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A Mutation Altering Auxin Homeostasis and Plant Morphology in Arabidopsis

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Many aspects of plant development are associated with changing concentrations of the phytohormone auxin. Several stages of root formation exhibit extreme sensitivities to exogenous auxin and are correlated with shifts in endogenous auxin concentration. In an effort to elucidate mechanisms regulating development of adventitious roots, an ethyl *methanesulfonate*-mutagenized M_2 population of Arabidopsis was screened for mutants altered in this process. A recessive nuclear mutant, *rooty* (*rty*), displayed extreme proliferation of roots, inhibition of shoot growth, and other alterations suggesting elevated responses to auxin or ethylene. Wild-type Arabidopsis seedlings grown on auxin-containing media phenocopied *rty*, whereas *rty* seedlings were partially rescued on cytokinin-containing media. Analysis by gas chromatography–selected ion monitoring–mass spectrometry showed endogenous indole-3-acetic acid concentrations to be two to 17 times higher in *rty* than in the wild type. Dose–response assays with exogenous indole-3-acetic acid indicated equal sensitivities to auxin in tissues of the wild type and *rty*. Combining *rty* with mutations conferring resistance to auxin (*axr1-3*) or ethylene (*etr1-1*) suggested that root proliferation and restricted shoot growth are auxin effects, whereas other phenotypic alterations are due to ethylene. Four mutant alleles from independently mutagenized populations were identified, and the locus was mapped using morphological and restriction fragment length polymorphism markers to 3.9 centimorgans distal to marker m605 on chromosome 2. The wild-type *RTY* gene product may serve a critical role in regulating auxin concentrations and thereby facilitating normal plant growth and development.

INTRODUCTION

Temporal and spatial changes in phytohormone concentrations are believed to regulate many aspects of plant development. Elucidating mechanisms modulating hormone concentrations is therefore critical to our understanding of certain developmental processes. The phytohormone auxin has been associated with deetiolation (O'Brien et al., 1985), root initiation and elongation (Pilet and Saugy, 1985; Norcini and Heuser, 1988; Blakesley et al., 1991a), vascular differentiation (Jacobs, 1976), gravitropism and phototropism (Li et al., 1991), endosperm development (Lur and Setter, 1993), somatic embryogenesis (Michalczuk et al., 1992), and fruit ripening (Slovin and Cohen, 1993). Changes in auxin concentration are often associated with these processes, although the coincident or regulatory nature of auxin concentration is not known. Exogenous applications of auxin affect many of these processes, implying regulatory roles for auxin and perhaps changes in auxin concentration.

The major endogenous auxin in plants, indole-3-acetic acid (IAA), exists in three principal forms: free IAA, believed to be the bioactive form (Taiz and Zeiger, 1991); conjugated to sugars or myoinositol through ester linkages; or conjugated to amino acids or small peptides through amide linkages (Cohen and Bandurski, 1982). Concentrations of free IAA are regulated through the interplay of biosynthesis, formation of conjugates, hydrolysis of conjugates to free IAA, irreversible oxidation, and transport (Cohen et al., 1988). Although formation and hydrolysis of conjugates (Cohen and Bandurski, 1982) and oxidation (Bandurski, 1984; Bandurski et al., 1992) have been well characterized biochemically and have recently been the subject of molecular analyses (Campos et al., 1992; Szerszen et al., 1994), pathways of auxin biosynthesis are less certain (Cohen and Bialek, 1984). The previously accepted role of tryptophan as the principal precursor of IAA is being challenged (Baldi et al., 1991; Wright et al., 1991; Normanly et al., 1993), and alternative pathways remain unresolved. Genetic and biochemical analyses of mutants with auxin overproduction (Slovin and Cohen, 1988; Wright et al., 1991; Normanly et al., 1993; Boerjan et al., 1995) should define processes affecting auxin concentration.

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Our long-term goal is to elucidate mechanisms regulating adventitious root formation, a strongly auxin-dependent developmental process (Thimann and Went, 1934; Haissig, 1974). Adventitious roots arise from shoots or older roots (Esau, 1977) and generally initiate from parenchyma cells associated closely with the vascular system (Lovell and White, 1986). Their formation represents respecification of cellular fates in response to endogenous or exogenous signals. The process is divided generally into a primordium initiation phase when auxin is critical and a root emergence phase when auxin is unnecessary or inhibitory (James, 1983). The dual roles of auxin suggest changes in auxin concentration or sensitivity to auxin during root development. In cuttings of apple rootstocks, high concentrations of IAA were found in easy-to-root genotypes, and low concentrations of IAA were found in difficult-to-root genotypes (Alvarez et al., 1989). Seasonal variations in the ease of root formation have shown similar correlations with IAA concentration in *Cotinus coggygria*, and a change from high to low concentrations of IAA was found between the time of cutting excision to root emergence (Blakesley et al., 1991b).

The genetic basis of root formation is poorly understood. Characterizing mutants with unique root phenotypes could elucidate mechanisms controlling root development, some of which may involve interactions with auxin. Numerous mutations that alter the presence, morphology, or physiology of roots have been described (reviewed in Schiefelbein and Benfey, 1991). In many cases, mutant root phenotypes are due to pleiotropic effects of altered sensitivities to auxin (Maher and Martindale, 1980; Muller et al., 1985; Estelle and Somerville, 1987; Wilson et al., 1990; Hobbie and Estelle, 1995; Muday et al., 1995). Recently, in Arabidopsis, development of wild-type roots was described (Dolan et al., 1993), and mutations were identified that prevent formation of the embryonic root (Barton and Poethig, 1993; Berleth and Jürgens, 1993), enhance proliferation of secondary roots (Boerjan et al., 1995), alter root morphologies or maintenance of root meristems, or reduce root growth rates (Baskin et al., 1992; Benfey et al., 1993; Galway et al., 1994; Cheng et al., 1995).

In an effort to understand adventitious root formation better, selection was initiated for mutations of Arabidopsis that altered this process. We present here an initial characterization of a recessive ethyl methanesulfonate (EMS)–induced mutant, *rooty* (*rty*), that exhibits extreme proliferation of adventitious and lateral roots, inhibition of shoot growth, and numerous morphological alterations suggestive of auxin effects. We demonstrate that the mutation causes elevated concentrations of endogenous IAA but does not alter tissue sensitivity to auxin. We also provide a map location for the locus and evidence for multiple mutant alleles.

Table 1. Mean Lengths of Hypocotyls and Roots of 3-Day-Old Dark-Grown Wild-Type and *rty* Seedlings

	Length (mm)	
Organ	Wild Type	*rty*
Hypocotyl	9.8 ± 0.3[a]	3.9 ± 0.4
Root	8.4 ± 0.5	4.1 ± 0.3

[a] Mean of 40 observations ± SE.

RESULTS

Time Course of Phenotype Development

Seed from an EMS-mutagenized population (Sheahan et al., 1993) was sown onto sterile medium, grown for 5 days in darkness, treated with the auxin indole-3-butyric acid (IBA), and scored after 14 days for variations in number of adventitious roots on etiolated hypocotyls. From this screen, a mutant line was selected expressing the single nuclear recessive mutation *rty*. Wild-type and *rty* embryos, dissected from mature seed, are indistinguishable at a gross phenotypic level. As shown in Table 1 and Figure 1A, the two genotypes become distinct phenotypically after 3 days on sterile medium in a dark growth chamber, when roots and hypocotyls are shorter in *rty* seedlings relative to the wild type. Hypocotyls of *rty* are expanded radially and hookless, whereas dark-grown wild-type seedlings form distinct apical hooks on elongated hypocotyls (Figure 1A). Within 24 hr after transfer to light, seedlings of both genotypes are hookless with unfolded, green cotyledons. Further development is identical to that of light-germinated seedlings described below.

After 4 days on sterile medium in Petri plates, light-germinated *rty* seedlings have short roots and epinastic cotyledons (Figure 1B). At 7 days, relative to the wild type, *rty* shows underdeveloped first true leaves, emergence of lateral roots, and exfoliation of epidermal and cortical tissues at hypocotyl bases (Figure 1C). Exfoliation of hypocotyl tissues was never observed in wild-type seedlings (Figures 1D and 1E), but in *rty*, it precedes adventitious root emergence by ~24 hr and progresses acropetally until the entire hypocotyl produces adventitious roots (Figures 1G and 1H). By 9 days, epinasty increases in *rty*, causing margins of cotyledons to roll inward (Figure 1G). Between 12 and 21 days, leaves of *rty* are expanding (Figures 1H and 1I) but remain small and epinastic compared with the wild type (Figures 1E and 1F). Based on the fact that new leaf production is sustained in advance of acropetal adventitious root proliferation, it appears that shoot apical meristems of *rty* remain functional. When grown in sterile conditions on hormone-free, agar-solidified nutrient medium, *rty* maintains this status for months and rarely flowers. If bolting occurs, adventitious roots emerge on primary inflorescence shoots, most flowers abort, and seed do not set. When grown in soil, seedlings of *rty* lodge and die after hypocotyl integrity is compromised by adventitious root proliferation.

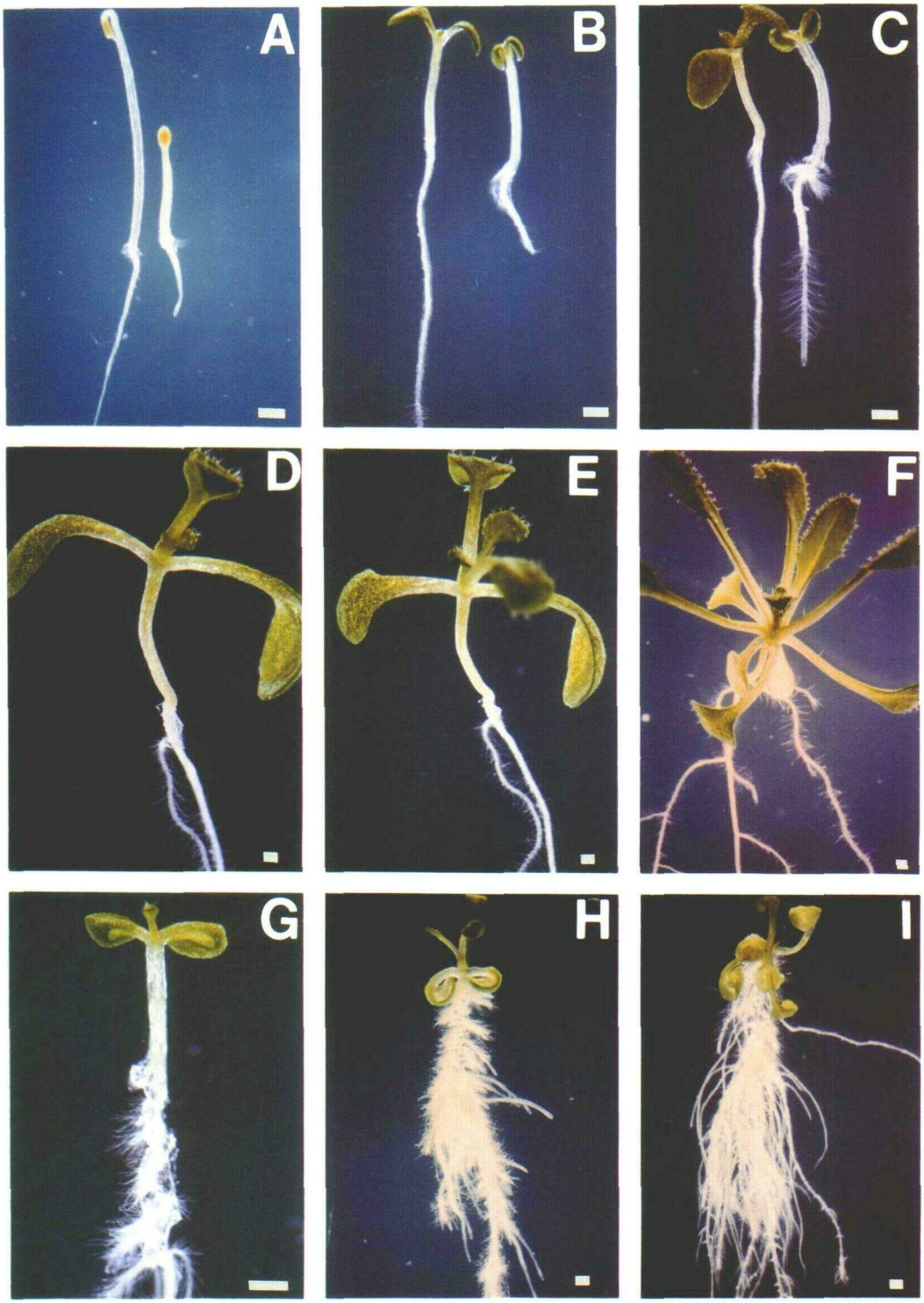

Figure 1. Development of Wild-Type and *rty* Seedlings Grown in Darkness or Light.

(A) Wild type (left) and *rty* (right) after 3 days in darkness.
(B) Wild type (left) and *rty* (right) after 4 days in light.
(C) Wild type (left) and *rty* (right) after 6 days in light.
(D) and **(G)** Wild type and *rty*, respectively, after 9 days in light.
(E) and **(H)** Wild type and *rty*, respectively, after 12 days in light.
(F) and **(I)** Wild type and *rty*, respectively, after 21 days in light.
Bars = 1 mm.

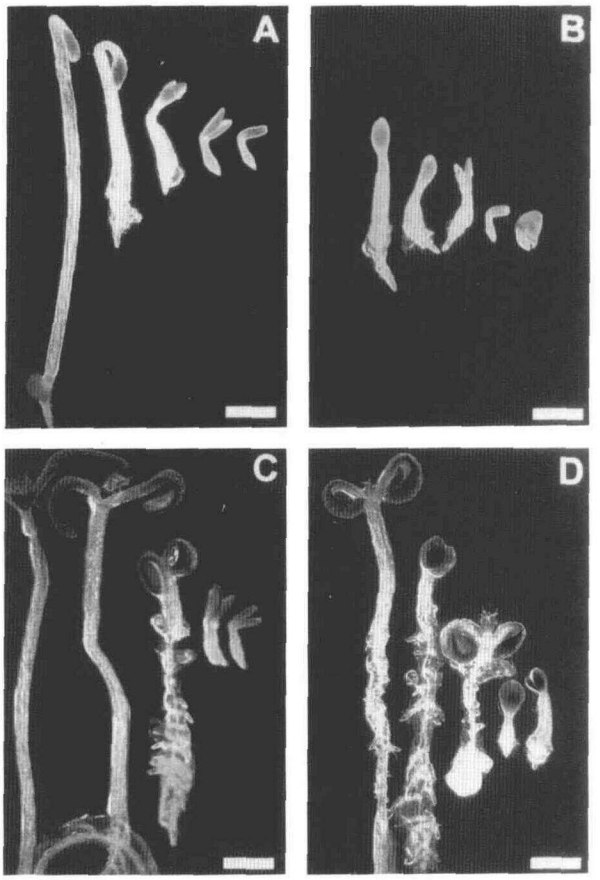

Figure 2. Morphologies of Wild-Type and *rty* Seedlings Grown in Darkness or Light.

Seedlings were grown on media containing 0, 1, 10, 55, and 100 μM NAA and are shown from left to right.
(A) and **(B)** Wild type and *rty*, respectively, after 3 days in darkness.
(C) and **(D)** Wild type and *rty*, respectively, after 3 days in darkness plus 6 days in light.
Bars = 1 mm.

Responses to Exogenous Auxins

Proliferation of secondary roots, inhibition of shoot development, and epinastic leaves in *rty* seedlings suggest the effects of elevated auxin. To determine whether the mutant phenotype can be copied in the wild type and to test sensitivities to auxin, wild-type and *rty* seedlings were grown on media containing the auxins naphthaleneacetic acid (NAA) or IAA. Wild-type seedlings grown on 10 μM NAA (Figures 2A and 2C) phenocopied *rty* seedlings grown on 0 or 1 μM NAA (Figures 2B and 2D). Morphological alterations typical of *rty*, which occurred in the wild type, included short, radially expanded, hookless hypocotyls and restricted root elongation after 3 days in darkness (Figure 2A); cotyledon and leaf epinasty; cortical and epidermal exfoliation on hypocotyls; and lateral and adventitious root proliferation after 3 days in darkness plus 6 days in light (Figure 2C).

Tissue sensitivities to auxin were determined as concentrations of IAA causing one-half maximum inhibition (I_{50}) of root and hypocotyl elongation in 3-day-old dark-grown seedlings. Response curves in Figure 3 illustrate that threshold concentrations for inhibition of hypocotyl elongation were 0.1 μM for the wild type and 1.0 μM for *rty* (Figure 3A). Hypocotyl inhibition was greater in the wild type than in *rty* at 0.01 to 100 μM. At 100 μM, nearly complete inhibition was observed in both genotypes. Responses of roots to IAA were similar in both genotypes. No inhibition of root elongation occurred at 0.0001 and 0.001 μM IAA (Figure 3B). Between 0.001 and 1.0 μM, root lengths declined from 100% to ~10% of controls, and nearly complete inhibition occurred at 10 and 100 μM. The I_{50} concentrations of IAA for hypocotyls were 1.7 ± 0.5 μM for the wild type and 3.3 ± 0.5 μM for *rty*; the I_{50} concentrations for roots were 0.02 ± 0.002 μM for the wild type and 0.04 ± 0.009 μM for *rty*. Within tissue types, these values did not differ significantly ($P > 0.05$).

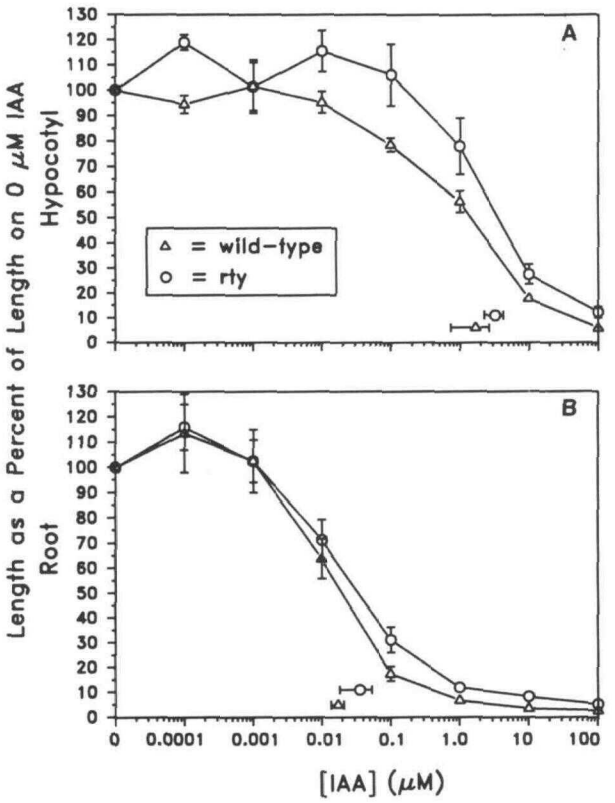

Figure 3. Responses of Wild-Type and *rty* Seedlings to Exogenous IAA.

(A) Relative elongation of hypocotyls.
(B) Relative elongation of roots.
Values represent length as a percentage of length when grown on control treatments. Vertical bars represent ± SE. Means ± SE of I_{50} concentrations of IAA are indicated as symbols above the *x* axes.

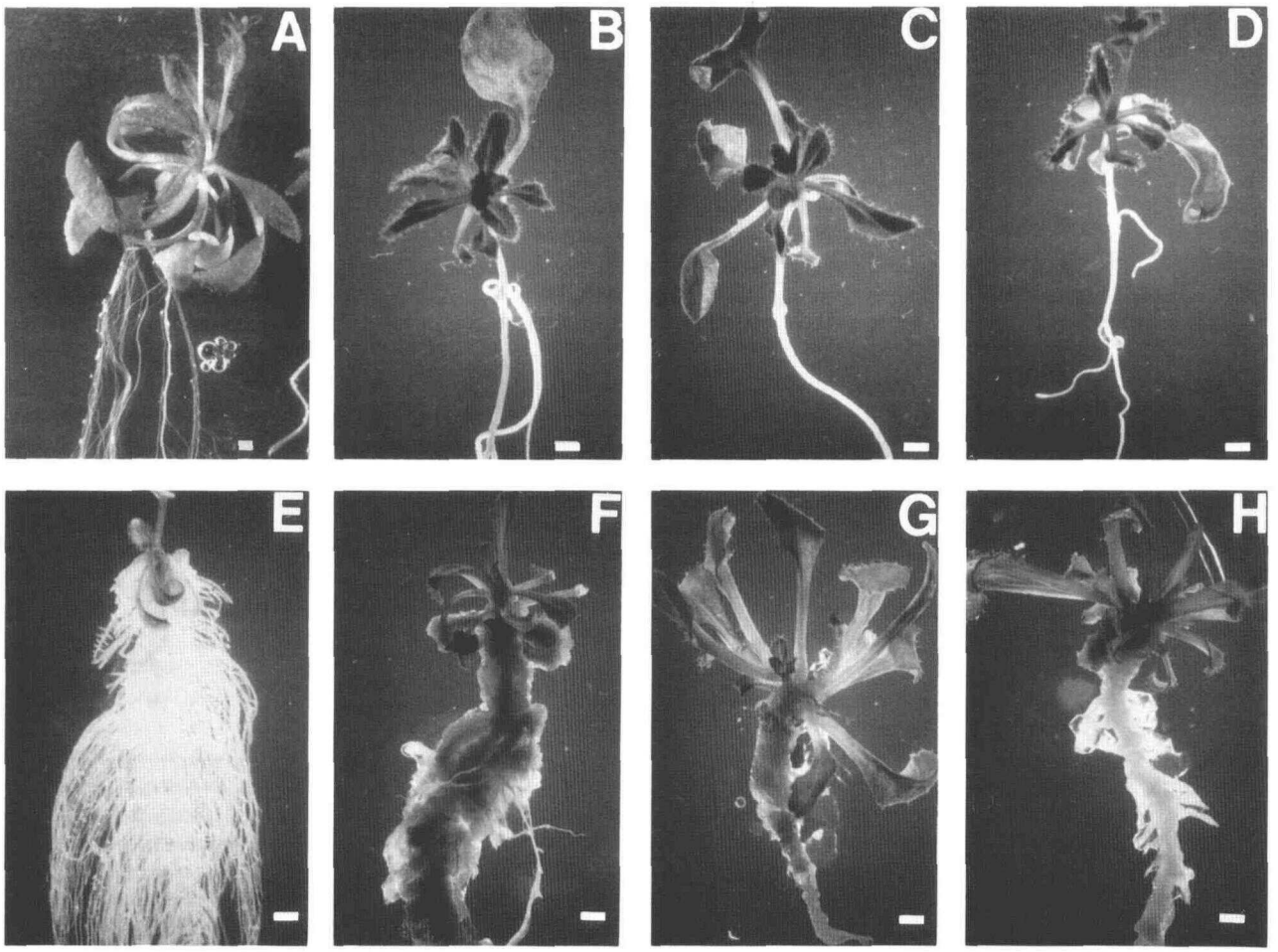

Figure 4. Morphologies of Wild-Type and *rty* Seedlings on Hormone-Free and 2iP-Containing Media.

(A) and **(E)** Wild type and *rty*, respectively, on hormone-free medium.
(B) and **(F)** Wild type and *rty*, respectively, on 6 μM 2iP.
(C) and **(G)** Wild type and *rty*, respectively, on 12 μM 2iP.
(D) and **(H)** Wild type and *rty*, respectively, on 24 μM 2iP.
Bars = 1 mm.

Preliminary experiments testing sensitivities to exogenous auxin were performed with cultures of excised roots, and results of these experiments suggested auxin autotrophy in isolated roots of *rty* (data not shown). In an additional test, 5-mm root tips of *rty* and the wild type were grown in auxin-free liquid medium for 42 days. Every seventh day, the apical 5 mm of each root culture was excised and transferred to fresh medium. During the experiment, *rty* roots sustained production of one to three lateral roots per 7-day cycle, whereas wild-type roots produced fewer than 0.5 lateral roots per cycle until day 21 and then ceased to proliferate. After 42 days, cultures were maintained without excision of root tips by refreshing the media every 14 days. Without added auxin, *rty* formed proliferating root cultures, whereas comparable growth in the wild type required addition of at least 0.01 μM NAA. These data support our hypothesis that, in contrast to wild-type roots, isolated roots of *rty* are auxin autotrophic.

Response to Exogenous Cytokinin

Responses of wild-type and *rty* seedlings to the cytokinin N^6-(2-isopentenyl)adenine (2iP) are shown in Figure 4. Root growth was inhibited in both genotypes (Figures 4A, 4B, 4E, and 4F), and shoot growth was inhibited in the wild type (Figures 4C and 4D) but stimulated in *rty* (Figures 4G and 4H). All concentrations of 2iP caused dwarfing and anthocyanin pigmentation in rosette leaves of the wild type. The same

Table 2. IAA Forms and Concentrations in Shoots and Roots of 16-Day-Old Wild-Type and *rty* Seedlings

Tissue	IAA Form	ng IAA g^{-1} FW[a]		*rty*/Wild Type Ratio
		Wild Type	*rty*	
Shoot	Free[b]	8.4 ± 1.9[c]	54.2 ± 8.6	6.4
Shoot	Free + ester[d]	59.1 ± 2.1	221.9 ± 18.0	3.8
Shoot	Total[e]	2,593.0 ± 380.4	44,614.8 ± 4,705.0	17.2
Root	Free	11.9 ± 1.1	26.0 ± 5.3	2.2
Root	Free + ester	98.5 ± 12.5	217.9 ± 32.4	2.2
Root	Total	4,611.0 ± 1,953.0	21,834.0 ± 1,274.0	4.7

[a] FW, fresh weight.
[b] Includes only free IAA.
[c] Mean of three observations ± SE.
[d] Includes free IAA plus ester-conjugated IAA.
[e] Includes free IAA plus ester-conjugated IAA plus amide-conjugated IAA.

treatments stimulated shoot growth and leaf expansion in *rty*. Anthocyanin was not visible in *rty* leaves except at 24 μM. Callus growth occurred in *rty* on 6 and 12 μM 2iP (Figures 4F and 4G) but never occurred in the wild type. The greatest amelioration of the *rty* phenotype was achieved with 12 μM 2iP (Figure 4G).

Determination of Endogenous IAA Concentrations

Because the phenotype of *rty* suggested enhanced auxin effects and was copied by applying auxin exogenously to wild-type seedlings, we analyzed endogenous IAA in root and shoot tissues of 16-day-old wild-type and *rty* seedlings. Concentrations of free, ester-conjugated, and amide-conjugated IAA are shown in Table 2. All forms of IAA were present at higher concentrations in *rty* than in the wild type. Free IAA in *rty* was 6.4 times higher in shoot tissue and 2.2 times higher in root tissue than in the wild type. The greatest difference in IAA levels was a 17-fold increase in amide-conjugated IAA in shoots of *rty* versus the wild type. In both genotypes, the dominant form of IAA conjugation was via amide linkages. Amide conjugates, ester conjugates, and free IAA comprised 97.7, 2, and 0.3%, respectively, of total IAA in wild-type shoots and 99.5, 0.4, and 0.1%, respectively, of total IAA in *rty* shoots. Within genotypes, proportions of IAA forms in roots were consistent with those of shoots; however, whole-plant distributions of total IAA between shoots and roots were different between the two genotypes. In wild-type seedlings, higher IAA levels were detected in roots, whereas in *rty* seedlings, higher IAA levels were detected in shoots.

Auxin and Ethylene Insensitivities Reduce Expression of the *rty* Phenotype

To discern more accurately the contributions of auxin and ethylene to development of the *rty* phenotype, double mutants were constructed by crossing *rty* with the auxin-resistant mutant *axr1-3* and the dominant, ethylene-insensitive mutant *etr1-1*. Individuals segregating for *rty* and *axr1-3* or *etr1-1* are shown in Figure 5. In F_2 populations, phenotypic ratios did not deviate significantly from 9 wild type:3 *axr1*:3 *rty*:1 *axr1/rty* and 9 *etr1*:3 wild type:3 *etr1/rty*:1 *rty* (data not shown). Resistance to auxin reduced but did not eliminate exfoliation of hypocotyl tissue, root proliferation, and epinasty and promoted root elongation and leaf expansion (Figure 5A). Inhibition of hypocotyl elongation caused by *rty* was not normalized by expression of *axr1-3*. Insensitivity to ethylene reduced epinasty, moderately promoted leaf expansion, and blocked inhibition of hypocotyl elongation but had no effect on proliferation of adventitious and lateral roots (Figure 5B).

Segregation and Allelism Tests

To determine the genetic basis of the *rty* phenotype, segregation of the wild type and *rty* was scored among progenies from self-pollination of heterozygous M_3 plants (M_4), a backcross of Arabidopsis ecotype Columbia × +/*rty* (BC_1S_1), and a cross of +/*rty* × Arabidopsis ecotype Landsberg homozygous for the *erecta* mutation (F_2). Segregation ratios, shown in Table 3, did not differ significantly from 3:1 (wild type to *rty*), indicating *rty* is inherited as a single, nuclear recessive gene.

Four mutants with *rty*-like phenotypes were isolated from independently mutagenized populations: *rty*, isolated in this laboratory; *invasive root-1* and *invasive root-2*, isolated by Z.R. Sung (University of California, Berkeley); and *hookless3*, isolated by A. Lehman and J.R. Ecker (University of Pennsylvania, Philadelphia). These were tested for allelism by scoring the segregation of the wild type and *rty* in F_1 families from crosses of parents heterozygous for the mutant alleles. Heterozygosity of all parents was confirmed with progeny tests from self-pollinations. As shown in Table 4, in all families tested, segregation occurred at a ratio of 3:1 (wild type to *rty*). Heterogeneity chi square values indicated no differences among

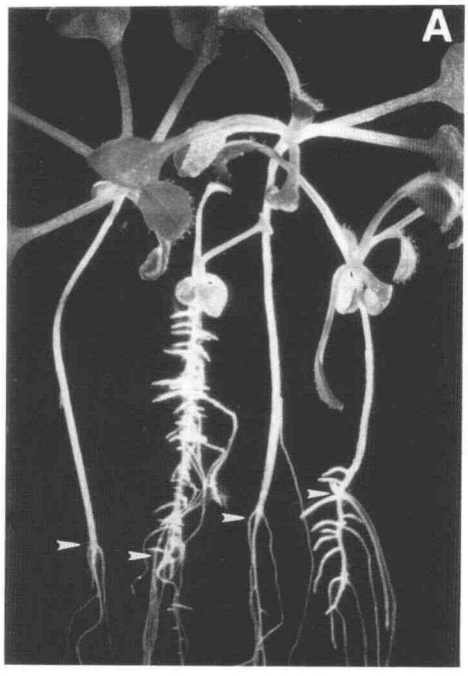

Figure 5. Phenotypes of the Wild Type, *rty*, *axr1-3*, *etr1-1*, and Double Mutants with *rty*.

(A) Representative seedlings of the wild type (WT), *rty*, *axr1-3*, and *rty/ axr1-3* grown on hormone-free medium for 3 days in darkness plus 6 days in light.

(B) Representative seedlings of the wild type, *rty*, *etr1-1*, and *rty/etr1-1* grown on hormone-free medium for 3 days in darkness in an atmosphere containing 10 ppm ethylene plus 6 days in light without added ethylene.

Arrowheads indicate hypocotyl–root junctions.

Table 3. Chi Square Tests for 3:1 Segregation of the Wild Type to *rty*

	Observed Number				
Family	Wild Type	*rty*	Total	χ^2 (3:1)	P
M_4[a]	1583	494	2077[b]	1.69	0.21 NS[c]
BC_1S_1[d]	104	27	131	1.35	0.25 NS
F_2[e]	312	94	406	0.73	0.42 NS

[a] Self-pollinated progeny of heterozygous M_3 plants.
[b] Summation of four replications.
[c] NS, not significant at P = 0.05.
[d] Self-pollinated progeny from a backcross of Columbia × +/*rty*.
[e] Self-pollinated progeny from a cross of +/*rty* × Landsberg *erecta*.

families within cross combinations, and data for these families were pooled. Results indicated that the four mutant isolates represent alleles at a single locus.

Mapping of *rty*

A preliminary map location of *rty* was determined relative to the 10 recessive phenotypic markers of the W100 tester line (Koorneef and Hanhart, 1983) by determining frequencies of W100 markers in an F_2 population (see Methods). Frequencies of four W100 markers deviated significantly from the expected 25%: on chromosome 2, *erecta* (*er*) (31.0%); on chromosome 3, *glabra* (*gl1*) (32.7%); and on chromosome 5, *male sterile* (*ms1*) (12.6%) and *transparent testa* (*tt3*) (18.1%). Frequencies >25% suggested linkage of *er* and *gl1* with *rty*.

In F_3 families generated by self-pollinating F_2 individuals expressing *er*, *gl1*, and *py* (linked to *er* and occurring at 28.6% in the F_2 generation), ratios of families segregating for *rty* to those not segregating (+/*rty* versus +/+ F_2 genotypes) deviated significantly from the 2:1 ratio expected for unlinked loci. Families representing chromosome 2 markers *er* and *py* showed lower than expected segregation of *rty* (0.6:1 and 0.5:1, respectively), and families representing the chromosome 3 marker *gl1* showed higher than expected segregation of *rty* (4:1). These data favored the location of *rty* on chromosome 2. Calculated map distances are illustrated in Figure 6A.

To map *rty* with higher resolution, restriction fragment length polymorphism (RFLP) markers on chromosome 2 (Chang et al., 1988) were evaluated for linkage. The order of three markers and *rty* on chromosome 2 determined by MapMaker was

Table 4. Allelism Tests among Four Mutants with *rty*-like Phenotypes

Genotype	No. of Families	Observed No. Wild Type	*rty*	Total	χ^2 (3:1)	P
BC_2S_1 × *hls3*[a]	2	121	44	165	0.24	>0.50 NS[b]
BC_2S_1 × *ivr-1*[c]	3	265	100	365	1.12	>0.25 NS
ivr-2 × BC_2S_1	3	97	35	132	0.16	>0.50 NS

[a] *hookless3.*
[b] NS, not significant at P = 0.05.
[c] *invasive root.*

m497, *rty*, m605, and m283, with distances to *rty* of 27.5 (m497), 3.9 (m605), and 32.3 (m283) centimorgans (cM) (Figure 6B).

DISCUSSION

The *RTY* locus of Arabidopsis appears to be critical for regulating IAA concentrations. We demonstrated that a recessive mutation at *RTY*, when homozygous, yields endogenous IAA concentrations from two to 17 times higher than in the wild type and causes several phenotypic alterations suggestive of auxin effects. Based on responses to exogenous auxin, sensitivity does not appear to be elevated in *rty/rty* individuals, and therefore, the phenotype is most likely caused by increased auxin concentration. The most extreme phenotypic effects of *rty* are proliferation of adventitious and lateral roots and restriction of shoot development. Four recessive mutant alleles were identified at *RTY*, and the locus mapped to 3.9 cM distal to RFLP marker m605 on chromosome 2. Further characterization of the structure and expression patterns of *rty* may provide insight to direct or indirect regulation of auxin concentration and its role in plant development.

Auxin Concentration and Sensitivity in *rty* and Wild-Type Tissues

Concentrations of all forms of IAA tested were higher in all tissues of *rty* relative to the wild type, with free IAA approximately six- and 2.2-fold higher in *rty* shoots and roots, respectively (Table 2). Distributions of IAA within plants differed in the two genotypes, with *rty* containing more IAA in shoots than in roots and the wild type containing more IAA in roots than in shoots. For *rty* tissues, this could reflect higher biosynthetic rates or lower turnover rates in shoots relative to roots, suboptimal auxin transport in shoots, or restricted expansion of shoot tissues yielding higher IAA concentrations on fresh weight bases.

Concentrations of free IAA are regulated through biosynthesis of IAA, formation and hydrolysis of conjugates, oxidation of IAA, and transport to or away from sites of action. Recessive inheritance of *rty* implies a loss-of-function allele and suggests a role for the wild-type gene product in repressing IAA biosynthesis or conjugate hydrolysis or in stimulating conjugation or oxidation of IAA. Feedback repression of IAA biosynthesis is not documented; however, this might reflect the poorly defined status of pathways leading to IAA rather than an absence of regulation. Without greater resolution of IAA biosynthetic pathways, we cannot discount the possibility of *RTY* acting as a repressor of some step in this process.

Conjugate formation and hydrolysis are pivotal in altering IAA levels (Cohen and Bialek, 1984). Quantities of ester and amide conjugates were greater in *rty* tissues than in the wild type but represented similar proportions of total IAA in both genotypes, suggesting that IAA conjugation is not altered in *rty*. It is also unlikely that the mutation at *rty* yields constitutive hydrolysis of conjugated IAA to free IAA, because under these conditions, one or both conjugated forms should be underrepresented. As discussed previously, this was not observed. Oxidative catabolism also serves as a significant irreversible route for reduction of IAA levels (Bandurski, 1984). Activities of IAA oxidation pathways and presence of oxidation products in *rty* were not evaluated in these studies but remain viable hypotheses for future analysis.

In several plant systems, isolated mutants or transgenic lines show increased IAA levels and phenotypic changes similar to *rty* (Klee et al., 1987; Slovin and Cohen, 1988; Romano et al., 1991, 1993; Sitbon et al., 1992), although the magnitude of effects appears to be greater in *rty*. This suggested a factor, such as elevated sensitivity to auxin concomitant with increased IAA levels, contributing to the *rty* phenotype. We tested responses of dark-grown wild-type and *rty* seedlings to exogenous auxin and found that IAA-induced inhibition of hypocotyl and root elongation did not differ significantly for the two genotypes (Figure 3), although a slight shift toward reduced sensitivity in *rty* hypocotyls was apparent (Figure 3A). These

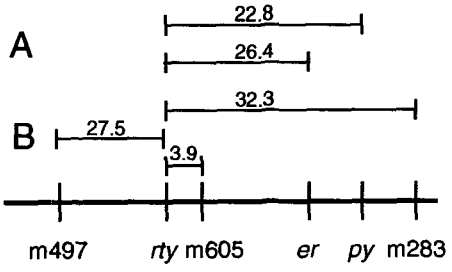

Figure 6. *Genetic Map Location of rty on Arabidopsis Chromosome 2.*

(A) Phenotypic markers.
(B) RFLP markers.
Map distances in centimorgans relative to *er*, *py*, and RFLP markers are based on 82, 71, and 51 observations, respectively.

data suggest that *rty* does not elevate sensitivity to auxin, but they cannot discount the possibility that other growth-promoting factors contribute to the *rty* phenotype.

Dissecting Causes of the *rty* Phenotype

All morphological alterations observed in *rty* were copied in wild-type seedlings exposed to exogenous auxin (10 μM NAA) (Figure 2), whereas *rty* seedlings exposed to exogenous cytokinin appear more like the wild type (Figure 4). These observations implicate elevated endogenous auxin in development of the *rty* phenotype and suggest direct or indirect regulation of auxin levels by the wild-type *RTY* gene product.

If altered auxin homeostasis is the primary defect in *rty*, the observation that wild-type and *rty* embryos are indistinguishable morphologically may indicate nonexpression of *RTY* during embryogenesis, expression only when it has minimal effect on embryo morphology, or existence of embryo-specific auxin concentration regulators maintaining normal levels of IAA in *rty/rty* embryos. Without higher resolution experiments, the above inferences remain equivocal but are supported in part by the existence of multiple IAA biosynthetic pathways during somatic embryogenesis in carrot (Michalczuk et al., 1992) and stage-specific differences in sensitivity to IAA during zygotic embryogenesis in Indian mustard (*Brassica juncea*) (Liu et al., 1993).

After germination, seedlings of *rty* and the wild type are first distinguishable after 3 days of dark growth, when *rty* exhibits reduced root and hypocotyl elongation (Table 1) and radially expanded, hookless hypocotyls (Figure 1A). These effects are reminiscent of the triple response (Neljubow, 1901) observed in wild-type Arabidopsis seedlings exposed to ethylene (Bleecker et al., 1988; Guzmán and Ecker, 1990), except that ethylene exaggerates apical hooks in the wild type. Formation of apical hooks has been attributed to asymmetric distributions of auxin (Migliaccio and Rayle, 1989; Evans, 1991; Li et al., 1991), ethylene (Schierle and Schwank, 1988), and a putative antagonist of ethylene action (Guzmán and Ecker, 1990). Hookless seedlings are characteristic of Arabidopsis mutants with reduced sensitivities to auxin (Mirza, 1987), ethylene (Bleecker et al., 1988; Guzmán and Ecker, 1990; Harpham et al., 1991), auxin and ethylene (Pickett et al., 1990), or auxin, ethylene, and abscisic acid (Wilson et al., 1990). If ethylene acts in concert with asymmetric distributions of auxin concentration or perception in apical hook formation and maintenance, hookless seedlings of *rty* could result from high internal auxin concentrations negating effects of auxin redistribution and elevating ethylene biosynthesis uniformly, rather than asymmetrically, in hypocotyl tissues.

Stimulation of ethylene biosynthesis by auxin (Abeles, 1966; Yang and Hoffman, 1984) complicates identification of specific effects of these two hormones. Some insight into the causes of the *rty* phenotype was derived by constructing double mutants combining auxin resistance from the *axr1-3* allele (Estelle and Somerville, 1987) or ethylene insensitivity from the *etr1-1* allele (Bleecker et al., 1988) with *rty*. In these combinations, insensitivity to auxin reduced epinasty and root proliferation and promoted root elongation (Figure 5A). Insensitivity to ethylene reduced epinasty and inhibition of hypocotyl elongation but had no apparent effect on root proliferation (Figure 5B). Neither of these mutations completely blocked the effects of *rty*. The *axr1-3* allele also confers partial resistance to cytokinins and ethylene (Estelle and Klee, 1994), and therefore, the reduction of epinasty in *rty* by both *axr1-3* and *etr1-1* prevents resolution of the cause of this phenotype. It is unlikely that cytokinin contributes to the *rty* phenotype, because wild-type seedlings exposed to cytokinin do not resemble *rty* (Figures 4B to 4D) and *rty* seedlings exposed to cytokinin appear more like the wild type (Figures 4F to 4H). In a similar experiment with transgenic Arabidopsis plants expressing the *iaaM* gene, which elevated endogenous IAA concentrations approximately fourfold, *axr1-3* was completely epistatic to effects of *iaaM* (Romano et al., 1995). These experiments suggest that the *rty* phenotype may be caused by a factor other than auxin, and elevated auxin is a secondary effect amplifying the phenotype. Alternatively, auxin concentrations in *rty* could be high enough to elicit some auxin responses in the *axr1-3* background, where auxin sensitivity is reduced but not abolished (Estelle and Somerville, 1987; Lincoln et al., 1990).

Genetic Analysis

Scoring phenotypic marker frequencies in wild-type individuals of the F_2 generation and segregation of *rty* in F_3 families indicated that *rty* is on chromosome 2 at 26.4 and 22.8 cM from *er* and *py*, respectively (Figure 6A). Map distances determined by RFLP analysis (Figure 6B) are comparable to other published maps for Arabidopsis. This analysis estimated 28.7 cM between m283 and m605, and this has been reported to be 30 to 35 cM (Reiter et al., 1992). Total map distance in this population was 59.8 cM from m497 to m283; this distance has been reported to be 48.3 cM (Chang et al., 1988). Location of *rty* to 3.9 cM from m605 provides a starting point for future molecular analyses of this locus.

Allelism tests suggested that four independently isolated mutants with *rty*-like phenotypes are alleles at a single locus. A fifth allele, generated by T-DNA mutagenesis, has been identified recently and should facilitate cloning of the *RTY* gene (N. Olszewski, personal communication). The *superroot* (*sur1*) mutant of Arabidopsis contains elevated auxin levels, expresses a phenotype similar to *rty*, and has been mapped to the same region of chromosome 2 (Boerjan et al., 1995). Complementation tests are needed to determine whether *rty* and *sur1* represent mutant alleles of a single locus.

It is of interest that the *rty* and *sur1* phenotypes can be copied by elevating auxin concentrations. If we assume that increased auxin causes this phenotype and we know of multiple biochemical steps at which auxin concentration could be

regulated, why do all known mutants with this phenotype represent alleles at a single locus or tightly linked loci? Genetic or biochemical redundancy at other points in auxin biosynthetic and metabolic pathways or lethality of mutations affecting these pathways could explain the dearth of mutants with altered auxin concentrations. This suggests that *rty* represents either a locus with only secondary effects on auxin concentration or a unique regulatory point in maintaining auxin homeostasis. Cloning and further characterization should resolve the function of this locus.

METHODS

Plant Growth Conditions

For in vitro screenings and analyses, seeds were surface sterilized for 20 min in 1.6% (v/v) NaOCl plus 0.1% (v/v) Tween 20, followed by three rinses in sterile deionized H_2O, and then sown onto basal medium (Haughn and Somerville, 1986) supplemented with 0.6 μM thiamine hydrochloride and 0.28 mM *myo*-inositol, hereafter referred to as the medium. Sucrose, agar (Difco Bacto), and hormone concentrations varied and are specified for each experiment. After sowing, seeds were stratified at 4°C in darkness for 24 hr and then transferred to 23°C in constant darkness or light from 0800 to 2400 hours at 50 $\mu E\ m^{-2}\ sec^{-1}$ provided by cool-white fluorescent bulbs. Unless specified otherwise, plants were grown in vertically oriented 100 × 15–mm Petri plates sealed with Parafilm (American National Can, Greenwich, CT). In the greenhouse, plants were grown in 1:1:1 soil-peat-perlite (v/v), hereafter referred to as potting mix. Greenhouse temperature was set to 20°C. A daily light regime as described above at a minimum of 550 $\mu E\ m^{-2}\ sec^{-1}$ was provided by natural light supplemented by 1000-W high-pressure sodium lamps.

Mutagenesis

An ethyl methanesulfonate (EMS)–mutagenized M_2 population of *Arabidopsis thaliana* ecotype Columbia was provided by J. Sheahan (Boyce Thompson Institute, Ithaca, NY). Procedures for mutagenesis have been described by Sheahan et al. (1993).

Mutant Selection Strategy

A screen was designed to select mutations altering development of adventitious roots on seedling hypocotyls. Selection was based on the number of new roots produced and root morphology, indicating competence to initiate root meristems and the ability to maintain actively growing meristems, respectively. This screen was expected to detect mutations affecting root development, hormone (particularly auxin) perception or metabolism, general meristem organization or maintenance, and possibly cell division factors.

Twenty-five milliliters of medium containing 1% (w/v) sucrose and 0.7% (w/v) agar was placed into 125-mL round glass jars. Before autoclaving, a polycarbonate ring ~3 cm tall and of a diameter ~5 mm less than the inside diameter of the jar was placed into the medium in the jar. Jars were covered with 60 × 10–mm glass Petri dish tops and autoclaved. Immediately after autoclaving, rings were centered in the jars, leaving a channel of 2 to 3 mm between the rings and inner walls of the jars. Bulked M_2 seeds were surface disinfested, and ~50 seeds were sown into channels in the jars as a suspension in 1 mL of cooled liquid medium containing 0.7% (w/v) agar. Seeds were stratified and then germinated in darkness at 23°C for 5 days, producing seedlings with etiolated hypocotyls 7 to 10 mm in length. Jars were opened in a laminar air flow hood, and 4 mL of cooled liquid medium containing 10 or 75 μM indole-3-butyric acid (IBA) and 0.7% (w/v) agar was added to the channels. This flooded the hypocotyls to a depth of 5 mm to induce adventitious root formation. Two concentrations of IBA were chosen to enhance detection of elevated rooting (10 μM) and reduced rooting (75 μM) variants against backgrounds of wild-type seedlings producing low (10 μM) and high (75 μM) numbers of roots. Jars were resealed and placed at 23°C in the light. After 14 days, seedlings were scored for the number and morphology of emerged adventitious roots.

Population Development

From a bulked M_2 population, a single seedling with an adventitious root count in the upper extreme of the population distribution was selected and self-pollinated to produce an M_3 family in which the *rooty* (*rty*) mutant segregated. This mutation is inherited as a single, nuclear recessive gene and, when homozygous, causes abnormal growth and prevents flowering. Therefore, transmission of the *rty* allele occurs only through heterozygous individuals that are phenotypically normal. Five wild-type segregants (+/+ or +/*rty* genotypes) from the M_3 family carrying *rty* were crossed to ecotype Columbia to generate backcross 1 (BC_1) seed. Flowers to be crossed were emasculated before or as petals emerged above sepals. Pollen was transferred immediately and again after 24 hr to stigmatic surfaces of emasculated flowers. Flowers not crossed were allowed to self-pollinate. Genotypes of the M_3 parents were determined by scoring self-pollinated seeds for segregation of *rty*. When these genotypes were known, 6 to 10 BC_1 seeds from M_3 parents heterozygous for *rty* were sown, and plants were allowed to self-pollinate to generate BC_1S_1 families, which were scored for segregation of *rty*. With a BC_1S_1 family segregating for *rty*, the above process was repeated to produce BC_2S_1 families. Physiological and biochemical analyses were performed on BC_1S_1 or BC_2S_1 families derived from sibling backcrossed plants after testing S_1 seedlings for segregation of *rty*. Families not segregating were used as wild-type controls, and *rty* individuals were selected for experiments from segregating families. Within experiments, genotypes of BC_1S_1 and BC_2S_1 individuals are referred to generically as wild type or *rty*. For genetic analyses, F_1 and F_2 generations were produced via methods analogous to BC_1 and BC_1S_1 in the above description.

Time Course of Phenotype Development

Seeds of two BC_2S_1 families were surface disinfested and sown onto 30 mL of medium with 1.05% (w/v) agar in Petri plates. Seedlings were grown in darkness or light at 23°C. Photographs of representative dark-grown seedlings were taken after 3 days. Light-grown individuals were photographed every 24 hr beginning after 4 days in light.

Responses to Exogenous Auxins

Seeds of sibling BC_2S_1 families were surface disinfested and sown onto 30 mL of medium supplemented with 1% (w/v) sucrose; 0, 1, 10,

55, or 100 μM α-naphthaleneacetic acid (NAA); and 1.05% (w/v) agar in Petri plates. Seedlings were grown at 23°C in darkness. After 3 days, seedling morphologies were observed, embryonic root and hypocotyl lengths were measured, and Petri plates were placed at 23°C in the light. Morphological observations were made every 3 days through day 15.

To test auxin sensitivity, sibling families were sown onto medium as given above and supplemented with 0, 0.0001, 0.001, 0.01, 0.1, 1.0, 10, or 100 μM indole-3-acetic acid (IAA). Medium preparation and seed sowing occurred under 0.5 $\mu E\ m^{-2}\ sec^{-1}$ incandescent light to minimize photooxidation of IAA. Seedlings were grown at 23°C in darkness in two randomized complete blocks. After 3 days, hypocotyl and root lengths were measured on 10 plants/replication. Data were normalized to lengths as a percentage of the control treatment and subjected to analysis of variance. The experiment was performed twice with similar results; therefore, data were pooled to yield four replications per treatment. Concentrations of IAA causing 50% inhibition of root and hypocotyl growth (I_{50}) were calculated for each replication by solving regression equations with $y = y$ intercept $\div$ 2.

Responses to Exogenous Cytokinin

Seeds of sibling BC_2S_1 families were surface disinfested and sown onto medium supplemented with 1% (w/v) sucrose and 1.05% (w/v) agar in Petri plates. After 7 days at 23°C in the light regime, wild-type and *rty* individuals were transferred to glass vials containing 10 mL of medium supplemented with 1% (w/v) sucrose, 0.7% agar, and N^6-(2-isopentenyl)adenine (2iP) at 6, 12, or 24 μM. After 2 weeks, plants were transferred to fresh medium for the same treatment. After 4 weeks, morphological observations were made.

Determination of Endogenous IAA Concentrations

Sibling BC_1S_1 families were sown onto medium supplemented with 1% (w/v) sucrose and 1.05% (w/v) agar in Petri plates and grown until *rty* segregants could be identified (6 days). Wild-type and *rty* seedlings were transferred to new Petri plates and grown for 10 days at 23°C in the light regime. Root and shoot portions were separated, weighed, frozen in liquid nitrogen, and stored at −80°C. Extraction and purification procedures followed Chen et al. (1988). Tissues were homogenized with cold 65% isopropanol/35% 0.2 M imidazole buffer, pH 7.0, in the presence of the following labeled standards: 50 ng of $^{13}C_6$-IAA as the internal standard for isotope dilution calculations and 1 kBq of ^{3}H-IAA (specific activity of 803 GBq mol^{-1}; Amersham, Arlington Heights, IL) as a radioactive tracer for chromatographic separations.

After incubation for at least 1 hr in homogenization buffer, cellular debris was removed by centrifugation at 10,000*g*. The pellet was washed and centrifuged three times with 1.0 mL of isopropanol, and supernatants were pooled. The organic phase was evaporated from the supernatant in vacuo. Aqueous residue was divided into three parts: one used directly for analysis of free IAA; the second subjected to mild alkaline hydrolysis (1 N NaOH at ~25°C for 1 hr) for analysis of free plus esterified IAA; and the third subjected to strong alkaline hydrolysis (7 N NaOH at ~100°C for 3 hr under N_2) for analysis of total (free plus ester-conjugated plus amide-conjugated) IAA (Cohen et al., 1986). Purification was initiated on a Fisher (Pittsburgh, PA) PrepSep disposable amino column (free IAA) or a Fisher PrepSep disposable C_{18} column (ester- and amide-conjugated IAA) followed by HPLC on a 4.6 × 50–mm C_{18} column (Ultracarb 30; Phenomenex, Torrence, CA) that was equilibrated for 30 min with 100% methanol and for 15 min with 1% acetic acid in 25% methanol. After sample injection, the column was eluted isocratically for 15 min with 1% acetic acid in 25% methanol.

Fractions containing IAA were collected, pooled, evaporated in vacuo, and methylated with CH_2N_2. Samples were analyzed by 70 eV electron impact gas chromatography–selected ion monitoring–mass spectrometry with a Hewlett-Packard (Palo Alto, CA) 5890 GC equipped with a 15-m × 0.32-mm i.d. DB-1701 fused silica column (J & W Scientific, Folsom, CA) coupled to a Hewlett-Packard 5971A mass selective detector. Ions at an *m/z* of 130 and 136 (unlabeled and ^{13}C-labeled quinolinium fragment ions) and at 189 and 195 (unlabeled and ^{13}C-labeled molecular ions) were monitored for IAA analysis. Peak areas for ions at an *m/z* of 130 and 136 were used for calculation of IAA concentrations with an isotope dilution equation (Cohen et al., 1986). Gas chromatography conditions were as follows: injector temperature at 250°C and oven temperature at 140°C for 1 min and then increased at 16°C per min up to 280°C. Helium at 1 mL min^{-1} was the carrier gas. Under these conditions, retention time for IAA methyl ester was 5.2 min. Triplicate determinations were performed for free IAA, free plus ester-conjugated IAA, and total (free plus ester-conjugated plus amide-conjugated) IAA in root and shoot tissues of wild type and *rty*. From these data, percentages of total IAA accounted for by free, ester-conjugated, and amide-conjugated forms were calculated as follows: free/total × 100; [(free + ester) − free]/total × 100; and [total − (free + ester)]/total × 100, respectively.

Double Mutant Analysis

Families segregating for *rty* and *axr1-3* (auxin-resistant mutant) or *etr1-1* (ethylene-insensitive mutant) were developed by crossing BC_2S_1 individuals heterozygous for *rty* to individuals homozygous for *axr1-3* or *etr1-1*. Seeds were sown in single lines onto Petri plates and scored as follows. For *rty*/*axr1-3*, plates were oriented vertically for 3 days in darkness, scored for phenotypes, transferred to light, and scored again after 6 days. Auxin insensitivity was confirmed with methods described by Estelle and Somerville (1987) on media containing 0.2 μM 2,4-D after completion of phenotypic analysis to avoid the possibility of 2,4-D confounding phenotypic effects of *rty*. For *rty*/*etr1-1*, plates were oriented horizontally in darkness in jars purged with air or 10 ppm ethylene. After 3 days, *rty*-associated phenotypes and ethylene insensitivity were scored, plates were transferred to light, and phenotypes were scored again after 6 days. Segregation ratios of phenotypic classes were tested with chi square analysis.

Segregation Analysis and Allelism Tests

Segregation of *rty* was tested in F_2, BC_2S_1, and M_4 families. Seeds were sown onto medium in Petri plates, and after 10 days in the light, counts of *rty* and wild-type individuals were made. Data were analyzed by chi square for goodness of fit to a 3:1 segregation ratio and a chi square heterogeneity test (Mather, 1951) to determine whether data from replicate analyses of the M_4 family could be pooled.

Four mutants expressing *rty*-like phenotypes were tested for allelism. These included *rty*, isolated in this laboratory, and *invasive root-1*, isolated by Z.R. Sung (University of California, Berkeley, CA) and generated by EMS mutagenesis; *invasive root-2*, isolated by Z.R. Sung and generated by γ-ray mutagenesis; and *hookless3*, isolated by A. Lehman and J.R. Ecker (University of Pennsylvania, Philadelphia, PA) and generated

by T-DNA mutagenesis (Feldmann, 1991). The *invasive root-1*, *invasive root-2*, and *hookless3* mutants were crossed reciprocally with *rty* using procedures described above. Self-pollinated seeds of parents were sown onto potting mix and tested for segregation of mutant (*rty*-like) phenotypes. The F_1 seeds for which both parents were heterozygous for the mutant allele were sown onto medium in Petri plates, and after 10 days, counts of *rty*-like and wild-type individuals were made. Segregation data for self-pollinated parental and F_1 families were analyzed by chi square for goodness of fit to a 3:1 ratio and heterogeneity chi square to determine whether family data could be pooled.

Mapping of *rty*

Seeds of Arabidopsis Wageningen tester line W100 (Koorneef and Hanhart, 1983) were obtained from the Nottingham Arabidopsis Stock Center, Nottingham, U.K. This line is homozygous for two recessive phenotypic markers on each chromosome. The W100 markers are all morphological, and some affect flower structures or fertility. Because the *rty* mutation reduces plant stature, restricts shoot development, and prevents flowering, only two W100 markers could be scored in F_2 homozygous combinations with *rty*. Therefore, linkages with W100 markers were determined in an F_2 population grown in soil in which *rty* seedlings are inviable beyond the cotyledon stage and are therefore eliminated from the population scored for markers. This distorts segregation of any markers linked in repulsion to *rty*, inflating their frequencies in the F_2 generation to a maximum of 33%, whereas unlinked markers occur at 25%.

Crosses of W100 × *rty* were made, and F_1 plants were self-pollinated to generate F_2 seed. An F_2 population of 307 individuals was grown in potting mix in a greenhouse and scored for the presence of phenotypic markers. Individuals in which a marker phenotype could not be scored clearly were omitted from the population for that marker, yielding population sizes of 196 to 301 that could be scored. Data were analyzed by chi square for goodness of fit to a 3:1 ratio. Markers occurring at frequencies >25% were investigated further for indications of linkage with *rty* by self-pollinating F_2 plants expressing these markers. Seeds of the resulting F_3 families were sown onto the surface of potting mix in 9 × 13.5–cm plastic pots and scored for segregation of *rty*. Because the portion of the F_2 population that could be scored represented only individuals of +/+ or +/*rty* genotypes, the expected frequency of +/*rty* individuals was 66% among individuals expressing unlinked markers and <66% among those expressing linked markers. Recombination frequencies (p) between *rty* and marker loci were calculated with the formulas described by Koorneef and Stam (1992).

$$\text{If} \quad x = \frac{a/a,\ B/b}{n} = \frac{\text{number of familes segregating for } b}{\text{number of } a/a \text{ familes tested}}$$

$$\text{then} \quad p = 0.01\, r = \frac{x}{2 - x}$$

$$\text{with SE} \quad S_p = \frac{2\sqrt{x\,(1 - x)}}{(2 - x)^2\sqrt{n}}$$

Recombination frequencies were converted to centimorgans with the Kosambi mapping function (Kosambi, 1944).

For mapping with molecular markers, a population was generated by crossing a +/*rty* individual × Arabidopsis ecotype Landsberg homozygous for the *erecta* mutation. Tissue for DNA extraction included single F_2 *rty*/*rty* plants and F_3 families from +/+ and +/*rty* F_2 individuals. Seeds of the F_2 plants were disinfested, imbibed in sterile deionized water at 3°C for 24 hr, and sown onto medium in Petri plates. When *rty* individuals could be scored, one plant was transferred to each of 45 125-mL Erlenmeyer flasks containing 50 mL of liquid medium. Liquid cultures were grown on an orbital shaker at 90 rpm at 23°C in the light at 30 $\mu E\ m^{-2}\ sec^{-1}$ provided by cool-white fluorescent bulbs. Seeds of F_3 families were treated similarly except that after surface sterilization, an aliquot of seed from each family was sown onto a Petri plate to score for segregation of *rty*. Liquid cultures were grown for 14 to 21 days, blotted to remove free liquid, wrapped in aluminum foil, frozen in liquid N_2, and stored at −80°C.

Tissues representing 51 F_2 individuals consisting of 25 *rty*/*rty* F_2 plants and 26 F_3 families (20 +/+ and six +/*rty*) were prepared for restriction fragment length polymorphism (RFLP) analysis. Total DNA was isolated from 2 to 5 g of frozen tissues by the CTAB procedure (Murray and Thompson, 1980). Three micrograms of DNA from each sample was cut with 40 units of EcoRI (No. R4014; Promega Corp., Madison, WI), size fractionated in 0.7% (w/v) agarose gels for ~700 $V\ hr^{-1}$ in 0.5 × TBE (0.045 M Tris-borate, 0.001 M EDTA), transferred overnight to nylon membranes (No. NT4HY00010; Micron Separations, Westboro, MA) by capillary action (Southern, 1975), and fixed to membranes by baking at 80°C for 1 hr.

Three RFLP markers were chosen from the Arabidopsis genetic map (Chang et al., 1988) based on proximity to the location for *rty* relative to morphological markers. Marker DNA was denatured for 5 min at 95°C and placed immediately on ice. Radioactive probes were prepared by random priming (Feinberg and Vogelstein, 1983) using the Prime-A-Gene labeling kit (No. U1100; Promega). Radioactivity was added as 5 μL of α-^{32}P-dCTP (10 $\mu Ci\ \mu L^{-1}$) (No. PB10475; Amersham), and reactions were run overnight at room temperature. One volume (50 mL) of Tris-EDTA was added to the reactions, and labeled probe DNA was separated from unincorporated deoxynucleotide triphosphate precursors by spin column chromatography.

Nylon membranes were prehybridized for 12 to 16 hr at 65°C in a hybridization solution containing 6 × SSPE (1 × SSPE is 150 mM NaCl, 10 mM NaH_2PO_4, 1.0 mM EDTA), 5 × Denhardt's reagent (1 g Ficoll, 1 g PVP, and 1 g BSA per L), 0.5% SDS, and 100 $\mu g\ mL^{-1}$ of denatured salmon sperm DNA. Labeled probe was added to the solution, and hybridization was at 65°C for 24 hr. Membranes were washed twice at 65°C for 45 min each in 1 × SSPE plus 0.1% SDS and then in 0.2 × SSPE plus 0.1% SDS, wrapped in Saran Wrap, and exposed to x-ray film at −80°C. After 2 to 6 days, films were developed, and sample lanes were scored for genotypes at marker loci. Data were analyzed by MapMaker/Exp Version 3.0b (Lander et al., 1987).

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