thus, although the *AGO1* gene is involved in the regulation of many genes, including various transcription factors, the variability observed in the 2-D profile of soluble proteins of the *ago1-3* hypocotyl remained in the same range as that observed in the hypocotyls of the two *superroot* mutants.

We also compared variations observed in *ago1-3* versus wild type (Col-0) to those in *sur1-3* versus wild type (Col-0) and in *sur2-1* versus wild type (Ws) after removal of the spots variable between the two wild-type ecotypes Ws and Col-0 (Table I). Interestingly, we could see that 37 spots were commonly affected by the *ago1-3* and *sur1-3* mutations, which represents on average 50% of the variable spots in *ago1-3* and *sur1-3* compared to their wild types. Three spots were absent in the wild type (Col-0) and appeared in both *sur1-3* and *ago1-3*, and one spot varied in opposite ways in *sur1-3* and *ago1-3*. In the comparison between *ago1-3*/wild type (Col-0) and *sur2-1*/wild type (Ws), 14 spots were commonly affected by *sur2-1* and *ago1-3* mutations. This indicates that there are more than 50% less common variable spots in *ago1-3*/wild type (Col-0) and *sur2-1*/wild type (Ws) than in *ago1-3*/wild type (Col-0) and *sur1-3*/wild type (Col-0). Three of them varied in opposite ways in *sur2-1* and *ago1-3* compared to their respective wild types.

The *sur2-1ago1-3* double mutant derived from a cross between a homozygote *sur2-1* plant in a Ws ecotype and a plant heterozygote for the *ago1-3* mutation in a Col-0 background. This recombinant genetic background will be referred to as recombinant genotype (RG) in the remainder of the article. The characterization of the double mutant *sur2-1ago1-3* (RG) showed that they produced more auxin than the wild type and the single *ago1-3* mutant, but less than *sur2-1* (RG) and developed no, or very few, adventitious roots (Table II; Sorin et al., 2005). We thus investigated the protein variation in the double mutant *sur2-1ago1-3* (RG). The 2-D protein pattern of *sur2-1ago1-3* (RG) was compared to that of siblings homozygous for *sur2-1* in the same genetic background (i.e. RG). Quantitative and qualitative variations are summarized in Table I. Only 4% of the spots were shown to be variable between *sur2-1ago1-3* (RG) and *sur2-1* (RG), although 7.4% were variable in *sur2-1* (Ws) compared to Ws and 7.1% in a*go1-3* (Col-0) compared to Col-0. A possible reason is that the *ago1* mutation has no effect on the level of accumulation of several proteins in a *sur2-1* background, whereas it has in a wild-type background. After elimination of spots significantly variable between genetic backgrounds, seven were shown to be variable only in the *sur2-1ago1-3* (RG) versus *sur2-1* (RG) comparison and neither in the *ago1-3* (Col-0) versus wild-type (Col-0) comparison nor in the *sur2-1* (Ws) versus Ws comparison. These variations reflect the effects of the *ago1-3* mutation that can only be seen in a *sur2-1* genetic background. Nine other spots were significantly affected by the *ago1-3* mutation in a wild-type background and a *sur2-1* background. This result fits with the previously observed phenotypic effect of *ago1* in a *sur2-1* background. Indeed, the double mutant *sur2-1ago1-3* displays an *ago1-3*-like phenotype

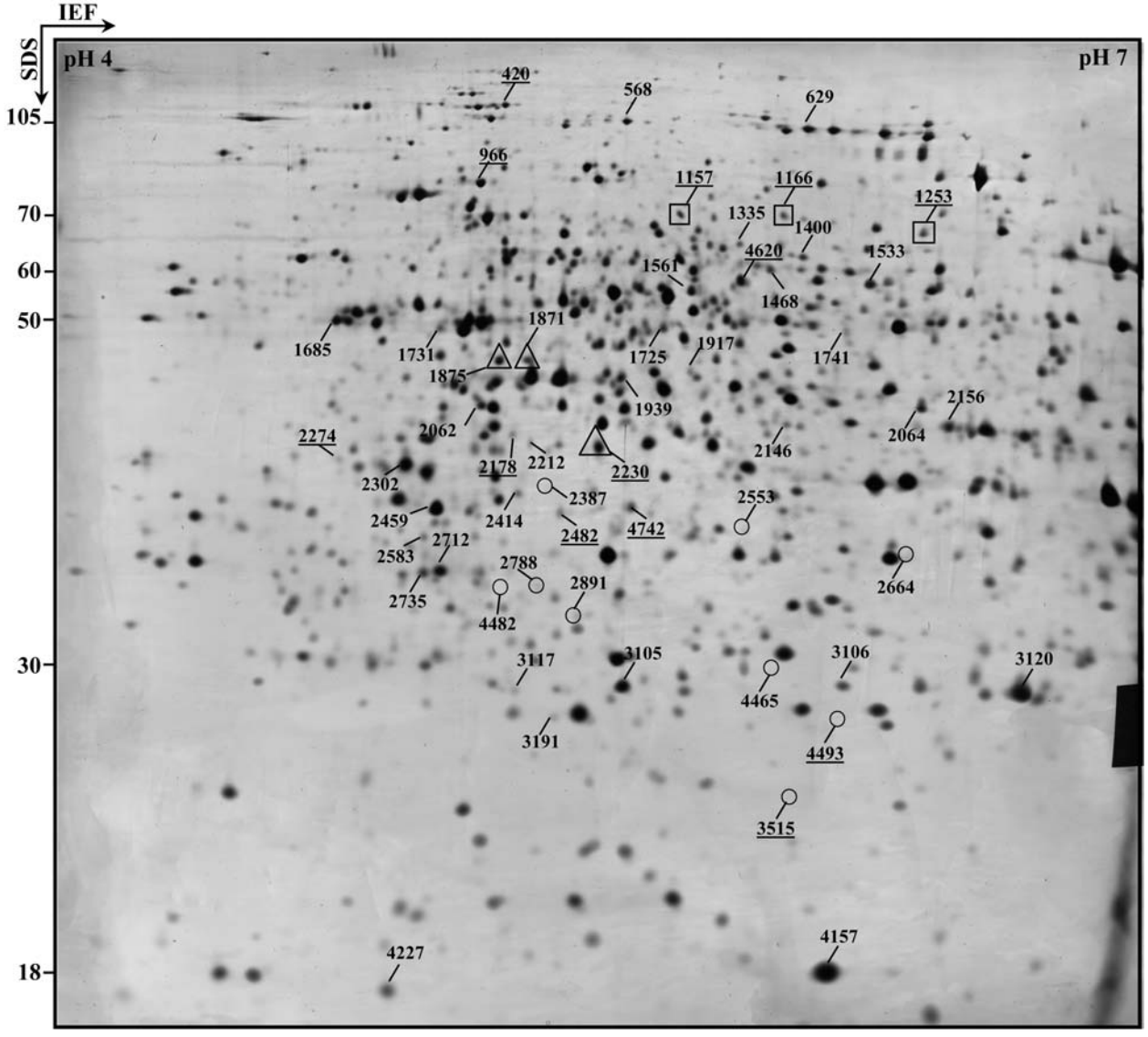

Figure 1. Representative 2-D gel pattern of hypocotyl proteins from Arabidopsis seedlings etiolated and transferred to light for 48 h. A typical gel of the genotype *sur2-1* (RG) is presented as a representative 2-D gel. The spots that could be identified are indicated. Rings indicate spots that cannot be seen on this gel. The spots underlined have been correlated to one or more parameters (Table III; Fig. 2) The GH3-like proteins (square boxes) are spots 1,157 (GH3-6/DFL1), 1,166 (GH3-5/AtGH3a), and 1,253 (GH3-3). Spots in triangle, 2,230 is CORI-7/AtST5a; 1,871 and 1,875 are two putative isoforms of the JR1 lectin (At3g16470). Proteins were prepared as described in ''Materials and Methods,'' subjected to 2-D PAGE, and the resulting gels were then silver stained.

in the aerial parts of the plant (Sorin et al., 2005). Thus, groupings of variable spots can be made according to their behavior.

Correlation of Spot Intensity with the Auxin Content and/or the Adventitious Rooting Process

To identify proteins associated with adventitious root formation, we computed Pearson correlations of spot intensities with free IAA content, number of adventitious root primordia 2 d after seedling transfer to light, and number of adventitious roots 7 d after transfer to light. These different parameters are reported in Table II and were defined either based on a previous characterization of the mutants (Sorin et al., 2005) or as described in ''Materials and Methods.''

Because *sur1-3* and *ago1-3* are sterile, the double mutant *sur1-3ago1-3* could only be unambiguously identified in the 2-week-old progeny of double heterozygotes grown in light (Camus, 1999). It was impossible to distinguish the phenotype of the double mutant *sur1-3ago1-3* from the single mutant *ago1-3* at the young stages of development used for proteomic analysis (data not shown). In addition, as a consequence of the strong phenotype of *sur1-3*, it was impossible to determine the number of adventitious

Table I. *Number of proteins variable in pairwise comparisons between wild-type and mutant hypocotyls*

Comparisons were performed between *ago1-3* (Col-0) and its wild-type (Col-0) siblings, *sur1-3* (Col-0) and its wild-type (Col-0) siblings, *sur2-1* (Ws) and its wild-type (Ws) siblings, and *sur2-1ago1-3* (RG) and its *sur2-1* (RG) siblings. Gels were performed with soluble proteins of hypocotyls from seedlings etiolated and transferred to light for 48 h as described in "Materials and Methods" and compared pairwise. The percentage of variable protein spots (number given in parenthesis) was calculated by dividing the number of spots that displayed qualitative or quantitative variations by the average number of total spots (1,147) used for comparisons. For analysis of common variable spots between *sur1-3* (Col-0)/wild type (Col-0) and *sur2-1* (Ws)/wild type (Ws), spots displaying significant ecotype variation (i.e. significantly variable between Col-0 and Ws) were removed from this comparison. Thus, after this correction, 59 remaining spots that were variable in the comparison *sur1-3* (Col-0)/wild type (Col-0) were compared to 62 remaining spots that were variable in the comparison *sur2-1* (Ws)/wild type (Ws). The numbers given in the corresponding column are spots variable in the two genetic backgrounds. The same correction was performed for the comparison *sur2-1* (Ws)/wild type (Ws) versus *ago1-3* (Col-0)/wild type (Col-0). After removal of the ecotype variable spots, 62 spots variable in the comparison *sur2-1* (Ws)/wild type (Ws) were compared to 58 variables in the comparison *ago1-3* (Col-0)/wild type (Col-0).

Comparisons	*sur1-3* (Col-0) versus Wild Type (Col-0)	*sur2-1* (Ws) versus Wild Type (Ws)	*sur1-3* (Col-0)/Wild Type (Col-0) versus *sur2-1* (Ws)/Wild Type (Ws) (59 Spots versus 62 Spots after Subtraction of Ecotype Variations)	*ago1-3* (Col-0) versus Wild Type (Col-0)	*sur2-1ago1-3* (RG) versus *sur2-1* (RG)	*sur1-3* (Col-0)/ Wild Type (Col-0) versus *ago1-3* (Col-0)/Wild Type (Col-0)	*sur2-1* (Ws)/Wild Type (Ws) versus *ago1-3* (Col-0)/Wild Type (Col-0) (62 Spots versus 58 Spots after Subtraction of Ecotype Variations)
Variable spots	76 (6.6%)	85 (7.4%)	19 (1.7%)	82 (7.1%)	46 (4%)	37 (3.2%)	14 (1.2%)
Up-regulated	33	26	8	47	17	21	6
Down-regulated	36	55	11	28	19	12	5
Spots that appeared in the mutant background	7	1	0	6	7[a]	3	0
Spots that disappeared in the mutant background	0	3	0	1	2[b]	0	0
Spots varying in opposite ways			0			1	3

[a]Spots that were present in *sur2-1ago1-3* (RG) but not in *sur2-1* (RG). [b]Spots that were absent in *sur2-1ago1-3* (RG) but not in *sur2-1* (RG).

roots 7 d after transfer to light because there were too many. Therefore, we preferred to eliminate *sur1-3* from the correlation analysis. We are in the process of creating an inducible *sur1* mutant that hopefully should allow easier identification of double mutants and completion of this analysis.

Only the spots that were shown to be significantly variable after variance analysis were used for correlation analysis. Pearson correlation coefficients were computed between the mean of the relative intensity in the six genotypes and the different parameters. The results of the correlations are reported in Table III. Among the variable proteins that were affected by at least one mutation, 18 showed a significant correlation ($P < 0.01$) with at least one parameter. Positively or negatively correlated spots were in approximately the same amount for each parameter.

Identification of Variable Spots and Correlated Spots

From the 192 spots that were significantly variable, only 110 were identifiable on preparative gels stained with SYPRO ruby protein gel stain (see "Materials and Methods") for identification via liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis. Among these 110, several did not give enough protein for proper sequencing or were degraded. After LC-MS/MS analysis, only 50 could be unambiguously identified (Table IV). For several spots, several proteins matched the sequence data and were not reported in Table IV. Nevertheless, they can be found in Supplemental Table I, where detailed sequence data are provided. These data will be made available together with quantitative data on the freely accessible database PROTICdb (Ferry-Dumazet et al., 2005; http://

Table II. *Parameters used in correlation analyses with protein spot intensity*

IAA, Endogenous free IAA levels (pg mg^{-1} fresh weight) were previously measured in the apical part of seedlings, 48 h after transfer to light in wild type (Col-0), wild type (Ws), *ago1-3* (Col-0), *sur2-1* (RG), and *sur2-1ago1-3* (RG) (Sorin et al., 2005). As no significant difference in IAA content between Col-0 and Ws ecotypes was detected and no significant difference in the number of adventitious root primordia or number of roots between *sur2-1* (Ws) and *sur2-1* (RG), we gave the same content to *sur2-1* (Ws) as in *sur2-1* (RG). Three biological replicates were performed ±SD. P-2, Average number of adventitious root primordia in the hypocotyl of seedlings 2 d after transfer to light. Three biological replicates were performed ±SE. Ro-7, Average number of adventitious roots emerging from the hypocotyl 7 d after transfer to light. The experiment was repeated three times ±SD.

	Wild Type (Col-0)	Wild Type (Ws)	*ago1-3* (Col-0)	*sur2-1* (Ws)	*sur2-1* (RG)	*sur2-1ago1-3* (RG)
IAA	17 ± 1.86	15 ± 2.16	9 ± 0.32	59 ± 9.36	59 ± 9.36	29 ± 9.36
P-2	0.36 ± 0.09	0.32 ± 0.08	0.04 ± 0.04	6.03 ± 0.37	6.63 ± 0.35	3.5 ± 0.24
Ro-7	1.82 ± 0.08	1.88 ± 0.09	0.04 ± 0.02	11.36 ± 0.41	11.91 ± 0.47	1.6 ± 0.13

Table III. *Number of protein spots correlated to IAA content, primordia number, and/or adventitious root number parameters*

Correlation analysis was performed with protein spots that were significantly variable in one of the comparisons shown in Table I. Their correlation with at least one of the three parameters was considered to be significant when the absolute value of the Pearson correlation coefficient $|r|$ was ≥ 0.92. When a spot was missing in at least one genotype, the correlation was considered to be significant for $|r| > 0.98$. ns, Not significant.

Spot No.	Locus Accession No.	Arabidopsis Protein Name	IAA	P-48	Ro-7
1,253	At2g23170	GH3-3	ns	ns	0.93
1,166	At4g27260	AtGH3a	ns	ns	0.94
1,157	At5g54510	DFL1	ns	ns	0.95
3,515	At5g20630	Germin-like protein GLP3b	ns	ns	−1.00
4,493	At4g25100	Iron superoxide dismutase	ns	−0.95	ns
2,274	At3g18490	Aspartyl protease/putative chloroplast nucleoid DNA-binding protein	−0.94	ns	−0.98
2,178	At1g32060	Phosphoribulokinase (chloroplast isoform)	−0.98	−0.95	−0.94
4,742	At4g38970	Putative Fru-bis-P aldolase	−0.95	ns	−0.97
966	At5g28540	Luminal binding protein, putative HSP	ns	0.94	ns
420	At1g79930	Putative HSP	ns	0.93	ns
2,230	At1g74100	Putative flavonol sulfotransferase	0.95	0.99	ns
790		Not identified	ns	0.95	ns
4,728		Not identified	ns	ns	−0.99
2,037		Not identified	−0.93	ns	−0.93
2,251		Not identified	−0.94	ns	−0.95
2,351		Not identified	ns	0.99	ns
2,650		Not identified	0.93	ns	0.96
2,885		Not identified	−0.98	−0.95	−0.97

moulon.inra.fr/~bioinfo/PROTICdb), designed for storage and analysis of plant proteome data obtained by 2-D PAGE and MS.

Seventeen proteins were related to metabolism, 10 of which were putatively involved in energy/carbon metabolism and five linked to nitrogen metabolism. Nine proteins were putatively involved in stress responses, four related to oxidative stress, and another four to the glucosinolate metabolism and biosynthesis. Four proteins associated with hormone metabolism were identified as well as proteins involved in protein folding, protein degradation, protein glycosylation, cytoskeleton, cell wall biosynthesis, and few proteins of unknown function.

Four (At3g01500, ATCG00490, At2g21330, and At1g53240) out of the 10 proteins related to energy and carbon metabolism were up-regulated in *ago1-3*, and one (At2g21330) was up-regulated in *sur2-1ago1-3*. At1g31390, a putative fructokinase, was also found up-regulated in *sur2-1ago1-3*, but since the induction level (1.45) was just below the cutoff level of 1.5, it appears in Table IV as nonsignificantly variable. In contrast, five proteins (At1g06680, At1g32060, At2g21330, At2g38970, and At1g31390) of the same category were down-regulated in *sur2-1*.

Twelve proteins were significantly variable in *sur1-3* compared to Col-0 but were not affected by the *sur2-1* mutation, whereas 18 proteins were significantly variable in *sur2-1* compared to Ws but were not affected by the *sur1-3* mutation. Seven proteins were affected by both mutations and were always modified in the same way (i.e. either up-regulated or down-regulated) in both mutant genotypes. At1g62380 and At3g11830 (a putative 1-aminocyclopropane-1-carboxylic acid oxidase and a putative T-complex protein 1 β-subunit, respectively) were also up-regulated in *sur1-3*, but since their induction levels were just below the cutoff level (1.35 and 1.45, respectively), they appear in Table IV as nonsignificantly variable.

Interestingly, two lectins (At3g16450 and At3g16470) similar to myrosinase-binding proteins were identified. One of them (At3g16470) may have been post-translationally modified as it was found in two different locations (see spots 1,871 and 1,875 in Fig. 1). They were both significantly variable in *ago1-3*, but only At3g16450 was significantly down-regulated in *sur2-1ago1-3* (RG). At3g16450 was significantly down-regulated in *sur1-3* compared to Col-0, but no significant variation was observed in *sur2-1* compared to Ws. Both putative At3g16470 isoforms were down-regulated in *ago1-3* compared to Col-0. Interestingly, the isoform corresponding to spot 1,875 was down-regulated in *sur1-3* compared to Col-0 but not in *sur2-1* compared to Ws, and it was the opposite for spot 1,871.

Three different auxin-inducible GH3-like proteins (GH3-3/At2g23170, GH3-5/AtGH3a/At4g27260, and GH3-6/DFL1/At5g54510) were up-regulated in *sur2-1* and down-regulated in *sur2-1ago1-3* (RG; Table IV;

Table IV. *Identification of proteins significantly correlated to IAA content, adventitious root primordia number, and/or adventitious root number parameters*

Arbitrary cutoff levels of 1.5 and 0.66 were considered to determine up- or down-regulation. For each comparison, the factor of induction is indicated when the variation is significant, otherwise a nonsignificant variation is indicated. EIP, Experimental pI; TIP, theoretical pI; EMM, experimental molecular mass; TMM, theoretical molecular mass; SAM, *S*-adenosylmethionine-dependent methyltransferase; ACC, 1-aminocyclopropane-1-carboxylic acid; ns, not significant.

Spot No.	Arabidopsis Genome Initiative Locus Name	Protein Name	EIP	TIP	EMM	TMM	*ago1-3* (Col-0) versus Wild Type (Col-0)	*sur1-3* (Col-0) versus Wild Type (Col-0)	*sur2-1ago1-3* (RG) versus *sur2-1* (RG)	*sur2-1* (Ws) versus Wild Type (Ws)
					kD	*kD*				
Hormone related										
1,253	At2g23170	GH3-3	5.99	5.98	67.05	67.54	ns	ns	0.15	3.6
1,166	At4g27260	GH3-5/AtGH3a	5.77	5.72	70.01	69.28	ns	ns	0.26	6.7
1,157	At5g54510	GH3-6/DFL1	5.61	5.53	70.41	68.94	ns	3.12	0.24	3.14
2,302	At1g62380	Putative ACC oxidase	5.18	4.98	39.92	36.18	ns	ns	ns	1.63
Metabolism										
Energy and C metabolism										
4,465	At3g01500	Carbonic anhydrase (chloroplast isoform)	5.76	6.14	26.39	25.57[a]	3.11	ns	ns	ns
3,191	At1g06680	Oxygen evolving complex 23	5.42	5.27	24.07	20.21[a]	ns	ns	ns	0.15
1,741	ATCG00490	Rubisco (large subunit)	5.87	5.88	52.69	52.74[a]	2.97	2.97	ns	ns
2,178	At1g32060	Phosphoribulokinase (chloroplast isoform)	5.35	5.16	42.53	39.20[a]	ns	ns	ns	0.41
2,482	At2g21330	Putative Fru-bis-P aldolase	5.42	6.48	36.28	42.89	1.55	ns	2.07	0.34
4,742	At4g38970	Putative Fru-bis-P aldolase	5.53	6.79	36.72	42.99	ns	ns	ns	0.43
2,414	At1g31390	Putative fructokinase	5.36	5.30	37.65	35.26	ns	ns	ns	0.56
1,561	At2g36530	Enolase 2-phospho-D-glycerate hydrolase	5.63	5.54	57.87	47.72	0.5	ns	ns	ns
4,482	At1g53580	Putative glyoxalase II	5.34	5.43	31.13	25.07	0.52	0.44	ns	ns
2,664	At1g53240	NAD-dependent malate dehydrogenase	5.97	6.00	33.23	35.29[a]	1.6	ns	ns	ns
N assimilation and metabolism										
1,335	At2g15620	Ferredoxin nitrite reductase	5.33	5.68	68.31	62.81[a]	ns	ns	ns	2.42
2,064	At5g07440	GDH2 (glutamate dehydrogenase)	5.99	6.07	45.02	44.7	2.74	2.63	ns	ns
629	At2g05710	Aconitate hydratase (cytosolic isoform)	5.81	6.72	93.14	108.2	ns	ns	0.45	ns
2,146	At3g45300	Isovaleryl-CoA dehydrogenase precursor (IVD)	5.78	5.6[a]	43.32	42.24[a]	1.76	ns	2.2	0.46
2,459	At1g79230	Mercaptopyruvate sulfur transferase	5.23	5.95	36.66	41.89	ns	ns	ns	1.53
Others										
2,788	At5g54770	Thiazole biosynthetic enzyme, chloroplast precursor	5.39	5.82	31.35	36.66	ns	0.55	ns	0.31
2,062[b]	At3g09820 At5g03300	Adenosine kinase	5.29	5.29 5.14	45.05	37.84 37.85	ns	ns	1.83	ns
Stress related										
Oxidative stress										
2,553	At5g16970	Quinone oxydoreductase-like protein	5.72	5.81	35.15	38.13	0.65	0.42	ns	0.45
3,105	At1g19550	Putative GSH-dependent dehydroascorbate reductase1	5.52	5.58	25.66	23.64	0.45	0.65	ns	ns

(*Table continues on following page.*)

Table IV. (*Continued from previous page.*)

Spot No.	Arabidopsis Genome Initiative Locus Name	Protein Name	EIP	TIP	EMM	TMM	*ago1-3* (Col-0) versus Wild Type (Col-0)	*sur1-3* (Col-0) versus Wild Type (Col-0)	*sur2-1ago1-3* (RG) versus *sur2-1* (RG)	*sur2-1* (Ws) versus Wild Type (Ws)
3,120	At1g78380	GST-like	6.14	5.8	25.45	25.65	ns	ns	ns	3.06
4,493	At4g25100	Iron superoxide dismutase	5.86	6.06	23.79	23.79	ns	ns	0.38	0.2
Related to glucosinolate biosynthesis and metabolism										
2,712	At3g16450	Putative lectin (Jacalin family)	5.24	5.06	32.33	32.02	0.3	0.63	0.3	ns
1,875	At3g16470	JR1 lectin (Jacalin family)	5.33	5.12	49.56	48.5	0.5	0.64	ns	ns
1,871	At3g16470	JR1 lectin (Jacalin family)	5.37	5.12	49.73	48.5	0.29	ns	ns	0.61
2,230	At1g74100	CORI-7/AtST5a	5.48	5.42	41.29	39.22	ns	1.73	ns	1.81
Others										
3,515	At5g20630	Germin-like protein GLP3b	5.79	5.84[a]	19.82	21.01[a]	10.05	ns	ns	ns
Protein degradation										
3,106	At3g27430	20S proteasome β1 subunit	5.87	6.66	25.66	29.56	5.36	ns	ns	5.05
2,583	At5g42790	20S proteasome PAF1 subunit	5.21	4.99	34.65	30.48	ns	ns	1.54	ns
1,468	At3g14067	Putative cucumisin-like Ser protease (subtilase family)	5.75	6.29	60.88	81.82	1.97	2.21	ns	ns
568	At1g63770	Putative aminopeptidase (family of M1 peptidase)	5.53	5.43	97.15	99.16	1.93	2.82	ns	ns
2,274	At3g18490	Aspartyl protease/putative chloroplast nucleoid DNA-binding protein	5.08	5.26	40.38	53.22	ns	0.53	2.1	0.32
Cytoskeleton										
1,685	At1g20010	Putative β-tubulin1	5.07	4.66	54.2	50.34	0.57	0.52	ns	ns
1,731[b]	At1g04820/ At1g50010	Tubulin α2/α4 chain	5.23	4.93	53.12	49.54	ns	0.58	ns	ns
	At5g19770/ At4g14960	Tubulin α3/α5 chain		4.93 4.95		49.54 49.65				
		Tubulin α6 chain		4.93		49.64				
Chaperone										
966	At5g28540	Luminal binding protein, putative HSP	5.3	5.05[a]	77.34	70.95[a]	ns	ns	ns	2.81
420	At1g79930	Putative HSP	5.34	5.15	105.77	91.75	ns	3.75	ns	ns
4,620	At5g20890	T complex protein1 β-subunit	5.71	5.59	59.34	57.29	ns	2.52	ns	ns
1,533	At3g11830	Putative T complex protein1 β-subunit	5.91	6.03	58.71	59.78	ns	ns	ns	1.5
Lignin biosynthesis										
2,735	At4g34050	Caffeoyl-CoA methyltransferase-like protein	5.21	5.13	32.12	29.16	ns	ns	ns	0.48
Protein glycosylation										
1,725	At5g57655	Xylose isomerase	5.58	5.74	55.31	53.15	ns	ns	ns	4.21
1,400	At5g64570	β-Xylosidase	5.81	7.75[c]	62.95	75.02[d]	1.75	2.25	ns	1.85
1,917	At5g13980	α-Mannosidase	5.62	6.00	48.66[e]	115.90	1.94	ns	1.96	ns
Unknown function or process										
4,157	At4g23670	Latex protein related	5.84	5.91	13.2	17.52	ns	ns	0.3	ns
4,227[b]	At3g26450	Latex protein related	5.15	5.01	12.57	17.8	0.55	0.54	0.49	0.37
	At3g26460			5.02		17.77				
2,212	At1g66700	Unknown protein (SAM carboxymethyl transferase family)	5.38	5.34	41.7	23.8	ns	ns	2.46	ns

(*Table continues on following page.*)

Table IV. (*Continued from previous page.*)

Spot No.	Arabidopsis Genome Initiative Locus Name	Protein Name	EIP	TIP	EMM	TMM	*ago1-3* (Col-0) versus Wild Type (Col-0)	*sur1-3* (Col-0) versus Wild Type (Col-0)	*sur2-1ago1-3* (RG) versus *sur2-1* (RG)	*sur2-1* (Ws) versus Wild Type (Ws)
2,891	At2g37660	Unknown protein	5.45	8.37[c]	29.67	34.88	ns	ns	ns	0.38
2,387	At1g49660	Unknown protein	5.41	5.32	38.08	35.2[d]	ns	ns	3.33	ns

[a]Estimated molecular mass without signal peptide. [b]Some spots are shown with several accession numbers due to equal number of peptides matching to several proteins having the same function (see Supplemental Table I). When several proteins of different functions were identified in the same spot, only the one represented by the highest number of peptides is shown. The complete identification information can be found in Supplemental Table I. [c]IP overestimated due to a potential signal peptide of unknown length. [d]Data not available by Swissprot; data taken from the Munich Information Center for Protein Sequences. [e]The observed difference between the EMM and the TMM might be due to degradation of the protein during the experiment.

Figs. 2A and 3B). Surprisingly, only one of these GH3-like proteins appeared to significantly accumulate in *sur1-3* (Table IV).

The behavior of the two lectin proteins and two GH3-like proteins again highlighted differences between the *sur1-3* and *sur2-1* mutants, although both mutations affect genes sequentially involved in the same biosynthesis pathway and induce auxin overproduction.

Out of the 18 correlated spots, 11 could be identified (Tables III and IV; Fig. 2). Five of these were negatively correlated with at least one parameter (Table III). Two proteins from the energy/carbon metabolism group (At1g32060 and At4g38970) were negatively correlated to adventitious root number. At4g38970 was also negatively correlated to the free IAA content. This was also the case for one protein related to protein degradation (At3g18490), also annotated as a putative chloroplast nucleoid DNA-binding protein. At1g32060 was negatively correlated to the three parameters as it correlated also with free IAA content and the number of adventitious root primordia. Two proteins related to stress responses were negatively correlated, the first one (At4g25100) to the number of primordia 2 d after transfer to light and the second (At5g20630) to the number of adventitious roots 7 d after transfer to light.

Among the identified proteins, six were positively correlated with one or more parameters. One protein related to glucosinolate metabolism (CORI-7/AtST5a/At1g74100), which was up-regulated in both *sur1-3* and *sur2-1*, was positively correlated with both free IAA content and adventitious root primordia 2 d after transfer to light. Two putative chaperone proteins were positively correlated to the number of primordia 48 h after transfer to light. The three GH3-like proteins were all positively correlated with the number of adventitious roots 7 d after germination but were surprisingly not correlated with free IAA content, although they are known to be auxin-inducible proteins.

Expression of *GH3*-Like Genes Is Down-Regulated in the *ago1-3* Background But Still Inducible by Exogenous Auxin

Because GH3-like proteins (GH3-3/At2g23170, GH3-5/AtGH3-a/At4g27260, and GH3-6/DFL1/At5g54510) that were positively correlated with adventitious root number belong to an auxin-inducible protein family and were recently shown to be auxin-conjugating enzymes (Staswick et al., 2002, 2005), we decided to complete the analysis by checking their expression at the transcriptional level in the wild type (Col-0), *ago1-3* (Col-0), *sur2-1* (RG), and *sur2-1ago1-3* (RG) using a semiquantitative reverse transcription (RT)-PCR technique (Fig. 3A). All three genes were down-regulated in *ago1-3* compared to the wild type (Col-0; $P < 0.001$ for *GH3-6/DFL1* and *GH3-5/AtGH3a* and $P < 0.01$ for *GH3-3*). Although no significant difference in protein accumulation could be detected between *ago1-3* (Col-0) and wild type (Col-0; Fig. 3B), most likely because the spot intensity was at the limit of the detection level (Fig. 2A), this suggests that difference in protein accumulation might occur between the two genotypes. *GH3-5/AtGH3a*, *GH3-3*, and *GH3-6/DFL1* transcription was down-regulated in the double mutant *sur2-1ago1-3* (RG) compared to the *sur2-1* (RG) mutant ($P < 0.001$). Indeed, we found that the mRNA level of these three genes was almost the same in *sur2-1ago1-3* (RG) as in *ago1-3* (Col-0) and were significantly lower than in Col-0 ($P < 0.05$). Nineteen *GH3* genes have been described in Arabidopsis (Hagen and Guilfoyle, 2002) and they are clustered in three groups based on their sequence homology (Staswick et al., 2002). There are eight GH3 proteins in group II, seven of which have been shown to adenylate IAA in vitro (Staswick et al., 2002, 2005). These include GH3-5/AtGH3a, GH3-3, GH3-6/DFL1, as well as GH3-2/YDK1/At4g37390 (Takase et al., 2004) and GH3-4/At1g59500. We checked the transcription level of the latter two by semiquantitative PCR and showed that they were also down-regulated in the double mutant *sur2-1ago1-3* (RG) compared to the *sur2-1* (RG) mutant (Fig. 4A). Nevertheless, no significant difference in RNA levels between wild type (Col-0) and *ago1-3* could be detected for *GH3-4* or *GH3-2/YDK1*. Although the *sur2-1ago1-3* (RG) double mutant contains less endogenous auxin than *sur2-1* (RG), it still contains more auxin than the wild type. Since all these genes were induced by exogenous auxin (Nakazawa et al., 2001; Tanaka et al., 2002; Tian et al., 2002; Zhao et al., 2003; Takase et al., 2004), it was important to check whether or not a mutation in the

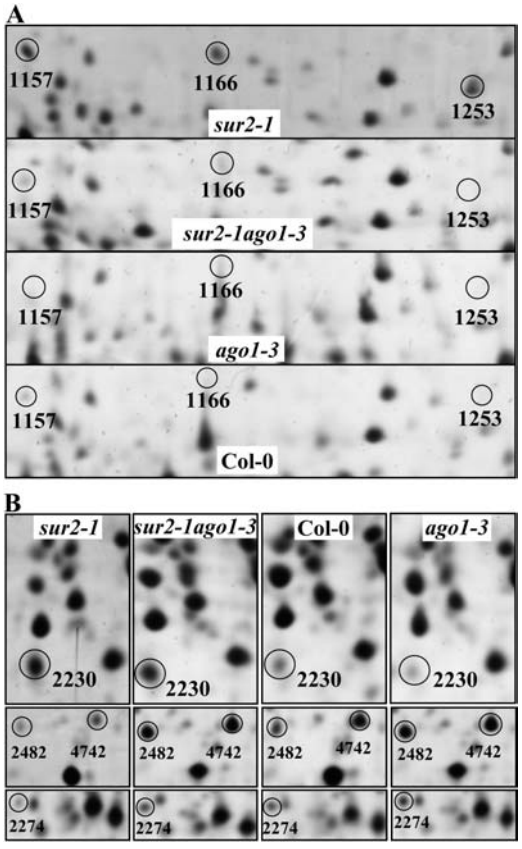

Figure 2. Example of the differential accumulation of seven correlated spots identified in the comparative analysis of protein profiles of etiolated Arabidopsis seedling transferred to light for 48 h. Spots 1,157 (At5g28540), 1,166 (At1g79930), and 1,253 (At5g20890), three GH3-like proteins, were positively correlated with the number of mature adventitious roots (A). Spot 2,230 (At1g74100, CORI-7/AtST5) was positively correlated with the free IAA content and the number of adventitious root primordia 2 d after transfer to light. Spots 2,274 (At3g18490) and 4,742 (At4g38970) were negatively correlated with the free IAA content and the number of adventitious root primordia 2 d after transfer to light (B). Proteins were prepared as described in "Materials and Methods," subjected to 2-D electrophoresis, and the resulting gels were then silver stained.

AGO1 gene would modify auxin inducibility of these *GH3*-like genes. We therefore checked their induction by exogenously applied 1-naphthalene acetic acid in the wild type and in the *ago1-3* mutant and showed that all five genes were normally induced in the *ago1-3* mutant by exogenous auxin (Fig. 4).

DISCUSSION

The objectives of the proteomic analysis presented here were to identify gene markers regulating adventitious rooting in Arabidopsis. We analyzed 2-D patterns of soluble proteins of Arabidopsis mutants differing in their auxin content and/or their adventitious rooting ability. The statistical analysis allowed us to restrict the initial 1,147 spots to 50 that showed significant variation and that could be identified by LC-MS/MS. Eleven of them were correlated to either the endogenous free IAA content, the number of adventitious root primordia after 2 d in light, and/or the number of adventitious roots 7 d after transfer to light.

The protein pattern of the different mutants was first compared to their respective wild types, and this led to interesting observations regarding the effect of their mutations. Comparing 2-D gels, the *ago1-3* mutant and its wild type (Col-0) revealed that less than 10% of the spots varied. Since *AGO1* is involved in the regulation of gene expression through the miRNA pathway (Vaucheret et al., 2004), we a priori expected to see many more proteins affected in this mutant background. One explanation could be that only the most abundant proteins are revealed on a 2-D gel and that variations affecting the less abundant proteins might not be detected. Nevertheless, the proportion observed was similar to that for the *superroot* mutants that are affected in enzymes involved sequentially in the same biosynthesis pathway leading to auxin overproduction (Barlier et al., 2000; Bak et al., 2001; Mikkelsen et al., 2004). Considering that auxin would also modify the expression of many genes, it is likely that the proportion of variable proteins observed in *ago1-3* compared to Col-0 reflects the effect of the mutation. This suggests that the pleiotropic phenotype of *argonaute* mutants observed at later stages (Bohmert et al., 1998) might be due to a cascade of events occurring downstream of the direct targets of AGO1.

Identification of variable proteins showed that several cellular functions were affected by *ago1-3*. One of the interesting features was the up-regulation of several proteins involved in energy and carbon metabolism. Up-regulation of some of the proteins linked directly or indirectly to photosynthesis in the *ago1-3* mutant is in agreement with the *ago1-3* mutant being hypersensitive to light (Sorin et al., 2005). The accumulation of some photosynthetic proteins is regulated by light. For example, carbonic anhydrase is regulated at the mRNA level (Fett and Coleman, 1994), and there are interactions between mitochondrial metabolism and photosynthetic carbon assimilation (Raghavendra and Padmasree, 2003). Consequently, up-regulation of proteins linked to photosynthesis and to the TCA cycle (such as malate dehydrogenase) fits with the light hypersensitivity of the *ago1-3* mutant.

The analysis of the *sur1-3* and *sur2-1* mutants interestingly showed that, although both mutants were altered in the same biosynthesis pathway and displayed very similar phenotypes in young seedlings (Boerjan et al., 1995; Delarue et al., 1998), they displayed differences in protein profiles. A similar number of polypeptides was affected by the two mutations; nevertheless, only 30% of the variable proteins in *sur1-3* and *sur2-1* were commonly variable. For example, three proteins of the GH3 family (GH3-3/At2g23170, GH3-5/AtGH3-a/At4g27260, and GH3-6/DFL1/At5g54510) were significantly up-regulated in the *sur2-1* mutant compared to the wild type (Ws), but only GH3-6/DFL1 accumulation increased significantly in *sur1-3*

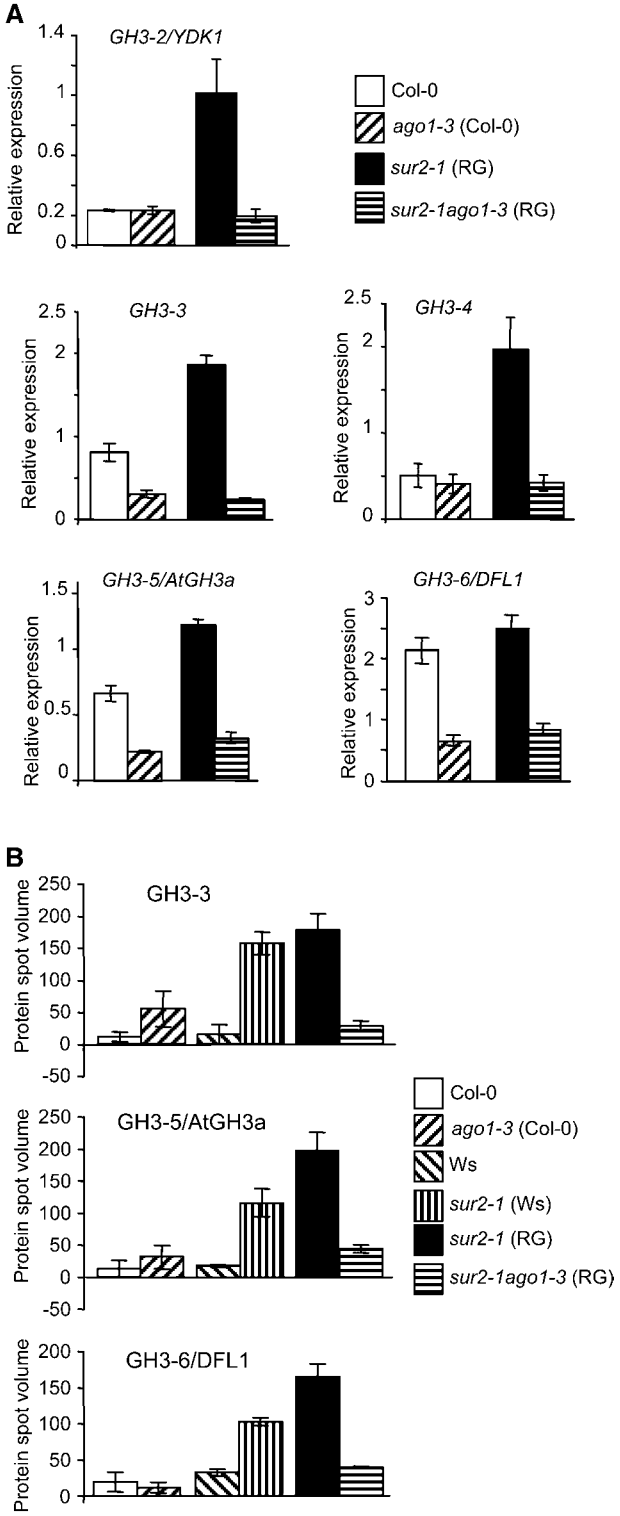

Figure 3. GH3-like protein and RNA level in wild-type and mutant seedlings 2 d after transfer to light. A, Analysis of expression of the *GH3* genes by RT-PCR in wild type (Col-0), *ago1-3* (Col-0), *sur2-1* (RG), and *sur2-1ago1-3* (RG). Total RNAs were extracted from hypocotyls of wild-type (Col-0), *ago1-3* (Col-0), *sur2-1* (RG), and *sur2-1ago1-3* (RG) siblings etiolated in the dark until the hypocotyl reached a 5-mm length and then transferred to light for 48 h. The relative expression of the genes was estimated by semiquantitative RT-PCR using 18S RNA as an internal control. For each genotype, expression of each gene was presented as a ratio between the value for the gene and the value of the corresponding 18S RNA. Units are arbitrary. Errors bars = SD of three PCR reactions. The experiment was performed on two independent biological replicates. B, Differential accumulation of the three GH3 proteins in different genotypes. Proteins were prepared as described in "Materials and Methods" and subjected to 2-D PAGE. Gels were analyzed as described in "Materials and Methods," and the volume value was estimated for each protein spot. Histograms were performed with the mean value of the volume for each genotype (mean of three or four values depending on the genotype) and for each spot. When a spot was absent in one of the gels, a value of zero was attributed to the volume of the spot (relative amount of protein) in the corresponding gel. Error bars = SE.

compared to the corresponding wild type (Col-0). Since these three *GH3* genes are induced by auxin (this work; Nakazawa et al., 2001; Tanaka et al., 2002; Tian et al., 2002; Zhao et al., 2003; Takase et al., 2004), we would expect to observe as strong an accumulation of all of them in the *sur1-3* mutant as in a *sur2-1* background because SUR1 acts downstream of SUR2. This suggests that auxin is not the only factor controlling the expression of *GH3* genes, but that other mechanisms are also involved in regulating their expression. A second interesting differential effect of the *sur1-3* and *sur2-1* mutations is highlighted by the two lectins similar to myrosinase-binding proteins that have been suggested to play roles in glucosinolate degradation (Rask et al., 2000). At3g16450 accumulation was affected in *sur1-3* but not in *sur2-1*. One isoform of At3g16470 was affected in *sur1-3* and the other one was affected in *sur2-1*. Both *sur1* and *sur2* are affected in glucosinolate biosynthesis but not to the same extent. In 5-week-old *sur1-3* mutants, glucosinolates were not detectable, whereas in *sur2-1* the glucosinolate content was decreased by 50% (Mikkelsen et al., 2004). This could explain the differential expression of glucosinolate degradation enzymes in these mutants. The fact that two isoforms of the same enzyme differentially accumulated in the *sur1* and *sur2* mutants suggests that they may target different types of glucosinolates. Interestingly, the CORI-7/AtST5a (At1g74100) protein that was recently proposed to be involved in the last step of indole-glucosinolate biosynthesis downstream of the SUR2 and SUR1 proteins (Piotrowski et al., 2004) accumulated normally in *sur2-1* and *sur1-3* mutants. While it is not surprising to see down-regulation of glucosinolate degradation enzymes in mutants that contain less or no glucosinolates, it is intriguing to see accumulation of a biosynthetic enzyme in the absence of precursors. It suggests that feedback loop regulation of this gene might exist (i.e. a low level of glucosinolates would stimulate the accumulation of enzymes of the biosynthetic pathway). Proteins of energy/carbon metabolism were also differentially affected by *sur1* and *sur2*. Out of 10 proteins, five were down-regulated in the *sur2-1* background, whereas only two (that were not affected by *sur2-1* mutation) were differentially expressed in the *sur1-3* background. One possible explanation for this could be the different behavior of *sur1* and *sur2* mutants

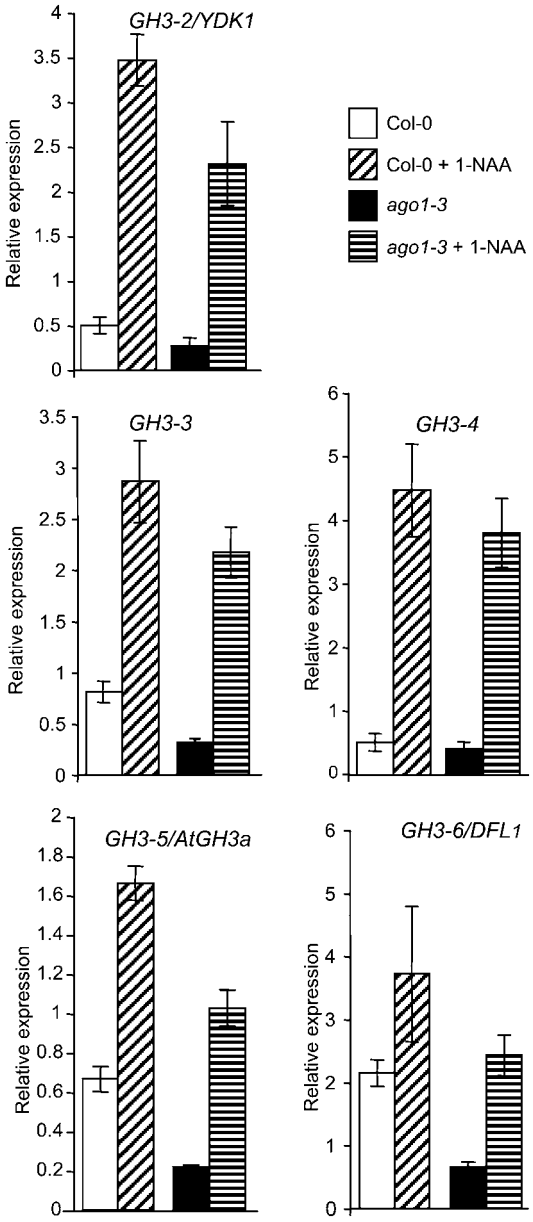

Figure 4. *GH3*-like genes are still inducible by auxin in the *ago1-3* mutant. Total RNAs were extracted from hypocotyls of wild-type (Col-0) and *ago1-3* (Col-0) siblings etiolated in the dark until the hypocotyl reached a 5-mm size, then transferred to light for 48 h with or without an auxin treatment as described in "Materials and Methods." The relative expression of the genes was estimated by semiquantitative RT-PCR using 18S RNA as an internal control. For each genotype, expression of each gene was presented as a ratio between the value for the gene and the value of the corresponding 18S RNA. Units are arbitrary. Errors bars = SD of three PCR reactions. The experiment was performed on two independent biological replicates.

toward light perception and/or signaling. Indeed, *sur2* was recently shown to be allelic to *red1*, a mutant that has an abnormal de-etiolation response in continuous red light but not in continuous far-red light (Hoecker et al., 2004). Nevertheless, although we were careful to eliminate variations due to ecotype effect (i.e. variable between Col-0 and Ws) from the comparison between *sur1-3* and *sur2-1*, we cannot rule out that some of the differences we observed between *sur1-3* and *sur2-1* may indeed be due to an ecotype effect. Indeed, this would also be supported by the fact that *ago1-3* and *sur1-3*, which are in the same ecotype, share more similarities than *sur2-1* and *sur1-3* or *sur2-1* and *ago1-3*, which are in different ecotypes. Since a *sur2* allele is now available in a Col-0 background (Smolen and Bender, 2002), we will be able to verify the differences described here.

The proteins that varied significantly were submitted to correlation analysis in order to find those that were related to adventitious root formation. A Pearson correlation analysis of spot intensity with the three physiological parameters (free IAA content, mean number of adventitious root primordia after 2 d of light, and mean number of adventitious roots after 7 d of light) was performed. Eleven proteins were correlated with at least one parameter.

As one could expect in the case of a developmental process regulated by auxin, proteins involved in the control of auxin homeostasis were identified. The three GH3 proteins, GH3-3/At2g23170, GH3-5/AtGH3-a/At4g27260, and GH3-6/DFL1/At5g54510, were significantly positively correlated with adventitious root number 7 d after transfer to light. Expression of these *GH3* genes was lower in the *ago1-3* mutant compared to Col-0 and in the *sur2-1ago1-3* (RG) mutant compared to *sur2-1* (RG). The lower level of expression in the *ago1-3* background could be explained by the lower level of endogenous free IAA (Table II; Sorin et al., 2005). Nevertheless, the level of expression was never above the wild-type level in the double mutant *sur2-1ago1-3* (RG), although it contained twice as much auxin as the wild type. In addition, we have shown that the five *GH3*-like genes analyzed were still induced by exogenous auxin in *ago1-3* mutant hypocotyls under the conditions described in "Materials and Methods." Therefore, if *GH3*-like gene expression was strictly linked to the level of auxin, we would have expected to observe an up-regulation of *GH3*-like gene expression in the *sur2-1 ago1-3* double mutant compared to the wild type. This was not the case, and our results suggested that the down-regulation in the *ago1-3* background was independent of the auxin level and likely due to other regulatory mechanisms. We have recently shown that the auxin response factor *ARF17*, a repressor of auxin-inducible genes, is regulated by a miRNA and accumulated in *ago1-3* hypocotyls (Sorin et al., 2005). We also showed that the expression of three *GH3*-like genes correlated with adventitious root number was down-regulated in a transgenic line overexpressing *ARF17* (Sorin et al., 2005). The up-regulation of *ARF17* would be expected to repress *GH3*-like gene expression in an *ago1-3* hypocotyl. Interestingly, the *ARF17* overexpresser line was also affected in adventitious root formation, strengthening the hypothesis that *GH3*-like genes might be involved in the regulation of adventitious root development. *GH3-2/YDK1*, *GH3-6/DFL1*, and *GH3-5/AtGH3a* have also been shown to be associated with the light control of development (Nakazawa et al., 2001; Tanaka

et al., 2002; Takase et al., 2004). Since *ago1-3* is altered in light sensitivity, we cannot exclude a combined effect on the regulation of the *GH3*-like genes. The ongoing characterization of mutants altered in the expression of *GH3*-like and/or *ARF17* genes will hopefully lead to a better understanding of the relative importance of the different genes in the regulation of adventitious rooting.

Among the other correlated proteins, two were related to energy and carbon metabolism and they were both negatively correlated to adventitious rooting. This is in agreement with data obtained by microarray analyses from Brinker et al. (2004) during adventitious rooting of hypocotyl cuttings of *P. contorta*. These authors proposed that the down-regulation of plastid proteins was due to the loss of photosynthetic capacity of hypocotyl cells during adventitious root formation. Indeed, in our case, several plastid-encoded proteins or proteins predicted to be localized to plastids (At3g01500, ATCG00490) accumulated in the genotypes that made less adventitious roots (i.e. in the *ago1-3* background). In contrast, several plastid proteins (At1g06680, At1g32060, and At4g38970) were down-regulated in *sur2-1*, a genotype that makes many adventitious roots, strengthening the hypothesis that light regulates adventitious rooting (Sorin et al., 2005).

Finally, several proteins either positively or negatively correlated were putative chaperones or stress-related proteins. Although it is difficult at this point to discuss the potential role of these proteins in adventitious root development, our results confirm at the protein level the observations made at the transcriptional level by Brinker et al. (2004). Indeed, they showed modification of the expression of a similar family of genes, suggesting a role in adventitious rooting.

Interestingly, and although these proteins were not correlated to adventitious rooting, we could find some overlap among the protein identified here and the genes described to be potentially associated with adventitious root formation in *P. contorta* (Brinker et al., 2004). This is the case of the 20S proteasome subunits, the putative β-tubulin and the tubulin $\alpha 2/\alpha 4$ chain, and the caffeoyl-CoA methyltransferase-like protein. A *PINHEAD/ZWILLE*-like gene was found up-regulated in young adventitious rooting stages in *P. contorta*. Since in Arabidopsis *PINHEAD/ZWILLE* is the closest gene related to *AGO1*, this suggests that the function of these genes is at least partially conserved among different plant species.

In conclusion, the proteomic analysis of *ago1-3*, *sur1-3*, *sur2-1*, and the *sur2-1ago1-3* double mutant allowed us to identify proteins whose expression was altered by the mutations, particularly in regard to the adventitious rooting process. We identified auxin-related proteins and light-related proteins positively or negatively correlated to adventitious root formation. Several other proteins related to stress responses, protein degradation, or cytoskeleton function were correlated to at least one of the studied parameters. As similar functions were affected during adventitious root formation in *P. contorta* (Brinker et al., 2004), these results strongly suggest that the identified proteins will be valuable markers for further quantitative genetic analysis of adventitious root development.

MATERIALS AND METHODS

Plant Material

ago1-3, *sur1-3*, and *sur2-1* mutants were identified in Col-0 and Ws Arabidopsis (*Arabidopsis thaliana* L. Heyhn.) ecotypes, respectively (Boerjan et al., 1995; Delarue et al., 1998). *sur2-1ago1-3* double mutant was obtained by crossing a homozygote *sur2-1/sur2-1* plant with a heterozygote +/*ago1-3* plant followed by several generations of selfing of the *sur2-1/sur2-1*, +/*ago1-3* plants. Double mutants were selected in the progeny of recombinant lines homozygous for the *sur2-1* mutation heterozygote for *ago1-3*. They are in a recombinant Ws/Col-0 genetic background.

ago1-3 and *sur1-3* mutants, which are sterile and kept as heterozygotes, were compared to their wild-type siblings, Col-0; *sur2-1* was compared to the Ws wild-type ecotype. *ago1-3sur2-1* double mutants were compared to their *sur2-1* siblings in the recombinant Ws/Col-0 background (RG). For simplification, the background (Col-0, Ws, or RG) will always be indicated in parentheses after the genotype.

Growth Conditions and Preparation of Biological Samples for Protein Extraction

Seeds from the different genotypes were sterilized and sown in vitro as described previously (Santoni et al., 1994). They were kept at 4°C for 72 h to homogenize germination and then transferred to light (130–140 μmol m^{-2} s^{-1}) for 10 h to induce germination. Seedlings were germinated and grown in the dark until the hypocotyl reached an average size of 5 mm. To create darkness, the petri dishes were wrapped in four layers of aluminum foil. Seedlings were then transferred to light, in long-day conditions, for 48 h (130–140 μmol m^{-2} s^{-1}, 16 h light, 20°C average day temperature, 15°C average night temperature). For observation of adventitious root initiation and counting the number of adventitious root primordia 2 d after transfer to light, complete seedlings were cleared overnight with a buffer described by Herr (1982) and observed with a Nikon microphot FXA microscope with Nomarski optics. For proteomic analysis, cotyledons and roots were removed, and hypocotyls were harvested, dried on tissue paper, and frozen in liquid nitrogen. Three to four independent biological replicates (of at least 300 hypocotyls each) were prepared for each genotype.

Protein Extraction

The protein extraction method was derived from Damerval et al. (1986). Frozen hypocotyls were ground into a fine powder in liquid nitrogen. The powder was mixed with cold acetone containing 10% (w/v) TCA and 0.07% (v/v) β-mercaptoethanol. Proteins were left to precipitate for 1 h at −20°C. They were then centrifuged at 10,000 rpm for 10 min at −20°C. Pellets were washed twice with cold acetone containing 0.07% (v/v) β-mercaptoethanol, then dried, weighed, and solubilized in R2D2 buffer (300 μL mg^{-1} dry pellet; R2D2, 5 M urea, 2 M thiourea, 2% 3-[(3-cholamidopropyl) dimethyl-ammonio]-1-propane-sulfonate, 2% *N*-decyl-*N*,*N*-dimethyl-3-ammonio-1-propane-sulfonate, 20 mM dithiothreitol, 5 mM Tris (2-carboxyethyl)phosphine, 0.5% carrier ampholytes 4–6.5, 0.25% carrier ampholytes 3–10; Mechin et al., 2003). Cellular debris was eliminated by centrifugation at 12,000 rpm. Protein amounts were estimated with the PlusOne 2D quant kit (Amersham Biosciences).

2-D Electrophoresis

Thirty-five micrograms of protein were mixed together with R2D2 buffer (Mechin et al., 2003) and used for isoelectric focusing (IEF). IEF was performed in 24-cm-long immobilized pH gradient (IPG) strips (pH range from 4–7; Amersham Biosciences). Migration was performed at 20°C with limited amperage of 50 μA on a Protean IEF cell (Bio-Rad) as follows: after an active rehydration at 50 V during 13 h, steps at 200 V, 500 V, and 1,000 V were run for 30 min, 30 min, and 60 min, respectively. Voltage was then increased to 10,000 V and IEF was stopped when 84,000 Vh were reached. After IEF, IPG strips were

equilibrated after Görg et al. (1987). Strips were then sealed on top of a 1-mm-thick 2-D gel (24 × 24 cm) with the help of 1% low-melting agarose in electrophoresis buffer. Continuous gels (11% acrylamide gels with potato dextrose agar as a cross-linking agent) were used. SDS-PAGE was carried out with running buffer in a PROTEAN Plus Dodeca cell (Bio-Rad) electrophoresis tank at 14°C at 20 V for 1 h followed by 120 V overnight (limited by a maximum of 30 mA per gel) until the bromphenol blue front reached the end of the gel. A silver-staining procedure was performed according to Mechin et al. (2003). Three to four gels per genotype (i.e. independent biological replicates) were used for the analysis. Stained gels were scanned and gel analysis was performed with Progenesis software (Nonlinear Dynamics). After spot detection, 2-D gels were aligned and matched to a reference gel created by the software on which were identified a total number of 1,174 reproducible spots. Optical density calibration was performed with a Kodak density calibration strip (21-step photographic step tablet, density range 0.05–3.05). After background subtraction, normalization was performed (mode total spot volume).

Protein Identification by LC-MS/MS

For protein identification, a preparative gel containing 150 μg of protein was prepared and stained with SYPRO ruby protein gel stain (Bio-Rad). Staining visualization was performed with the use of a dark reader transilluminator DR190M (blue light source 400–500 nm; Clare Chemical Research). Spots were picked out and the acrylamide pieces were collected in 96-well microplates. In-gel digestion was performed with the Progest system (Genomic Solution) according to a standard trypsin protocol. Briefly, gel pieces were washed and then subsequently digested with 125 ng of modified trypsin (Promega) during 5 h. The peptides were extracted with 30 μL of 5% trifluoroacetic acid (TFA), 10% acetonitrile (ACN), then 30 μL of 0.2% TFA, 83% ACN. Peptide extracts were dried in a vacuum centrifuge and resuspended in 20 μL of 0.1% TFA, 3% ACN. The peptides were separated on HPLC Famos-Switchos II-Ultimate (LC Packings-Dionex). Sample (5 μL) was loaded on a PEPMAP C18 column (5 μm, 75 μm × 15 cm; LC Packings-Dionex) after a 3-min preconcentration step at 5 μL min^{-1} on a micro precolumn cartridge (300 μm × 5 mm). The separation was achieved with a linear gradient from 5% to 30% B for 25 min at 200 nL min^{-1}. Buffers were 0.1% HCOOH, 3% ACN (A) and 0.1% HCOOH, 0.95% ACN (B). The LCQ deca xp+ (Thermofinnigan) was used with a nanoelectrospray interface. Ionization (1.2- to 1.4-kV ionization potential) was performed with liquid junction and a noncoated capillary probe (New Objective). Peptide ions were analyzed by the *n*th-dependent method as follows: (1) full MS scan (*m/z* 500–1500); (2) ZoomScan (scan of the two major ions with higher resolution); and (3) MS/MS of these two ions. Data were then analyzed with Bioworks 3.1 and the Arabidopsis protein sequences database downloaded from the National Center for Biotechnology Information (NCBI; http://www.ncbi.nlm.nih.gov) or the plant genome database (http://www.plantgdb.org). Identified tryptic peptides were filtered according to their cross-correlation (Xcorr) score and their charge state (Xcorr > 1.7 for +1 and Xcorr > 2.2 for +2 charge). Annotation of the identified proteins was obtained on http://www.arabidopsis.org/servlets/Search; http://www.tigr.org and http://www.genome.ad.jp/kegg were also used as a complement in protein function identification. Molecular weights were obtained from http://au.expasy.org/sprot and http://mips.gsf.de/proj/thal/db.

Data Analysis

Statistical analysis was performed on 1,147 reproducible spots. ANOVA was performed for each of the 1,147 reproducible proteins. The factor was the combination between genetic background (Col-0, Ws, and RG) and the genotype at the locus of the different studied mutations. Thus, the different levels of the factor were wild type (Col-0), *sur1-3* (Col-0), wild type (Ws), *ago1* (Col-0), *sur2-1* (Ws), *sur2-1* (RG), and *sur2-1ago1-3* (RG). This analysis allowed the computation of the residual variance of the spots over the complete dataset. For proteins showing a significant variation ($P < 0.01$), contrasts were computed between the following couples: *sur1-3* (Col-0) versus wild type (Col-0), *ago1* (Col-0) versus wild type (Col-0), *sur2* (Ws) versus wild type (Ws), and *sur2ago1* (RG) versus *sur2* (RG). Contrasts were considered significant at $P < 0.01$. Thus, only proteins showing a significant quantitative difference at least between a mutant and its corresponding wild type were selected. Then spots showing an induction factor below 1.5 or above 0.66 were not considered further. The GLM procedure of the SAS software package (SAS Institute, version 8.1) was used for ANOVA and contrast analysis.

Spots were described as showing qualitative variations when they were present (or absent) in all the gels of a mutant genotype and absent (or present) in all the gels of the corresponding wild type or *sur2* (in the case of the *sur2ago1* [RG] versus *sur2* [RG] comparison).

Correlation Analysis

For each protein that was significantly variable, Pearson correlation coefficients (correlation procedure of the SAS package) were computed between means of relative intensity in the six genotypes and IAA (IAA concentration), number of adventitious root primordia 2 d after transfer to light, and number of adventitious roots emerging from the hypocotyl 7 d after transfer to light. When the protein was not detected in a genotype, the genotype was excluded from the computation of the correlation with this protein. When the correlation was significant ($P < 0.01$), consistence of the correlation with the absence of the protein in this genotype was verified.

Endogenous free IAA levels (pg mg^{-1} fresh weight) were previously measured in the apical part of seedlings, 48 h after transfer to light in wild type (Col-0), wild type (Ws), *ago1-3* (Col-0), *sur2-1* (RG), and *sur2-1ago1-3* (RG) (Sorin et al., 2005).

The average number of primordia 2 d after transfer to light was determined in *sur2-1* (RG) mutants and *sur2-1ago1-3* (RG) double mutants by analyzing the expression of the *CyclinB1:uidA* reporter gene as described by Sorin et al. (2005). For wild-type Ws, wild-type Col-0, and *sur2-1* (Ws), the average number of primordia was determined on seedlings cleared overnight in Herr's buffer (Herr, 1982) and observed with a Nikon microphot FXA microscope with Nomarski optics. An average of 40 seedlings was analyzed in three independent biological replicates.

Adventitious roots emerging from the hypocotyls were counted on seedlings 7 d after transfer to light using a stereomicroscope. An average of 40 seedlings was analyzed in three independent biological replicates.

RNA Analysis

Siblings from Col-0 and *ago1-3* or *sur2-1* (RG) and *sur2-1ago1-3* (RG) were grown in the same conditions as for protein extraction. For auxin induction of *GH3*-like genes, the seedlings were transferred, after 44 h in the light, into liquid culture medium with or without 10 μM of 1-naphthylacetic acid for 4 h in the same environmental conditions. RNA was extracted from dissected frozen hypocotyls.

For semiquantitative RT-PCR, total RNA was extracted using the RNAqueous kit (Ambion). Five hundred nanograms or 1 μg of RNA was transcribed into cDNA by using iScript reverse transcriptase (Bio-Rad). PCR amplification was carried out in triplicate with each cDNA and primer pairs 5′-CCTATGCTGGGCTTTACAGG-3′ and 5′-ACCAGGGGACCATTTAGGAC-3′ for *GH3-6/DFL1*/At5g54510; 5′-AAGTTTGTGCGGAGGAAGAA-3′ and 5′-AAAGCGGGCTGAAGTGTGT-3′ for *GH3-3*/At2g23170; 5′-AATGCCAACAATCGAAGAGG-3′ and 5′-CTTGCACTCAAATTCCACGA-3′ for *GH3-5/AtGH3a*/At4g27260; 5′-GAAATGACTCGGAACCCTGA-3′ and 5′-GCAGAGGATGGCTTCGTTAG-3′ for *GH3-2/YDK1*/At4g37390; and 5′-AGCCATCCTCTGCTGTGACT-3′ and 5′-ACTCCTCCATCTCCATCGTG-3′ for *GH3-4*/At1g59500. Quantum 18S RNA internal standard kit (Ambion) served as an internal control. PCR products were analyzed on a 1.5% agarose gel. The experiment was performed on two independent biological replicates.

ACKNOWLEDGMENTS

We thank John D. Bussell, Brian Jones, Thomas Moritz, and Gunnar Wingsle for discussions and critical reading of the manuscript.

Received July 1, 2005; revised November 2, 2005; accepted November 2, 2005; published December 23, 2005.

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