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The *axr6* mutants of *Arabidopsis thaliana* define a gene involved in auxin response and early development

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SUMMARY

The indolic compound auxin regulates virtually every aspect of plant growth and development, but its role in embryogenesis and its molecular mechanism of action are not understood. We describe two mutants of *Arabidopsis* that define a novel gene called *AUXIN-RESISTANT6* (*AXR6*) which maps to chromosome 4. Embryonic development of the homozygous *axr6* mutants is disrupted by aberrant patterns of cell division, leading to defects in the cells of the suspensor, root and hypocotyl precursors, and provasculature. The homozygous *axr6* mutants arrest growth soon after germination lacking a root and hypocotyl and with severe vascular pattern defects in their cotyledons. Whereas previously described mutants with similar developmental defects are completely recessive, *axr6* heterozygotes display a variety of morphological and physiological alterations that are most consistent with a defect in auxin physiology or response. The *AXR6* gene is likely to be important for auxin response throughout the plant, including early development.

Key words: Auxin, *Arabidopsis thaliana*, Embryogenesis

INTRODUCTION

The plant hormone auxin (indole-3-acetic acid) has been shown to regulate a wide variety of plant processes, including tropisms, vascular development and formation of lateral shoots and roots (for reviews see Aloni, 1995; Malamy and Benfey, 1997; Chen et al., 1999). At the cellular level, auxin affects elongation, division and differentiation, but the mechanisms by which it produces these cellular effects remain poorly understood. The auxin-binding protein ABP1 appears to function as an auxin receptor regulating cell elongation (Jones et al., 1998); roles as receptors for other auxin-binding proteins have not been demonstrated. The products of the *AXR1* (Leyser et al., 1993) and *TIR1* genes (Ruegger et al., 1998) are likely to regulate auxin response through controlling protein modification by ubiquitin or ubiquitin-like molecules (del Pozo et al., 1998). Possible targets of such ubiquitin- or ubiquitin-like modification are the proteins encoded by the Aux/IAA family of genes (Abel et al., 1994), short-lived, nuclear-localized proteins whose transcription is induced by auxin and which may encode transcriptional regulators (Abel and Theologis, 1996). The related Auxin Response Factor (ARF) genes encode proteins demonstrated to bind to and regulate transcription at auxin-responsive promoters (Ulmasov et al., 1999). The importance of these proteins in auxin response has been demonstrated by genetic approaches: three *Arabidopsis* mutants with defects in auxin-related processes, *auxin-resistant3* (Rouse et al., 1998), *short hypocotyl2* (Tian and Reed, 1999), and *monopteros* (Hardtke and Berleth, 1998) were found to have defects in Aux/IAA or ARF genes. There is also some evidence that the auxin signal transduction cascade may include components of a mitogen-activated protein kinase (MAPK) cascade (Kovtun et al., 1998; Mizoguchi et al., 1994), although not all experiments supports this model (Tena and Renaudin, 1998).

The factors controlling embryonic development in plants are still poorly understood, but evidence suggests that short-range or direct cell-cell interactions may play important roles (Laux and Jürgens, 1997). Putative auxin gradients established by auxin polar transport (Lomax et al., 1995) may be important for establishing bilateral symmetry and polarity during embryonic development (Schiavone and Cooke, 1987; Liu et al., 1993; Fischer and Neuhaus, 1996; Hardtke and Berleth, 1998), although the only direct evidence that auxin acts as a positional morphogen comes from studies of cambial development (Uggla et al., 1998). Auxin has been further implicated in embryonic pattern formation by characterizaton of three embryonic mutants. *fass* mutants (Torres-Ruiz and Jürgens, 1993) were shown to have defects in auxin homeostasis (Fisher et al., 1996). Homozygous *monopteros* mutants, with mutations in an Auxin Response Factor gene

(Hardtke and Berleth, 1998), show severe defects in development of the basal region of the embryo, resulting in seedlings that sometimes possess only a single cotyledon after germination, and lack roots and hypocotyl; the mutants also show dramatically aberrant vascular development (Berleth and Jürgens, 1993; Przemeck et al., 1996). The recently isolated *bodenlos* mutant has phenotypic similarities to *monopteros* and a reduced response to auxin in a tissue culture assay (Hamann et al., 1999).

In the course of a series of screens for auxin-resistant mutants, we isolated two mutants in a novel gene called *AXR6*. The unique phenotype of these mutants, described in this paper, indicates that the gene is likely to be important for auxin response in all parts of the plant, and supports a role for auxin in pattern formation and cell determination during embryogenesis.

MATERIALS AND METHODS

Plant material

Arabidopsis thaliana plants were grown at 20-23°C on the soilless mixture MetroMix 360 (GraceSierra) with continuous light at a photon fluence rate of 70-120 μmol/m^2/second. To score for seedling resistance to hormones and antibiotics and to perform root growth and gravitropism assays, seedlings were grown under sterile conditions on Petri plates containing nutrient solution (Wilson et al., 1990) supplemented with 7 g/l agar and 10 g/l sucrose (minimal medium). Hormones or antibiotics (Sigma) were added after the solution was autoclaved. Seeds were surface sterilized for 15-25 minutes in 30% (v/v) bleach, 0.01% Triton X-100, washed 3 times with sterile water, and spread using 0.7% agar. Seeds were cold-treated for at least 2 days at 4°C before plates were placed in a CU-32L growth chamber (Percival Scientific) at 20-22°C on a 16-hour light/ 8-hour dark cycle with a photon fluence rate of 70-100 μmol/m^2/second (standard conditions). The day of placing into the growth chamber is considered day 0. Auxin sensitivity or resistance was routinely scored on day 7 of growth on minimal medium containing 8×10^{-8} M 2,4-dichlorophenoxyacetic acid (2,4-D). When using segregating populations of seed (as in the assays of heterozygous physiology and morphology), the genotype of viable plants (heterozygous *axr6* or wild type) was determined by one of the following criteria: auxin resistant root growth of seedlings, rosette morphology, and testing progeny for auxin resistance and presence of homozygous mutants. The tetraploid plant CS3432 was obtained from the *Arabidopsis* Biological Resource Center.

Mutagenesis

Columbia ecotype seeds (10,000) were allowed to imbibe overnight (~18 hours) in water, and then were treated with 10 mM diepoxybutane (DEB; Sigma) for 5 hours. Seeds were washed extensively with water and planted in two independent populations in flats at 5000 seeds/flat. M_2 seeds were harvested in bulk.

Mutant isolation

Approximately 25,000 seed from each independent DEB-mutagenized populations were sterilized, spread on plates containing 1×10^{-7} M 2,4-D, cold-treated for 2 days, and then screened for seedlings with long roots up to the 9th day after placing in the growth chamber. 36 putative auxin-resistant mutants were isolated and their M_3 progeny retested. After back-crossing and complementation tests (data not shown), nine of these mutants were found to be allelic to *aux1* (Maher and Martindale, 1980; Pickett et al., 1990), two are allelic to *axr1* (Lincoln et al., 1990), one is the *axr3-3* allele (Leyser et al., 1996), and two are alleles of *ein2* (Guzman and Ecker, 1990; Masucci and Schiefelbein, 1996). The initial isolates of the *axr6* mutants were backcrossed to wild type at least 5 times (*axr6-1*) or 6 times (*axr6-2*) before genetic and physiological characterization.

Mapping

Heterozygous *axr6* mutants were crossed to Landsberg *erecta* plants and auxin-resistant F_1 plants identified. DNA was isolated from individual auxin-sensitive F_2 plants and scored for the set of 13 ARMS markers (Fabri and Schäffner, 1994). No linkage was found to markers other than m326 (map position 4:49.1; 11/96 recombinant F_2 chromosomes) and m448a (map position 4:18.7; 4/96 recombinant F_2 chromosomes; genome-www3.stanford.edu/cgi-bin/Webdriver?MIval=atdb_rimap_con). The *AXR6* gene thus appears to be 4/96 ≃4.2 cM distal from m448a. The map distance between *axr6* and *prl* was determined to be ~7.3 cM by analysis of 602 F_2 seedlings from a cross between *axr6-2* and *prl* (Cesarski, 1997).

Analysis of embryogenesis

Siliques from wild-type and heterozygous mutant plants were prepared for whole-mount visualization as in Berleth and Jürgens (1993). Ovules were photographed using differential interference contrast optics on a Zeiss Axiophot microscope.

Analysis of seedling vascular patterns

Homozygous seedlings were identified by their morphology after germination on hormone-free minimal medium, fixed with ethanol:acetic acid: water (6:3:1), and then cleared and mounted in Hoyer's solution. Vascular patterns were photographed using dark-field optics on a Zeiss Axiolab microscope (×2.5, ×5 and ×10 objectives). Photographs were projected and seedlings measured using a map tool, calibrated with a photograph of a stage micrometer.

Growth assays

Root growth assays were performed essentially as described by Hobbie and Estelle (1995) and Wilson et al. (1990). Data are presented as the elongation on the hormone relative to the elongation on hormone-free medium.

Assays of hypocotyl elongation were performed by cold-treating sterilized seeds for 3-5 days and then spreading directly onto minimal medium plates without sucrose and with or without IAA, exposing to room light for 45 minutes, and then placing in a light-tight box in the growth chamber. After 7 days, seedling hypocotyl length was measured.

Lateral root growth and root elongation were measured daily under a dissecting microscope on seedlings that had been germinated on hormone-free medium in a vertical orientation and transferred on day 3 to large (150 mm) plates containing minimal medium, the root tip marked, and the plates replaced in the growth chamber in a vertical position.

Morphometric analysis

Seeds were placed in 5-inch clay pots (4 plants/pot), cold-treated for 9 days, and then placed under constant illumination. Pots were watered with 1× nutrient solution (Wilson et al., 1990) at 7, 13 and 24 days, at other times as needed with tap water. Bolting time was analyzed on plants grown in a similar manner. Bolting was defined as the appearance of an inflorescence at least 5 mm high.

Gravitropic response

On day 3 of growth on hormone-free medium in a vertical orientation, seedlings were transferred to square plates of hormone-free medium and returned to the growth chamber in vertical orientation. After two additional days of growth, plates were rotated by 90 degrees, photographed, and then photographed at the indicated

times thereafter. Negatives were scanned and root positions traced in Adobe Photoshop; the angle of root tip reorientation was measured by comparing the 0 time point with each subsequent photograph.

Analysis of BA3-GUS expression

Plants containing the BA3-GUS construct (Oono et al., 1998; obtained from A. Theologis) were crossed to *axr6* mutant plants and lines homozygous for the BA3-GUS construct and containing the *axr6* mutant allele or *AXR6* wild-type allele were identified by testing F_3 families for kanamycin resistance and auxin response. GUS expression was analyzed by a modified version of the procedure of Oono et al. (1998). After the indicated growth on plates with or without auxin, seedlings were washed twice and then vacuum-infiltrated for 30 minutes with buffer containing 1 mM 5-bromo-4-chloro-3-indolyl β-D-glucuronide (LabScientific), 10 mM $NaPO_4$, pH 7, 2 mM EDTA, 0.1% TritonX-100, then incubated at 37°C in the dark for the indicated times, mounted in 50% glycerol, and photographed.

Tissue culture

Seedlings were grown on hormone-free minimal medium for 7 days, and then *axr6* homozygotes or excised wt cotyledons were transferred to MSN medium consisting of 1× Murashige-Skoog salts (Sigma), pH 5.7, 1× Murashige-Skoog vitamins (Sigma), 0.5% agar, 0.5% sucrose, and 64.5 μM naphthaleneacetic acid and grown in standard conditions for 32 days.

Photography and figures

Photos were taken using Kodak Ektachrome T160 color slide film or Agfa APX25 black-and-white film and scanned using a Nikon LS-1000 35 mm film scanner. Adobe Photoshop 4.0 was used to process photographs and assemble figures.

ANOVA statistical analysis used the Analysis ToolPak of Microsoft Excel 5.0 for Macintosh.

RESULTS

Mutant isolation and genetic characterization

In an effort to identify new mutants affected in auxin response, diepoxybutane-mutagenized *Arabidopsis* seeds were screened for auxin-resistant root growth on nutrient agar medium containing 1×10^{-7} M of the synthetic auxin 2,4-D (see Materials and Methods). Two mutants with similar novel phenotypes, described below, were isolated from independent populations; the mutants subsequently proved to be produced by alleles of the same gene, and were named *auxin-resistant6-1* (*axr6-1*) and *axr6-2*.

Seedlings derived from self-fertilization of the auxin-resistant *axr6* plants fell into three classes: wild type auxin-sensitive, mutant auxin-resistant, and rootless, in approximately a 1:2:1 ratio (Table 1). Crosses to wild-type plants demonstrated that the auxin-resistant plants are heterozygous for the *axr6* mutation (Table 1); the rootless seedlings are the *axr6* homozygotes. Crosses between *axr6-1* and *axr6-2* heterozygotes gave 1/4 rootless progeny, demonstrating allelism (Table 1). A reduced frequency of homozygotes in the progeny of *axr6-1* heterozygotes was shown not to be due to reduced transmission of the mutant allele, and thus probably results from reduced viability of the homozygotes during embryogenesis or germination (Table 1). Crossing the *axr6-2* mutant to a tetraploid plant produced triploid seedlings (*axr6-2/AXR6/AXR6*) that were auxin resistant (Table 1); this suggests that at least this allele is a gain-of-function mutation. No other mutants with the homozygous rootless morphology that also show a mutant phenotype in the heterozygote have been reported.

The location of the *AXR6* gene was determined by a combination of molecular and classical mapping approaches (see Materials and Methods). The *AXR6* gene maps to the interval between m448a and *PRL* on the short arm of chromosome 4. No other auxin-resistant or seedling-lethal mutations map to this region (genome-www3.stanford.edu/cgi-bin/AtDB/). The *MONOPTEROS* and *BODENLOS* genes, which have mutant phenotypes similar to those of *axr6* homozygotes, both map to chromosome 1 (Hardtke and Berleth, 1998; Hamann et al., 1999). Therefore, the *axr6* mutants define a novel gene.

Homozygote morphology

Wild-type seedlings have two cotyledons (seed leaves) connected by the hypocotyl to the primary root; by 14 days, they develop true leaves and lateral roots (Fig. 1).

Table 1. Genetic analysis of *axr6*

Genotypes of parents		Progeny: Auxin-sensitive (wt)	Progeny: Auxin-resistant (mutant)	Progeny: Rootless (1 cot., >1 cot.)	(Hypothesis) χ^2
axr6-1/AXR6 (self-fertilization)	*axr6-1/AXR6*	221	304	87 (72, 15)	(1:2:1) 58.7*
axr6-2/AXR6 (self-fertilization)	*axr6-2/AXR6*	135	253	108 (5, 103)	(1:2:1) 3.14
axr6-1/AXR6	*axr6-2/AXR6*	80	150	95	(1:2:1) 3.51
axr6-1/AXR6 (female)	*AXR6/AXR6*	179	164	0	(1:1) 0.66
axr6-1/AXR6 (male)	*AXR6/AXR6*	60	51	0	(1:1) 0.73
axr6-2/AXR6 (male)	*AXR6/AXR6*	25	22	0	(1:1) 0.19
axr6-2/AXR6 (male)	*AXR6/AXR6/AXR6/AXR6* (tetraploid)	67	47	0	(1:1) 3.51

*P<<0.01; for all other results, P>0.05

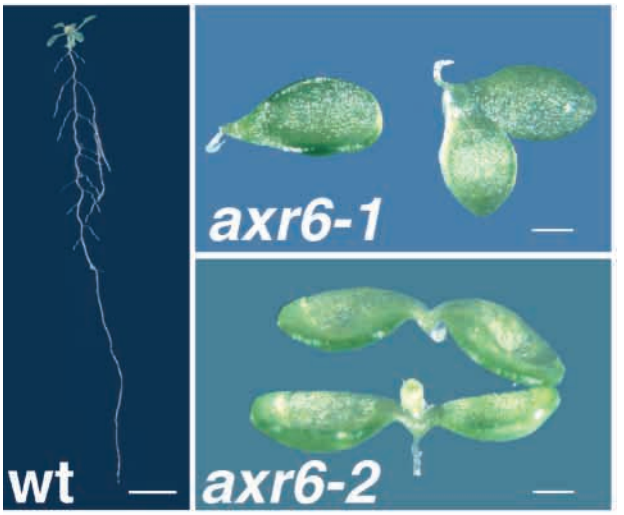
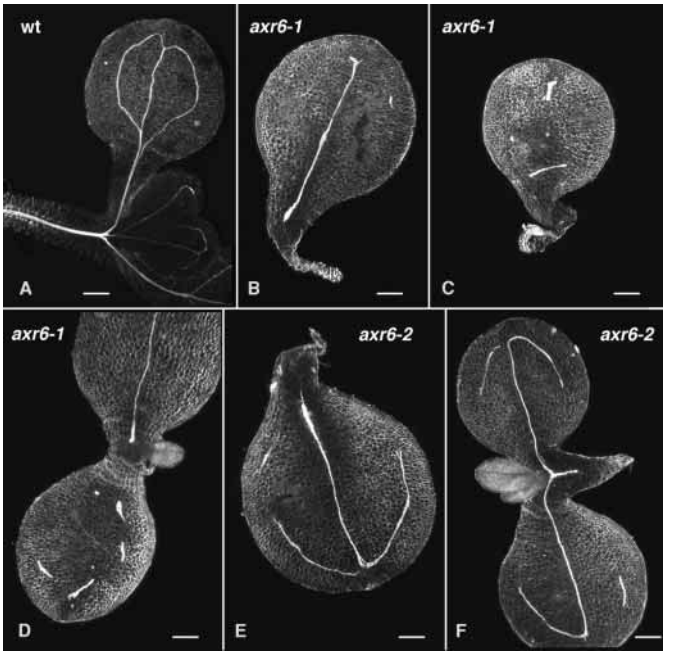


Fig. 1. Wild-type and homozygous *axr6* seedlings. Seedlings were grown on hormone-free medium under standard conditions for 14 days. Bar in wt, 10 mm; bars in mutants, 0.5 mm.

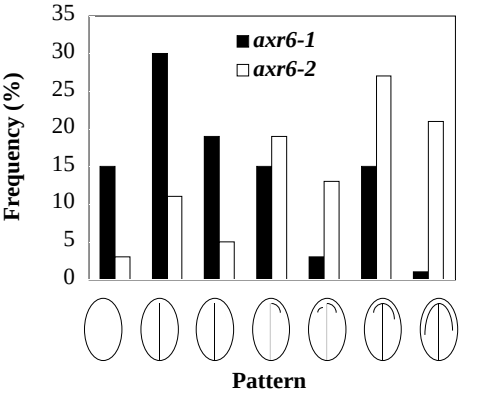
Homozygous *axr6* seedlings display a dramatically different morphology: one or two cotyledons are attached to a small basal stub or peg of whitish tissue. The *axr6-1* homozygotes generally produce only a single cotyledon, but are occasionally found with two cotyledons (Fig. 1; Table 1). The *axr6-2* homozygotes generally produce two cotyledons but are very rarely found with just a single cotyledon, and may sometimes even produce a few tiny true leaves (Fig. 1; Table 1). The homozygous seedlings of either allele with two cotyledons usually show a normal 180 degree angle between the two cotyledons; an unusual *axr6-1* exception is shown in Fig. 1. Rarely, homozygous seedlings with exceptional morphologies are found; these include irregularly shaped single cotyledons and two partially separated cotyledons. Homozygous seedlings also frequently develop a purplish color assumed to be anthocyanin accumulation.

Vascular development

Auxin's importance for vascular development is well known (Aloni, 1995), and consistent with the abnormal vascular patterns found in the cotyledons of the *mp* mutants (Berleth and Jürgens, 1993). Fig. 2 compares the vascular development in wild type and in homozygous *axr6* mutant seedlings at 7 days of growth. Whereas wild-type cotyledons have a single central strand and at least one semicircular strand running around the periphery of each half of their cotyledons, the homozygous *axr6* mutants have a variety of incomplete vascular patterns (Fig. 2A-F). Most, but not all, of the homozygous *axr6* seedlings have a complete central strand, but the peripheral strands are in general partial and incomplete, and in some cases consist of only a short segment completely detached from the central vasculature. Quantitative analysis of the vasculature in mutant and wild-type seedlings revealed the following: the frequency of the most severely



G. Frequency of Vascular Patterns



H. Vein Length

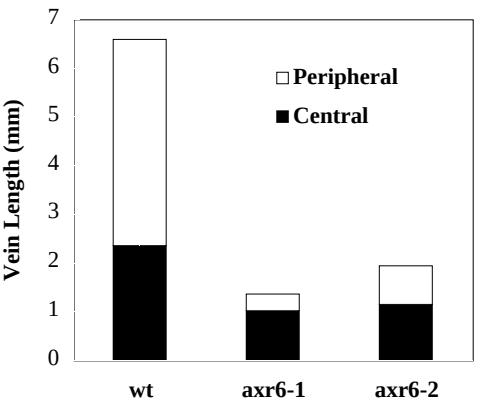


Fig. 2. Vascular development in wild-type and *axr6* homozygous cotyledons. Seedlings were grown on hormone-free medium for 7 days, cleared, and photographed under dark-field illumination. (A-F) Representative patterns. Bar, 0.1 mm. (G) Frequency of vascular patterns in 7 day seedlings. Complete patterns (wild type) are included in the last category. No. of cotyledons measured: 85 for wt, 102 for *axr6-1*, and 150 for *axr6-2*. (H) Length of central and peripheral vasculature in 7 day seedlings. Differences between wild-type and both mutants in total length, peripheral length and central length were all highly statistically significant ($P<<0.001$) by an ANOVA test. Same sample size as for G.

defective patterns was higher in *axr6-1* than in *axr6-2* (Fig. 2G), both central and peripheral vein length showed significant decreases in both mutants, compared to wild type, and the reduction in length of peripheral veins was much greater than that of the central vein in the mutants (Fig. 2H). Similar defects were seen in seedlings of different ages (data not shown), indicating that the mutant phenotypes reflect defects rather than mere delays in development. To summarize, seedlings homozygous for the *axr6-1* and *axr6-2* mutations displayed dramatic deficiencies in vascular development.

Morphology of the basal stub

Stained cross sections of the basal stub of the *axr6* homozygotes revealed that it was composed of large morphologically undifferentiated cells (not shown), and was very similar to the basal stub of *monopteros* homozygotes (Berleth and Jürgens, 1993). Occasionally root hairs were present at the very tip of the stub (not shown), as seen in *bodenlos* homozygotes (Hamann et al., 1999).

Homozygote development

The variety of abnormalities found in the *axr6* homozygous seedlings after germination suggested that these defects must originate during embryonic development. To determine how embryonic development of the *axr6* homozygotes deviates from that of wild type, the morphology of embryos in siliques from heterozygous *axr6* plants was analyzed by differential interference contrast microscopy of cleared whole embryos at different stages. Fig. 3 presents a developmental series of control wild-type and homozygous *axr6* embryos, showing some of the patterns that were consistently seen in the mutants.

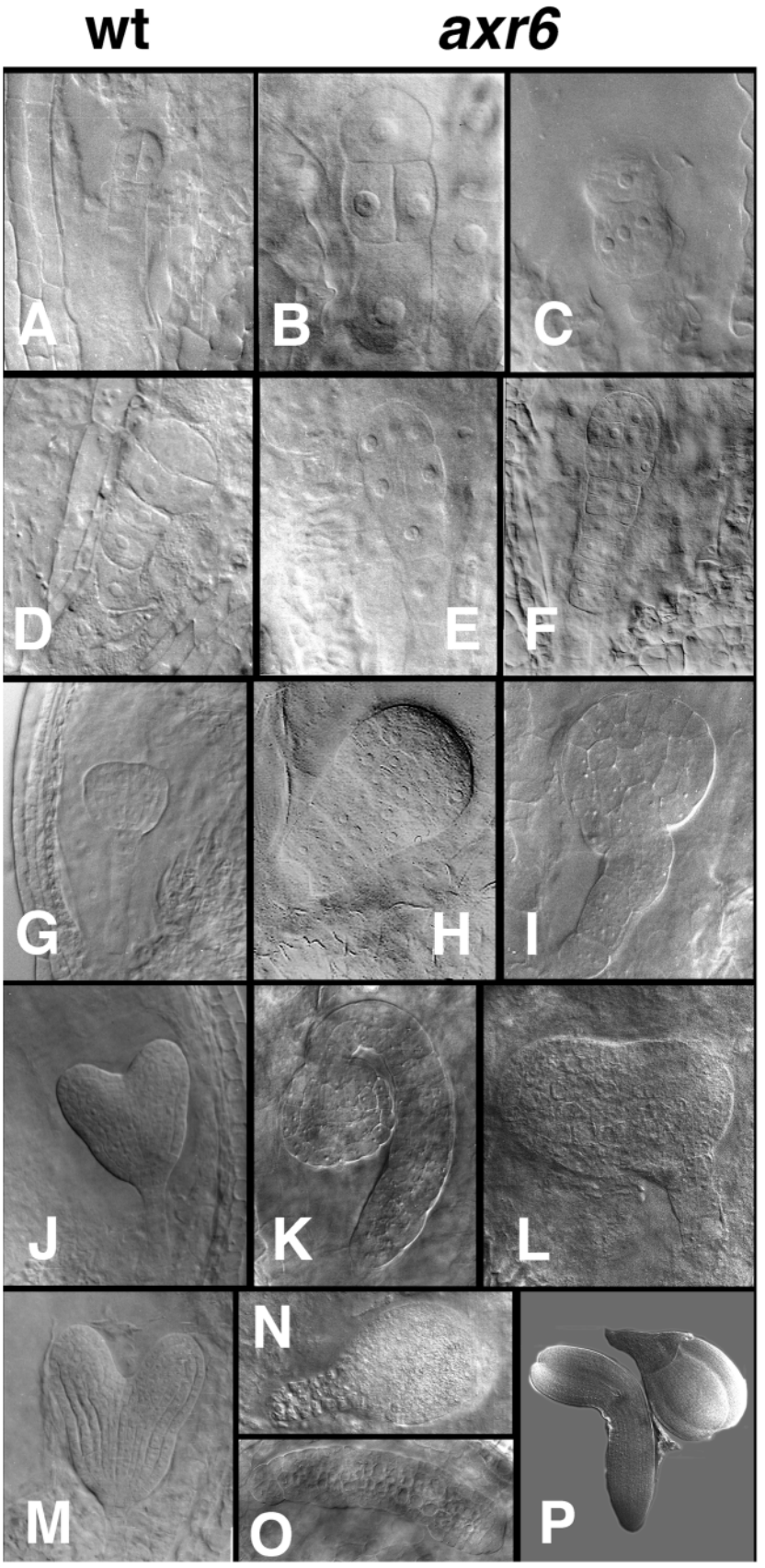


Fig. 3. Embryonic development of wild type and *axr6* homozygotes. Embryos were cleared and visualized by differential interference microscopy. (A) Wild type, 4-cell stage, showing the vertical division of the apical cell. (B) *axr6-1*, showing abnormal periclinal division of second cell. (C) *axr6-1*, showing abnormal arrangement of three cells in position of the uppermost cell of suspensor. (D) Wild type, 8-cell stage. (E) *axr6-1*, with five cells in embryo (three in front are visible; two behind are out of plane of focus) and two cells in position of the uppermost suspensor cell. (F) *axr6-1*, showing two-cell-wide suspensor that has lengthened by repeated transverse divisions. (G) Wild type, triangular stage, showing 3 distinct tiers of cells. (H) *axr6-1* late globular stage, with abnormal multiple tiered embryo. (I) *axr6-1* globular stage, with poor cell alignment and abnormal uppermost suspensor cell. (J) Wild type, heart stage. (K) *axr6-1* globular stage, with several-cell-wide suspensor and poorly aligned cell files. (L) *axr6-1* heart stage, with poorly aligned cells. (M) Wild type, late heart stage, with clear cell tiers and aligned cell files. (N) *axr6-1*, mature, three-cell-wide suspensor. (O) *axr6-1*, no defined embryo visible. (P) Mature wild type (left) and *axr6-1* (right).

The wild-type zygote divides asymmetrically to give rise to a small cytoplasmically dense apical cell and an elongated basal cell. The apical cell divides in a regular pattern to produce most of the embryo proper; the basal cell divides transversely several times, yielding an elongated single-cell-wide suspensor which connects the embryo to the maternal tissue (Fig. 3A,D,G,J,M). The most striking differences in the early embryogenesis of the *axr6* homozygotes are in the divisions of the suspensor, which divides periclinally one or more times before the normal anticlinal orientation of divisions resumes. The earliest abnormal division in *axr6* was seen at the second cell division (Fig. 3B). However, it is possible that an abnormality at the first cell division stage would have been missed because of the small number of embryos of this stage that were examined. The periclinal divisions of the suspensor cells in the mutants result in a suspensor that is two or more cells wide along part or most of its length (Fig. 3F,H,I,K,N). In wild type, the uppermost cell of the suspensor gives rise to the hypophyseal cell, the lens-shaped cell at the base of the embryo proper which is the precursor of the root cap and the root quiescent center (Fig. 3G; Scheres et al., 1994). In the *axr6* homozygotes the hypophyseal cell is not produced, presumably as a result of the abnormal suspensor development.

In the wild-type embryo, the pattern of divisions is synchronized, regular and reproducible, resulting in an embryo that at the triangular stage has an outer layer of protodermal cells and inner cells organized in three tiers (Fig. 3G). At slightly later stages, central files of cells can be seen, the precursors of the vascular tissue (Fig. 3J,M). In the homozygous mutant embryos, the divisions are frequently asynchronous and irregular in pattern, giving embryos with unusual cell numbers (Fig. 3C,E) and misaligned or disorganized cell patterns (Fig. 3I,L).

During later stages in wild-type embryos, increased cell division on both flanks of the upper tiers leads to development of the cotyledons. This was seen only on one flank in the *axr6-1* homozygotes (not shown), giving rise to the characteristic one cotyledon morphology (Fig. 3L).

Thus, the *axr6* homozygotes can be inferred to show abnormal orientation and timing of cell divisions starting early in development, and affecting especially those cells in the suspensor and the basal half of the embryo. There does not appear to be just a single division that is abnormal in the mutants; rather, throughout development the divisions in the embryo deviate from the wild-type pattern.

Homozygous mutant root development in tissue culture

Arabidopsis produces the primary root meristem during embryogenesis, and produces other roots post-embryonically (lateral and adventitious), and from callus in tissue culture. Although the *axr6* homozygous mutant seedlings lack roots, we found that *axr6* homozygous callus developed morphologically normal roots when grown on 64.5 μM naphthaleneacetic acid (not shown). Wild-type tissue remained as callus under identical conditions. Thus, the *axr6* mutations, when homozygous, do permit root development in tissue culture and show a difference in hormonal response from wild type.

Phenotype of *axr6* heterozygotes

To characterize the effects of the *axr6* mutations more fully, we analyzed the morphology, auxin responses, and gravitropism of the *axr6* heterozygotes.

Morphology

Compared to wild type, rosette leaves of heterozygous *axr6* plants are shorter and more wrinkled (Fig. 4A), and the plants are shorter and produce more inflorescences (Fig. 4B; Table 2). The siliques of the *axr6* mutants lie at a more acute angle relative to the stem than those of wild-type Columbia plants (Fig. 4C).

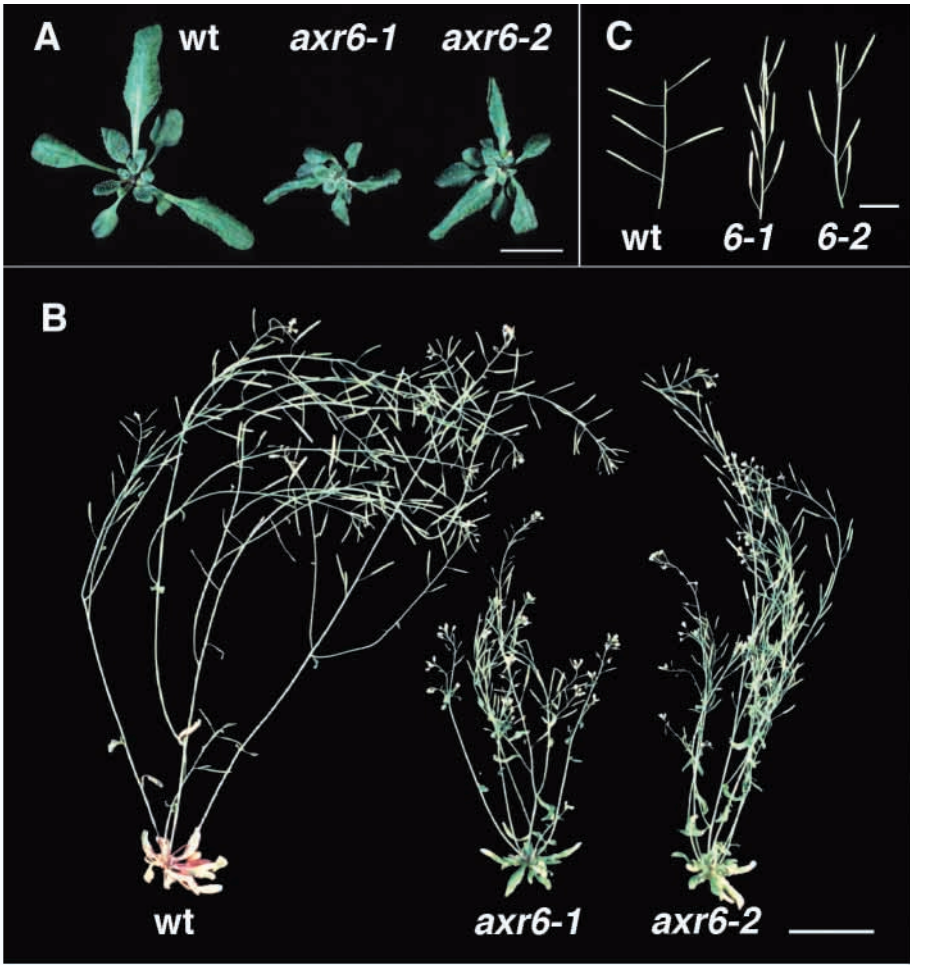


Fig. 4. Wild-type and *axr6* heterozygote morphology. (A) Rosettes at 26 days; bar, 1 cm. (B) Mature plants at 49 days; bar, 5 cm. (C) Siliques at 54 days; bar, 1 cm. Plants were grown under continuous illumination.

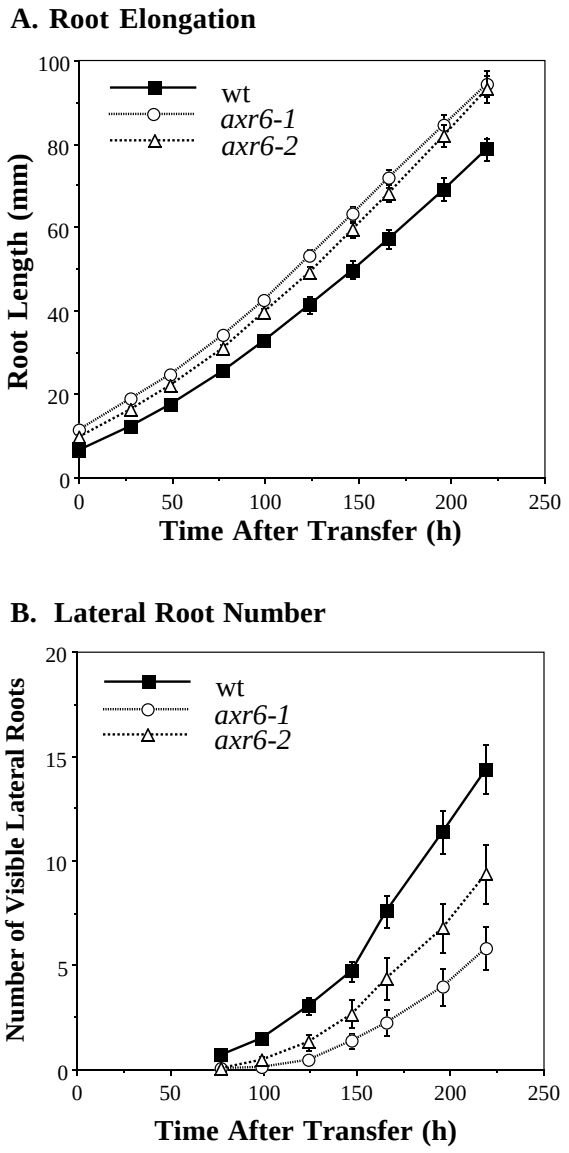


Fig. 5. Root phenotype of wild-type and *axr6* heterozygotes. One day after germination on hormone-free medium, seedlings were transferred to 150 mm plates of hormone-free minimal medium, grown vertically, and measured daily ($n \geq 16$). (A) Root length. (B) Number of visible lateral roots. Differences between wt and *axr6-1* root lengths were highly signficant ($P << 0.001$) at all time points; differences between wt and *axr6-2* were highly signficant ($P << 0.001$) at 77 hours and earlier; at later time points, $P < 0.005$, except for 147 hours where $0.01 < P < 0.05$. In lateral root number, differences between wt and *axr6-1* were highly signficant ($P << 0.001$) at 99 hours and later; differences between wt and *axr6-2* were signficant ($P < 0.01$) at all time points except for 166 hours, where $0.01 < P < 0.05$.

Root growth

Auxin is important for root development and lateral root formation, as shown both by physiological experiments and by alterations in these processes seen in other auxin-related mutants (Boerjan et al., 1995; Hobbie and Estelle, 1995; Malamy and Benfey, 1997). Seedlings heterozygous for the *axr6* mutations, when grown on hormone-free medium, were found to have slightly but signficantly increased rates of root elongation (Fig. 5A) and substantially decreased lateral root growth (Fig. 5B) over a 9-day period.

Hormone resistance

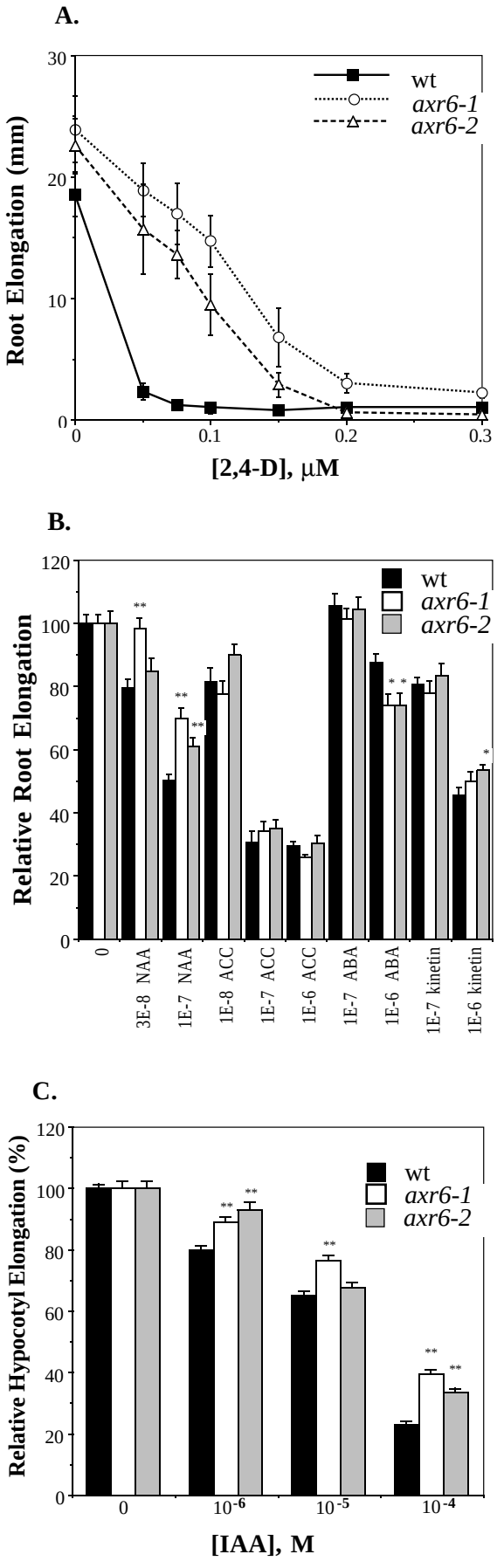
To assess quantitatively the level of resistance to auxins and other hormones in the *axr6* heterozygotes, the root elongation of seedlings grown on different concentrations of various hormones was determined. The *axr6* mutant seedlings were found to be about two-fold to four-fold more resistant to the synthetic auxin 2,4-D compared to wild type (Fig. 6A). The *axr6* mutants were also resistant to the synthetic auxin NAA (Fig. 6B) and the natural auxin IAA (not shown), but did not show reproducible and signficant differences from wild type in their growth responses to the ethylene precursor ACC, to abscisic acid, or to kinetin (a cytokinin) (Fig. 6B). These results indicate that the *axr6* mutations produce auxin resistance but do not appear to cause cross-resistance to other tested plant hormones.

The *axr6* mutations also affected hypocotyl growth in dark-grown seedlings. On hormone-free medium, the hypocotyls of dark-grown *axr6-1* and *axr6-2* heterozygous seedlings were slightly but significantly longer than those of wild type: wild type, 17.5±0.17 mm; *axr6-1*, 19.3±0.31 mm; *axr6-2*, 18.5±0.41 mm ($n \geq 40$ for each genotype; P for *axr6-1* $<<0.001$, P for *axr6-2* <0.005). The elongation of the heterozygous mutant hypocotyls was more resistant to inhibition by IAA in the medium than was hypocotyl elongation of wild-type seedlings (Fig. 6C). Thus the mutants are auxin-resistant in both root and hypocotyl elongation, and show small intrinsic differences in hypocotyl elongation in the dark.

Table 2. Measurement of *axr6* heterozygotes

	AXR6/AXR6	*axr6-1/AXR6*	*p* for *axr6-1* same as wt	*axr6-2/AXR6*	*p* for *axr6-2* same as wt
n	10	13		13	
Height, main inflorescence (cm)	46.6±1.1	22.5±1.8	$< 10^{-9}$	34.2±1.6	$<10^{-5}$
No. of lateral branches from main inflorescence	3.4±0.4	3.1±0.4	0.57	2.8±0.2	0.21
No. of inflorescences	5.8±0.6	9.5±0.8	0.001	9.0±0.8	0.008
Total no. of siliques	406±56	272±42	0.06	373±48	0.65
Bolting time, days after placing in growth room	29±1 (n=16)	31±1 (n=12)	0.30	31±1 (n=12)	0.29

Mean±s.e.m. is given. Plants were grown directly from seed in 5 inch clay pots under 24 hour illumination until senescence (54 days). Genotype was determined by progeny testing. Bolting time was measured in a separate experiment, in which genotype was determined by rosette morphology and silique angle. Statistical analysis used the ANOVA test.



Gravity response

Auxin is believed to be important in the differential cell growth necessary for gravitropic response; other auxin-resistant mutants have been found to be altered in their response to gravity (reviewed in Chen et al., 1999). The *axr6* heterozygous mutants were tested for such defects by determining the rate of root tip curvature in seedlings grown on hormone-free medium in the light after the seedlings were reoriented from vertical to horizontal. The results show that the *axr6* mutant seedlings are indeed substantially delayed in their gravitropic response (Fig. 7).

Expression from an auxin-regulated promoter

A number of other auxin-resistant mutants have been found to have changes in the expression or inducibility of auxin-regulated genes (e.g. Abel et al., 1995; Timpte et al., 1994). To test for such differences in *axr6-1* heterozygotes, the β-glucuronidase (GUS) gene under the control of a synthetic auxin-regulated promoter (Oono et al., 1998) was introduced into lines carrying the mutant or wild-type alleles of *AXR6* (see Materials and Methods). In the absence of hormone treatment, wild-type seedlings displayed weak expression of GUS in the cotyledons and hypocotyl (Fig. 8A and C), as well as at the hypocotyl-root junction and in the root elongation zone (not shown). *axr6-1* heterozygous seedlings showed dramatically increased expression in the the first true leaves and in the cotyledons, in particular at the tips (Fig. 8B and D). *axr6-2* seedlings showed an expression pattern that was overall very similar to that of the wild-type seedlings (not shown). After 2 days' growth on plates containing 8×10^{-8} M 2,4-D, to induce expression of the reporter gene, wild-type seedlings showed high levels of GUS expression localized to the root elongation zone (Fig. 8E), as observed previously (Oono et al., 1998). *axr6-1* heterozygous seedlings, by contrast, showed only weak and diffuse expression of GUS in the root under these conditions (Fig. 8F). The *axr6-1* mutants therefore show reduced auxin inducibility of an auxin-regulated gene in their roots, along with a strikingly altered pattern of expression in the apical regions of the seedlings in the absence of auxin induction.

DISCUSSION

We have described the isolation and characterization of two mutant alleles of a novel gene, *AXR6*. The *axr6* mutants show a unique combination of phenotypes: heterozygous mutant plants are viable but altered in a variety of auxin-related

Fig. 6. Hormone response of wild-type and *axr6* heterozygous seedlings. (A) Root elongation on 2,4-D over 3 days. (B) Relative root elongation on different hormones over 3 days. NAA, α-naphthalene acetic acid; ACC, 1-amino-1-cyclopropanecarboxylic acid; ABA, abscisic acid. (C) Hypocotyl elongation in the dark. In A, mean ± s.d. is presented. In B, elongation is presented as mean ± s.e.m., relative to elongation on hormone-free medium (values in mm: wt, 21.5±0.62, *axr6-1*, 23.3±0.68, *axr6-2*, 23.4±0.91). Statistical significance for comparison to wt: *0.001<*P*<0.01; **$P<0.001$. In C, seedlings ($n\geq30$) were grown on minimal medium without sucrose, but with IAA, in the dark for 7 days before measurment were taken. Elongation was normalized to elongation observed for the same genotype on hormone-free medium (see text for values). ** indicates $P<<0.001$ for comparison to wt.

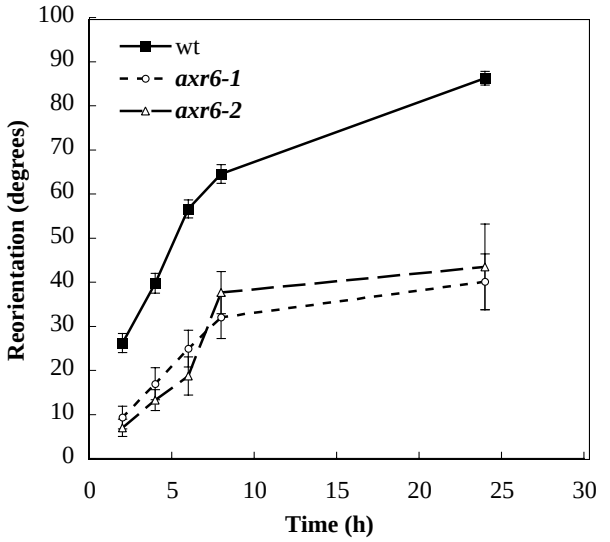


Fig. 7. Root gravitropic response of wild-type and *axr6* heterozygous seedlings. Seedlings grown on hormone-free minimal medium on vertically oriented square plates were turned by 90 degrees, and root tip reorientation was measured from photographs taken at the indicated times (wt, n=65; *axr6-1*, n=15; *axr6-2*, $n \geq 7$). Differences between wt and both mutants were statistically highly significant ($P<0.001$) at all time points.

processes, whereas homozygous mutants arrest growth after germination due to dramatic defects in embryonic development. The phenotype of the heterozygous *axr6* mutants directly implicates defective auxin response as the cause of the abnormal embryonic development of the *axr6* homozygotes.

The *axr6-1* allele is stronger than the *axr6-2* allele, a difference seen in both homozygotes and heterozygotes. The *axr6-1* homozygotes generally have only a single cotyledon, versus two for *axr6-2*, and show more severe vascular defects; the *axr6-1* heterozygotes are shorter, bushier, more auxin-resistant, and more altered in lateral root formation and gravity response than *axr6-2* heterozygotes. The *axr6* mutations are likely to be gain-of-function, as they are dominant in heterozygotes and the *axr6*-2 mutation is dominant in triploid seedlings of genotype *axr6/AXR6/AXR6*. Although both dominant (*axr2*: Wilson et al., 1990) and semidominant (*tir1*: Ruegger et al., 1998; *axr3*: Leyser et al., 1996; *shy2*: Tian and Reed, 1999) auxin-related mutations have been described, to our knowledge this is the first description of auxin-related mutants showing such dramatic differences in phenotype between the heterozygote and homozygote. Isolation and characterization of loss-of-function alleles of *axr6*, perhaps in screens for revertants, will give further insights into the normal physiological and developmental role of the *AXR6* gene.

The *axr6* homozygous embryos show altered patterns of cell division starting almost as early as could be observed, at the second cell division (Fig. 3B). These abnormal divisions in the early embryo especially affect the distinction between the cells of the embryo and the suspensor, giving rise to unusual suspensors that are several cells wide and not clearly delineated from the embryo proper. Other defects in the embryonic development of the *axr6* homozygotes include failure to generate the vascular precursors (procambial cells), failure to undergo the cell proliferation on one upper flank of the embryo that normally produces one of the cotyledons (in the *axr6-1* homozygotes), and failure to differentiate vasculature in the cotyledons. As much other evidence suggests that morphogenesis in plants is regulated at the regional or organ level rather than by control of cell division patterns (Smith et al., 1996; Traas et al., 1995), the aberrant timing and orientation of cell divisions are not likely to be the primary defects in *axr6* homozygotes, but may instead reflect underlying defects in auxin-mediated positional signaling or cell determination.

The importance of auxin in plant embryonic development has been previously demonstrated by the disruptions of normal developmental patterns produced by auxin-related compounds (Schiavone and Cooke, 1987; Fischer and Neuhaus, 1996; Hadfi et al., 1998) and by the alterations in auxin physiology found in several embryonic mutants of *Arabidopsis*, and is further supported by our characterization of *axr6* mutants. The phenotypic similarities among the *monopteros* (Berleth and Jürgens, 1993), *bodenlos* (Hamann et al., 1999), and homozygous *axr6* mutants (this paper) are so striking as to suggest that the three genes could act in a common pathway of auxin response during embryonic development. Eventual molecular characterization may help clarify the function of the *AXR6* gene in embryogenesis and in auxin response in general.

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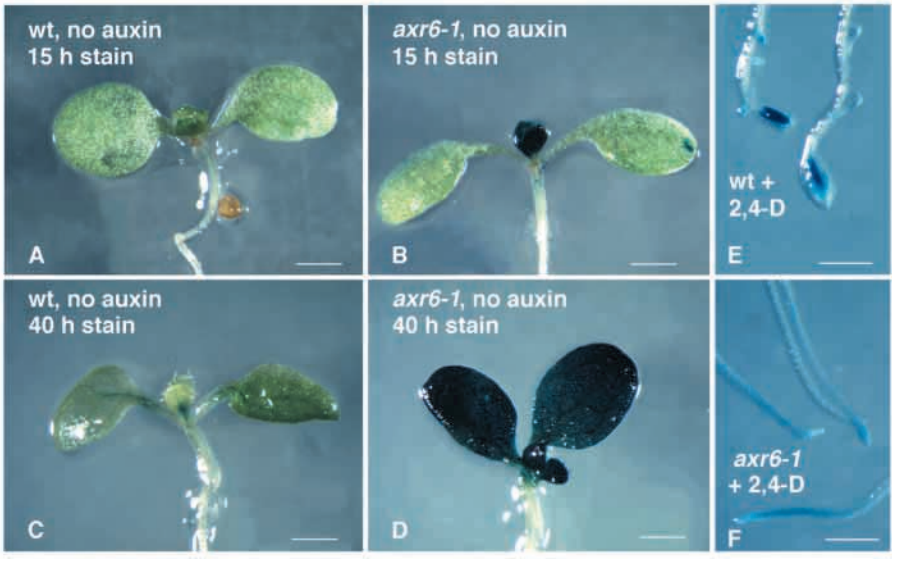

Fig. 8. BA3/GUS expression in wild-type and *axr6-1* heterozygotes. Seedlings homozygous for the BA3/GUS construct and of the indicated genotypes were grown on hormone-free medium and incubated in 1 mM X-Gluc for the 'staining' periods indicated (A-D). The seedlings in E and F were transferred to medium containing 8×10^{-8} M 2,4-D for 2.5 days (to induce expression and determine genotype) before incubating with X-Gluc for 45 hours. Bar, 1 mm.

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