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A Strong Loss-of-Function Mutation in *RAN1* Results in Constitutive Activation of the Ethylene Response Pathway as Well as a Rosette-Lethal Phenotype

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A recessive mutation was identified that constitutively activated the ethylene response pathway in Arabidopsis and resulted in a rosette-lethal phenotype. Positional cloning of the gene corresponding to this mutation revealed that it was allelic to *responsive to antagonist1* (*ran1*), a mutation that causes seedlings to respond in a positive manner to what is normally a competitive inhibitor of ethylene binding. In contrast to the previously identified *ran1-1* and *ran1-2* alleles that are morphologically indistinguishable from wild-type plants, this *ran1-3* allele results in a rosette-lethal phenotype. The predicted protein encoded by the *RAN1* gene is similar to the Wilson and Menkes disease proteins and yeast Ccc2 protein, which are integral membrane cation-transporting P-type ATPases involved in copper trafficking. Genetic epistasis analysis indicated that RAN1 acts upstream of mutations in the ethylene receptor gene family. However, the rosette-lethal phenotype of *ran1-3* was not suppressed by ethylene-insensitive mutants, suggesting that this mutation also affects a non-ethylene-dependent pathway regulating cell expansion. The phenotype of *ran1-3* mutants is similar to loss-of-function ethylene receptor mutants, suggesting that RAN1 may be required to form functional ethylene receptors. Furthermore, these results suggest that copper is required not only for ethylene binding but also for the signaling function of the ethylene receptors.

INTRODUCTION

The gaseous hormone ethylene influences many aspects of plant growth and development, including germination, leaf and flower senescence and abscission, fruit ripening, nodulation, and root hair development (Abeles et al., 1992). Many components of ethylene signaling have been identified, principally through the use of a simple genetic screen that uses the ethylene-mediated seedling morphology known as the triple response. The triple response of Arabidopsis is characterized by an inhibition of elongation of the seedling root and hypocotyl, radial expansion of the hypocotyl, and exaggeration of the curvature of the apical hook (Figure 1). Three classes of response mutants have been identified: ethylene-insensitive mutants, constitutive ethylene response mutants, and those mutants that affect only a subset of organs (reviewed in Kieber, 1997; Johnson and Ecker, 1998).

The *ETR1* gene was identified by a series of dominant, ethylene-insensitive mutations (Bleecker et al., 1988). *ETR1* encodes a protein that is similar to bacterial two-component histidine kinases (Chang et al., 1993). Two-component systems are the major route by which bacteria sense and respond to external stimuli, such as phosphate availability and osmolarity (Stock et al., 1990). The C terminus of ETR1 is similar to both the histidine kinase domain of the sensor component and the receiver domain of the response regulators. Yeast cells expressing wild-type, but not mutant, ETR1 bound ethylene with an affinity consistent with the observed concentrations that cause physiological effects. This, coupled with other genetic and molecular data, indicates that *ETR1* encodes an ethylene receptor (Schaller and Bleecker, 1995). ETR1 forms disulfide-linked dimers in vivo and has been shown to possess intrinsic histidine kinase activity in vitro (Schaller et al., 1995; Gamble et al., 1998).

ETR1 is part of a small gene family of ethylene receptors in Arabidopsis, and dominant, ethylene-insensitive mutations have been found in several of the other genes in this family (Hua et al., 1995, 1998; Sakai et al., 1998). Loss-of-function mutations in *ETR1* result in little or no phenotype, but disruption of multiple homologs results in a constitutive ethylene response phenotype that becomes increasingly strong as additional homologs are disrupted (Hua and Meyerowitz, 1998). This suggests that ETR1 and its homologs function as negative regulators of ethylene signaling

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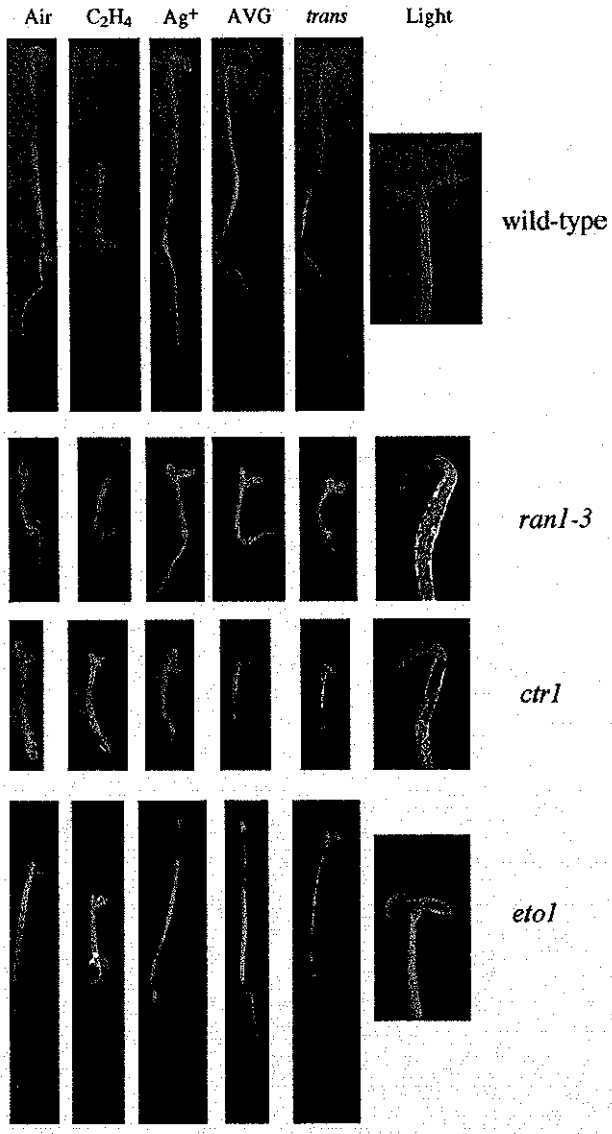

Figure 1. The *ran1-3* Mutation Constitutively Activates the Ethylene Response Pathway in Seedlings.

Phenotypes of 3-day-old etiolated wild-type, *ran1-3*, *ctr1*, and *eto1* (as indicated at right) seedlings were grown on Murashige and Skoog (MS) medium in air, ethylene (C_2H_4), or *trans*-cyclooctene (*trans*) or were grown in air on MS medium containing $AgNO_3$ (Ag^+) or aminoethoxyvinylglycine (AVG) as indicated above each column of seedlings. At right are seedlings that were grown for 3 days in the dark on MS medium in air and then placed in a lighted growth chamber for 12 hr (Light).

and that binding of ethylene serves to inactivate receptor function.

Recessive *ethylene-insensitive3* (*ein3*) mutations result in a weak ethylene-insensitive phenotype (Roman et al., 1995). *EIN3* is the founding member of the *EIN3/EIL* gene family, the members of which encode transcription factors that are involved in regulating ethylene-mediated gene expression (Chao et al., 1997). Overexpression of *EIN3* or the other *EIL* homologs results in a constitutive ethylene response phenotype. Strong alleles of the recessive *ein2* mutation completely abolish all ethylene responses that have been tested (Roman et al., 1995). *EIN2* encodes a 12-pass transmembrane protein that is most similar to the Nramp family of metal transporters (Alonso et al., 1999). However, although EIN2 has been demonstrated to be an integral membrane protein, its biochemical function remains unknown.

CONSTITUTIVE TRIPLE RESPONSE1 (*CTR1*), a negative regulator of ethylene signaling, acts downstream of *ETR1* and encodes a protein with similarity to the Raf family of protein kinases (Kieber et al., 1993). Recessive mutations in *CTR1* have pleiotropic effects on plant development, including a reduction in the size of roots, leaves, and stems; delayed flowering; delayed opening of the apical hook; and ectopic formation of root hairs (Kieber et al., 1993). These phenotypes can be linked to a constitutive activation of the ethylene response pathway and are all suppressed by downstream ethylene-insensitive mutations, such as *ein2*. The smaller organs in *ctr1* mutants have been linked to a reduction in cell expansion. In many plant tissues, this process is regulated by ethylene. Interestingly, when four of the five Arabidopsis *ETR1* homologs are disrupted simultaneously, the resulting plants have a phenotype more severe than that of *ctr1* null alleles (Hua and Meyerowitz, 1998).

responsive to antagonist1 (*ran1*) was identified in a screen for mutants that displayed a triple response in response to *trans*-cyclooctene, a compound that inhibits ethylene action in wild-type plants (Sisler et al., 1990; Hirayama et al., 1999). *ran1* mutations are recessive and do not affect the response to exogenous ethylene. Importantly, neither the *ran1-1* nor the *ran1-2* mutants have any appreciable phenotype in the absence of *trans*-cyclooctene. Analysis of ethylene-regulated gene expression revealed that *ran1* also confers on adult plants the ability to respond positively to *trans*-cyclooctene. RAN1 has significant similarity to the human Wilson and Menkes disease proteins and yeast Ccc2p, which are involved in copper trafficking (Chelly et al., 1993; Mercer et al., 1993; Vulpe et al., 1993; Fu et al., 1995). Consistent with a role in copper trafficking, the phenotype of the *ran1* mutant was partially reverted by growth on copper-supplemented medium, and RAN1 was able to suppress the phenotype of a yeast *ccc2* mutant. Biochemical analysis of the ethylene receptor ETR1 has demonstrated that it contains a copper cofactor (Rodriguez et al., 1999), which may be added by a RAN1-dependent mechanism.

Here, we describe *ran1-3*, a mutation that results in the constitutive activation of ethylene responses in seedlings and adults. Analysis of the interaction with other mutants affected in ethylene action indicates that dominant, ethylene-insensitive receptor mutations are epistatic to *ran1-3*, indicating that the *RAN1* gene product acts very early in signal-

ing. In addition to its role in ethylene signaling, our analysis indicates that RAN1 function is required for cell elongation in an ethylene-independent manner.

RESULTS

Isolation and Phenotypic Characterization of *ran1-3*

We screened small families of mutagenized Arabidopsis for lines that segregated seedlings displaying a constitutive triple response (Ctr⁻) phenotype. The use of a family screen allowed the recovery of lethal or infertile mutants. From a screen of 6300 individual ethyl methanesulfonate–mutagenized M_2 seeds (in 2100 families), two new *ctr1* alleles were identified (data not shown). In addition, a single line was identified that segregated for a Ctr⁻ seedling phenotype that died after growing for 2 weeks in soil. We identified a sibling from this family that segregated for the Ctr⁻ mutant phenotype. The mutation in this line was originally called *constitutive triple response2* but has been renamed *ran1-3* because it is allelic to the *ran1* mutation. *ran1-3* complemented *ctr1* (42 of 42 F_1 seedlings from a *ctr1/ctr1* × *RAN1/ran1-3* cross displayed a wild-type phenotype, and the F_2 from all F_1 plants segregated both wild-type and Ctr⁻ mutant seedlings).

Inhibitors of ethylene biosynthesis (aminoethoxyvinylglycine) or binding ($AgNO_3$ and *trans*-cyclooctene) did not revert the seedling phenotype of *ran1-3*, indicating that the mutation probably affects ethylene signaling rather than ethylene biosynthesis (Figure 1). Consistent with this, *ran1-3* mutant and wild-type 3-day-old etiolated seedlings produced comparable amounts of ethylene (0.17 ± 0.07 pL of ethylene seedling^{-1} hr^{-1} for *ran1-3* versus 0.19 ± 0.05 pL of ethylene seedling^{-1} hr^{-1} for wild-type seedlings).

When grown in air, the hypocotyls of 3-day-old etiolated *ran1-3* seedlings were shorter than those of the *ctr1-5* mutant, which is a strong *ctr1* allele (2.4 ± 0.5 mm for *ran1-3* versus 3.1 ± 0.7 mm for *ctr1*). In contrast, the roots of *ran1-3* mutant seedlings were not significantly shorter (1.5 ± 0.3 mm) than those of *ctr1-5* (1.4 ± 0.4 mm). Like *ctr1* mutant and ethylene-treated wild-type seedlings, the apical hook and cotyledons of *ran1-3* mutants take longer to open when etiolated seedlings are shifted to the light, and the cotyledons are darker green (Figure 1).

ran1-3 segregated as a single-gene, recessive mutation. However, the progeny of a self-fertilized *ran1-3* heterozygote segregated a Ctr⁻ phenotype at a frequency that deviated from the expected 25% (554 wild type: 115 *ran1-3*, $\chi^2 = 22$, $P < 0.05$). We examined reciprocal crosses between *ran1-3* heterozygotes and wild-type plants to determine if the transmission of *ran1-3* is reduced through the male, the female, or both gametes (Table 1). Transmission of *ran1-3* through the female gamete did not differ from the expected 1:1 segregation ratio in the F_1 in crosses to wild-type males.

Table 1. The *ran1-3* Allele Is Transmitted Poorly through the Male Gamete

Cross[a]	F_1 Genotype[b]		
	RAN1/RAN1	*RAN1/ran1-3*	χ^{2c}
ran1-3/RAN1 × *RAN1/RAN1*	49	44	0.54 ($P > 0.5$)
RAN1/RAN1 × *RAN1/ran1-3*	31	16	4.8 ($P < 0.5$)

[a] The first plant listed in each cross is the female.
[b] Determined by progeny testing each F_1 plant.
[c] Calculated for an expected 1:1 ratio.

However, when *ran1-3* heterozygotes were used as males in crosses to wild-type females, the F_1 showed a marked departure from the expected 1:1 ratio; wild-type alleles were approximately twice as likely as *ran1-3* to be transmitted to the F_1 through the pollen. In contrast, *ctr1* mutations display a defect in transmission through the female gametes (Kieber et al., 1993).

When grown on soil, *ran1-3* mutants die after forming only two to three sets of true leaves (Figures 2A and 2B). The cotyledons and leaves of *ran1-3* senesce much earlier than those of wild-type plants and display high amounts of anthocyanins (Figure 2B). The rosette of *ran1-3* is quite compact (<5.0 mm in diameter), and the true leaves are much smaller than those of the wild type or the *ctr1-5* mutant. *ran1-3* mutants do not bolt, and they rarely survive for >21 days on soil. Like *ctr1*, *ran1-3* mutants have a greatly reduced root system and display an increase in the number of root hairs (data not shown). These adult phenotypes cosegregate with the seedling phenotype (i.e., within <1.9 map units of the seedling phenotype with a 95% degree of confidence), suggesting that they result from a single mutation. This was confirmed by complementation of both the seedling and the adult phenotypes with the same gene (see below).

When grown in vitro on Gamborg's media, *ran1-3* mutants were more robust than their soil-grown counterparts (Figure 2C). The rosette was slightly larger, and early senescence was not observed. In contrast to soil-grown *ran1-3* plants, *ran1-3* mutants grown in vitro produced an inflorescence, although flowering was delayed relative to the wild-type plants, and the inflorescence was very small (Figure 2E). *ran1-3* mutants formed flowers in vitro, but these never fully developed or opened, and the plants were not fertile (Figure 2G). These rudimentary *ran1-3* flowers displayed high concentrations of anthocyanins.

The *ran1-3* Mutation Affects Cell Size and Ethylene-Regulated Gene Expression

To determine the basis for the reduced size of *ran1-3* mutants, we examined the size of leaf epidermal cells by using

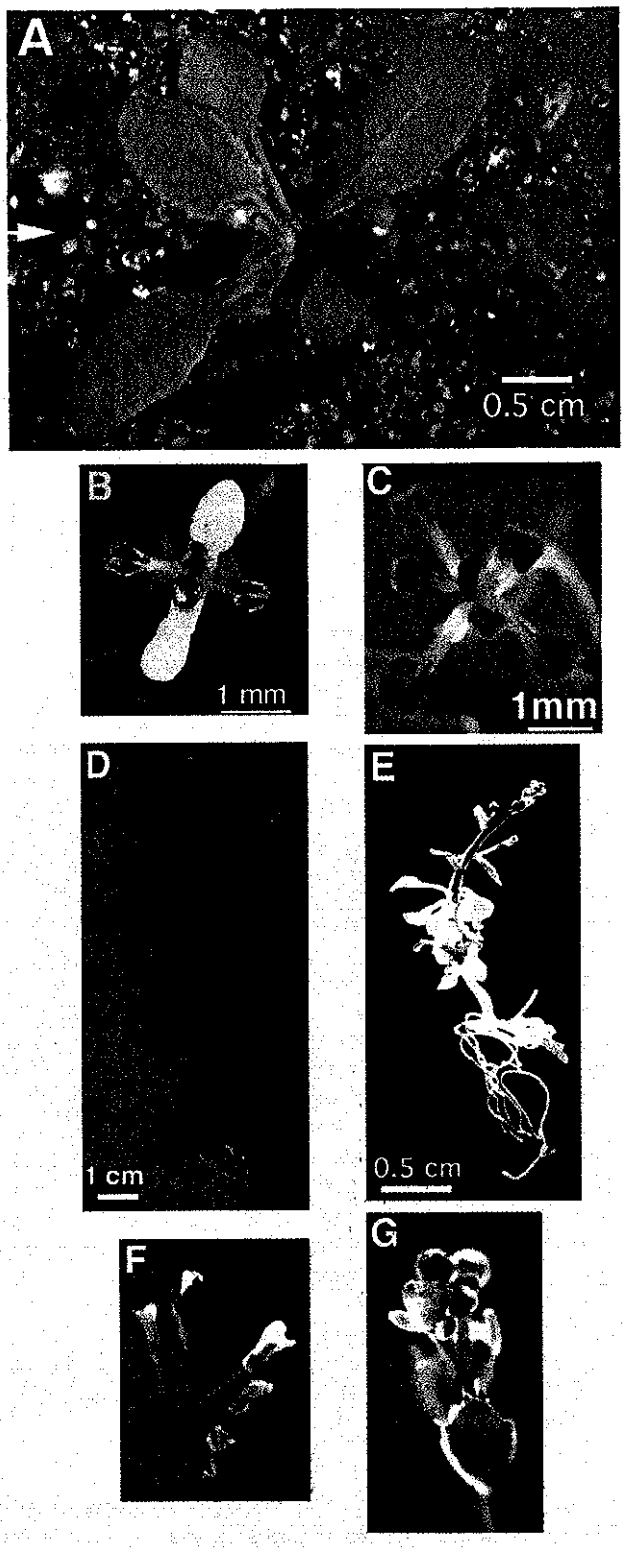

Figure 2. Phenotypes of the Adult *ran1-3* Mutants.

(A) Phenotype, from left to right, of *ran1-3*, wild-type, and *ctr1* 18-day-old adult plants grown in soil during long days. The *ran1-3* plant is indicated by an arrow.

(B) Close-up of a *ran1-3* mutant grown in soil as given in **(A)**. Note the senescence of the cotyledons.

(C) Phenotype of a 21-day-old *ran1-3* plant grown in vitro on Gamborg's medium.

(D) Phenotype of a 28-day-old wild-type plant grown in vitro on Gamborg's medium.

(E) Phenotype of a 28-day-old *ran1-3* mutant grown in vitro on Gamborg's medium.

(F) Close-up of a flower from a 28-day-old wild-type plant grown in vitro on Gamborg's medium.

(G) Close-up of a flower from a 28-day-old *ran1-3* mutant grown in vitro on Gamborg's medium. The *ran1-3* mutant floral structures do not develop past this point. Note the accumulation of anthocyanin in the *ran1-3* floral structures.

scanning electron microscopy. As shown in Figure 3, *ran1-3* epidermal cells were much smaller than those of the wild type and were only slightly larger than stomatal cells. The *ran1-3* epidermal cells were also considerably smaller than those from the *ctr1* mutant or from wild-type plants grown in ethylene (data not shown), consistent with the smaller size of the *ran1-3* leaves. *ran1-3* epidermal cells were much rounder than wild-type cells, similar to unexpanded cells. In addition to the cell size difference, there were considerably fewer trichomes on *ran1-3* mutant leaves, and the ones that did form were generally misshapen, often developing only a single point, in contrast to the three found in wild-type trichomes.

The expression of the β-chitinase gene is induced by ethylene in adult Arabidopsis plants (Samac et al., 1990). We examined the steady state amount of β-chitinase mRNA in air- and ethylene-grown wild-type and mutant plants by using RNA gel blot analysis (Figure 4). As previously observed (Kieber et al., 1993), the β-chitinase gene was expressed to a greater extent in *ctr1* mutants in both the presence and the absence of ethylene. This is consistent with the constitutive activation of the ethylene response pathway in the *ctr1* mutant. In both control and ethylene-treated *ran1-3* mutants, the steady state expression of β-chitinase mRNA was very high, exceeding that observed in wild-type plants treated with high amounts of ethylene or in the *ctr1-2* mutant. This indicates that like the *ctr1* mutation, the *ran1-3* mutation results in the constitutive activation of the ethylene response pathway in adult plants.

Genetic Interaction between *ran1-3* and Other Ethylene Action Mutants

To determine the order of action of RAN1 relative to other ethylene-signaling components, we analyzed the epistasis between *ran1-3* and various *ein* mutations (Figure 5). These *ein* mutations all affect ethylene perception or signal transduction in both seedlings and adult plants. Double mutants of *ran1-3* with *etr1-3*, *ein2*, *ein3*, *ein4*, *ein5*, and *ein6* were generated, and their seedling and adult phenotypes were

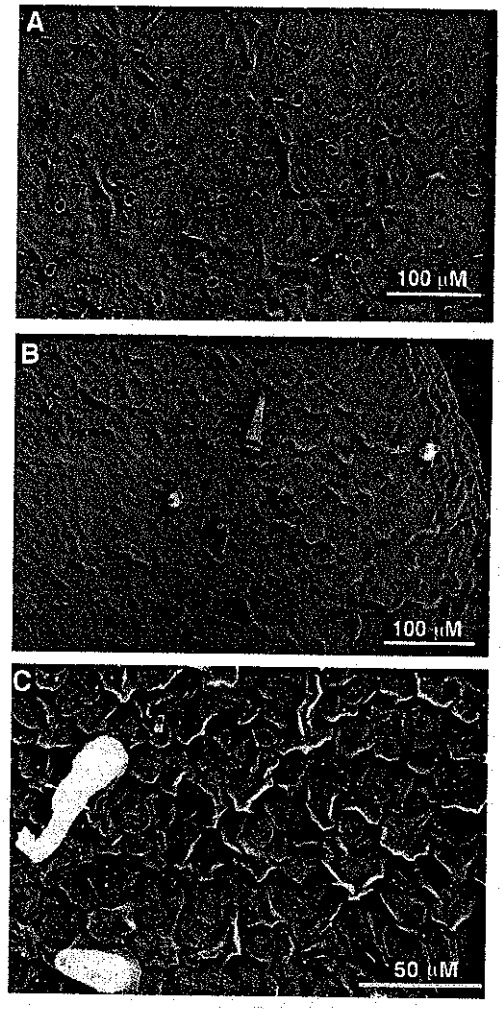

Figure 3. The *ran1-3* Mutant Displays a Reduction in the Size of Epidermal Cells.

(A) and **(B)** Wild-type **(A)** or *ran1-3* **(B)** plants were grown on Gamborg's medium for 13 days, and leaf epidermal cells were observed by scanning electron microscopy, as described in Methods. Note that the *ran1-3* epidermal cells are much smaller than their wild-type counterparts. The spikes protruding from the *ran1-3* leaves are the trichomes, which in wild-type leaves have three points.

(C) Close-up of *ran1-3* epidermal cells. The doughnut-shaped stomata are clearly visible, as are two trichomes (white spikes).

examined. All the double mutants displayed ethylene insensitivity as 3-day-old etiolated seedlings (Figure 5; data not shown), indicating that the *RAN1* gene product acts at the same place as or upstream of the products of these genes. Seedlings doubly mutant for both *ran1-3* and each of these ethylene-insensitive mutations could not be visually distinguished from the ethylene-insensitive single mutants grown under the same conditions, indicating that the suppression of the *ran1-3* phenotype was essentially complete. However, when all six of these *ran1-3 ein* double mutants were transferred to soil, the adult plants displayed the phenotype of the *ran1-3* mutant (Figure 5; data not shown). The rosettes of double mutant plants were similar to *ran1-3* single mutants in size and appearance. These results were confirmed with multiple F_2 lines for each double mutant combination.

To determine whether the phenotype of the double mutants reflected constitutive activation of the ethylene response pathway, we examined expression of the β-chitinase gene (Figure 4). As expected, the steady state amount of β-chitinase mRNA was not induced by ethylene in the *etr1-3* or *ein2* mutants grown in either air or ethylene. Despite their *ran1-3* morphology, the adult plants of the *ran1-3 etr1-3*, *ran1-3 ein2*, *ran1-3 ein3*, and *ran1-3 ein5* double mutants did not have elevated expression levels of β-chitinase. This suggests that the double mutant adult plants do not constitutively activate the ethylene response pathway, which is consistent with the phenotypes of the double mutant etiolated seedlings.

Cloning of Gene Corresponding to *ran1-3*

We mapped the *ran1-3* mutation to chromosome 5 by using a series of simple sequence length polymorphic (SSLP)

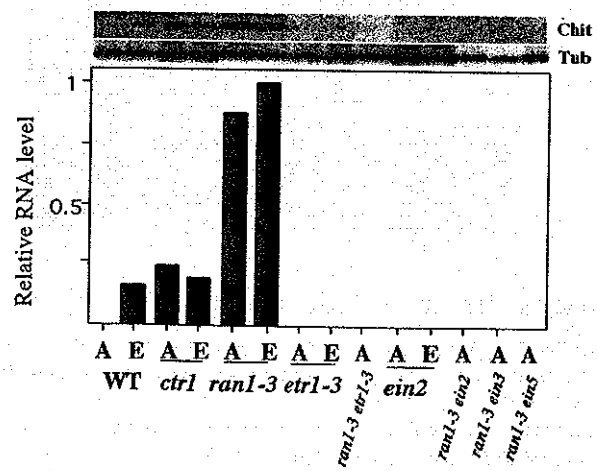

Figure 4. The *ran1-3* Mutation Results in Increased Expression of β-Chitinase.

RNA gel blot analysis of wild-type (WT), *ctr1*, *ran1-3*, *etr1-3*, *ran1-3 etr1-3*, *ein2*, *ran1-3 ein2*, *ran1-3 ein3*, and *ran1-3 ein5* (as indicated) adult tissues grown on soil. Plants were placed in an enclosed chamber containing either air (A) or 10 μL L^{-1} ethylene (E) for 48 hr as indicated. Total RNA was extracted, separated by gel electrophoresis, blotted to a nylon membrane, and hybridized with either a β-chitinase probe (Chit) or a β-tubulin probe (Tub). The signals were quantified by using a PhosphorImager. The signal from the β-chitinase blot was normalized to the β-tubulin signal, and the highest ratio was then assigned a value of 1; the others were plotted relative to that. The original image of the gel blot is shown at top, and the lanes correspond to the labeling of the graph's x axis.

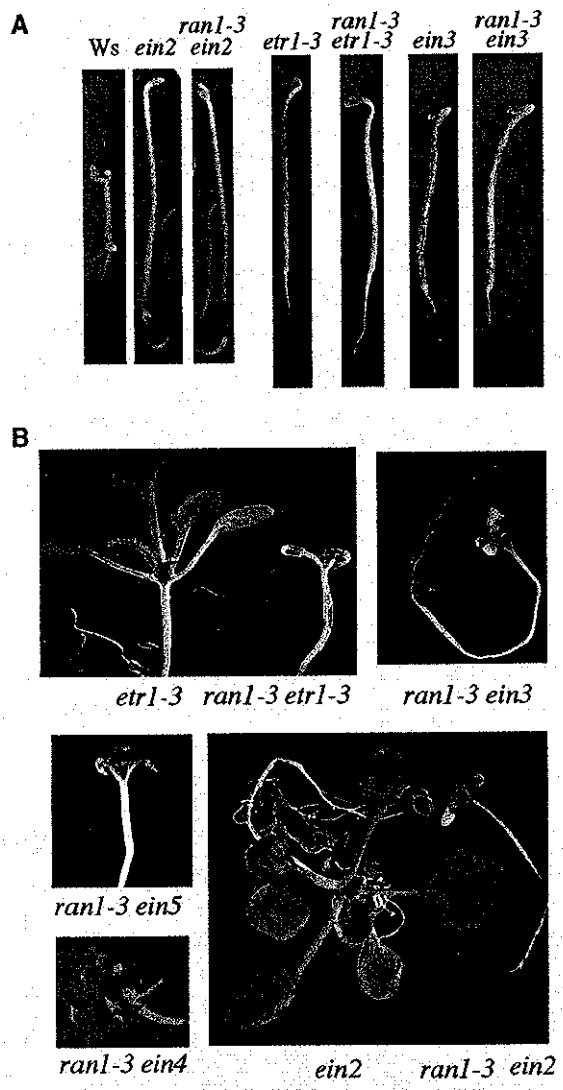

Figure 5. Phenotype of Various Double Mutants.

(A) Seedling phenotype of wild-type (Ws) and single and double mutant seedlings (as indicated at the top) grown in ethylene in the dark for 3 days.

(B) Eighteen-day-old wild-type and single and double mutant plants (as indicated below each photograph). All the adult plants were grown under long days on soil, except for the *ran1-3 ein4* double mutant, which was grown under long days on Gamborg's media.

markers. This map position was refined by using cleaved amplified polymorphic sequences (CAPS) markers that we generated using sequence information obtained from the Arabidopsis genome sequencing project (see Methods). We found that *ran1-3* mapped very close to the *RAN1* gene, which also had been linked to ethylene signaling (Hirayama et al., 1999). We thus sequenced the *RAN1* gene amplified from *ran1-3* mutant as well as wild-type Wassilewskija (Ws) genomic DNA (because the original *RAN1* sequence was from the Columbia ecotype). Comparison of the sequences revealed that the *ran1-3* mutation was correlated with a single nucleotide change in the *RAN1* coding region (Figure 6). This mutation is a G-to-A transition at nucleotide 3061 of the genomic sequence (in which the A of the predicted start codon is defined as residue 1), which results in the replacement of Gly-759 with an Arg residue. Gly-759 is in the loop of RAN1 that contains the predicted ATPase domain, even though it does not occur within the highly conserved ATPase signature sequence that surrounds the Asp residue involved in the aspartyl phosphate intermediate (Figure 6D). This Gly is invariant in all sequenced cation transport P-type ATPases, including genes from eubacteria and archaebacteria (Figure 6E).

To confirm that this gene corresponds to the *ran1-3* mutations, we tested whether a genomic fragment spanning the *RAN1* gene could complement the *ran1-3* mutation. A population segregating for *ran1-3* mutants was transformed with a *RAN1* genomic fragment comprising the entire coding region plus 1 kb of the 5′ and 0.5 kb of the 3′ flanking DNA. Transformants were selected, and a line (TCt-12) was identified that was segregating ~17% Ctr⁻ seedlings. The kanamycin resistance marker, which was contained on the T-DNA, cosegregated 100% with wild-type progeny (40 of 40 wild-type seedlings were kanamycin resistant, and 120 of 120 Ctr⁻ seedlings were kanamycin sensitive). We conclude that the *RAN1* gene complements the *ran1-3* mutation and that line TCt-12 was a homozygous mutant at the endogenous *RAN1* locus and heterozygous for the transgene. We confirmed that the endogenous *RAN1* locus in this line was homozygous mutant in the phenotypically wild-type seedlings by using a CAPS marker created by the *ran1-3* mutation (see Methods). Importantly, the *RAN1* gene complemented both the seedling and adult *ran1-3* phenotypes, indicating that this mutation is responsible for all the phenotypes observed in this line (Figures 6B and 6C).

Copper Ion Partially Suppresses the *ran1-3* Seedling Phenotype

Addition of copper ions to the media suppresses the phenotype of both the *ran1* and the yeast *ccc2* mutants (Fu et al., 1995; Yuan et al., 1995; Hirayama et al., 1999), consistent with their role in mediating copper trafficking. To test whether copper could suppress the phenotype of *ran1-3*, we examined seedlings grown on Murashige and Skoog (MS) media supplemented with various concentrations of $CuSO_4$ (Figure 7). Addition of as much as 20 μM $CuSO_4$ to the media had little or no effect on the germination or growth of 3-day-old etiolated wild-type or *eto1* seedlings (data not shown). The roots of 3-day-old etiolated *ran1-3* mutant seedlings were progressively longer when greater concentrations of $CuSO_4$ were included in the media (up to 20 μM),

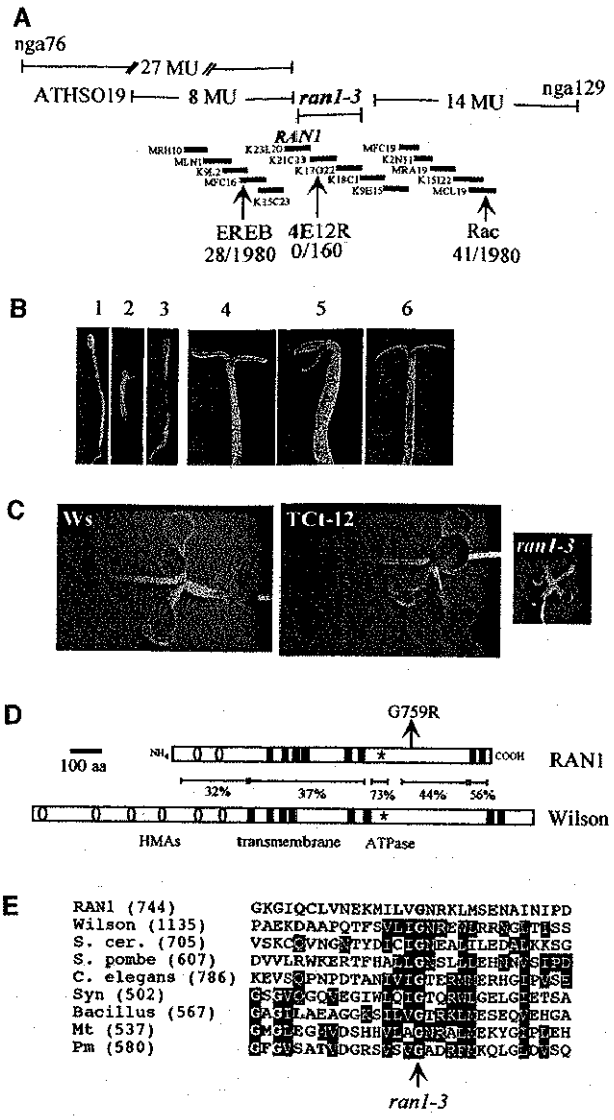

Figure 6. Cloning of *RAN1-3*.

(A) Physical mapping of *ran1-3*. The thick bars represent ordered bacterial artificial chromosome (BAC) clones from the physical map of the 16- to 18-Mb region of chromosome 5 as determined by the Kazusa sequencing group. The BAC containing the *RAN1* gene is indicated. The identities of each BAC are indicated to the left of the bars. Shown above are the map distances from the initial mapping with the SSLP markers. The distances from the *ran1-3* mutation to each marker are indicated. Note that the lines are not to scale. The results of fine-structure mapping of 990 *ran1-3* seedlings plants (1980 chromosomes) from the F_2 progeny of a cross between *ran1-3* and the wild type (ecotype Columbia) are indicated below. The Rac and the EREB CAPS markers are described in Methods. MU, map units.

(B) The *RAN1* gene complements the seedling phenotype of *ran1-3*. Phenotype of 3-day-old etiolated seedlings (seedlings 1 to 3) and etiolated seedlings shifted to the light for 14 hr (seedlings 4 to 6). Wild-type (seedlings 1 and 4), *ran1-3* (seedlings 2 and 5), and TCt-12 seedlings (seedlings 3 and 6) were grown on MS media at 23°C, and representative seedlings were photographed. The TCt-12 seedlings are T_2 progeny of a transformed line that harbors a wild-type *RAN1* transgene and which is homozygous mutant at the endogenous *ran1-3* locus, as determined by molecular analysis (see Methods). Note that the *RAN1* gene complements both the Ctr⁻ and the delayed hook opening phenotypes of *ran1-3*.

(C) The *RAN1* gene complements the adult phenotype of mature *ran1-3* plants. Phenotype of wild-type (Ws), TCt-12 (see **[B]**), and *ran1-3* 12-day-old plants grown on Gamborg's medium are shown.

(D) Diagram of RAN1 and the Wilson's disease proteins. The numbers represent the percentage identities between the various domains of the two protein sequences. The various domains are indicated below the Wilson's (Wilson) gene. The heavy metal binding domains (HMAs) are represented by open circles, the transmembrane domains are indicated by black boxes, and the P-type cation transport ATPase signature motif is indicated with asterisks. The position of the amino acid alteration predicted from the *ran1-3* mutation is indicated with an arrow. The HMAs and the ATPase signature motif were identified by using a Prosite search. The transmembrane domains were predicted with the Tmpred program at EMBnet (www.ch.embnet.org/index.html). aa, amino acids.

(E) Lineup of the region surrounding the *ran1-3* mutation. Various P-type cation transport ATPases were aligned by using the Clustal program; the region surrounding the *ran1-3* mutation is shown. The numbers within parentheses indicate the residue number of the first amino shown in the lineup. The protein sequences were from the predicted amino acid sequences from genes from the following species (GenBank accession numbers are given in parentheses): Wilson, human (4502323); S. cer., *Saccharomyces cerevisiae CCC2* gene (P38995); S. pombe, *Schizosaccharomyces pombe* (CAA18378); C. elegans, *Caenorhabditis elegans* (BAA20550); Syn, *Synechoccus* sp *PCC7942* (P37279); Bacilius, *Bacillus subtilis* (CAB15355); Mt, *Methanobacterium thermoautotrophicum* (AAB86009); and Pm, *Proteus mirabilis* (AAB01764). The residues in reverse type indicate identity to the RAN1 amino acid sequence.

which indicates that cupric ion is able to partially suppress this aspect of the triple response phenotype of the *ran1-3* mutant. In contrast to the roots, there was little or no change in the length of the hypocotyl or the curvature of the apical hook of etiolated *ran1-3* seedlings, even at 20 μM of $CuSO_4$. Greater concentrations of copper had toxic effects on seedling growth (data not shown). We detected no amelioration of the delayed hook opening phenotype or the adult phenotypes of *ran1-3* mutants by supplementing with various concentrations of $CuSO_4$ up to 20 μM (data not shown).

DISCUSSION

We identified *ran1-3*, a constitutive ethylene response mutant with a rosette-lethal phenotype, by using a family screen for seedlings displaying the triple response in the absence of exogenous ethylene. Several lines of evidence

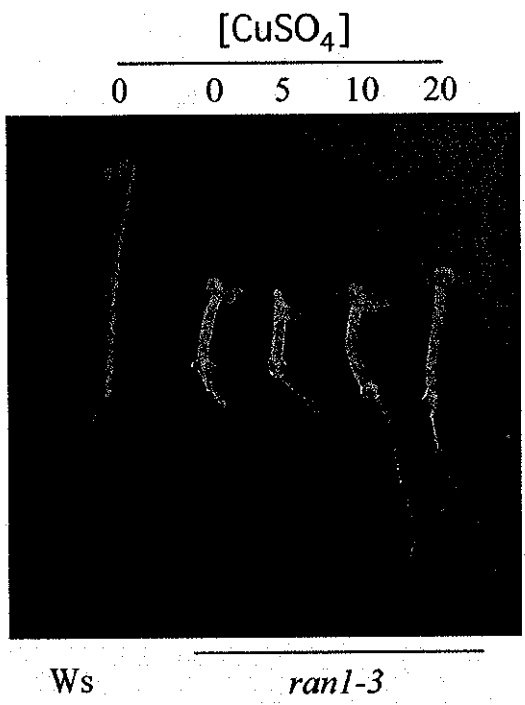

Figure 7. Effect of $CuSO_4$ on the Phenotype of *ran1-3* Seedlings.

Seeds were plated on MS media containing the indicated amounts (in μm) of $CuSO_4$. After a 4-day cold treatment, the plates were incubated in the dark for 3 days, and representative seedlings were photographed.

indicate that *ran1-3* is a constitutive ethylene-signaling mutant. First, *ran1-3* mutant seedlings display a Ctr⁻ phenotype. This phenotype is not reverted by inhibitors of ethylene biosynthesis or binding, and *ran1-3* seedlings produce wild-type amounts of ethylene. The seedling phenotype of *ran1-3* mutants is suppressed by ethylene-insensitive mutants, indicating that the Ctr⁻ phenotype results from an activation of the ethylene response pathway. Finally, *ran1-3* adult plants display a constitutive activation of an ethylene-inducible gene, and this phenotype also is suppressed by ethylene-insensitive mutations. However, the morphological changes that result from the *ran1-3* mutation are not observed in plants grown continuously in the presence of saturating concentrations of ethylene (Kieber et al., 1993) and are not suppressed by ethylene-insensitive mutations, suggesting that *ran1-3* also affects a non-ethylene-dependent signaling pathway that affects cell expansion.

Molecular analysis indicated that *ran1-3* disrupts the *RAN1* locus. This result was surprising because the previously identified *ran1* mutants are phenotypically distinct from the *ran1-3* mutant. *ran1-1* and *ran1-2* were identified as seedlings that displayed a triple response when exposed to the ethylene antagonist *trans*-cyclooctene (Hirayama et al., 1999). Apart from their altered response to *trans*-cyclooctene, *ran1-1* and *ran1-2* have no discernable effect on the morphology of seedlings or adult plants. *RAN1* encodes a protein with substantial amino acid similarity to the human Menkes and Wilson disease proteins (Bull et al., 1993; Chelly and Monaco, 1993; Chelly et al., 1993; Mercer et al., 1993; Tanzi et al., 1993; Vulpe et al., 1993; Yamaguchi et al., 1993) and to yeast Ccc2p (Fu et al., 1995). These proteins are P-type cation transport ATPases that are involved in copper trafficking. Ccc2p is required in yeast to provide copper to the Fet3 protein (Yuan et al., 1995), which is a multicopper oxidase involved in high-affinity iron transport (Askwith et al., 1994). In the absence of Ccc2p, no functional Fet3 protein is made, and the yeast cells become iron deficient. Hirayama et al. (1999) have proposed that RAN1 functions to add copper ions to the ETR1 family of ethylene receptors.

Copper is required for the full ethylene binding activity of ETR1 expressed in yeast (Rodriguez et al., 1999), and the *ran1-1* and *ran1-2* mutations have been suggested to cause decreased loading of copper ions to the ethylene receptors, resulting in a change in receptor output in response to *trans*-cyclooctene (Hirayama et al., 1999). The *ran1-1* and *ran1-2* mutations are not amorphic, because cDNAs containing these mutations still can complement a yeast *ccc2* mutant, albeit not as efficiently as the wild-type *RAN1* gene (Hirayama et al., 1999). In contrast to *ran1-3* mutants, the *ran1-1* and *ran1-2* mutants have no observable distinct adult phenotype, consistent with the notion that these two mutations represent very subtle changes in the function of RAN1. Although it is unclear whether *ran1-3* is an amorphic mutation, it does disrupt a residue that is absolutely conserved in all described cation-transporting P-type ATPases across several kingdoms (Solioz et al., 1994; Lutsenko and Kaplan, 1995; Solioz and Vulpe, 1996). This, coupled with its striking phenotype and the lack of morphological phenotype of the *ran1-1* and *ran1-2* mutations, suggests that if not amorphic, *ran1-3* represents at least a very strong loss-of-function allele. Phenotypically, *ran1-3* mutants resemble cosuppressed *RAN1* lines, although the latter have not been well characterized (Hirayama et al., 1999).

In yeast, Ccc2p acts downstream of a high-affinity copper transporter, CTR1 (not related to the Arabidopsis *CTR1* gene), and Atx1p, a copper chaperone protein that binds copper en route to Ccc2p (Dancis et al., 1994; Lin et al., 1997). Yeast two-hybrid analysis demonstrated that Atx1p, which contains one copper binding motif, was able to interact directly with the putative copper binding domain of Ccc2p (Pufahl et al., 1997; Hamza et al., 1999). Because this interaction was dependent on copper ions, it suggested that Atx1p could donate copper to Ccc2p by direct interaction and copper exchange. Homologs of both the yeast *CTR1* and *Atx1* genes have been identified in Arabidopsis (Kampfenkel et al., 1995; Himelblau et al., 1998). Using the yeast system as a guide, the model for RAN1 function presented in Figure 8 postulates that copper enters the cell by way of COPT1, the Arabidopsis homolog of the yeast *CTR1*

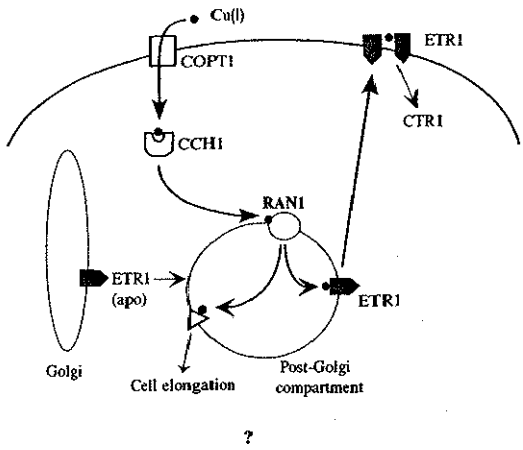

Figure 8. Model of RAN1 Function.

Copper ion (filled circle) is hypothesized to be transported into the cell and brought to the RAN1 protein by the Arabidopsis homologs of the yeast *CTR1* and *ATX1* genes, called *COPT1* and *CCH1*, respectively. The RAN1 protein (open circle) then delivers copper ion to the ETR1 apoprotein (apo), which then becomes functional and possibly is transported to the plasma membrane. In the absence of ethylene, ETR1 functions to activate CTR1, which is a negative regulator of the ethylene response pathway. Analysis of the *ran1-3* mutant suggests that the protein also is required for the function of additional cuproenzymes, which are represented by the open triangle. Arrows indicate either direct or indirect interactions between elements. See the text for additional details.

gene. The CCH1 protein (the Atx1 homolog) then brings the copper to RAN1, which may reside in a post-Golgi compartment vesicle. Here, RAN1 delivers copper ion to the ethylene receptors, which then become functional and ultimately may be sent to the plasma membrane. RAN1 also may act to deliver copper to other cuproenzyme(s), which when defective may lead to the rosette-lethal phenotype observed in the *ran1-3* mutant.

The constitutive ethylene response phenotype of *ran1-3* mutants is consistent with a disruption in the function of the ethylene receptors (Hua and Meyerowitz, 1998). This suggests that RAN1 is required to form functional ethylene receptors and, by extension, that copper is required for their function. Copper has been shown to be required for high-level ethylene binding of ETR1 purified from yeast; however, the results presented here indicate that copper also is required for the signaling function of the ethylene receptors. The suppression of the *ran1-3* constitutive ethylene phenotype by the *ein4* and *etr1-3* mutants suggests that that the phenotype of *ran1-3* mutants is not likely to be due to the receptors getting caught up in the secretory pathway through a lack of copper addition. Furthermore, this suppression indicates that the *etr1-3* and *ein4* mutations result in a catalytically hyperactive receptor that can function in the absence of copper. This is consistent with the results of Hall et al. (1999), who found that some *etr1* alleles that resulted in dominant ethylene insensitivity did not impair ethylene binding. However, the *etr1-3* mutation did markedly reduce ethylene binding, which suggests that this mutation both disrupts ligand binding and results in a catalytically hyperactive receptor. Interestingly, although gain-of-function receptor mutations suppressed *ran1-3*, silver ion did not. How silver ion blocks ethylene responses is unclear (Beyer, 1976), but it has been shown to enhance the binding of ethylene to purified ETR1 (Rodriguez et al., 1999). The lack of reversion of the *ran1-3* phenotype by silver ion suggests that silver ion does not act by constitutively activating the signaling function of the receptors, or if it does, then this activation requires copper ion.

The *ran1-3* mutation could cause rosette lethality in several ways. Copper, an essential micronutrient required for the activity of several cuproenzymes (Linder, 1991), is, however, very reactive in biological systems; disruption of copper homeostasis results in the rapid generation of reactive oxygen species. The Wilson and Menkes disease proteins are localized to the *trans*-Golgi network and relocalize to the plasma membrane when intracellular copper concentrations increase, presumably facilitating copper efflux (Vulpe et al., 1993; Petris et al., 1996; Hung et al., 1997). Thus, these proteins have been postulated to act both in the assembly of cuproenzymes and in removing excess copper from the cell. Perhaps the RAN1 protein plays a similar role in plant cells. If so, then the *ran1-3* mutation could disrupt copper homeostasis, resulting in an increase of intracellular copper to toxic concentrations. However, this seems unlikely because *ran1-3* mutants do not display increased sensitivity to copper (K.E. Woeste and J.J. Kieber, unpublished observations). Alternatively, the lethality of *ran1-3* could result from the loss of catalytic activity of an essential cuproenzyme or enzymes, as we have postulated in the model presented in Figure 8. Also, disruption of the function of all the ethylene receptors could result in the rosette lethality. Although disruption of four of the five Arabidopsis ethylene receptors is not lethal, it results in a stronger phenotype than null *ctr1* mutations, raising the possibility that these proteins may act in pathways other than just ethylene signaling.

The *ran1-3* mutation, like *ctr1*, demonstrates reduced genetic transmission. However, in contrast to *ctr1*, which shows reduced transmission only through the female gamete (Kieber and Ecker, 1994), *ran1-3* appears to affect transmission through male gametes. This unusual effect on one gamete but not the other by different constitutive ethylene-signaling mutants seems to suggest that the defects in transmission seen in each case are not the result of constitutive ethylene signaling or of ethylene-mediated inhibition of cell expansion. Otherwise, one would expect that both mutations would affect gametes of the same gender. The role of *ctr1* and *ran1-3* in gamete development or fertilization (or both) may be related to pleiotropic effects of these genes that remain to be elucidated.

METHODS

Growth of Plants

The Columbia and Wassilewskija (Ws) ecotypes of *Arabidopsis thaliana* were used in this study. The *ein6* and *ein7* mutants were obtained from the Arabidopsis Biological Resource Center (Ohio State University, Columbus). Seeds were surface-sterilized and grown on sterile Murashige and Skoog (MS) media (GIBCO), or they were grown on Gamborg's B5 medium plus 0.8% agar as described in Vogel et al. (1998b), or they were sown directly onto Metro mix 200 (Grace, Boca Raton, FL) as indicated. $CuSO_4$, aminoethoxyvinylglycine (10 μM) and $AgNO_3$ (17 μg/mL) were added directly to MS agar before the seeds were plated. For *trans*-cyclooctene treatment, plates were placed in a sealed 3-L polystyrene chamber (Billups-Rothenberg, Del Mar, CA) containing 4 μL of liquid *trans*-cyclooctene. Adult plants were grown in soil under continuous illumination at 23°C.

Isolation of Mutants by Family Screen

Arabidopsis seeds (ecotype Ws) were mutagenized as described by Vogel et al. (1998b) and sown directly onto soil in 4-inch-diameter (~10 cm) pots. Plants were thinned to three or four plants per pot and grown to maturity. Seeds from each pot were harvested separately, and a portion of the seeds from each family was screened for triple response mutants as described previously (Kieber et al., 1993). Lethal *Ctr*⁻ mutants were rescued by identifying heterozygous siblings from the family by progeny testing.

Genetic Analysis

ran1-3 heterozygotes were crossed to *etr1-3*, *ein2*, *ein3*, *ein4*, *ein5*, and *ein6* mutants to create double mutant stocks for analysis of epistasis. The *ran1-3* mutations were isolated from the Ws ecotype, *ein6* from the Landsberg *erecta* ecotype, and the other ethylene-insensitive mutants from the Columbia ecotype. Plants used in crosses were verified as *ran1-3* heterozygotes by progeny testing. To identify *ran1-3 ein* double mutants, we identified homozygous Ein⁻ F_2 plants either by their phenotype (for recessive mutations) or by progeny testing (for dominant mutations). We examined progeny from multiple homozygous *ein* lines for adults that displayed the phenotype of the *ran1-3* mutant. Plants that exhibited both the seedling Ein⁻ phenotype and the adult *ran1-3* phenotype were used as the double mutants for the RNA gel blot analysis.

Measurement of Ethylene Production

Wild-type and *ran1-3* heterozygous seeds were surface-sterilized, plated on MS agar, and incubated at 4°C for 4 days. They then were incubated in the dark for 48 hr at 23°C. Wild-type and *ran1-3* mutant plants were identified, and ~15 were transferred to 22-mL gas chromatography vials containing 3 mL of MS medium. The vials were flushed with hydrocarbon-free air and sealed for 24 hr. The accumulated ethylene was measured with a gas chromatograph (Perkin-Elmer) as described previously (Vogel et al., 1998b). All observations were from at least three replicates, and each experiment was repeated at least once with comparable results.

RNA Gel Blot Analysis

Seeds were germinated on MS agar, and 3-day-old seedlings then were transferred to soil and grown for 1 to 2 weeks under constant light at 23°C. For the *ran1-3 ein* double mutants, Ein⁻ seedlings were picked, and those that displayed a *ran1-3* mutant adult phenotype were chosen for RNA isolation. For *ran1-3* mutants, Ctr⁻ seedlings were picked from the progeny of a selfed heterozygote. Plants treated with ethylene were grown in soil and placed in an illuminated incubator at 23°C with a continuous flow of ethylene (10 μL L^{-1}) for 48 hr. Total RNA was isolated, separated by agarose gel electrophoresis, and blotted to a nylon membrane as described (Ausubel et al., 1994). The filters were hybridized with radiolabeled probes by using Rapid-hyb buffer as described by the manufacturer (Amersham). Signals were quantified by using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA). The β-chitinase gene was obtained from plasmid pMON8817 (Samac et al., 1990), and the β-tubulin gene was obtained by polymerase chain reaction (PCR) amplification from genomic Arabidopsis DNA (Vogel et al., 1998a).

Organ and Cell Size

The roots and hypocotyls of 3-day-old etiolated seedlings grown on MS agar were measured to the nearest 0.5 mm with a dissecting microscope. At least 23 individuals of each genotype were measured.

For the evaluation of epidermal cell size, wild-type Ws and *ran1-3* mutant plants were grown on Gamborg's agar under sterile conditions with continuous illumination for 13 days after germination at 23°C. Plants were prepared for scanning electron microscopy by immersion in 2.5% gluteraldehyde in 50 mM sodium cacodylate buffer for 2 hr at room temperature, followed by two 10-min washes in 50 mM sodium cacodylate. Tissues then were dehydrated in an ethanol concentration gradient of 25, 50, 75, 95, and 100% ethanol. After dehydration, the tissue was dried by using a critical-point drier and then coated with gold to a thickness of 300 Å with a sputter coater and mounted on Al stumps. Micrographs were taken with a DS 130 scanning electron microscope (Topcon Corp., Paramus, NJ).

Cloning the Gene Corresponding to the *ran1-3* Mutation

ran1-3 heterozygotes (ecotype Ws) were backcrossed to wild-type plants of the Columbia ecotype. Three-day-old etiolated F_2 seedlings were isolated that displayed the Ctr⁻ phenotype and then were grown for 10 days on Gamborg's medium agar to confirm their phenotype. DNA was prepared as described (Vogel et al., 1998a) and used in PCRs to amplify simple sequence length polymorphisms (SSLPs; Bell and Ecker, 1994) and cleaved amplified polymorphic sequences (CAPS; Konieczny and Ausubel, 1993) markers. The SSLPs were used to map *ran1-3* to chromosome 5. To further refine the map position of *ran1-3*, we generated a series of CAPS markers near the region of *ran1-3* by sequencing genomic DNA isolated by PCR amplification with primers derived from the Arabidopsis genomic sequencing project. We identified sequence differences between the two ecotypes that resulted in restriction polymorphisms. Two sets of primers then were designed, one nested relative to the other, to amplify the DNA surrounding the polymorphic site. These primers were used in a single PCR or in two sequential PCRs (to increase the robustness of the markers) as indicated, with 1 μL of 1:100 dilution of the first reaction used as a substrate for the second. The two reac-

tions consisted of 30 cycles of 94°C for 30 sec, 58°C for 1 min, and 72°C for 2 min. The reactions then were cleaved with the appropriate enzymes, and the products were analyzed by gel electrophoresis. The primers and the restriction enzymes used for the CAPS markers are as follows. For the EREB marker, the primers for the first round of PCR were 5′-CCCTCGTTGACCGTCTCTT-3′ and 5′-CCTCTT-ACTTAGCCAAAAACATAGGA-3′, and for the second round of PCR the primers were 5′-GTCGTCGGTGGTTATGCTTC-3′ and 5′-TAATTA-TTTTCCGGCAATGTAAAATAC-3′. The products were cleaved with TaqI to yield DNA fragments of 148, 75, and 35 bp for Ws and 129, 75, 35, and 19 bp for the Columbia ecotype. For the Rac CAPS marker, the primers for the first round of PCR were 5′-CAGAGA-CATCCTCCTTACCAAT-3′ and 5′-GCGTACCGGCAGTATCCCA-3′, and for the second round of PCR the primers were 5′-CCACCACT-TCTCTTCTCTTCTG-3′ and 5′-GGTACTCCCGTCGACCAC-3′. The products were cleaved with DdeI to yield DNA fragments of 366 and 211 bp for Ws and 298, 211, and 68 bp for the Columbia ecotype.

Our analysis indicated that *ran1-3* mapped very close to the *RAN1* gene (Hirayama et al., 1999). We thus amplified the *RAN1* gene from *ran1-3* homozygotes and from Ws (because *RAN1* was sequenced from the Columbia ecotype) and identified a sequence change in the *ran1-3* mutants by directly sequencing the PCR products, by using a series of gene-specific primers. We then isolated a genomic fragment corresponding to the full-length, wild-type *RAN1* gene by PCR amplification of the entire coding region with 1 kb of 5′ and 250 bp of 3′ flanking DNA. The oligonucleotide primers used for this contained added SalI sites (lowercase letters) at the ends to simplify subsequent cloning. The primers used were 5′-aaagtcgacCTCGGGATC-AGACTAGGCTACATCC-3′ and 5′-aaagtcgacATTAGAGGAATTCAC-TACTTTCACCATTTTAC-3′. The PCR fragment was cloned into the SalI site of a binary plant transformation vector (a pBI101 derivative that had the β-glucuronidase gene deleted). This construct, called pJK495, was transformed into a *ran1-3* segregating population by the floral dip method (Clough and Bent, 1998). We identified kanamycin-resistant seedlings from the progeny of the transformed plants and allowed these to self. We identified a line (TCt-12) that was segregating 5:1 wild type to Ctr⁻, with 100% of linkage of the kanamycin resistance gene marker to the wild-type phenotype. This line then was checked to be homozygous for the *ran1-3* mutation at the endogenous *RAN1* locus by analysis of products derived from nested PCR amplification of the endogenous genes with use of primers flanking the site of the *ran1-3* mutation. The primers were designed so that the 3′ antisense primer was complementary to sequences outside of the DNA present in the transgene. The *ran1-3* mutation introduces an HphI restriction polymorphism, which simplified this analysis.

We amplified a full-length cDNA by using primers directed to the predicted 5′ and 3′ untranslated regions of the predicted *RAN1* open reading frame. Sequence analysis (not shown) confirmed that the intron/exon structure previously described by Hirayama et al. (1999), which is distinct from that predicted by the Arabidopsis genome sequencing project, was correct.

The heavy metal binding domains (HMAs) and the ATPase signature motif in the predicted RAN1 protein were identified by using a ScanProsite program search at the Expert Protein Analysis System proteomics server of the Swiss Institute of Bioinformatics (www.expasy.ch/tools/scnpsit1.html). The transmembrane domains of RAN1 were predicted by using the Tmpred program at EMBnet (www.ch.embnet.org/index.html) and the default values. The lineup of the various P-type cation transport ATPases was generated by using the ClustalW 1.8 program at the Baylor College of Medicine Search Launcher (http://dot.imgen.bcm.tmc.edu:9331/multi-align/multi-align.html) also with the default parameters.

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