

COS1: An *Arabidopsis coronatine insensitive1* Suppressor Essential for Regulation of Jasmonate-Mediated Plant Defense and Senescence

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The *Arabidopsis thaliana* CORONATINE INSENSITIVE1 (COI1) gene encodes an F-box protein to assemble SCF^{COI1} complexes essential for response to jasmonates (JAs), which are a family of plant signaling molecules required for many essential functions, including plant defense and reproduction. To better understand the molecular basis of JA action, we screened for suppressors of *coi1* and isolated a *coi1 suppressor1* (*cos1*) mutant. The *cos1* mutation restores the *coi1*-related phenotypes, including defects in JA sensitivity, senescence, and plant defense responses. The *COS1* gene was cloned through a map-based approach and found to encode lumazine synthase, a key component in the riboflavin pathway that is essential for diverse yet critical cellular processes. We demonstrated a novel function for the riboflavin pathway that acts downstream of *COI1* in the JA signaling pathway and is required for suppression of the *COI1*-mediated root growth, senescence, and plant defense.

INTRODUCTION

Jasmonates (JAs), which include jasmonic acid and its cyclopentane derivatives as well as cyclopentenones (Reymond and Farmer, 1998), are synthesized from the octadecanoid/hexadecanoid pathways and widely distributed throughout the plant kingdom. JAs modulate the expression of numerous genes (Reymond et al., 2000; Schenk et al., 2000; Sasaki et al., 2001; Feng et al., 2003) and mediate responses to stress, wounding, insect attack, pathogen infection, and UV damage (Staswick, 1992; McConn et al., 1997; Wasternack and Parthier, 1997; Reymond and Farmer, 1998; Farmer, 2001). They also play pivotal roles in reproduction (Feys et al., 1994; McConn and Browse, 1996; Xie et al., 1998) and regulate many other plant developmental processes (Creelman and Mullet, 1997). Without JA, *Arabidopsis thaliana* plants, such as the JA biosynthetic mutants *fad* (McConn and Browse, 1996), *opr3/dd1* (Sanders et al., 2000; Stintzi and Browse, 2000; Stintzi et al., 2001), and *aos* (Park et al., 2002), are unable to generate viable pollen and therefore fail to complete their life cycle. Without JA signaling or JA adenylation, *Arabidopsis* plants, including *jar1* (Staswick et al., 1992, 1998), *coronatine insensitive1* (*coi1*) (Feys et al., 1994), and *jln* (Berger et al., 1996), are unresponsive to JA-inhibitory root growth, unable to accumulate JA-inducible genes, and susceptible to pest attack and pathogen infection.

The *coi1* mutation defined a key regulator in the JA signaling pathway. The recessive *coi1* mutants fail to respond to JA and coronatine, a phytotoxin structurally similar to jasmonic acid (Feys et al., 1994), displaying defects in JA-regulated gene expression, exhibiting male sterility, and showing susceptibility to insect attack and pathogen infection (Feys et al., 1994; McConn et al., 1997; Vijayan et al., 1998; Reymond et al., 2000; Stintzi et al., 2001; Feng et al., 2003). The *COI1* gene encodes a 592-amino acid protein containing an F-box motif and 16 leucine-rich repeat sequences (Xie et al., 1998), which interact with *Arabidopsis* CULLIN1, RBX1, and Skp1-like proteins ASK1 or ASK2 to assemble SCF^{COI1} complexes in planta (Xu et al., 2002). SCF^{COI1} is assumed to regulate the abundance of the substrate proteins, which may suppress a set of transcription factors and/or affect the expression of appropriate target genes essential for JA responses (Xie et al., 1998; Xu et al., 2002).

Genetic screens for suppressors have been used to further investigate gene functions and to dissect signaling transduction pathways. In *Arabidopsis*, suppressor screens have been used to identify genes/mutants functional in many pathways, including the auxin (Cernac et al., 1997), gibberellin (Jacobsen and Olzewski, 1993; Wilson and Somerville, 1995), and abscisic acid (Koorneef et al., 1982) pathways as well as the systemic acquired resistance pathway (Li et al., 1999; Zhang et al., 2003).

To better understand the molecular mechanism via which *COI1* regulates JA responses, we conducted a screen for suppressors of the *coi1* mutant. A *coi1 suppressor1* (*cos1*) recessive mutant was identified and found to regain wild-type-like phenotypes of JA-sensitive root elongation, gene expression, senescence, and defense response in the *coi1* background. The *COS1* gene was cloned using a map-based approach and

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found to encode lumazine synthase, a key component in the riboflavin pathway that is essential for diverse yet critical cellular processes. The riboflavin pathway appears to act downstream of *COI1* and to be required for suppression of the *COI1*-mediated root growth, senescence, and plant defense.

RESULTS

Genetic Screening for the *coi1* Suppressors

Because null mutant alleles of *COI1* are male sterile (Feys et al., 1994; Xie et al., 1998) and unsuitable for suppressor screening, we isolated the leaky allele *coi1-2* (Xu et al., 2002), which is resistant to JA but partially fertile and able to produce a small quantity of seeds. The *coi1-2* seeds were mutagenized with ethyl methanesulfonate (EMS), and the M2 seeds harvested from ~20,000 M1 EMS-mutagenized *coi1-2* plants were then used in a screen for *coi1* suppressors based on the suppression of JA resistance in the elongating root of *coi1-2*. Approximately 100,000 M2 seeds were germinated on plant growth medium containing 25 μ M methyl JA (MeJA) that inhibits root growth in wild-type seedlings but not in *coi1-2* seedlings.

Fourteen seedlings were isolated from the mutagenized *coi1-2* M2 population because they regained the JA-sensitive phenotype displaying short roots and stunted growth when grown on medium containing MeJA. These fourteen plants were therefore selected as suppressor candidates of *coi1*. Each suppressor candidate was backcrossed to the *coi1-2* plant, and all the F1 plants were found to be resistant to MeJA, demonstrating that they are all recessive mutations. Among the fourteen F2 populations, four F2 progeny clearly showed 3:1 segregation of JA resistance/sensitivity, indicating that the restoration of JA inhibitory root growth in each of these four mutants results from a single recessive mutant locus. Genetic crosses between these four lines demonstrate that they are mutated at different loci because all the F1 progeny show resistance to MeJA. One locus, which is named *cos1*, was chosen for further studies because of the strong suppression phenotypes (Figure 1A). This *cos1 coi1-2* mutant (homozygous for both *cos1* and *coi1-2* mutations) was backcrossed to *coi1-2* four times to segregate away other potential mutations.

Suppression of *cos1* on *coi1*-Mediated JA Insensitivity

The basis for the initial screen for *coi1* suppressors was the suppression of JA insensitivity in the elongating *coi1-2* root. To further confirm the effect of the *cos1* mutation, we measured the root length of seedlings grown on MS medium supplemented with various concentrations of MeJA (0, 1, 5, and 25 μ M). As expected, the *coi1-2* is resistant to JA (Figures 1A and 1B). However, root growth of the *cos1 coi1-2* seedlings was inhibited by MeJA (Figures 1A and 1B), demonstrating that the *cos1* mutation fully suppresses *coi1*-mediated JA insensitivity. In contrast with JA-sensitive root growth rescued by *cos1* in *coi1-2*, the *cos1* mutation was unable to restore the male fertility defect of *coi1-2* plants (data not shown).

Restoration of JA-Inducible Gene Expression in the *coi1* Mutant by *cos1*

When assayed for JA-inducible expression of Arabidopsis *VEGETATIVE STORAGE PROTEIN* (*AtVSP*) (Benedetti et al., 1995) and *LIPOXYGENASE2* (*LOX2*) (Bell et al., 1995), the *cos1* mutation is able to restore defect of the JA-inducible gene expression in *coi1-2*. Figure 1C shows that the levels of *AtVSP* and *LOX2* transcripts upon JA induction are higher in *cos1 coi1-2* than those in *coi1-2*.

Mutations in *COI1* were found to cause a defect in JA-inducible expression of senescence-associated genes (Y. He et al., 2002; S. Xiao and D. Xie, unpublished data). To investigate if the *cos1* mutation can restore the defect in JA-inducible expression of senescence marker genes in *coi1-2*, the rosette leaves excised from 20-d-old plants were treated with MeJA for 48 h and then used for RNA extraction. A senescence marker gene, *SEN4* (Nam, 1997), was used as a probe in RNA gel blot hybridization. As shown in Figure 1D, *SEN4* transcript is highly accumulated in the wild type upon JA induction (Figure 1D, lane 2); however, the *SEN4* expression upon JA induction was severely reduced in the *coi1-2* mutant (Figure 1D, lane 4), consistent with previous observations that mutations in *COI1* lead to defect of JA-inducible senescence-associated gene expression. Interestingly, *cos1 coi1-2* highly accumulated *SEN4* transcript even without JA induction (Figure 1D, lanes 5 and 6), suggesting that the *cos1* mutation constantly suppresses the defect of JA-inducible expression of senescence-associated genes in the *coi1* background. When assayed for expression of other senescence marker genes using RT-PCR, *cos1* also was found to constantly suppress the *coi1* mutation (Figure 1E; data not shown). For example, the *cos1 coi1-2* (Figure 1E, lanes 5 and 6), but not *coi1-2* (Figure 1E, lanes 3 and 4), constantly accumulated the senescence-associated gene *SAG12* that is believed to be a good marker of senescence (Weaver and Amasino, 2001) (Figure 1E).

Restoration of JA-Dependent Senescence in the *coi1* Mutant by *cos1*

JA was found to induce leaf senescence in wild-type Arabidopsis but not in the JA-insensitive mutant *coi1* plant (Y. He et al., 2002). Having shown the suppression of *cos1* on the defect of JA-inducible expression of senescence genes, we attempted to investigate whether *cos1* can rescue the defect of the age-dependent expression of senescence genes. The soil-grown *coi1-2*, *cos1 coi1-2*, and wild-type plants at various developmental stages were harvested for RNA extraction and then used in RNA gel blot analysis with the senescence marker genes as probes. As shown in Figure 2A, the *SEN4* transcript was found not to express in the 20-d-old wild type or *coi1-2* plants; with respect to the 30-d-old plants, a high level of *SEN4* expression was detected in the wild type but not in the *coi1-2* mutant, suggesting that mutations in *COI1* affect the age-dependent senescence. Interestingly, the *cos1 coi1-2* plants constantly accumulated a high level of *SEN4* transcript as indicated at the age of 20 and 30 d (Figure 2A), suggesting that the *cos1* mutation continuously suppresses the defect of age-dependent expression of senescence genes in *coi1-2*. Similar conclusion was

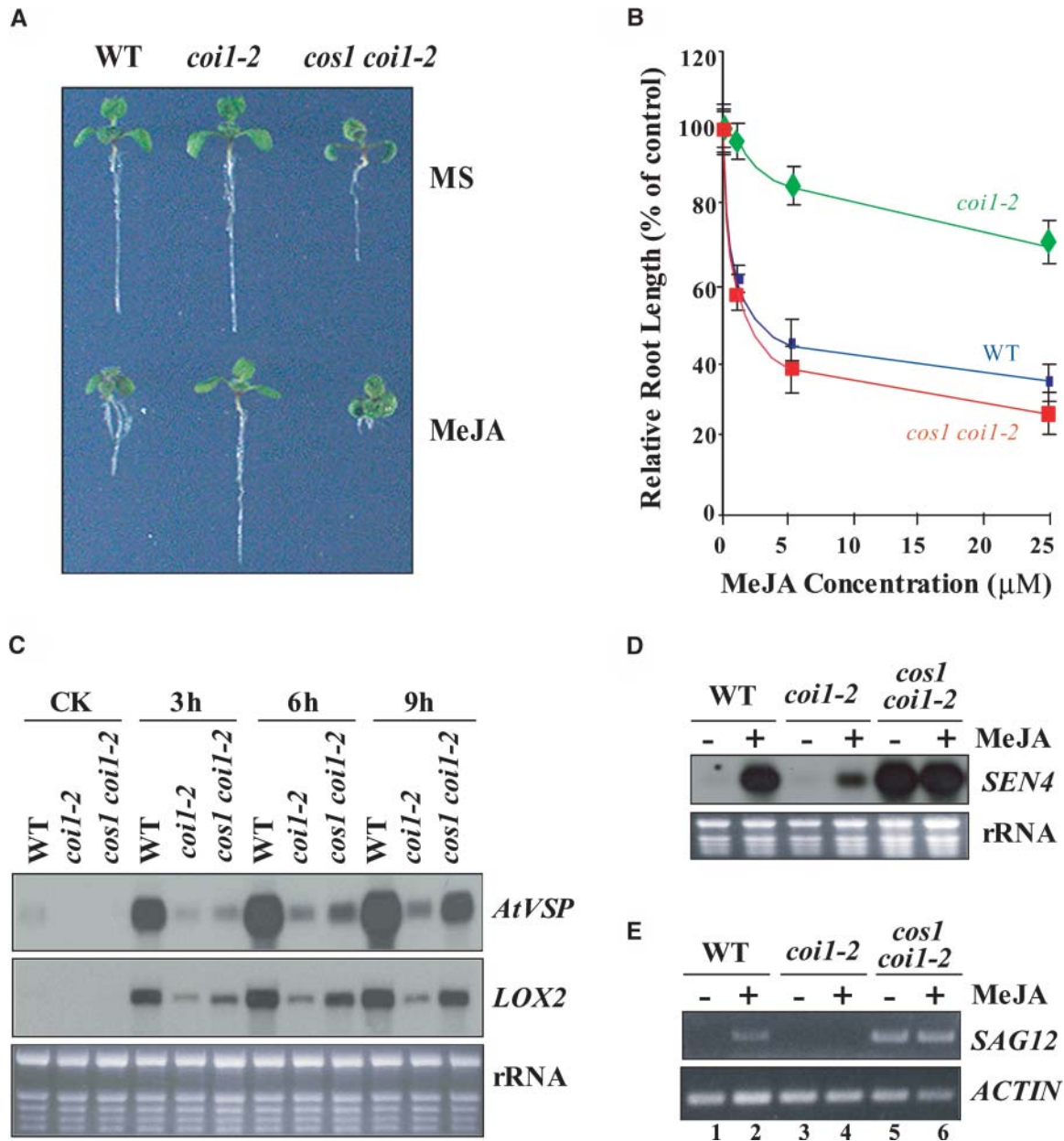


Figure 1. *cos1* Restores the Wild-Type-like JA Sensitivity and JA-Inducible Gene Expression in *coil-2*.

(A) Phenotypes of 10-d-old wild type (ecotype Columbia), *coil-2*, and *cos1 coil-2* grown on MS medium without (MS) or with 10 μ M MeJA.

(B) MeJA dose-response curve of root growth. Root length of the seedlings grown on MS containing 1, 5, or 25 μ M MeJA is expressed as a percentage of root length on MS (control). The experiment was repeated five times. Error bars represent SE ($n > 15$).

(C) RNA gel blotting of the JA-inducible gene *AtVSP* and *LOX2* in 20-d-old seedlings untreated (CK) or treated with 100 μ M MeJA for 3, 6, and 9 h. Ethidium bromide staining of rRNAs shown at bottom indicates the loading amount of total RNA on the gel.

(D) RNA gel blotting of JA-inducible senescence marker gene *SEN4* in plants treated with MeJA (+) or water (-) for 48 h.

(E) The total RNA described in (D) was used in RT-PCR to examine the expression of the senescence-associated gene *SAG12*. An *ACTIN* fragment was amplified as a control.

obtained when other senescence marker genes were examined (data not shown).

Senescence also was assessed by measuring chlorophyll content, a typical senescence-associated physiological marker, which is known to decline with the progression of age-dependent

senescence (Yoshida et al., 2002). The rosette leaves of 20-d-old plants grown in soil were harvested and used in the chlorophyll content measurement. As shown in Figure 2B, the relative chlorophyll content of the *coil-2* mutant (100%) is higher than that of the wild type (90%). The chlorophyll content in *cos1 coil-2*

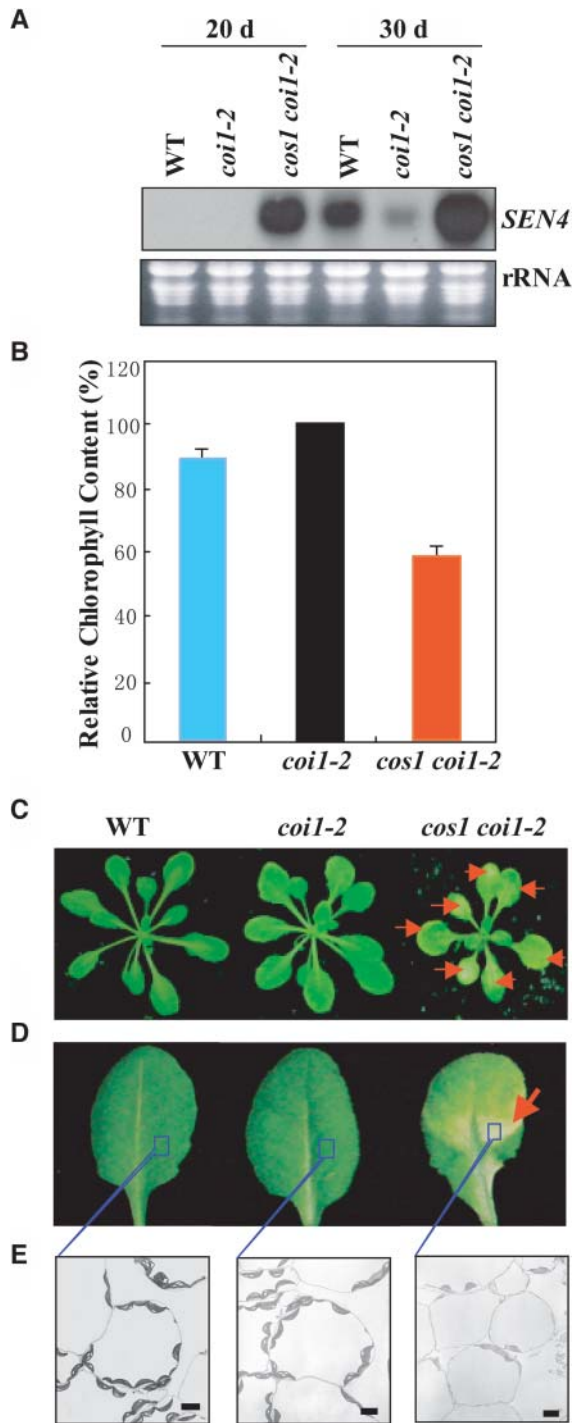


Figure 2. The *cos1* Mutation Suppresses the Defect of Senescence in *coi1-2*.

(A) The wild-type, *coi1-2*, and *cos1 coi1-2* plants grown in soil for 20 or 30 d were harvested for extraction of total RNAs used in RNA gel blot analysis with the senescence marker gene *SEN4* as a probe.

(B) Chlorophyll content of leaves from the plants grown in soil for 20 d. The chlorophyll content in *coi1-2* was set to 100%, and the relative chlorophyll content in the wild type and *cos1 coi1-2* was calculated

is severely reduced (60%), suggesting that *cos1* severely suppresses the defect of leaf senescence in *coi1-2*.

The leaves of Arabidopsis wild-type seedlings treated with JA displayed visible yellowing, which is one of the striking precocious symptoms associated with leaf senescence (Y. He et al., 2002). This kind of yellowing is mostly caused by the preferential breakdown of chlorophyll and chloroplasts (Gut et al., 1987). The *cos1 coi1-2* plant was found to constantly display the visible yellowing phenotypes in the leaves, stems, and siliques (Figures 2C and 2D; data not shown). We used electron microscopy to investigate if these visible yellowing phenotypes in *cos1 coi1-2* are caused by a senescence-related preferential breakdown of chloroplasts. As shown in Figure 2E, the chloroplasts within the yellowing leaves from the 20-d-old *coi1-2* plants were significantly broken down, whereas no preferential breakdown of chloroplasts was observed in the wild type and *coi1-2* at similar developmental stages. These results were consistent with the RNA gel blot analysis data that senescence marker genes were expressed in 20-d-old *cos1 coi1-2* but not in the wild type or *coi1-2*. Taking all the data together, we conclude that *cos1* may act downstream of *COI1* and constantly suppresses the defect of the JA-dependent senescence in *coi1-2*.

Restoration of the JA-Regulated Defense Response in the *coi1* Mutant by *cos1*

The *coi1* mutations previously were found to cause loss of resistance against insects and pathogens in Arabidopsis (McConn et al., 1997; Vijayan et al., 1998; Stintzi et al., 2001). To investigate whether the *cos1* mutation restores the defense responses in *coi1-2*, we inoculated the fungus pathogen (*Botrytis cinerea*) onto the wild type, *coi1-2*, and *cos1 coi1-2* (Figure 3). By 6 d after inoculation, 60% of *coi1-2* plants had died, whereas the *cos1 coi1-2* plants showed resistance to fungal infection and <10% of *cos1 coi1-2* had died, which was similar to the wild type (7%). By 9 d after inoculation, all of the *coi1-2* plants (100%) were dead; however, the *cos1 coi1-2* has only ~35% of death rate similar to that of the wild type (Figures 3A and 3B). These results clearly demonstrate that the *cos1* mutation restores the resistance against pathogens in *coi1-2*.

The Effect of *cos1* on transport inhibitor response1

In addition to *COI1*, the *JAR1* gene was the only cloned gene in the signaling pathway defined by the JA-insensitive mutants (Staswick et al., 2002). To examine the effects of the *cos1* mutation in the JA-insensitive mutant *jar1* (Staswick, 1992; Staswick et al., 1998), we attempted to generate the *cos1 jar1*

accordingly. Experiments were repeated five times. Error bars represent SE ($n > 10$).

(C) and **(D)** Phenotypes of the wild type, *coi1-2*, and *cos1 coi1-2* grown in soil for 20 d. The red arrows indicate the senescence-associated yellowing phenotypes in resettle leaves.

(E) Electronic microscopic examination on chloroplasts of resettle leaves described in **(D)**. Scale bars = 5 μ m.

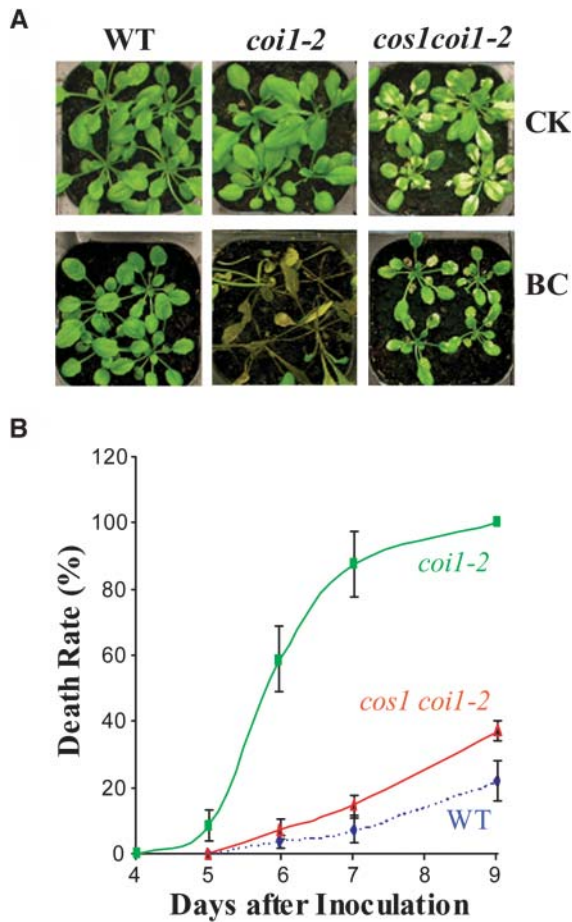


Figure 3. The *cos1* Mutation Restores the Defect of Disease Resistance in *coi1-2*.

(A) Fourteen-day-old plants were inoculated with the fungus pathogen *B. cinerea* (BC) or with water as control (CK) and photographed by 9 d after inoculation.

(B) The death rate for each line (WT, *coi1-2*, and *cos1 coi1-2*) was recorded at 4, 5, 6, 7, or 9 d after inoculation. The experiment was repeated three times. Error bars represent SE ($n > 40$).

double mutant through sequence verification of the *jar1-1* allele among *cos1* homozygous plants, which were identified from F2 progeny of a cross between *jar1-1* and *cos1 coi1-2* based on *cos1*-conferred lesion-like yellow spots (Figures 2C and 2D). Surprisingly, we were unable to identify the *cos1 jar1-1* mutants because none of the *cos1* homozygous F2 plants contained the *jar1-1* allele, though >100 plants were sequence verified. These data indicate that the *cos1* locus has tight genetic linkage with the *JAR1* locus, which makes it difficult to generate the *cos1 jar1-1* double mutants. Similarly, we also failed to generate *cos1* in the wild-type *COI1* or *coi1-1* null mutant background through genetic crossing (data not shown), suggesting a tight linkage of *cos1* with *COI1* that is physically close to the *JAR1* gene (Arabidopsis genome sequencing project).

We then attempted to investigate the role for the *cos1* mutation in *transport inhibitor response1* (*tir1*), an auxin-insensitive

mutant, which defines the F-box protein TIR1 that is most closely related to COI1 (Ruegger et al., 1998; Xie et al., 1998). The *tir1-1 coi1-2* mutant was identified through sequence verification of the *tir1-1* allele among the *cos1 coi1-2* homozygous plants from F2 progeny of a cross between *tir1-1* and *cos1 coi1-2*. When assayed for auxin inhibitory root elongation, the *tir1-1* and *tir1-1 coi1-2* mutant seedlings are resistant to the synthetic auxin 2,4-D as expected, and the *tir1-1 coi1-2* mutant seedlings exhibit auxin resistance similar to the *tir1-1* or *tir1-1 coi1-2* mutant seedlings (Figure 4). These data indicate that the *cos1* mutation is unable to alter the auxin responses in the *tir1-1* mutant and therefore has no suppression on *tir1*.

Map-Based Cloning of *COS1*

To genetically map the *COS1* locus, we identified a Landsberg erecta JA-insensitive mutant allele *coi1-12*, which contains a single amino acid replacement from Phe 359 to Lys in COI1 (D. Xie, unpublished data) and has a polymorphic genetic background from the Columbia *cos1 coi1-2* mutant. We made the genetic crossing between the *coi1-12* and *cos1 coi1-2* plants and subsequently screened for their F2 progeny (homozygous for both *cos1* and *coi1*) those that are sensitive to JA. Based on the linkage analysis among molecular markers and the *cos1*-conferred JA-sensitive phenotype using these JA-sensitive F2 progeny, we localized the *COS1* locus on chromosome II between two amplified fragment length polymorphism (AFLP) markers, A18000 and A18300. As shown in Figure 5A, the *COS1* locus was mapped between the *JAR1* and *COI1* loci, consistent with our previous observation that *cos1* has tight genetic linkage with *JAR1* and *COI1*. Further mapping using a cleaved amplified polymorphic sequence marker, C18150 (one recombinant), and AFLP marker A18250 (four recombinants) placed *COS1* on a BAC, F6E13, which contains a 110-kb Arabidopsis genomic insert (Figure 5B).

The *COS1* gene was finally localized onto an ~4-kb *Bam*HI fragment (B4) by functional complementation with the subcloned

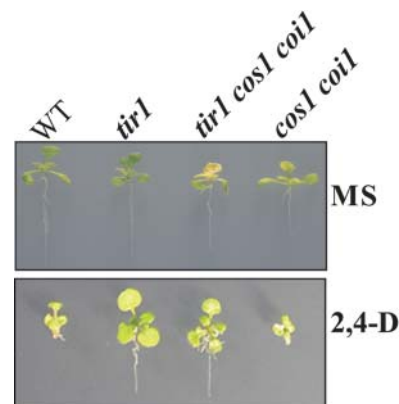


Figure 4. *cos1* Is Not a Suppressor of the *tir1* Mutation.

Phenotypes of 10-d-old wild type (ecotype Columbia), *tir1-1*, *tir1-1 coi1-2*, and *cos1 coi1-2* grown on MS medium or MS containing 0.1 μ M 2,4-D.

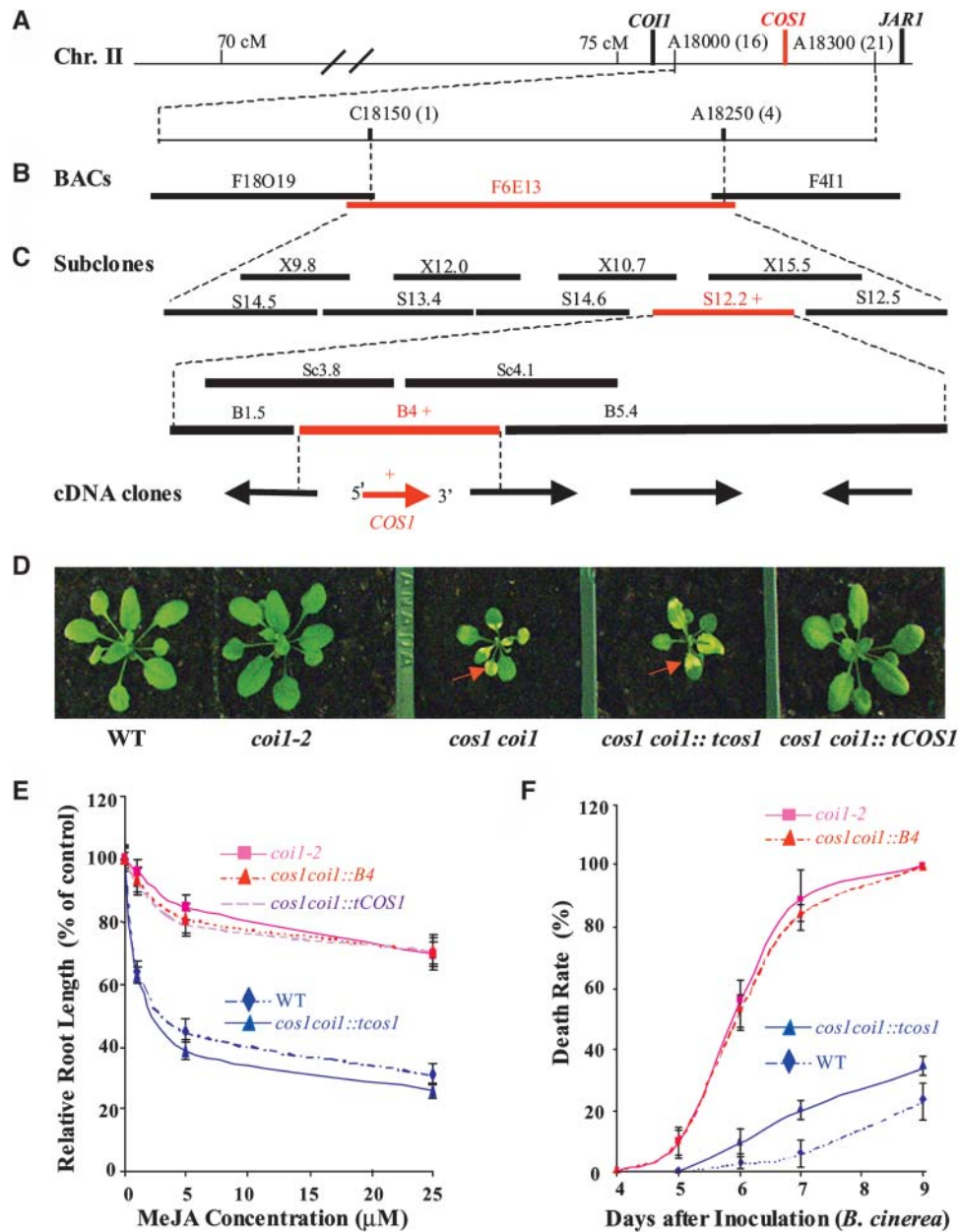


Figure 5. Map-Based Cloning of *COS1*.

(A) to (C) The *COS1* locus is located on chromosome II between A18000 (16 heterozygous recombinants) and A18300 (21 recombinants), further mapped onto a single BAC (F6E13), and was finally placed onto an ~ 4 -kb genomic fragment (B4) corresponding to *COS1* by a complementation test with subcloned BAC inserts. Restriction endonucleases used to generate subclones (B, *Bam*HI; Sc, *Sac*I; S, *Spe*I; X, *Xba*I) are shown. The fragments marked with red and plus signs can complement *cos1*.

(D) Phenotype of the 20-d-old plants of the wild type, *coi-2*, *cos1 coi-2*, and *cos1 coi-2* transgenic for either the mutant *cos1* cDNA (*cos1 coi-2::tcos1*) or the wild-type *COS1* cDNA (*cos1 coi-2::tCOS1*). The red arrows indicate the senescence-associated yellowing phenotypes.

(E) *COS1* complements *cos1*-conferred JA sensitivity. Root length of the indicated seedlings grown on MS containing MeJA (1, 5, or 25 μM) is expressed as a percentage of root length on MS medium. The experiment was repeated five times. Error bars represent SE ($n > 15$).

(F) *COS1* complements *cos1*-conferred disease resistance in *coi-2*. The death rate for each indicated line was recorded at various days after inoculation with *B. cinerea*. The experiment was repeated three times. Error bars represent SE ($n > 40$).

F6E13 fragments (Figure 5C). The B4 fragment identifies a single 684-bp full-length cDNA (Figure 5C). Thus, the 684-bp cDNA is the *COS1* gene. Further analysis of the *cos1 coi1-2* plants transgenic for either the B4 fragment or the *COS1* cDNA that was 5' myc-tagged under the control of the 35S promoter of *Cauliflower mosaic virus* (CaMV 35S) (referred to as *tCOS1*) demonstrates that both the B4 fragment and the *COS1* cDNA can complement the *cos1*-conferred senescence (Figure 5D; data not shown), JA sensitivity (Figure 5E), and defense response (Figure 5F).

The corresponding sequence from the *cos1* mutation deviated from that of the wild type by a single nucleotide change (G238A), G to A, at position + 238 relative to the translation start codon of the *COS1* genomic DNA (Figure 6). This single nucleotide, G238, is the first nucleotide of the second exon and therefore is crucial for RNA splicing. The G238A point mutation abolished the splicing recognition sequence (from TA/GG to TA/AG), and the TA/GC sequence located 18 bp downstream of G238A mutation serves as a new RNA splicing recognition site resulting in an 18-nucleotide deletion in the mutant *cos1* cDNA (Figure 6),

which was confirmed by sequence verification of the RT-PCR-amplified *cos1* mutant cDNA.

To investigate whether the 18-bp deletion in the mutant *cos1* mRNA affects the protein expression of the mutant *cos1*, we made transgenic plants expressing the mutant *cos1* cDNA that was 5' myc-tagged under the control of the CaMV 35S constitutive promoter (referred to as *tcos1*). Protein gel blotting with α -Myc antibody shows that the *tcos1*-encoded mutant protein was accumulated at a level similar to the *tCOS1*-encoded protein in the transgenic lines (data not shown); however, *tCOS1* is able to fully complement the *cos1* mutation, whereas *tcos1* could not (Figures 5D to 5F; data not shown). The result indicates that the 18-bp deletion in *cos1* does not disrupt *COS1* protein stability; however, the six amino acids encoded by the deleted 18 nucleotides are crucial for *COS1* function.

Sequence analysis reveals that the *COS1* gene encodes lumazine synthase, which was previously shown to functionally complement the bacterial lumazine synthase-deficient mutant (Jordan et al., 1999) and shares high identity at amino acid levels with lumazine synthases from bacteria (~53%), yeast (~57%),

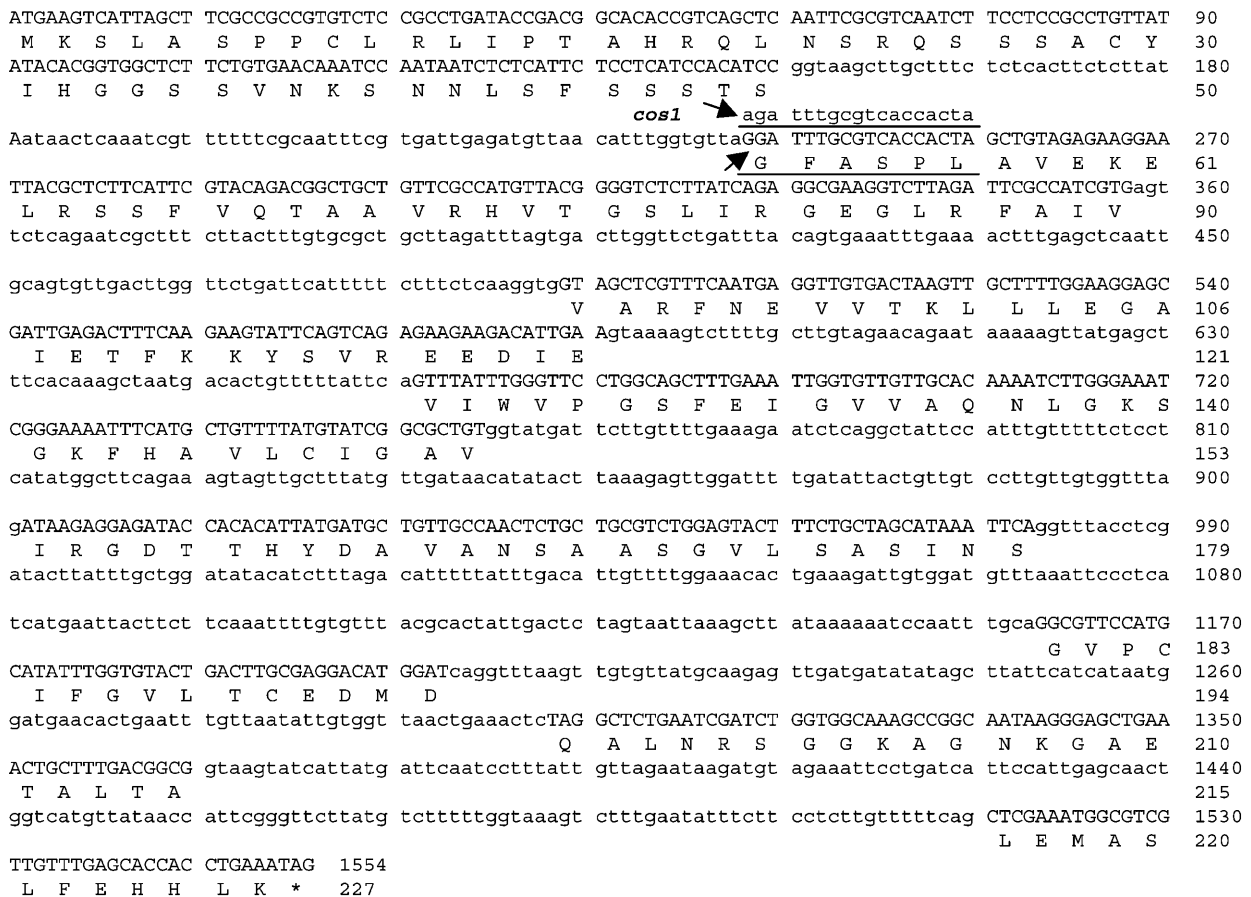


Figure 6. Sequence Comparison of the Wild Type and Mutant *COS1* Gene.

DNA sequence of exons is indicated with capital letters; the *cos1* mutation G238A is indicated by arrows; the 18-bp DNA sequence absent from the *cos1* mutant cDNA is underlined; and the six amino acids encoded by the 18-bp DNA is also underlined.

and other plants, including spinach (*Spinacia oleracea*) and tobacco (*Nicotiana tabacum*; >70%) (Jordan et al., 1999; Persson et al., 1999).

DISCUSSION

Null mutations in *COI1* abolish all the JA responses exhibiting resistance to JA inhibition of plant growth, defect in pollen fertility, reduction in senescence, and susceptibility to pathogen infection and insect attack. The *COI1* protein previously was found to assemble into SCF^{COI1} complexes that were speculated to recruit substrate proteins, which may function as suppressors to negatively regulate the expression of appropriate downstream genes essential for JA responses (Xie et al., 1998; Xu et al., 2002). To further dissect *COI1*-mediated JA responses and to gain insight into the mechanism of *COI1* function, we have isolated a *coi1* suppressor mutant, here referred as to *cos1*. The *cos1* mutation can restore the *coi1*-related phenotypes, including defects in JA sensitivity, senescence, and plant defense responses in the *coi1* background. However, the *cos1* mutation failed to restore the male fertility defect of the *coi1-2* plants, which further dissects the JA signal transduction pathway and indicates that JA-mediated pollen development is regulated by other component(s) independent of *COS1*. The *cos1* mutation also failed to restore the auxin insensitivity in the *tir1-1* mutant, demonstrating that *cos1* is not a suppressor of *tir1*.

The *COS1* gene was cloned via a map-based approach and found to encode lumazine synthase. Lumazine synthase catalyzes the penultimate step in biosynthesis of riboflavin (vitamin B2) in plants, fungi, and microorganisms (Persson et al., 1999), which is a key component in the riboflavin pathway essential for diverse yet critical cellular processes. Riboflavin is essential for the basic metabolism and serves as a precursor of coenzymes riboflavin monophosphate and flavin adenine dinucleotide; the riboflavin pathway-derived flavocoenzymes were found to function as indispensable redox cofactors in all cells and also serve

a variety of other roles, such as DNA photorepair, light sensing, and bioluminescence (Muller, 1992; Sancar, 1994; Ahmad et al., 1998; Christie et al., 1998; Vande Berg and Sancar, 1998; Briggs and Huala, 1999; Aubert et al., 2000; Briggs and Olney, 2001; Salomon et al., 2001; Q.Y. He et al., 2002). The evidence that *cos1* restores the defects in *coi1-2* demonstrates a novel function for the riboflavin pathway in JA signaling transduction, suggesting that the wild-type *COS1* represents the riboflavin pathway essential for suppression action exerted by unidentified negative regulator(s) (such as SCF^{COI1} substrates) of JA responses.

Because the riboflavin pathway is essential for diverse yet critical cellular processes, a complete abolishment of the riboflavin pathway would cause pleiotrophic phenotypes and probably affect the survival of plants. It is likely that the *cos1* mutant identified in this work is a leaky allele and that the *cos1* mutant protein is partially functional. In the *cos1 coi1-2* mutant plants, the 6-amino acid deletion in the *cos1* mutant protein appears to attenuate the riboflavin pathway but not to completely disrupt this pathway. Therefore, the *cos1* mutation does not affect the plant survival but suppresses the defects in the JA sensitivity, senescence, and plant defense responses in the *coi1-2* mutant background via attenuation of the unidentified negative regulator(s) that require the riboflavin pathway to exert their suppression action.

Here, we propose a working model for the function of the riboflavin pathway in the JA signaling transduction chain. As shown in Figure 7, different SCF^{COI1} substrates (indicated as groups S1 and S2), which will be degraded through SCF^{COI1}-26S proteasome in responsive to JA signaling, may negatively regulate different transcription factors and/or appropriate downstream genes (indicated as two sets of genes, G1 and G2) that mediate their corresponding JA responses (indicated as response I and response II). The first group of substrates (S1) may require the riboflavin pathway (indicated as RIB) to exert their suppression action on the downstream G1 target genes, whereas the action of the second group of substrates (S2) may not require the riboflavin pathway. In wild-type plants (Figure 7,

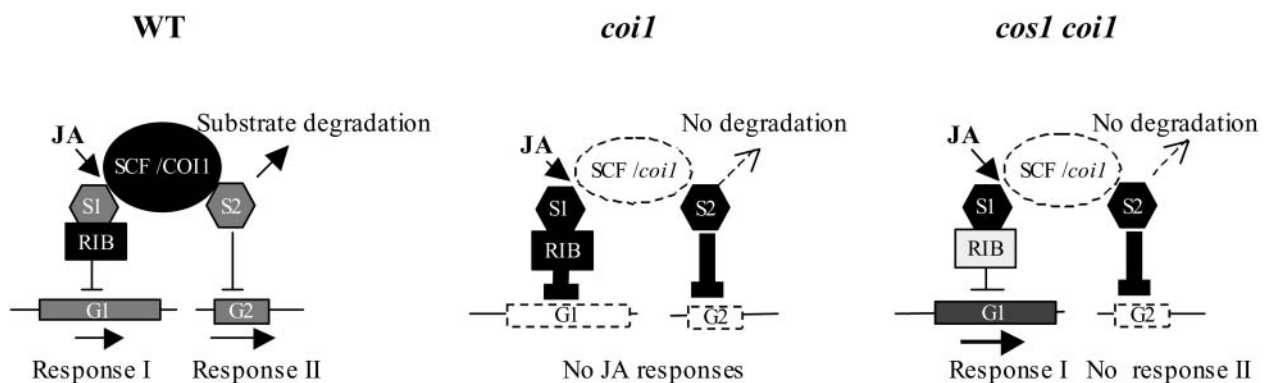


Figure 7. Model of the Function of the Riboflavin Pathway in the JA Signal Transduction Chain.

S1 and S2 represent two groups of substrates that are activated by the JA signals (JA) for ubiquitylation and degradation via SCF^{COI1}-26S proteasome (SCF/COI1). G1 and G2 represent different sets of JA response genes that mediate distinct JA responses (I and II). Arrows indicate positive regulation action; blunted lines indicate negative regulation; the weight of each line indicates the predicted regulation level. The shading, which was used to mark the putative components, indicates abundance of these components. RIB, riboflavin pathway.

left panel), the endogenous JA, and developmental and environmental cues (such as flowering and insect attack) that activate JA signaling, promote the ubiquitylation and degradation of substrate proteins. As a result, the downstream genes are expressed to maintain JA responses. In the *coi1* null mutant plants (Figure 7, middle panel), mutation of *COI1* disrupts SCF^{COI1}, resulting in a high level of accumulation of the SCF^{COI1} substrates, which constantly exert suppression on the downstream genes, leading to loss of all the JA responses. In the *cos1 coi1* double mutants (Figure 7, right panel), the *cos1* mutation attenuates the riboflavin pathway and therefore decreases the suppression action exerted by the S1 substrates and finally activates the expression of their downstream genes (G1), leading to restoration of their corresponding JA responses, including JA sensitivity, senescence, and defense (response I). However, *cos1* is unable to restore fertility in *coi1* because the riboflavin pathway is not required for the suppression exerted by the S2 substrate that suppresses the G2 downstream genes, leading to defect in pollen fertility (response II).

In the *cos1 coi1-2* double mutants, application of exogenous JA was found to cause enhanced inhibition of root growth (response I) and induction of gene expression (Figure 1). This induction of JA response by exogenous JA in the *cos1 coi1-2* double mutants may result from activation of substrate (S1) degradation because *coi1-2* is a leaky mutant allele. Alternatively, JA-responsive G1 genes may be repressed by substrate S1 but also activated by some unknown JA-regulated positive components, probably including dephosphorylated regulator(s) previously proposed (Rojo et al., 1998); the exogenous JA was therefore able to activate the unknown JA-regulated positive components that modulate the expression of JA-responsive G1 genes in the *cos1 coi1-2* double mutants. More experiments will be required to test our working model and explanation. Identification and functional analysis of the SCF^{COI1} substrates will be useful to understanding the novel role for the riboflavin pathway in JA signaling and will be essential for illustrating the molecular mechanism via which JA regulates plant defense and development.

METHODS

Plant Growth Conditions

Seeds were surface sterilized, plated on plant growth medium (MS; Sigma, St. Louis, MO), chilled at 4°C for 3 d, and then transferred to a growth chamber under a 16-h-light (22 to 24°C)/8-h-dark (16 to 19°C) photoperiod. For root length measurement experiments, the seedlings were grown on MS medium supplemented with various concentrations of MeJA (Aldrich, Milwaukee, WI) for 9 d before measurement.

Mutant Screening

The *coi1-2* leaky mutant was identified previously (Xu et al., 2002). The *coi1-2* seeds (~30,000) were mutagenized with 0.3% EMS following routine procedures. About 70% of the mutagenized seeds (referred as to the M1 population) could grow in soil and generate M2 seeds under the growth conditions of a 16-h-light (21 to 23°C)/8-h-dark (16 to 19°C) photoperiod.

M2 Seeds were routinely plated on MS medium containing 25 μM MeJA to screen for mutants sensitive to MeJA exhibiting phenotypes of short root and stunted growth.

Generation of the *tir1-1 cos1 coi1-2* Mutant

The *tir1-1* mutant, which harbors a point mutation from guanine 440 to adenine (g440a) leading to an amino acid replacement from G147 to D (G147D) (Ruegger et al., 1998), was crossed to the *cos1 coi1-2* mutant plant. The *cos1* homozygous plants were identified from the F2 progeny based on *cos1*-conferred lesion-like yellow spots, and all of the *cos1* homozygous plants were also homozygous for the *coi1-2* allele because *cos1* has close genetic linkage with *coi1-2*. These *cos1 coi1-2* homozygous plants were then used in sequence verification of the *tir1-1* g440a mutation; the plants containing the homozygous *tir1-1* allele were the *tir1-1 cos1 coi1-2* mutants. Seeds harvested from the *tir1-1 cos1 coi1-2* mutant plant were used to assay for auxin inhibitory elongation in MS medium containing 0.1 μM 2,4-D.

RNA Gel Blot and RT-PCR Analysis

Specific primers were designed based on their DNA sequences to PCR amplify *AtVSP* (Benedetti et al., 1995), *LOX2* (Bell et al., 1995), and *SEN4* (Nam, 1997) and used as RNA gel blot probes. The probe labeling and RNA gel blot hybridization methods were described previously (Xu et al., 2001).

RT-PCR analysis was performed following routine procedures. The senescence marker gene *SAG12* was amplified with primers 5'-CAG-CTGCGGATGTTGTTG-3' and 5'-CCAATTCTCCCATTTTG-3', and the *ACTIN* gene was amplified with primers 5'-CACCGCTTAACCCGAA-3' and 5'-GTGAGGTCACGACCAG-3'.

Total RNA used in Figure 1 was extracted from 20-d-old plants that were untreated (CK) or treated with 100 μM MeJA in daytime for 3, 6, and 9 h (Figure 1C) or for 48 h (Figures 1D and 1E).

Molecular Markers

The cleaved amplified polymorphic sequence marker C18150 reveals a DNA polymorphism between Columbia and Landsberg *erecta* when Sau3AI is used to digest a PCR fragment amplified with the primers 5'-CGTTACAAGATCTGATAATT-3' and 5'-TCTCGCCATTAGCAGGTTA-3'. The AFLP markers A18000, A18250, and A18300 reveal a polymorphism (difference in size of 15, 29, and 58 bp, respectively) when the PCR fragment was amplified with their corresponding primers (A18000, 5'-AGAGCTATGTTGTGCCTGATA-3' and 5'-ATCCTCCATCGATGCGGCT-3'; A18250, 5'-TTGGGGATTGATAACGACAA-3' and 5'-ACCAACCATTCTAAAGTGA-3'; A18300, 5'-TATGCATATGCATTGAGCGTAA-3' and 5'-TCTTTTATACAAGAAACCTCAACCTT-3').

Complementation Test

The F6E13 inserts were digested with the indicated restriction enzymes. Each digested fragment was recovered and cloned into *pCambia 1300* vector. The mutant and wild-type *COS1* cDNAs were amplified with the primers 5'-ATGAAGTCATTAGCTTCGCC-3' and 5'-CTATTCAGGTGG-TGCTCAA-3' using RT-PCR from RNAs isolated from *cos1 coi1-2* and the wild type, respectively. The cDNA fragment was in-frame tagged with the myc epitope at 5' and driven by the CaMV 35S promoter in the *pMYC2* vector (Xu et al., 2002). The constructs were mobilized into *Agrobacterium tumefaciens* by electroporation and then introduced into *cos1 coi1-2* by in planta vacuum infiltration.

The *cos1 coi1-2* plants transgenic for the mutant *cos1* cDNA, the wild-type *COS1* cDNA, and an ~4-kb genomic fragment containing

COS1 were identified and referred as to *cos1 coi1-2::tcos1*, *cos1 coi1-2::tCOS1*, and *cos1 coi1-2::B4*.

Pathogen Infection

Fourteen-day-old plants were inoculated with the fungus pathogen *Botrytis cinerea* (500,000 spores/mL) or with water as the control, placed in a growth chamber at the appropriate temperature (22°C) and high humidity (~100%) for 2 d to stimulate infection, and then transferred to a growth room under the growth conditions of a 16-h-light (21 to 23°C)/8-h-dark (16 to 19°C) photoperiod. The death rate for each line was recorded at various days after inoculation. In each treatment, at least 40 plants from each line (the wild type, *coi1-2*, or *cos1 coi1-2*) were investigated. The experiment was repeated three times.

Measurement of Chlorophyll and Transmission Electron Microscopy

Chlorophyll was extracted from equal volumes of leaf discs by immersion in 1 mL of N,N-dimethylformamide for 48 h in the dark at 4°C. Absorbance was recorded at 664 and 647 nm, and total chlorophyll concentration was calculated according to methods described previously (Yoshida et al., 2002).

Samples examined by transmission electron microscopy were fixed in 2% paraformaldehyde and 4% glutaraldehyde in 100 mM cacodylate buffer for 3 h and then postfixed with 2% osmium tetroxide in 100 mM sodium cacodylate buffer for 1 h at 4°C. Samples were then dehydrated through a series of 30, 50, 70, 90, and 100% ethanol and finally in propylene oxide. Samples were embedded in 100% Spurr's resin (Electron Microscopy Sciences, Fort Washington, PA) and polymerized at 65°C overnight. Ultrathin sections were cut on a Jung Reichert ultramicrotome and examined with a transmission electron microscope (JEM1010; JEOL, Tokyo, Japan) at 100 kV.

Sequence data from this article have been deposited with the GenBank data libraries under accession number NM_129967.

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