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COI1-Dependent Expression of an Arabidopsis Vegetative Storage Protein in Flowers and Siliques and in Response to Coronatine or Methyl Jasmonate[1]

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The phytotoxin coronatine and the plant growth regulator methyl jasmonate (MeJA) inhibit the growth of Arabidopsis seedlings. Coronatine and MeJA induced the accumulation of an approximately 29-kD protein in wild-type seedlings but not in seedlings of the *coi1* mutant, which is insensitive to both compounds. The approximately 29-kD protein was recognized not only by antibodies raised against the partially purified polypeptide, but also by antibodies raised against vegetative storage proteins (VSPs) from soybean (29 kD) and poplar (32 kD). In the absence of added MeJA/coronatine, the VSP-like protein was highly expressed in flowers and siliques but not in seeds, seedlings, or mature leaves of wild-type Arabidopsis. By contrast, this protein could not be detected in *coi1* seedlings treated with coronatine or MeJA, and it was found in very low levels in the male sterile flowers of *coi1*. A transcript corresponding to the gene of the Arabidopsis 27-kD VSP precursor shows the same pattern of expression as the VSP-like protein. Significantly, the VSP-like protein was not detected in green siliques or seeds obtained from *coi1* flowers fertilized with wild-type pollen. We conclude that the VSP-like protein is normally expressed in maternal tissues, where it is regulated by *COI1*, but is not essential for the development of siliques.

The phytotoxin coronatine produced by pathovars of *Pseudomonas syringae* (Ichihara et al., 1977; Mitchell and Young, 1978) causes diverse responses in plants. Coronatine causes chlorosis and promotes senescence in tobacco leaves (Kenyon and Turner, 1990), inhibits root growth in wheat (Sakai, 1980), stimulates ethylene production by leaves of bean (Ferguson and Mitchell, 1985) and tobacco (Kenyon and Turner, 1992), increases amylase activity in potato tubers (Sakai et al., 1979a), and causes hypertrophy of potato tubers (Sakai et al., 1979b). Some of the responses caused by coronatine are similar to responses induced by the plant growth regulator MeJA (for reviews, see Koda, 1992; Sembdner and Parthier, 1993; Gross and Parthier, 1994; Reinbothe et al., 1994). Coronatine and MeJA have similar chemical structures and cause identical responses in Arabidopsis seedlings (Feys et al., 1994), cell culture of *Eschscholtzia californica*, and tendrils of *Bryonia dioica* (Weiler et al., 1994), suggesting that coronatine mimics the action of MeJA on plants. This was confirmed by the isolation of an Arabidopsis mutant, *coi1*, selected for resistance to coronatine, which was also found to be insensitive to MeJA (Feys et al., 1994). Unexpectedly, *coi1* plants were male sterile. *coi1*, however, differs from the previously described MeJA-insensitive mutant of Arabidopsis, *jar1* (Staswick et al., 1992), which is partially insensitive to MeJA, is male fertile, and has increased sensitivity to ABA (Staswick et al., 1992).

Coronatine and MeJA inhibit root growth, stimulate anthocyanin accumulation, and increase the level of two proteins of approximately 31 and 29 kD detected by SDS-PAGE in wild-type Arabidopsis but not in the *coi1* mutant. Also, male-sterile flowers of *coi1* have a reduced level of an approximately 31-kD protein, which is abundant in wild-type flowers (Feys et al., 1994). We report here that this protein, originally detected in two-dimensional gel electrophoresis, migrates around approximately 29 kD in SDS-PAGE.

To examine whether the protein induced by coronatine is related to the MeJA-induced protein or to the flower protein, we have raised antiserum against the partially purified approximately 29-kD coronatine-induced protein. Two heterologous sera specific for the soybean and poplar VSPs were also used to test the identity of the Arabidopsis proteins. We report here that the Arabidopsis approximately 29-kD protein induced by coronatine or MeJA is immunologically related to the approximately 29-kD protein of Arabidopsis flowers and to the approximately 29-kD VSP of soybean (Wittenbach, 1983; Franceschi and Grimes, 1991) and the 32-kD bark storage protein of poplar (van Cleve and Apel, 1993). The pattern of expression of the approximately 29-kD protein in wild-type Arabidopsis was similar to that of the soybean VSPs (29 and 27 kD), which accumulate at flowering and decline during seed development (Staswick, 1990). However, the Arabidopsis approximately 29-kD protein was not detected in *coi1* plants treated with MeJA or coronatine. A transcript cor-

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Abbreviations: MeJA, methyl jasmonate; MS, Murashige and Skoog; VSP, vegetative storage protein.

responding to the Arabidopsis VSP gene, homologous to the soybean and tomato VSPs, is induced by MeJA and coronatine in seedlings of wild-type Arabidopsis, but it was not detected in seedlings of *coi1*. The transcript was at high levels in wild-type flowers but at low levels in *coi1* flowers. These results suggest that the approximately 29-kD coronatine protein of Arabidopsis is a VSP-like protein that is regulated by MeJA, and that *COI1* controls a step in this signal pathway.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Seeds of wild-type *Arabidopsis thaliana* ecotype Columbia and seeds from an F_2 population segregating for the *coi1* mutant were soaked in water, sterilized for 10 min in 10% hypochlorite, washed in three changes of sterile water, and sown on MS medium (Sigma) (Murashige and Skoog, 1962) supplemented with 10 g L^{-1} Suc and vitamins (Sigma). Seedlings were grown in white light (150 μE m^{-2} s^{-1}) with a 16-h-day/8-h-night photoperiod at 22°C. Wild-type seeds were first germinated in MS medium and then transferred to fresh MS plates containing either 1 μM coronatine or 10 μM MeJA (Bedoukian Research, Inc., Danbury, CT). An F_2 population segregating for *coi1* was germinated in 1 μM coronatine to select *coi1* plants (Feys et al., 1994), which were then moved to fresh MS plates and grown for 3 d before they were transferred to MS plates or to MS plates containing coronatine or MeJA. Arabidopsis plants were grown in peat-based compost in a controlled environment with an 18-h-day (white light 150 μE m^{-2} s^{-1})/6-h-night photoperiod at 20°C.

Preparation of Coronatine

Coronatine was isolated from 14-d-old cultures of *Pseudomonas syringae* pv *Glya* 4180 grown at 18°C in modified Hointink's medium (Palmer and Bender, 1993) with orbital agitation at 200 cycles/min. The organic acid fraction containing coronatine was obtained by phase-partition of acidified, concentrated, cell-free culture supernatant against ethyl acetate, according to Mitchell (1982). Coronatine was purified by reversed-phase HPLC as described previously (Feys et al., 1994).

Protein Extraction, Gel Electrophoresis, Preparation of Antiserum, and Immunoblots

Seeds, seedlings, mature leaves, flowers, and siliques were homogenized in 0.1 M Tris-HCl, pH 6.8, containing 5% (v/v) β-mercaptoethanol, 2% (w/v) SDS, and leupeptin (25 μg mL^{-1}) and held on ice for 5 min. Insoluble material was removed by centrifugation, and proteins were precipitated with 3 volumes of cold acetone. After 1 h at −20°C, proteins were collected by centrifugation, resuspended on ice in 0.1 M Tris-HCl, pH 6.8, containing 1% (v/v) β-mercaptoethanol and leupeptin (25 μg mL^{-1}), and reprecipitated in acetone as described above. Protein pellets were washed with cold acetone, dried, resuspended in Laemmli sample buffer (Laemmli, 1970), heated to 90°C for 5 min, and centrifuged to remove insoluble material. Protein concentration was estimated using the Bio-Rad protein assay reagent according to the manufacturer's instructions. Proteins were separated in 12% SDS-polyacrylamide gels (Laemmli, 1970), transferred onto nitrocellulose membranes, and visualized by Pounceau "S" staining, and the appropriate region of the membrane containing the approximately 29-kD coronatine-induced protein was cut out. Pieces of membrane containing the protein were dissolved in DMSO, mixed with an equal volume of complete Freund's adjuvant, and immediately injected subcutaneously into a white rabbit (Sambrook et al., 1989). The rabbit was injected three more times at 30, 60, and 90 d after the first injection, except that incomplete Freund's adjuvant was used. The rabbit was bled 15 d after the last injection.

Plant proteins separated by SDS-gel electrophoresis were transferred onto nitrocellulose membranes and incubated with antiserum raised against the SDS gel-purified, coronatine-induced protein, and with two heterologous sera specific for the 29-kD soybean and the 32-kD poplar VSPs, according to Towbin et al. (1979). Blots were developed using the peroxidase/diaminobenzidine reaction (Sambrook et al., 1989) or the ECL detection system (Amersham).

RNA Extraction and Analyses

Two-week-old seedlings of *coi1* and wild-type Arabidopsis grown in MS medium were transferred to fresh MS plates containing either 1 μM coronatine or 10 μM MeJA. Seedlings were collected and frozen at 4, 8, 12, 24, 48, and 72 h after the start of the treatment. Total RNA was extracted from seedlings and flowers of *coi1* and wild-type Arabidopsis according to Verwoerd et al. (1989). RNA was denatured in 40% (v/v) formamide, 18% (v/v) formaldehyde at 65°C for 15 min, separated in a 1.4% agarose/formaldehyde gel, and transferred onto a nylon membrane (Sambrook et al., 1989). RNA blots were hybridized to a 330-bp cDNA fragment corresponding to the end of the gene of the Arabidopsis 27-kD VSP precursor (Höfte et al., 1993). The DNA was amplified by PCR from a cDNA library of Arabidopsis flower constructed in λ ZAP II (Yanofsky et al., 1990). The library was obtained from The Ohio State University. One primer (5′-GCAGAGCTCGTGTGACAAAATGGAAGC) was designed from the sequence of the Arabidopsis VSP gene, except that a *Sac*I site was included at the 5′ end. The second primer (3′-CCATGGGTTAAGCGGGATA) was designed from the sequence of the pBluescript flanking the *Xho*I cloning site at the 3′ end. A single 330-bp PCR fragment was amplified, cloned into *Sac*I/*Kpn*I pBluescript, and sequenced. The sequence corresponded to the 3′ end of the Arabidopsis expressed sequence tag (ATTS0751) deposited in the data base and described as having homology to the soybean and tomato VSPs (Höfte et al., 1993). After hybridization, membranes were washed three times for 15 min in 2% (w/v) SSC, 0.5% (w/v) SDS, and for 10 min in 0.2% (w/v) SSC, 0.1% (w/v) SDS at 65°C (Sambrook et al., 1989).

RESULTS

Accumulation of a VSP-Like Protein in Arabidopsis Seedlings Treated with Coronatine or MeJA

Both coronatine and MeJA induced the accumulation of an approximately 29-kD protein in wild-type Arabidopsis seedlings but not in *coi1* seedlings (Fig. 1A). Polyclonal antibodies raised against the approximately 29-kD coronatine-induced protein recognized both the coronatine- and the MeJA-induced protein. The antibodies also cross-reacted with an approximately 29-kD protein that was expressed constitutively in wild-type flowers but was not detected in the male-sterile flowers of *coi1* (Fig. 1B). The induced approximately 29-kD protein and the flower protein were also detected by antisera raised against the approximately 29-kD soybean VSP (Franceschi and Grimes, 1991) (Fig. 1C) and the 32-kD poplar VSP (van Cleve and Apel, 1993) (Fig. 1D). These antisera detected very low levels of the approximately 29-kD protein in *coi1* flowers and in untreated wild-type seedlings, but did not detect this protein in *coi1* seedlings (Fig. 1, C and D). An approximately 45-kD protein detected by the antiserum against the poplar protein is thought to be a nonspecific cross-reaction, although this protein was not observed in *coi1* seedlings (Fig. 1D).

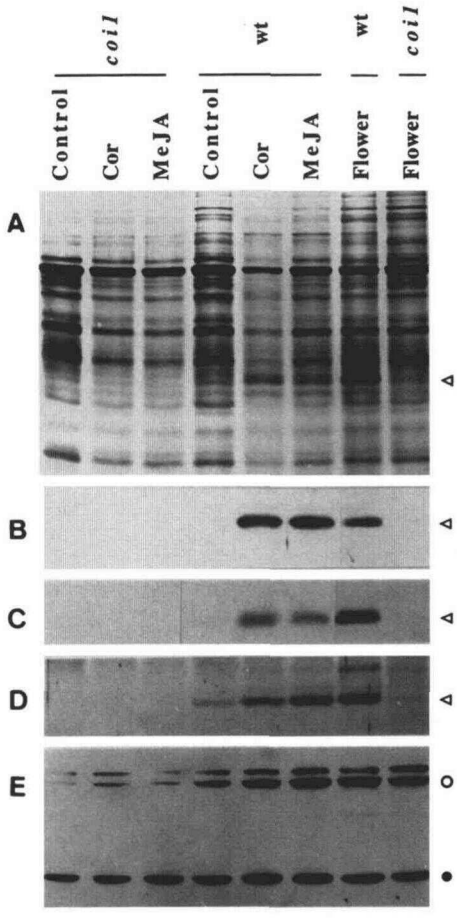

Figure 1. Ten-day-old seedlings of wild-type Arabidopsis (wt) and *coi1* were transferred to MS plates (Control) or to plates containing 1 μM coronatine (Cor) or 10 μM MeJA and grown for 72 h, after which proteins (approximately 1 μg) were extracted and separated by SDS-PAGE (A) and blotted and probed with different antisera (B–E). The approximately 29-kD coronatine/MeJA-inducible protein and a flower protein of similar molecular mass are indicated (arrowheads). B, Western blot of proteins probed with the antiserum (1:1500) raised against the partially purified, coronatine-induced approximately 29-kD protein. C and D, Blots of proteins probed with the antisera raised against the soybean (29 kD) (1:3000) and the poplar (32 kD) (1:1000) VSPs, respectively. E, Western blot of proteins probed with anti-LOX1 serum (1:3000). An approximately 98-kD band corresponding to the Arabidopsis lipoxygenase is indicated (○); a nonspecific band (approximately 45 kD) was present at the same levels in all samples (●).

To test whether the *coi1* mutant could express other MeJA-regulated proteins, protein blots were also probed with an antiserum raised against the Arabidopsis lipoxygenase (anti-LOX1, Peterman et al., 1994), which detects lipoxygenase from Arabidopsis, soybean, and pea tissues (Peterman et al., 1994). *LOX*, which encodes lipoxygenase, is a MeJA-regulated gene (Bell and Mullet, 1991) and is expressed in inflorescences (Bell and Mullet, 1993; Melan et al., 1993). Figure 1E shows an approximately 98-kD band that we interpret to be the Arabidopsis lipoxygenase. It is interesting that this band was present at low levels in *coi1* seedlings but at higher levels in wild-type seedlings and in flowers of both *coi1* and wild-type plants. Treatments with MeJA and coronatine caused a slight induction of the approximately 98-kD band in wild-type seedlings but not in the *coi1* mutant. LOX1 has been shown to be spatially and temporally expressed in roots of 1- to 6-d-old seedlings of Arabidopsis (Melan et al., 1994) and was detected at low levels in Arabidopsis inflorescences (Peterman et al., 1994), whereas *LOX2* mRNA was detected at high levels in Arabidopsis inflorescences (Bell and Mullet, 1993). Because the anti-LOX1 antiserum may not distinguish between LOX1 and LOX2 (Peterman et al., 1994), it is likely that the approximately 98-kD band shown in Figure 1E corresponds to both of these proteins.

Expression Pattern of the VSP-Like Protein in Wild-Type Arabidopsis

Figure 2 shows that in the absence of added coronatine or MeJA, the approximately 29-kD coronatine- and MeJA-induced protein was highly expressed in flowers and green siliques but was not detected in seeds, developing seedlings, and mature leaves (Fig. 2). This pattern of expression is very similar to that of the soybean VSP, which accumulates at flowering and declines during seed formation (Staswick, 1990). It has been suggested that the soybean VSP may function as a temporary store during flowering, contributing to the pool of nutrients mobilized from siliques to developing seeds (Staswick, 1989).

To test the importance of this protein during silique formation and seed development, we fertilized male-sterile flowers of *coi1* with wild-type pollen and observed that green siliques and seeds obtained from these crosses developed normally, but that the VSP-like protein could not be detected (Fig. 3). Therefore, development of siliques and seeds does not depend on accumulation of VSP.

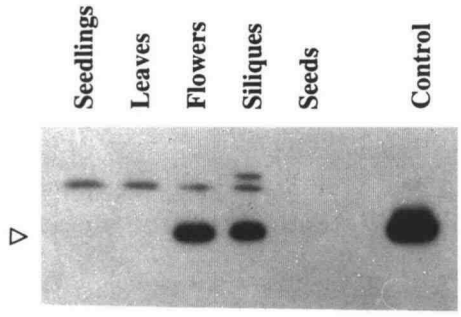

Figure 2. Western blot of wild-type Arabidopsis proteins (approximately 1 μg) probed with antiserum against the soybean VSP (1:3000), showing high levels of the VSP-like protein in flowers and green siliques (arrowhead) compared to seeds, seedlings, and mature leaves where the protein was not detected. Control, Proteins from 10-d-old seedlings of wild-type Arabidopsis treated with 1 μM coronatine for 72 h. Similar results were obtained when samples were probed with antiserum raised against the partially purified coronatine-induced protein (not shown). Minor bands are thought to indicate a nonspecific cross-reaction.

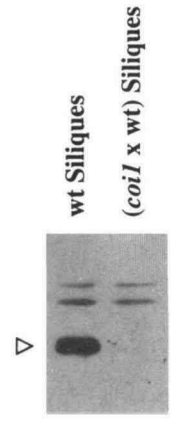

Figure 3. Western blot of proteins (approximately 1 μg) from wild-type (wt) siliques and *coi1* siliques obtained from *coi1* flowers fertilized with wild-type pollen probed with the anti-soybean VSP serum (1:3000). The VSP-like protein present at high levels in wild-type siliques (arrowhead) was not detected in (*coi1* × wt) siliques. The two minor bands are thought to indicate a nonspecific cross-reaction.

A VSP mRNA Present in Flowers of Wild-Type Arabidopsis Was Induced by Coronatine and MeJA in Wild-Type Seedlings but Not in *coi1* Seedlings

Seedlings of wild-type Arabidopsis treated with either coronatine or MeJA for 4, 8, 12, 24, 48, or 72 h accumulated high levels of two mRNAs corresponding to the gene of the 27-kD VSP precursor (Höfte et al., 1993). These transcripts could not be detected in *coi1* seedlings treated with MeJA or coronatine (Fig. 4, A and C). The two mRNAs (approximately 1.1 and 1.2 kb) were present in low abundance in control seedlings, but the bands could not be resolved where high amounts of the transcripts were present following treatment with coronatine or MeJA. The transcripts were detected in wild-type flowers at higher levels than in untreated seedlings and in the male-sterile flowers of *coi1* plants (Fig. 4C). The VSP-like protein was detected 20 h after seedlings were exposed to either coronatine or MeJA (data not shown), and it accumulated during treatment for 72 h (Fig. 1) or longer (data not shown). These results show that the Arabidopsis VSP gene, homologous to the soybean VSPs (29 and 27 kD), is coronatine/MeJA induced, its expression is associated with the appearance of the VSP-like protein, and its regulation is dependent on *COI1*.

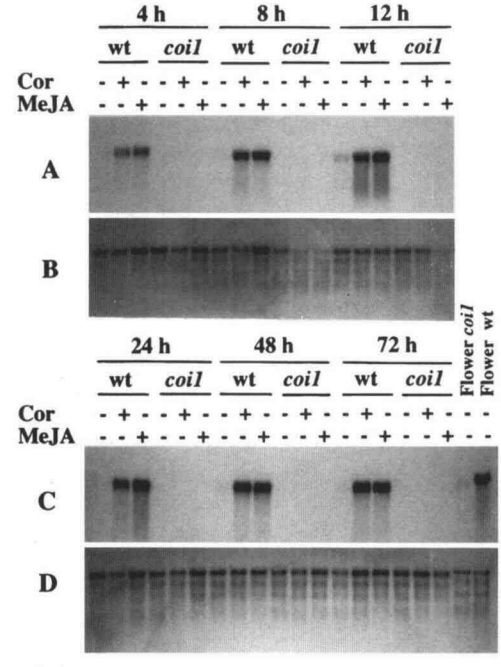

Figure 4. Ten-day-old seedlings of wild-type Arabidopsis (wt) and *coi1* were transferred to MS plates containing 1 μM coronatine (Cor) or 10 μM MeJA or neither compound, as control. Presence or absence of Cor or MeJA is indicated by "+" and "−," respectively. Plates were incubated for 4, 8, or 12 h (A), or 24, 48, or 72 h (C), after which total RNA (approximately 10 μg) was extracted and separated by gel electrophoresis, transferred to nylon membrane, and hybridized to a probe for the Arabidopsis VSP gene as described. An approximately 1.2-kb band was induced by coronatine or MeJA in wild-type seedlings, but it was not detected in *coi1* seedlings (A and C). A band of similar size present at higher levels in wild-type flowers was present in *coi1* flowers at very low levels (C). Similar amounts of the same RNA preparation corresponding to the treatments in A and C were probed with a tobacco ubiquitin gene (B and D, respectively).

DISCUSSION

We report here that the approximately 29-kD coronatine-induced protein is immunologically related to the MeJA-induced protein and to the approximately 29-kD protein constitutively present in wild-type flowers. The coronatine-induced protein and the approximately 29-kD flower protein were recognized by antibodies raised against VSPs from soybean (Wittenbach, 1983; Franceschi and Grimes, 1991) and poplar (van Cleve and Apel, 1993) (Fig. 1). This protein was present at high levels in flowers and siliques, but could not be detected in seeds, seedlings, and mature leaves of wild-type Arabidopsis (Fig. 2), a pattern of expression similar to that of the soybean VSPs (Staswick, 1990).

Coronatine and MeJA induced the accumulation of transcripts corresponding to the gene for the Arabidopsis 27-kD VSP precursor (Höfte et al., 1993) in wild-type seedlings but not in *coi1* seedlings (Fig. 4). Moreover, transcripts accumulated in wild-type flowers but not in *coi1* flowers. Thus, the expression of the Arabidopsis VSP gene, which has homology to the soybean and tomato VSP genes (Höfte et al., 1993), is similar to that of the approximately 29-kD protein. In wild-type plants, both are at low levels in seedlings and at high levels in flowers or in seedling tissues treated with coronatine or MeJA; in *coi1* plants, neither transcript nor protein is at a high level in flowers or in seedlings treated with coronatine or MeJA or in untreated seedlings. This strongly suggests that the approximately 29-kD protein detected in immunoblots is likely to be encoded by the gene for the Arabidopsis 27-kD VSP precursor. Several attempts to micro-sequence this protein were unsuccessful, apparently because the amino terminus of the protein was blocked. Taken together, the results suggest that the coronatine/MeJA-induced protein in Arabidopsis is a VSP-like protein that is regulated by *COI1*.

Although the Arabidopsis VSP-like protein cross-reacted with the poplar VSP antiserum, and the poplar VSP was detected by the antiserum raised against the Arabidopsis coronatine-induced protein (not shown), no DNA or protein homology was observed between these proteins. A common feature among VSPs is that they are glycosylated proteins. It is possible that the poplar antiserum detected glycosylation sites in the Arabidopsis protein.

MeJA-induced proteins in Arabidopsis leaves were also detected by an antiserum raised against a MeJA-inducible VSP from soybean, which was shown to accumulate in smaller quantities in the *jar1* mutant than in wild-type plants (Staswick et al., 1992). Arabidopsis proteins of 29 and 30 kD that cross-reacted with antibodies against soybean VSP are abundant in flowers and accumulate in roots and leaves of plants treated with MeJA (Berger et al., 1995). Moreover, a mRNA encoding a protein homologous to the soybean VSPs was also found at high levels in flowers and buds and in leaves of illuminated Arabidopsis plants treated with MeJA and Suc (Berger et al., 1995). We also find that two proteins of approximately 29 and approximately 31 kD are induced by coronatine or MeJA in seedlings of wild-type Arabidopsis. However, the resolution of the 12% acrylamide gels and western blots presented here does not allow us to clearly distinguish between the two proteins. In Figure 1A, showing the sample from wild-type plants treated with MeJA, it is just possible to see the two bands, one right above the band indicated by the arrow. Both proteins are detected by the antiserum raised against the coronatine-induced protein, but in western blots the two bands could not be resolved unless the blots were developed for a much shorter time. A similar problem occurred with the resolution of two transcripts of the Arabidopsis VSP gene (Fig. 4).

VSPs have been suggested to act as temporary stores of amino acids during pod filling in soybean (Staswick, 1989). However, siliques and seeds developed normally in cross-fertilized *coi1* plants even though the VSP-like protein could not be detected. Apparently, the VSP-like protein is not essential for silique and seed development in Arabidopsis. Alternatively, this protein might function in pollen development, since the only apparent phenotype associated with *coi1* is very low levels of this protein and male sterility. This protein was detected in anthers of wild-type flowers but not in anthers of the *coi1* flowers (not shown). It will therefore be of interest to examine the phenotype of plants transgenic for antisense constructs of the VSP gene.

We have shown that two proteins induced by MeJA, the VSP-like protein and LOX, were not induced in the *coi1* mutant. In *coi1*, LOX was detected in seedlings at reduced levels but at normal levels in flowers, whereas the VSP-like protein was not detected in seedlings and was present in flowers only at very low levels. This suggests that the developmental expression of LOX is affected by *coi1* in seedlings but not in flowers. These results also suggest the possibility that the gene for the VSP-like protein is in fact *COI1*. However, this gene is likely not to be *COI1* because a yeast artificial chromosome containing the *COI1* locus (J.G. Turner, unpublished data) did not hybridize to the DNA probe corresponding to the Arabidopsis VSP (not shown).

The LOXA and VSP genes in soybean are regulated by MeJA and are modulated similarly by Suc and phosphates (Mason et al., 1992; DeWald et al., 1994; Sadka et al., 1994). The Arabidopsis VSP gene seems to be regulated in a similar way (Berger et al., 1995), which might suggest that *cis*-acting elements responsive to MeJA in the Arabidopsis VSP promoter cannot be activated in *coi1*, perhaps because specific regulatory proteins are absent or inactive in this mutant.

In *coi1* flowers a basal level of the approximately 29-kD protein and the mRNA was detected, indicating that the developmental expression of this protein in flowers is regulated differently from that in vegetative tissue, which seems to be entirely dependent on *COI1*. The characterization of the promoter region of the Arabidopsis VSP gene will therefore be necessary to identify elements controlling the expression of this gene under different environmental stimuli.

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