Differential Expression of a Novel Gene in Response to Coronatine, Methyl Jasmonate, and Wounding in the \textit{Coi1} Mutant of Arabidopsis\textsuperscript{1}

Celso E. Benedetti*, Cinthia L. Costa, Silvia R. Turcinelli, and Paulo Arruda

Centro de Biologia Molecular e Engenharia Genética–Universidade Estadual de Campinas, Campinas, São Paulo 13083–970, Brazil (C.E.B., C.L.C., S.R.T., P.A.); and Departamento de Genética e Evolução, Instituto de Biologia, Universidade Estadual de Campinas, 13083–970, Campinas, São Paulo, Brazil (P.A.)

Coronatine is a phytotoxin produced by some plant-pathogenic bacteria. It has been shown that coronavirus mimics the action of methyl jasmonate (MeJA) in plants. MeJA is a plant-signaling molecule involved in stress responses such as wounding and pathogen attack. In \textit{Arabidopsis thaliana}, MeJA is essential for pollen grain development. The \textit{coi1} (for coronatine-insensitive) mutant of Arabidopsis, which is insensitive to coronavirus and MeJA, produces sterile male flowers and shows an altered response to wounding. When the differential display technique was used, a message that was rapidly induced by coronavirus in wild-type plants but not in \textit{coi1} was identified and the corresponding cDNA was cloned. The coronavirus-induced gene \textit{ATHCOR1} (for \textit{A. thaliana} coronavirus-induced) is expressed in seedlings, mature leaves, flowers, and siliques but was not detected in roots. The expression of this gene was dramatically reduced in \textit{coi1} plants, indicating that \textit{COI1} affects its expression. \textit{ATHCOR1} was rapidly induced by MeJA and wounding in wild-type plants. The sequence of \textit{ATHCOR1} shows no strong homology to known proteins. However, the predicted polypeptide contains a conserved amino acid sequence present in several bacterial, animal, and plant hydrolases and includes a potential ATP/GTP-binding-site motif (P-loop).

JA and its methyl ester, MeJA, and related compounds derived from linolenic acid are recognized as signaling molecules synthesized by plants in response to wounding and herbivore and pathogen attack (Creelman et al., 1992; Farmer and Ryan, 1992; Mueller et al., 1993). These substances can activate the expression of several genes, leading to the accumulation of their products, which are referred to as jasmonate-induced proteins. The best-studied jasmonate-induced proteins include proteinase inhibitors, thionins, vegetative storage proteins, lipoxygenases, ribosome-inactivating proteins, enzymes of phenylpropanoid metabolism, and others (Koda, 1992; for review, see Reinbothe et al., 1994). The jasmonates can also repress the expression of genes related to photosynthesis at the transcriptional and translational levels (Reinbothe et al., 1994). It has been demonstrated that MeJA induces a shift in the length of the plastid \textit{rbcL} transcript in barley, thus impairing translation initiation (Reinbothe et al., 1993). However, little is known about the mechanisms by which jasmonates control gene expression and the signal cascade that mediates this response. New evidence suggests that protein phosphorylation is required for the activation of certain wound-inducible genes that respond to JA (Damman et al., 1997).

Coronatine is a phytotoxin produced by several pathogens of \textit{Pseudomonas syringae} (Ichihara et al., 1977; Mitchell and Young, 1978; Mitchell, 1982). Its biological effects include induction of leaf chlorosis and inhibition of root growth (Nishiyama et al., 1976; Ferguson and Mitchell, 1985; Kenyon and Turner, 1990), and it has been suggested to play a role in disease development as a virulence factor produced by the bacteria during infection. Mutations that abolished coronatine production in \textit{P. syringae pv tomato} reduced the capacity of this pathogen to produce necrotic lesions on tomato (\textit{Lycopersicon esculentum}) leaves (Bender et al., 1987). Moreover, coronatine production was required for the successful infection of Arabidopsis leaves by \textit{P. syringae pv tomato}, and this was attributed to suppression of defense-related genes by the toxin (Mittal and Davis, 1995).

Coronatine acts as a mimic of MeJA in plants (Weiler et al., 1994), and the \textit{Arabidopsis thaliana} mutant insensitive to coronatine (\textit{coi1}) is also insensitive to MeJA and is male sterile (Feys et al., 1994; Benedetti et al., 1995). In addition, \textit{coi1} plants are more sensitive to wounding than are the wild type, but they are resistant to \textit{P. syringae} infection (Feys et al., 1994). These findings suggest that both coronatine and MeJA may interact with a common receptor and that MeJA is required for pollen development and mediates at least part of the wound response. However, there is evidence that one of the MeJA responses in Arabidopsis is needed to induce the symptoms caused by a coronatine-
producing strain of \textit{P. syringae} (Fey et al., 1994). This apparent paradox remains to be clarified, since MeJA is suggested to play a role in plant-defense responses (Coehn et al., 1993; Mueller et al., 1993; Reinbothe et al., 1994). Nevertheless, no correlation could be found between jasmonates and defense responses in plant-pathogen interactions (Schweizer et al., 1993; Kogel et al., 1995; Schweizer et al., 1997). How coronatine could function as a virulence factor by mimicking MeJA in a bacteria-plant interaction is an open question.

In an attempt to clarify this issue we are studying coronatine and MeJA responses in Arabidopsis by identifying genes that are rapidly activated by coronatine, MeJA, or wounding. To detect and clone such genes we are using the mRNA differential display technique (Liang and Pardee, 1992). We present here the initial characterization of a novel Arabidopsis gene that is induced by coronatine, MeJA, and wounding, the expression of which is affected by the COII gene.

**MATERIALS AND METHODS**

**Biological Material and Chemicals**

Wild-type \textit{Arabidopsis thaliana} ecotype Columbia (Col-0) was used. The \textit{coi1} mutant was described previously (Fey et al., 1994) and was donated by Dr. John G. Turner (University of East Anglia, UK). Coronatine and MeJA were obtained as previously described (Feys et al., 1994).

**Plant Growth**

Seeds of wild-type Arabidopsis were germinated in MS medium (Murashige and Skoog, 1962), whereas \textit{coi1} seeds from an \textit{F}2 population segregating for the \textit{Coi} phenotype were first germinated in MS containing 1 \textmu M coronatine to select for homozygous \textit{coi1} plants. Seedlings were grown in white light (70 \textmu E m\textsuperscript{−2} s\textsuperscript{−1}) for 1 week in a growth cabinet with a 16-h day/8-h night photoperiod at 22\degree C, after which they were transferred to fresh MS and grown for another 1 week. Seedlings were then either transferred to fresh MS for coronatine and MeJA treatments or moved to soil to grow to maturity.

**Coronatine and MeJA Treatments**

Two-week-old seedlings were transferred to MS (control seedlings) or MS containing either 1 \textmu M coronatine or 10 \textmu M MeJA. After different periods of incubation, they were frozen in liquid nitrogen and total RNA was extracted.

**Wounding**

For the wounding experiment, seeds were germinated in MS and grown for 2 weeks under short-day conditions (9-h day/15-h night at 22\degree C). Seedlings were then transferred to soil and grown for 2 weeks. Leaves of individual plants were wounded once with scissors. After the treatment, plants were returned to the growth cabinet, and wounded leaves were collected at different times, frozen in liquid nitrogen, and stored at −70\degree C for RNA extraction.

**RNA Extraction and Differential Display**

Total RNA from roots, seedlings, leaves, flowers, silique, and wounded leaves was extracted according to the method of Verwoerd et al. (1989).

Differential display of mRNA was performed according to the method of Liang and Pardee (1992), with minor modifications. Total RNA (1 \textmu g) from control and coronatine-treated seedlings of wild-type and \textit{coi1} plants were reverse transcribed with 100 units of reverse transcriptase in the presence of 2.5 \textmu M T\textsubscript{12}V\textsubscript{N} as anchored primers and 20 \textmu M dNTPs for 10 min at 25\degree C, followed by a 50-min incubation at 37\degree C. Two microliters of the reaction was then added to 18 \textmu L of the PCR mixture consisting of 1× PCR buffer, 1.25 mm MgCl\textsubscript{2}, 1 \textmu M anchored primer (T\textsubscript{12}V\textsubscript{N}, 1 \textmu M arbitrary primer (10-mer from Operon, Alameda, CA), 2.0 \textmu M dNTPs, 10 \textmu Ci [\alpha\textsuperscript{32}P]ATP, and 1.5 units of Taq polymerase. PCR conditions were 40 cycles of 94\degree C for 30 s, 40\degree C for 2 min, and 72\degree C for 30 s, followed by 5 min of elongation at 72\degree C. PCR products were analyzed on a 6% acrylamide denaturing DNA-sequencing gel. Gels were dried at 80\degree C for 2 h, and radiographic films were aligned to them and exposed overnight at −70\degree C. Differentially displayed bands were cut off and eluted in 200 \textmu L of water at 95\degree C for 15 min. DNA was ethanol precipitated to remove urea and reamplified by PCR using the same conditions as above, except that the final concentration of dNTPs was 20 \textmu M. Reamplified DNA was analyzed on agarose gels, and bands of the expected size were purified and cloned into pMOSBlue vector (Amersham). Cloned fragments were sequenced and used to probe RNA blots and to screen a cDNA library of Arabidopsis flowers.

**Northern Analysis**

Total RNA (20 \textmu g of each sample) was electrophoresed on formaldehyde-agarose gels (Sambrook et al., 1989), transferred onto nylon membranes (Hybond N+, Amersham) by capillary blot, and fixed by UV cross-linking according to the manufacturer’s instructions. Blots were hybridized using the cloned fragments obtained from the display gels or fragments of the full-length cDNA clones as probes. Membranes were washed twice with 2× SSC containing 0.1% SDS for 10 min at 42\degree C and twice with 0.2× SSC containing 0.1% SDS for 10 min at 42\degree C.

**cDNA Library Screening**

A cDNA library of Arabidopsis (ecotype Landsberg erecta) flowers constructed in Lambda Zap II cloning vector (Stratagene) was kindly donated by Dr. Elliot M. Meyerowitz (California Institute of Technology, Pasadena). The library was screened following the manufacturer’s protocols using the cloned fragments that showed differential expression as probes on northern analysis.
RACE

The 5' end of the isolated cDNA was cloned by RACE according to the method of Frohman et al. (1988). Total RNA from wild-type flowers and from MeJA- and coronatine-treated seedlings was reverse transcribed using the P1 primer (5'-CCATTCTTACACATACAACC-3'). First-strand cDNA was purified and amplified by PCR using P1 and the (dT17)-adaptor primer (Frohman et al., 1988). After 20 PCR cycles (94°C for 30 s, 45°C for 1 min, and 72°C for 1 min), an aliquot was reamplified using the internal P2 primer (5'-CGTGATGGATGGGTCTAATG-3') and the adapter primer in 20 PCR cycles of 94°C for 30 s, 55°C for 1 min, and 72°C for 1 min (Frohman et al., 1988). PCR fragments were gel purified, cloned into pMOSBlue vector (Amersham), and sequenced.

RESULTS

A DNA fragment of about 280 bp was detected by differential display in wild-type but not coil seedlings upon induction with coronatine (not shown). DNA from the corresponding region of the display gel was reamplified, cloned, and sequenced.

This clone, TGCOPP9–280, was used to probe a northern blot of total RNA extracted from seedlings and flowers of wild-type and coil mutant plants (Fig. 1). The probe confirmed the differential expression of a major transcript of approximately 1.3 kb that was induced by coronatine in wild-type but not in coil seedlings. Apparently, two transcripts of similar molecular weight were also detected in lower levels in untreated wild-type seedlings and flowers but not in male-sterile flowers of coil (Fig. 1).

The clone TGCOPP9–280 was used to screen a cDNA library from Arabidopsis flowers. Five independent cDNA clones were isolated and sequenced. The sequences of all clones were identical and contained the entire sequence of TGCOPP9–280. However, unexpectedly, we found that the anchored primer T12GC did not prime at the poly(A+1) tail of the mRNA but within the gene in an A-rich region. In addition, all of the isolated cDNAs were truncated at their 5' ends. To obtain a full-length clone, the 5' end of the gene was generated by RACE from mRNAs extracted from flowers or seedlings treated with either coronatine or MeJA. The sequences of these different RACE products were identical, indicating that the transcripts induced by coronatine or MeJA and those expressed in flowers were the same. The RACE products were also identical to the corresponding 5' end of the cDNAs isolated from the library, except they contained an extra 50 bp in their 5' ends (Fig. 2). A full-length cDNA clone was then obtained by ligating the 5' end of the RACE product to the original cDNA isolated from the library using a SacI site upstream of the P2 primer (not shown).

Figure 2. Sequence of ATHCOR1 cDNA (AF021244) and its predicted protein. The 5' end of the untranslated sequence was found in the genomic clone (not shown) and was interpreted as the mRNA cap. Amino acids in bold represent a possible N-glycosylation site and the bold, underlined sequence represents a potential ATP-/GTP-binding site motif A (P-loop).
mass of 34.8 kD (Fig. 2). The predicted polypeptide has no significant homology to other proteins in the database, except in two small and conserved domains (A and B) found in different hydrolases. These include the 

\textit{Synechocystis} sp. dienelactone hydrolase, \textit{Moraxella} sp. haloacetate dehalogenase, guinea pig platelet-activating factor acetylhydrolase, and epoxide hydrolases from soybean, potato, and Arabidopsis (Fig. 3). In the literature, no biochemical function has been attributed to domain A in these different hydrolases; however, domain B is a potential ATP-/GTP-binding site known as the P-loop, which is common to many ATP-/GTP-binding proteins (Saraste et al., 1990).

Northern blots using ATHCOR1 as a probe confirmed the same pattern of coronatine induction observed with the TGCOPP9–280 probe shown in Figure 1, except that the cDNA detected a single, approximately 1.3-kb band on northern analysis. To determine whether this gene was also induced by MeJA, wild-type and \textit{coi1} seedlings were grown in the presence of 100 \textmu M MeJA, which produces the same phenotype as 1 \textmu M coronatine (e.g. inhibition of root growth and anthocyanin accumulation; Feys et al., 1994). Figure 4 shows that the gene is rapidly induced by MeJA and that high levels of the transcript accumulate in the first 4 h of treatment. A similar pattern of induction was observed with 1 \mu M coronatine; however, the toxin was apparently a less efficient inducer of the gene in comparison with MeJA (Fig. 4).

ATHCOR1 was used to probe northern blots of total RNA extracted from different plant organs (Fig. 5). The coronatine-/MeJA-induced gene was normally expressed in low levels in young and mature leaves and was apparently expressed in higher levels in flowers, but not detected at all in roots. Its expression was dramatically reduced in all tissues of \textit{coi1} plants (Fig. 5).

Wounding of wild-type leaves produced a rapid induction of ATHCOR1 (Fig. 6). Its expression peaked about 30 min after wounding and returned to basal levels in the following 4 h. Wounded leaves of \textit{coi1} showed a similar pattern of induction, but transcripts accumulated at much lower levels (Fig. 6).

**DISCUSSION**

The \textit{coi1} mutant of Arabidopsis has proved to be an excellent model for the identification and analysis of...
It is possible that $ATHCOR1$ belongs to a family of related enzymes involved in the biosynthesis/hydrolysis of plant cell wall components. $ATHCOR1$ may also function during another development, since male sterile-flowers of $coi1$ have much lower levels of its transcripts.

It is interesting to note that the predicted protein of $ATHCOR1$ has a potential ATP/GTP-binding site. This would suggest that the protein could hydrolyze ATP or GTP to exert its function or it could simply be modulated by the binding of nucleotides in a regulatory fashion. We are currently investigating these possibilities. Further characterization of $ATHCOR1$ will be necessary to understand its function and regulation by MeJA, as well as its possible role in the wounding response and perhaps in disease development of Arabidopsis infected by coronatine-producing strains of $P. syringae$.

ACKNOWLEDGMENTS

We wish to thank Dr. John G. Turner for donating the $coi1$ mutant and Dr. Elliot M. Meyerowitz for providing the Arabidopsis cDNA library. We also thank Dr. Adelson Leite and Dr. Ivan Maia for their helpful discussions.

Received September 2, 1997; accepted November 19, 1997.

Copyright Clearance Center: 0032–0889/98/116/1037/06.

LITERATURE CITED


Koda Y (1995) Resistance in barley against the powdery mildew fungus (Erysiphe graminis f. sp. hordei) is not associated with enhanced levels of endogenous jasmonates. Eur J Plant Pathol 101: 319–332