The Plant Cell, Vol. 15, 1083–1094, May 2003, www.plantcell.org © 2003 American Society of Plant Biologists

The COP9 Signalosome Interacts Physically with SCF^{COI1} and Modulates Jasmonate Responses

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The COP9 signalosome (CSN) is an evolutionarily conserved, nucleus-enriched multiprotein complex. CSN plays roles in photomorphogenesis, auxin response, and floral organ formation, possibly via the regulation of ubiquitin-proteasome–mediated protein degradation. *COI1* encodes an F-box protein, which is a subunit of SCF^{COI1} E3 ubiquitin ligase, and is required for jasmonate (JA) responses. Here, we demonstrate using coimmunoprecipitation and gel-filtration analyses that endogenous as well as epitope-tagged COI1 forms SCF^{COI1} and associates directly with CSN in vivo. Like the *coi1-1* mutant, CSN reduction-of-function plants exhibited a JA-insensitive root elongation phenotype and an absence of JA-induced–specific gene expression. Genome expression profile analyses indicated that JA-triggered genome expression is critically dependent on COI1 dosage. More importantly, most of the *COI1*-dependent JA-responsive genes also required CSN function, and CSN abundance was shown to be important for JA responses. Furthermore, we showed that both *COI1* and CSN are essential for modulating the expression of genes in most cellular pathways responsive to JA. Thus, CSN and SCF^{COI1} work together to control genome expression and promote JA responses.

INTRODUCTION

The COP9 signalosome (CSN) was characterized genetically as a repressor of photomorphogenesis in darkness. Ten pleiotropic *COP*/*DET*/*FUS* loci were identified by screening for mutants that showed light-grown phenotypes when grown in complete darkness (Chory, 1993; Deng, 1994; Kwok et al., 1996) and for mutants with purple cotyledons (high level of anthocyanin) in young seedlings or mature seeds (Miséra et al., 1994). To date, molecular and genetic studies have shown that 6 of the 10 loci—*COP9*, *FUS6*/*COP11*, *FUS5*, *FUS4*/*COP8*, *FUS11*, and *FUS12*—encode subunits of the COP9 signalosome, which are CSN8, CSN1, CSN7, CSN4, CSN3, and CSN2, respectively (Chamovitz et al., 1996; Staub et al., 1996; Karniol et al., 1999; Serino et al., 1999, 2003; Peng et al., 2001b).

CSN is conserved in diverse organisms, including mammals, flies, and fission yeast (Seeger et al., 1998; Wei et al., 1998; Freilich et al., 1999; Mundt et al., 1999). Evidently, the function of CSN goes far beyond repressing photomorphogenesis. One of the most recent findings about CSN function is its regulatory role in ubiquitin-proteasome–mediated protein degradation.

The 26S proteasome is a proteolytic machine that degrades proteins conjugated with a multiubiquitin chain and plays a central role in selective proteolysis and the regulation of a wide variety of biological processes (Baumeister et al., 1998; Hershko and Ciechanover, 1998). In the ubiquitin-proteasome–mediated protein degradation pathway, ubiquitin is joined reversibly to target proteins by an isopeptide linkage, a process that requires the action of a series of distinct enzymes, including E1 ubiquitin-activating enzyme, E2 ubiquitin-conjugating enzyme, and E3 ubiquitin ligase (Hochstrasser, 1996). SCF complexes are the largest family of E3 ubiquitin ligases and are composed of four subunits: SKP1; a cullin family member (e.g., CUL1); a small RING finger protein, RBX1/ROC1/HRT1; and an F-box protein. Within this complex, the F-box protein directly binds the substrate through protein–protein interaction domains, the cullin binds the RING finger protein RBX1, together they recruit E2 ubiquitin-conjugating enzyme, and SKP1 helps to link the F-box protein and cullin (Deshaies, 1999). The cullins are modified covalently by NEDD8/RUB1, a ubiquitin-like protein, in a process called neddylation. This modification has been found to stimulate SCF ubiquitin ligase activity in mammalian cells (Furukawa et al., 2000; Podust et al., 2000; Read et al., 2000; Wu et al., 2000; Kawakami et al., 2001) and in plants (del Pozo et al., 1998, 2002; Gray and Estelle, 2000).

CSN has been shown to associate with multiple SCF-type E3 ubiquitin ligases in vivo (Lyapina et al., 2001; Schwechheimer et al., 2001; Zhou et al., 2001; Wang et al., 2003). Besides physical interaction, CSN also harbors deneddylation activity toward the neddylated cullin subunit of SCF complexes, which has been shown in different organisms to be important for SCF ubiquitin ligase activity (Lyapina et al., 2001; Schwechheimer et al., 2001; Zhou et al., 2001; Cope et al., 2002; Yang et al.,

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Article, publication date, and citation information can be found at www.plantcell.org/cgi/doi/10.1105/tpc.010207.

2002). In analyzing Arabidopsis CSN partial loss-of-function lines, it has been found that CSN plays important roles in regulating auxin response and petal and stamen formation, which are mediated at least in part by modulating SCF^{TIR1} and SCF^{UFO} activities, respectively (Schwechheimer et al., 2001; Wang et al., 2003).

Jasmonates (JAs) not only mediate plant defense responses to both mechanical trauma and pathogenesis but also regulate stamen and pollen development (Liechti and Farmer, 2002). Both pathways are dependent on the *COI1* gene. The Arabidopsis null mutant for *COI1*, *coi1-1*, is insensitive to JAs and male sterile (Feys et al., 1994). The *COI1* gene encodes a 66-kD protein with an F-box motif and 16 Leu-rich repeats (Xie et al., 1998). COI1 has been shown to form a functional E3 ubiquitin ligase, SCF^{COI1}, in plants (Devoto et al., 2002; Xu et al., 2002). Thus, SCF^{COI1} is thought to target key regulators of the JA pathway for ubiquitination and subsequent degradation by the 26S proteasome. Initial gene expression profile changes specifically in response to JA and the role of *COI1* have been reported using DNA arrays with 150, 2375, and 2880 genes, respectively (Reymond et al., 2000; Schenk et al., 2000; Sasaki et al., 2001).

Here, we report that CSN and SCF^{COI1} interact physically and are capable of forming a large complex in vivo. We also show that Arabidopsis lines partially deficient in CSN exhibit JA-insensitive phenotypes that are analogous to those of *coi1-1* mutants. Using an EST microarray with 6126 genes, we demonstrate that both *COI1* and CSN are required for JA-responsive genome expression in Arabidopsis and that the regulation of gene expression is critically dependent on COI1 and CSN dosage. Moreover, we reveal that the *COI1* and CSN requirement for expression changes in genes that encode several distinctive cellular pathway components. Our results strongly suggest that CSN and SCF^{COI1} associate with each other in vivo and mediate JA responses collaboratively.

RESULTS

Construction and Expression of the COI1-Flag Fusion Protein in Wild-Type and *coi1-1* Mutant Arabidopsis Plants

As a first step in the biochemical analysis of *COI1*, we constructed a chimeric gene using the 35S promoter of *Cauliflower mosaic virus* to drive the expression of the full-length COI1 protein with three copies of the flag epitope tag at the C terminus (Figure 1A). The chimeric gene was first transformed into wild-type Arabidopsis plants, and T2 progeny carrying a single-locus transgene were selected. The transgene was introduced subsequently into the *coi1-1* mutant (Feys et al., 1994) by genetic crossing. Protein gel blot analysis using polyclonal antibodies against COI1 resulted in the identification of the 66-kD COI1 protein as well as the slightly low-mobility tagged COI1 protein, COI1-flag, in wild-type or *coi1-1* mutant plants carrying the *COI1-flag* transgene (Figure 1B).

Interestingly, the level of the COI1-flag protein was lower in the *coi1-1* mutant background than in the wild-type background, which makes the CF/*coi1* line a reduction-of-function strain for COI1 (Figure 1B). However, the male-sterile phenotype of the *coi1-1* mutant was rescued completely in the CF/*coi1* line (data not shown), suggesting that the low COI1-flag level in the CF/*coi1* line is sufficient to properly regulate stamen and pollen development. Thus, this CF/*coi1* line provides a via-

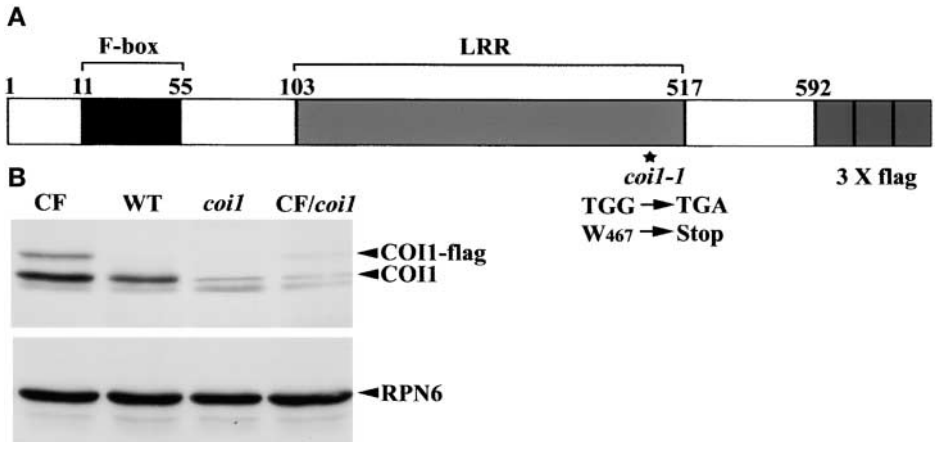

Figure 1. Construction and Expression of the COI1-Flag Fusion Protein in Arabidopsis Plants.

(A) Scheme of the C-terminal flag-tagged COI1 protein. The F-box domain, Leu-rich repeat (LRR), and three copies of the flag epitope tag fused to the COI1 C terminus are illustrated with shaded boxes and labeled at top. *COI1* encodes a 592–amino acid protein with an approximate molecular mass of 66 kD. The *coi1-1* null mutation takes place at codon 467, as indicated.

(B) Expression of the COI1-flag fusion protein. Protein samples were extracted from flowers of wild-type Arabidopsis expressing the *COI1-flag* transgene (CF), wild-type Arabidopsis (WT), *coi1-1* mutant Arabidopsis (*coi1*), and *coi1-1* mutant Arabidopsis expressing the *COI1-flag* transgene (CF/*coi1*). The protein extracts were run on SDS-PAGE, blotted, and hybridized with anti-COI1 antibody to monitor endogenous COI1 and transgenic COI1-flag (top gel) and with anti-RPN6 antibody to monitor total protein amount (bottom gel). The *coi1-1* mutation results in the absence of COI1 protein in *coi1-1* flower extracts (*coi1*) when hybridized with anti-COI1 antibody. The two faint bands detected in *coi1* are the result of nonspecific cross-reaction.

ble and low-level COI1 strain with which to analyze the COI1 protein dosage effect on JA responses (see below).

The COP9 Signalosome Associates Physically with SCF^{COI1} in Vivo

It has been reported that COI1 forms an SCF-type E3 ubiquitin ligase in vivo with CUL1, RBX1, and ASK1 or ASK2 (SKP1 homologs in Arabidopsis) (Devoto et al., 2002; Xu et al., 2002). To examine a possible physical association of CSN with SCF^{COI1} in vivo, we immunoprecipitated COI1 protein from JA-untreated wild-type flower extract using anti-COI1 antibody–conjugated beads. As expected, anti-COI1 antibody can readily bring down endogenous CUL1, the scaffold component of SCF complexes, confirming the existence of SCF^{COI1} in vivo (Figure 2A). Importantly, four representative CSN subunits, but not the TATA binding protein (TBP; which results in the negative control of immunoprecipitation), were brought down by the immunoprecipitation as well (Figure 2A). In a reciprocal experiment, anti-CSN4 antibody not only immunoprecipitated the COP9 signalosome, as shown by the existence of three other subunits in the immunoprecipitate, but they also pull down endogenous COI1 and CUL1, but not TBP (Figure 2A). These results demonstrate that CSN and SCF^{COI1} associate in vivo.

The COI1-Flag Fusion Protein Also Forms SCF$^{COI1\text{-}flag}$ and Associates Physically with the COP9 Signalosome in Vivo

We next examined whether the COI1-flag fusion protein can act properly in forming the SCF complex and its association with CSN. We used monoclonal anti-flag antibody to precipitate the COI1-flag fusion protein from JA-untreated CF flower extract and then examined the pull-down of the SCF and CSN components. As shown in Figure 2B, immunoprecipitation of COI1-flag readily pulls down the SCF complex scaffold component CUL1, implying the formation of an SCF complex with the COI1-flag fusion protein. Furthermore, five representative CSN subunits also were detected clearly in the immunoprecipitate from the CF flower extract. This pull down was specific, because the same flag antibodies did not reduce CSN subunits or CUL1 from the wild-type flower extract, and the endogenous COI1 and TBP were not reduced in either wild-type or transgenic *COI1-flag* flower extracts (Figure 2B). These data demonstrate that the COI1-flag fusion protein also forms SCF$^{COI1\text{-}flag}$ and associates physically with the COP9 signalosome in vivo.

We further analyzed the association of SCF$^{COI1\text{-}flag}$ and CSN by comparing the gel-filtration profiles of the COI1-flag fusion protein, CUL1, and CSN5 in JA-untreated CF flower extract. As shown in Figure 3, the COI1-flag fusion protein eluted from fractions 2 to 16, with a minor peak centered on fractions 3 and 4 and a major peak centered on fractions 10 to 12. CUL1 exhibited an elution profile over a similar range of fractions. Interestingly, we observed three peaks of CSN5 in the gel-filtration profiles. Two of the peaks are similar to the previously reported observations for CSN5 (Kwok et al., 1998), one from fractions 6 to 9, which corresponds to the COP9 signalosome, and one from fractions 16 to 17, which corresponds to the CSN5 monomers (Figure 3). In addition to these two peaks, we also observed that CSN5 has a third peak from fractions 2 to 4 (Figure 3). The coelution of COI1-flag, CUL1, and CSN5 in higher molecular mass fractions (>700 kD) supports the existence of a large complex containing CSN and SCF^{COI1} in vivo. It is possible that this large complex is labile and dynamic; thus, only a small fraction of cellular CSN and SCF was caught in this complex. It is worth noting that among the relatively small fraction of neddylated CUL1, most of them cofractionate with higher

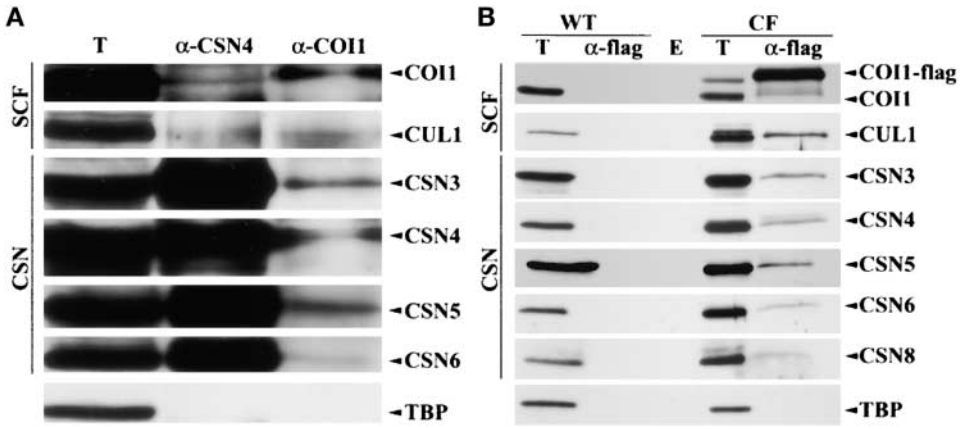

Figure 2. Coimmunoprecipitation Analysis of the Physical Association of CSN and SCF^{COI1} in Vivo.

(A) Flower extract prepared from wild-type Arabidopsis was incubated with anti-CSN4 antibody–conjugated beads (α-CSN4) or anti-COI1 antibody–conjugated beads (α-COI1). The immunoprecipitates were run on SDS-PAGE, blotted, and subjected to hybridization with antibodies against different SCF and CSN subunits as shown. Anti-TBP antibody was used as an immunoprecipitation control. T, total protein extract.

(B) Flower extracts prepared from wild-type Arabidopsis (WT) and wild-type Arabidopsis expressing the *COI1-flag* transgene (CF) were incubated with anti-flag antibody–conjugated beads (α-flag). The immunoprecipitates were run on SDS-PAGE, blotted, and subjected to hybridization with antibodies against different SCF and CSN subunits as shown. Anti-TBP antibody was used as an immunoprecipitation control. E, empty-bead immunoprecipitation control; T, total protein extract.

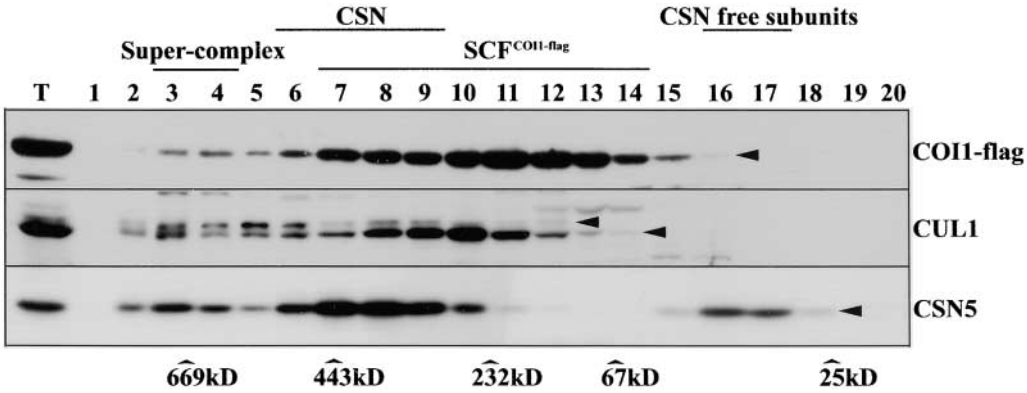

Figure 3. CSN and SCF^{COI1} Form a Large Complex in Vivo, as Shown by Gel-Filtration Analyses.

Flower extract prepared from wild-type Arabidopsis expressing the *COI1-flag* transgene was fractionated on a Superdex-200 gel-filtration column. Immediately after the void volume (7.0 mL) was reached, individual fractions of 0.5 mL (numbered from 1 to 20) were collected, run on SDS-PAGE, blotted, and subjected to hybridization with anti-flag antibody, anti-Cul1 antibody, and anti-CSN5 antibody. The fractions that correspond to CSN free subunits, SCF$^{COI1-flag}$, CSN, and the CSN-SCF supercomplex are marked at top. The protein bands of COI1-flag, neddylated CUL1 (top band), unneddylated CUL1 (bottom band), and CSN5 are indicated by arrowheads at right. The positions of the molecular mass standards are labeled at bottom. T, total protein extract.

molecular mass fractions (Figure 3, top arrowhead in the CUL1 gel), as observed in human cells (Yang et al., 2002).

JA Responses Are Dependent on *COI1*

To test a possible role of CSN in JA responses, we first set out to establish the effect of exogenously applied JA on Arabidopsis seedling root growth and gene expression under our experimental conditions. JA-conferred root growth inhibition in wild-type Arabidopsis seedlings was dose dependent, with 60% root length reduction on 10 μM JA, 70% on 50 μM JA, and 90% on 100 μM JA (Figures 4A and 4B). The *coi1-1* mutants showed insensitivity to root growth inhibition by JA, with only ~10% length reduction on 100 μM JA (Figures 4A and 4B). Consistently, the *VSP2* (*Vegetative Storage Protein2*) gene that is induced normally by JA in wild-type Arabidopsis seedlings was not induced at all in *coi1-1* mutants (Figure 4C). Similarly, we found that *PDF1.2*, a plant defensin gene, can be induced by JA in wild-type Arabidopsis seedlings but not in *coi1-1* mutants (Figure 4C), as reported previously (Penninckx et al., 1998). By contrast, the expression level of *BGL2* (*β-1,3-glucanase*), which is inducible by salicylic acid but not by JA (Cao et al., 1994), was the same in wild-type and *coi1-1* seedlings treated or untreated with JA (Figure 4C). These data indicate that our experimental conditions can adequately reproduce the previously reported JA responses and the effect of the *coi1-1* mutation. Thus, we examined the effect of low COI1 level and the role of CSN on JA responses.

The *coi1-1* mutant with the *COI1-flag* transgene, CF/*coi1*, which contains only a low level of the functional COI1-flag protein, was used to analyze the JA responses. Unlike *coi1-1*, CF/*coi1* seedlings showed root growth inhibition by JA. However, compared with wild-type seedlings, they were less sensitive to JA, with ~75% (instead of 90%) root length reduction on 100 μM JA (Figures 4A and 4B). In addition, *VSP2* transcript induction upon JA treatment was reduced significantly in CF/*coi1* seedlings compared with wild-type seedlings (Figure 4C), suggesting that a sufficient protein level of COI1 is important to maintain proper JA responses. Surprisingly, *PDF1.2* was induced by JA to an even greater level in CF/*coi1* than in the wild type (Figure 4C). It seems that *PDF1.2* induction by JA is optimal at lower COI1 levels and decreases in magnitude at higher COI1 levels. Those observations imply that distinct JA responses have different sensitivities to COI1 abundance.

In an independent experiment, a transgenic line expressing wild-type levels of a flag-COI1-hemagglutinin fusion protein in the *coi1-1* mutant background was generated. In contrast to CF/*coi1*, the *coi1-1* mutant phenotypes, including JA insensitivity and sterility, were rescued fully in this line (D. Xie, unpublished data). This finding suggests that the partial rescue of the *coi1-1* mutant phenotype in CF/*coi1* is attributable to the low COI1-flag protein level.

Proper Responses to JA Require CSN Function

To examine a possible role for CSN in JA responses, we used a previously described Arabidopsis line called *fus6/CSN1-11* (hereafter referred to as FL), which has a low expression level of *CSN1* transgene in a *fus6-1* background and, thus, low abundance of CSN (Wang et al., 2003). We selected this line because its phenotype is stable and it is capable of completing its life cycle, in contrast to the severely retarded seedling development and late lethality of the available *csn* mutants. Examination of root growth inhibition by JA indicated that FL seedlings exhibited partial resistance to JA. The FL seedlings had only ~50% root length reduction on 100 μM JA, in contrast to a 90% reduction in wild-type seedlings (Figures 4A and 4B). Furthermore, *PDF1.2* transcript induction by JA also was abolished in FL seedlings (Figure 4C). These results indicate that those tested JA responses require CSN function.

However, there was no detectable change in *VSP2* transcript induction by JA in FL seedlings (Figure 4C). It is interesting that both CSN5 (Schwechheimer et al., 2002) and CSN6 (Z. Peng and X.W. Deng, unpublished data) reduction-of-function lines, which exhibited more pleiotropic phenotypes and dramatic reduction of CSN levels, showed defects in the JA induction of *VSP* transcripts. Thus, it is possible that the reduction of CSN in the FL line is not great enough to affect *VSP2* induction. This idea is examined further below.

Expression Profile Analyses Show That JA-Responsive Gene Expression Is Largely *COI1* Dependent

We have shown that *coi1-1* mutants are defective in JA responses (Figure 4). To further substantiate the physiological observations, we compared the genome expression profiles of JA-treated wild-type seedlings with those of untreated wild-type seedlings using an EST microarray. The microarray contains 9216 Arabidopsis ESTs that correspond to an estimated 6126 unique genes (Ma et al., 2001, 2002). Of the 6126 genes, 290 (4.7%) displayed differential expression of twofold or more. Among these, 167 exhibited upregulation in wild-type seedlings by JA, and 123 exhibited downregulation (see supplemental data at http://plantgenomics.biology.yale.edu). This group of genes was designated JA-responsive genes.

Next, we compared the genome expression profiles of JA-treated wild-type seedlings with those of JA-treated *coi1-1* mutant seedlings. This time, 290 (4.7%) of the 6126 genes were upregulated or downregulated by at least twofold. Of these genes, 130 had higher expression in JA-treated wild-type seedlings, and 160 had higher expression in JA-treated *coi1-1* seedlings (Table 1; see also supplemental data at http://plantgenomics.biology.yale.edu). This group of genes was designated *COI1*-dependent JA-responsive (or *COI1*-regulated) genes, because their responsiveness to JA required *COI1* function.

Cluster analysis showed a significant overlap between JA-responsive and *COI1*-regulated genes. Indeed, most genes showed similar regulation patterns in the two clusters, although differences in magnitude often were observed (Figure 5A, compare lanes 1 and 2). Our data confirmed the previous observation that most JA-induced responses require *COI1* function.

JA Control of Genome Expression Is Critically Dependent on COI1 Dosage

To further analyze the role of *COI1* in JA responses, we examined the effect of reduced COI1 abundance on JA-triggered genome expression profiles. To this end, the genome expression profiles of CF/*coi1* (low COI1 activity) with or without JA treatment (Figure 5A, lane 3) were obtained. A careful comparison with the JA-responsive and *COI1*-regulated genome expression profiles (Figure 5A, lanes 1 and 2) revealed that most of the JA-responsive and *COI1*-regulated genes displayed reduced responses to JA in the CF/*coi1* line (see Table 1 for statistics). These genes can be categorized further into two groups. First, for the majority of JA-responsive and *COI1*-regulated genes, their responses to JA in CF/*coi1* were compromised partially to varying degrees (Figure 5A), indicating that a reduced level of

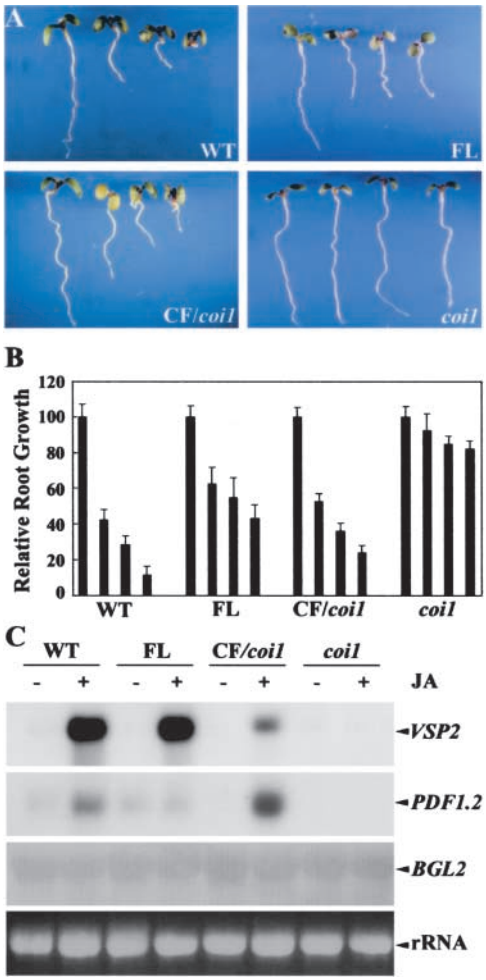

Figure 4. Proper Responses to JAs Require Both *COI1* and CSN.

(A) Seedlings of wild-type Arabidopsis (WT), *fus6/CSN1-11* (FL), *coi1-1* mutant Arabidopsis expressing the *COI1-flag* transgene (CF/*coi1*), and *coi1-1* mutant Arabidopsis (*coi1*) were grown on Murashige and Skoog (1962) (MS) plates for 4 days and transferred to new MS plates containing 0, 10, 50, or 100 μM JA (left to right). Photographs were taken 3 days after transfer.

(B) Relative root growth of the seedlings generated in **(A)** was measured. Root growth in the absence of JA was set to 100%. Bars from left to right represent relative root growth in the absence of JA and with 10, 50, or 100 μM JA.

(C) Seedlings of wild-type Arabidopsis (WT), *fus6/CSN1-11* (FL), *coi1-1* mutant Arabidopsis expressing the *COI1-flag* transgene (CF/*coi1*), and *coi1-1* mutant Arabidopsis (*coi1*) were grown on MS plates without (−) or with (+) 50 μM JA for 7 days and extracted for RNA. RNA (10 μg) was loaded on each lane. The RNA gel blots were hybridized by probes against *VSP2*, *PDF1.2*, and *BGL2* transcripts. The total amount of RNA in each lane is shown at bottom (rRNA).

COI1 is able to regulate their responses to JA but a high level of COI1 is required for optimal responses. Second, the rest of the JA-responsive and *COI1*-regulated genes no longer respond to JA in the low COI1 abundance line (Figure 5A), suggesting that their responses to JA require normal high levels of COI1. Se-

Table 1. Statistical Summary of the Microarray Analysis of Genome Expression in Relation to JA, *COI1*, and CSN

Regulation	Number of *COI1*-Regulated JA-Responsive Genes	Number of JA-Responsive Genes			
		CF/*coi1*	FL	*cop9*	*fus11*
Upregulated (twofold or greater)	130	17	42	18	18
Downregulated (twofold or greater)	160	3	3	1	0

lected genes from the two groups are represented in Figures 5B and 5C, respectively. Our results indicate that most *COI1*-dependent JA-responsive genes require COI1 in a dose-dependent manner and that different genes exhibit distinct sensitivities to COI1 abundance.

The *COI1*-Dependent JA-Responsive Genes Require Fully Functional CSN

To investigate the role of CSN in JA responses, JA-triggered genome expression profiles were analyzed in the low-level CSN FL line and two *csn* null mutants (*cop9-1* and *fus11-1*) using the same microarray system. We compared the gene expression profiles of JA responses in FL, *cop9*, and *fus11* seedlings (with or without JA treatment) with those of the *COI1*-dependent JA-responsive genes and observed a clear correlation among them (Figure 6A). Although a small number of *COI1*-regulated JA-responsive genes were not affected, the majority of the genes had reduced responsiveness to JA in FL seedlings, some of which were no longer induced or repressed by JA (Figure 6A, lane 2). This phenomenon was more severe in *cop9* and *fus11* mutants, in which JA failed to trigger significant expression level changes for almost all of the *COI1*-regulated JA-responsive genes (Figure 6A, lanes 3 and 4). Statistics summarizing these findings are presented in Table 1. These results suggest a clear requirement for CSN in *COI1*-dependent JA-responsive gene expression.

A closer examination of gene regulation patterns reveals clear differential sensitivities of different *COI1*-dependent JA-responsive genes to CSN abundance. One group of genes is very sensitive to CSN level, because the genes do not respond to JA in the FL line, which has a low CSN level (Figure 6B, lane 2). On the contrary, JA still can regulate some other genes in the FL line, although the magnitude is reduced to variable extents (Figure 6C, lane 2). Furthermore, genes in both clusters are no longer responsive to JA in *cop9* and *fus11* mutants (Figures 6B, lanes 3 and 4, and 6C, lanes 3 and 4). Together, the microarray data and the physiological studies (Figure 4) demonstrate that, as with COI1, the requirement for CSN in JA responses also is dose dependent.

A Small Group of *COI1*-Dependent JA-Responsive Genes Do Not Depend on CSN and Are Insensitive to COI1 Dosage

Overall, of the 130 genes that exhibited *COI1*-dependent upregulation in response to JA, 17 retained twofold or more upregulation by JA in the COI1 low-level line (CF/*coi1*), 42 retained it in the CSN low-level line (FL), and 18 each retained it in *cop9* and *fus11* (Table 1; see also supplemental data at http://plantgenomics.biology.yale.edu). Interestingly, comparison of those genes that still responded to JA in CF/*coi1*, FL, and *cop9* or *fus11* revealed an obvious overlap (Figure 7). Among them, seven common genes exhibited significant JA-responsive expression in the low-level COI1 line, the low-level CSN line, or

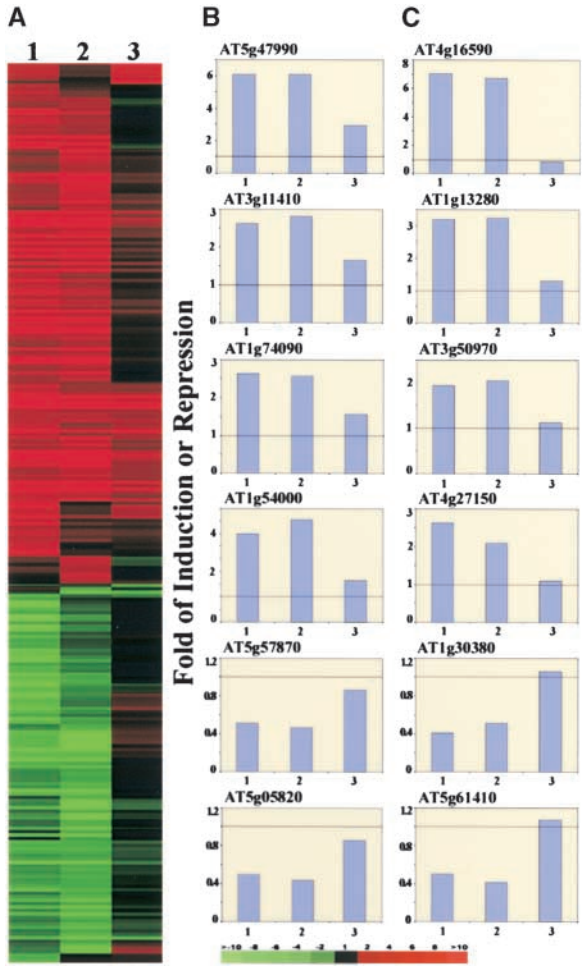

Figure 5. Genome Expression Profiles of JA-Responsive Genes and Their Dependence on COI1 Dosage and Function.

(A) Overview of the hierarchical cluster display. Lane 1, expression ratios of JA-treated wild-type seedlings and untreated wild-type seedlings; lane 2, expression ratios of JA-treated wild-type seedlings and JA-treated *coi1-1* mutant seedlings; lane 3, expression ratios of JA-treated CF/*coi1* seedlings and untreated CF/*coi1* seedlings. The color scale is shown at bottom right. Only those genes that exhibited twofold or greater differential expression in at least one of the three experimental sets were included for comparison. A total of 261 genes were included in the cluster.

(B) and **(C)** Differential expression of 12 genes in the three microarray experiments described in **(A)**. The AGI code for each gene is indicated at the top of each graph. The bar numbers in **(B)** and **(C)** correspond to the lane numbers in **(A)**. A line is drawn in each graph corresponding to the ratio of 1.

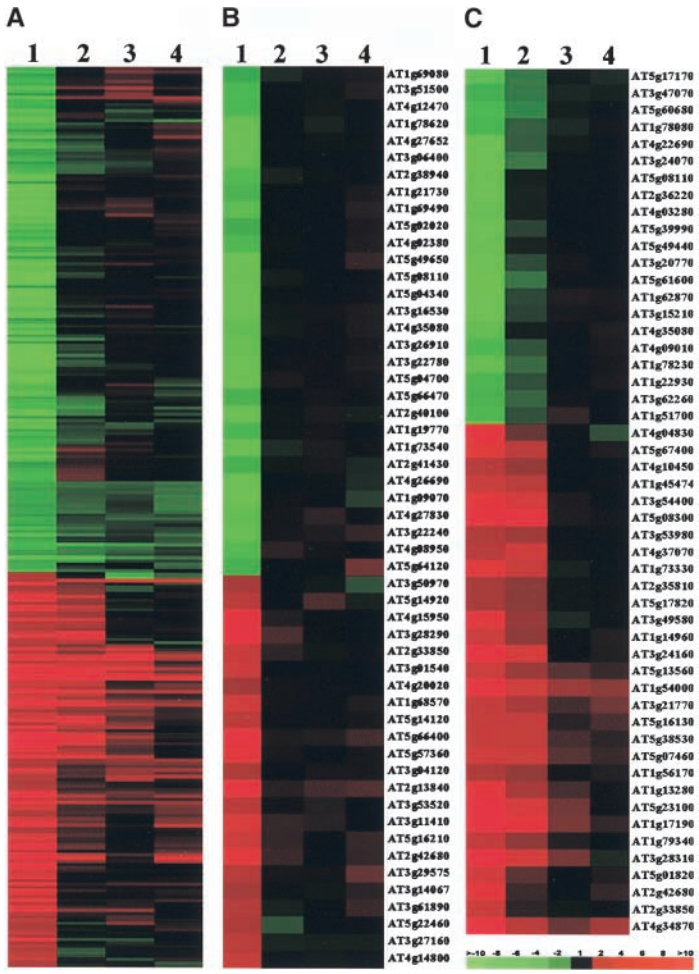

Figure 6. Microarray Analyses Show Functional Overlaps of *COI1*- and CSN-Regulated Gene Expression in Response to JA and the Effect of the CSN Dosage on JA Responses.

(A) Overview of the hierarchical cluster display. Lane 1, expression ratios of JA-treated wild-type seedlings and JA-treated *coi1-1* mutant seedlings; lane 2, expression ratios of JA-treated FL seedlings and untreated FL seedlings; lane 3, expression ratios of JA-treated *cop9* seedlings and untreated *cop9* seedlings; lane 4, expression ratios of JA-treated *fus11* seedlings and untreated *fus11* seedlings. Only those genes that exhibited twofold or greater differential expression in JA-treated wild-type seedlings versus JA-treated *coi1-1* mutant seedlings were included for comparison. A total of 290 genes were included in the cluster. The color scale is shown at bottom right.

(B) and **(C)** Hierarchical clusters of selected genes. The lane numbers in **(B)** and **(C)** correspond to the lane numbers in **(A)**. The AGI code for each gene is indicated to the right of the clusters. See text for details of the selected genes.

the null *csn* (*cop9-1*) mutant. Those seven genes include a cytochrome P450 (At5g47990), a putative RNA helicase (At3g48120), and the unknown proteins. Most of the remaining genes also exhibited similar JA responsiveness in these lines, but the expression ratios decreased to less than the twofold cutoff in some cases. These results have interesting implications. First, most JA-responsive genes required adequate COI1 and CSN (Figures 5 and 6). Second, the genes that were not sensitive to COI1 level also were likely to be insensitive to CSN level as well. Third, the genes that were not controlled by CSN were likely to be insensitive to COI1 level. It appears that there are few *COI1*-dependent JA-responsive genes that require only minimal SCF^{COI1} activity and do not need the COP9 signalosome.

COI1- and CSN-Dependent JA-Responsive Genes Are Involved in Specific Cellular Processes

Various genes involved in JA responses and *COI1* regulation had been identified previously using arrays containing selected groups of genes (Reymond et al., 2000; Schenk et al., 2000; Sasaki et al., 2001). Those studies involved arrays with 150, 2375, and 2880 genes, respectively. Our data agree well with the previous results (see supplemental data at http://plantgenomics.biology.yale.edu) but provide a more comprehensive analysis as a result of our large gene coverage (6126 genes). We looked for functional groups in the *COI1*- and CSN-regulated JA-responsive genes, and those genes with an assigned functional category

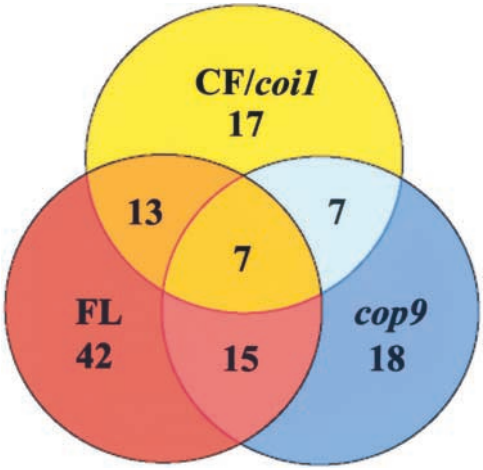

Figure 7. Interloping Diagram of the Numbers of *COI1*-Regulated JA-Responsive Genes That Show Twofold or More Upregulation by JA in CF/*coi1*, FL, and *cop9* Lines.

The numbers in the overlapping area indicate the shared number of genes in either two or three lines. In fact, most of those genes that did not overlap also exhibited similar expression pattern changes, except that in some cases the JA-responsive gene expression regulation was below the twofold cutoff.

are summarized in Table 2. Ten putative stress-related genes were found in the genes that were upregulated, which suggests that *COI1* and CSN work together to promote stress responses triggered by JA (Table 2). Another upregulated pathway is polysaccharide metabolism, in which six genes were identified (Table 2). Both *COI1* and CSN are required for the JA-responsive expression of genes involved in amino acid metabolism, signal transduction, and transcription. For different genes in these pathways, the regulation could be either stimulatory or inhibitory (Table 2). Photosynthesis, energy generation, and resistance to certain diseases seem to be downregulated by *COI1* and CSN in response to JA (Table 2).

DISCUSSION

The COP9 Signalosome Associates Physically with SCF^{COI1} in Vivo and Is Essential for Its Function

In this study, we used coimmunoprecipitation experiments to show that CSN is associated with CUL1 and COI1 or COI1-flag in wild-type and *COI1-flag* transgenic Arabidopsis plants (Figure 2), respectively. Moreover, COI1-flag, CUL1, and CSN5 were found to cofractionate in a large complex (>700 kD) in gel-filtration analysis (Figure 3). The size of this large complex is greater than that of CSN and SCF^{COI1} combined, which indicates that it contains other components. It has been reported that neddylated CUL1 was enriched in cellular SCF complexes immunoprecipitated by antibodies against SCF substrates (Read et al., 2000; Kawakami et al., 2001). Here, we observed that most of the neddylated CUL1 exists in higher molecular mass fractions corresponding to the large complex. It is reasonable to speculate that this large complex, with SCF and CSN, may be the active form and that it contains SCF substrates.

Phenotypic and gene expression studies support the idea that the observed CSN and SCF^{COI1} physical association is functionally significant. The CSN partial loss-of-function line, *fus6/CSN1-11* (FL), was found to be partially defective in JA responses as well (Figure 4). Furthermore, microarray analyses confirm that most of the *COI1*-regulated JA-responsive genes are controlled by CSN (Figure 6). Thus, our data suggest that CSN associates physically with SCF^{COI1} in vivo and plays an essential regulatory role in its function.

In an exhaustive genome-wide search, 694 potential F-box proteins were identified in the Arabidopsis genome, constituting the largest gene superfamily currently known in plants (Gagne et al., 2002). Considering the fact that Arabidopsis also has 2 RBXs and 19 ASKs (Callis and Vierstra, 2000; del Pozo and Estelle, 2000; Shen et al., 2002), a combinatorial arrangements of subunit isoforms would produce a huge number of SCF complexes. Thus, SCF complexes are able to regulate enormous developmental processes by specifically targeting important regulators for degradation.

With this study, three Arabidopsis SCF complexes, SCF^{TIR1}, SCF^{UFO}, and SCF^{COI1}, have been demonstrated to associate with CSN in vivo. In all cases, CSN has been found not only to interact with but also to directly modulate the activities of SCF^{TIR1}, SCF^{UFO}, and SCF^{COI1} and the developmental responses they regulate (Schwechheimer et al., 2001; Wang et al., 2003; this work). Thus, it is reasonable to conclude that CSN interacting with and regulating multiple SCF complexes is a common scheme in Arabidopsis, and possibly in all other CSN-containing eukaryotes, to achieve its role in many biological processes. Considering the huge number of potential SCF-type E3 ubiquitin ligases in Arabidopsis, it is not surprising that mutations in CSN have pleiotropic effects and lethality after seedling development.

The Dosage Effect of SCF^{COI1} and the COP9 Signalosome in JA Responses

We provide evidence that proper responses to JA are dependent on COI1 dosage. We generated an Arabidopsis line that underexpressed the COI1-flag protein, CF/*coi1* (Figure 1B). The pollen and stamen development of the CF/*coi1* line was normal (data not shown), but its response to exogenous JAs was partially defective (Figure 4). In accordance with this finding, microarray analyses showed that expression profiles of most *COI1*-regulated JA-responsive genes were sensitive to COI1 level. In this CF/*coi1* line, the expression of some *COI1*-regulated genes in response to JA was defective, whereas expression of the others was at least compromised to different levels (Figure 5).

Like the COI1 dosage effect, we found that the abundance of CSN also is important for JA responses. The FL line, with a low CSN level, displayed reduced responsiveness to JA (Figure 4). It turns out that the expression of a majority of the *COI1*-regulated genes in response to JA was affected in the FL line, whereas almost all of these genes were affected in severe *csn* mutants (Figure 6, Table 1). Furthermore, *COI1*-regulated JA-

Table 2. Microarray Data for Selected Genes Involved in Various Pathways

Gene Identification and Putative Function	AGI Code	Fold Induction or Repression by JA				
		Wild Type versus *coi1*	CF/*coi1*	FL	*cop9*	*fus11*
Stress response						
Dehydrin RAB18-like protein	At5g66400	5.258	1.479	1.177	1.205	1.353
Myrosinase-associated protein	At1g54000	4.442	1.63	2.682	1.921	1.687
Putative monodehydroascorbate reductase (NADH)	At3g09940	3.075	1.227	4.449	1.487	1.195
Peroxidase	At5g67400	3.01	1.207	2.299	0.799	1.184
Putative peroxidase	At3g21770	2.398	1.276	2.06	1.361	1.548
Homeobox-Leu zipper protein ATHB-12	At3g61890	2.191	0.978	0.857	0.961	1.317
Alcohol dehydrogenase	At1g77120	2.063	1.113	1.323	1.494	1.457
Peroxidase ATP13a	At5g17820	2.047	0.9795	1.692	0.707	0.926
Dehydrin Xero2	At3g50970	2.043	1.128	0.976	0.884	0.728
Quinone oxidoreductase-like protein	At5g37940	2.019	0.998	0.959	0.811	1.095
Polysaccharide metabolism						
Cellulose synthase-like protein	At4g16590	6.698	0.828	6.485	3.289	2.37
β-Glucosidase	At1g66280	6.545	2.809	6.623	2.261	2.434
β-Glucosidase	At1g66270	6.387	1.738	4.547	2.426	2.166
Mannosyltransferase	At3g10630	3.439	1.21	1.479	0.968	1.645
UDP-glucuronic acid decarboxylase	At3g53520	2.246	1.462	1.1035	1.226	1.021
Quinone oxidoreductase-like protein	At5g37940	2.019	0.998	0.959	0.811	1.095
Amino acid metabolism						
Trp synthase β-chain	At5g38530	2.437	1.293	2.4	1.368	1.255
3-Isopropylmalate dehydratase-like protein	At3g58990	2.104	2.026	1.395	1.28	1.552
Arg decarboxylase	At4g34710	2.094	1.279	1.58	1.895	1.103
Gln synthetase	At1g66200	0.268	0.94	1.223	1.043	1.171
Branched-chain α-keto acid dehydrogenase E1 β-subunit	At3g13450	0.473	0.408	0.681	0.843	1.365
Signal transduction						
Phosphatidylinositol-4-phosphate 5-kinase	At1g21920	2.531	1.183	1.63	1.342	1.283
Receptor-like protein glossy1	At1g02200	2.452	0.748	1.171	0.739	1.256
Protein phosphatase type 2C	At1g22280	2.199	1.153	2.062	1.132	1.111
DAG-like protein	At4g20020	2.004	1.145	1.122	1.086	1.065
Putative receptor-like protein kinase	At2g31880	0.254	1.256	1.15	1.323	1.025
Putative protein kinase	At1g67470	0.257	0.674	0.777	0.941	1.054
Mitogen-activated protein kinase 3	At3g45640	0.257	0.765	1.378	1.147	0.842
Dof zinc finger protein	At1g51700	0.38	0.816	0.73	1.287	1.055
Putative protein inositol polyphosphate 5-phosphatase	At4g18010	0.385	1.313	1.288	0.79	0.977
Putative phosphoprotein phosphatase	At3g62260	0.404	0.452	0.633	1.104	1.007
Putative mitogen-activated protein kinase	At2g01450	0.467	0.957	0.763	0.828	0.937
Putative protein kinase	At2g05940	0.478	0.785	1.061	1.351	1.121
Receptor protein kinase-like protein	At4g08850	0.498	1.15	1.055	1.063	1.016
Transcription factors						
Homeobox-Leu zipper protein ATHB-12	At3g61890	2.191	0.978	0.857	0.961	1.317
Putative transcription factor	At1g56170	2.117	1.329	1.837	1.11	1.201
Putative zinc finger transcription factor	At3g55980	0.081	1.275	1.226	1.459	0.961
Putative C2H2 zinc finger transcription factor	At5g04340	0.235	0.97	0.936	1.063	1.087
AP2 domain–containing protein	At1g78080	0.393	0.682	0.683	0.84	1.116
Basic domain/Leu zipper transcription factor	At1g42990	0.399	1.236	1.357	1.135	0.959
Putative basic domain/Leu zipper transcription activator	At3g58120	0.443	0.913	0.646	0.623	1.006
Photosynthesis						
Putative chlorophyll *a*/*b* binding protein	At2g40100	0.215	0.7985	0.905	1.176	0.862
Putative thylakoid lumen rotamase	At3g01480	0.413	0.887	0.556	0.603	0.79
Photosystem I reaction center subunit psaN precursor	At5g64040	0.466	0.9995	0.494	0.761	0.768
Putative photosystem I reaction center subunit IV	At2g20260	0.498	0.752	0.579	0.674	0.774
Energy generation						
Putative mitochondrial uncoupling protein	At4g24570	0.058	0.386	0.87	1.177	1.165
Putative component of cytochrome b_6/f complex	At4g03280	0.308	0.972	0.862	1.005	0.994
Ferrochelatase-I	At5g26030	0.312	0.906	1.291	1.294	1.087
Putative ribulose-1,5- bisphosphate carboxylase/oxygenase small subunit *N*-methyltransferase I	At5g14260	0.432	0.9785	1.0085	0.84	0.912
Disease resistance						
Putative nematode resistance protein	At2g40000	0.052	1.114	0.79	0.969	0.828
Disease resistance protein	At1g72910	0.193	0.821	0.722	0.827	1.042
Flax rust resistance protein	At1g72930	0.198	0.938	0.748	0.704	0.87
Disease resistance protein-like	At5g40910	0.359	0.731	0.795	0.676	0.849
Putative disease resistance protein	At3g50950	0.47	0.788	0.708	0.687	0.614

responsive genes showed different sensitivities to CSN level (Figure 6).

Based on these findings, we hypothesize that protein ubiquitination and degradation mediated by SCF^{COI1} is a process that affects most aspects of the JA pathway and that regulation by CSN is essential for SCF^{COI1} to perform its function. Thus, adequate COI1 and CSN protein levels are required for proper JA responses. In some rare cases, in which minimal COI1 activity is sufficient for their JA-responsive gene expression, CSN function is not essential (Figure 7).

Specific JA-Triggered Pathways That Are Regulated by Both *COI1* and CSN

Using EST microarray analyses, we identified 290 *COI1*-dependent JA-responsive genes among approximately one-fourth of the Arabidopsis genome (Table 1). Most of these genes also are controlled by CSN (Figure 6, Table 1; see also supplemental data at http://plantgenomics.biology.yale.edu/).

Among these genes, we found several interesting functional groups. Clearly, stress responses were increased by *COI1* and CSN when plants encountered exogenous JA (Table 2). By contrast, photosynthesis and energy-generating processes were suppressed (Table 2). These findings are consistent with the current understanding of JA-responsive pathways. JA is produced when plants suffer from mechanical trauma and pathogenesis. Under such circumstances, stress responses are of high priority to plant cells, whereas photosynthesis and energy metabolism should be limited.

Because signal transduction and transcription processes are involved in various aspects of cellular activities, it is not surprising that the regulation of different genes in these pathways by *COI1* and CSN could be either stimulatory or inhibitory (Table 2). Interestingly, *COI1* and CSN can downregulate certain disease resistance genes (Table 2). This finding implies that JA pathways and other defense-related pathways might have antagonistic effects to each other.

Overall, EST microarray analyses provide an overview of how plant cells, using *COI1* and CSN, respond to JA, adjust genome expression patterns, and affect necessary cellular pathways.

METHODS

Plant Materials and Growth Conditions

The *cop9-1*, *fus11-U203*, *coi1-1*, and *fus6/CSN1-11* mutants were described previously (Feys et al., 1994; Wei et al., 1994; Kwok et al., 1996; Wang et al., 2003). When not specified, we use *cop9* to indicate *cop9-1*, *fus11* to indicate *fus11-U203*, *coi1* to indicate *coi1-1*, and FL to indicate *fus6/CSN1-11* throughout the text. The wild-type *Arabidopsis thaliana* used in this study was the Columbia ecotype. Arabidopsis seeds were surface-sterilized, plated on Murashige and Skoog (1962) (MS) medium (GIBCO) containing 1% sucrose, and vernalized by incubating at 4°C for 3 to 5 days before being placed in a standard long-day growth chamber at 22°C. After 7 to 9 days of incubation, seedlings were transferred to soil and grown to adult plants in a standard long-day growth room.

For exogenous jasmonate treatment experiments, Arabidopsis seedlings were grown on MS medium containing 50 μM methyl jasmonate (Bedoukian, Danbury, CT) for 7 days. For root growth inhibition experiments, Arabidopsis seedlings were grown on normal MS medium for 4 days and then transferred to MS medium containing 10, 50, or 100 μM methyl jasmonate. Photographs of the seedlings were taken 3 days after transfer, as were root length measurements.

Plasmid Construction, Arabidopsis Transformation, and Antibodies

An XhoI-SalI fragment containing three copies of flag tag was subcloned into pBluescript SK+ (Stratagene), generating a construct named pF3BS. The BamHI-KpnI fragment from pF3BS then was subcloned into the Arabidopsis transformation vector pPZPY122 (Yamamoto et al., 1998). The resulting construct was designated pF3PZPY122.

Full-length cDNA of *COI1* was amplified by reverse transcriptase–mediated PCR with the forward primer COI1-5NKS (5′-ATAAGAATGCGGCCGCAAGGTACCATTCCCGGGATGGAGGATCCTGATATCAAG-3′) and the reverse primer COI1-3SSS (5′-ACGCGTCGACGAGCTCTCACCCGGGTATTGGCTCCTTCAGGACTC-3′), both of which contain a SmaI site. The resulting PCR product was cloned into pCR2.1-TOPO (Invitrogen, Carlsbad, CA). The SmaI fragment of the PCR product then was subcloned into pF3PZPY122, which, besides three copies of flag tag, also carries a gentamicin-resistant marker and a 35S promoter of *Cauliflower mosaic virus* to drive the expression of the transgene (Yamamoto et al., 1998).

Arabidopsis plants of Columbia ecotype were used in the transformation of the *COI1-flag* transgene. The methods of plant transformation were described separately (Wang et al., 2002). Transgenic plants were selected with gentamicin (100 μg/mL; Sigma).

The primary antibodies used in this study include anti-CSN3 (Peng et al., 2001b), anti-CSN4 (Serino et al., 1999), anti-CSN5 (Kwok et al., 1998), anti-CSN6 (Peng et al., 2001a), anti-CSN8 (Wei et al., 1994), anti-CUL1 (Wang et al., 2002), anti-COI1 (Xu et al., 2002), anti-TBP (Schwechheimer et al., 2001), anti-RPN6 (Kwok et al., 1999), and anti-flag (Sigma). Except for the anti-flag antibody that is a mouse monoclonal antibody, all other antibodies are rabbit polyclonal antibodies.

Immunoblot, Immunoprecipitation, and Gel-Filtration Analyses

For direct immunoblot analysis of plant extracts, Arabidopsis flowers were homogenized in an extraction buffer containing 50 mM Tris-HCl, pH 7.4, 10 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 10% glycerol, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 1× complete protease inhibitor (Roche, Basel, Switzerland). The extracts were centrifuged twice at 4°C for 10 min each, and the protein concentration in the supernatant was determined by the Bradford assay (Bio-Rad).

Experimental conditions for the immunoprecipitation experiments were as described previously (Staub et al., 1996), except that the formula for the extraction/washing buffer was 50 mM Tris-HCl, pH 7.4, 100 mM NaCl, 10 mM $MgCl_2$, 1 mM EDTA, 10% glycerol, 1 mM DTT, 1 mM PMSF, and 1× complete protease inhibitor (Roche) and the antibodies coupled with protein A beads were mixed with the protein extracts for 4 h instead of 6 h. For flag immunoprecipitation, anti-flag antibody–conjugated agarose beads (Sigma) were used.

Gel-filtration chromatography was performed as described previously (Peng et al., 2001b) with minor modifications. The protein homogenization buffer was 50 mM Tris-HCl, pH 7.4, 100 mM NaCl, 10 mM $MgCl_2$, 1 mM EDTA, 10% glycerol, 1 mM DTT, 1 mM PMSF, and 1× complete protease inhibitor (Roche), and the column equilibrium/elution buffer was 50 mM Tris-HCl, pH 7.4, 100 mM NaCl, 10 mM $MgCl_2$, 1 mM EDTA, and 10% glycerol. For fast protein liquid chromatography, a Superdex-200 (Pharmacia) column was used with a flow rate of 0.3 mL/min.

All protein samples were run on SDS-PAGE (8 to 12%), transferred to nylon Immobilon membranes (Millipore, Bedford, MA), and subjected to immunoblot analysis.

RNA Gel Blot Analysis

Arabidopsis seedlings were ground in liquid nitrogen, and the RNA was extracted using the RNeasy kit (Qiagen, Valencia, CA). The RNA was loaded onto an agarose-formaldehyde gel and then transferred to a Hybond N^+ membrane (Amersham) for standard RNA gel blot hybridization in a buffer containing 5× Denhardt's solution (1× Denhardt's solution is 0.02% Ficoll, 0.02% polyvinylpyrrolidone, and 0.02% BSA), 5× SSC (1× SSC is 0.15 M NaCl and 0.015 M sodium citrate), 50% formamide, and 1% SDS.

PDF1.2 and *BGL2* probes were prepared by PCR amplification from Columbia wild-type genomic DNA. For *PDF1.2*, the forward primer was 5′-ACGCACCGGCAATGGTGGAAGCAC-3′ and the reverse primer was 5′-CGGGAAAATAAACATTAAAACAG-3′. For *BGL2*, the forward primer was 5′-TCAGAGCTACAGAGATGGTGTCAG-3′ and the reverse primer was 5′-AACAGTGGACTGGGCGGTGAACAG-3′. The SalI-NotI fragment from a *VSP2* EST clone was used as the *VSP2* probe. All of the probes were radiolabeled using the Rediprime kit (Amersham).

Microarray Analysis

The procedures used in the microarray experiments and data analysis were described previously (Ma et al., 2002). Briefly, 50 μg of total mRNA for each sample was prepared and used to synthesize Cy3- and Cy5-labeled cDNA. Pair-wise combinations of two selected samples were used to simultaneously probe a glass slide containing duplicate arrays in three microarray experiments. The hybridized microarray slides were scanned with the GenePix 4000B scanner (Axon, Union City, CA), and the data were processed by GenePix Pro4.0 (Axon).

Upon request, all novel materials described in this article will be made available in a timely manner for noncommercial research purposes.

Accession Number

The accession number for the *VSP2* EST clone is T22924.

ACKNOWLEDGMENTS

We thank Elizabeth Strickland and Vicente Rubio for reading and commenting on the manuscript, the members of our laboratory for technical assistance and helpful discussion, and J.G. Turner for *coi1-1* mutants. This research was supported by grants from the U.S. Department of Agriculture (2001-35304-10852) and the National Science Foundation (MCB-0077217 and MCB-0115870) to X.W.D. and by grants from the National Science Foundation of China (30170489 and 39725002).

Received December 22, 2002; accepted February 21, 2003.

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