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Hormone-Resistant Mutants of *Arabidopsis* Have an Attenuated Response to *Agrobacterium* Strains[1]

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ABSTRACT

We have examined the response of the hormone-resistant mutants *axr1* and *axr2* of *Arabidopsis thaliana* to inoculation by *Agrobacterium tumefaciens* and *Agrobacterium rhizogenes*. Our results indicate that recessive mutations in the *axr1* gene affect the frequency of tumor formation after inoculation with either *Agrobacterium* strain. In addition, tumors produced on *axr1* plants were smaller than those growing on wild-type plants. These results indicate that the product of the *AXR1* gene is important for both crown gall and hairy root tumor formation. In contrast, the dominant *axr2* mutation has a more severe effect on the development of crown gall tumors than on hairy root tumors. Crown gall tumors produced on *axr2* plants had a different morphology than wild-type tumors and did not grow when they were removed from the explant. In contrast, a large number of hairy root tumors were produced on wild-type and *axr2* plants, and both types of tumors grew when they were removed from the explant. Like the roots of *axr2* plants, roots produced on *axr2* explants lacked root hairs.

Virulent strains of *Agrobacterium tumefaciens* and *A. rhizogenes* cause the formation of crown gall and hairy root tumors, respectively, on susceptible dicotyledonous plants (2, 5, 12). In both cases, tumorigenesis is due to the action of plant-specific oncogenes that are present on the Ti or Ri plasmid and transferred from the bacteria into the plant genome during infection (23, 29). T-DNA transfer involves a set of genes called the *vir* genes and appears to be identical in both *A. tumefaciens* and *A. rhizogenes* (29). However, the oncogenes differ between the two bacterial species (5). The formation of a crown gall tumor involves the action of three genes on the T-DNA called *iaaM, iaaH,* and *ipt* (1, 6). The products of the *iaaM* and *iaaH* genes produce IAA from tryptophan in a two-step process (6), whereas the *ipt* gene product synthesizes isopentenyl AMP, a precursor to a number of biologically active cytokinins (1). Hence, tumor formation is primarily the result of a change in the levels of IAA and cytokinin in the affected plant tissue. Until recently, changes in hormone level were thought to have a limited role in the formation of a hairy root tumor (5). A number of groups have shown that tumor formation by *A. rhizogenes* depends on the synergistic action of three genes called *rolA, rolB,* and *rolC* (18, 21, 22, 27). Some experiments indicated that the *rol* genes, particularly *rolB,* act by increasing auxin sensitivity in transformed tissue (19, 21). However, experiments by Spena and his colleagues (3, 4) indicate that *rolB* and *rolC* encode enzymes that hydrolyze IAA and cytokinin glucosides, respectively. Thus, the *rol* genes appear to promote hairy root tumor formation either by increasing the intracellular levels of IAA and cytokinin or by decreasing the levels of hormone glucosides. It is not clear how these changes also result in an increase in auxin sensitivity.

Very little is known about the plant genes required for tumorigenesis. Increased levels of IAA and cytokinin probably stimulate cellular proliferation by acting through hormone reception/signal transduction pathways. However, the components of these pathways have not been identified. In addition, the differences in morphology between crown gall and hairy root tumors suggest that different plant genes may be important for development of these two types of tumors.

Phytohormone-resistant mutants have been identified in a number of plant species including *Arabidopsis thaliana,* tobacco, and tomato (7). An analysis of the response of these mutant plants to inoculation with *Agrobacterium* species may provide new information on the mechanisms of tumorigenesis as well as the function of the mutant genes. For example, an auxin-resistant mutation in tobacco has been shown to affect response to inoculation by *Agrobacterium* (25). In *Arabidopsis,* the *axr1*[2] and *axr2* mutations both confer resistance to auxin (8, 9, 28). The *axr1* mutations are recessive and confer a number of morphological abnormalities, including defects in stem elongation and apical dominance (8). In addition, experiments with stem explants indicate that mutant tissue requires a higher concentration of auxin to induce callus growth than does wild type (9). The *axr2* mutation is dominant and confers resistance to cytokinin, ABA, and ethylene in addition to auxin (28). Mutant plants also display a number of growth changes, including defects in shoot and root gravitropism, reduced root length, and lack of root hairs. In this study, we examine the effects of the *axr1* and *axr2* mutations on the process of crown gall and hairy root tumor formation. Our results show that these mutations affect the frequency of tumor formation as well as tumor morphology. In addition,

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[2] Genetic nomenclature: according to guidelines established at the Third International *Arabidopsis* Meeting, Michigan State University, April 1987, wild-type gene symbols are capitalized (*i.e. AXR2*) and mutants are represented with lower case symbols (*i.e. axr2*).

our experiments confirm that auxin response is required for normal development of these tumors.

MATERIALS AND METHODS

All strains of *Arabidopsis thaliana* used in this study were ecotype Columbia. The *axr1* and *axr2* mutants have been previously described (8, 9, 28). Sterile plants were grown on MSO[3] in 100 × 25 mm plastic Petri dishes. Seeds were surface-sterilized for 20 min in 30% (v/v) bleach and 0.01% Triton X-100 with periodic agitation and then rinsed at least three times with sterile distilled water. Twelve seeds were placed on each Petri dish and plants were grown under constant illumination (50 μE m^{-2} s^{-1}) at 21°C.

Two strains of *Agrobacterium* were used in this study. Strain R1000 is *A. tumefaciens* strain C58 cured of the Ti plasmid and carrying wild-type Ri plasmid pRiA4b (15). Strain ACH5 is a virulent octopine-type strain of *A. tumefaciens* (15). Both strains were grown in 1% tryptone, 1% NaCl, 0.5% yeast extract.

Plants were used for inoculations when they were 4 to 6 weeks old. Stem explants were made by making a cut a few millimeters above and below any node that had an actively growing axillary bud. Cauline leaves were not removed, and all possible nodes on a stem were used. The basal end of each explant was dipped in a suspension of *Agrobacterium* cells (1.5×10^9 cells/mL) immediately after cutting. The explants were then blotted thoroughly on sterile Whatman No. 1 paper and placed basal end up into MSO in a 100 × 25 mm plastic Petri dish. The next day, the explants were transferred, still in the inverted orientation, to MSO medium containing cefotaxime (200 mg/L). Plates were sealed with Parafilm and placed at 21°C under constant illumination. Explants were examined with a dissecting microscope after 21 d and placed into one of three categories: no tumorous growth, tumorous growth, or tumorous growth with roots. Each experiment included at least 30 explants, except for one R1000/*axr2* experiment that consisted of 16 explants. Statistical significance was evaluated by one-way analysis of variance.

RESULTS

axr1 Mutants Have an Altered Response to *Agrobacterium* Infection

Previous studies suggested that the *AXR1* gene of *Arabidopsis* is required for auxin response in most tissues of the plant (8, 9). To determine if this gene is also important for growth of hairy root and crown gall tumors, we have examined the response of wild-type and mutant plants to inoculation with either *A. rhizogenes* strain R1000 or *A. tumefaciens* strain ACH5. Two different mutant alleles were used for these experiments, *axr1-3* and *axr1-12*. We have shown previously that *axr1-12* plants are more resistant to auxin than are *axr1-3* plants (8).

Sterile stem explants from wild-type, *axr1-3*, and *axr1-12* plants were inoculated with *A. rhizogenes* strain R1000. After 21 d, the explants were examined and placed into one of three categories. In one group of explants, no sign of cell proliferation was observed, and frequently the inoculated ends of the explants became necrotic. In the second group, tumors were observed, but these tumors failed to produce roots. Tumors with agravitropic roots characteristic of a hairy root tumor were produced in the final group of explants. Uninoculated control explants from either wild-type or mutant plants consistently produced a small amount of callus tissue that was clearly distinguishable from tumorous growth. When tumors were removed from the explants and placed on hormone-free MSO medium, only those tumors with roots grew. Tumors without roots did not grow, nor did the small amount of callus produced by uninoculated control explants.

The response of wild-type and *axr1* plants to inoculation by *A. rhizogenes* is shown in Table I. Both *axr1-3* and *axr1-12* explants displayed an attenuated response. The percentage of mutant explants that produced a hairy root tumor was reduced compared to wild type. In the case of *axr1-12* plants, this difference was statistically significant at a 99% confidence level. Examples of tumors from wild-type, *axr1-3*, and *axr1-12* explants are shown in Figure 1, A–C. In general, hairy root tumors produced on mutant plants were smaller and had fewer roots than wild type.

When wild-type explants were inoculated with *A. tumefaciens* strain ACH5, approximately 68% of the explants produced a tumor (Table I). The majority of these tumors had gravitropic roots growing from the underside of the tumor. Inoculation of *axr1* explants produced a lower frequency of tumors (Table I), and only a small percentage of these tumors produced roots. The difference in percentage of rooty tumors produced between wild type and either *axr1-3* or *axr1-12* was statistically significant. When wild-type and mutant tumors were excised and placed on MSO medium without hormones, virtually all of the root-producing tumors grew, whereas most of the tumors that had not produced roots died after they had been excised (data not shown). Examples of wild-type and mutant tumors are shown in Figure 2, A–C.

Table I. *Tumor Formation after Inoculation with Agrobacterium Strains*

Sterile stem explants were scored 21 d after inoculation. Values are the means ± SE.

Strains	Number of Experiments	Explants with Rooty Tumors	Explants with Nonrooty Tumors	Explants with No Tumors
		%		
R1000				
Wild type	8	35.7 ± 5.2	10.2 ± 1.7	54.1 ± 6.2
axr1-3	3	16.2 ± 4.6[a]	16.8 ± 11.5	67.0 ± 15.9
axr1-12	5	4.8 ± 2.1[b]	24.1 ± 4.9	71.1 ± 4.9
axr2	2	49.8 ± 12.3[a]	6.0 ± 6.1	44.2 ± 18.3
ACH5				
Wild type	6	44.1 ± 7.9	23.7 ± 7.0	32.2 ± 5.4
axr1-3	3	2.8 ± 0.8[b]	33.1 ± 10.1	64.1 ± 10.6
axr-12	3	4.1 ± 0.7[c]	45.0 ± 11.6	50.9 ± 12.2
axr2	2	3.8 ± 3.8[c]	53.9 ± 2.9	42.3 ± 26.9

[a] Not significantly different from wild type. [b] Significantly different from wild type at 99% confidence level. [c] Significantly different from wild type at 95% confidence level.

[3] Abbreviation: MSO, Murashige and Skoog salts, Gamborgs B-5 vitamins, 2% sucrose, 1% agar.

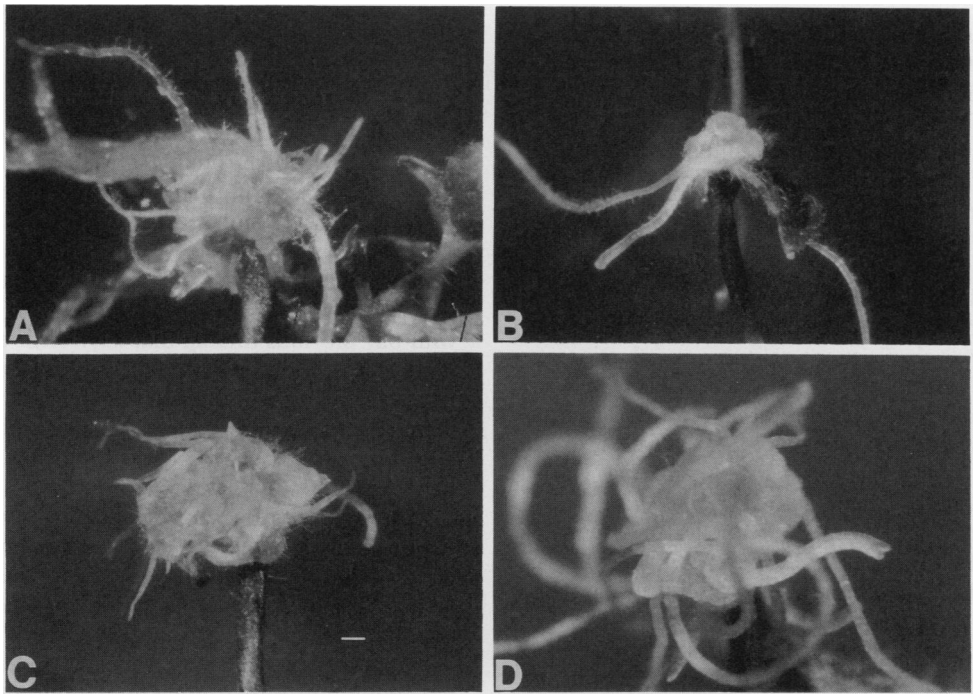

Figure 1. Hairy root tumors produced on wild-type and mutant explants. A, Wild type; B, *axr1-3*; C, *axr1-12*; D, *axr2*. Tumors were photographed with a microscope at a magnification of 7.5× for A, B, and C, and at 15× for D.

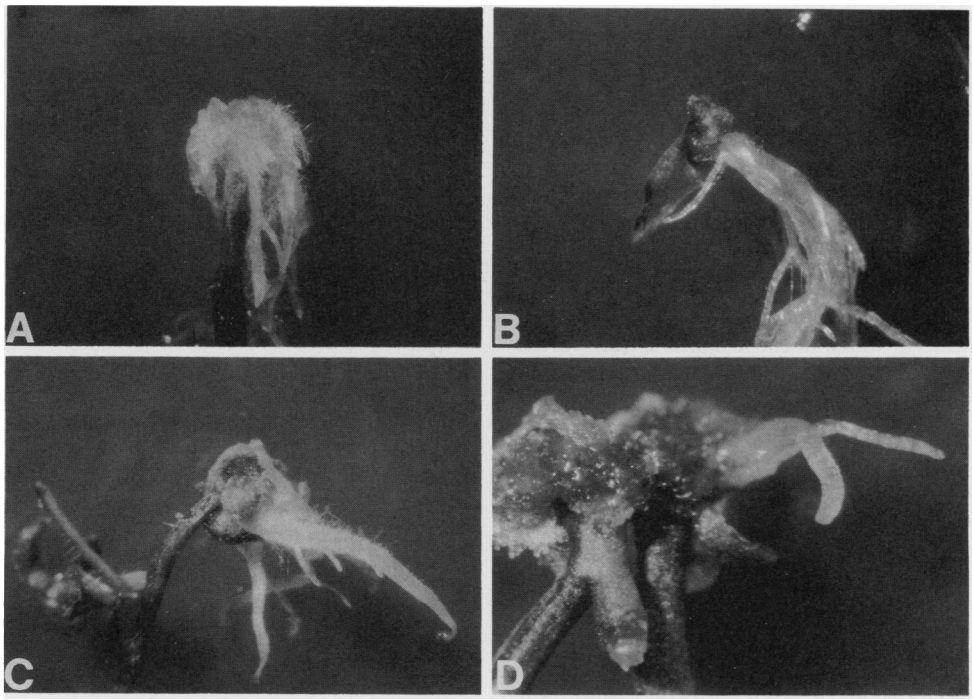

Figure 2. Crown gall tumors produced on wild-type and mutant explants. A, Wild type; B, *axr1-3*; C, *axr1-12*; D, *axr2*. Tumors were photographed with a dissecting microscope at a magnification of 7.5× for A, B, and C, and at 15× for D.

axr2 Mutation Affects Crown Gall and Hairy Root Tumor Morphology

The dominant mutation *axr2* has a number of dramatic effects on plant development (28). Stem elongation is reduced relative to wild type, and stems and roots both display defects in gravitropism. The roots of mutant plants are shorter than wild type and lack root hairs. The *axr2* mutant was originally identified because of its auxin resistance. Subsequent experiments have shown that the mutant is also resistant to ethylene and ABA (28). To determine the effects of the *axr2* mutation on tumor development, explants from homozygous *axr2* plants were inoculated with either *A. rhizogenes* R1000 or *A. tumefaciens* ACH5. When *axr2* explants were inoculated with *A. rhizogenes* R1000, the frequency of tumor production was similar to that of wild type and most of the *axr2* tumors produced agravitropic roots (Table I). Like wild-type tumors, these tumors all grew when placed on MSO. However, unlike wild-type tumors, *axr2* tumors lacked root hairs (Fig. 1D).

Inoculation of *axr2* explants with *A. tumefaciens* ACH5 resulted in approximately the same frequency of tumor formation as on wild type (Table I). However, unlike wild-type tumors, most of the *axr2* tumors did not produce roots (Fig. 2D). The difference in percentage of rooty tumors between wild type and *axr2* was statistically significant at the 99% confidence level. Any roots that did develop were short and lacked root hairs. When mutant tumors were placed on MSO medium, only those tumors that had roots were able to grow.

DISCUSSION

We have used an axenic inoculation procedure to examine the response of wild-type and auxin-resistant *Arabidopsis* plants to inoculation with *Agrobacterium* species. The response of wild-type plants to inoculation with *A. rhizogenes* R1000 is similar to that described previously in *Arabidopsis* and in other species (5, 26). In contrast, the morphology of crown gall tumors produced by strain ACH5 was somewhat unusual, because octopine strains normally cause the production of undifferentiated tumors (12). However, Ondrej *et al.* (13) also report the formation of teratogenic tumors on *Arabidopsis* plants inoculated with strain ACH5. The fact that we are infecting the basal end of stem explants may also result in increased formation of roots. Because auxin is transported in an apical-to-basal direction in the stem, there may be an unusually high concentration of auxin at the basal cut site. Polarity of inoculation response on carrot discs has been explained in a similar way (17). The nature of the tumors that do not grow on MSO medium is unclear. It is possible that cellular proliferation in these tumors is due to production of phytohormones by the infecting bacterial cells (10). The level of hormone produced in these tumors may not be sufficient to maintain growth. Alternatively, these tumors may contain transformed cells, but the tumorigenic loci may be expressed at a reduced level.

The *AXR1* and *AXR2* genes of *Arabidopsis* play important roles in auxin response and normal plant development. In this study, we demonstrate that these genes are also important for crown gall and hairy root tumor formation. Plants that are homozygous for either the *axr1-3* or the *axr1-12* mutation have an attenuated response to both *A. tumefaciens* and *A. rhizogenes*. Upon inoculation, the proportion of tumors that develop roots and are viable after excision from the explant is much smaller in the mutants compared with the wild type. Although there are a number of possible explanations for this behavior, we believe that the attenuated response is best explained by the general reduction in auxin response exhibited by the *axr1* mutants. In the case of *A. tumefaciens*, it is clear that tumor formation is a consequence of an increase in auxin and cytokinin levels, and recent studies suggest that this may also be true for *A. rhizogenes* (3–5). In *axr1* tissue, this increase in auxin level is probably not sufficient to induce the same amount of cellular proliferation. This is consistent with our previous finding that *axr1* stem explants require a higher concentration of auxin in the growth medium to initiate callus growth than do wild-type stem explants (9).

In contrast with *axr1*, the dominant mutation *axr2* does not affect the frequency of hairy root tumor formation. Instead, this mutation has a clear effect on tumor morphology. Like the normal roots of *axr2* plants, tumorous roots lack root hairs, indicating that the mutation prevents root hair development regardless of the origin of the root. Our results suggest that the product of the *AXR2* gene does not play a direct role in hairy root tumor formation. It is possible that the auxin responses that are deficient in the *axr2* mutant are not important during tumor initiation but are required for development of root structures such as root hairs.

The response of *axr2* explants to inoculation with *A. tumefaciens* is significantly different from wild type. Tumors developed on mutant explants with a similar frequency to that of wild type. However, the majority of these tumors did not develop roots like wild type and were unable to grow when excised from the explant. It is possible that roots are required for tumor viability on hormone-free medium. If this is the case, the *axr2* mutation may affect growth of excised tumors simply by inhibiting root development. Alternatively, the inability of *axr2* tumors to grow on hormone-free media may reflect a change in the way *axr2* tissue responds to the increase in auxin and cytokinin levels that occur in transformed cells.

Naturally occurring variation in susceptibility to *Agrobacterium* inoculation has been described in cucurbits (20), pea (16), soybean (14), grapevine (24), and interspecific hybrids of *Nicotiana* (11). In grapevine, resistance to crown gall disease seems to be inherited as a single gene, but in the other species differences in susceptibility are not inherited in a simple fashion. Tourneur *et al.* (25) have shown that an auxin-resistant mutant in *Nicotiana tabacum* displays a modified response to *Agrobacterium* species. Here we show that mutations in the *AXR1* or *AXR2* genes cause significant changes in both the frequency of tumor formation as well as tumor morphology. We expect that the molecular characterization of these two genes will provide new insight into the mechanism of tumorigenesis in plants.

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