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Arabidopsis Mutants Selected for Resistance to the Phytotoxin Coronatine Are Male Sterile, Insensitive to Methyl Jasmonate, and Resistant to a Bacterial Pathogen

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The phytotoxin coronatine and the plant growth regulator methyl jasmonate (MeJA) caused similar growth-inhibitory effects on Arabidopsis seedlings. To test whether these two compounds have similar action, 14 independent *coi1* (coronatine-insensitive) mutants of Arabidopsis were selected. The mutants segregated as single recessive Mendelian markers, and all were alleles at the *coi1* locus. All *coi1* mutants were also insensitive to MeJA and were male sterile. Both coronatine and MeJA inhibited root growth, stimulated anthocyanin accumulation, and increased the level of two proteins of ~31 and ~29 kD detected in SDS-polyacrylamide gels of wild-type Arabidopsis but caused none of these effects in the *coi1* mutant. Coronatine and MeJA also induced the systemic appearance of proteinase inhibitor activity in tomato. The male-sterile flowers of the *coi1* mutant produced abnormal pollen and had reduced level of an ~31-kD protein, which was abundant in the wild-type flowers. A coronatine-producing strain of *Pseudomonas syringae* grew in leaves of wild-type Arabidopsis to a population more than 100 times greater than it reached in the *coi1* mutant. We conclude that coronatine mimics the action of MeJA and that *coi1* controls a step in MeJA perception/response and in flower development.

INTRODUCTION

Coronatine, shown in Figure 1, is a chlorosis-inducing toxin produced by several pathovars of the plant pathogen *Pseudomonas syringae* (Ichihara et al., 1977; Mitchell and Young, 1978; Mitchell et al., 1983). It is responsible for the chlorotic halo that typically surrounds leaf infections caused by these bacteria. Coronatine production appears to be important for infection because mutants that do not produce this toxin have reduced virulence (Bender et al., 1987).

Coronatine has diverse effects on plants. It promotes senescence in tobacco (Kenyon and Turner, 1990), inhibits root growth in wheat (Sakai, 1980), stimulates stomatal opening in ryegrass but not in broad bean (Mino et al., 1987), causes anomalous cell growth in mature leaves of tobacco (Kenyon and Turner, 1990), and increases amylase activity (Sakai et al., 1979a) and causes hypertrophy (Sakai et al., 1979b) of potato tuber tissues. Coronatine also stimulates ethylene production from leaves of bean (Ferguson and Mitchell, 1985) and tobacco (Kenyon and Turner, 1992). Although the mode of action of coronatine is not known, its effects on plants are clearly different from those of other chlorosis-inducing toxins produced by plant pathogenic *P. syringae* pathovars, which act as metabolic inhibitors or biocides and inhibit the growth of or directly kill plant cells (Mitchell, 1984).

The jasmonates are recognized as a new class of plant growth regulators (Parthier, 1991), and some of their effects on plants are similar to those caused by coronatine. For example, methyl jasmonate (MeJA; Figure 1) promotes senescence of oat leaves (Ueda and Kato, 1980), inhibits root growth of Arabidopsis (Staswick et al., 1992), and stimulates ethylene production from tomato fruits (Saniewski et al., 1987); 3-oxo-2-[5-hydroxy-2-*cis*-pentenyl]-cyclopentane-1-acetic acid promotes hypertrophy and tuberization in potato (Koda et al., 1991). Moreover, there is some similarity between the structures of coronatine and the jasmonates: both compounds possess a cyclopentanone ring, and the keto group at C3 of jasmonate, shown in Figure 1, is also found in coronatine. The groups at C1, C2, and C3 are required for activity of the jasmonates (Yamane et al., 1980; Koda et al., 1991). However, coronafacic acid (Figure 1) does not promote senescence (Mitchell, 1984) or hypertrophy (Shirashi et al., 1979), indicating that the conjugated amino acid in coronatine is required for these activities.

To compare the activity of coronatine with that of MeJA, we sought mutants of Arabidopsis that were resistant to growth inhibition by coronatine. We report here the genetic characterization of these mutants, their response to coronatine and MeJA, and a description of their striking, pleiotropic phenotype.

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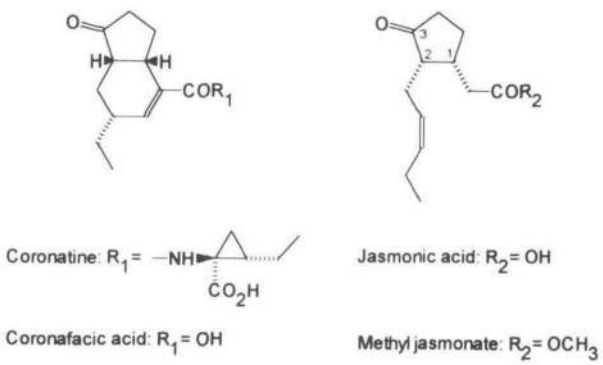

Figure 1. Structures of Coronatine, Coronafacic Acid, Jasmonic Acid, and MeJA.

R_1 groups are for coronatine and coronafacic acid (left). R_2 groups are for jasmonic acid and methyl jasmonate (right). 1, 2, 3, indicate the positions of the functional groups required for activity of the jasmonates.

RESULTS

Isolation of Mutants of Arabidopsis

Coronatine and MeJA inhibited the growth of wild-type Arabidopsis seedlings shown in Figure 2. The cotyledons of affected seedlings had distinct purple margins, due to the accumulation of anthocyanin. We screened ~200,000 seedlings from M_2 populations of ethyl methanesulfonate–mutagenized seeds of Arabidopsis germinating on Murashige and Skoog (MS; Sigma, U.K.) plates containing 1 μM coronatine and isolated 14 *coi1* (coronatine insensitive) mutants. Root and shoot growth of these mutants was not visibly inhibited by coronatine or MeJA (Figure 2), even at 50 and 100 μM respectively, the highest concentrations tested. The *coi1* mutant and the stunted wild-type Arabidopsis seedlings grew vigorously upon transfer from selection plates to MS plates or soil. Anthocyanin did not visibly accumulate in the *coi1* mutants growing on MS plates containing coronatine. However, these mutants were not generally defective in their capacity to produce anthocyanin because it could accumulate in leaves of *coi1* plants germinating under continuous white light or transplanted to soil and subjected to water stress.

All of the *coi1* mutants were male sterile. The flowers set seed when fertilized with pollen from wild-type plants, but pollen from the *coi1* mutants did not fertilize emasculated flowers from wild-type plants.

Genetic Characterization of the *coi1* Mutants

Because the 14 mutants were from independently mutagenized seed lots, it appeared that mutations at a single locus were responsible for insensitivity to coronatine and MeJA and for male sterility. When the 14 *coi1* mutants were used as females with wild-type pollen, they were fertile and produced F_1 progeny that were phenotypically wild type with regard to sensitivity to coronatine and MeJA and male fertility. F_2 progeny were collected from each of the F_1 plants, and batches of >200 were screened separately for sensitivity to coronatine, sensitivity to MeJA, and for male fertility. For all mutants and for all three phenotypes, the Chi-square test for the expected ratio 3 wild type:1 Coi1 was $P > 0.2$ to $P > 0.9$. Thus, the three mutant phenotypes were inherited as single recessive Mendelian markers.

Tests for complementation between the *coi1* mutants were made by crossing fertile F_1 plants heterozygous for each of the 14 mutations to male-sterile plants homozygous for *coi1*-1, *coi1*-4, and *coi1*-11. More than 50 seedlings from each cross were scored for the Coi1 phenotype, and the Chi-square test for the expected ratio of 1 wild type:1 Coi1 ranged from $P > 0.1$ to $P > 0.85$. Thus, the 14 mutants were alleles at the *coi1* locus. Further work therefore concentrated on the *coi1*-1 mutant that was back-crossed two times to the parental Arabidopsis ecotype Columbia, with selection for the Coi1 phenotype.

Genetic linkage between the three mutant phenotypes was confirmed. From MS plates containing coronatine or MeJA seeded with F_2 progeny segregating for *coi1*-1, 100 sensitive

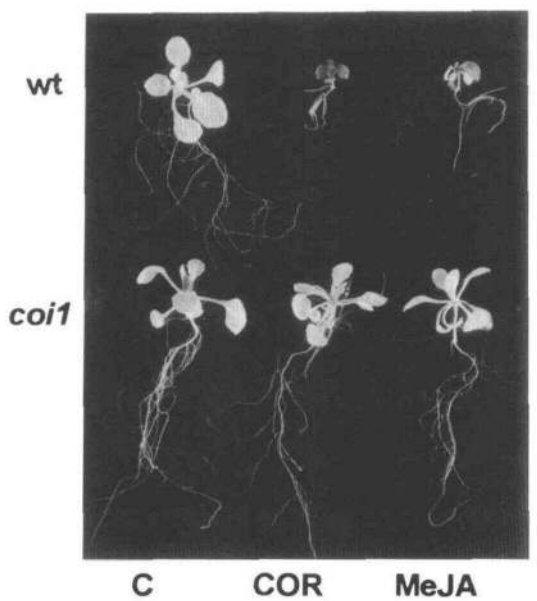

Figure 2. The Coi1 Phenotype.

Seeds of Arabidopsis wild type (wt) and of the *coi1* mutant were germinated on control MS plates (C) and on MS plates containing 1 μM coronatine (COR) or 10 μM MeJA. The seedlings shown were removed from the plates after 10 days. Because the *coi1* mutant was from an F_2 population segregating for the Coi1 phenotype, seedlings from the control and MeJA plates shown here were subsequently transferred to MS plates containing coronatine (1 μM) to confirm that these were *coi1* mutants.

and 100 insensitive seedlings were transferred to compost and the plants grown to maturity. For both treatments, sensitive seedlings produced flowers that were self-fertile and insensitive seedlings produced male-sterile flowers. Also, batches of 100 seedlings sensitive and insensitive to coronatine were transferred to MS plates containing MeJA. All seedlings sensitive to coronatine were also sensitive to MeJA, and all seedlings insensitive to coronatine were also insensitive to MeJA. In tests on batches of >200 F_3 progeny from 100 fertile F_2 plants, only seedlings insensitive to coronatine and to MeJA gave rise to male-sterile plants. For each mutant phenotype, the Chi-square test for the expected ratio of 1 mutant:5 wild type in F_3 populations was $P > 0.85$. In all of these experiments, no recombination was detected between the three mutant phenotypes.

Germination and growth of the *coi1* seedlings on MS plates containing zeatin, benzylaminopurine, abscisic acid (ABA), indole-3-acetic acid, or 1-aminocyclopropane-1-carboxylic acid were not different from that of wild-type Arabidopsis seedlings (data not shown).

Male Sterility of the *coi1* Mutant

Stamens of the *coi1* flower shown in Figure 3A had shorter filaments than those of the wild-type flower. The stigma and style of the *coi1* flowers appeared normal and siliques developed normally when wild-type Arabidopsis was the pollen donor. In Figure 3C, thin sections through the *coi1* anthers show that these had not dehisced and contained pollen grains with conspicuous vacuoles. By contrast, flowers of similar age from wild-type Arabidopsis contained dehiscing anthers and larger pollen grains that lacked any vacuoles visible at this magnification (Figure 3B). As well as these anatomical differences, the flowers of *coi1* and wild-type Arabidopsis plants also differed in their content of soluble proteins detected in two-dimensional gels. In Figure 4A, two proteins of ~61 and ~31 kD are indicated; they were abundant in wild-type flowers but apparently absent from *coi1* flowers. One protein of ~55 kD is indicated in Figure 4B; this protein was present in *coi1* flowers but could not be detected in wild-type flowers. Quantitative differences between other proteins from these flowers could also be detected. However, there was no detectable difference between the soluble proteins, separated in two-dimensional gels, from 14-day-old seedlings of wild-type Arabidopsis and the *coi1* mutant (data not shown).

Comparison of the Effect of Coronatine and MeJA on Wild-Type Arabidopsis, the *coi1* Mutant, and Tomato

Figure 5 shows that root growth in the wild-type plants was inhibited by coronatine and MeJA at concentrations >10 nM. However, growth of *coi1* roots was not affected by either compound at concentrations up to 50 μM. Roots of untreated wild-type seedlings were slightly but significantly shorter than

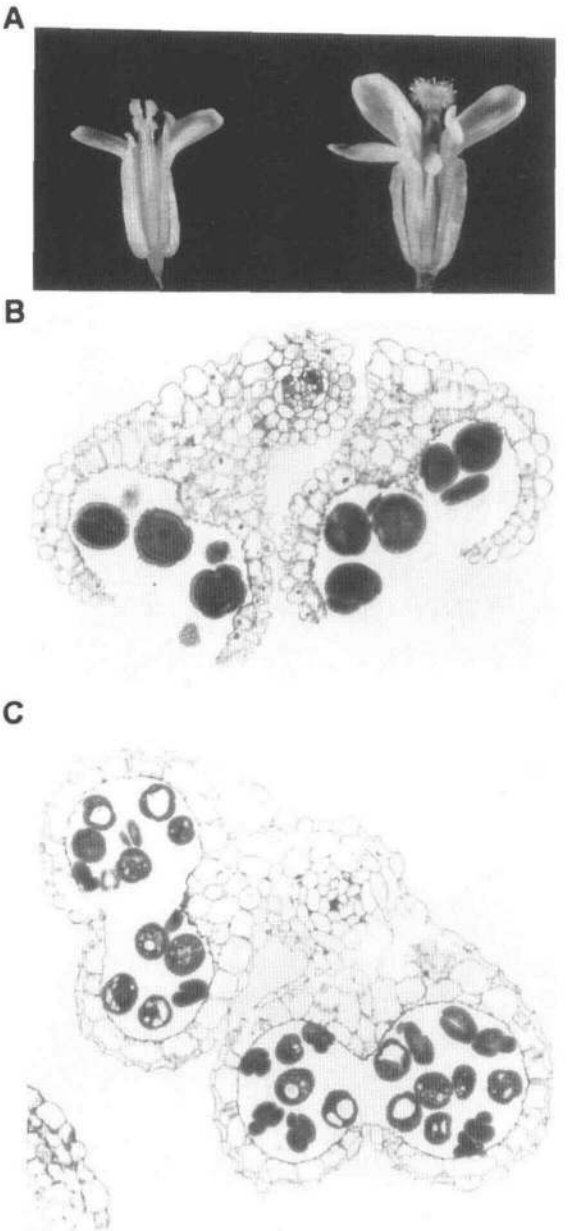

Figure 3. The Male-Sterile *coi1* Flower.

(A) Partially dissected, mature flowers of the male-fertile Arabidopsis wild type (left) and the male-sterile *coi1* mutant (right). Flowers were collected 3 to 4 days after the buds had opened and were from the primary inflorescence of 4-week-old plants grown under an 18-hr-day and 8-hr-night photoperiod.

(B) Transverse section through a dehiscing anther of an Arabidopsis wild-type flower similar to that shown in **(A)** at left; uniformly stained pollen grains are visible. Magnification ×260.

(C) Transverse section through an anther of a flower of the *coi1* mutant similar to that shown in **(A)** at right. The anther has not dehisced. The pollen grains are of irregular shape, contain conspicuous unstained vacuoles, and are somewhat smaller than those of the wild-type pollen. Magnification ×260.

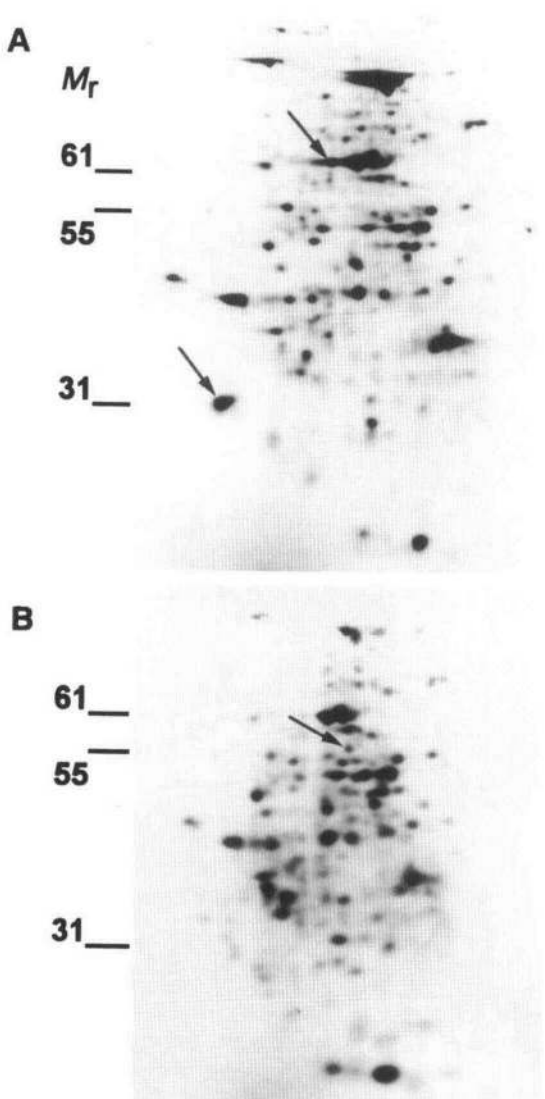

Figure 4. Two-Dimensional Electrophoresis of Proteins from Flowers of Arabidopsis Wild-Type and *coi1* Mutant Plants.

(A) Protein extracted from wild-type flowers. Arrows indicate two major peptides of ~61 and ~31 kD that were apparently absent from flowers of the *coi1* mutant.

(B) Protein from *coi1* mutant flowers. Arrow indicates one protein of ~55 kD that was apparently absent from the wild-type flowers.

Proteins were extracted from Arabidopsis flowers of similar age to those shown in Figure 3A. Proteins were separated by electrophoresis in the first dimension in a gradient from pH 10 at left to pH 3 at right and in the second dimension, top to bottom, in an SDS–polyacrylamide gel.

those of the *coi1* mutant. Coronafacic acid also inhibited root growth of wild-type Arabidopsis (Figure 5), but it was less effective than coronatine or MeJA. Coronafacic acid did not inhibit root growth of the *coi1* mutants (data not shown).

Anthocyanin accumulates visibly in Arabidopsis leaves treated with coronatine (Bent et al., 1992). We compared the effect of coronatine, MeJA, and coronafacic acid on the anthocyanin content of Arabidopsis. Eight-day-old seedlings of wild-type plants germinated on MS plates containing 1 μM coronatine, coronafacic acid, or MeJA contained 320, 300, and 170% more anthocyanin per seedling, respectively, than the controls germinating on MS plates. By contrast, the anthocyanin content in 8-day-old *coi1* seedlings germinated in MS plates containing 1 μM coronatine, coronafacic acid, or MeJA was not different from that of wild-type or *coi1* seedlings grown on MS plates alone (data not shown).

Some plant species respond to MeJA by producing new proteins, referred to as jasmonate-induced proteins (Koda, 1992). Wild-type Arabidopsis and *coi1* seedlings were treated with coronatine or MeJA for 48 hr, and proteins were extracted and separated by electrophoresis in SDS–polyacrylamide gels. Figure 6 indicates two proteins migrating at ~29 kD and ~31 kD, which accumulated in wild-type Arabidopsis exposed to coronatine or MeJA. These bands were absent from the *coi1* mutant, which was either untreated or exposed to MeJA or coronatine.

MeJA induces proteinase inhibitor (PIN) activity in tomato (Farmer and Ryan, 1990). Table 1 shows that 48 hr after application of MeJA or coronatine to unwounded tomato leaves, PIN

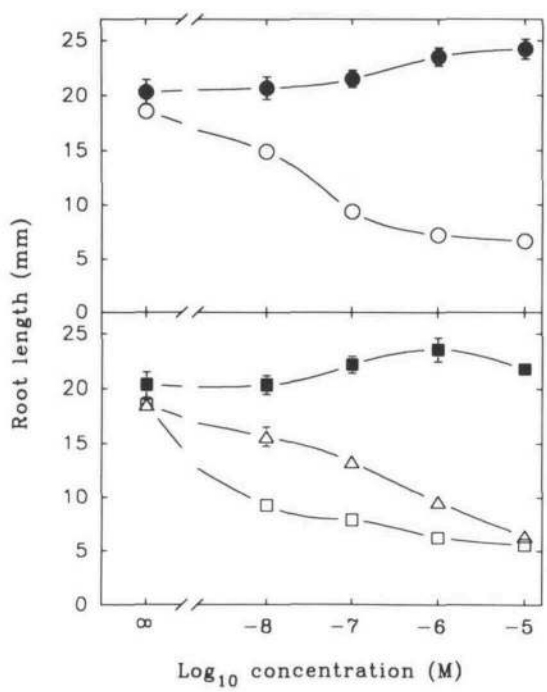

Figure 5. Effect of Coronatine and MeJA on the Growth of Roots of Arabidopsis Wild-Type and *coi1* Mutant Seedlings.

Root lengths are for 10-day-old seedlings of the Arabidopsis wild type (○, □, and △) or the *coi1* mutant (● and ■) germinated and grown on MS plates containing the indicated concentration of MeJA (○, ●), coronatine (□, ■), or coronafacic acid (△). Each point is the mean of at least 20 measurements, and bars show the standard error of the mean.

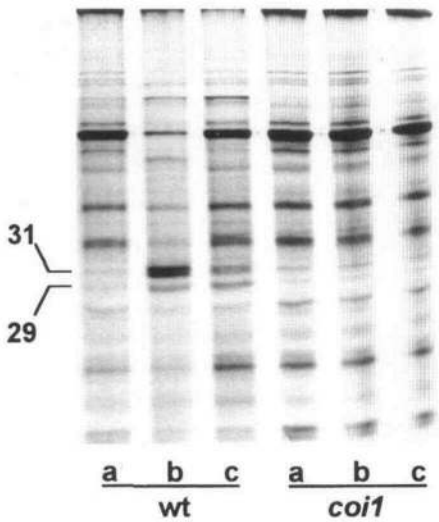

Figure 6. Effect of Coronatine and MeJA on Proteins from 10-Day-Old Seedlings of Arabidopsis Wild Type and the *coi1* Mutant.

Eight-day-old seedlings of the Arabidopsis wild type (wt) or the *coi1* mutant were transferred to MS plates as control (a) or to plates containing 1 μM coronatine (b) or 10 μM MeJA (c) and incubated for a further 2 days, after which proteins were extracted and separated by SDS–polyacrylamide electrophoresis. The positions of two bands of ~31 and ~29 kD are indicated.

activity increased locally and systemically. Application of coronafacic acid also increased PIN activity both locally and systemically.

Growth of *P. s.* pv *atropurpurea* in Arabidopsis Leaves

In preliminary tests, we observed that coronatine-producing strains of *P. s. atropurpurea* caused spreading lesions in leaves of wild-type Arabidopsis. The susceptibility of the *coi1* mutant and wild-type Arabidopsis to one such strain, Atr1, was compared. Figure 7 shows that Atr1 grew to a population more than 100 times greater than that recovered from leaves of the *coi1* mutant. At 6 days after inoculation, the infected leaves of wild-type Arabidopsis were chlorotic, but this symptom was absent from inoculated leaves of the *coi1* mutant at 14 days after inoculation when the final observations were made.

DISCUSSION

The mutant allele *coi1* conferred male sterility, insensitivity to the phytotoxin coronatine, and insensitivity to the phytohormone MeJA. The principal evidence that a single mutant allele was responsible was that 14 independent mutants insensitive to coronatine all displayed the three mutant phenotypes. Moreover, these mutations segregated as single recessive Mendelian markers that did not show recombination in the F_2 and F_3 populations. We consider below how a single mutation could be directly responsible for the pleiotropic phenotype of the *coi1* plants.

Coronatine and MeJA had similar effects on the plants tested. Both compounds induced systemic PIN activity in tomato plants and inhibited root growth, stimulated anthocyanin accumulation, and caused the accumulation of two proteins in wild-type Arabidopsis. Significantly, neither compound produced any of these effects in the *coi1* plants. Coronatine was more effective than MeJA in each of these assays, but because the MeJA used contained only ~10% of the more active *cis*-isomer (Koda, 1992), the significance of quantitative differences in these responses cannot be determined from these experiments. Thus, coronatine and MeJA affect the same response pathway. The structural similarity of the cyclopentanone ring in coronatine and MeJA (Figure 1) suggests that these compounds act at the same site in this pathway. A difficulty with this argument is that coronafacic acid (Figure 1) also possesses the cyclopentanone ring found in coronatine but does not stimulate hypertrophy of potato tuber tissue (Shirashi et al., 1979) or cause chlorosis of bean leaves (Mitchell and Young, 1978), two effects characteristic of coronatine. It is significant, therefore, that in this study we showed that coronafacic acid, coronatine, and MeJA are similar in other biological activities: each induced local and systemic PIN activity in tomato leaflets and inhibited root growth and stimulated anthocyanin accumulation in wild-type Arabidopsis but not in the *coi1* mutant. These results suggest that coronatine and coronafacic acid exhibit species- or tissue-specific differences, similar to those that have been shown for the jasmonates (Koda, 1992). Our results are therefore consistent with coronatine acting as a molecular mimic of natural phytohormones, the jasmonates.

The *coi1* allele also affected flower development. The flowers of *coi1* plants were male sterile, produced abnormal anthers

Table 1. Inhibition of Chymotrypsin by Extracts of Tomato Leaflets Treated with Coronatine, Coronafacic Acid, or MeJA

	Chymotrypsin Activity[a]	
Leaf Treatment[b]	Treated Leaf[b]	Upper Leaf[c]
Water	105.1[d]	100.7[d]
Coronatine	22.9[e]	61.1[e]
Coronafacic Acid	29.4[e]	74.7[e]
MeJA	87.3[f]	93.2[f]

[a] Chymotrypsin was added to plant extracts. Chymotrypsin activity is given as the percent of activity in non-plant controls and is the mean of five replicates.

[b] A 3-μL drop of water alone or containing 3 nmol of the indicated compound was placed on the terminal leaflet of leaf 4.

[c] Leaf 5 of the treated plant.

[d–f] For each column, different letter superscripts indicate significant differences ($P < 0.05$).

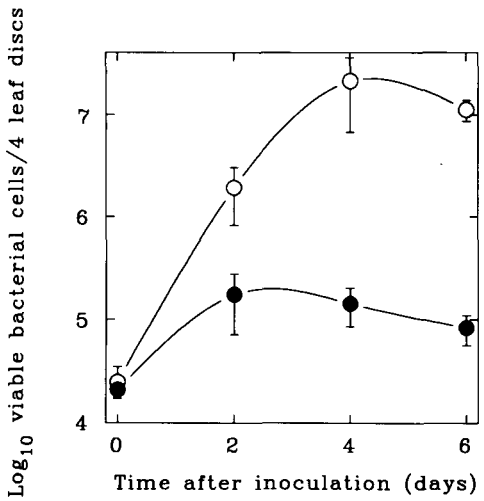

Figure 7. Growth of *P. s. atropurpurea* in Leaves of Arabidopsis Wild-Type and *coi1* Mutant Plants.

Suspensions of Atr1, a coronatine-producing strain of *P. s. atropurpurea*, were infiltrated into leaves of 5-week-old Arabidopsis wild-type (○) and the *coi1* mutant (●) plants. Samples consisting of four leaf discs were harvested at the indicated intervals, and bacteria were extracted to determine the viable cell number. Points are the means of four independent determinations, and bars represent the standard error of the mean.

and pollen, and lacked a protein of ~31 kD, which was present constitutively in the wild-type flowers. A protein of similar size accumulated in wild-type seedlings, but not in the *coi1* seedlings, treated with coronatine or MeJA. Although it is not shown that these two 31-kD proteins are identical, they are immunologically related (C.E. Benedetti, unpublished results). Possibly, the insensitivity of the *coi1* mutant to MeJA results in the absence of one or more jasmonate-dependent gene products required for normal anther development. To our knowledge, jasmonates have not previously been implicated in anther and pollen development. However, jasmonates do occur in flowers (Meyer et al., 1991) and in the pollen and anthers (Yamane et al., 1982) of different species. Interestingly, two genes that are normally expressed in floral buds of potato are not normally expressed in leaves but can be induced by applying MeJA (Hildmann et al., 1992).

An alternative way in which jasmonates might be involved in flower development is through regulation of the synthesis of vegetative storage protein. In soybean, vegetative storage proteins and their transcripts are induced by MeJA in the vegetative parts (Mason and Mullet, 1990) and accumulate in the developing inflorescence (Staswick, 1989) where the protein may provide a reserve for flower and pod development. Proteins antigenically related to soybean vegetative storage protein accumulate in Arabidopsis leaves treated with MeJA but not in the treated leaves of a *jar* mutant that is partially insensitive to MeJA (Staswick et al., 1992). The *jar* mutants are self-fertile and have increased sensitivity to ABA. These are therefore phenotypically different from the *coi1* mutants, which are male sterile, and their response to ABA is indistinguishable from that of wild-type Arabidopsis.

The *coi1* allele conferred resistance to a coronatine-producing plant pathogen, Atr1. This is consistent with the reduced virulence of coronatine-minus mutants of *P. s.* pv *tomato* on their natural host, tomato (Bender et al., 1987). We concluded that coronatine predisposes Arabidopsis and tomato to infection. However, this is difficult to reconcile with evidence for an association between MeJA-induced responses and the expression of disease resistance (Melan et al., 1993; Mueller et al., 1993). Another apparent inconsistency is that coronatine induced PIN activity in tomato (Table 1), whereas the accumulation of PIN transcripts has been linked to the resistance of tomato to infection by *P. s. tomato* PT11 (Pautot et al., 1991). However, PT11 carries the genes for coronatine production (Bender and Cooksey, 1986; Bender et al., 1989), and it is possible therefore that PIN transcripts were induced by coronatine in the infected tissue.

We conclude that coronatine is a molecular mimic of MeJA. The *coi1* mutants may therefore provide a useful tool for dissection of the jasmonate signal perception/transduction pathway, its role in flower development, and its response to pathogens. Molecular characterization of the *COI1* allele is therefore an important goal for future research.

METHODS

Plants and Growth Conditions

Ten-day-old M_2 and wild-type *Arabidopsis thaliana* ecotype Columbia (product numbers M2E-1A-2 and WT-1A, respectively; Lehle Seed Co., Tucson, AZ) seedlings were used to screen for coronatine-insensitive mutants. Seeds were routinely soaked in water overnight, sterilized for 10 min in a solution containing 10% hypochlorite, washed with three changes of sterile water, and sown in Petri dishes on Murashige and Skoog (1962) (MS) basal medium (Sigma) supplemented with 30 g/L sucrose and solidified with 0.8% agar (Sigma). Seeds were germinated, and seedlings were grown in white light (150 $\mu E\ m^{-2}\ sec^{-1}$) with a 16-hr-day and 8-hr-night photoperiod at 20°C. For some experiments, seeds were germinated on or seedlings were transferred to MS plates containing 10 μM methyl jasmonate (MeJA; Bedoukian Research Inc., Danbury, CT) containing ~10% of the *cis* form and ~90% of the *trans* isomer or the following compounds at the indicated concentration: kinetin (10 μM), zeatin (10 μM), 6-benzylaminopurine (10 μM), abscisic acid (ABA; 1 μM), indole-3-acetic acid (10 μM), and 1-aminocyclopropane-1-carboxylic acid (20 μM). Arabidopsis plants for inoculations with bacteria were grown in white light (150 $\mu E\ m^{-2}\ sec^{-1}$) with a 10-hr-day and 14-hr-night photoperiod at 20°C. To score the male-sterile phenotype and for making crosses, seedlings were transplanted to pots containing peat-based compost plants and grown in a controlled environment chamber with 18-hr-day (white light 150 $\mu E\ m^{-2}\ sec^{-1}$) and 6-hr-night photoperiod at 20°C. For anthocyanin determination, seedlings were germinated on MS plates containing coronatine (1 μM), MeJA (10 μM),

or coronafacic acid (1 μM) and grown for 8 days in a 19-hr-day (white light 150 μE m^{-2} sec^{-1}) and 5-hr-night photoperiod at 20°C.

Tomato (*Lycopersicon esculentum* var Moneymaker) plants for the proteinase inhibitor (PIN) assay and Italian ryegrass (*Lolium multiflorum* var Atalja) for pathogenicity tests were raised from seed in pots containing compost in a controlled environment at 20°C at 50% relative humidity with a 12-hr-day (150 μE m^{-2} sec^{-1}) and 12-hr-night photoperiod.

Preparation of Coronatine

Coronatine was isolated from 14-day-old cultures of *Pseudomonas syringae* pv *glycinea* 4018 grown at 18°C in 1 L of modified Hointink's medium (Palmer and Bender, 1993) with orbital agitation at 200 cycles per min. The crude organic acid fraction was isolated according to Mitchell (1982) by phase-partition of acidified, concentrated cell-free culture supernatant against ethyl acetate and back-extraction into alkaline aqueous solution. Coronatine and coronafacic acid were purified by reverse phase HPLC (Palmer and Bender, 1993) with the following modifications: the column was of Spherisorb ODS2 (10-mm internal diameter × 250 mm long; HPLC Technology, Macclesfield, U.K.); the volume of sample injected was 20 μL; and the mobile phase was at 2 mL/min and contained 0.05% (v/v) trifluoroacetic acid and a varying concentration of acetonitrile in water. The acetonitrile was at 35% (v/v) for the first 5 min and increased linearly to 55% (v/v) after 20 min. Peaks detected at 208 nm corresponding to coronafacic acid and coronatine were identified by comigration with the authentic standards (a gift from R.E. Mitchell, Auckland, New Zealand) and were collected. The MS of the purified toxin gave the molecular ion at m/z 319 and prominent fragment ions at m/z 191, 163, and 145, corresponding to the MS of authentic coronatine (Ichihara et al., 1977; Mitchell and Young, 1978). Concentrations of coronatine and coronafacic acid were confirmed by their molar adsorption coefficients in methanol at 208 and 217 nm, respectively (Ichihara et al., 1977).

Fixing and Embedding Arabidopsis Flowers

Young, opened flowers from the primary inflorescences of Arabidopsis plants were fixed overnight in 0.1 M Pipes buffer, pH 7.2, containing 5% (v/v) glutaraldehyde and then dehydrated for 30 min in each of five solutions containing increasing concentrations of ethanol up to 100%. Buds were placed overnight in LR White resin (London Resin Company, London, U.K.), transferred to capsules containing fresh resin, and incubated at 60°C for 24 hr. Sections that were 1 μm thick were cut with a glass knife, heat-fixed to a glass slide, and stained for 30 sec with toluidine blue.

Gel Electrophoresis

Entire seedlings were ground in liquid nitrogen and homogenized in 63 mM Tris-HCl, pH 6.8, containing 5% (v/v) β-mercaptoethanol and 2% SDS (w/v) and held on ice for 5 min. Four volumes of acetone was added, and after 1 hr at −20°C, precipitated proteins were collected by centrifugation and washed twice with acetone at −20°C. Pellets were resuspended in Laemmli sample buffer (Laemmli, 1970), heated to 90°C for 5 min, and centrifuged to remove insoluble material. The proteins were separated on 10% (w/v) SDS–polyacrylamide gels according to Laemmli (1970) at 100 V for 2.5 hr. Proteins were stained with silver reagent according to Blum et al. (1987).

For two-dimensional gels, flowers were harvested and immediately stored in liquid nitrogen. Samples were ground in liquid nitrogen, homogenized in 0.1 M Tris-HCl, pH 7.0, containing 1% (v/v) Triton X-100 and 50 mM dithiothreitol, and held on ice for 5 min. Insoluble material was removed by centrifugation, and proteins were precipitated in acetone as described above. Pellets were resuspended in sample buffer containing 9.5 M urea, 2% (w/v) Nonidet P-40, and 100 mM dithiothreitol. Ampholines in the pH range of 3 to 10 (Pharmacia) were added at 2% (v/v) final concentration prior to loading the gel. Samples containing ~30 μg protein, determined according to Bradford (1976), were separated in the first dimension in rod gels with a 2.5-mm inside diameter, as described by O'Farrell (1975), except that the ampholines were in the pH range of 3 to 10 at a 2% final concentration and with electrophoresis at 350 V for 14 hr. After isoelectric focusing, gels were equilibrated for 2 hr in 63 mM Tris-HCl buffer, pH 6.8, containing 2% (w/v) SDS, 20% (v/v) glycerol, and 50 mM dithiothreitol. Equilibrated rod gels were assembled onto 10% SDS–polyacrylamide gels (Laemmli, 1970), and electrophoresis in the second dimension was at 140 V for 3 hr. Proteins were stained with the silver reagent (Blum et al., 1987).

Anthocyanin Extraction and Determination

Anthocyanins were extracted from 50, 8-day-old seedlings in 3 mL of 1% (v/v) HCl-methanol overnight at 4°C in the dark, with back-extraction with chloroform to remove chlorophyll, according to Rabino and Mancinelli (1986). Absorbance at 530 nm minus absorbance at 657 nm was used as a measure of anthocyanin content and was normalized per seedling. The experiment was repeated three times.

Proteinase Inhibitor Assay

A 3-μL drop containing 3 nmol of MeJA, coronafacic acid, coronatine, or water, as the control, was placed on the surface of the terminal leaflet of the fourth-oldest leaves of 4-week-old tomato plants, and the plants were held in continuous light for the following 48 hr. The treated leaflet and the terminal leaflet from the fifth-oldest leaf of the treated plant were removed and immediately frozen in liquid nitrogen. PIN activity was assayed by the inhibitory effect of leaf extracts on added chymotrypsin, exactly as described by Wildon et al. (1992). The experiment was repeated three times.

Maintenance of Bacteria

P. s. pv *atropurpurea* (No. 2396; National Collection of Plant Pathogenic Bacteria, Harpenden, U.K.) was grown on King's B medium (King et al., 1954) shaken at 28°C or on King's B agar plates at 28°C. Atr1 was a spontaneous rifampicin-resistant mutant selected on a King's B agar plate containing rifampicin (100 μg/mL) seeded with ~10^9 cells *P. s. atropurpurea.*

Inoculation of Plants

Cultures of Atr1 were grown for 18 hr in King's B medium, harvested by centrifugation at 12,000*g*, and resuspended in PBS (Sambrook et

al., 1989). With the aid of a standard curve relating A_{550} to the viable cell number, the suspension was diluted in PBS to give ~10^6 cells per mL. Fully expanded leaves of 5-week-old Arabidopsis plants were uniformly infiltrated with these suspensions according to Swanson et al. (1988) as modified by Dong et al. (1991). At intervals after inoculation, samples comprising four discs, 0.5-cm diameter, were removed from the infiltrated portion of leaves from four plants and homogenized in a plastic pestle. Serial dilutions were made in King's B liquid medium, and the number of viable cells was determined on King's B plates containing 100 μg/mL rifampicin. The experiment was repeated two times. To test for pathogenicity on its native host, 5-μL drops containing ~5 × 10^5 cells of Atr1 were placed on pin-prick wounds made in leaves of 3-week-old Italian ryegrass plants. After 8 days, the 20 sites inoculated with Atr1 were each surrounded by spreading chlorotic lesions.

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