Changes in Auxin Response from Mutations in an *AUX/IAA* Gene

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Transcription of the *AUX/IAA* family of genes is rapidly induced by the plant hormone auxin, but evidence that *AUX/IAA* genes mediate further responses to auxin has been elusive. Changes in diverse auxin responses result from mutations in the *Arabidopsis AXR3* gene. *AXR3* was shown to be a member of the *AUX/IAA* family, providing direct evidence that *AUX/IAA* genes are central in auxin signaling. Molecular characterization of *axr3* gain-of-function and loss-of-function mutations established the functional importance of domains conserved among AUX/IAA proteins.

Plant growth and development are regulated by the hormone auxin. Mutational analysis in *Arabidopsis* has identified genes important in auxin action (*1*). One such gene, *AXR3*, is defined by three semidominant mutations that result in increased auxin responses (*2*). Here, we describe intragenic suppressors of the *axr3-1* phenotype and the sequence of the *AXR3* gene. *AXR3* encodes a member of the auxin-inducible AUX/IAA family of short-lived nuclear proteins (*3–5*), demonstrating that *AUX/IAA* genes are central in auxin signaling.

Seeds homozygous for the *axr3-1* mutation and a second marker, *gl1* (*6*), were subjected to mutagenesis, and the resultant M_2 population was screened for revertants on the basis of shoot morphology (*7*). Five independent revertant lines were recovered, all of which contained the *gl1* mutation, indicating that they were not wild-type contaminants. When the revertants were crossed with the wild type, no *axr3-1* plants segregated in the resulting F_2 populations. When the revertants were crossed with *axr3-1* mutants, the F_1 plants were phenotypically similar to *axr3-1* heterozygotes, and *axr3-1* and revertant plants segregated in the F_2 populations in a ratio not significantly different from 3:1 (*8*). Because the data suggested that the revertant phenotypes resulted from *axr3-1* intragenic mutations, the corresponding lines were named *axr3-1R1* to *axr3-1R5*.

None of the revertants is completely wild type (Fig. 1). With primary root length as a measure of allelic strength, the rank order of reversion from strongest to weakest was *axr3-1R4* > *axr3-1R3* > *axr3-1R2* = *axr3-1R5* > *axr3-1R1* (Fig. 1B). With the exception of *axr3-1R4*, all the revertants have agravitropic roots. The roots of *axr3-1R4* plants grow downward but are abnormally straight, indicating that the root wave response is defective (*9*). Shoot phenotypes such as leaf curling persist only in *axr3-1R1* and *axr3-1R5*.

The *AXR3* gene maps to chromosome 1, ~1.5 centimorgans distal to *AXR1* (Fig. 2) (*2*). The flanking markers *dis1*, *ga4*, and *cer1* (*6*) in the Landsberg genetic background were used to identify lines with recombinational break points flanking *AXR3*. With the use of these lines, *AXR3* was mapped with respect to DNA polymorphisms in the region (*10*). *AXR3* maps immediately distal to the genomic clone 0846A, which was then used to probe bacterial artificial chromosome (BAC) and yeast artificial chromosome (YAC) libraries. The ends of the 0846A-positive BACs and YACs were used to probe the existing 0846A-positive BACs and YACs, as well as the BAC and YAC libraries (*10*), and contiguous clones extending distal to 0846A were collected (Fig. 2). YAC and BAC end clones were tested for polymorphisms between the Landsberg and Columbia genetic backgrounds. The distal end of BAC IGF20G19 was shown to include a polymorphic Tsp5091 site that maps distal to *AXR3*, delimiting a 30-kb region that includes *AXR3* (Fig. 2).

The BAC IGF19P19 extends 16 kb into the proximal portion of the delimited region and has been sequenced (*11*) as part of the *Arabidopsis* genome initiative. This 16-kb sequence includes two members of the *AUX/IAA* gene family, *IAA3* (*4*) and *IAA17* (*12*), both of which were then subcloned and sequenced from *axr3-1* DNA (*13*). The *IAA3* gene of *axr3-1* was identical to that of the wild type, whereas *IAA17* contained a single nucleotide difference, predicted to convert the proline at position 88 to leucine (Fig. 3).

To confirm that *IAA17* is *AXR3*, we amplified fragments encompassing the gene by the polymerase chain reaction (PCR) and sequenced other *axr3* alleles (*14*). The *axr3-4* and *axr3-1* mutations are identical, and the *axr3-3* mutation affects the adjacent valine (Fig. 3). The five revertant alleles retain the *axr3-1* mutation and include an additional point mutation that in three alleles (*axr3-1R1*, *axr3-1R2*, and *axr3-1R3*) results in a single amino acid change, and in two alleles (*axr3-1R4* and *axr3-1R5*) affects a splice site. RNA was extracted from *axr3-1R4* and *axr3-1R5* plants and subjected to reverse transcription and PCR to amplify fragments corresponding to the affected exon boundaries (*14*). Sequence analysis of the PCR products revealed that, in both instances, splicing occurs to a cryptic site within the affected intron. For *axr3-1R5*, such splicing results in the insertion of 33 nucleotides and, hence, 11 amino acids (Fig. 3). For *axr3-1R4*, four nucleotides are inserted, resulting in a shift in the reading frame and deletion of half of domain IV of the protein (Fig. 3). Because *AXR3* and *IAA17* map to the same 30-kb region and eight independent *axr3* alleles all have mutations in *IAA17*, we conclude that

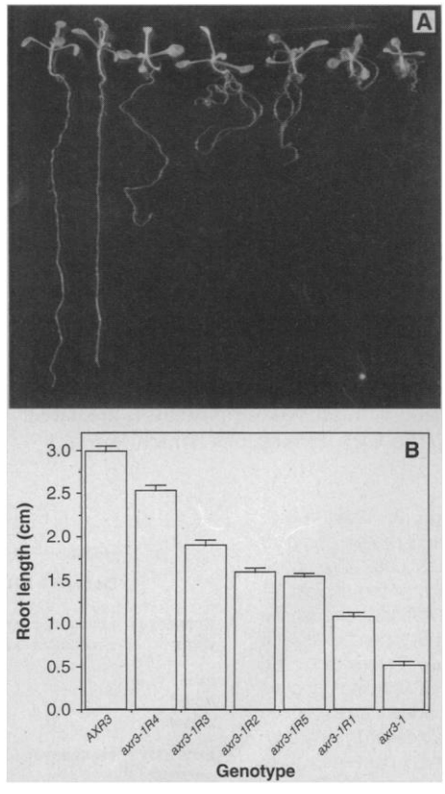

Fig. 1. The phenotype of *axr3-1* intragenic revertants. (**A**) Ten-day-old plants (*18*) homozygous for *axr3-1* intragenic revertant mutations. From left to right, the plants are *AXR3*, *axr3-1R4*, *axr3-1R3*, *axr3-1R2*, *axr3-1R5*, *axr3-1R1*, and *axr3-1*. (**B**) Mean root lengths for each genotype after growth for 7 days under the same conditions. Data are means ± SEM (n = 15).

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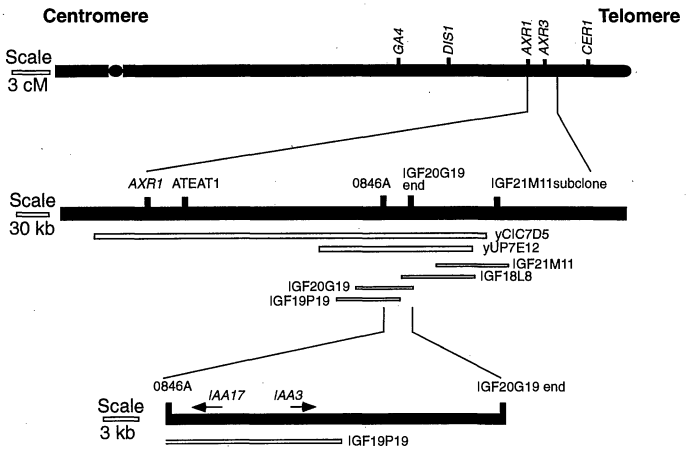

Fig. 2. Map-based cloning of the *AXR3* gene. Six clones encompassing the *AXR3* region; two YAC clones (prefixed y) and four BAC clones (prefixed IGF), are shown (open boxes). The position of the *AXR3* locus is shown relative to the visible markers *axr1*, *ga4*, *dis1*, and *cer1* (*2*, *6*) and to DNA markers (ATEAT1, 0846A, IGF20G19 distal end, and an IGF21M11 subclone) (*10*). Of ~1760 F_2 chromosomes, 17 showed recombination between *AXR3* and ATEAT1; only one of these 17 showed recombination between *AXR3* and 0846A. Of ~750 F_2 chromosomes, two showed recombination between *AXR3* and the IGF21M11 subclone; only one of these two showed recombination between *AXR3* and the IGF20G19 distal end. The delimited region includes two members of the *AUX/IAA* gene family (*11*), *IAA17* and *IAA3*, which are shown with arrows indicating the direction of their transcription. cM, centimorgan.

IAA17 and *AXR3* are the same gene.

Members of the auxin-inducible *AUX/IAA* gene family have been isolated from several species (*3*, *4*). They vary with respect to tissue specificity of expression, the kinetics of auxin induction, and the auxin dose-response relation (*4*). They encode short-lived nuclear proteins that contain four highly conserved domains (Fig. 3). Domain III shows similarity to βαα dimerization and DNA binding domains (*4*, *5*). AUX/IAA proteins form homodimers and heterodimers through domains III and IV (*12*). Furthermore, these proteins interact with the auxin response factors (ARFs) ARF1 and IAA24 (*12*, *15*), which bind to the auxin response element present in the promoters of many auxin-inducible genes (*15*). DNA binding by ARFs is mediated through the NH_2-terminus, whereas interaction with AUX/IAA proteins is mediated by the COOH-terminus, which shows sequence similarity to domains III and IV of AUX/IAA proteins.

Semidominant mutations in the *IAA17/AXR3* gene result in a wide range of auxin-related phenotypes, consistent with an increase in the amplitude of auxin responses and including ectopic expression from the *SAUR-AC1* promoter (*2*). This promoter contains the auxin response element to which ARFs bind (*16*). The ectopic *SAUR-AC1* expression thus supports the hypothesis that AUX/IAA proteins interact with ARFs to regulate gene expression directly.

The *axr3* mutations affect the four conserved AUX/IAA protein domains, confirming their functional significance. The tight clustering of the semidominant mutations contrasts with the scattered distribution of the intragenic revertant mutations, indicating that the revertant mutations cause loss of or a reduction in gene function, negating the gain of function conferred by the *axr3-1* mutation. Proteins containing the semidominant mutations may act in a dominant negative manner—for example, by forming nonfunctional complexes with AUX/IAA or ARF proteins. However, the increased auxin responses of *axr3* plants suggest that the mutations are hypermorphic, resulting, for example, in increased IAA17/AXR3 stability.

Arabidopsis contains at least 25 *AUX/IAA* genes (*12*). Presumably, each encoded protein is capable of interacting with other family members and with ARFs. Furthermore, each may bind DNA directly as heterodimers or homodimers through the βαα domain. This complex web of interactions links auxin to its downstream responses. The molecular characterization of the diverse *axr3* mutations offers an opportunity to understand better the molecular basis of AUX/IAA-mediated auxin signaling.

REFERENCES AND NOTES

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7. An M_1 population of 50,000 *axr3-1* plants was generated by ethyl methanesulfonate mutagenesis (*17*). The M_2 population was harvested as 40 subpopulations, and 2500 plants from each subpopulation were screened. Revertant mutants were selected on the basis of their shoot phenotype when grown at 22°C under a 16-hour-light, 8-hour-dark cycle on Fison's Levington F2 compost. The revertants were recovered from five different subpopulations.

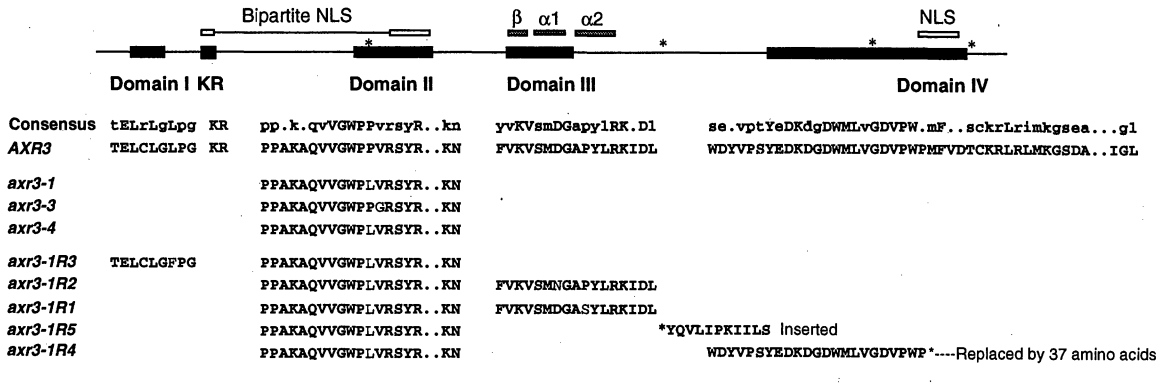

Fig. 3. The IAA17/AXR3 protein and *axr3* mutations. The positions of the nuclear localization sequences (NLS) (open boxes), the βαα dimerization and DNA binding domain (shaded boxes), exon boundaries (*), and conserved domains I to IV are shown (*4*). The domain consensus sequences are shown with uppercase letters indicating complete conservation, lowercase letters indicating partial conservation, and dots indicating nonconserved residues. The amino acid sequences for domains I to IV of IAA17/AXR3 are shown under the consensus (*12*). Amino acid substitutions in the *axr3* alleles are highlighted (*14*). Amino acid insertions resulting from the splice site mutations are shown after the relevant asterisk (*14*). Abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.

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10. The relative positions of *axr3* and several genomic sequences were determined by analyzing the segregation of *axr3* from restriction fragment length polymorphisms (RFLPs) and simple sequence length polymorphisms (SSLPs). F_2 plants with recombinational break points in the *dis1-axr3*, *ga4-axr3*, or *cer1-axr3* intervals were identified, and their RFLP and SSLP genotypes were determined with DNA extracted from the F_2 recombinants or from their F_3 progeny as described [S. L. Dellaporta, J. Wood, J. B. Hicks, *Plant Mol. Biol. Rep.* **1**, 19 (1983)]. Polymorphisms between the Columbia and Landsberg ecotypes were detected with the use of (i) the ATEAT1 SSLP previously described [C. J. Bell and J. R. Ecker, *Genomics* **19**, 137 (1994)], (ii) a subclone from BAC IGF21M11 as a probe on Southern blots of Hap II–digested genomic DNA, (iii) Tsp5091 cleavage of a PCR product amplified from the distal end of IGF20G19 with primers 5′-CAGAGGAAAATACATGGAC-3′ and 5′-AATGGCTACACTAGGCAC-3′, and (iv) Dra I cleavage of a PCR product amplified from 0846A (http://nasc.nott.ac.uk/new_ri_map.html) with primers 5′-ACTCACTTCCGCATCAAC-3′ and 5′-CAAGAAAAACGGGTGATAG-3′. The IGF20G19 end sequence and 0846A sequence were obtained with the use of an Amersham Sequenase kit. The 0846A clone was provided by C. Lister and C. Dean. YAC clones were identified by P. Dunn and J. R. Ecker with ^{32}P-labeled 0846A, and ends were isolated as described [J. Putterill, F. Robson, K. Lee, G. Coupland, *Mol. Gen. Genet.* **239**, 145 (1993)]. BAC clones were identified by D. Berger and T. Altman as described (http://194.94.225.1/private_workgroups/pg_101/igf_bac_lib.html) with ^{32}P-labeled probes. BAC ends were isolated by inverse PCR as described (http://194.94.225.1/private_workgroups/pg_101/bac_end_prep.html#ipcr) or by internal deletion by restriction enzyme digestion and religation.
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13. An *axr3-1* genomic library was constructed and screened with 0846A as described [N. E. Olszewski, F. B. Martin, F. M. Ausubel, *Nucleic Acids Res.* **16**, 10765 (1988)]. The *IAA17* and *IAA3* genes were subcloned into pUC19 by standard restriction enzyme digestion and ligation protocols. The subclones were sequenced with an ABI automated sequencer and an ABI Prism dye terminator cycle sequencing kit.
14. Four primer pairs were used to amplify overlapping fragments of the *IAA17/AXR3* gene from mutant allele DNA: 5′-ATCTTCCTCATCACCTTCCC-3′ and 5′-GGACAAAGCATTAGAAAGC-3′, 5′-TGTGACATCACGTCAAAG-3′ and 5′-AAGGGAGAAGAAGAAGACG-3′, 5′-GCACCATAAAAGAAAACCAC-3′ and 5′-CAAGTTATGCGGTTGAGG-3′, and 5′-AGCTTACAAAAGAGATTTGC-3′ and 5′-TTTCACTTTCAAGACGAACC-3′. RNA was extracted from 3-day-old *axr3-1R4* and *axr3-1R5* etiolated seedlings as described [J. Logemann *et al.*, *Anal. Biochem.* **163**, 16 (1987)]. Complementary DNA synthesis was performed with an oligo(dT) primer and reverse transcriptase (Superscript, Gibco-BRL). A region including the boundaries of the second and third introns was amplified with 5′-CATACCGGAAGAACGTGATG-3′ and 5′-CCAATGGCATCCGATCCTTTC-3′ as primers. PCR products were purified with a QIAGEN QIA quick PCR purification kit and sequenced (*12*) with the use of the PCR primers and additional internal primers. GenBank accession numbers for the semidominant and intragenic revertant mutations are AF040631 and AF040632, respectively.
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18. The plants were grown on vertically oriented petri dishes under a 16-hour-light, 8-hour-dark cycle at 22°C on antithymocyte serum medium (*17*) solidified with 1% agar.
19. We thank J. R. Ecker and T. Altman for sharing clones and hybridization results, S. Theologis for providing unpublished data, horticultural technicians at the University of York for plant care, the *Arabidopsis* Biological Resource Center Ohio and members of the *Arabidopsis* community for supplying seeds and clones, S. Freeman and W. Cooper for contributions to the work, and S. Day for critical reading of the manuscript. Supported by the Biotechnology and Biological Sciences Research Council of the United Kingdom.

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