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JASMONATE-INSENSITIVE1 Encodes a MYC Transcription Factor Essential to Discriminate between Different Jasmonate-Regulated Defense Responses in Arabidopsis [W]

Oscar Lorenzo, Jose M. Chico, Jose J. Sánchez-Serrano, and Roberto Solano[1]

Departamento de Genética Molecular de Plantas, Centro Nacional de Biotecnología, Consejo Superior de Investigaciones Cientificas, Campus Universidad Autónoma, 28049 Madrid, Spain

In spite of the importance of jasmonates (JAs) as plant growth and stress regulators, the molecular components of their signaling pathway remain largely unknown. By means of a genetic screen that exploits the cross talk between ethylene (ET) and JAs, we describe the identification of several new loci involved in JA signaling and the characterization and positional cloning of one of them, *JASMONATE-INSENSITIVE1* (*JAI1*/*JIN1*). *JIN1* encodes AtMYC2, a nuclear-localized basic helix-loop-helix-leucine zipper transcription factor, whose expression is rapidly upregulated by JA, in a CORONATINE INSENSITIVE1–dependent manner. Gain-of-function experiments confirmed the relevance of AtMYC2 in the activation of JA signaling. AtMYC2 differentially regulates the expression of two groups of JA-induced genes. The first group includes genes involved in defense responses against pathogens and is repressed by AtMYC2. Consistently, *jin1* mutants show increased resistance to necrotrophic pathogens. The second group, integrated by genes involved in JA-mediated systemic responses to wounding, is activated by AtMYC2. Conversely, Ethylene-Response-Factor1 (ERF1) positively regulates the expression of the first group of genes and represses the second. These results highlight the existence of two branches in the JA signaling pathway, antagonistically regulated by AtMYC2 and ERF1, that are coincident with the alternative responses activated by JA and ET to two different sets of stresses, namely pathogen attack and wounding.

INTRODUCTION

Jasmonates (JAs) are fatty acid–derived signaling molecules involved in the regulation of many physiological and developmental processes in plants, including root growth, tuberization, fruit ripening, senescence, tendril coiling, and pollen development. They are also important regulators of plant responses to environmental stress, such as ozone exposure, wounding, water deficit, and pathogen and pest attack (Penninckx et al., 1996; Creelman and Mullet, 1997; McConn et al., 1997; Pieterse et al., 1998; Reymond and Farmer, 1998; Staswick et al., 1998; Overmyer et al., 2000; Berger, 2002; Rao et al., 2002; Turner et al., 2002; Farmer et al., 2003; Rojo et al., 2003).

For most of these stress situations, the precise plant response is not activated only by JAs but is the result of a network of interactions between different signaling pathways. Several examples of cross talk between JAs and other hormonal pathways, such as ethylene (ET), salicylic acid, auxins, or abscisic acid (ABA), have been reported (Turner et al., 2002; Farmer et al., 2003; Rojo et al., 2003). JA has been shown to inhibit seed germination in several species and to have a synergistic effect with ABA in this process in *Arabidopsis thaliana* (Wilen et al., 1991; Staswick et al., 1992; Ellis and Turner, 2002). Mutants impaired in auxin signaling, such as *auxin resistant1* (*axr1*), have also been found to have altered JA responses (Tiryaki and Staswick, 2002; Xu et al., 2002). Positive and negative interactions between JA and salicylic acid signaling pathways have been broadly documented (Turner et al., 2002; Farmer et al., 2003; Rojo et al., 2003), and MPK4 has been identified as a key regulator of this cross talk in Arabidopsis (Petersen et al., 2000). Finally, JA and ET have been shown to either cooperate or antagonize in the regulation of different stress responses, including pathogen attack, wounding (mechanical or biotic), ozone exposure, or exaggerated apical hook development (Turner et al., 2002; Farmer et al., 2003; Rojo et al., 2003). In the case of necrotrophic pathogens, ERF1 (Ethylene-Response-Factor1) plays a key role in the integration of JA and ET signals, thus explaining at the molecular level the cooperation between both hormones in the activation of plant defenses (Berrocal-Lobo et al., 2002; Lorenzo et al., 2003). However, in the case of the wound response in Arabidopsis, an antagonistic interaction between JA and ET in the activation of local responses (in the damaged tissues) has been described (Rojo et al., 1999).

Understanding hormonal cross talk is thus essential to elucidate how plants activate the correct set of responses to a particular stress. The first task to achieve this goal is to identify the molecular components of signaling pathways, mainly those implicated in the cross talk regulation.

The biosynthetic (octadecanoid) pathway of JAs from linoleic and linolenic acid is being thoroughly studied, and much information about the type and subcellular localization of the

[1] To whom correspondence should be addressed. E-mail rsolano@cnb.uam.es; fax 0034-91-5854506.

The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (www.plantcell.org) is: Roberto Solano (rsolano@cnb.uam.es).

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enzymes involved is now available (Mueller, 1997; Berger, 2002; Turner et al., 2002). However, in spite of their relevance as plant growth and stress regulators, the current knowledge about the JA signaling pathway is limited. The few signaling components described so far have been identified in most cases by mutant screens for plants displaying either reduced sensitivity to JA, methyl JA, and/or the JA analog coronatine or constitutive or enhanced response to JA (Turner et al., 2002).

Among the identified mutants in Arabidopsis, *coronatine insensitive1* (*coi1*) is fully insensitive to JA, and the COI1 protein is required for all JA-dependent responses tested so far (Feys et al., 1994; Xie et al., 1998). COI1 has been molecularly identified as an F-box protein, suggesting the involvement of ubiquitin-mediated protein degradation in JA signaling (Xie et al., 1998). This hypothesis has been further supported by the demonstration that COI1 is present in a functional SKIP-CULLIN-F-box-type E3 ubiquitin ligase complex. Moreover, plants deficient in other components of SKIP-CULLIN-F-box complexes also show impaired JA responses (Devoto et al., 2002; Xu et al., 2002; Feng et al., 2003). The existence of a conserved COI1 function in other species has been demonstrated recently by the identification of a *COI1* homolog in tomato (*Lycopersicon esculentum*) (*LeCOI1*). Interestingly, mutations in *LeCOI1* demonstrated that, at least in this species, JA is involved in developmental processes, such as ovule and trichome development, which are not impaired in the Arabidopsis *coi1* mutant (Li et al., 2003).

In addition to *coi1*, other JA-insensitive mutants have been described, including *jar1* (*methyl-jasmonate resistant1*; Staswick et al., 1992), *jin1* and *jin4* (*methyl-jasmonate insensitive1/4*; Berger et al., 1996), and *jue1*, *jue2*, and *jue3* (*jasmonate underexpressing1/2/3*; Jensen et al., 2002). All alleles of these mutants show weaker phenotypes than *coi1*, suggesting that the corresponding genes are not required for all JA responses and/or that these mutations may affect members of gene families with partially redundant functions. Among these JA-insensitive mutants, only *jar1*, which is allelic to *jin4*, has been identified at the molecular level. *JAR1* encodes an enzyme with JA adenylation activity, indicating that this modification modulates JA signal transduction (Staswick et al., 2002).

Mutants with constitutive or enhanced responses to JAs have also been described and include *cev1* (*constitutive expression of vsp1*), *cex1*, *cet1/9*, and *joe1/2* (Ellis and Turner, 2001; Hilpert et al., 2001; Xu et al., 2001; Jensen et al., 2002). Among them, only *cev1* (Ellis and Turner, 2001) has been molecularly identified. The phenotype of this mutant is a result of an increased production of JAs and ET that can be suppressed by mutations that block these signaling pathways (*coi1* and *etr1*). *CEV1* encodes a cellulose synthase, indicating that the cell wall may be involved in stress signaling (Ellis et al., 2002).

Finally, gain-of-function experiments in transgenic Arabidopsis have shown that two ERF transcription factors (ORCA3 and ERF1; van der Fits and Memelink, 2000; Lorenzo et al., 2003) regulate the expression of JA-inducible genes.

Although the above-mentioned screens have identified some steps in JA signaling, our knowledge of this pathway is still very limited. Thus, the identification and characterization at the molecular level of new components is essential to get a deeper insight into this pathway.

Here, we report on the identification of five *jasmonate-insensitive* (*jai*) loci and on the molecular characterization of one of them, *jai1*/*jin1*. We show that *JIN1* encodes a nuclear-localized helix-loop-helix-leucine zipper (bHLHzip)-type transcription factor (AtMYC2) that differentially regulates two branches in the JA signaling pathway. One of these branches, positively regulated by AtMYC2, induces the expression of genes involved in the response to wounding (mechanical or biotic). The other branch, negatively regulated by AtMYC2, is required for the expression of pathogen defense genes. Moreover, we show that these two branches are also differentially regulated by ERF1. Therefore, the interplay between AtMYC2 and ERF1 may explain, at the molecular level, how plants select the correct response to two different albeit related stresses, pathogen attack or wounding.

RESULTS

Identification of *jai* Mutants

It has been demonstrated previously that ET represses some JA-regulated responses to stress (Rojo et al., 1999). We therefore reasoned that elimination of ET sensitivity could enhance some plant responses to JA. To test this idea, we compared the sensitivity to JA of ET-insensitive mutants with that of wild-type (Columbia-0 [Col-0]) plants. As shown in Figure 1A, ET-insensitive *ein3-3* mutants grown in plates containing JA show an increased response to the hormone compared with wild-type plants, as observed by the enhanced accumulation of anthocyanins in the aerial parts of the mutant plants and the enhanced inhibition of root growth.

Based on this observation, we developed a screening strategy to find novel *jai* mutants. Using an *ein3-3* mutant background, different media and JA concentrations were tested to establish the growth conditions in which the largest differences between the *ein3-3* parental plants and the strong JA-insensitive *coi1* mutant could be observed in response to JA. In these conditions, mutants with a weaker phenotype than *coi1*, not easily detectable in other media, were identified (see below).

A screen of 150,000 M2 seedlings from 30,000 M1 ethyl methanesulfonate (EMS)-mutagenized *ein3-3* plants, yielded 32 M2 new putative *jai* mutants. M3 seeds from only 11 of these putative mutants were recovered and confirmed to be insensitive to JA; all showing a phenotype intermediate between *coi1* and the *ein3-3* parental plants. The remaining 21 putative mutants showed a much stronger phenotype, including sterility. This developmental defect has also been described for the strong JA-insensitive *coi1* mutant (Feys et al., 1994). In fact, crosses between seven of these *jai* mutants and a heterozygous *coi1* rendered F1 plants that segregated *coi*:wild-type in a 1:1 ratio, indicating that they represented new alleles of *coi1*. This was further supported by the recessive nature of all seven mutants (in backcrosses with the parental *ein3-3* and with Landsberg *erecta* [L*er*] wild-type plants) and by their map position near COI1 (Figure 1B; data not shown).

Genetic Characterization of *jai* Mutants

Backcrosses of the 11 remaining *jai* mutants with their parental plants (*ein3-3*; Col-0 background) yielded F1 and F2 segregation

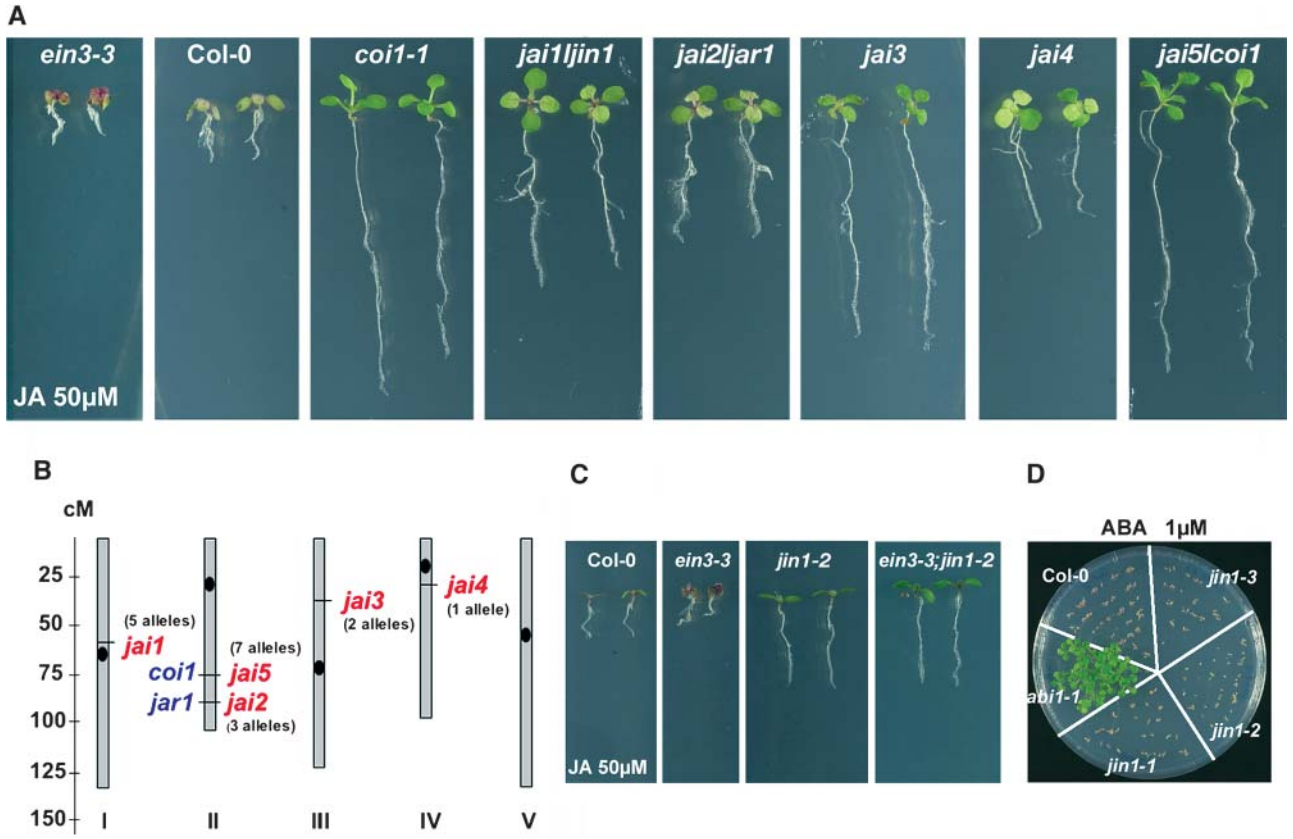

Figure 1. Identification and Characterization of *jai* Mutants.

(A) Sensitivity to JA of the ET-insensitive *ein3-3* mutant, wild-type (Col-0), *coi1-1*, and alleles of all five complementation groups (*jai1*/*jin1* to *jai5*/*coi1*) grown for 10 d on agar plates supplemented with 50 μM JA.

(B) Chromosomal position of the five identified loci (*jai1*/*jin1*, *jai2*/*jar1*, *jai3*, *jai4*, and *jai5*/*coi1*). The position of *jar1* and *coi1* is also shown. cM, centimorgan.

(C) Phenotypic comparison of the JA sensitivity of *jin1-2* in wild-type and *ein3-3* backgrounds. Plants were grown for 7 d on plates containing 50 μM JA.

(D) Sensitivity of *jin1* alleles (in wild-type background) to ABA. Seeds were germinated on plates containing 1 μM ABA, and the ABA-insensitive *abi1-1* mutant and wild-type plants were included for comparison.

ratios consistent with single nuclear traits being responsible for all 11 mutant phenotypes, scored as reduction of root-growth inhibition in the presence of JA. All F1 seedlings from the backcrosses of four of these mutants had a wild-type (parental) phenotype, and the F2 obtained from the self-pollination of these F1s segregated mutant plants in a 1:3 ratio (mutant:wild type), indicating that all these mutations had a recessive character. In the other seven mutants, all F1 seedlings from the backcross displayed an intermediate root-length phenotype between mutant and parental plants, and the corresponding F2s segregated three different phenotypic classes in a 1:2:1 ratio (mutant: intermediate:wild type). These mutations were thus considered to be semidominant. Complementation tests among mutants in each group demonstrated that all semidominant mutants belonged to two independent complementation groups, named *jai1* (five alleles) and *jai3* (two alleles). The recessive mutants also belonged to two independent complementation groups, *jai2* (three alleles) and *jai4* (one allele). Figure 1A shows the phenotype of all five complementation groups (*jai1* to *jai5*) grown in the presence of JA, compared with that of the wild type, *ein3-3*, and *coi1-1*. As scored by JA-dependent inhibition of root growth, *jai5*/*coi1* has the strongest JA-insensitive phenotype, followed by *jai3* and *jai1*, whereas *jai2* and *jai4* represent the weakest mutations.

Consistent with the complementation tests, genetic mapping of the five loci yielded five independent chromosomal positions: *jai1* is located in chromosome I (Chr I) close to the centromere; *jai2* and *jai5* localize to the bottom arm of Chr II near *jar1* and *coi1*, respectively; *jai3* mapped in the top arm of Chr III; and *jai4* in Chr IV (Figure 1B).

The proximity of the map position of *jai2* to that described for *jar1* (encoding a JA adenylase) in the bottom arm of Chr II suggested their possible allelism. Complementation tests between these mutants confirmed this possibility because all crosses performed rendered only F1 plants with mutant phenotype, and no wild-type phenotypes were detected in the F2 progenies.

No JA-related mutants have been described near the chromosomal positions determined for *jai3* and *jai4*, indicating that

both represent new loci required for JA signaling. The cloning and molecular characterization of these two new mutants is underway.

In this work, the *jai1* mutation was characterized in more detail. Two JA-related mutants have been described in Chr I, *axr1*, and *jin1*. *axr1* is located on top of Chr I, far away from the map position of *jai1*. Because a more precise map position of *jin1* was not known, we tested whether it could be allelic to *jai1*. Crosses between different alleles of *jai1* (*jai1-1*, *jai1-2*, and *jai1-3*) and *jin1* rendered 100% of F1 and F2 plants with the mutant phenotype, indicating that, indeed, *jin1* is allelic to *jai1*. Therefore, we renamed all our alleles as *jin1-2* to *jin1-6*.

Sensitivity of *jin1* Alleles to JA and to Other Hormones

Comparisons of the (JA-dependent) root-growth inhibition in response to JA demonstrated that all *jin1* alleles show a similar JA-insensitive phenotype intermediate between wild-type and *coi1*, with only little allelic differences (data not shown).

When segregated from the *ein3-3* background (see Methods), *jin1-2* showed a similar phenotype to *jin1-2*;*ein3-3*, and this is also the case for most of the identified mutants (Figure 1C; data not shown). Therefore, because *ein3* is more sensitive to JA than wild-type plants, the phenotypic differences between mutant and parental plants are greater in the *ein3-3* than in wild-type background, demonstrating that the use of the *ein3* background favored the identification of the mutants in our screening conditions. Moreover, in at least one case (*jai4*), the presence of *ein3-3* enhances its JA-insensitive phenotype, which is very weak in the wild-type background and easily distinguishable in the *ein3-3* background (data not shown; O. Lorenzo and R. Solano, unpublished data). Therefore, this result constitutes a proof of concept for the rationale of the screening.

Because root growth and anthocyanin accumulation are affected by other hormones, the specificity of *jin1* in the JA-signaling pathway was addressed by testing the sensitivity of all alleles to other hormones. To this end, alleles of *jin1* as well as *ein3-3* and Col-0 plants were germinated on plates containing 10 μM 1-aminocyclopropane-1-carboxylic acid, 2 μM indoleacetic acid, 0.3 μM naphtalenacetic acid, 1 μM kinetin, and different concentrations of ABA (1 and 3 μM). No significant differences in seedling development were observed in these assays between *jin1* alleles and their corresponding parental plants (Col-0 or *ein3-3* in the double mutants), suggesting that JIN1 is a specific regulator of JA responses. The ABA response is shown in Figure 1D as an example.

jin1 Plants Show Increased Resistance to Necrotrophic Pathogens

JA has been described as an essential signal in the activation of defenses to necrotrophic pathogens (Turner et al., 2002). To further characterize JA-dependent responses in *jin1* plants, the susceptibility of different *jin1* alleles to necrotrophic fungi, such as *Botrytis cinerea* and *Plectosphaerella cucumerina*, was tested. Four-week-old wild-type plants, *coi1* and *ein3-3* mutants, as well as *jin1* alleles in wild-type or *ein3-3* backgrounds

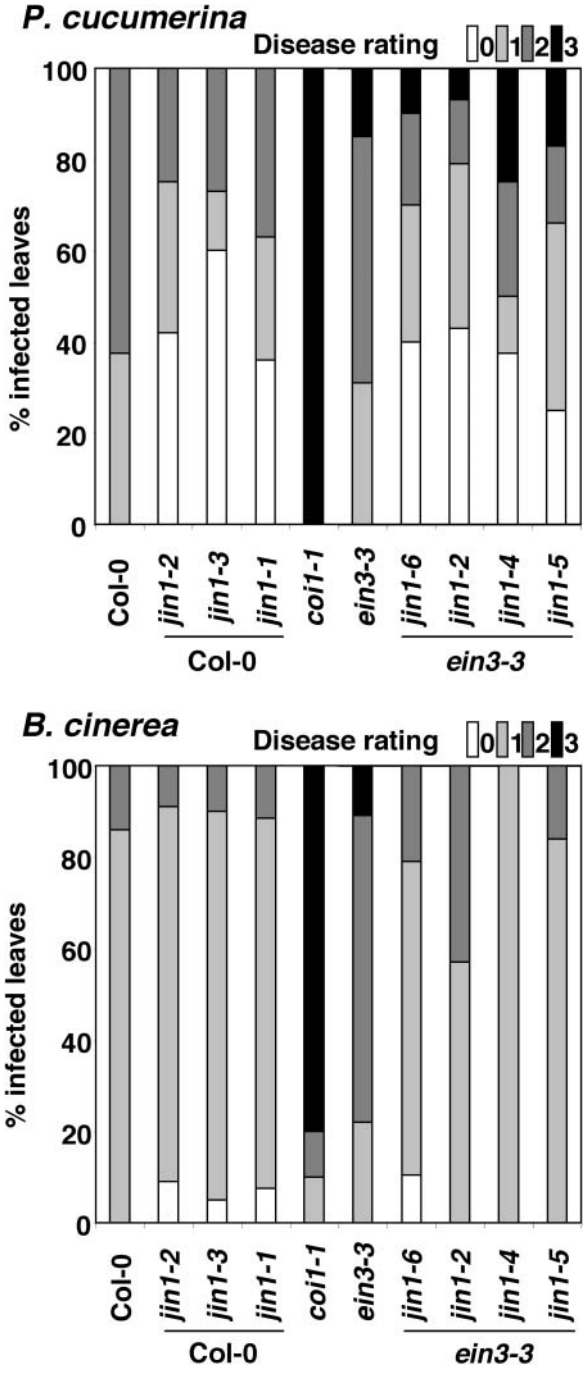

Figure 2. Susceptibility of *jin1* Alleles to Necrotrophic Fungi.

Graphical representation of disease symptoms 10 d after inoculation of the leaves with 2×10^6 spores/mL of *P. cucumerina* (top graph) or 5 d after inoculation of the leaves with 10^5 spores/mL of *B. cinerea* (bottom graph). Four-week-old plants of *jin1* alleles in wild-type (Col-0) or *ein3-3* backgrounds were used as well as the indicated mutants and wild-type plants. Disease rating was determined at the indicated days as described in Methods (0, resistance; 3, highly susceptible). Data values represent one of three independent experiments with similar results.

were challenged with either of these fungi, and infection symptoms scored 5 or 10 d after inoculation, respectively. In contrast with wild-type plants, *coi1* mutants were heavily affected upon infection of either fungi, with necrotic lesions spreading throughout most of the surface of the infected leaves (Figure 2; Thomma et al., 1998; Berrocal-Lobo et al., 2002). As previously described, *ein3-3* mutants also showed an increased sensitivity to both fungi compared with wild-type plants (Berrocal-Lobo et al., 2002). In contrast with *coi1* and in spite of the defect in JA signaling, all *jin1* alleles tested, either in wild-type (Col-0) or *ein3-3* backgrounds, showed reduced infection symptoms compared with their corresponding parental strains (Col-0 or *ein3-3*; Figure 2). The decrease was low but highly reproducible because similar results were obtained in three independent assays. These results indicate that although JIN1 is required for some JA-dependent responses (root-growth inhibition or anthocyanin accumulation), it actually represses others, such as resistance to necrotrophic pathogens. Hence, JIN1 may have opposite effects in the regulation of different JA-mediated responses.

JIN1 Differentially Regulates Two Branches in the JA Pathway

To further understand these contrasting phenotypes of *jin1* mutants, we analyzed the expression of JA-regulated genes in alleles of *jin1* by RNA gel blot analysis. Because JA regulates different defense responses, for example, responses to necrotrophic pathogens and to wounding (mechanical or biotic), we monitored the expression of genes activated by JA in response to these two stresses.

As shown in Figure 3, the JA-induced expression of three marker genes of the wound response (*VSP2*, *LOX3*, and *TAT*) was largely prevented in two independent alleles of *jin1* (*jin1-1* and *jin1-2*) compared with wild-type plants. This result indicates that JIN1 is required for JA-dependent transcriptional regulation, likely participating in the activation of the wound response.

However, in agreement with the enhanced resistance of *jin1* to necrotrophic fungi, we found the opposite behavior in the case of the pathogen-responsive genes. As shown in Figure 3, the JA-induced expression of *PR4*, *PR1*, and *PDF1.2* was even greater in the mutant alleles than in the wild-type plants. An extreme example is the case of *PDF1.2*. Although the expression of this gene is clearly induced by JA treatment in plants grown in soil, it is only weakly induced by JA in plants grown in agar plates. Even in these suboptimal conditions, the induction by JA of PDF1.2 in the *jin1* mutants is very high (Figure 3). This result helps to explain at the molecular level the increased resistance of *jin1* alleles to necrotrophic pathogens and suggests that JIN1 normally represses responses to pathogens.

In summary, the *jin1* mutation prevents the activation of some JA-induced genes (at least those involved in JA-mediated plant defense against insects, herbivores, or mechanical damage), while it enhances the expression of other JA-induced genes that are related to defense responses against pathogens. Hence, these data illustrate the existence of at least two branches in the JA signaling pathway that are differentially regulated by JIN1.

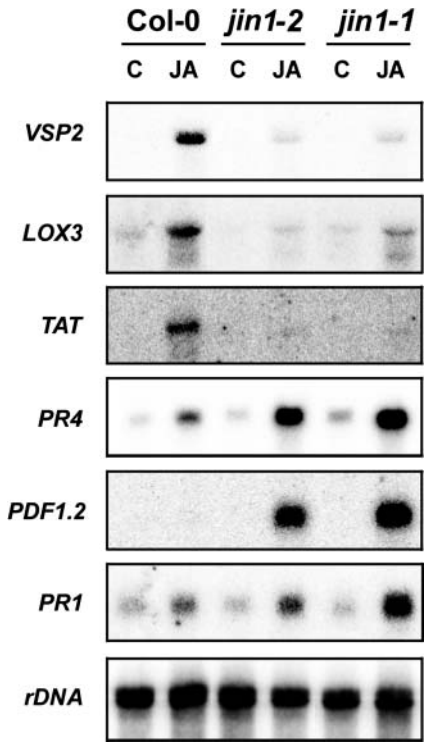

Figure 3. RNA Gel Blot Analysis of the Expression of JA-Regulated Genes in *jin1* Alleles Compared with Wild-Type Plants.

Fourteen-day-old wild-type plants and *jin1-1* and *jin1-2* mutants were not treated (C) or treated with 50 μM JA during 6 h. Ten micrograms of total RNA were loaded per lane, and the blot was hybridized with the indicated probes and *rDNA* as loading control.

jin1 Encodes the AtMYC2 Transcription Factor

Cleaved-amplified polymorphic sequence and simple sequence length polymorphism (SSLP)-assisted analysis was used to refine the aforementioned chromosomal position of *jin1* and identify the mutated gene (see Methods). The analysis of 1600 F2 segregants of the mapping population described above restricted the location of the mutant to an interval comprised of seven BACs (F27G20, F5D14, T9G5, F6N18, F9L11, T9L6, and T16O9; see Supplemental Figure S1 online). The presence of the centromere close to this region, or possible rearrangements within the region, may be responsible for the low recombination rates observed that prompted us to design new strategies for the identification of the mutated gene. With this aim, we started three different approaches in parallel: (1) the construction of a contig of TACs that would allow the direct complementation of the mutant (see Supplemental Figure S1 online), (2) RNA gel blot, DNA gel blot, and sequence analyses of candidate genes, and (3) identification of insertional mutants in candidate genes. As candidates we first looked for those genes whose expression would be regulated by JA in wild-type plants. As a result of this combined strategy, we identified a defect in the JA-induced expression of one such candidate gene, *AtMYC2*, in some of the alleles, as well as an RNA band of smaller size in one allele (*jin1-1*) (Figures 4B

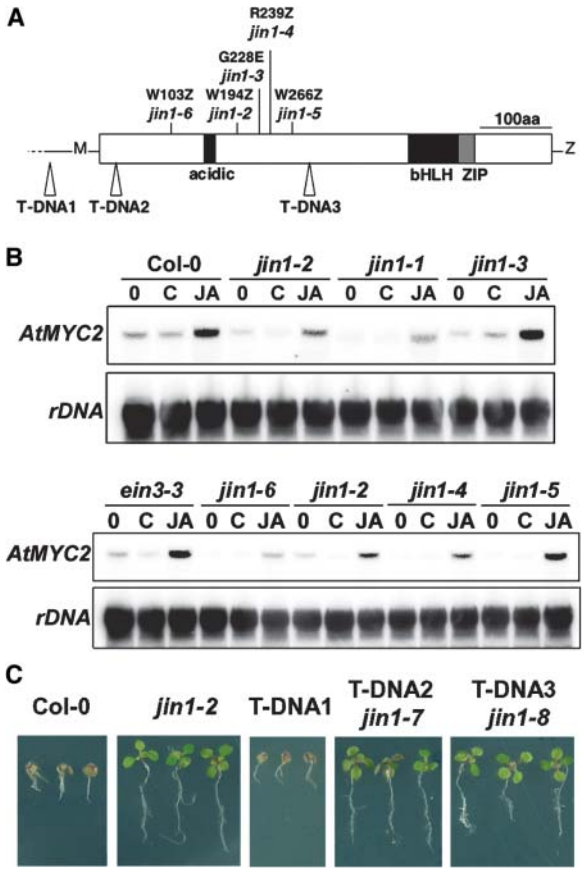

Figure 4. Molecular Identification of *JIN1* by Positional Cloning.

(A) Schematic representation of point mutations and T-DNA insertions in the *jin1* alleles. Domains in the protein are indicated. aa, amino acids; M, Met; Z, stop codon.

(B) RNA gel blot analysis of the defect in the level of JA-induced expression of *AtMYC2* in *jin1* alleles. All alleles in the top blot are in wild-type background, whereas the alleles in the bottom blot are in the *ein3-3* background. Fourteen-day-old wild-type Arabidopsis seedlings were treated with water (C), 50 μM of JA, or nontreated (0) for 15 min. Twenty micrograms of total RNA were loaded per lane, and blots were hybridized with *AtMYC2* probe and *rDNA* as loading control.

(C) JA-insensitive phenotype of T-DNA insertion lines compared with wild-type (Col-0) and *jin1-2* mutant grown on agar plates containing 50 μM JA.

and 7B). DNA gel blot analysis showed the absence of a band corresponding to part of *AtMYC2* in *jin1-1* (data not shown), whereas sequence analysis identified point mutations in all other *jin1* alleles (Figure 4A). In most cases, these point mutations generate stop codons that would eliminate a high portion of the coding region. Further confirmation of *AtMYC2* being the mutated gene was obtained by analysis of three independent insertional mutants from the SALK collection obtained from Nottingham Arabidopsis Stock Centre (T-DNA1:SALK_039235, T-DNA2:SALK_040500, and T-DNA3:SALK_061267; Alonso et al., 2003). As shown in Figure 4C, two of these three lines, those with the T-DNA insertion inside the coding region (SALK_040500 in amino acid Asn^{18} and SALK_061267 in amino acid Ser^{290} that we renamed *jin1-7* and *jin1-8*) had a JA-insensitive phenotype similar to the EMS *jin1* alleles. Taken together, these data demonstrate that *AtMYC2* is the gene mutated in the alleles of *jin1*.

As shown in Figure 4A, AtMYC2 contains a bHLH domain characteristic of the MYC family of transcription factors from amino acid 447 to 496. This domain is followed in AtMYC2 by a Leu zipper domain (from amino acid 497 to 525). A short stretch of acidic amino acid that could conform a putative activation domain is also present in this protein (from amino acid 154 to 165; Figure 4A).

Constitutive Expression of AtMYC2 Confers Hypersensitivity to JA and ABA

Transgenic plants that constitutively express the full-length AtMYC2 (*35S:AtMYC2*) were obtained in wild-type and mutant backgrounds (*jin1-1* and *jin1-2*) to elucidate whether AtMYC2 may be sufficient to activate the JA pathway or at least would complement the mutant phenotypes. Because *AtMYC2* has also been described as an ABA response factor (RD22BP1) that regulates the expression of the ABA-responsive gene *RD22* (Abe et al., 1997), we also tested whether AtMYC2 would be sufficient to activate the ABA pathway. Several independent T2 transgenic lines in each genetic background were plated on media without or with different concentrations of JA or ABA. Root-growth inhibition and anthocyanin accumulation induced by JA and inhibition of seed germination by ABA in the transgenic lines was compared with that of the corresponding parental plants (wild type, *jin1-1*, and *jin1-2*). As shown in Figures 5A and 5C, constitutive expression of AtMYC2 in the wild-type background induced an exaggerated response to both JA and ABA that correlated with the level of AtMYC2 expression (Figure 5B). In the case of JA, transgenic plants showed an enhanced inhibition in growth, not only of the root but also of the aerial parts, and an increased accumulation of anthocyanins (Figure 5A; data not shown). In the case of ABA, seed germination was largely reduced even in concentrations as low as 0.3 μM (Figure 5C). Constitutive expression of *AtMYC2* in the *jin1-1* and *jin1-2* mutant backgrounds not only complemented the mutation but also enhanced the response to both JA and ABA to the same level observed in the wild-type background (Figures 5A and 5C).

However, in the absence of any treatment, all transgenic lines showed a phenotype indistinguishable from their corresponding parental plants (wild type, *jin1-1*, and *jin1-2*; Figure 5C; data not shown), indicating that AtMYC2 alone is not sufficient to activate the JA or ABA pathways, and additional factors induced by the corresponding treatments are also required, together with AtMYC2, for the activation of these pathways.

Subcellular Localization of AtMYC2

The cellular localization of AtMYC2 and its possible hormonal regulation were investigated using C-terminal green fluorescent protein (GFP) fusions of full-length AtMYC2 and a truncated derivative corresponding to the predicted protein in *jin1-2*.

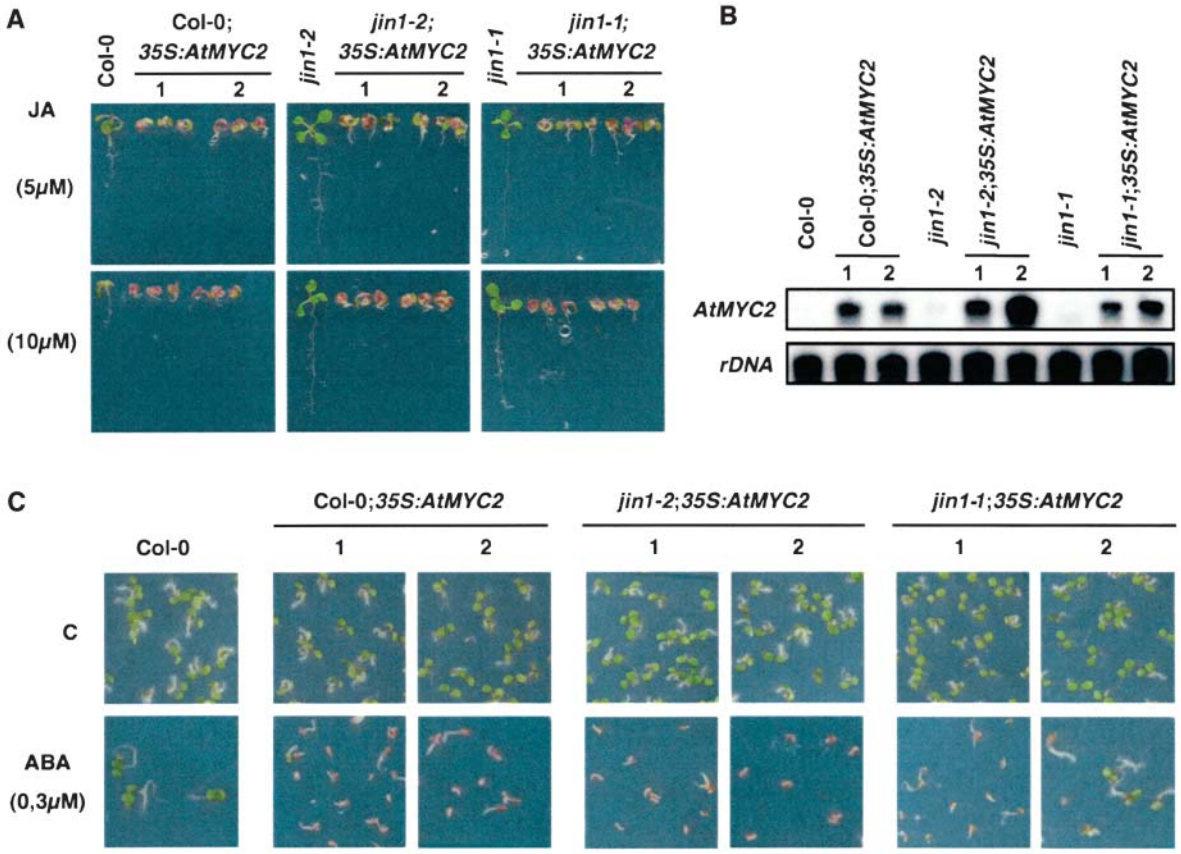

Figure 5. Constitutive Expression of AtMYC2.

(A) Root growth inhibition and anthocyanin accumulation in two independent transgenic lines in each background (Col-0;*35S:AtMYC2*, *jin1-1*;*35S:AtMYC2*, and *jin1-2*;*35S:AtMYC2*) compared with parental plants (wild-type, *jin1-1*, and *jin1-2*) in the presence of different concentrations of JA (5 and 10 μM).

(B) RNA gel blot analysis of the expression of *AtMYC2* in the transgenic lines. Total RNA was extracted from 10-d-old wild-type plants, *jin1-1* and *jin1-2* mutants, and two independent transgenic lines in each genetic background (Col-0;*35S:AtMYC2*, *jin1-1*;*35S:AtMYC2*, and *jin1-2*;*35S:AtMYC2*). Ten micrograms of total RNA were loaded per lane, and the blot was hybridized with the *AtMYC2* probe and *rDNA* as loading control.

(C) Effect of ABA (0.3 μM) in seed germination and seedling development of 6-d-old wild-type Arabidopsis plants and two independent T2 transgenic lines (1 and 2) in each genetic background. C, control plants (no ABA treatment).

Transient expression of these constructs in BY2 tobacco (*Nicotiana tabacum*) cells and detached Arabidopsis leaves from wild-type plants and *coi1* mutants (Figure 6; data not shown) demonstrated that, consistent with its role as a transcription factor, the full-length protein localized to the nucleus. However, the truncated version, missing the bHLHzip domain, showed a cellular localization similar to that of the native GFP, suggesting that this protein lacks the nuclear localization signal(s).

Similar results were obtained when wild-type or *coi1* leaves had been pretreated for 2 h with either JA (50 μM), ABA (100 μM), or both hormones simultaneously, suggesting that the nuclear localization of the protein is not hormonally regulated (at least by JA or ABA).

Hormonal and Stress Regulation of *AtMYC2* Expression

The expression of *AtMYC2* in response to JA and ABA was analyzed in alleles of *jin1* and their corresponding parental plants (Col-0 and *ein3-3* mutants). As shown in Figure 7A, *AtMYC2* expression is rapidly induced in wild-type plants by both JA and ABA either alone or in combination. This induction is transient in the case of ABA with *AtMYC2* mRNA returning to basal levels 6 h after treatment. The induction by JA is more steady and still clearly visible 6 h after JA treatment. In the *jin1* alleles tested, *AtMYC2* expression was also induced by both treatments (JA and ABA) independently of the background (Col-0 or *ein3-3*). A lower level of mRNA accumulation was, however, detected in most of the alleles. In addition, a lower migrating band was detected in *jin1-1*, likely as a consequence of the genomic deletion in this allele (Figures 4B and 7B; data not shown).

No synergism was observed between both treatments, suggesting that activation of *AtMYC2* expression by both hormones may occur through the same mechanism. To test this idea, the expression of *AtMYC2* was analyzed in mutant backgrounds impaired in JA or ABA signaling (*coi1* and *abi1*, respectively). As

shown in Figure 7B, JA or ABA treatments induced *AtMYC2* expression in *abi1-1* to a similar level than in wild-type plants. In *coi1* mutants, however, JA or ABA treatment did not induce *AtMYC2* expression. These results indicate that activation of *AtMYC2* expression by JA and ABA occurs through signaling pathways that share at least one of their components, COI1, but is independent of ABI1. Therefore, the simplest explanation of these results is that ABA regulates *AtMYC2* expression by the activation of the JA pathway.

Because gene expression analysis in the *jin1* alleles suggested that AtMYC2 is a regulator of JA-dependent wound responses, we also tested whether *AtMYC2* expression is regulated by mechanical damage. As shown in Figure 7C, *AtMYC2* expression is rapidly upregulated by wounding both in damaged (local) and nondamaged (systemic) tissues, further supporting a role for AtMYC2 in the activation of wound responses. Consistent with this hypothesis, the wound induction of *AtMYC2* precedes that of *VSP*, one of its likely targets as shown by the gene expression analysis (Figure 7C).

ERF1 Represses Wound-Inducible Gene Expression Downstream of AtMYC2

The pathogen-response genes repressed by AtMYC2 are known to be regulated by ERF1 (Lorenzo et al., 2003). It has also been shown that ET represses local responses to wounding (Rojo et al., 1999). To get a further insight into the molecular mechanisms underlying these mutually exclusive (antagonistic) responses, we tested whether the ET-mediated repression affects *AtMYC2* expression or its downstream targets and whether it is executed through ERF1. Expression of *AtMYC2* and *VSP2*, a putative downstream target of AtMYC2, was monitored by RNA gel blot analysis after JA treatment in three independent

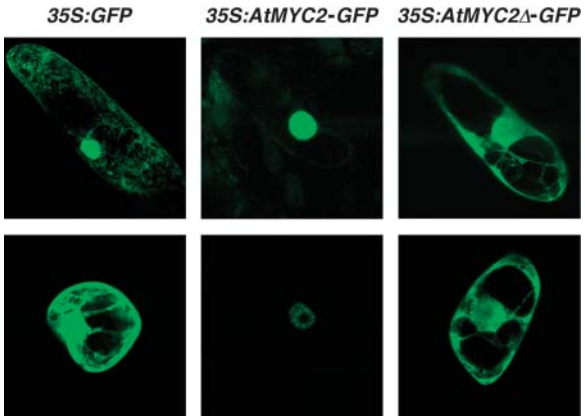

Figure 6. Subcellular Localization of AtMYC2 in Transiently Transformed BY2 Tobacco Cells.

Constructs delivered correspond to C-terminal GFP fusions of full-length AtMYC2 (AtMYC2-GFP) and a truncated version corresponding to the mutation in the *jin1-2* allele (AtMYC2Δ-GFP). The three top panels correspond to a longitudinal view of the cells, whereas the bottom panels represent transversal views. Nuclear and cytosolic distribution of the GFP protein alone is shown as control.

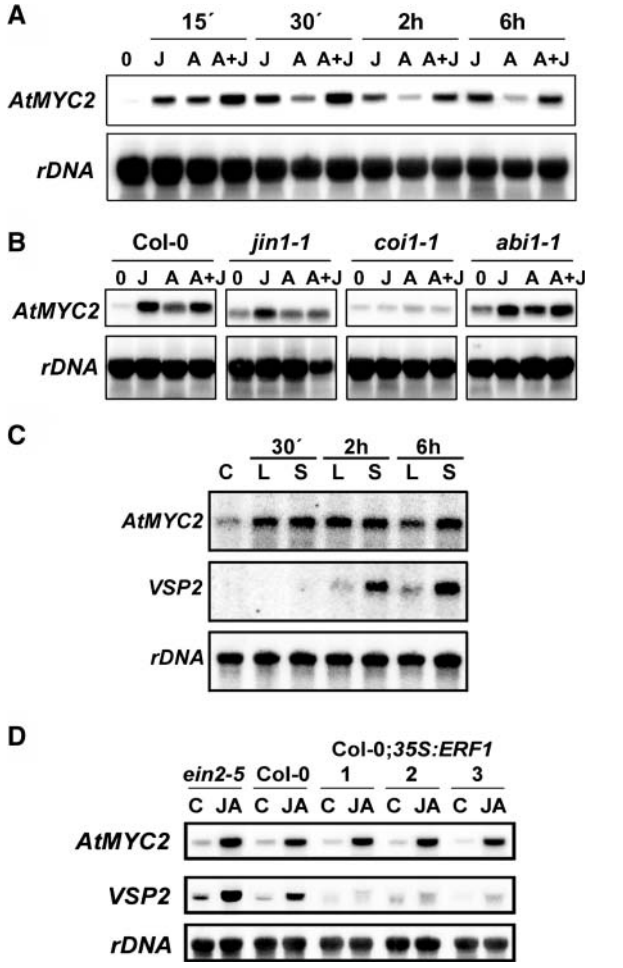

Figure 7. Stress and Hormonal Regulation of *AtMYC2* Expression.

(A) RNA gel blot analysis of the induction of *AtMYC2* expression by JA and ABA. Ten-day-old wild-type Arabidopsis seedlings were treated with either 50 μM JA (J), 100 μM ABA (A), both (A+J), or nontreated (0) and tissue collected at the indicated times. Twenty micrograms of total RNA were loaded per lane, and blots were hybridized with *AtMYC2* probe and *rDNA* as loading control.

(B) RNA gel blot analysis of *AtMYC2* induction by JA and ABA in different mutant backgrounds. Four-week-old wild-type plants and *coi1*, *jin1-1*, and *abi1-1* mutants were treated with either 50 μM of jasmonic acid (J), 100 μM of ABA (A), both (A+J), or nontreated (0) and tissue collected after 30 min of treatment. Twenty micrograms of total RNA were loaded per lane, and blots were hybridized with the *AtMYC2* probe and *rDNA* as loading control.

(C) RNA gel blot analysis of *AtMYC2* and *VSP2* induction by wounding. Three-week-old wild-type plants were wounded, and samples from control leaves (C), local (L) damaged leaves, and systemic (S) nondamaged leaves were collected at the indicated times after wounding.

(D) RNA gel blot analysis of the induction of *AtMYC2* and *VSP2* expression in 14-d-old wild-type, *ein2-5*, and three independent transgenic lines constitutively expressing *ERF1*, treated with JA (50 μM) for 30 min.

transgenic lines constitutively overexpressing *ERF1* (Lorenzo et al., 2003) as well as wild-type plants and *ein2-5* mutants. As shown in Figure 7D, the lack of ET sensitivity in the *ein2-5* mutant or the constitutive activation of the ET pathway in the transgenic *ERF1*-expressing lines had little effect on the induction of *AtMYC2* expression by JA, if at all. These results are consistent with the lack of local (ET-dependent) repression of *AtMYC2* expression by wounding (Figure 7C). However, JA induction of *VSP2* expression was largely prevented in all three *ERF1*-expressing lines. Taken together, these results suggest that ET repression of wound-inducible genes occurs downstream of *AtMYC2* and is executed through ERF1.

DISCUSSION

In spite of the importance of JAs as plant growth and stress regulators, their signaling pathway is still poorly understood. Although several screens for JA-related mutants in Arabidopsis have been performed in many laboratories, only a low number of mutants have been described and characterized so far (see Introduction). In this work, we designed a new search strategy that exploited the cross talk between ET and JA signaling pathways, with the idea that alterations in one of them may influence the sensitivity of the plant to the other. The result has been the identification of five loci involved in JA sensitivity in the ET-insensitive *ein3-3* background. For all mutations, the phenotypic differences with the parental plant are larger in the *ein3-3* than in wild-type background. This result serves as a proof of concept and demonstrates that alterations in the genetic constitution of an individual may enhance, and thus help to uncover, phenotypes not detectable in the wild-type plant. This concept, commonly used in other research systems (i.e., Drosophila), has been largely unexploited in plants so far.

In this work, the gene altered in one of the five loci described, *jin1*, has been identified by positional cloning and found to encode AtMYC2, a member of the bHLHzip family of transcription factors. All alleles of *jin1* showed a decreased sensitivity to JA compared with wild-type plants, as shown by the reduction in root-growth inhibition and anthocyanin accumulation by JA and by the defect in JA-regulated gene expression, demonstrating that AtMYC2 is required for JA responses in Arabidopsis. Gain-of-function experiments also supported the key role of this protein in the JA pathway because constitutive expression of *AtMYC2* renders transgenic plants hypersensitive to the hormone. Nevertheless, overexpression of *AtMYC2* does not promote a constitutive response to JA in the absence of the hormonal signal, suggesting that additional JA-regulated factors cooperate with AtMYC2 in the activation of the responses to this hormone.

Although most *jin1* alleles seem to be loss-of-function or severely hypomorphic mutants (the mutations result in most cases in stop codons that eliminate a large portion of the coding region, and *jin1-7* and *jin1-8* are T-DNA insertions in amino acids Asn^{18} and Ser^{290}, respectively), the phenotype of this mutant is, however, weaker than that of *coi1*. This may be explained by (partial) functional redundancy of AtMYC2-related proteins. In fact, AtMYC2 shares a high sequence similarity with three other MYC proteins in the Arabidopsis genome (At4g17880, At5g46760, and At5g46830), and a dominant mutation in one of these proteins (*atr2D*) promotes a constitutive high-level expression of *PDF1.2* (Smolen et al., 2002), suggesting that AtMYC2 and ATR2 may share common targets. Loss-of-function mutations in these three genes are currently being analyzed in our laboratory. Preliminary tests of these mutants do not show clear defects in their response to JA; therefore, generation and analysis of double or multiple mutants in combination with *jin1* alleles will be required to assess their role in JA signaling.

In addition to a phenotype weaker than *coi1*, *jin1* mutants do not show a defect in all COI1-dependent responses to JA. For instance, male fertility, a character regulated by JA and impaired in *coi1* (Feys et al., 1994; Turner et al., 2002), is not affected in any of the alleles of this mutant, suggesting that AtMYC2 acts downstream of COI1 in the JA signaling pathway and only regulates a subset of the COI1-dependent responses. Whether any other of the above-mentioned MYC homologs is responsible for this function awaits further characterization.

Although some JA-dependent responses are impaired in *jin1*, as discussed above, other JA-activated responses are enhanced in this mutant. Thus, whereas it has often been shown that JA is necessary and sufficient for resistance to necrotrophic pathogens (Thomma et al., 2001; Turner et al., 2002; Farmer et al., 2003; Rojo et al., 2003), *jin1* alleles exhibit a significant increase in their resistance to pathogens of this type, such as *B. cinerea* and *P. cucumerina*. These results provide evidence for the existence of two branches in the JA signaling pathway that are antagonistically regulated by AtMYC2 (Figure 8). Analysis of JA-regulated gene expression in *jin1* mutants supports this conclusion because AtMYC2 differentially regulates two distinct sets of JA-inducible genes. Consistent with increased resistance to necrotrophic pathogens, *jin1* mutants show enhanced expression (in response to JA) of pathogen-response genes such as *PR4*, *PR1*, and *PDF1.2*. These genes have been previously shown to be regulated by a positive interaction between JA and ET through ERF1 (Figure 8; Penninckx et al., 1996, 1998; Berrocal-Lobo et al., 2002; Lorenzo et al., 2003). The enhanced expression of these ERF1-regulated genes in *jin1* indicates that, indeed, AtMYC2 represses this JA-dependent response. Moreover, this repression by AtMYC2 is likely to occur downstream of ERF1 because expression of this transcription factor is not altered with respect to the wild type in the transcriptional profiles obtained for *jin1* mutants (data not shown).

The second set of genes regulated by AtMYC2 (those positively regulated by this protein and therefore downregulated in the mutant), includes genes such as *VSP2*, *LOX3*, and *TAT*, previously shown to be activated after wounding (because of mechanical damage or insect/herbivore feeding) that are induced by JA alone (Figure 8; Reymond et al., 2000; Cheong et al., 2002; Turner et al., 2002; Farmer et al., 2003; Rojo et al., 2003). Interestingly, in contrast with the cooperation of ET and JA in the regulation of pathogen-defense genes through ERF1, this set of JA-induced genes is negatively regulated by ET (Rojo et al., 1999, 2003; Turner et al., 2002); here, we show that this negative regulation is executed through ERF1. Thus, as in the case of AtMYC2, ERF1 also differentially regulates the two branches of the JA signaling pathway leading to defenses against pathogens or wounding (Figure 8). The transcriptional activation of ERF1

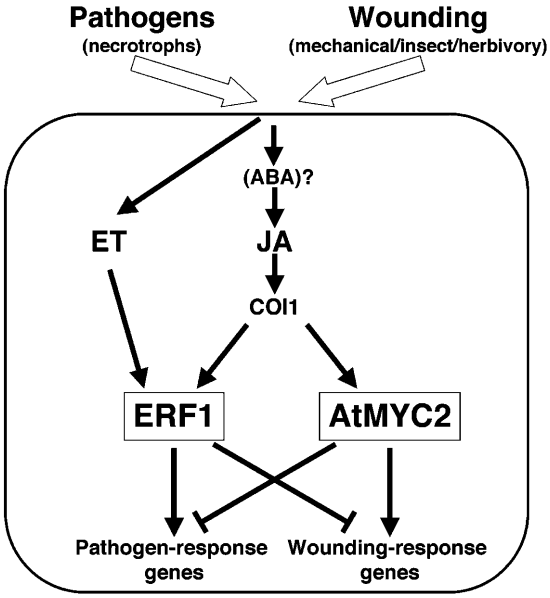

Figure 8. Schematic Representation of the AtMYC2- and ERF1-Dependent Activation of Arabidopsis Responses to Pathogens and Wounding.

Different types of stresses, such as wounding (mechanical or biotic) or necrotrophic pathogen infection, induce the synthesis and subsequent activation of the ET and JA pathways. JA alone will induce the expression of *AtMYC2* that is responsible for the activation of wound-response genes and for the repression of pathogen-response genes. However, the cooperation of the ET and JA signals through the transcriptional induction of ERF1 drives to the activation of pathogen-response genes and to the repression of wounding-response genes (Lorenzo et al., 2003; this article). Therefore, the interplay between ERF1 and AtMYC2 allows the plant selection of the correct set of genes in response to these two stresses.

and AtMYC2 by necrotrophs and wounding, respectively, is consistent with their role in the regulation of each set of responses (Lorenzo et al., 2003; this article). The fact that local induction of *AtMYC2* expression is not impaired in *ERF1*-expressing plants further supports that this repression occurs downstream of AtMYC2. Therefore, local responses to wounding or necrotrophs are mutually antagonistic, and the interplay between AtMYC2 and ERF1 may explain how plants select distinct responses to these two different stresses even though both types of responses are regulated by the same signal molecules (ET and JA; Figure 8). Thus, in our view (Figure 8), the selection by the plant of the correct set of responses to each stress depends on the differential activation of AtMYC2 and ERF1. These transcription factors, which are mutually antagonistic, will activate one set of defense responses and repress the other. Because AtMYC2 and ERF1 do not influence each other's levels of expression, as demonstrated by microarray (data not shown) and RNA gel blot analyses, the mutual antagonism may involve a direct transcriptional repression of each other's targets or the activation by AtMYC2 and ERF1 of repressor molecules acting downstream of them. Alternatively, the antagonism may also be explained by mutual repression of protein activity. Further analysis will be required to discriminate between these possibilities.

All alleles of *jin1*, except *jin1-1* and *jin1-3*, eliminate the bHLHzip motif in AtMYC2, suggesting that the DNA binding activity in this protein is essential to modulate JA responses. This is consistent with the nuclear localization of the AtMYC2-GFP fusion protein. AtMYC2 was shown to recognize a G-box-like element (CATGTG) in the promoter of the ABA-responsive gene *rd22* (Abe et al., 1997). In the case of JA, two G-box-like elements have been previously reported to drive JA-mediated expression of *VSP1* and *PDF1.2* in Arabidopsis (AACGTG, Guerineau et al., 2003; CATGTG, Brown et al., 2003).

Although most alleles of *jin1* seem to be loss-of-function or severely hypomorphic, all alleles tested (*jin1-2*, *jin1-3*, *jin1-4*, and *jin1-5*) were semidominant. In several instances, MYC proteins have been shown to homodimerize and heterodimerize through the HLH domain and also to interact with proteins of the MYB family (Martin and Paz-Ares, 1997; Grotewold et al., 2000; Payne et al., 2000). These MYB–MYC interactions occur through the N-terminal part of the MYC protein and the MYB DNA binding domain. Because all the alleles identified in our screening retain this N-terminal part of the protein, but not the HLHzip domain, it is tempting to speculate that the semidominant character of *jin1* mutations reflects the interference of this truncated protein with its corresponding MYB partner that would lead to the formation of inactive MYC–MYB complexes.

In addition to the existence of different alternative branches in the JA signaling pathway, *jin1* mutants also highlight the existence of a cross talk between JA and ABA pathways. This cross talk has been suggested to influence several physiological and developmental processes (Moons et al., 1997; Carrera and Prat, 1998; Hays et al., 1999). In particular, JA has been shown to inhibit seed germination in several species and to have a synergistic effect with ABA in this process in Arabidopsis (Wilen et al., 1991; Staswick et al., 1992; Ellis and Turner, 2002). Consistent with this synergism, constitutive expression of AtMYC2 in Arabidopsis, which leads to an increased response to JA, also promotes an increased inhibition of germination in response to ABA. Whether this ABA hypersensitivity is a direct effect of AtMYC2 in the activation of the ABA pathway or results from the indirect effect of the enhanced sensitivity to JA remains to be elucidated. Nevertheless, a direct effect of AtMYC2 in the ABA pathway would be consistent with the reported activation of ABA-dependent gene expression in transgenic plants consti tutively expressing AtMYC2 and AtMYB2 simultaneously, as observed by microarray analysis (Abe et al., 2003). However, these microarray data also showed upregulation of JA-responsive genes, such as Myrosinase, Myrosinase-associated protein, or VSP2, indicating that the combination of AtMYC2 and AtMYB2 is also sufficient to induce the expression of a subset of JA-regulated genes.

An insertional allele of AtMYC2 has been reported to be weakly insensitive to ABA (Abe et al., 2003). By contrast, none of our alleles showed differences with the wild type in the insensitivity to this hormone. This apparent discrepancy is likely to be because of the different genetic backgrounds used in both studies (Nossen and Col-0). Alternatively, it may also be because of

allelic differences (no expression of AtMYC2 is detected in their insertional allele) or to the different media/conditions in which insensitivity was analyzed.

A cross talk between JA and ABA pathways is also illustrated by the fact that both hormones induce the expression of AtMYC2. Nevertheless, because induction by ABA depends on COI1, the activation of AtMYC2 by ABA is likely to be mediated by the JA-signaling pathway. Consistent with this, the induction of JA biosynthesis and signaling by ABA in response to wounding has already been reported in several instances (Hildmann et al., 1992; Pena-Cortes et al., 1995; Leon et al., 2001).

In summary, in this work we have identified, cloned, and characterized a novel component of the JA signaling pathway (AtMYC2) that has uncovered the existence of two antagonistic branches differentially regulated by AtMYC2 and ERF1. This conceptual change in our current view of the JA pathway (from linear to branched) is also essential to understand the cross talk between the JA and ET pathways and, thus, to decipher the molecular mechanisms underlying the plant decision to select the correct set of responses to different stresses (pathogens or wounding) that are both mediated by the same signaling pathways, the JA and ET pathways.

METHODS

Biological Materials and Growth Conditions

Arabidopsis thaliana Col-0 is the genetic background for all wild-type, *ein3-3*, *coi1-1*, and transgenic AtMYC2-expressing plants used in this work, except for the *abi1-1* mutants (and their corresponding wild-type control) that are in L*er* background. Plants were grown in vitro (in Johnson's media) and in soil as previously described (Berrocal-Lobo et al., 2002; Lorenzo et al., 2003). The fungal pathogen strains were kindly provided by E. Pérez-Benito (University of Salamanca, Spain; *Botrytis cinerea*), and B. Mauch-Mani (University of Fribourg, Switzerland; *Plectosphaerella cucumerina*). *B. cinerea* and *P. cucumerina* were grown on potato dextrose agar medium (Difco, Detroit, MI) at 28°C for 8 d, and spores were collected in sterile water and stored at −80°C in 20% glycerol. Wound-response experiments were performed as described by Rojo et al. (1999).

Plant Infection with Pathogens

All infections have been performed as previously described (Berrocal-Lobo et al., 2002) except that instead of spray we infected with a 5-μL drop on top of the leaves of the spore suspension containing 10^5, or 2×10^6 spores/mL, of *B. cinerea* and *P. cucumerina*, respectively. Fungal progression and infection symptoms were monitored for 5 to 10 d, and infection ratings from 0 to 3 were assigned to the inoculated plants (0, no infection/necrosis; 1, leaves showing some necrosis; 2, leaves showing severe necrosis; 3, dead/decayed leaves). At least 15 plants per genotype were inoculated in each experiment. Experiments were repeated at least three times with similar results.

Screening Conditions and Mutant Isolation

EMS-mutagenized *ein3-3* M2 seeds were grown on Johnson's media plates containing 0.8% agar, 1% sucrose, and 50 μM JA (Apex Organics, Honiton, UK) at 21°C. Defects in root-growth inhibition by JA were scored after 7 to 12 d of growth, and putative mutant seedlings were transferred to soil. Segregation of the mutants from the *ein3-3* background was performed by backcrossing with Col-0 and identifying the JA insensitive plants that were sensitive to ET in Johnson's media containing 1-aminocyclopropane-1-carboxylic acid (10 μM).

Mapping and Cloning of the *JAI* Loci

Genetic mapping of the mutants was performed by crossing alleles of each of the five complementation groups with the Arabidopsis L*er* ecotype and scoring recombination events in the F2 segregants of the mapping population using SSLP and cleaved-amplified polymorphic sequence markers (Konieczny and Ausubel, 1993; Bell and Ecker, 1994). New SSLP mapping markers on F27G20, F5D14, T9G5, F6N18, F9L11, T9L6, and T16O9 BAC clones (see Supplemental Figure S1 online) were developed based on insertion/deletions identified from the Cereon Arabidopsis polymorphism and L*er* sequence collection (http://www.arabidopsis.org). Genomic DNA corresponding to candidate genes was amplified by PCR from *jin1* mutant plants and sequenced to identify the *jin1* mutations in all alleles.

RNA Gel Blot Analysis

Total RNA was extracted from 12-d-old frozen seedlings using RNAwiz as described by the manufacturer (Ambion, Austin, TX). Extracted RNAs were subjected to electrophoresis on 1.5% formaldehyde/agarose gels and blotted to Hybond N+ membranes (Amersham, Buckinghamshire, UK). All probes were labeled with 50 μCi of [α-^{32}P]dCTP. Blots were exposed for 24 h on a PhosphorImager screen (Molecular Dynamics, Sunnyvale, CA).

The AtMYC2 gene-specific probe was generated by PCR with the following primer pairs: forward primer, 5′-GAGCGTGTGATACACGTGCGA-3′; reverse primer, 5′-CTTGCTCTGAGCTGTTCTTGC-3′. The probes for PDF1.2, VSP2, TAT, LOX3, PR4, and PR1 were a fragment of the available Arabidopsis EST clones. The 18S gene was used as a loading control.

Generation of Transgenic Plants and Complementation of *jin1*

The AtMYC2 sequence for the *35S:AtMYC2* constructs was PCR amplified by Pfu Taq polymerase (Promega, Madison, WI) using wild-type genomic DNA as template. The PCR primers used were as follows: MYC2 forward primer, 5′-GACGCTCTGCAGTTTTCTCCACTACGAAG-3′; MYC2 reverse primer, 5′-CACTAAAACGAATTAATTAAGATCTGACCCC-3′. The resulting PCR product was digested with *Pst*I and *Bgl*II and cloned into the pCAMBIA 3300 vector under the control of two copies of the 35S promoter of *Cauliflower mosaic virus* between the *Pst*I and *Bam*HI sites. All constructs were completely sequenced to ensure that they did not contain PCR or cloning errors. The pCAMBIA-*35S:AtMYC2* construct was transferred to *Agrobacterium tumefaciens* C58C1 (pGV2260; Deblaere et al., 1985) by freeze thawing, and Arabidopsis plants (Col-0, *jin1-2*, and *jin1-1*) were transformed by the floral dip method (Clough and Bent, 1998).

Basta-resistant transgenic plants were selected, and their T2 progenies tested for JA sensitivity and *AtMYC2* expression.

GFP Fusions and Transient Expression Assays

PCR fragments corresponding to the full-length AtMYC2 and the deletion mutant corresponding to *jin1-2* were obtained from genomic DNA using the following primers: forward primer, 5′-GACGCTCTGCAGTTTTCTCCACTACGAAG-3′ for both PCR products; reverse primers, 5′-CACACCCATGGAACCGATTTTTGAAAT-3′ for wild-type fragment and 5′-CGAACCATGGTAATAAGGTCCGAACTC-3′ for truncated fragment. The resulting PCR fragments were digested with *Pst*I and *Nco*I. The coding region of the GFP cDNA was excised from the pMON 30063 (Pang

et al., 1996) vector using an *Nco*I-*Bgl*II double digestion. Both fragments (AtMYC2 and GFP) were cloned into the *Pst*I-*Bam*HI doubly digested binary vector pCAMBIA 3300 downstream of the 35S promoter of *Cauliflower mosaic virus*. In addition, the GFP coding region was also PCR amplified from pMON30063 vector using as forward primer 5′-GTCGCATCCATGGCACCTCCTCCCTTGTAGAGTTCATCCA-3′ and as reverse primer 5′-CGGATCCTGCAGCAACCATGGGCAAGGGCG-3′. The obtained fragment was digested with *Pst*I and *Bgl*II and subcloned into *Pst*I-*Bam*HI sites of the pCAMBIA vector described.

GFP fusion and control constructs were transiently expressed by particle bombardment into tobacco BY2 cells and Arabidopsis leaves. DNA absorption to gold particles and bombardment using a helium-driven particle accelerator (PDS-1000/He; Bio-Rad, Hercules, CA) was performed according to the manufacturer's recommendations. Five micrograms of plasmid was used for transformation, and all target materials were bombarded twice.

Fluorescence Microscopy

The fluorescence photographs of cells and leaves expressing the GFP reporter gene under control of the 35S promoter were taken using a Zeiss Axiovert 200 confocal microscope (Jena, Germany) and Bio-Rad Radiance 2100 laser scanning confocal imaging system with LaserSharp version 5 Image software acquisition. For GFP detection, the excitation source was an argon ion laser at 488 nm and detection filters from 515 to 530 nm.

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