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Arabidopsis *PAD3*, a Gene Required for Camalexin Biosynthesis, Encodes a Putative Cytochrome P450 Monooxygenase

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Phytoalexins are low molecular weight antimicrobial compounds that are synthesized in response to pathogen attack. The phytoalexin camalexin, an indole derivative, is produced by Arabidopsis in response to infection with the bacterial pathogen *Pseudomonas syringae.* The *phytoalexin deficient 3* (*pad3*) mutation, which causes a defect in camalexin production, has no effect on resistance to *P. syringae* but compromises resistance to the fungal pathogen *Alternaria brassicicola.* We have now isolated *PAD3* by map-based cloning. The predicted PAD3 protein appears to be a cytochrome P450 monooxygenase, similar to those from maize that catalyze synthesis of the indole-derived secondary metabolite 2,4-dihydroxy-1,4-benzoxazin-3-one. The expression of *PAD3* is tightly correlated with camalexin synthesis and is regulated by *PAD4* and *PAD1.* On the basis of these findings, we conclude that *PAD3* almost certainly encodes an enzyme required for camalexin biosynthesis. Moreover, these results strongly support the idea that camalexin does not play a major role in plant resistance to *P. syringae* infection, although it is involved in resistance to a fungal pathogen.

INTRODUCTION

Plants have evolved a battery of biochemical and molecular weapons to defend themselves against invasion by microbial pathogens. Pathogen invasion triggers activation of defense responses, including the synthesis of reactive oxygen species and signal molecules such as salicylic acid (SA), accumulation of antimicrobial metabolites, and expression of many defense-related genes, such as those encoding pathogenesis-related (PR) proteins. In some instances, a potential pathogen may trigger a form of strong resistance called gene-for-gene resistance, which is mediated by the specific recognition of a pathogen avirulence gene product by the corresponding plant resistance gene. In such cases, plant defense responses are activated rapidly, disease does not ensue, and the pathogen is termed avirulent. Gene-for-gene resistance is not triggered by virulent pathogens, however, with the result that defense responses to these pathogens are activated more slowly; consequently, the responses are less effective, and disease ensues. During the past few years, much attention has been given to elucidating the mechanisms by which defense responses are activated and inhibit pathogen growth (Hammond-Kosack and Jones, 1996; Glazebrook et al., 1997a). Arabidopsis is an ideal model system for such studies, and many mutations that affect a wide variety of specific defense responses have been identified in that species (Glazebrook et al., 1997a).

Phytoalexins are low molecular weight antimicrobial metabolites produced by plants in response to pathogen attack (Paxton, 1981). The chemical structures of phytoalexins vary among different plant families and include flavonoids, terpenoids, and indoles (Darvill and Albersheim, 1984). The antimicrobial properties of phytoalexins suggest their potential function in the host defense machinery. Despite the fact that phytoalexins have been studied extensively for many years, little direct evidence indicates whether they make important contributions to plant defenses against particular pathogens. The only phytoalexin that has been detected in Arabidopsis is an indole derivative called camalexin (3-thiazol-2′-yl-indole) (Tsuji et al., 1992). Accumulation of camalexin was found in tissue exposed to infection by either avirulent or virulent strains of the bacterium *Pseudomonas syringae* (Tsuji et al., 1992; Glazebrook and Ausubel, 1994) and after inoculation with the fungus *Cochliobolus carbonum* (Glazebrook et al., 1997b). In vitro studies demonstrated that camalexin inhibited bacterial and fungal growth (Jejelowo et al., 1991; Tsuji et al., 1992; Rogers et al., 1996).

Very little is known about camalexin biosynthesis. Analysis of camalexin accumulation in tryptophan-deficient Arabidopsis mutants and the incorporation of radiolabeled compounds into camalexin suggested that the camalexin

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biosynthetic pathway originates with an intermediate of the tryptophan pathway that lies between anthranilate and indole (Tsuji et al., 1993). Recent studies have revealed that indole is the precursor to camalexin and that the thiazole ring of camalexin is derived from cysteine (Zook and Hammerschmidt, 1997; Zook, 1998).

The Arabidopsis *phytoalexin deficient* (*pad*) mutants are defective in phytoalexin production and show specific alterations in their responses to a number of pathogens (Glazebrook and Ausubel, 1994; Glazebrook et al., 1996, 1997b; Zhou et al., 1998). Mutations in *pad1*, *pad2*, and *pad4* but not in *pad3* or *pad5* show significantly enhanced susceptibility to virulent *P. syringae* strains (Glazebrook and Ausubel, 1994; Glazebrook et al., 1997b). The *pad4* mutation causes plants to become more susceptible than wild-type plants to normally incompatible isolates of the oomycete *Peronospora parasitica* (Glazebrook et al., 1997b) and the fungus *Erisyphe orontii* (Reuber et al., 1998). Moreover, *pad3* plants are considerably more susceptible to the fungus *Alternaria brassicicola* than are wild-type plants (Thomma et al., 1998).

We have proposed a model to explain the phenotypes of *pad* mutants (Glazebrook and Ausubel, 1994; Glazebrook et al., 1997b; Zhou et al., 1998). Mutations in *PAD3* or *PAD5* cause a defect only in camalexin synthesis, and camalexin does not play a major role in resistance to *P. syringae.* The other *PAD* genes encode regulatory factors that control camalexin synthesis as well as other defense responses. It is these other defense responses that contribute to plant resistance to *P. syringae.* In support of this model, we found that *PAD4* affects regulation of camalexin synthesis and the pathogenesis-related gene *PR-1* (Zhou et al., 1998). Activation of camalexin synthesis and *PR-1* expression requires the signal molecule SA. SA is sufficient for *PR-1* expression and is necessary, but not sufficient, for camalexin synthesis (Ryals et al., 1996; Zhao and Last, 1996; Zhou et al., 1998). *PAD4* seems to act upstream of SA in the activation of defense responses to virulent *P. syringae* strains, but it is not required for responses to isogenic avirulent strains, such as those carrying the avirulence gene *avrRpt2* (Zhou et al., 1998).

Our model predicts that *PAD3* encodes a biosynthetic enzyme or a regulatory factor that affects only camalexin synthesis and other defense responses that do not restrict growth of *P. syringae.* In this study, we isolated *PAD3* by using a map-based cloning approach and found that it encodes a putative cytochrome P450 monooxygenase similar to four maize gene products required for biosynthesis of the indole-derived metabolites 2,4-dihydroxy-1,4-benzoxazin-3-one (DIBOA) and 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one (DIMBOA) (Frey et al., 1997). *PAD3* expression is pathogen inducible and requires *PAD4* and *PAD1.* Our results strongly suggest that PAD3 is an enzyme involved in camalexin biosynthesis. Therefore, camalexin does not seem to be a crucial factor in limiting growth of *P. syringae* but is required for resistance to *A. brassicicola.*

RESULTS

Map-Based Cloning of *PAD3*

To gain insight into the role of *PAD3* in camalexin synthesis, we decided to isolate *PAD3.* The only known *pad3* alleles were induced by ethyl methanesulfonate (Glazebrook and Ausubel, 1994; Glazebrook et al., 1997b), so we used a map-based approach to clone the gene. *GLABROUS1* (*GL1*) was known to lie close to the *PAD3* locus on chromosome 3 (Glazebrook and Ausubel, 1994). To map *pad3*, we crossed *pad3-2 gl1-1* Columbia plants and wild-type plants of the polymorphic accession Landsberg *erecta.* In the F_2 generation, homozygous *gl-1* plants were scored for *pad3-2* homozygosity by using a camalexin assay. *PAD3* was mapped by using the cleaved amplified polymorphic sequences (CAPS) mapping technique (Konieczny and Ausubel, 1993) to measure the frequency of recombination between *PAD3* and nearby markers. Figure 1 shows that *PAD3* was found

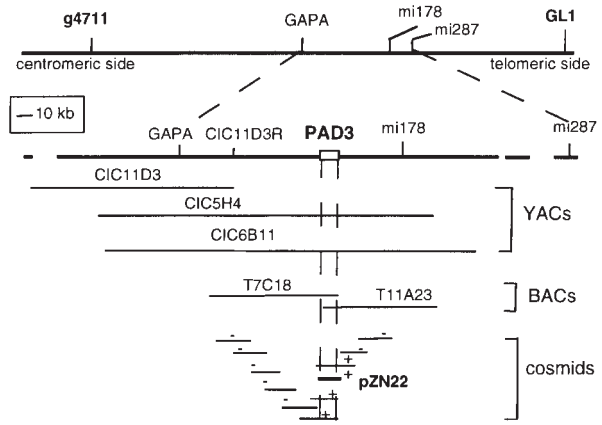

Figure 1. Map of the *PAD3* Region on Chromosome 3.

Thirty-three recombination events were detected between marker *GL1* and *PAD3* among 948 chromosomes examined, and 31 recombination events were detected between marker g4711 and *PAD3* among 694 chromosomes examined, indicating that *PAD3* is between these two markers, 3.5 centimorgans from *GL1* and 4.5 centimorgans from g4711. Among g4711 recombinants, there were three recombination events between *PAD3* and *GAPA.* Among 16 recombinants for marker mi287, all were recombinant at *GL1*, whereas none was recombinant at *GAPA.* Therefore, *PAD3* was placed between *GAPA* and mi287. The 19 F_3 families that were recombinant at *GAPA* or mi287 were scored for marker mi178, and one recombinant, an mi287 recombinant, was detected; therefore, *PAD3* must lie between *GAPA* and mi178 on YAC clones CIC5H4 and CIC6B11 and on one of the BAC clones T7C18 or T11A23. Cosmids that complement or fail to complement *pad3* are labeled (+) and (−), respectively. The position of *PAD3* is indicated by the dashed lines. The cosmid pZN22 is shown in boldface.

between g4711 (4.5% recombination) and *GL1* (3.5% recombination) on chromosome 3.

A physical map of this region was used to identify yeast artificial chromosomes (YACs) containing *PAD3* (Camilleri et al., 1998). Scoring the g4711 and *GL1* recombinants with the CAPS marker *GAPA* and with the restriction fragment length polymorphism markers mi287 and mi178 showed that *PAD3* resides on two YACs, CIC5H4 and CIC6B11 (Figure 1). The Texas A&M University bacterial artificial chromosome (BAC) library (Choi et al., 1995) was screened by filter hybridization to identify BACs that hybridize with mi178 and CIC11D3R (Figure 1). Two BACs, T7C18 and T11A23, were found to span the interval from CIC11D3R to mi178. A cosmid library was constructed from the yeast strain carrying CIC6B11. BACs T7C18 and T11A23 were labeled and used as hybridization probes to identify cosmids corresponding to the BACs. These cosmids were arranged into a contig by using hybridization to identify overlapping cosmids.

Cosmids containing *PAD3* were identified by transforming *pad3-2* plants with various cosmids and testing the transformants for camalexin synthesis after infection with *P. s.* pv *maculicola* ES4326. Six cosmids complemented and 13 did not. Common to all six complementing cosmids was a section of Arabidopsis DNA of ~11 kb (Figure 1). Consequently, this is the section that must contain *PAD3.*

PAD3 Encodes a Putative Cytochrome P450 Monooxygenase

The nucleotide sequence of the Arabidopsis DNA in one of the cosmids, pZN22, was determined. A BLAST (Altschul et al., 1990) search of the GenBank database revealed that part of the sequence was similar to those of cytochrome P450 monooxygenases. To determine whether this sequence was derived from the *PAD3* gene, we amplified the cytochrome P450-like sequence from wild-type, *pad3-1*, and *pad3-2* plants. The DNA sequences of the amplified fragments were determined and compared to identify any mutations in the *pad3-1* and *pad3-2* plants. As shown in Figure 2, both *pad3-1* and *pad3-2* plants have mutations in this region. In *pad3-1* plants, a single nucleotide deletion causes a frameshift and early stop codon in the predicted open reading frame, whereas in *pad3-2* plants, there is a G-to-A point mutation in the predicted open reading frame (Figure 2). DNA fragments corresponding to the entire complementing region of pZN22 were amplified from wild-type, *pad3-1*, and *pad3-2* plants. DNA sequencing of these fragments demonstrated that no other nucleotide changes were present in this region in *pad3-1* or *pad3-2* plants. Therefore, we concluded that the gene encoding the putative cytochrome P450 monooxygenase must be *PAD3.*

The genomic sequence was used to isolate a *PAD3* cDNA clone from wild-type plants by rapid amplification of cDNA ends–polymerase chain reaction (RACE-PCR). DNA sequencing of the cDNA clone revealed a 1.7-kb full-length mRNA (Figure 2). Comparison of the cDNA sequence and the genomic sequence revealed the presence of one intron in the *PAD3* gene. The encoded protein, which consists of 490 amino acid residues, has a proline-rich motif near the N terminus and a heme binding motif near the C terminus (Figure 2), structures characteristic of all eukaryotic cytochrome P450 monooxygenases. The *pad3-1* mutation causes a translation stop before the heme binding site, whereas the *pad3-2* mutation causes substitution of glutamic acid for glycine at amino acid 176. The results of the DNA gel blot analysis shown in Figure 3 demonstrate that *PAD3* is a single-copy gene.

The sequence that we identified as *PAD3* (GenBank accession number AB016889) was assigned the name CYP71B15 by D. Nelson (University of Tennessee, Memphis). CYP71 is a large family of plant cytochrome P450 monooxygenases. Typically, all members of a CYP family share at least 40% amino acid sequence identity and are divided into subfamilies (A, B, C, and so forth) composed of members with greater sequence identity (Chapple, 1998). For historical reasons, the large CYP71 family is broader than this, and the identity between members of different subfamilies can be as low as 30%. *PAD3* shares 47 to 61% amino acid sequence identity with five Arabidopsis cytochrome P450 monooxygenases of unknown function: CYP71B2, CYP71B3, CYP71B4, CYP71B5, and CYP71B7. The only members of the CYP71 family for which in vivo functions are known are CYP71E from *Sorghum bicolor*, which converts (*Z*)-*p*-hydroxyphenylacetaldoxime to *p*-hydroxymandelonitrile in the dhurrin biosynthetic pathway (Bak et al., 1998), and CYP71C1, CYP71C2, CYP71C3, and CYP71C4 from maize (encoded by *Bx4*, *Bx3*, *Bx5*, and *Bx2*, respectively), which synthesize DIBOA from indole (Frey et al., 1997). *PAD3* is 39% identical to CYP71E and 31 to 34% identical to the maize enzymes.

Figure 4 shows an alignment of the structures of PAD3 and the CYP71C proteins. The similarity between the CYP71C enzymes and PAD3 probably results from a common affinity for indoles, because both camalexin and DIBOA are derived from indole. Given these sequence similarities between PAD3 and the cytochrome P450 monooxygenases, we concluded that PAD3 is almost certainly an enzyme required for camalexin biosynthesis.

PAD3 Expression Is Induced by *P. s. maculicola* ES4326 Infection and SA Treatment

If *PAD3* encodes an enzyme for camalexin biosynthesis, then the expression of *PAD3* might be induced by stimuli that trigger camalexin accumulation. To test this idea, we used RNA gel blot hybridization to examine *PAD3* expression in plants challenged with *P. s. maculicola* ES4326. Figure 5 shows a time course of the amounts of *PAD3* mRNA in infected tissue. In response to infection, *PAD3* mRNA was induced by 12 hr after infection (Figure 5) and reached a maximum by 36 hr after infection (data not shown). *PAD3*

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GGGCAAGGAAAATGTCGGTTTTCCTCTGTTTCCTCGTCCTCTTACCCCTG 50
          M  S  V  F  L  C  F  L  V  L  L  P  L
ATCTTAATCTTCTTGAACGTTTTGAAACCTTCGAAATATAAGCTTCCTCC 100
I  L  I  F  L  N  V  L  K  P  S  K  Y  K  L  P  P
GGGTCCAAAGAAGCTTCCCATCATCGGAAACTTACACCAACGCAGGACAC 150
G  P  K  K  L  P  I  I  G  N  L  H  Q  R  R  T  L
TACATCCGAGGAATCGCCGTAATCTCGCCGAAATGTATGGACCAGTGGCG 200
  H  P  R  N  R  R  N  L  A  E  M  Y  G  P  V  A
CTTCTCCAATACGGATTCGTCCCCGTGGTCGCGATCTCTTCGAAAGAAGC 250
L  L  Q  Y  G  F  V  P  V  V  A  I  S  S  K  E  A
AGCAGAGGAAGTGCTAAAGATCAACGATCTTGAGTGTTGTAGTCGACCAG 300
 A  E  E  V  L  K  I  N  D  L  E  C  C  S  R  P  E
AGGCGGCCGGAATGAGAGCAACTTTTTACAACTTCAAAGACATCGGGATG 350
  A  A  G  M  R  A  T  F  Y  N  F  K  D  I  G  M
GCACCCTTCGGTGACGAGTGGAGTCTGATGCGGAAGCTCTCGGTGGTCGA 400
A  P  F  G  D  E  W  S  L  M  R  K  L  S  V  V  E
                    ▼
GCTCTTCAGCGTGAAAAAGCTTCAATCTTTCAAGTATATCATAGAGGAAG 450
 L  F  S  V  K  K  L  Q  S  F  K  Y  I  I  E  E  E
AGAATAACTTGTGTGTCAAGAAACTCTCTGAGTTTGCTACGAGACAATCT 500
  N  N  L  C  V  K  K  L  S  E  F  A  T  R  Q  S
                                  ▽
CCGGTGAATCTTGAGAGAGCCATTTTCACTTTAGTCGGAAATATAGTGTG 550
P  V  N  L  E  R  A  I  F  T  L  V  G  N  I  V  C
TCGTATCGGGTACGGGATAAATCTCTATGAGTGTGATTTCTTTGAAGCTG 600
 R  I  G  Y  G  I  N  L  Y  E  C  D  F  F  E  A  D
ATAGAGTCGTGGATCTTGTGCTAAAGGCTGAAGCGGTCATAAGAGAAACT 650
  R  V  V  D  L  V  L  K  A  E  A  V  I  R  E  T
GTGTTCTCTGATTTCTTTCCCGGAAGAATCGGTAGGTTCATCGACTGTAT 700
V  F  S  D  F  F  P  G  R  I  G  R  F  I  D  C  I
CTCCGGTCAGAACAGGAGATTGAAGAATAATTTCTCGGTAGTAGACACTT 750
 S  G  Q  N  R  R  L  K  N  N  F  S  V  V  D  T  F
TCTTTCAGAATGTTCTTAACGAGCATCTTAAGCCTGGAAGAGAGAGCTCT 800
  F  Q  N  V  L  N  E  H  L  K  P  G  R  E  S  S
ACTATTGTTGACTTGATGATCGATATGAAGAAGAAGCAAGAGAACGATGG 850
T  I  V  D  L  M  I  D  M  K  K  K  Q  E  N  D  G
AGATGCTCTCAAGTTCACCACTGATCATCTCAAAGGAATGATCTCGgtaa 900
 D  A  L  K  F  T  T  D  H  L  K  G  M  I  S
gcctttagtctttttcaagttatatatattccacagtggttaaaaaatct 950
tgcaacaacataccaatatatttgtgactttgtgtttatgtcagGACATA 1000
                                              D  I
TTTGTAGCAGGGATTGGTGGAGTCGCTGGCATAACACTATGGGGAATGAC 1050
F  V  A  G  I  G  G  V  A  G  I  T  L  W  G  M  T
CGAGCTGATCAGAAACCCAAGAGTGATGAAGAAAGTGCAAGACGAGATTC 1100
 E  L  I  R  N  P  R  V  M  K  K  V  Q  D  E  I  R
GGACAACACTTGGGGACAAGAAGGAGAGAATCAAAGAAGAAGATCTAAAC 1150
  T  T  L  G  D  K  K  E  R  I  K  E  E  D  L  N
CAACTTCACTACTTTAAGCTCGTGGTCAAGGAGACATTAAGGTTACATCC 1200
Q  L  H  Y  F  K  L  V  V  K  E  T  L  R  L  H  P
AACAACTCCACTCTTGCTCCCAAGACAGACAATGTCTCATATCAAGATTC 1250
 T  T  P  L  L  L  P  R  Q  T  M  S  H  I  K  I  Q
AAGGCTACGATGTTCCTGCGAAAACACAGATCTTAGTCAACGTTTATGCG 1300
  G  Y  D  V  P  A  K  T  Q  I  L  V  N  V  Y  A
ATGGGTCGTGATCCAAAACTCTGGGAAAACGCAGATGAGTTTAACCCTGA 1350
M  G  R  D  P  K  L  W  E  N  A  D  E  F  N  P  D
CAGGTTTCTCGACAGTTCCGTTGATTTTAAAGGAAAAAACTACGAGTTTA 1400
 R  F  L  D  S  S  V  D  F  K  G  K  N  Y  E  F  I
TACCGTTTGGATCTGGTAGGAGAATATGTCCAGGGATGACAATGGGTACC 1450
  P  F  G  S  G  R  R  I  C  P  G  M  T  M  G  T
ATACTTGTTGAGATGGCATTGTTGAATTTGCTTTACTTCTTCGATTGGGG 1500
I  L  V  E  M  A  L  L  N  L  L  Y  F  F  D  W  G
ATTGGCGAAGCAGGAGGAAGCCAAAGAGATCATCAATGGGGAAGAAAATT 1550
 L  A  K  Q  E  E  A  K  E  I  I  N  G  E  E  N  F
TTCTAGCCTTCTTTCAAGTTCTTCACCACTGAATATATGGTTTGGTTCCT 1600
  L  A  F  F  Q  V  L  H  H
ATCAAAAAAAATATATATATATGGTTTTGTTATGTACACTAGTTTCTAAG 1650
ATAATAAATGAAAATTGTATCTGCTTTTGATTTAGTTTCAATAAAAATAA 1700
AAAGTGATGATCCAGTTGGAGTCAGCTTCTAAA  1733
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Figure 2. Nucleotide Sequence and Predicted Amino Acid Composition of *PAD3.*

A 98-bp intron is shown in lowercase letters. The proline-rich and heme binding motifs are shown in boldface and underlined. The mutations in *pad3-1* (a single nucleotide deletion) and *pad3-2* (G-to-A transition) are indicated by the filled and open arrowheads, respectively. The sense and antisense primer sequences used for making the *PAD3* hybridization probe are underlined.

was also induced in plants infected with avirulent bacteria. As shown in Figure 5, *PAD3* mRNA started to accumulate in amounts exceeding that in the uninfected control plants by 3 hr after infection and reached a peak by 12 hr after infection. In previous work, we found that camalexin accumulated within 12 hr after infection by avirulent bacteria, whereas it accumulated more slowly in response to virulent bacteria, reaching noticeable amounts between 12 and 24 hr after infection (Zhou et al., 1998). The observation that accumulation of *PAD3* transcripts precedes camalexin accumulation after infection with either virulent or avirulent strains is consistent with the idea that PAD3 plays a role in camalexin synthesis.

Because SA is necessary but not sufficient for camalexin synthesis, we tested the effect of SA treatment on *PAD3* expression. As Figure 6 shows, the amounts of *PAD3* mRNA increased rapidly in response to SA treatment, demonstrating that SA is sufficient for *PAD3* expression, even though it is not sufficient for activation of camalexin synthesis. Camalexin synthesis must be therefore subject to additional control beyond regulation of *PAD3* expression. Certain mutations that cause formation of spontaneous lesions resembling disease lesions, such as the *accelerated cell death* (*acd2-2*) mutation, also cause high SA concentrations and camalexin synthesis in the tissue with lesions (Greenberg et al., 1994). We found that *PAD3* was also expressed in *acd2-2* plant tissue with lesions (Figure 6).

Jasmonic acid (JA) is sufficient to activate phytoalexin synthesis in some plants, but it does not activate camalexin synthesis in Arabidopsis (Thomma et al., 1999). Nevertheless, because JA signaling might be required, we investigated the possibility. Plants homozygous for the *coronatine insensitive1* (*coi1*) mutation are insensitive to JA and are male sterile (Feys et al., 1994). Camalexin concentrations in a population segregating for *coi1* homozygotes and in wild-

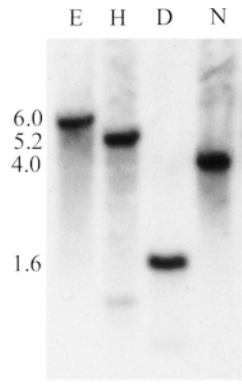

Figure 3. *PAD3* Is a Single-Copy Gene.

Approximately 1.5 μg of digested DNA from Arabidopsis ecotype Columbia was loaded in each lane and hybridized with the *PAD3* probe. Lengths of fragments (in kilobases) are shown at left. D, DraI; E, EcoRI; H, HindIII; N, NdeI.

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          1                                                  50
CYP71C1   ~~~~~~MALE AAY.D..YLH VAVVQCTPTQ AAAVLGVLLL LAIRLAAAAR
CYP71C2   ~~~~~~MALG AAYHH..YLQ LAGDHGTATH .ALLLGVLIF LVIRLVSARR
CYP71C3   ~~~~~~~ALQ AAY.E..YLQ QAVGHGAWSS TQTL..TLLL IAVPTVLLLL
CYP71C4   MAAQLHHALY ELLHEAAAAQ RALLLAIPFS LLLLPLLLRY LAASASASAT
   PAD3   ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ MSVFLCFLVL

          51                                                 100
CYP71C1   SSSATSPKWK QHR...LPPT PPGKLPIIGH LHLIGSHPHV SFRDLHAKYG
CYP71C2   TGTTSANKRK QQQRLPLPPW PPGKLPIIGH LHLIGAETHI SIRDLDAKHG
CYP71C3   ASLAKSTSSS GRGKPPLPPS PPGTLPIVGH LHHIGPQTHI SLQELVAKYG
CYP71C4   KNDGAAPASD PDKLLSLLPS PPMKLPIIGH LHLMGDIPYV SLAALATRYG
   PAD3   LPLILIFLNV LKPSKYKLPP GPKKLPIIGN LHQRRTLHPR NRRNLAEMYG

          101                                                150
CYP71C1   HNGLMLVQVG AVPTIVVSTP QAAEAVLRTH DHVLASRPRN PVADIIRYNC
CYP71C2   RNGLLLLRIG AVPTLFVSSP SAADAVLRTQ DHIFASRPPW MAAEIIRYGP
CYP71C3   HNGFLFLRAG AVPTLIVSSP SAAEAVMRTH DHICASRPWS MASHILRYNT
CYP71C4   PD.LMLLRLG AVPTVVVSSP RVAEAVLRTY DHVFSSRPRS LVSDIIMYGA
   PAD3   P..VALLQYG FVPVVAISSK EAAEEVLKIN DLECCSRPEA AGMRATFYNF

          151                                                200
CYP71C1   TDVAFAPYGE YWRTARKVVN THLLSAKMVF SKRRREREEV RLVVARIRDA
CYP71C2   SDVAFVPYGE YGRQGRKLLT THMLSTKKVQ SFRHGRQEEV RLVMDKIRAA
CYP71C3   CDVAFSPLGE YWQQTRKLMN THLLSNKKVY SFRHGREEEV CLVVDNLREA
CYP71C4   TDSCFAPYGD HFRKARKLVT VHLLNASKVR SQRPAREEEV RGALDRVRRA
   PAD3   KDIGMAPFGD EWSLMRKLSV VELFSVKKLQ SFKYIIEEEN NLCVKKLSEF

          201                                                250
CYP71C1   AEASPGTALD MTELLGGYAS DFVCRAVLGE SHRKQGRNKL FR..ELTETS
CYP71C2   ATAAPPAAVD LSDLLSGYTN DVVSRAVLGA SHRNQGRNRL FS..ELTEIN
CYP71C3   AAKSPSTAVD MSEVLAAYTN DVVSRSVLGS THRKKGRNTL FR..EMTMTN
CYP71C4   AAAREP..VD MSELLHSFVN NLVCRAVSGK FSMEEGRNRL FR..ELTDIN
   PAD3   ATRQSP..VN LERAIFTLVG NIVCRIGYGI NLYECDFPEA DRVVDLVLKA

          251                                                300
CYP71C1   AALLGGFNVE DYF.PKLADV DLFLRIICAK AKGVSKRWDS LFNELLSEYA
CYP71C2   VSLLAGFNLE DYFPPNMAMA DVLLRLVSVK ARRLNQRWND VFDELIQEHV
CYP71C3   VDLLVGFNLE .YYIPRWPLT DLLFRLVCWK VTRHLKRWDA LLEEVIHEHV
CYP71C4   AGLLGGFHIQ DYF.PRLGRI ELVRKVACAK TRRVRKRWDD LLDKLIDDHA
   PAD3   EAVIRETVFS DFFPGRIGRF IDCISGQNRR LKNNFSVVDT FFQNVLNEHL

          301                                                350
CYP71C1   LSGGKQGDHN SE..DFVHLL LSLQKDYGLT TDNIKGILVN MFEAAIETSF
CYP71C2   QSRPSGESEE SEA.DFIHVL LSIQQEYGLT TDNLKAILVD MFEAGIETSY
CYP71C3   EMRKLSGDKE KESDDFIDIF LSRYEEYGFT MDNVKSLLMN VFEAAIETSY
CYP71C4   .ARMATHQDE DDDKDFIYVL LSLQKEYGLT RDHIKAILID MFEAGTDTSY
   PAD3   KPGRESSTIV DLMIDMKKKQ ENDGDALKFT TDHLKGMISD IFVAGIGGVA

          351                                                400
CYP71C1   LVLEYSMSEL MNNRHVLAKL QKEVRT..AT PDGR...MVM EEDLSRMPYL
CYP71C2   LTLEYGMAEL INNRHVMEKL QTEVRTTMGS PDGKKLDMLA EEDLGSMPYL
CYP71C3   LVLESAMAEL MNHRRVMKKL QAEVRAYGAE ...KKLDMIR EDDLSSLPYL
CYP71C4   MTLEFAMTEL IRKPHLMKKL QEEVRRNVPA GQ....EMVT EDNLPGMTDL
   PAD3   GITLWGMTEL IRNPRVMKKV QDEIRTTL.. ..GDKKERIK EEDLNQLHYF

          401                                                450
CYP71C1   KATIKESMRI HPPAPFLLPH FSTHDCEING YTIPAGTRVI VNAWALARDP
CYP71C2   KATIKETLRL HPPAPFLLPH YSTADSEIDG YFVPAGTRVL VHAWALGRDR
CYP71C3   KASMKEALRL HPPGPLLLPH YSTADCQIDG YHIPANPRVL VNGWAIGRDP
CYP71C4   KAVIKETLRL HPPVPLLLPH YSLDACEVAG YTIPANTRVV VNAWALGRHS
   PAD3   KLVVKETLRL HPTTPLLLPR QTMSHIKIQG YDVPAKTQIL VNVYAMGRDP

          451                                                500
CYP71C1   TCWDKAEEFF PERFLEQGRD AEVDMYGKDI RFVPFGAGRR ICAGATFAIA
CYP71C2   TTWEKPEEFM PERFVQEPGA VDVHMKGKDL RFIPFGSGRR ICPGMNFGFA
CYP71C3   AVWEKPEEFM PERFMRDGWD KSNSYSGQDF RYLPFGSGRR ICPGANFGLA
CYP71C4   GYWERENEFV PERFLSGDVA GGVDLKPNEF QFLAFGSGRR MCPGVHSASA
   PAD3   KLWENADEFN PDRFL....D SSVDFKGKNY EFIPFGSGRR ICPGMTMGTI

          501                                                550
CYP71C1   TVEIMLANLI YHFDWEMPAE MERTGAKVDM SDQFGMTLRR TQKLYLVPRI
CYP71C2   TMEVMLANLM YHFDWEVPG. ...SGAGVSM EESFGLTLRR KEKLLLVPRI
CYP71C3   TMEIMLANLM YHFDWEVPNE KEDGCWKVSM DEKFGLMLRR NELLYLVPRA
CYP71C4   TIEAMLSNLM YRFDWQLPAG MK..AEDVDM TEVFGITVSR KEKLLLVPQA
   PAD3   LVEMALLNLL YFFDWGL.AK QEEAKEIING EENFLAFFQV LHH~~~~~~~

          551
CYP71C1   PK~
CYP71C2   AS~
CYP71C3   SSY
CYP71C4   A~~
   PAD3   ~~~
```

Figure 4. Amino Acid Sequence Alignment of PAD3 and Other Cytochrome P450 Monooxygenases.

CYP71C1, CYP71C2, CYP71C3, and CYP71C4 are encoded by the maize genes *Bx4*, *Bx3*, *Bx5*, and *Bx2*, respectively (Frey et al., 1997).

type plants were determined 34 hr after infection with *P. s. maculicola* ES4326. Camalexin concentrations (ng/cm^2; means ±SD of three to five replicate samples) were as follows: wild-type, 375 ± 93; *coi1* homozygotes (male-sterile plants from the segregating population), 87 ± 19; and *COI1* homozygotes and *COI1*/*coi1* heterozygotes (fertile plants from the segregating population), 489 ± 136. The decreased concentrations of camalexin in *coi1* plants are consistent with the idea that JA plays a role in the activation of camalexin synthesis in response to infection by *P. s. maculicola* ES4326.

PAD3 Expression Is Reduced in *pad1* and *pad4* Mutants

Among the five identified *PAD* genes, one (*PAD4*) is known to have a regulatory effect on camalexin synthesis (Zhou et al., 1998). Mutations affecting regulation of camalexin synthesis might cause defects in pathogen-induced *PAD3* expression. To test for such defects, we examined the amount of *PAD3* mRNA in *P. s. maculicola* ES4326–infected leaves in *pad* mutants and wild-type plants. As shown in Figure 7A, we observed a defect in accumulation of *PAD3* transcripts in *pad3-1* plants. This could result from the premature stop codon in the coding sequence, causing instability of the transcribed RNA (Culbertson, 1999). Interestingly, after infection by virulent *P. s. maculicola* ES4326, the amount of *PAD3* mRNA expressed in the *pad1* and *pad4* mutants was much less than in the wild-type plants (Figure 7A). However, this decrease was not observed in *pad4-1* plants inoculated with avirulent bacteria (Figure 7B), in agreement with our previous findings that the *pad4* mutation does not affect camalexin synthesis in *P. s. maculicola* plants infected by *avrRpt2*-containing strains (Zhou et al., 1998). On the basis of these results, *PAD1* and *PAD4* most likely contribute to control of camalexin synthesis by mediating increased amounts of *PAD3* mRNA in response to infection with *P. s. maculicola* ES4326.

DISCUSSION

Sequence analysis of *PAD3* revealed that it encodes a putative cytochrome P450 monooxygenase. Cytochrome P450 monooxygenases make up a superfamily of heme-thiolate proteins that are found in various organisms, including

The proline-rich motif near the N terminus and the heme binding motif near the C terminus are underlined. Residues conserved in all protein sequences are in boldface. Multiple sequence alignment was performed with PILEUP (version 9.1; Genetics Computer Group, Madison, WI). Dots represent gaps introduced into the sequence to maximize the sequence similarities. The symbol ~ marks the ends of protein sequences.

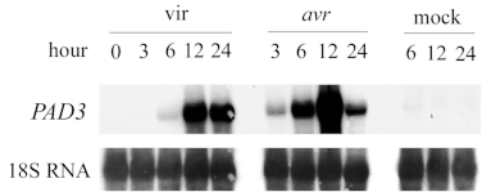

Figure 5. *PAD3* Expression Is Induced by *P. s. maculicola* ES4326 Infection.

Total RNA was extracted from tissue inoculated with *P. s. maculicola* ES4326 (vir) or *P. s. maculicola* ES4326 carrying *avrRpt2* (*avr*), or mock-inoculated with 10 mM $MgSO_4$ (mock), at the indicated times after inoculation. An 18S RNA probe was used as a control for equal loading.

bacteria, plants, and mammals. Most of these proteins catalyze NADPH- and O_2-dependent hydroxylation reactions (Chapple, 1998). Plant cytochrome P450 monooxygenases participate in myriad biochemical pathways, including biosynthesis of phenylpropanoids, alkaloids, terpenoids, lipids, and plant growth regulators, such as gibberellins, JA, and brassinosteroids (Chapple, 1998). We found that PAD3 is closely related to the Arabidopsis cytochrome P450 monooxygenases CYP71B2 through CYP71B5 and CYP71B7. However, no biochemical functions have been reported for these proteins.

PAD3 shares 31 to 34% amino acid sequence identity with the maize cytochrome P450 monooxygenase proteins encoded by *Bx2* (CYP71C4), *Bx3* (CYP71C2), *Bx4* (CYP71C1), and *Bx5* (CYP71C3) (Figure 4). Expression of these genes in yeast revealed that each of them catalyzes one of the oxidation steps between indole and DIBOA in the pathway of DIMBOA biosynthesis (Frey et al., 1997). DIMBOA and its precursor, DIBOA, are believed to be involved in pathogen defense and disease resistance in maize (Niemeyer, 1988). The *Bx1* gene encodes a tryptophan synthase α homolog that appears to provide indole for DIBOA synthesis (Frey et al., 1997; Melanson et al., 1997). Presumably, this activity is needed because in plants, tryptophan synthase α and β are tightly associated, and indole is immediately converted to tryptophan without being released from the enzyme complex. Together, these five *Bx* genes constitute a branch pathway that converts indole to DIBOA rather than tryptophan (Frey et al., 1997). Like DIBOA, camalexin is also an indole derivative; therefore, the sequence similarity between the DIBOA cytochrome P450 monooxygenases and PAD3 strongly suggests that PAD3 functions in camalexin biosynthesis. Indole is a precursor to camalexin, and an Arabidopsis sequence with 80% sequence identity to Arabidopsis tryptophan synthase α has been identified; therefore, Arabidopsis is also likely to have a Bx1-like activity that provides free indole for camalexin synthesis (Zook, 1998). Similar pathways for synthesis of secondary metabolites with protective roles may have evolved in both Arabidopsis and maize.

Most cytochrome P450 monooxygenases catalyze O_2- and NADPH-dependent hydroxylation reactions. Camalexin does not contain any oxygen atoms, so the role for a cytochrome P450 enzyme in camalexin synthesis is not immediately obvious. However, the finding that indole and cysteine are precursors to camalexin (Zook and Hammerschmidt, 1997; Zook, 1998) is consistent with a proposed pathway for camalexin biosynthesis beginning with the condensation of indole-3-carboxaldehyde and cysteine (Browne et al., 1991; Zook, 1998). The cytochrome P450 monooxygenase encoded by *PAD3* could be involved in the synthesis of indole-3-carboxaldehyde from indole.

In addition to camalexin synthesis, the tryptophan pathway leads to the biosynthesis of many secondary metabolites, including the auxin indole-3-acetic acid and indole glucosinolates (Radwanski and Last, 1995). The importance of these secondary metabolites in plants suggests the need for regulatory mechanisms that control the metabolic flow and production of precursors in the tryptophan pathway to accommodate the biosynthesis of these diverse secondary metabolites. Zhao and Last (1996) pointed out that the amounts of the enzymes participating in tryptophan biosynthesis and camalexin synthesis are coordinately regulated. Infection by a pathogen and elicitor treatment induced both activity by the tryptophan pathway enzymes and accumulation of camalexin (Zhao and Last, 1996). On the other hand, because mutations in the *trp* genes did not cause major changes in camalexin accumulation (Zhao and Last, 1996), flux through the tryptophan biosynthetic pathway is apparently not rate limiting for camalexin synthesis.

We have shown that the accumulation of *PAD3* transcripts is correlated with and precedes camalexin accumulation after infection with *P. s. maculicola* ES4326 or ES4326 carrying *avrRpt2* and is correlated with camalexin synthesis in *acd2-2* plants. However, SA treatment, although sufficient for activating expression of *PAD3*, is not sufficient for acti-

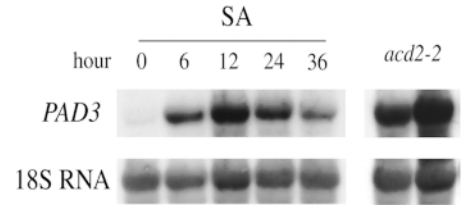

Figure 6. *PAD3* Expression Is Induced by SA Treatment and by the *acd2-2* Mutation.

RNA was prepared from wild-type Columbia plants sprayed with 5 mM SA in 0.02% (v/v) Silwet L-77 at various times after treatment (five lanes at left) and from leaves with lesions from 7-week-old *acd2-2* plants (two lanes at right).

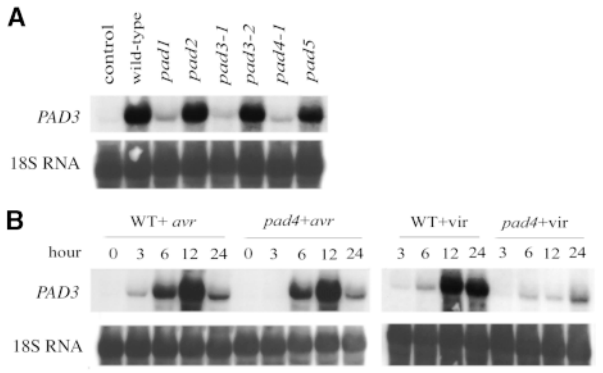

Figure 7. *PAD3* Expression in *pad* Mutants.

(A) *PAD3* mRNA accumulation in *pad* and wild-type plants. Leaf samples were collected 8 hr after infection with virulent *P. s. maculicola* ES4326. Uninfected tissue of wild-type plants was used as the control.

(B) *PAD3* expression in the *pad4-1* mutant compared with wild-type (WT) plants in response to avirulent or virulent bacteria. Total RNA was extracted from tissue inoculated with *P. s. maculicola* ES4326 (vir) or *P. s. maculicola* ES4326 carrying *avrRpt2* (*avr*) at the indicated times after infection.

vating synthesis of camalexin. Apparently, camalexin synthesis is regulated partly by controlling the expression of a biosynthetic enzyme and partly by an unknown mechanism.

The idea that camalexin synthesis is controlled at the mRNA level is supported by the observation that the accumulation of *PAD3* mRNA in response to virulent *P. s. maculicola* ES4326 is greatly decreased in *pad1* and *pad4* mutants—an indication that *PAD3* expression is regulated by *PAD1* and *PAD4.* Our finding that *PAD3* expression requires *PAD1* and *PAD4* is consistent with our model, because it predicts that *PAD1* and *PAD4* should control camalexin synthesis as well as other defense responses. Previous work has shown that *PAD4* encodes a regulatory factor that is required to promote SA synthesis in response to *P. s. maculicola* ES4326 infection (Zhou et al., 1998). We have now shown that *PAD3* expression is enhanced by SA treatment. Therefore, the requirement of *PAD4* for *PAD3* induction and camalexin synthesis is expected, and the action of *PAD4* is likely to be mediated by SA.

Our finding that *PAD1* is required for *PAD3* expression indicates that *PAD1* has a role in regulating the expression of defense genes. *PR-1* is expressed normally in *pad1* mutants (J. Glazebrook, unpublished data); therefore, *PAD1* is unlikely to affect SA signaling. The effect of *PAD1* on camalexin synthesis could conceivably be mediated by the JA-dependent signaling pathway, however. This pathway (Creelman and Mullet, 1997), which leads to induction of the plant defensin gene *PDF1.2*, is important for resistance to certain fungal and oomycete pathogens (Penninckx et al., 1996; Bowling et al., 1997; Thomma et al., 1998). In *pad1* plants, expression of *PDF1.2* in response to *A. brassicicola* infection was diminished (Thomma et al., 1999). Interestingly, *coi1* mutants showed a defect in camalexin production in response to *P. s. maculicola* ES4326 infection, consistent with the idea that activation of camalexin biosynthesis requires components of the JA signaling pathway. Curiously, *coi1* did not block camalexin synthesis in response to *A. brassicicola* infection (Thomma et al., 1999), suggesting that camalexin synthesis may be regulated by different mechanisms in response to different pathogens. Clearly, many more experiments are needed to explore the possible role of JA in activating camalexin synthesis.

Because *PAD3* is expressed normally in *pad2* and *pad5* mutants, the defects in camalexin synthesis in these plants must have some other cause; for instance, *PAD2* or *PAD5* may regulate genes encoding other camalexin biosynthetic enzymes. Based on the enhanced susceptibility of *pad2* mutants to *P. s. maculicola* ES4326, our model predicts that *PAD2* affects a regulatory factor influencing camalexin synthesis and some other defense response that limits growth of *P. s. maculicola* ES4326. If so, the regulatory effect must occur by a mechanism other than control of *PAD3* expression. The *pad5* mutation does not affect *P. s. maculicola* ES4326 growth; therefore, we predict that *PAD5* encodes another biosynthetic enzyme or affects regulation of camalexin synthesis without affecting defense responses required for limiting *P. s. maculicola* ES4326 growth. Cloning and characterization of other *PAD* genes should help resolve these issues.

The facts that *PAD3* almost certainly encodes an enzyme for camalexin biosynthesis and that *pad3* mutations do not cause disease susceptibility to the bacterial pathogen *P. syringae* (Glazebrook and Ausubel, 1994) imply that camalexin does not play a major role in plant resistance to bacterial infection. In support of this idea, the concentration of camalexin that is toxic to *P. syringae* is much higher than the concentration in infected plants, and camalexin-resistant mutants of *P. s. maculicola* ES4326 are not more virulent than wild-type bacteria (Rogers et al., 1996). Although it is possible that other host defense responses are enhanced in *pad3* mutants and that this compensatory stimulation of alternate defense responses may diminish the impact of restricted phytoalexin synthesis in the mutants, thus far no such compensatory defense responses have been found.

The enhanced sensitivity of *pad3* to the fungal pathogen *A. brassicicola* suggests a role for camalexin in resistance to some fungal pathogens (Thomma et al., 1998). Consistent with our conclusion that *PAD3* encodes a biosynthetic enzyme, the expression of *PR-1*, *PR-4*, and *PDF1.2* in response to *A. brassicicola* was unaffected in *pad3* plants (Thomma et al., 1999). Consequently, the only defect in *pad3* plants probably involves camalexin synthesis. Therefore, camalexin seems to be an important factor contributing to resistance to *A. brassicicola.* Alterations in camalexin

concentrations might have dramatic effects on other fungal pathogens as well.

METHODS

Plants and Growth Conditions

Plants (*Arabidopsis thaliana* ecotype Columbia) with the *pad3-1*, *pad3-2*, and *acd2-2* mutations have been described previously (Glazebrook and Ausubel, 1994; Greenberg et al., 1994; Glazebrook et al., 1997b). Plants were grown in pots in Metro-Mix 200 soil (Scotts-Sierra Horticultural Products, Marysville, OH) either in a plant room (23 ± 2°C at 15 to 50% humidity and 60 μmol m^{-2} sec^{-1} fluorescent illumination) or in a growth chamber (22 ± 2°C at 85% relative humidity and 100 μmol m^{-2} sec^{-1} fluorescent illumination) on a 12-hr-light/12-hr-dark cycle. Fully expanded leaves of 4-week-old plants were used for all experiments.

Inoculations with Bacteria

Pseudomonas syringae pv *maculicola* ES4326 has been described previously (Dong et al., 1991). The avirulence gene *avrRpt2* was carried on plasmid pLH12, as described previously (Dong et al., 1991; Whalen et al., 1991). *P. syringae* strains were grown in King's B medium supplemented with appropriate antibiotics (Glazebrook and Ausubel, 1994). Bacteria were infiltrated into Arabidopsis plants, as described previously (Glazebrook and Ausubel, 1994). Unless stated otherwise, for camalexin assays, the bacterial dose for *P. syringae* strains was 3×10^4 colony-forming units per cm^2 leaf area (equivalent to $OD_{600} = 0.006$). For experiments involving extraction of total RNA from infected leaves, strains *P. s. maculicola* ES4326 and *P. s. maculicola* ES4326 carrying *avrRpt2* were introduced at a dose of 10^4 colony-forming units per cm^2 leaf area (equivalent to $OD_{600} = 0.002$).

Camalexin Determination

Camalexin assays were performed as described previously (Glazebrook and Ausubel, 1994).

Preparation of DNA

Plant genomic DNA samples were isolated from frozen leaf tissues, according to standard procedures (Dellaporta et al., 1983). Yeast strains carrying appropriate yeast artificial chromosome (YAC) clones were grown in 5 mL of AHC medium (6.7 g/L yeast nitrogen base with amino acids or ammonium sulfate, 10 g/L acid-hydrolyzed casein, 20 g/L glucose, and 20 μg/mL adenine sulfate) at 30°C until late-log phase. After being washed with 1 M sorbitol, the cell pellet was resuspended in a solution of 1 M sorbitol, 10 mM EDTA, 100 mM sodium citrate, pH 5.8, and 30 mM β-mercaptoethanol and then incubated with 10 mg/mL lyticase at 30°C for 1 hr. DNA was extracted in a 300-μL solution containing 50 mM Tris, 20 mM EDTA, pH 7.5, and 1% SDS at 65°C for 30 min. After the addition of 250 μL of 5 M potassium acetate and incubation on ice for 30 min, DNA in the supernatant was precipitated in 500 μL of isopropanol, treated with RNaseA, extracted with 50:50 (v/v) phenol:chloroform, and precipitated with ethanol. The final yield of yeast DNA was ~1 μg/mL. This procedure was scaled up to obtain large quantities of yeast DNA for subcloning. Bacterial artificial chromosome (BAC) DNA was purified by using the protocol provided by the Arabidopsis Biological Resource Center (Columbus, OH).

Construction of the Cosmid Library

Yeast DNA from YAC clone CIC6B11 was partially digested with Taq I and fractionated on a sucrose gradient. The fraction containing 19- to 22-kb fragments was ligated with ClaI-digested pCLD04541 (Bent et al., 1994) and packaged by using Gigapack II Gold Packaging Extract (Stratagene, La Jolla, CA).

Generation of Probes from YAC and BAC Ends

To obtain the right end (CIC11D3R) from YAC CIC11D3, we digested ~200 ng of yeast DNA with HincII and treated it with the Klenow fragment of DNA polymerase I followed by T4 DNA ligase. Inverse polymerase chain reaction (PCR) was conducted by using primers that hybridize with the pYAC4 vector (GenBank accession number U01086): P8, 5′-TCTGGGAAGTGAATGGAG-3′; and P13, 5′-TGGGCTGCTTCCTAATGCA-3′. BAC ends from T7C18 and T11A23 were obtained by using a modified version of the adapter ligation protocol (Siebert et al., 1995). Probes were labeled with 11-digoxygenin-dUTP, according to the instructions of the supplier (Boehringer Mannheim).

Plant Transformation

Plasmids were introduced into *Agrobacterium tumefaciens* GV3101 pMP90 (Koncz and Schell, 1986) by triparental mating in the presence of the helper strain of *Escherichia coli* MM294 pRK2013. Plants were transformed by the method of Bechtold et al. (1993), except that 0.005% Silwet L-77 (Lehle Seeds, Round Rock, TX) was added to the bacterial suspension and the vacuum step was omitted. Transformants were selected on plates containing 0.5 × Murashige and Skoog salts (Life Technologies, Gaithersburg, MD), 1 × Gamborg's B5 vitamins (Sigma), and 50 μg/mL kanamycin.

DNA Sequencing

Cosmid DNA containing *PAD3* was digested with HindIII and subcloned in Bluescript II SK+ (Stratagene). DNA sequencing was performed by using standard dye-terminator sequencing procedures and automated sequencers (models 373 and 377; Applied Biosystems, Foster City, CA).

Isolation of cDNA

The *PAD3* cDNA was obtained by using a Marathon cDNA amplification kit (Clontech, Palo Alto, CA) and mRNA prepared from wild-type plants infected with *P. s. maculicola* ES4326. The gene-specific primer for 5′ rapid amplification of cDNA ends (RACE)–PCR was 5′-GCTCTCTCTTCCAGGCTTAAGATGCTCG-3′; for 3′ RACE-PCR, it was 5′-CTGAGTTTGCTACGAGACAATCTCCGGTG-3′. A full-length

double-stranded cDNA was obtained by performing end-to-end amplification with primer pairs complementary to the ends of 5′ and 3′ RACE products: 5′-CCTTCGAAATATAAGCTTCCTCCGGGTCC-3′ and 5′-GGCTTCCTCCTGCTTCGCCAATCCCCAATC-3′.

RNA Gel Blot Analysis

RNA gel blots with 5 μg of total RNA per lane were prepared as described previously (Zhou et al., 1998). The *PAD3* probe was made from pZN22 cosmid DNA by PCR amplification with sense primer 5′-CCGGTGAATCTTGAGAGAGCC-3′ and antisense primer 5′-GATCAGCTCGGTCATTCCCC-3′; PCR was used to label the antisense single-strand DNA with digoxygenin-11-dUTP. Blots were stripped and reprobed with the 18S rRNA probe to assess equal loading of RNA samples (Zhou et al., 1998).

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