Specificity and Similarity of Functions of the *Aux/IAA* Genes in Auxin Signaling of Arabidopsis Revealed by Promoter-Exchange Experiments among *MSG2/IAA19*, *AXR2/IAA7*, and *SLR/IAA14*[1][C][OA]

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As indicated by various and some overlapped phenotypes of the dominant mutants, the *Aux/IAA* genes of Arabidopsis (*Arabidopsis thaliana*) concomitantly exhibit a functional similarity and differentiation. To evaluate the contributions of their expression patterns determined by promoter activity and molecular properties of their gene products to *Aux/IAA* function, we examined phenotypes of transgenic plants expressing the green fluorescent protein (GFP)-tagged *msg2-1/iaa19*, *axr2-1/iaa7*, or *slr-1/iaa14* cDNA by the *MSG2* or *AXR2* promoter. When driven by the *MSG2* promoter (*pMSG2*), each GFP-tagged cDNA caused the *msg2-1* phenotype, that is, the wild-type stature in the mature-plant stage, long and straight hypocotyls in the dark, reduced lateral root formation, relatively mild agravitropic traits in hypocotyls, and a normal gravitropic response in roots. However, development of one or two cotyledonary primordia was often arrested in embryogenesis of the *pMSG2*::*axr2-1*::*GFP* and *pMSG2*::*slr-1*::*GFP* plants, resulting in monocotyledonary or no cotyledonary seedlings. Such defects in embryogenesis were never seen in *pMSG2*::*msg2-1*::*GFP* or the *msg2-1*, *axr2-1*, or *slr-1* mutant. The *MSG2* promoter-*GUS* staining showed that expression of *MSG2* started specifically in cotyledonary primordia of the triangular-stage embryos. When driven by the *AXR2* promoter (*pAXR2*), each GFP-tagged mutant cDNA caused, in principle, aberrant aboveground phenotypes of the corresponding dominant mutant. However, either the *axr2-1*::*GFP* or *slr-1*::*GFP* cDNA brought about dwarf, agravitropic stems almost identical to those of *axr2-1*, and the *pAXR2*::*msg2-1*::*GFP* and *pAXR2*::*slr-1*::*GFP* hypocotyls exhibited complete loss of gravitropism as did *axr2-1*. These results showed functional differences among the msg2-1, axr2-1, and slr-1 proteins, though some phenotypes were determined by the promoter activity.

Auxin acts as a signaling molecule in many aspects of plant growth and development, including embryogenesis, root and shoot patterning, apical dominance, and tropic responses. These processes include the regulation of gene expression by two protein families, auxin response factors (ARFs) and the Aux/IAA proteins (Leyser, 2002; Hagen et al., 2004), that function directly downstream of the auxin F box receptors (AFBs; Dharmasiri et al., 2005; Kepinski and Leyser, 2005).

ARFs were initially identified by their ability to bind to auxin-responsive elements via an amino-terminal DNA-binding domain, and they regulate the expression of genes containing such promoter elements in an auxin-dependent manner (Ulmasov et al., 1997; Hagen et al., 2004). The *Aux/IAA* genes were first identified as genes whose transcripts were rapidly induced by auxin (Abel and Theologis, 1996). They encode short-lived nuclear proteins, and most of them contain four highly conserved domains (I–IV) that contribute to their functional properties. Domains I and II are unique to the Aux/IAA proteins, while domains III and IV are also conserved in the carboxyl-terminal domain (CTD) of ARFs and serve as protein-protein interaction domains that promote both homo- and heterodimerization between members of the Aux/IAA and ARF families (Kim et al., 1997; Ulmasov et al., 1997; Hagen et al., 2004). Domain I is a repressor domain that is dominant over the activation function of ARF (Tiwari et al., 2004). Domain II confers instability to the protein (Worley et al., 2000; Ouellet et al., 2001). Recent studies indicate that an AFB, TIR1, interacts with the Aux/IAA proteins and stimulates their degradation in an auxin-dependent manner (Dharmasiri et al., 2005; Kepinski and Leyser, 2005). Both the ARF and Aux/IAA proteins consist of

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large families with 23 and 29 members, respectively, in Arabidopsis (*Arabidopsis thaliana*).

Genetic studies of loss-of-function mutants of the *ARF* genes have revealed both distinct and redundant roles of *ARF*s in plant growth and development. For example, *NPH4/ARF7* plays a central role in tropistic responses in hypocotyls and lateral root formation (Liscum and Briggs, 1995; Watahiki and Yamamoto, 1997; Harper et al., 2000; Tatematsu et al., 2004), and so does *MP/ARF5* in embryo patterning and vascular tissue formation (Mattsson et al., 2003). However, *ARF19* is also involved in tropic responses and lateral root formation because the *arf19* mutation, which does not affect these processes alone, enhances the *nph4* defects significantly (Okushima et al., 2005; Wilmoth et al., 2005). Similarly, the *nph4 mp* double mutants exhibit more severe defects than the corresponding single mutants, indicating that *MP* and *NPH4* act redundantly (Hardtke et al., 2004). In the *Aux/IAA* genes, several dominant mutations in domain II, which increase the stability of the gene products, have been identified, such as *iaa1/axr5* (Park et al., 2002; Yang et al., 2004), *iaa3/shy2* (Tian and Reed, 1999), *iaa6/shy1* (Reed, 2001), *iaa7/axr2* (Nagpal et al., 2000), *iaa12/bdl* (Hamann et al., 2002), *iaa14/slr* (Fukaki et al., 2002), *iaa17/axr3* (Rouse et al., 1998), *iaa18* (Reed, 2001), *iaa19/msg2* (Tatematsu et al., 2004), and *iaa28* (Rogg et al., 2001). Various aberrant phenotypes involved in auxin-mediated growth and development, as well as altered gene expression in response to auxin, are found in these mutants. Each of the altered phenotypes is distinct in a different subset of the *aux/iaa* mutants, which indicates functional similarity and differentiation of the *Aux/IAA* family.

The similarity and differentiation of the *Aux/IAA* genes may result from their expression patterns produced by their promoter activity and/or molecular properties of their gene products. Knox et al. (2003) and Weijers et al. (2005) evaluated the contributions of the two factors to function of the *Aux/IAA* genes in transgenic plants expressing the mutant *Aux/IAA* (*mAux/IAA*) cDNA by the same promoter. Their results show that the specificity of the *Aux/IAA* action is primarily regulated at the level of gene transcription, while there exist some functional differences between the aux/iaa proteins. The *msg2* mutants provide a rare opportunity to address this question because the *msg2* defects appear to be very specific compared to the other dominant *Aux/IAA* mutants. *msg2* is defective only in tropic responses of hypocotyls, formation of lateral roots and fecundity, and looks almost normal at the mature stage in contrast to the pleiotropic defects displayed by most of the *aux/iaa* mutants. By taking advantage of the unique *msg2* phenotype, we also investigated the molecular basis of the similarity and differentiation among the *msg2-1*, *axr2-1*, and *slr-1* genes.

RESULTS

Growth and Development of Shoots

Adult plants of *msg2-1/iaa19* look similar to the wild type except for having a reduced fecundity (Fig. 1B; Tatematsu et al., 2004). *slr-1/iaa14* has small leaves and short and thin inflorescence stems. The number of inflorescence stems is also reduced (Fig. 1C; Fukaki et al., 2002). The *axr2-1/iaa7* is dwarf and has agravitropic stem (Fig. 1D; Nagpal et al., 2000). In an attempt to investigate whether the phenotype of each mutant was determined by the specific pattern of expression of each gene, we compared phenotype of transgenic Arabidopsis plants that expressed the *msg2-1*, *axr2-1*, or *slr-1* cDNA under the control of a 2-kb portion of the *MSG2* or *AXR2* promoter (*pMSG2* and *pAXR2*, respectively). The *mAux/IAA* cDNAs were also fused translationally with GFP to detect their expression.

Transgenic plants harboring the *pMSG2::msg2-1::GFP* transgene showed a normal morphology (Fig. 1E). The reduced fecundity as observed in *msg2-1* was found in five out of 50 independent T_1 plants. On the other hand, seedlings of multiple *pMSG2::axr2-1::GFP* and

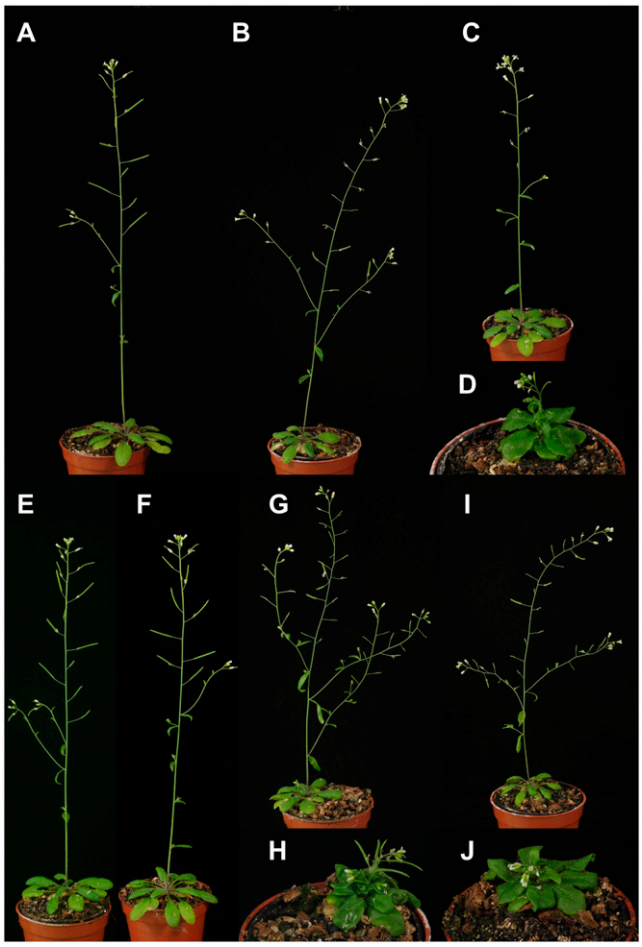

Figure 1. Phenotypes of 4-week-old transgenic plants that express mutant *Aux/IAA* genes tagged with *GFP*. A, Wild type; B, *msg2-1/iaa19*; C, *slr-1/iaa14*; D, *axr2-1/iaa7*; E, *pMSG2::msg2-1::GFP*; F, *pAXR2::msg2-1::GFP*; G, *pMSG2::axr2-1::GFP*; H, *pAXR2::axr2-1::GFP*; I, *pMSG2::slr-1::GFP*; and J, *pAXR2::slr-1::GFP*. All plants were sown in 55-mm-diameter pots. [See online article for color version of this figure.]

pMSG2::*slr-1*::*GFP* lines often lacked one or both cotyledons (Fig. 2, F, L, and M, and G, N, and O, respectively). In one *pMSG2*::*axr2-1*::*GFP* line, one and no cotyledon phenotypes appeared in 59% and 25% of the seedlings ($n = 334$); 44% and 27% were observed for a *pMSG2*::*slr-1*::*GFP* line, respectively ($n = 126$). The single cotyledon of defective seedlings was normal in shape and normally expanded after germination (Fig. 2, L and N). Plants with the defective cotyledons were smaller than wild type, but they produced true leaves with normal phyllotaxis except for the first leaf, which was sometimes fused (data not shown). The *pMSG2*::*axr2-1*::*GFP* and *pMSG2*::*slr-1*::*GFP* plants with two cotyledons looked almost normal, though they showed reduced fecundity that was more severe than that of *msg2-1* (Fig. 1, G and I).

To our knowledge, the aberrant development of cotyledons had never been seen in *msg2-1*, *slr-1*, *axr2-1*, or the *pMSG2*::*msg2-1*::*GFP* plants. The abnormality became evident from the torpedo-stage embryo, in which development of primordia of one or both cotyledons was arrested (Fig. 2P). Examination of the *MSG2* promoter-*GUS* line showed *MSG2* expression in primordia of the cotyledon in as early as the triangular stage of embryo (Fig. 2, Q and R). This suggests that ectopic expression of *axr2-1* or *slr-1* in cotyledon primordia tends to repress development of the cotyledon, but that native expression of *msg2-1* does not.

The *pAXR2*::*axr2-1*::*GFP* plants reproduced the *axr2-1* phenotype in respect of their dwarfism and agravitropism in stems (Fig. 1H). Out of 34 T_1 plants harboring the *pAXR2*::*axr2-1*::*GFP* transgene, 17 plants showed the *axr2*-like phenotype, nine plants were normal in morphology, and the others exhibited an intermediate phenotype with semi-dwarf traits. The *pAXR2*::*slr-1*::*GFP* plants also showed the *axr2* phenotype (Fig. 1J). In contrast, the *pAXR2*::*msg2-1*::*GFP* plants displayed normal morphology even though we examined 48 T_1 lines (Fig. 1F). These results suggest again that axr2-1 and slr-1 perform very similar functions, and that their functions differ from that of msg2-1 under the control of the *AXR2* promoter. This observation is consistent with phylogenetic evidence that AXR2 and SLR are most closely related in the Aux/IAA family (Remington et al., 2004).

Growth of Dark-Grown Hypocotyls

Dominant mutations in *Aux/IAA* genes result in varied phenotypes in hypocotyls grown in the dark. *axr2-1* exhibits a de-etiolated phenotype (Fig. 2C; Nagpal et al., 2000), but *msg2-1* and *slr-1* do not, except that they have a partially open hook structure. In addition, hypocotyls of *msg2-1* grow straight, whereas those of *slr-1* display curly growth (Fig. 2, B and D; Fukaki et al., 2002; Tatematsu et al., 2004). Under the control of the *MSG2* promoter, none of the three *mAux/IAA*::*GFP*s caused either de-etiolated phenotype or curly growth of hypocotyls. Their hypocotyls looked like those of *msg2-1* (Fig. 2, E–G). Interestingly, hypocotyls of etiolated seedlings with one or no cotyledon were shorter than normal ones by up to 30%, possibly due to a shortage of auxin or nutrients. On the other hand, growth in the dark of hypocotyls of the three transgenic plants expressing *mAux/IAA*::*GFP*s by the *AXR2* promoter were different. Seedlings of *pAXR2*::*axr2-1*::*GFP*, like those of *axr2-1*, were de-etiolated (Fig. 2I). Seedlings of *pAXR2*::*msg2-1*::*GFP*, like those of *msg2-1*, had straight hypocotyls (Fig. 2H). Seedlings of *pAXR2*::*slr-1*::*GFP*, like those of *slr-1*, had curly hypocotyls (Fig. 2J). It is thus concluded that when driven by the *AXR2* promoter, each mAux/IAA protein fused with GFP reproduced the phenotype of the corresponding dominant mutant from which it was derived. These results suggest that the three mAux/IAAs

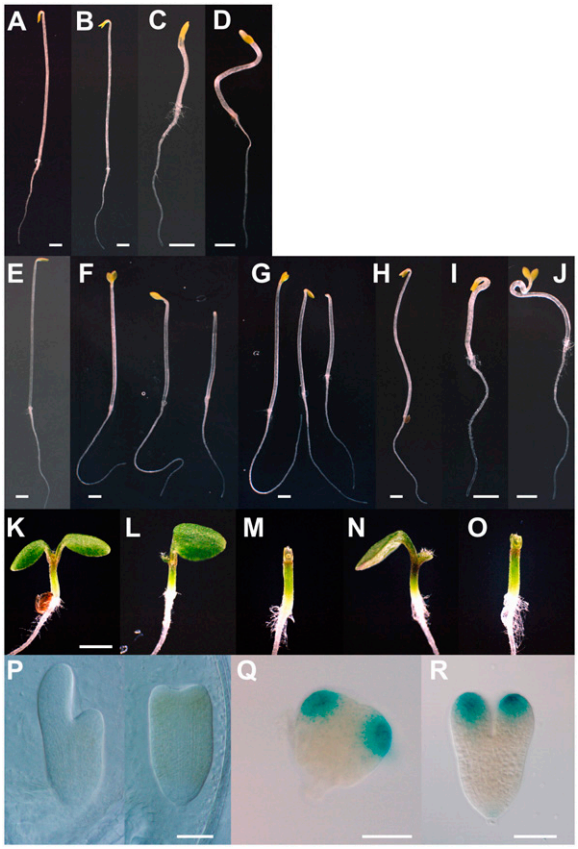

Figure 2. Phenotypes of transgenic plants in the seedling and embryo stages. A to J, Dark-grown seedlings; K to O, light-grown seedlings. A and K, Wild type; B, *msg2-1*; C, *axr2-1*; D, *slr-1*; E, *pMSG2*::*msg2-1*::*GFP*; F, L, and M, *pMSG2*::*axr2-1*::*GFP*; G, N, and O, *pMSG2*::*slr-1*::*GFP*; H, *pAXR2*::*msg2-1*::*GFP*; I, *pAXR2*::*axr2-1*::*GFP*; and J, *pAXR2*::*slr-1*::*GFP*. The seedlings of *pMSG2*::*axr2-1*::*GFP* and *pMSG2*::*slr-1*::*GFP* sometimes lacked one cotyledon (F [middle], G [middle], L, and N) or had no cotyledons (F [right], G [right], M, and O). All seedlings were grown for 3 d. P, Aberrant development of torpedo-stage embryos of *pMSG2*::*axr2-1*::*GFP* that lacked one (left) or two (right) cotyledon primordia. Q and R, Expression of the *MSG2* promoter-*GUS* at the triangular (Q) and early torpedo stages (R). Scale bar, 1 mm (A–K) and 100 μm (P–R). Scale in L to O is same as that in K.

had different effects on growth regulation in hypocotyls.

Growth and Development of Roots

msg2-1 and *slr-1* are defective in formation of lateral roots, but *msg2-1*, unlike *slr-1*, exhibits normal root hairs (Fig. 3; Fukaki et al., 2002; Tatematsu et al., 2004). On the other hand, *axr2-1* has more abundant lateral roots than wild type but has no root hairs (Fig. 3; Wilson et al., 1990; Nagpal et al., 2000). Under the control of the *MSG2* promoter, all three *mAux/IAA*::*GFP*s decreased the number of lateral roots, but had no effect on the abundance of root hairs. Thus, roots of the *pMSG2*::*mAux/IAA*::*GFP* plants looked similar to those of the *msg2* mutants (Fig. 3), suggesting that the *msg2* phenotype in roots is dependent on the activity of the *MSG2* promoter. On the other hand, the *axr2-1*::*GFP* cDNA driven by the *AXR2* promoter did not show significant effects on the formation of lateral roots ($P = 0.081$ in *t* test) or root hairs, which was distorted in *axr2-1*. Therefore, other cis-acting elements outside the 2-kb promoter used in this study must be required to control the *AXR2* gene expression in roots. The *pAXR2*::*msg2-1*::*GFP* and *pAXR2*::*slr-1*::*GFP* plants also showed a normal root phenotype ($0.18 < P < 0.34$ in *t* test for lateral root formation), which might be due to insufficient activity of the *AXR2* promoter that we used in this study.

Tropic Responses

The gravitropic response is reduced in most *aux/iaa* mutants, including *msg2*, *axr2*, and *slr*, but in a distinct manner. For example, although *axr2* and *slr* exhibit agravitropism in both roots and hypocotyls, *msg2* is defective only in hypocotyls (Tatematsu et al., 2004). We first examined growth orientation of hypocotyls and roots of seedlings, which reflects their ability to respond to gravity. Seedlings were grown on vertically held agar plates. Growth orientation of hypocotyls and roots was randomized in *axr2-1* and *slr-1* as revealed by their much greater SD than that of wild type, whereas it was normal in *msg2-1* (Fig. 4, top). However, when turned 90°, *msg2-1* hypocotyls did not respond to gravity as readily as wild type (Fig. 4, bottom), showing that *msg2-1* hypocotyls were also defective in gravitropism. The large SE of bending curvature of *axr2-1* hypocotyls indicated almost complete loss of gravitropism in *axr2-1*, while the smaller SE in *msg2-1* and *slr-1* showed that they retained some gravitropic sensitivity.

Growth orientation of hypocotyls with each *mAux/IAA*::*GFP* under the control of the *MSG2* or *AXR2* promoter tended to be random except for *pMSG2*::*msg2-1*::*GFP*. However, the randomness of hypocotyl orientation with *pMSG2*::*axr2-1*::*GFP* and *pMSG2*::*slr-1*::*GFP* was smaller than that of hypocotyls with *mAux/IAA*::*GFP*s driven by the *AXR2* promoter, which were almost perfectly agravitropic as judged from their SD values (Yamamoto and Yamamoto, 1998). The SD of growth orientation of *pMSG2*::*axr2-1*::*GFP* and *pMSG2*::*slr-1*::*GFP* was variable because hypocotyls with one or two cotyledons and with no cotyledons responded to gravity differently. The SD of the former hypocotyls was approximately 40°, while that of the latter was approximately 95°. Therefore, if all the *pMSG2*::*axr2-1*::*GFP* and *pMSG2*::*slr-1*::*GFP* seedlings have a pair of cotyledons, their gravitropic defects would be certainly weaker than those of the *pAXR2*::*mAux/IAA*::*GFP* plants. This clearly shows that the gravitropic responses in the transgenic plants were primarily determined by their promoter activities. However, even when driven by the same *MSG2* promoter, gravitropic defects were not the same between the mAux/IAA::GFP proteins: Defects of hypocotyls with axr2-1::GFP and slr-1::GFP proteins were larger than those observed in the *pMSG2*::*msg2-1*::*GFP* hypocotyls, which were normal with respect to growth orientation on the vertically oriented agar plates. This result indicates that the axr2-1 and slr-1 proteins have more deleterious effects on gravitropic responses than msg2-1.

Roots were normally orientated in all the transgenic plants tested, like those of *msg2-1* (Fig. 4, top, white bars). The gravitropic response also was not affected in

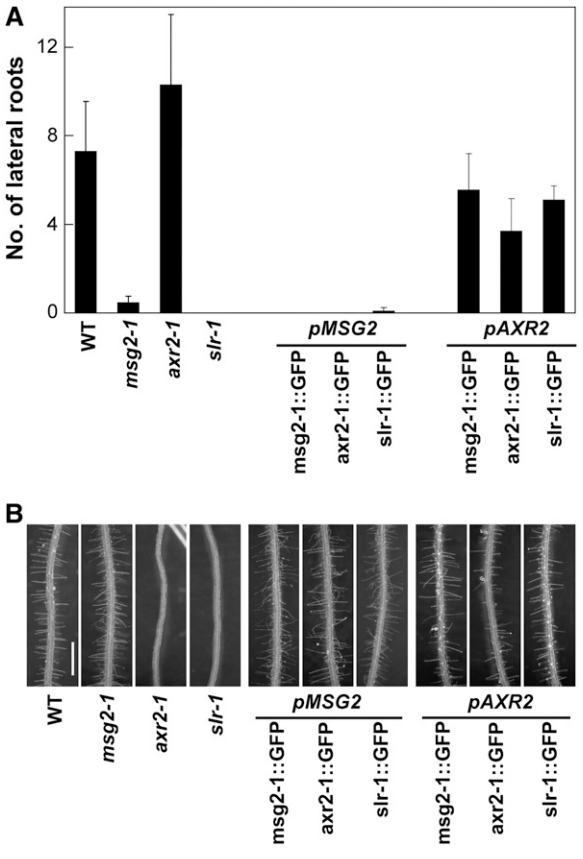

Figure 3. Formation of lateral root (A) and root hair (B) in transgenic plants expressing mutant *Aux/IAA* genes tagged with *GFP*. Seedlings were grown on vertically oriented plates for a week under continuous white light. A, Number of lateral roots; B, images of the primary roots of the seedlings. Scale bar, 1 mm.

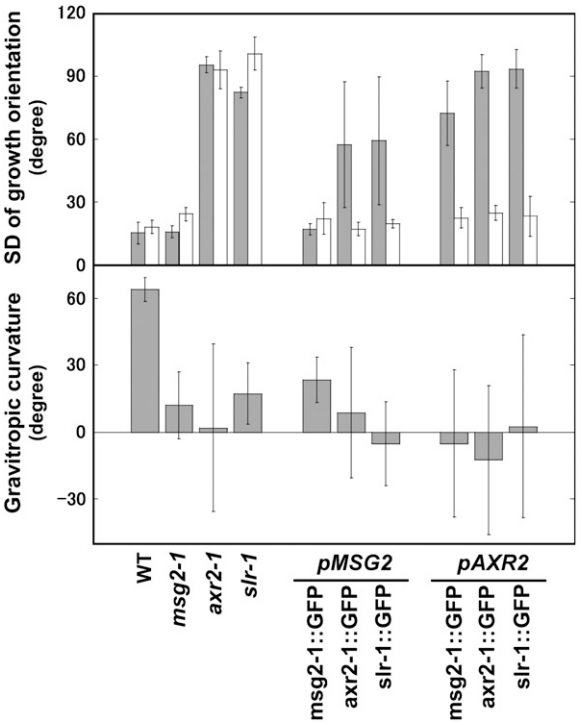

Figure 4. Growth orientation (top) and gravitropic responses (bottom) of transgenic plants that express the mutant *Aux/IAA* genes tagged with *GFP*. Top, Seedlings were grown on vertically oriented plates for 3 d in the dark, and growth angle was measured. Variation of growth orientation of hypocotyl (gray bars) or root tip (white bars) was represented by SD of growth angles. Values indicate mean and SD of three independent experiments, each using eight to 13 seedlings. Bottom, Curvature of hypocotyls of seedlings that were grown on vertically oriented plates for 3 d in the dark and then reoriented by 90°. Curvature was measured 15 h after the turn. Values indicate mean and SE of three independent experiments, each using seven to 13 seedlings.

roots (data not shown). *MSG2* has been shown to be expressed in the elongation zone and pericycle of roots (Tatematsu et al., 2004), indicating that expression of neither msg2-1, axr2-1, nor slr-1 there affects root gravitropism. Expression of *axr2-1*::*GFP* under the control of the *AXR2* promoter also did not affect the gravitropic response in roots, while in the *axr2-1* mutant the root was agravitropic. Since lateral roots and root hairs in *pAXR2*::*axr2-1*::*GFP* formed normally as described above, the *axr2* phenotype was produced only in aerial parts in this transgenic line, probably because the *AXR2* promoter that we used had insufficient activity. Consequently, the function of msg2-1 and slr-1 could not be determined in root when they were driven by the *AXR2* promoter.

Expression of Transgenes

Expression of the *mAux/IAA*::*GFP* transgenes was measured with fluorescent confocal microscopy (Fig. 5) and quantitative reverse transcription (RT)-PCR (Fig. 6). GFP fluorescence was detected in nuclei in all the transgenic plants (Fig. 5), except for *pAXR2*::*msg2-1*::*GFP* (Fig. 5, D and N). However, quantitative RT-PCR analysis revealed that all transgenic plants, including *pAXR2*::*msg2-1*::*GFP*, expressed the transgenes (Fig. 6). GFP fusion proteins whose expressions were driven by the *MSG2* promoter were found in hypocotyls (Fig. 5, A–C) and roots except for their meristematic zone (Fig. 5, G–M), which is consistent with the staining pattern reported for the *pMSG2-GUS* gene (Tatematsu et al., 2004). Under the control of the *AXR2* promoter, the expression of *axr2-1*::*GFP* and *slr-1*::*GFP* was observed in hypocotyls (Fig. 5, E and F) and the meristematic and elongation zones of roots (Fig. 5, O and P). In the case of the *pAXR2*::*axr2-1*::*GFP* roots, GFP signals were also obvious in the central stele of the apical region of the maturation zone (Fig. 5O). The expression of *AXR2* has been investigated by the use of the GUS fusion gene with the 2.3-kb *AXR2* promoter. GUS activity was found in shoot and root apical meristems of seedlings, but not in either hypocotyls or dark-grown roots except for the vasculature of hypocotyl/root junctions (Tian et al., 2002). Therefore, we observed a wider distribution of the *AXR2* promoter activity in this study. The use of the GFP-tagged axr2-1 or slr-1, both of which are stabilized by a mutation in domain II, may make it possible to detect a lower activity of the *AXR2* promoter.

Expression of *pMSG2*::*axr2-1*::*GFP* was seen in all the epidermal cells of the root hair initiation zone (Fig. 5Q). Root hair formation is regulated through interaction between two types of epidermal cells, trichoblasts and atrichoblasts (Kurata et al., 2005). The fact that the presence of the axr2-1 protein in all the epidermal cells of the root hair initiation zone of the transgenic plants did not inhibit root hair formation suggests that inhibitory effects of the *axr2-1* mutation on the root hair formation may not be epidermis autonomous, but may be brought about by global defects caused by the mutation. We also carried out confocal microscopic measurements of the *pMSG2*::*msg2-1*::*GFP* and *pMSG2*::*axr2-1*::*GFP* embryos. However, we have failed to detect their GFP fluorescence so far (data not shown).

Transcription level of the *mAux/IAA*::*GFP* transgene, determined with quantitative RT-PCR, was relatively similar when driven by the *MSG2* promoter. However, fluorescence of the axr2-1::GFP protein (Fig. 5, B and H) was stronger than that of msg2-1::GFP (Fig. 5, A, G, J, and K) or slr-1::GFP (Fig. 5, C, I, L, and M). When driven by the *AXR2* promoter, mRNA level varied 7-fold among the three transgenic plants (Fig. 6). The varied level was not proportional to level of the mAux/IAA proteins estimated from fluorescent intensity of GFP (Fig. 5) and severity of abnormal phenotypes of the transgenic plants as mentioned above. In particular, no fluorescence of the GFP-tagged msg2-1 protein was observed in *pAXR2*::*msg2-1*::*GFP* (Fig. 5, D and N), while the *pAXR2*::*slr-1*::*GFP* plants fluoresced significantly (Fig. 5, F and P). But, mRNA level of the latter transgene was lower than that of the former transgene (Fig. 6). These results suggest that the stability of the mAux/IAA proteins might differ, possibly

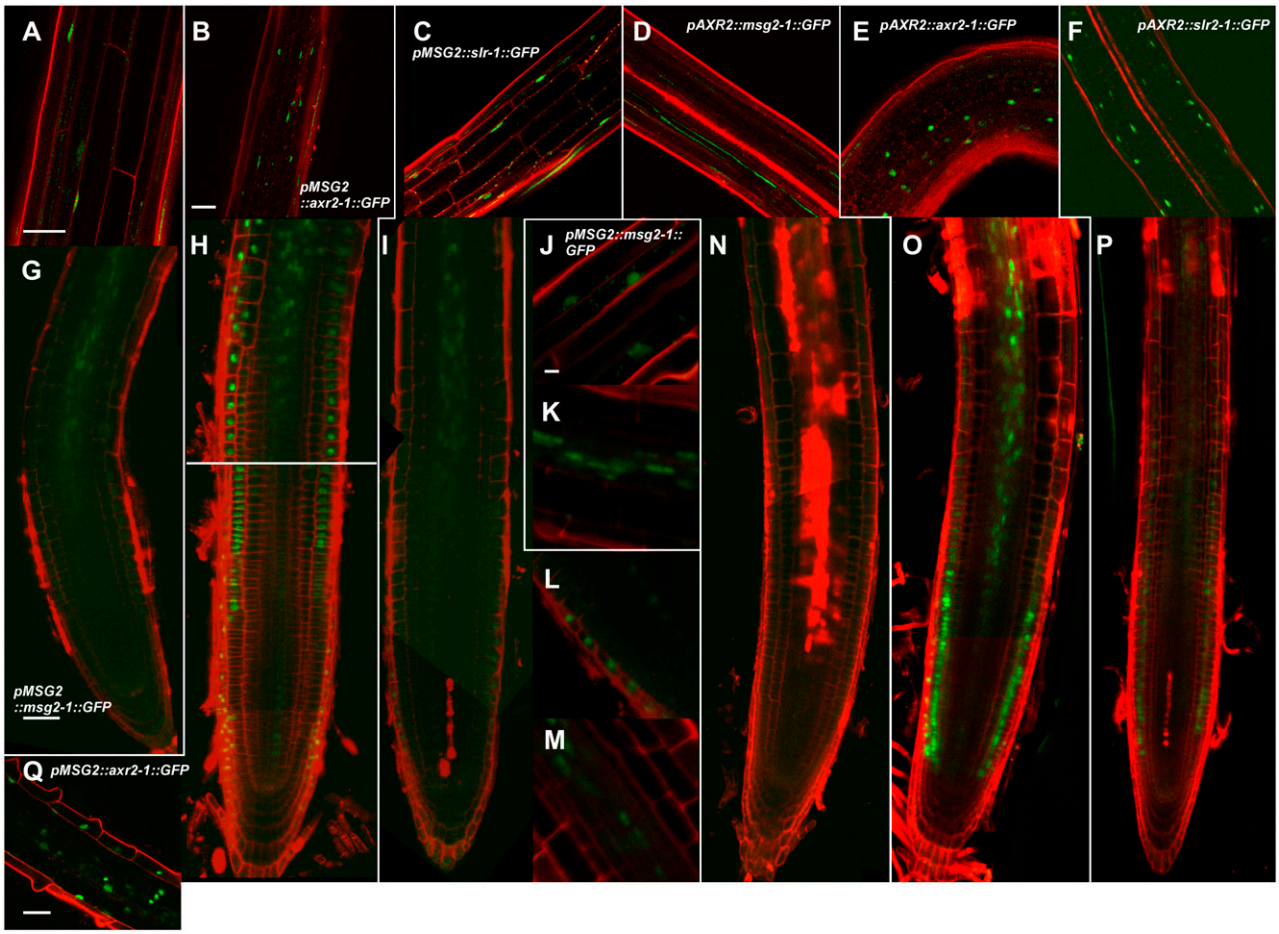

Figure 5. Expression of *mAux/IAA*::*GFP* transgenes observed with confocal fluorescent microscopy. Longitudinal images of GFP were observed in hypocotyls of 3-d-old etiolated seedlings (A–F) and roots of 1-week-old light-grown seedlings (G–Q) counterstained with 10 μg mL^{-1} propidium iodide. A, G, J, and K, *pMSG2*::*msg2-1*::*GFP*; B, H, and Q, *pMSG2*::*axr2-1*::*GFP*; C, I, L, and M, *pMSG2*::*slr-1*::*GFP*; D and N, *pAXR2*::*msg2-1*::*GFP*; E and O, *pAXR2*::*axr2-1*::*GFP*; F and P, *pAXR2*::*slr-1*::*GFP*. Images from A to C, E, J, L, and Q are lateral optical sections; the other images are medial sections. J to M and Q, Enlarged images of the central stele in the elongation zone (K and M), and epidermis in the elongation zone (J), the meristematic zone (L), and the root hair initiation zone (Q). Scale bar, 50 μm (A, B, G, and Q) and 10 μm (J). Scales in B to F are the same; scales in G to I and N to P are the same; and scales in J to M are the same.

with the msg2-1 protein being the least stable of the three.

DISCUSSION

Promoter Activity Often Determines Phenotype of Transgenic Plants

In this study, we created transgenic Arabidopsis plants harboring dominantly mutated *MSG2*, *AXR2*, or *SLR* cDNA driven by the *MSG2* or *AXR2* promoter. All the cDNAs were translationally fused with GFP. Their phenotypes, summarized in Table I, indicate that promoter activity often specifies the phenotype of transgenic plants. This promoter-dependent phenotype must result from the different expression patterns of *MSG2* and *AXR2*. Promoter-*GUS* analyses show that the expression patterns of these genes are rather complementary to each other in etiolated hypocotyls: *AXR2* is expressed only in the apical meristem (Tian et al., 2002), while *MSG2* is expressed in the epidermis, the cortex, and especially the central stele of hypocotyls. In particular, no obvious staining is observed in the apical meristem except for the procambium (Tatematsu et al., 2004; Saito et al., 2007; our unpublished data). These results suggest that the extent of gravitropic defects in hypocotyls may be determined by the expression patterns of the mAux/IAA proteins, and that each of the three proteins may be able to repress the activity of NPH4 and ARF19 (Okushima et al., 2005; Wilmoth et al., 2005) to a similar extent once expressed in the same region. Similar scenarios are conceivable for lateral root formation since all the *pMSG2*::*mAux/IAA*::*GFP* plants exhibit similar defects and since *MSG2* is expressed in root pericycles (Tatematsu et al., 2004) from which the primordia of lateral roots

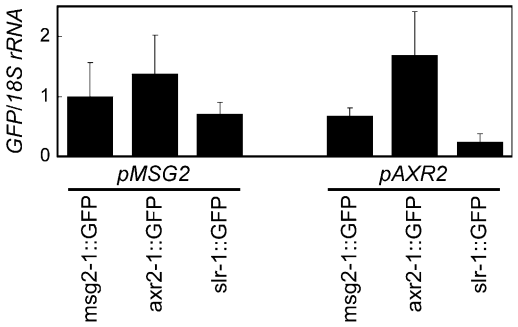

Figure 6. Expression of *mAux/IAA*::*GFP* transgenes determined with quantitative RT-PCR. RT-PCR was done for the sequence encoding GFP in transgenic seedlings grown for 1 week under white-light condition. Expression of the 18S ribosomal RNA was determined as a control. Transcript levels are shown as values relative to those in *pMSG2*::*msg2-1*::*GFP* after normalization to the 18S ribosomal RNA levels. Values indicate mean and SD of three independent experiments.

are formed. Similarly, normal root gravitropism in the *pMSG2*::*mAux/IAA*::*GFP* plants may be explained if we simply assume that root meristem is a critical tissue for root gravitropic response because *MSG2* does not appear to express there. The wild-type stature of the *pMSG2*::*mAux/IAA*::*GFP* plants may also be explained in a similar manner.

When expressed by the *AXR2* promoter, the axr2-1::GFP and slr-1::GFP proteins induced almost identical morphologies in mature plants. This is another example of promoter-dependent phenotypes. Because the two proteins are most closely related in the Aux/IAA family, the distinct shapes of mature plants and the opposite phenotypes in lateral root formation observed in the *axr2* and *slr* mutants (Wilson et al., 1990; Nagpal et al., 2000; Fukaki et al., 2002) must be determined by different activities of the *AXR2* and *SLR* promoters. Essentially the same promoter-dependent phenotypes have been reported in promoter-exchange experiments between *SHY2/IAA3* and *BDL/IAA12* (Weijers et al., 2005). Whereas *shy2-2* mutants have no embryonic phenotypes (Tian and Reed, 1999), *pBDL*::*shy2-2* plants showed a rootless phenotype similar to that of *bdl*. *pBDL*::*shy2-2* plants also showed *bdl*-like postembryonic growth abnormalities. Furthermore, *pSHY2*::*bdl* plants resemble *shy2-2* mutants at both the seedling and mature stages.

Different Functions Are Also Observed among Mutated Aux/IAA Proteins

These results also indicate functional differentiation among the three mAux/IAA proteins when driven by the *AXR2* promoter. Growth fashion of etiolated hypocotyls of each *pAXR2*::*mAux/IAA*::*GFP* was distinct and was similar to that of the original dominant *aux/iaa* mutant. The *msg2-1* and *pAXR2*::*msg2-1*::*GFP* hypocotyls were long and straight, and looked like those of *nph4* (Tatematsu et al., 2004). The *axr2-1* and *pAXR2*::*axr2-1*::*GFP* hypocotyls were short and similar to the *arf6 arf8* double mutants (Nagpal et al., 2005). The curly *slr-1* and *pAXR2*::*slr-1*::*GFP* hypocotyls were distinct from those of other *arf* and *aux/iaa* mutants that have been characterized (Fukaki et al., 2002). The distinct effects of the *mAux/IAA*::*GFP*s should be posttranslational because each transgene was driven by the same promoter with the same 5′ and 3′ untranslated regions. Therefore, the msg2-1 proteins may specifically interfere with the activities of NPH4 and the axr2-1 proteins may specifically interfere with the activities of ARF6 and ARF8, in the *AXR2* expression domain. At present, we cannot identify any ARFs that work with slr-1. These speculations about specific interference between Aux/IAA and ARF were also suggested by a comparison of the shape of transgenic plants at the mature stage when *mAux/IAA*::*GFP* was driven by the *AXR2* promoter. The specificity of mAux/IAA proteins could also arise from a possible difference in stability of the proteins as mentioned above. This suggests the difference of mAux/IAA proteins in AFB-dependent degradation pathway. In this

Table I. *Phenotypes of the transgenic lines examined in this study*

Expressed Protein	Driving Promoter	
	pMSG2	*pAXR2*
msg2-1::GFP	*msg2* phenotype	*msg2* phenotype except for root and severer agravitropism in hypocotyl
axr2-1::GFP	Largely *msg2* phenotype with abnormal cotyledon and more reduced fecundity	*axr2* phenotype except for root
slr-1::GFP	Largely *msg2* phenotype with abnormal cotyledon and more reduced fecundity	*axr2* phenotype in shoot morphology and hypocotyl gravitropism; *slr* phenotype in hypocotyl; wild-type phenotype in root
Phenotypes of Dominant *Aux/IAA* Mutants		
msg2	Agravitropic hypocotyl, fewer lateral roots, reduced fecundity	
axr2	Dwarf morphology, agravitropic hypocotyl and root, de-etiolation in the dark, increased number of lateral roots, no root hair	
slr	Agravitropic hypocotyl and root, no lateral root, no root hair, curly growth of etiolated hypocotyl	

assumption, msg2-1 proteins may have a higher affinity to AFBs than axr2-1 and slr-1 proteins.

Interestingly, expressions of *axr2-1*::*GFP* and *slr-1*::*GFP* under the control of the *MSG2* promoter resulted in aberrant cotyledon development, which had never been observed in *pMSG2*::*msg2-1*::*GFP* plants or the *msg2-1* mutant so far. Taken together, these results clearly show that msg2-1 functions differently from axr2-1 and slr-1, and there appear to be some differences between functions of axr2-1 and slr-1 even though they are most closely related with respect to their primary sequences (Remington et al., 2004).

Similar conclusions have been reached for the relationship between shy2/iaa3 and axr3/iaa17 (Knox et al., 2003) or bdl/iaa12 (Weijers et al., 2005). Transient expression of the *shy2-6* and *axr3-1* cDNAs by the same soybean (*Glycine max*) heat shock promoter reproduces the different root hair phenotype of the original mutant (Knox et al., 2003). On the other hand, expression of shy2-2 protein under the control of the *SHY2* promoter inhibits gravitropism, auxin sensitivity, and auxin-induced gene expression in roots. However, expression of bdl protein under the control of the *SHY2* promoter does not have these effects. Although embryonic defects are qualitatively determined by transcriptional regulation, there are clear quantitative differences in embryonic root initiation between shy2-2 and bdl action when driven by the *BDL* promoter: The shy2-2 protein is less effective than the bdl protein (Weijers et al., 2005). Although different properties are observed between mAux/IAA proteins that are stabilized by dominant mutations in domain II, these results suggest that the wild-type Aux/IAA proteins also have distinct functions as well as shared functions.

Weijers et al. (2005) found the promoter-dependent phenotype in embryo, hypocotyl, and shoot, and the protein-dependent phenotype in root development. In this study, we observed both the promoter-dependent and the expressed protein-dependent phenotypes in one organ, the hypocotyl. However, our finding is not surprising considering that hypocotyls consist of a few cell layers and a dozen cell types. Recently, Fukaki et al. (2005) investigated the *slr* defects in roots using tissue-specific promoters and succeeded in identifying the cell types in which *slr* functioned. Their results highlight the importance of knowledge on cells crucial for each auxin phenotype. In conclusion, it is necessary to know the sites of *Aux/IAA* action at the cell level, as well as the strength of the molecular interaction between Aux/IAAs and target ARFs or AFBs to further understand the molecular basis for specificity and similarity of the function of Aux/IAA proteins.

Cotyledons Develop Aberrantly When axr2-1::GFP or slr-1::GFP Is Expressed by the *MSG2* Promoter

The *pMSG2*::*axr2-1*::*GFP* or *pMSG2*::*slr-1*::*GFP* plants with no cotyledons look very similar to the *pin-formed1* (*pin1*) *pinoid* (*pid*) double mutants (Furutani et al., 2004), which develop pin-like inflorescences. However, vegetative and reproductive development of the transgenic plants is relatively normal. The transgenic plants with only one cotyledon are somewhat similar to the single cotyledonous phenotype sometimes observed in the *mp* single mutants or the *nph4 mp* double mutants (Hardtke et al., 2004), although these mutations also affect embryonic development of axes and roots. During the transition from the globular stage to the heart stage of the embryo, cotyledonary primordia are formed in places where auxin accumulates, owing to the function of the auxin efflux facilitator PIN1 (Friml et al., 2004) and PID kinase that appears to determine subcellular positioning of PIN1 (Benková et al., 2003; Jenik and Barton, 2005). Our present results suggest that ectopic expression of axr2-1 or slr-1 in the cotyledonary primordia interferes with auxin signaling and arrests growth and development of the cotyledon. The molecular targets of both mutated proteins might be NPH4 and MP.

It is interesting that neither *pMSG2*::*msg2-1*::*GFP* nor *msg2-1* exhibits any defects in embryogenesis. msg2-1 might not be able to interact with MP as readily as axr2-1 or slr-1. However, the interaction between MSG2 and CTD of MP (MP-CTD) has been shown to be as strong as that between MSG2 and NPH4-CTD by yeast two-hybrid assay (Tatematsu et al., 2004) and fluorescence cross-correlation spectroscopy in HeLa cells (Muto et al., 2006). The interaction between MSG2 and NPH4-CTD appears strong enough to bring about tropistic defects in *msg2-1* hypocotyls (Tatematsu et al., 2004). Apparently, full-length MP and NPH4 proteins must be used in further analyses of molecular interaction with Aux/IAA proteins.

We found that *MSG2* was expressed in primordia of the cotyledon during the transition stage from globular to heart stage. The restricted expression of *MSG2* in the primordia is in sharp contrast to the largely uniform, low-level expression of *NPH4* and *MP* in the embryo (Hamann et al., 2002; Hardtke et al., 2004; Weijers et al., 2006). *NPH4* and *MP* are expressed uniformly in subepidermal tissues of early embryos, being gradually confined to the vascular precursor cells. *MSG2* expression in the embryo is also distinct from that of other *Aux/IAA* genes, *BDL/IAA12*, *SHY2/IAA3* (Hamann et al., 2002; Weijers et al., 2006), and *IAA13* (Weijers et al., 2005). The expression pattern of *BDL* is very similar to that of *MP*, while *SHY2* is not expressed until the mid-torpedo stage. *SHY2* is subsequently expressed in provascular cells. The distinct pattern of *MSG2* expression in embryogenesis suggests that MSG2, in addition to functioning in tropistic responses in hypocotyls and lateral root formation (Tatematsu et al., 2004; Saito et al., 2007), also functions in the formation of cotyledons.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

Seeds of Arabidopsis (*Arabidopsis thaliana*) were first imbibed in water in the dark at 4°C for 3 d. They were surface sterilized with 1% hypochlorite, and

sown on nutrient agar plates that contained half-strength Murashige and Skoog salts (Murashige and Skoog, 1962), 1% (w/v) Suc, and 1% (w/v) agar. Plants were grown at 23°C under continuous illumination at a fluence rate of 14 W m^{-2}, obtained from three 40-W white fluorescent tubes (FL40SW; NEC). In some experiments, plants were grown on a 1:1 (v/v) vermiculite:Metromix 350 (Scotts-Sierra).

Gravitropism of hypocotyls was examined according to Nakamoto et al. (2006). In brief, seedlings were grown on vertically oriented agar plates for 3 d in the dark and then turned 90° to a horizontal position. An image of the seedlings was taken 15 h after the turn with a digital camera (C-4040 Zoom; Olympus), and hypocotyl curvature was measured with Image Pro-Plus (Media Cybernetics).

Transgenic Plants

For promoter-swapping experiments, 2-kb promoter regions of *MSG2* and *AXR2*, which contained 5′ untranslated region, were amplified by PCR from DNA of wild-type Columbia using a pair of oligonucleotide primers: 5′-CACCTAAGAAACATGAGACATGTCACAA-3′ and 5′-GCATGCATATATATAGTCGACTTCTTGAACTTCTTTTTTTCCTCT-3′ for *MSG2*, and 5′-CACCGATCAAAACGGATCACAAAATTAA-3′ and 5′-GCATGCATATATATAGTCGACGTTACTTGTAATAGATTAGAAATA-3′ for *AXR2*. All the forward primers contained CACC sequence at the 5′ ends for directional TOPO-cloning (Invitrogen), and all the reverse primers contained the *Sal*I and *Sph*I sites at the 5′ ends. The PCR products were subcloned into pENTR/D-TOPO (Invitrogen). The sGFP gene (Niwa, 2003) was amplified from pGWB4 (Huang et al., 2006) by PCR using forward primers containing the *Sal*I and *Sma*I sites at the 5′ end and reverse primers with the *Sph*I site at the 5′ end. The PCR product was inserted between the *Sal*I and *Sph*I sites at the 3′ end of each promoter. The *Aux/IAA* cDNA that has a dominant mutation was amplified from total RNA prepared from *msg2-1*, *axr2-1*, and *slr-1* by RT-PCR using a pair of oligonucleotide primers: 5′-GTCGACATGGAGAAGGAAGGACTCGGGCTT-3′ and 5′-GCATGCCTCGTCTACTCCTCTAGGCTGCAG-3′ for *msg2-1*, 5′-GTCGACATGATCGGCCAACTTATGAACCTC-3′ and 5′-GCATGCAGATCTGTTCTTGCAGTACTTCTC-3′ for *axr2-1*, and 5′-GTCGACATGAACCTTAAGGAGACGGAGCTT-3′ and 5′-GCATGCTGATCTGTTCTTGAACTTCTCCAT-3′ for *slr-1*. All the forward and reverse primers contained the *Sal*I and *Sma*I sites at the 5′ ends, respectively. The PCR product was inserted between promoter and sGFP using the *Sal*I and *Sma*I sites. The DNA fragment, promoter::*mAux/IAA* cDNA::*GFP*, was inserted in T-DNA of the gateway binary vector pGWB1 (Huang et al., 2006) using LR clonase (Invitrogen). The constructs were introduced into *Agrobacterium tumefaciens* strain pGV3101 by electroporation, which was then used to transform Arabidopsis Columbia ecotype by flower dip method (Clough and Bent, 1998).

Microscopy

For GFP analysis, hypocotyls and roots were counterstained with 10 μg mL^{-1} propidium iodide (Dojindo) and placed on slides in a drop of water. Fluorescence was imaged by a confocal microscope (LSM510; Zeiss). GFP was excited at the 488 nm laser line of a CW Ar^{+} laser, and propidium iodide was excited at the 543 nm laser line of a CW He-Ne laser through a water immersion objective (C-Apochromat, 40×, 1.2NA; Zeiss). Emission signals were detected at 500 to 530 nm for GFP and >560 nm for propidium iodide by sequential scanning.

For GUS staining, young siliques were fixed with ice-cold 50% acetone for 15 min. After rinse with 50 mM sodium phosphate, pH 7.0, thrice, ovules were taken off from siliques and stained for GUS activity by incubation in 50 mM sodium phosphate, pH 7.0, containing 2 mM 5-bromo-4-chloro-3-indolyl-β-D-glucuronide, 10 mM $K_3Fe(CN)_6$, 10 mM $K_4Fe(CN)_6$, 1 mM EDTA, and 0.1% Triton X-100, at 37°C for 16 h. Then, they were rinsed with the above phosphate buffer and examined with a light microscope (Zeiss Axioplan) equipped with a digital camera (DXM1200; Nikon).

RT-PCR

Total RNA was prepared from 1-week-old seedlings grown under continuous white light using RNeasy Plant Mini kit (QIAGEN). After DNase treatment (RQ1 RNase-free DNase; Promega), RT was carried out using Moloney murine leukemia virus reverse transcriptase RNase H^{-} (ReverTra Ace; Toyobo) with random primers. Quantitative PCR was performed using Power SYBR Green PCR Master Mix (Applied Biosystems) and a real-time PCR system (model 7300; Applied Biosystems). The PCR primers 5′-AAGCAGAAGAACGGCATCAAG-3′ and 5′-GGACTGGGTGCTCAGGTAGTG-3′ were used to amplify the transgene. *ACTIN2* and *18S rRNA* genes were used as internal controls with primers 5′-CGCTCTTTCTTTCCAAGCTCATA-3′ and 5′-CCATACCGGTACCATTGTCACA-3′, and 5′-ACGCGCGCTACACTGATGTA-3′ and 5′-TGATGACTCGCGCTTACTAGGA-3′, respectively. Essentially the same results were obtained by use of either control gene. Experiments were carried out for three independently prepared total RNA samples.

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