A Role for a Menthone Reductase in Resistance against Microbial Pathogens in Plants[1][C][W][OA]

Hyong Woo Choi, Byung Gil Lee, Nak Hyun Kim, Yong Park, Chae Woo Lim[2], Hyun Kyu Song, and Byung Kook Hwang*

Laboratory of Molecular Plant Pathology (H.W.C., N.H.K., C.W.L., B.K.H.) and Laboratory of Structural Biology (B.G.L., Y.P., H.K.S.), School of Life Sciences and Biotechnology, Korea University, Anam-dong, Sungbuk-ku, Seoul 136–713, Republic of Korea

Plants elaborate a vast array of enzymes that synthesize defensive secondary metabolites in response to pathogen attack. Here, we isolated the pathogen-responsive *CaMNR1* [menthone: (+)-(3*S*)-neomenthol reductase] gene, a member of the short-chain dehydrogenase/reductase (SDR) superfamily, from pepper (*Capsicum annuum*) plants. Gas chromatography-mass spectrometry analysis revealed that purified CaMNR1 and its ortholog AtSDR1 from Arabidopsis (*Arabidopsis thaliana*) catalyze a menthone reduction with reduced nicotinamide adenine dinucleotide phosphate as a cofactor to produce neomenthol with antimicrobial activity. CaMNR1 and AtSDR1 also possess a significant catalytic activity for neomenthol oxidation. We examined the cellular function of the *CaMNR1* gene by virus-induced gene silencing and ectopic overexpression in pepper and Arabidopsis plants, respectively. *CaMNR1*-silenced pepper plants were significantly more susceptible to *Xanthomonas campestris* pv *vesicatoria* and *Colletotrichum coccodes* infection and expressed lower levels of salicylic acid-responsive *CaBPR1* and *CaPR10* and jasmonic acid-responsive *CaDEF1*. *CaMNR1*-overexpressing Arabidopsis plants exhibited enhanced resistance to the hemibiotrophic pathogen *Pseudomonas syringae* pv *tomato* DC3000 and the biotrophic pathogen *Hyaloperonospora parasitica* isolate Noco2, accompanied by the induction of *AtPR1* and *AtPDF1.2*. In contrast, mutation in the *CaMNR1* ortholog *AtSDR1* significantly enhanced susceptibility to both pathogens. Together, these results indicate that the novel menthone reductase gene *CaMNR1* and its ortholog *AtSDR1* positively regulate plant defenses against a broad spectrum of pathogens.

Plants constitutively synthesize a wide variety of secondary metabolites to aid fitness by preventing pathogen invasion and insect herbivory as well as by attracting pollinators and natural enemies of herbivores (Wink, 1998; Osbourn et al., 2003; Kliebenstein, 2004). Moreover, plants also induce defensive secondary metabolites in response to pathogen attack and stress (Litvak and Monson, 1998; Arimura et al., 2000; Biere et al., 2004), including compounds such as terpenoids, phytoalexins, and glucosinolates. It has been reported that the plant kingdom produces approximately 50,000 secondary metabolites of known structure, including 30,000 terpenoids, 12,000 alkaloids, 2,500 phenylpropanoids, and 2,500 other compounds (De Luca and St Pierre, 2000). In Arabidopsis (*Arabidopsis thaliana*), approximately 25% of the total 25,498 protein-encoding genes are predicted to be involved in secondary metabolism (Arabidopsis Genome Initiative, 2000). These findings suggest the significance of secondary metabolite production in plant defense responses to biotic and abiotic stresses. However, relatively little is known regarding the functions of the genes that control the biosynthesis of secondary metabolites.

Among these metabolites, terpenoids comprise one of the largest and most diverse groups, which include menthol, abscisic acid, chlorophyll, gibberellin, β-carotene, and rubber (Davis and Croteau, 2000). Terpenoids are frequently described as natural products active against a variety of herbivores (Litvak and Monson, 1998) and pathogens, including bacteria (Cichewicz and Thorpe, 1996; Trombetta et al., 2005; Schelz et al., 2006), fungi (Kubo et al., 1993; Rana et al., 1997), viruses (Harrigan et al., 1993; Sun et al., 1996), and protozoa (Ghoshal et al., 1996). For instance, conifer oleoresin, a complex mixture of terpenoids secreted in response to attack by insect predators, is toxic to insects and their symbiotic fungal pathogens (Philips and Croteau, 1999). The specific mechanisms

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[2] Present address: School of Bioresource Sciences, Dankook University, San 29, Anseo-dong, Cheonan-si, Chungcheongnam-do 330–714, Korea.

* Corresponding author; e-mail bkhwang@korea.ac.kr.

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of the antimicrobial action of terpenoids are not fully understood. However, the toxic effects of terpenoids on membrane structure and function have been studied (Mendoza et al., 1997; Trombetta et al., 2005; Pérez-Fons et al., 2006). The lipophilic property of terpenoids allows them to partition from the aqueous phase into membrane structures, resulting in membrane expansion, increased membrane fluidity and permeability, disturbance of membrane-embedded proteins, inhibition of respiration, and alteration of ion transport processes.

The significance of terpenoids in disease resistance has been reported for a number of plant-pathogen interactions. For instance, sesquiterpenoid phytoalexins, including 2,7-dihydroxycadalene, 2-hydroxy-7-methoxycadalene, lacinilene C, and lacinilene C 7-methyl ether, significantly accumulate in the leaves of resistant cotton (*Gossypium hirsutum*) lines but not in susceptible varieties after infection by *Xanthomonas campestris* pv *malvacearum* (Essenberg et al., 1982, 1990; Pierce et al., 1996). These compounds diffuse from cells exhibiting the hypersensitive response (HR) to arrest bacterial growth in resistant plants. Moreover, mutational analysis of a fungal pathogen of oat (*Avena sativa*) roots, *Gaeumannomyces graminis*, revealed that the triterpene saponin determines the host range of these pathogens (Bowyer et al., 1995). Mutation of *G. graminis* avenacinase, a saponin-detoxifying enzyme, leads to a loss of pathogenicity on oat, which produces saponins. However, the mutant fungal pathogen retains full pathogenicity on wheat (*Triticum aestivum*), which does not produce saponins. These findings underscore the significant roles of terpenoids in plant defense responses.

The biosynthetic pathway of terpenoids is well established in peppermint (*Mentha piperita*) by in vivo and cell-free studies (Davis and Croteau, 2000; Gershenzon et al., 2000; Mahmoud and Croteau, 2001; Ringer et al., 2005; Davis et al., 2005). Terpenoids (monoterpenes [C_{10}], sesquiterpenes [C_{15}], diterpenes [C_{20}], triterpenes [C_{30}], tetraterpenes [C_{40}], and polyterpenes [$>C_{40}$]) are synthesized by the condensation of isopentenyl diphosphate and its allylic isomer, dimethylallyl diphosphate, by prenyltransferase following conversion by terpenoid synthase and various enzymes, such as short-chain dehydrogenase/reductases (SDRs).

The primary pathway of monoterpene biosynthesis is well characterized, and a variety of enzymes is required in these reactions (Croteau et al., 2005; Davis et al., 2005; Ringer et al., 2005). The final product, (−)-menthol, which is synthesized as the most familiar monoterpene in this pathway, is used in a wide range of confectionery goods, pharmaceuticals, oral health-care products, cigarettes, and toiletries (http://www.leffingwell.com/menthol1/menthol1.htm). The conversion from (−)-menthone to (−)-menthol is catalyzed by stereospecific dehydrogenases, such as menthone reductases [MMR, (−)-menthone:(−)-(3*R*)-menthol reductase; MNR, (−)-menthone:(−)-(3*S*)-neomenthol reductase], in a NADPH-dependent manner (Davis et al., 2005). Sequence analysis of the cDNAs encoding MNR and MMR indicates that menthone reductases belong to a member of the SDR superfamily (Kallberg et al., 2002). SDRs are enzymes of great functional diversity. They typically share only 15% to 30% sequence identity, but specific sequence motifs are prominent, indicating common folding patterns (Kallberg et al., 2002). SDRs have approximately 250-residue subunits catalyzing NAD(P)(H)-dependent oxidation/reduction reactions. Most neomenthol is known to be glycosylated and transported to the rhizome in peppermint (Kjonaas et al., 1982). However, its biological function has not yet been determined in peppermint and other plant species.

Here, we isolated and functionally characterized a novel gene, menthone: (+)-(3*S*)-neomenthol reductase (*CaMNR1*), from pepper (*Capsicum annuum*) leaves infected by *Xanthomonas campestris* pv *vesicatoria* (*Xcv*) and its ortholog SDR gene from *Arabidopsis*, *AtSDR1*. The pepper *CaMNR1* and its ortholog Arabidopsis *AtSDR1* genes were overexpressed in *Escherichia coli* and purified to homogeneity. Enzyme activity of purified CaMNR1 and AtSDR1 was confirmed by gas chromatography-mass spectrometry (GC-MS) analysis of reaction products. The catalytic reactions of CaMNR1 and AtSDR1 yielded predominantly neomenthol as a reaction product at neutral pH. In this study, we used virus-induced gene silencing (VIGS) in pepper (Baulcombe, 1999) and ectopic expression in Arabidopsis (Clough and Bent, 1998) as efficient reverse genetics approaches to define the functions of the *CaMNR1* gene in plant defense. *CaMNR1*-silenced pepper plants were highly susceptible to infection by *Xcv* and *Colletotrichum coccodes*, accompanied by significantly lowered expression levels of the salicylic acid (SA)-responsive *CaBPR1* (basic PR1) and *CaPR10* and jasmonic acid (JA)-responsive *CaDEF1* (defensin) genes. Transgenic Arabidopsis plants that constitutively overexpressed the *CaMNR1* gene also exhibited enhanced basal resistance to *Pseudomonas syringae* pv *tomato* (*Pst*) DC3000 and *Hyaloperonospora parasitica*. In contrast, a T-DNA insertion in *AtSDR1*, a putative ortholog of *CaMNR1* in Arabidopsis, enhanced susceptibility to these pathogens.

RESULTS

CaMNR1 cDNA Encodes the MNR Protein

The *CaMNR1* cDNA was isolated from a cDNA library made from pepper leaves infected with the *Xcv* avirulent strain Bv5-4a using a macro cDNA array method (Jung and Hwang, 2000; Chung et al., 2007). The *CaMNR1* cDNA consists of 1,136 bp, including a poly(A) tail, and it encodes a protein of 314 amino acids with a predicted molecular mass of 34.7 kD and a pI of 5.39 (Supplemental Fig. S1). A database search (http://www.ncbi.nlm.nih.gov/blast/) using the translated CaMNR1 amino acid sequence as a query showed 59% identity to the MNR protein (accession no.

AAQ55959) from peppermint (Davis et al., 2005). CaMNR1 shares moderate amino acid identity (55%–57%) with Arabidopsis and peppermint SDRs (Fig. 1A). Computational analysis of the CaMNR1 peptide sequence using PROSITE (www.expasy.org/prosite) revealed that CaMNR1 possesses conserved short-chain dehydrogenase domains, including a coenzyme-binding domain (motif I: GxxxGxG), a structural domain of undefined function (motif II: [N/C]NAG), and an active site element (motif III: YxxxK; Kallberg et al., 2002; Davis et al., 2005; Ringer et al., 2005). A phylogenetic tree of CaMNR1 and its closest relatives illustrates its proximity to peppermint SDRs, including MNR, MMR, and isopiperitenone reductase, and to uncharacterized SDRs from Arabidopsis (Fig. 1B).

Overexpression and Purification of CaMNR1 in *E. coli*

The CaMNR1 protein was overexpressed in *E. coli* and purified to homogeneity in several chromatographic steps (Fig. 2A). Neomenthol was the predominant reaction product generated by CaMNR1, while menthol was produced extremely slowly and at very low yield. CaMNR1 shares slightly higher sequence identity with peppermint MNR than with MMR. Therefore, we designated CaMNR1 as a menthone neomenthol reductase. The final yield of recombinant CaMNR1 was approximately 10 mg of over 99% purity from a 3-L bacterial culture (Fig. 2A). Purified CaMNR1 is a monomeric enzyme in solution with a molecular mass of approximately 34 kD, as indicated by gel filtration analysis (Fig. 2A). As described previously, CaMNR1 belongs to the SDR superfamily, members of which adopt a unique α/β structure known as the Rossmann fold (Oppermann et al., 2003). Their coenzyme specificity can be predicted very accurately by a hidden Markov model-based method (Kallberg and Persson, 2006). When the CaMNR1 sequence was submitted to a prediction server (http://www.ifm.liu.se/bioinfo/), a NADP-binding domain (residues 4–46) was identified, which indicates the same coenzyme specificity as that of biochemically characterized MMR and MNR (Davis et al., 2005).

Enzyme Activity of CaMNR1

Monoterpene products were quantified and identified by their GC retention times in comparison with standards including (+)-camphor (Fig. 2B). CaMNR1 converted (−)-menthone to 93% (+)-(3*S*)-neomenthol and 7% (−)-(3*R*)-menthol at pH 7.5 and to 72% (+)-(3*S*)-neomenthol and 28% (−)-(3*R*)-menthol at pH 9.0 with NADPH as a cofactor. Although the amount of menthol generated was higher at pH 9.0 than at pH 7.5, neomenthol was the predominant reaction product (Fig. 2, C and D).

The oxidation of menthol isomers in the presence of $NADP^+$ was also evaluated. Only neomenthol was converted into menthone in the presence of the $NADP^+$ cofactor. The oxidation reaction displayed maximal activity at alkaline pH (9.0), whereas the reduction reaction was maximal at neutral pH 7.5 (Fig. 2, E and F). All kinetic parameters of CaMNR1 for monoterpene substrates and cofactors are summarized in Table I. Interestingly, the K_m values of CaMNR1 for the forward reaction (reduction) were lower and its turnover rates were much higher than those for the reverse reaction (oxidation); however, the catalytic efficiencies of CaMNR1 for both reactions were similar.

In Vitro Antimicrobial Activities of Menthone, Neomenthol, and Menthol

In vitro analysis of enzyme activity revealed that CaMNR1 converted (−)-menthone to (+)-(3*S*)-neomenthol and (−)-(3*R*)-menthol (Fig. 2). As described previously, most terpenoids are known to be active against a wide variety of microorganisms, including gram-positive and gram-negative bacteria and fungi (Cowan, 1999; Trombetta et al., 2005; Arfa et al., 2006). We analyzed the antimicrobial activities of (−)-menthone and its metabolites (+)-(3*S*)-neomenthol and (−)-(3*R*)-menthol using the microatmosphere method (Arfa et al., 2006). Interestingly, neomenthol and menthol strongly inhibited the growth of the phytopathogenic bacteria *Pst* DC3000 and *Xcv* at a concentration of 1 mg per plate (Fig. 3A). However, menthone, the precursor form of neomenthol and menthol, was not effective at inhibiting *Pst* and *Xcv* growth at a concentration of 5 mg per plate. Similarly, neomenthol and menthol completely inhibited spore germination and hyphal growth of the phytopathogenic fungi *Alternaria brassicicola* and *C. coccodes* at a concentration of 5 mg per plate; however, menthone was inhibitory against *A. brassicicola* and *C. coccodes* at higher concentrations (>10 mg per plate; Fig. 3, B and C).

CaMNR1 Is Expressed in an Organ-Specific Manner and Induced in Leaves by Bacterial Infection and Abiotic Elicitor Treatments

Expression of the *CaMNR1* gene was examined in different pepper organs by RNA gel-blot analysis (Supplemental Fig. S2A). The *CaMNR1* transcripts were detected in flowers and red fruit tissues of healthy pepper plants. However, transcripts were not present in healthy leaves, stems, roots, or green fruits. RNA gel-blot analysis of the *CaMNR1* gene was performed to determine whether it is induced in pepper leaves during compatible and incompatible interactions with *Xcv* (Fig. 4A). The *CaMNR1* gene was strongly induced in the leaves inoculated with the avirulent strain Bv5-4a (incompatible interaction). However, *CaMNR1* transcripts were only weakly detected in leaves inoculated with the virulent strain Ds1 (compatible interaction). Protein expression is often indicative of gene function. We next determined CaMNR1 protein levels by protein gel-blot analysis to investigate the possible role of CaMNR1 in enhancing plant resistance to *Xcv* infection. As shown in Figure 4B, infection with the avirulent *Xcv* strain

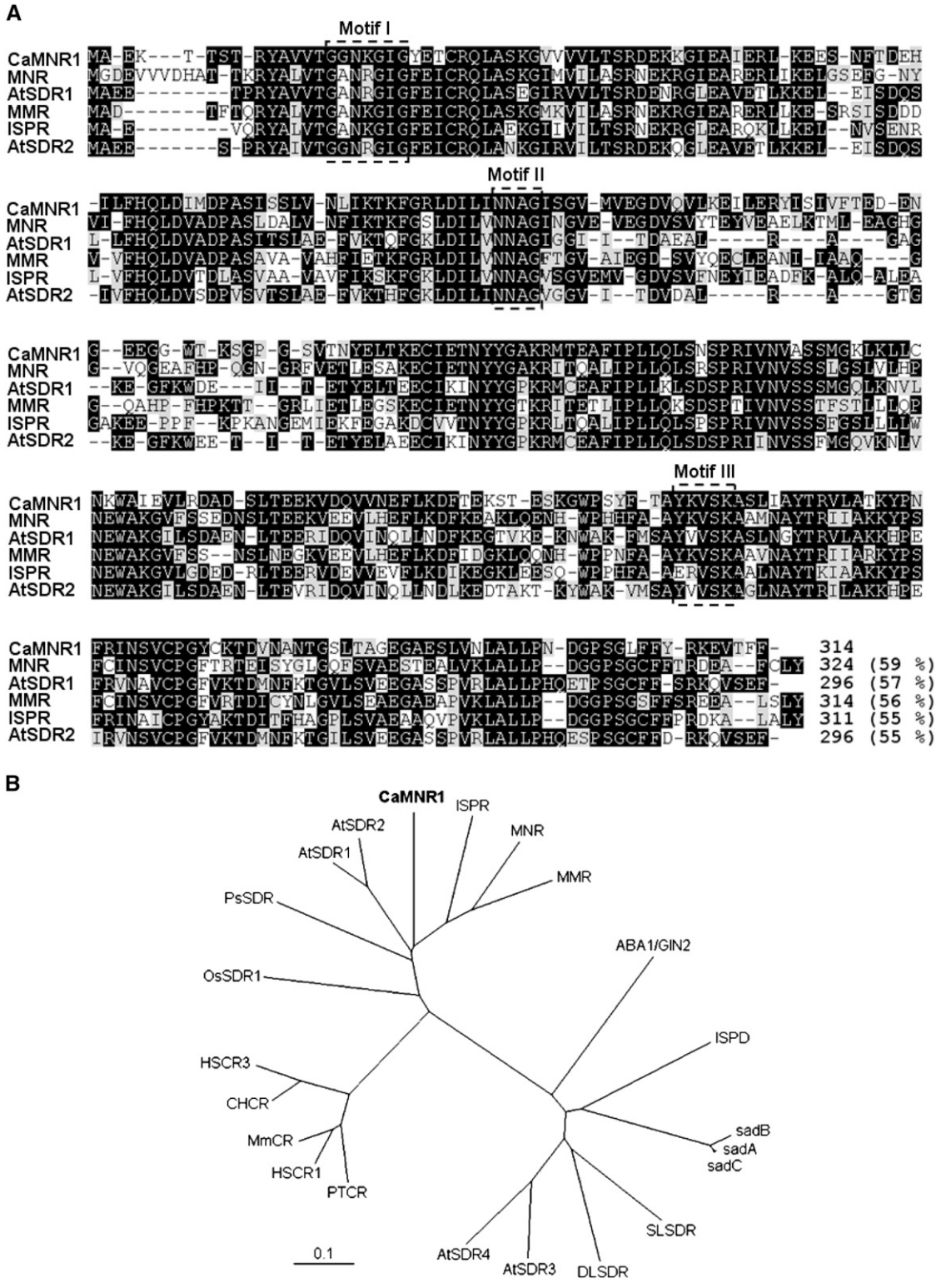


Figure 1. Amino acid sequence alignment and phylogenic analysis of *CaMNR1*. A, Comparison of the deduced amino acid sequences of the pepper *CaMNR1* cDNA (CaMNR1) with menthone: (+)-(3*S*)-neomenthol reductase protein (MNR), (−)-menthone: (−)-(3*R*)-menthol reductase (MMR), isopiperitenone reductase (ISPR), and Arabidopsis SDRs (AtSDR1 and AtSDR2). The conserved coenzyme-binding domain (motif I: GXXXGXG), the structural domain of undefined function (motif II: [N/C]NAG), and the active site element (motif III: YXXXK) are shown above the alignment. Black boxes indicate identical amino acid residues. Dashes indicate spacing in the amino acid sequences for proper alignment. B, Alignment of full-length pepper CaMNR1 with MNR (accession no. AAQ55959), MMR (accession no. AAQ55960), ISPR (accession no. AAQ75422), (−)-trans-

strongly induced the accumulation of CaMNR1 in pepper leaves, compared with that in leaves infected with the virulent *Xcv* strain. These data indicate that the *CaMNR1* gene plays a role in regulating the resistance of pepper plants at the mRNA and protein levels.

To determine whether *CaMNR1* expression is triggered by abiotic elicitors, pepper plants at the six-leaf stage were treated with SA, ethylene, methyl jasmonate (MeJA), and abscisic acid (ABA; Supplemental Fig. S2B). The *CaMNR1* gene was strongly induced by SA, ethylene, and ABA treatment. In pepper leaves treated with ethylene and ABA, *CaMNR1* transcripts began to significantly increase at 1 h after treatment, and they were markedly induced at 15 h after SA treatment. By contrast, MeJA slightly induced *CaMNR1* gene expression at 5 h after treatment. To determine whether *CaMNR1* expression is influenced by environmental stress, pepper plants were exposed to hydrogen peroxide (H_2O_2), drought, cold, and mechanical wounding stresses (Supplemental Fig. S2C). Under drought conditions, the *CaMNR1* transcript was rapidly induced within 1 h and thereafter gradually increased to 25 h. However, H_2O_2, cold, and mechanical wounding treatment did not cause *CaMNR1* transcripts to appear in treated pepper leaves.

Metabolite Profiles in Pepper Leaves

We tested the in vitro enzyme activities of CaMNR1 (Fig. 2) and the antimicrobial activities of menthone, neomenthol, and menthol (Fig. 3). The findings led us to check whether these monoterpenes are present in pepper leaves. We first used GC-MS analysis to evaluate the quantity of metabolic components induced by *Xcv* infection. In pepper plants, some pathogen-induced metabolic compounds were increased or newly induced at 18 h after inoculation with virulent and avirulent *Xcv* (Table II; Supplemental Figs. S3 and S4). The lipid-derived volatile compounds 2-hexenal, 3,5-dimethyl-3-hexanol, and cis-3-hexenol distinctly accumulated in pepper leaves infected with virulent and avirulent *Xcv* (Table II). The long-chain hydrocarbons 2,6,10-trimethyldodecane (farnesane) and 2-hexyl-1-decanol also accumulated in pepper leaves inoculated with *Xcv*. In particular, trans-2-cis-6-nonadienal, a product derived from a 9-hydroperoxide of linolenic acids (Matthew and Galliard, 1978), was de novo synthesized and induced mainly by infection with avirulent *Xcv* that triggered HR. Previously, HR-specific accumulation of trans-2-cis-6-nonadienal was reported in *Phaseolus vulgaris* leaves (Croft et al., 1993). Furthermore, some metabolic compounds with a benzene ring, including 1-ethly-2,4-dimethylbenzene, 1,2,4,5-tetramethyl benzene, and 2-hydroxy-methylester benzoic acid (methyl salicylate; MeSA), were increased during *Xcv* infection. Recently, MeSA was reported to be a key mobile signal in systemic acquired resistance in plants (Park et al., 2007). However, we failed to detect menthone, neomenthol, menthol, or their derivatives among the metabolic components of extracts of healthy or *Xcv*-infected pepper leaves (Supplemental Figs. S3 and S4).

VIGS of the *CaMNR1* Gene Alters Pepper Defense-Related Gene Expression

We revealed that the *CaMNR1* gene was strongly induced in pepper leaves during the incompatible interaction of pepper plants with the *Xcv* avirulent strain Bv5-4a (Fig. 4). To examine the effect of loss of function of *CaMNR1*, we silenced the gene in pepper plants using the tobacco rattle virus (TRV)-mediated VIGS technique and the full-length open reading frame (Liu et al., 2002; Chung et al., 2004; Choi et al., 2007). *CaMNR1* transcript levels were analyzed in unsilenced (TRV:00) and silenced (TRV:*CaMNR1*) leaves by reverse transcription (RT)-PCR (Fig. 5A). For the empty vector control, unsilenced leaves, the *CaMNR1* gene was strongly induced at 18 h after inoculation with virulent and avirulent strains of *Xcv* (5×10^6 colony forming units [cfu] mL^{-1}), but expression was compromised in *CaMNR1*-silenced pepper leaves. This suggests that silencing was effective for *CaMNR1*. We further tested whether the expression of several pepper pathogenesis-related (*PR*) genes was affected in *CaMNR1*-silenced leaves. Transcript levels of pepper *CaBPR1*, *CaPR4*, *CaPR10*, *CaDEF1*, and *CaSAR8.2*, but not of *CaPOA1* (ascorbate peroxidase) and *CaOSM1* (osmotin), were significantly lowered in *CaMNR1*-silenced leaves infected with *Xcv*, especially during the compatible interaction. Healthy pepper leaves did not produce any transcripts of the *PR* genes examined. In order to determine the relative abundance of the CaMNR1 protein, we next performed protein gel-blot analysis using rabbit polyclonal antibodies against CaMNR1 (Fig. 5B). Accumulation of the CaMNR1 protein was completely compromised in

Figure 1. (*Continued.*)
iso-piperitenol dehydrogenase (ISPD; accession no. AAU20370), Arabidopsis SDRs (AtSDR1, accession no. At3g61220; AtSDR2, accession no. At2g24190; AtSDR3, accession no. At2g47140; AtSDR4, accession no. At2g47130; and ABA1/GIN2, accession no. At1g52340), *Papaver somniferum* SDR (PsSDR; accession no. ABC47654), *Oryza sativa* SDR (OsSDR1; accession no. NP_001053402), *Pisum sativum* SDRs (sadA, accession no. AAF04193; sadB, accession no. AAF04194; and sadC, accession no. AAF04253), *Solanum lycopersicum* SDR (SLSDR; accession no. CAB91875), *Digitalis lanata* 3-β-hydroxysteroid-hydrogenase (DLSDR; accession no. CAC93667), human carbonyl reductases (HSCR1, accession no. J04056; and HSCR3, accession no. NP_001227), *Mus musculus* carbonyl reductase (MmCR; accession no. NP_031646), pig (*Sus scrofa*) carbonyl reductase (PTCR; accession no. M80709), and hamster (*Cricetulus griseus*) carbonyl reductase (CHCR; accession no. AB020238). The scale value of 0.1 represents 0.1 amino acid substitutions per site.

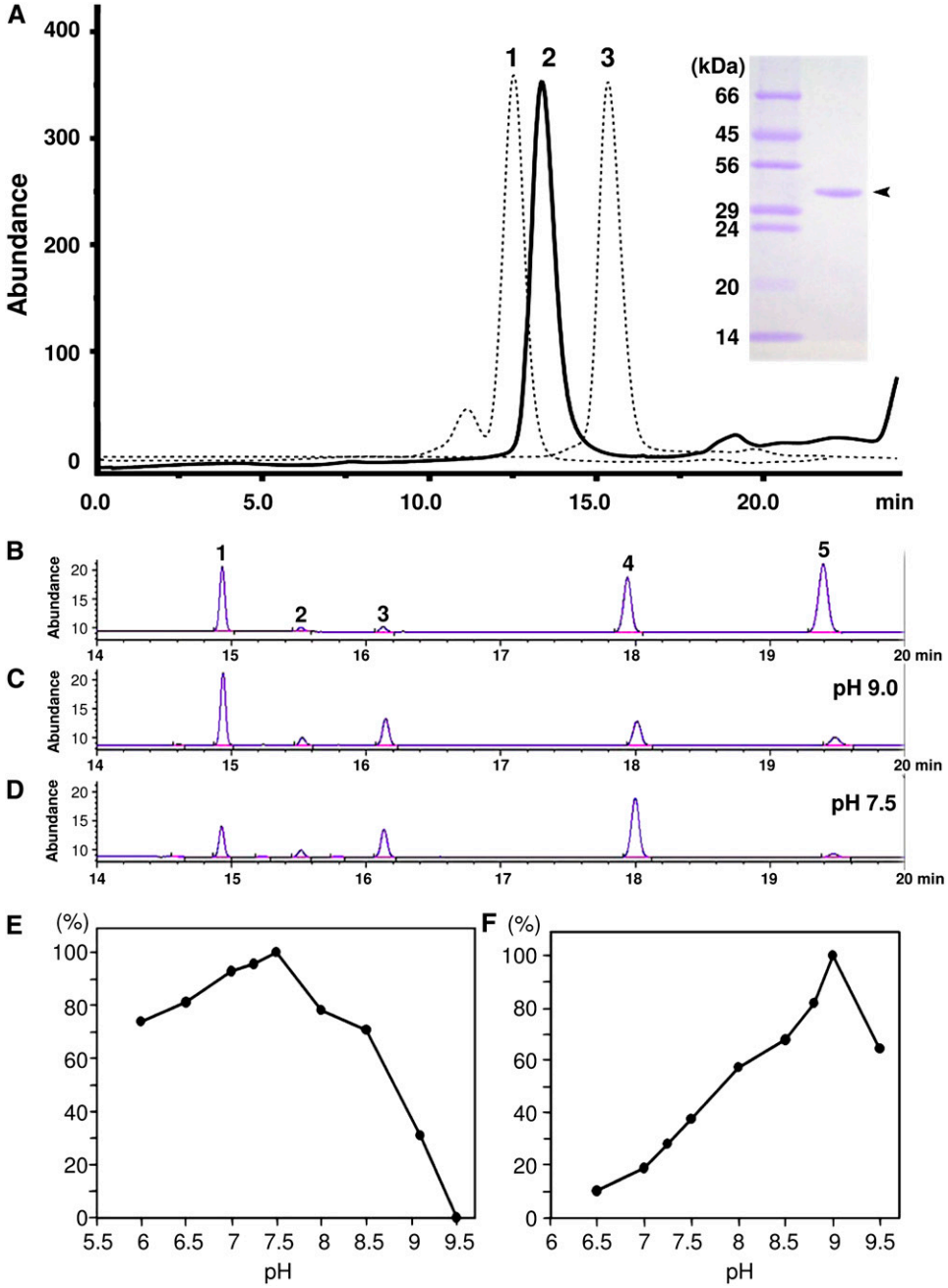


Figure 2. Purification and enzyme activity of CaMNR1. A, Gel filtration profile of recombinant CaMNR1 by Superose 12 column chromatography reveals that purified CaMNR1 (34 kD; peak 2) is a monomeric enzyme in solution. Standard molecular mass markers are bovine serum albumin (66 kD; peak 1) and chymotrypsinogen (25 kD; peak 3). The inset represents the final purity of recombinant CaMNR1 (arrowhead) and molecular mass markers. B, Gas chromatographic analyses of monoterpene standards: peak 1, menthone; peak 2, isomenthol; peak 3, camphor; peak 4, neomenthol; and peak 5, menthol. C and D, Chromatograms of enzymatic reaction products assayed at pH 9.0 (C) and pH 7.5 (D). The *y* axis is a detector response unit corresponding to the generated reaction products. E, Effects of pH on enzymatic activity using menthone as a substrate. F, Effects of pH on enzymatic activity using neomenthol as a substrate. Product values (*y* axis) are relative percentages when the maximal amount at optimal pH is taken as 100%. [See online article for color version of this figure.]

silenced leaves at 18 h after inoculation with virulent *Xcv* (compatible interaction); however, the CaMNR1 protein remained at a detectably visible level in the avirulent *Xcv*-infected leaves (incompatible interaction). These analyses indicated that TRV-mediated VIGS of *CaMNR1* compromised the accumulation of *CaMNR1* transcripts and protein and that this was accompanied by lower expression of some defense-related genes during the defense response of pepper plants.

Table I. *Kinetic parameters of CaMNR1*

Parameter	Forward Reaction		Reverse Reaction	
	Menthone	NADPH	Neomenthol	$NADP^+$
K_m (μM)	35.1 ± 4.3	21.7 ± 3.4	113.2 ± 14.9	76.7 ± 1.2
k_{cat} (s^{-1})	0.039 ± 0.003	0.053 ± 0.01	0.13 ± 0.01	0.13 ± 0.001
k_{cat}/K_m ($\mu M^{-1}\ s^{-1}$)	1.01 ± 0.05 × 10^{-3}	2.44 ± 0.28 × 10^{-3}	1.11 ± 0.04 × 10^{-3}	1.74 ± 0.02 × 10^{-3}

VIGS of *CaMNR1* Enhances the Susceptibility of Pepper against *Xcv* and *C. coccodes*

Silencing resulted in a susceptible response of pepper to infection by the *Xcv* virulent strain Ds1 and the avirulent strain Bv5-4a (Fig. 6). *CaMNR1*-silenced pepper leaves (TRV:*CaMNR1*) exhibit chlorosis at 3 d after virulent *Xcv* inoculation, whereas the empty vector, control leaves (TRV:00) became chlorotic at 5 d after inoculation (Fig. 6A). More severe chlorosis phenotypes appeared in *CaMNR1*-silenced leaves than in unsilenced leaves at 5 d after inoculation. However, we did not observe any apparent phenotypic changes in *CaMNR1*-silenced pepper leaves infected with the avirulent strain Bv5-4a compared with unsilenced leaves (Fig. 6C). Importantly, infection by virulent and avirulent strains of *Xcv* resulted in high levels of bacterial growth in *CaMNR1*-silenced plants compared with unsilenced plants at 3 and 5 d after inoculation (Fig. 6, B and D).

We also analyzed the response of *CaMNR1*-silenced pepper to *C. coccodes*, the causal agent of pepper anthracnose. *CaMNR1*-silenced pepper leaves exhibited enhanced susceptibility to *C. coccodes* infection (Fig. 7). Disease severity was rated on the basis of lesion numbers and areas of brown or dark brown

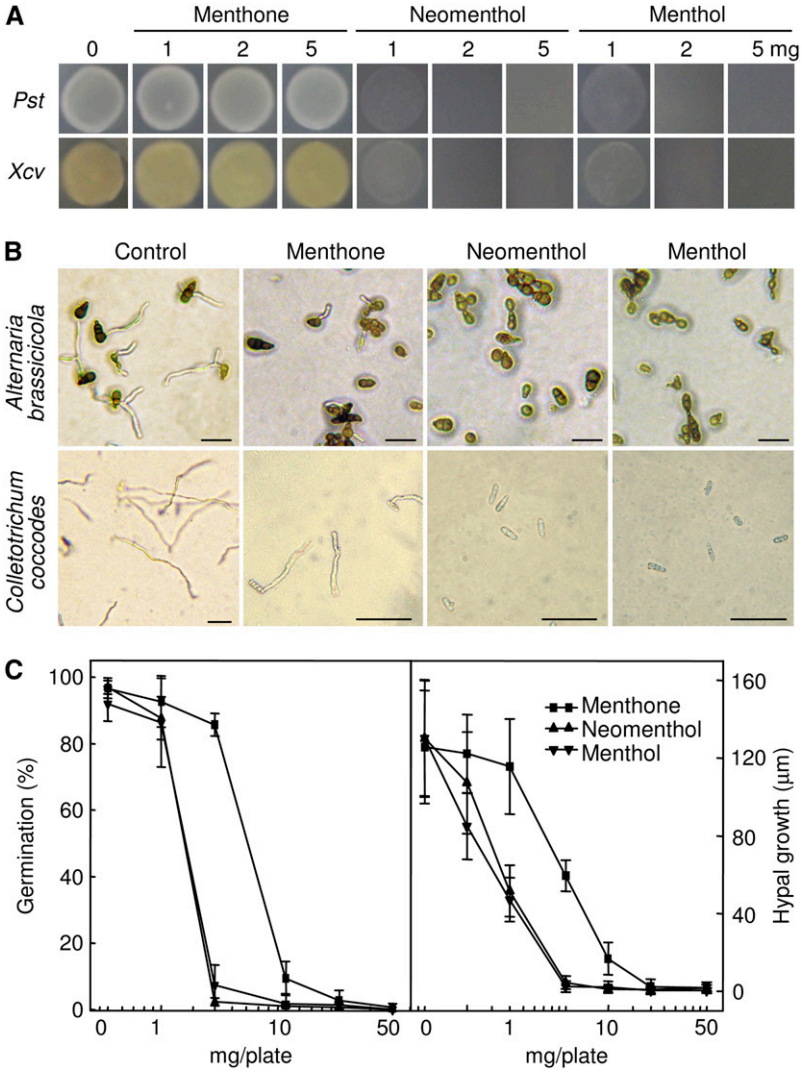


Figure 3. Antimicrobial activities of menthone, neomenthol, and menthol. A, Inhibition of growth of *Pst* DC3000 and *Xcv* at 48 h after treatment with various concentrations of menthone, neomenthol, and menthol. B, Micrographs of the inhibitory effects of menthone, neomenthol, and menthol on spore germination of *A. brassicicola* and *C. coccodes* at 24 h after treatment with 5 mg per plate (9 cm in diameter) of these compounds. Bars = 50 μm. C, Inhibitory effects of menthone, neomenthol, and menthol on spore germination and hyphal growth of *C. coccodes* at 12 and 24 h after treatment with various concentrations of these compounds. Data are means ± SD of three independent experiments.

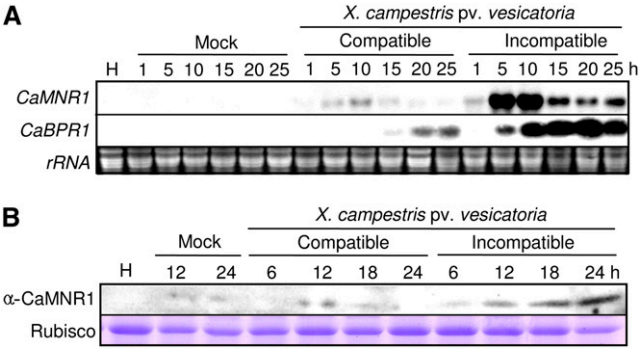


Figure 4. Pathogen-induced expression of *CaMNR1* in pepper leaves. A, RNA gel-blot analysis of the expression of the *CaMNR1* and *CaBPR1* genes in pepper leaves at various time points after inoculation with the *Xcv* virulent strain Ds1 (compatible) and the avirulent strain Bv5-4a (incompatible) at the six-leaf stage. B, Protein gel-blot analysis of the expression of CaMNR1 in pepper leaves at various time points after *Xcv* inoculation. Equal loading of proteins was confirmed by comparing the band intensities of the Rubisco protein stained with Coomassie blue. H, Uninoculated healthy leaves; Mock, 10 mM $MgCl_2$. [See online article for color version of this figure.]

spots at 5 d after inoculation (Fig. 7A). Approximately 45% of *CaMNR1*-silenced leaves showed severe disease symptoms (enlarged dark brown lesions with severe chlorosis), compared with 25% severely diseased, unsilenced leaves. These data indicated that *CaMNR1* expression is required for basal resistance of pepper plants against the bacterial pathogen *Xcv* and the fungal pathogen *C. coccodes*.

Overexpression of *CaMNR1* in Arabidopsis Confers Enhanced Expression of *AtPR1* and *AtPDF1.2*

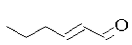
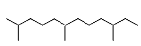
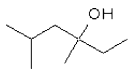
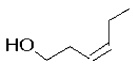
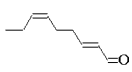
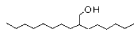
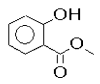
To investigate the effect of a gain of function of *CaMNR1*, transgenic Arabidopsis plants expressing *CaMNR1* were generated. The *CaMNR1* open reading frame was integrated between the cauliflower mosaic virus 35S promoter and the *nos* terminator region in the binary vector pBIN35S (Choi et al., 2007). Arabidopsis ecotype Columbia (Col-0) plants were transformed with the 35S:*CaMNR1* construct by the floral dipping method (Clough and Bent, 1998). We did not observe any visible phenotype difference between wild-type and *CaMNR1*-overexpression (OX) transgenic lines. Three Arabidopsis transgenic T2 lines were tested to determine whether overexpression of *CaMNR1* induces the expression of defense-related genes. The pathogenesis-related genes *AtPR1* and *AtPDF1.2* were strongly induced in the tested *CaMNR1*-OX transgenic lines (Fig. 8A). The transcript levels of the SA-responsive *AtPR1* gene were very high in *CaMNR1*-OX transgenic plants. However, the JA-responsive *AtPDF1.2* gene was induced at relatively low levels compared with *AtPR1*. In contrast, transcripts of the ABA-responsive gene *AtRD29a* were not detected in *CaMNR1*-OX transgenic plants. Wild-type plants produced no detectable transcripts for any of the genes examined. We also examined the expression of the *CaMNR1* gene at the protein level in transgenic Arabidopsis plants using rabbit polyclonal antibodies against CaMNR1 (Fig. 8B). In *CaMNR1*-OX transgenic leaves, CaMNR1 protein was strongly expressed. However, we could not detect any CaMNR1 protein in the wild-type leaves. This led us to conclude that ectopic expression of *CaMNR1* in a heterologous system triggers the expression of *PR* genes (*AtPR1* and *AtPDF1.2*) but not of the ABA-responsive gene *AtRD29a*.

Overexpression of *CaMNR1* in Arabidopsis Enhances Basal Resistance against *Pst* and *H. parasitica*

RT-PCR and protein gel-blot analysis showed that *CaMNR1*-OX transgenic lines constitutively expressed *CaMNR1* and defense-related genes, which suggests a possible role for *CaMNR1* in defense signaling activation in Arabidopsis (Fig. 8). To determine if the overexpression of *CaMNR1* in Arabidopsis confers enhanced resistance, we examined bacterial growth using the virulent (empty vector) or a near-isogenic avirulent (*avrRpt2*) strain of *Pst* DC3000. Seven days after inoculation, severe chlorosis symptoms developed on the leaves of wild-type plants infected with virulent *Pst* DC3000. However, *CaMNR1* transgenic lines exhibited only slight chlorotic symptoms compared with wild-type plants (Fig. 9A). Consistent with the increased expression of defense-related genes and no visible disease symptoms, *CaMNR1* transgenic lines exhibited significantly lower bacterial growth at 3 d after inoculation than did wild-type plants (Fig. 9B). *CaMNR1* transgenic lines also exhibited increased resistance to the avirulent strain *Pst* DC3000 carrying *avrRpt2* (Fig. 9C). The avirulent strain *Pst* DC3000 (*avrRpt2*) grew approximately 10-fold better in wild-type plants compared with *CaMNR1* transgenic lines.

To determine whether the overexpression of *CaMNR1* also enhances disease resistance to a biotrophic oomycete pathogen, we inoculated *H. parasitica* virulent isolate Noco2 onto cotyledons of 7-d-old Col-0 plants. Freshly harvested conidiospores (5×10^4 mL^{-1}) of downy mildew were sprayed onto cotyledons of 7-d-old wild-type (ecotype Col-0) and *CaMNR1*-OX transgenic plants. Seven days after inoculation, sporangiophores were counted on over 50 cotyledons with a stereomicroscope. Wild-type plants exhibited a heavy infestation of sporangiophores on cotyledon surfaces (Fig. 9D). However, the number of sporangiophores was significantly reduced in *CaMNR1* transgenic lines (Fig. 9F). Approximately 53% of wild-type cotyledons exhibited heavy sporulation (>20 sporangiophores per cotyledon). However, only 24% to 26% of *CaMNR1* transgenic cotyledons exhibited heavy sporulation. The average number of sporangiophores was significantly lower in *CaMNR1* transgenic lines (line 1, 13.36; line 2, 13.88; line 3, 12.59) than in wild-type plants (21.05). Trypan blue staining of infected leaves revealed that *CaMNR1*-OX transgenic lines exhibited not only reduced levels of sporulation but also retarded hyphal growth in cotyledons compared

Table II. *Secondary metabolites accumulated in pepper leaves infected with virulent strain Ds1 and avirulent strain Bv5-4a of Xcv*

Peak No.	Compound	Structure	Formula	Molecular Weight	Retention Time	Fold Change[a]	
						Xcv Strain Ds1	*Xcv* Strain Bv5-4a
					min		
4	2-Hexenal		$C_6H_{10}O$	98	16.64	1.58	1.54
6	2,6,10-Trimethyldodecane		$C_{15}H_{32}$	212	17.10	0.98	1.24
16	3,5-Dimethyl-3-hexanol		$C_8H_{18}O$	130	23.19	1.67	1.77
18	1-Ethyl-2,4-dimethylbenzene		$C_{10}H_{14}$	134	24.81	1.14	1.33
20	Cis-3-hexenol		$C_6H_{12}O$	100	25.54	2.71	2.99
25	1,2,4,5-Tetramethylbenzene		$C_{10}H_{14}$	134	28.84	1.21	1.51
40	Trans-2-cis-6-nonadienal		$C_9H_{14}O$	138	37.69	–	New
43	2-Hexyl-1-decanol		$C_{16}H_{34}O$	242	41.65	1.28	1.49
50	2-Hydroxy-methyl ester benzoic acid		$C_8H_8O_3$	152	48.43	1.14	1.26

[a]The fold changes are calculated by peak areas of metabolic compounds from extracts of infected leaves compared with those of uninoculated pepper leaves.

with wild-type plants (Fig. 9E). Thus, we conclude that overexpression of *CaMNR1* confers enhanced basal resistance against at least two taxonomically unrelated hemibiotrophic bacterial and biotrophic oomycete pathogens in Arabidopsis plants.

Altered Responses of *sdr1* Mutants to *Pst* and *H. parasitica*

Alignment and phylogenetic analysis of the deduced amino acid sequence of *CaMNR1* revealed its proximity to two uncharacterized *SDR* genes from Arabidopsis (Fig. 1). To determine the function of *AtSDR1* and *AtSDR2* during the defense response, we analyzed the expression of *AtSDR1* and *AtSDR2* in healthy and *Pst* DC3000-infected Arabidopsis leaves by RT-PCR. As shown in Figure 10A, we could not detect any *AtSDR1* and *AtSDR2* transcripts in non-inoculated healthy leaves. Interestingly, infection by *Pst* DC3000 distinctly induced the expression of *AtSDR1,* but not *AtSDR2*. Expression of *AtSDR1* was stronger in avirulent *Pst* DC3000 (*avrRpm1*)-infected plants than in virulent *Pst* DC3000 (empty)-infected plants. Expression patterns of *AtSDR1*, but not of *AtSDR2*, after infection with bacterial pathogens were similar to those of *CaMNR1* (Figs. 4A and 10A). Furthermore, *CaMNR1* shares higher sequence identity with *AtSDR1* than with *AtSDR2* (Fig. 1B). To test whether AtSDR1 has MNR activity, it was overexpressed in *E. coli* and purified to homogeneity following several chromatographic steps (Supplemental Fig. S6A). Purified AtSDR1 was a monomeric enzyme in solution, with a molecular mass of approximately 32 kD. Monoterpene products were quantified and identified by their GC retention times (Supplemental Fig. S6B). The optimal pH for AtSDR1 enzyme activity of menthone reduction to neomenthol was slightly higher

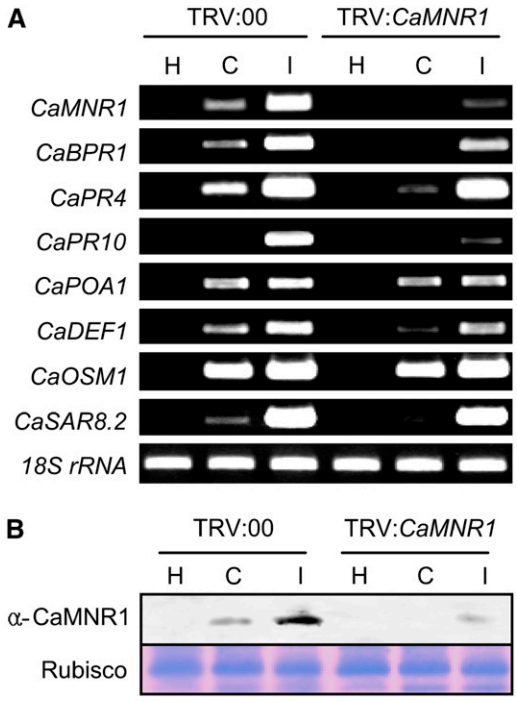


Figure 5. Expression of defense-related genes in *CaMNR1*-silenced pepper leaves. A, RT-PCR analyses of the expression of the *CaMNR1*, *CaBPR1*, *CaPR4*, *CaPR10*, *CaPOA1*, *CaDEF1*, *CaOSM1*, and *CaSAR8.2* genes in unsilenced (TRV:00) and *CaMNR1*-silenced (TRV:*CaMNR1*) pepper plants at 18 h after inoculation with *Xcv* (10^8 cfu mL^{-1}). 18S rRNA was the control. The experiment was replicated three times with similar results. H, Uninoculated healthy leaves; C, compatible interaction between the *Xcv* virulent strain Ds1 and pepper leaves; I, incompatible interaction between the *Xcv* avirulent strain Bv5-4a and pepper leaves. B, Protein gel-blot analysis of the expression of CaMNR1 in unsilenced (TRV:00) and *CaMNR1*-silenced (TRV:*CaMNR1*) pepper leaves at 18 h after inoculation with the *Xcv* virulent strain Ds1 and the avirulent strain Bv5-4a. Equal loading of proteins was confirmed by comparing the band intensities of the Rubisco protein stained with Coomassie blue. [See online article for color version of this figure.]

than that of CaMNR1 (Fig. 2; Supplemental Fig. S6E). AtSDR1 converted (−)-menthone to 92% (+)-(3*S*)-neomenthol and 8% (−)-(3*R*)-menthol at pH 8.0 with NADPH as a cofactor (Supplemental Fig. S6, C and E). Neomenthol was the predominant reaction product catalyzed by AtSDR1, like CaMNR1, because the amount of menthol generated was less than 10% at all pH ranges. The oxidation of menthol isomers in the presence of $NADP^+$ was also evaluated (Supplemental Fig. S6, D and F). Similar to the results of enzyme activity of CaMNR1, neomenthol was only converted into menthone by AtSDR1 in the presence of the $NADP^+$ cofactor. The oxidation reaction of AtSDR1 displayed maximal activity at pH 9.0 (Supplemental Fig. S6F). Thus, these findings enabled us to further analyze the Arabidopsis *AtSDR1* gene as a putative ortholog of *CaMNR1*.

The *sdr1-1* and *sdr1-2* Arabidopsis mutants obtained from the Salk T-DNA populations (http://www.arabidopsis.org) have a T-DNA insertion within their first and third exons, respectively (Fig. 10B). In wild-type plants, *AtSDR1* transcripts of expected sizes accumulated to a significant level at 18 and 24 h after inoculation with avirulent *Pst* DC3000. Therefore, we tested whether the *sdr1* mutant exhibits altered responses to pathogens. In the *sdr1* insertion homozygous mutants selected from the Salk T-DNA bulk lines, no transcripts of the expected size were detected at 18 and 24 h after avirulent *Pst* DC3000 infection (Fig. 10C). Furthermore, *AtPR1* expression was distinctly reduced in the *sdr1* mutant plants during infection with virulent and avirulent *Pst* DC3000 compared with wild-type plants. No obvious abnormal phenotypes in the *sdr1-1* and *sdr1-2* mutant plants were uncovered during plant growth or development (data not shown). To determine the role of *AtSDR1* in plant defense, we first evaluated responses of the *sdr1-1* and *sdr1-2* mutant plants to virulent and avirulent strains of *Pst* DC3000. As shown in Figure 10D, the homozygous *sdr1-1* and *sdr1-2* mutants exhibited 5- to 10-fold more bacterial growth compared with wild-type plants at 3 d after inoculation with virulent and avirulent *Pst* DC3000. Significantly enhanced bacterial growth and reduced expression of *AtPR1* in *sdr1* mutant leaves indicated that *AtSDR1* is required for basal defense or *R* gene-mediated resistance to the hemibiotrophic bacterial pathogen. We also examined the response of the *sdr1* mutants to the biotrophic oomycete pathogen *H. parasitica* isolate Noco2 (Fig. 10, E and F). Interestingly, *sdr1* mutant plants exhibited enhanced susceptibility to infection with *H. parasitica* isolate Noco2. Approximately 60% of the cotyledons of *sdr1* mutants exhibited heavy sporulation (>20 sporangiophores per cotyledon), while 50% of the cotyledons of wild-type plants were heavily sporulated. The average number of sporangiophores in *sdr1* mutant plants (*sdr1-1*, 25.1; *sdr1-2*, 25.3) was higher than in wild-type plants (21.3), but lower than in *eds1* mutant plants (Landsberg *erecta* background, 28.2). These data indicate that *AtSDR1* plays a crucial role in the defense response of Arabidopsis to the hemibiotrophic bacterial pathogen *P. syringae* and the biotrophic oomycete pathogen *H. parasitica*.

DISCUSSION

In this study, we identified a pepper *CaMNR1* gene using an array-based differential screening method (Jung and Hwang, 2000) and its putative ortholog *AtSDR1* in Arabidopsis. The amino acid sequences of CaMNR1 and AtSDR1 share 59% and 54% sequence identity, respectively, with peppermint MNR (Davis et al., 2005). The CaMNR1 and AtSDR1 proteins belong to the SDR family, based on length (300 ± 50 amino acids) and on conserved sequence motifs, including an N-terminal cofactor-binding domain (TGxxxGxG), a downstream structural domain (GxxDxxxNNAG) for stabilizing a central β-sheet, and a catalytic domain (YxxxK; Persson et al., 2003; Davis et al., 2005). SDRs are

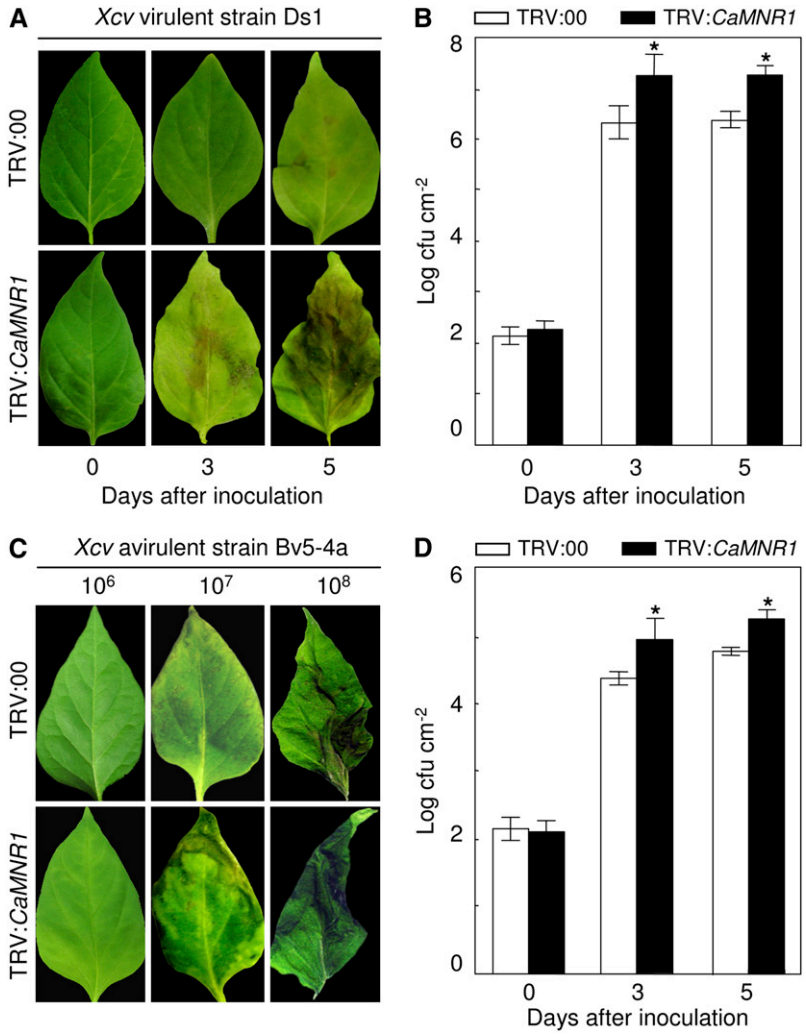


Figure 6. Enhanced susceptibility of *CaMNR1*-silenced pepper plants to *Xcv* infection. A, Disease symptoms at 0, 3, and 5 d after inoculation with virulent *Xcv* (5×10^6 cfu mL^{-1}). B, Bacterial growth in leaves of unsilenced (TRV:00) and *CaMNR1*-silenced (TRV:*CaMNR1*) pepper plants at 0, 3, and 5 d after inoculation (5×10^4 cfu mL^{-1}). Data are means ± SD from three independent experiments. Asterisks indicate significant differences as determined by Student's *t* test ($P < 0.05$). C, Disease symptoms at 1 d after inoculation with avirulent *Xcv* suspensions of different concentrations (10^6, 10^7, and 10^8 cfu mL^{-1}). D, Bacterial growth in leaves of unsilenced (TRV:00) and *CaMNR1*-silenced (TRV:*CaMNR1*) pepper plants at 0, 1, 3, and 5 d after inoculation (5×10^4 cfu mL^{-1}). Data are means ± SD from three independent experiments. Asterisks indicate significant differences as determined by Student's *t* test ($P < 0.05$).

enzymes of great functional diversity with a wide substrate spectrum, ranging from steroids, alcohols, sugars, and aromatic compounds to xenobiotics (Kallberg et al., 2002; Persson et al., 2003).

CaMNR1 and AtSDR1 exhibited an enzymatic activity for menthone reduction, as predicted by significant sequence identities with peppermint MNR and MMR. Indeed, CaMNR1 shares 59% and 56% sequence identity with peppermint MNR and MMR, respectively. CaMNR1 and AtSDR1 possess both enzyme activities, but neomenthol generation is predominant. Compared with peppermint MNR, CaMNR1 showed not only greater substrate specificity but also greater catalytic power, indicating a much higher enzyme turnover number (Davis et al., 2005). The optimal pH of CaMNR1 and AtSDR1 was also different from that of peppermint MNR. CaMNR1 and AtSDR1 displayed maximal activities at neutral pH 7.5 and 8.0, respectively, whereas the activity of peppermint MNR enzyme was maximal at alkaline pH 9.3 (Davis et al., 2005). However, a direct comparison should not be overestimated, due to different assay conditions, including pH and the concentration of each component. The capability of CaMNR1 and AtSDR1 to oxidize monoterpenols was also tested in the presence of $NADP^+$. Only oxidation of neomenthol to menthone was significantly detected by GC analysis. In contrast to peppermint MNR, the K_m value of CaMNR1 for $NADP^+$ was higher than that for NADPH. The cofactor binding affinity of CaMNR1 was significantly lower than that of peppermint menthone reductases. By contrast, the menthone-binding affinity of CaMNR1 was much higher than that of peppermint MNR but lower than that of peppermint MMR (Davis et al., 2005). Interestingly, CaMNR1 possessed significantly higher catalytic activity for neomenthol oxidation compared with peppermint menthone reductases (Davis et al., 2005). A very low level of neomenthol was detected at nearly all developmental stages, whereas a very high level of menthone was observed in peppermint oil. The very low activity of neomenthol oxidation by peppermint MNR is consistent with the in vivo level of each compound in peppermint (Davis et al., 2005). However, further studies on monoterpene metabolism in pepper and Arabidopsis plants are required to gain new insights into the enzymatic activity of CaMNR1 and AtSDR1 for neomenthol oxidation.

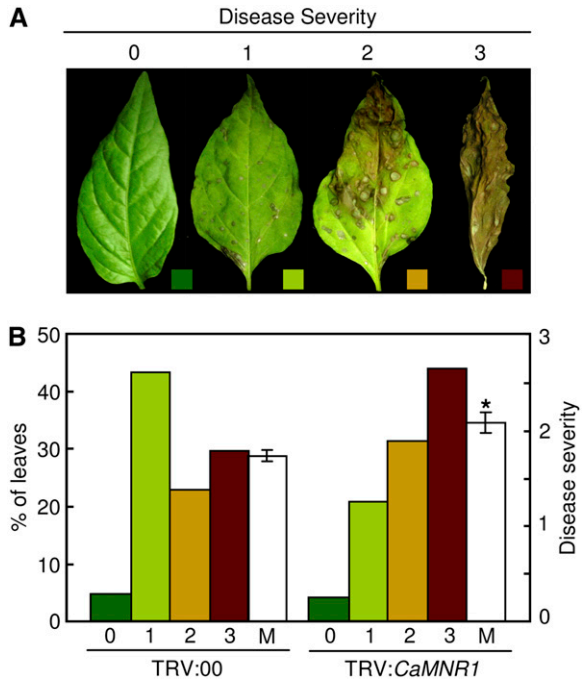


Figure 7. Enhanced susceptibility of *CaMNR1*-silenced (TRV:*CaMNR1*) pepper plants to *C. coccodes* infection. A, Disease severities were rated on a scale of 0 to 3 (0, no symptoms; 1, weakly visible symptoms; 2, dark brown lesions with mild chlorosis; 3, enlarged dark brown lesions with severe chlorosis). B, Percentages of pepper leaves exhibiting the indicated disease severities at 5 d after inoculation with *C. coccodes*. Disease severity was rated on more than 50 leaves. The asterisk indicates a significant difference between means as determined by Student's *t* test ($P < 0.05$). M, Means of disease severities.

The enzymatic activities of CaMNR1 described in this study and the well-known antimicrobial activities of monoterpenes (Cowan, 1999; Dixon, 2001; Arfa et al., 2006) led us to test the antimicrobial activities of menthone, neomenthol, and menthol. Neomenthol and menthol strongly inhibited phytopathogenic bacterial and fungal growth compared with their precursor form, menthone. The differential antimicrobial activities of monoterpene compounds may be related to their different hydrophobicities, which can be expressed as their log*P* values (partitioning behavior of the compound in octanol/water; Lanciotti et al., 2003; Arfa et al., 2006). The estimated octanol/water partition coefficient (log*P*) was 2.87 for menthone and 3.38 for both neomenthol and menthol (http://www.syrres.com/esc/est_kowdemo.htm). Hydrophobic compounds with log*P* values higher than 3 exhibit efficient antimicrobial activities due to their high affinity for cell membranes (Arfa et al., 2006). The insertion of these compounds between cell membranes induces changes in membrane physicochemical properties, including lipid ordering and bilayer stability (Sikkema et al., 1995; Weber and de Bont, 1996). Interestingly, neomenthol and menthol, both of which have a log*P* of 3.38, were more effective at inhibiting phytopathogenic fungal and bacterial growth than menthone, which has a log*P* of 2.87. These findings support the idea that the production of neomenthol or menthol from menthone by pathogen-responsive CaMNR1 may enhance the immunity of pepper plants against pathogen infections.

In attempts to determine the metabolite profile of the terpenoid pathway in pepper leaves, we used GC-MS analyses to detect certain metabolic compounds that are de novo synthesized and accumulated by virulent and avirulent *Xcv* infections. Lipid-derived volatiles, including 2-hexenal and cis-3-hexenol identified in this study, were reported not only to increase in *P. vulgaris* leaves inoculated with *Pseudomonas syringae* pv *phaseolicola* but also to exhibit in vitro antifungal and antiprotozoal activity (Croft et al., 1993). Interestingly, MeSA was detected and its levels increased in extracts of pepper leaves inoculated with virulent and avirulent *Xcv*. Recently, Park et al. (2007) reported that MeSA is a critical mobile signal for systemic acquired resistance in tobacco (*Nicotiana tabacum*) plants and that silencing of salicylic acid methyl transferase, which converts SA to MeSA, compromised systemic acquired resistance. These findings support the notion that MeSA may play an important role in the defense response of pepper plants. De novo synthesis of trans-2-cis-6-nonadienal in pepper plants during an incompatible interaction with an avirulent *Xcv* strain is also consistent

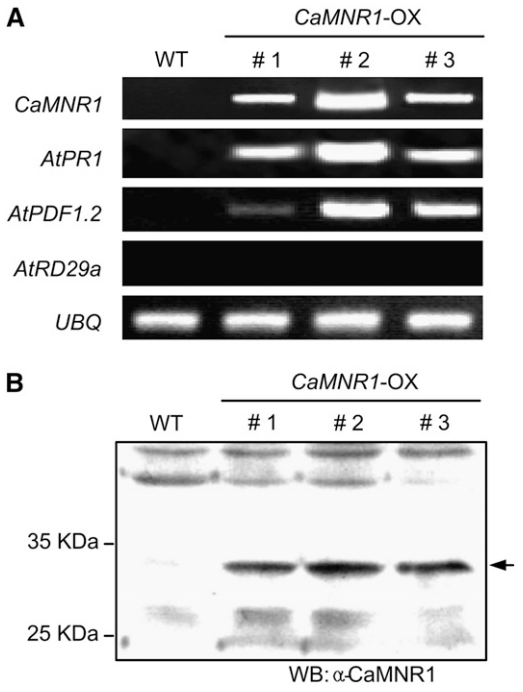


Figure 8. Generation of *CaMNR1*-OX Arabidopsis transgenic plants. A, RT-PCR analyses of expression of the *CaMNR1*, *AtPR1*, *AtPDF1.2*, and *AtRD29a* genes in leaves of wild-type Arabidopsis (ecotype Col-0; WT) and *CaMNR1*-OX (lines 1, 2, and 3) plants. B, Protein gel-blot analysis of CaMNR1 expression in leaves of wild-type and *CaMNR1*-OX plants. The arrow indicates the CaMNR1 band detected by the anti-CaMNR1 polyclonal antibody.

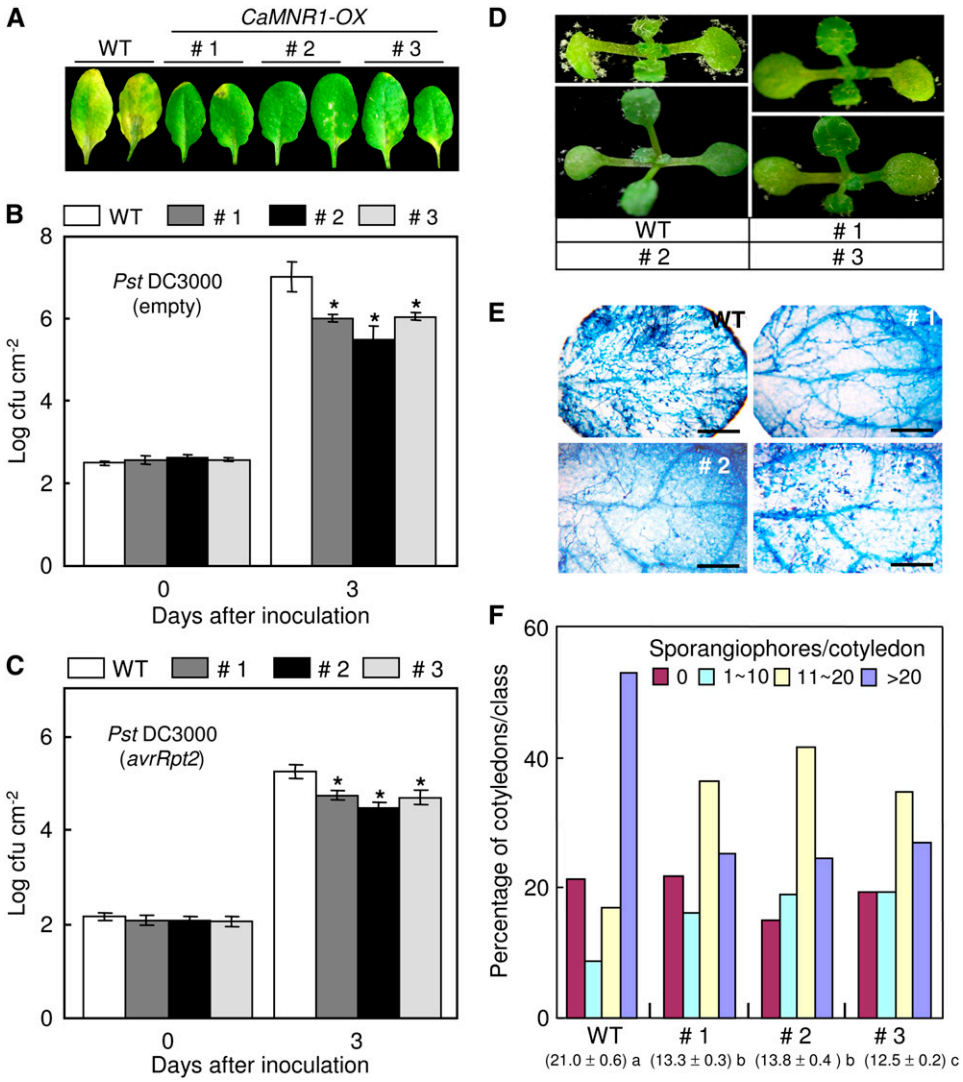


Figure 9. Enhanced basal disease resistance of *CaMNR1*-OX transgenic Arabidopsis. A, Disease symptoms on the leaves of wild-type and transgenic Arabidopsis plants at 5 d after spray inoculation with *Pst* DC3000 (10^8 cfu mL^{-1}). B and C, Bacterial growth in the leaves of wild-type and transgenic plants at 0 and 3 d after inoculation with a virulent (empty) strain (B) and an avirulent (*avrRpt2*) strain (C) of *Pst* DC3000 (5×10^4 cfu mL^{-1}). Data are means ± SD from three independent experiments. Asterisks indicate significant differences as determined by Student's *t* test ($P < 0.05$). D, Disease symptoms on the cotyledons of wild-type and transgenic Arabidopsis plants at 7 d after spray inoculation with *H. parasitica* isolate Noco2 (5×10^4 spores mL^{-1}). E, Trypan blue staining of cotyledons of wild-type and transgenic Arabidopsis plants at 7 d after inoculation with *H. parasitica* isolate Noco2. Bars = 200 μm. F, Numbers of sporangiophores per cotyledon of wild-type and transgenic plants at 7 d after inoculation with *H. parasitica*. Cotyledons were classified as follows: no sporulation (no sporangiophores per cotyledon), light sporulation (approximately 1–10 sporangiophores per cotyledon), medium sporulation (approximately 11–20 sporangiophores per cotyledon), or heavy sporulation (more than 20 sporangiophores per cotyledon). Sporangiophores were counted on more than 50 cotyledons per genotype. Average numbers of sporangiophores produced on the cotyledons of wild-type and transgenic lines are shown at bottom for each of the lines tested. Statistically significant differences between means were determined employing the LSD ($P = 0.05$).

with HR-specific accumulation of this compound in *P. vulgaris* leaves (Croft et al., 1993). Trans-2-cis-6-nonadienal is known to be synthesized from the 9-hydroperoxide of linolenic acid by hydroperoxide lyase activity (Matthew and Galliard, 1978). The absence of trans-2-cis-6-nonadienal in uninfected healthy or virulent bacterial pathogen-infected *P. vulgaris* (Croft et al., 1993) and pepper leaves in this study suggests that a highly specific and common mode of lipid peroxidation occurs during HR in plants. Despite our various efforts to detect monoterpenes in pepper, we were unable to detect menthone, neomenthol, or menthol in the extracts of pepper leaves infected by *Xcv*. Only one terpenoid pathway-related metabolic compound, 2,6,10-trimethyldodecane (farnesane), the parent compound of approximately 10,000 sesquiter-

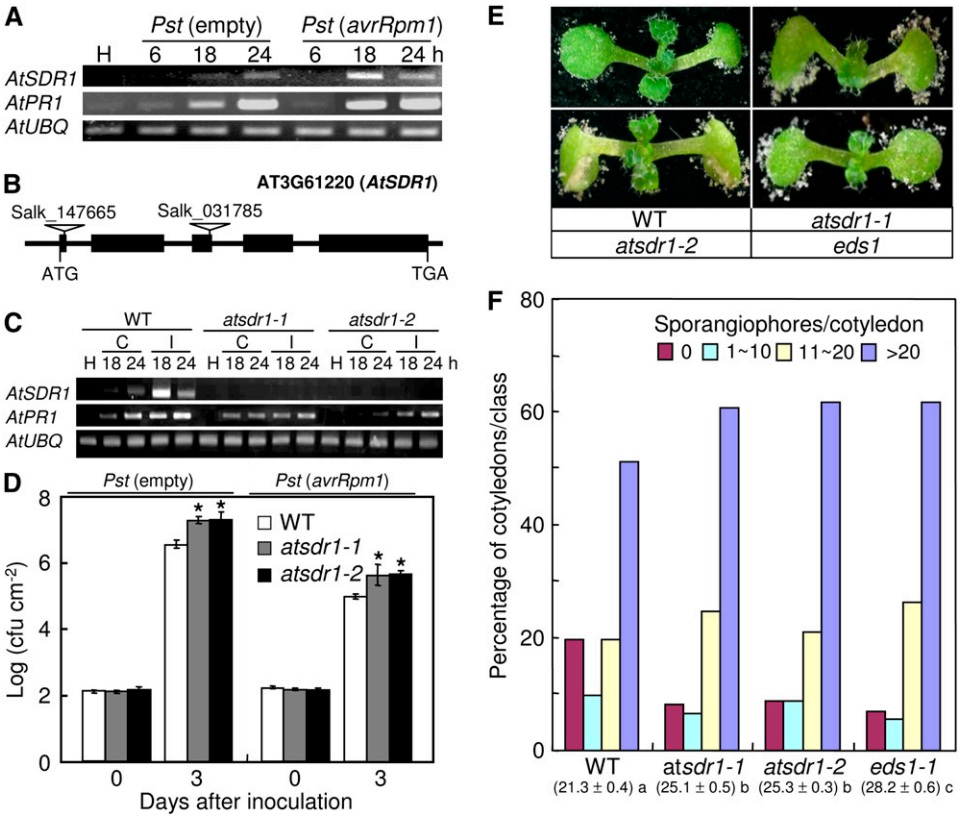


Figure 10. Enhanced disease susceptibility of *sdr1* Arabidopsis mutants. A, RT-PCR analyses of the expression of Arabidopsis *AtSDR1* and *AtPR1* in the leaves of wild-type Col-0 plants at 6, 18, and 24 h after inoculation with virulent (empty) and avirulent (*avrRpm1*) *Pst* DC30000. B, Schematic diagram showing *AtSDR1* and its T-DNA insertion mutants. C, RT-PCR analyses of transcript levels of *AtSDR1* and *AtPR1* in leaf tissues of wild-type Col-0 and *sdr1* mutant plants at 18 and 24 h after inoculation with virulent (compatible [C]) and avirulent (*avrRpm1*; incompatible [I]) *Pst* DC30000 (10^6 cfu mL^{-1}). The *AtSDR1* transcript is absent in *sdr1* plants. D, Bacterial growth in the leaves of wild-type and *sdr1* mutant plants at 0 and 3 d after inoculation with virulent (empty) and avirulent (*avrRpm1*) strains of *Pst* DC3000 (5×10^4 cfu mL^{-1}). Asterisks indicate significant differences as determined by Student's *t* test ($P < 0.05$). E, Disease symptoms on the cotyledons of wild-type and *sdr1* mutant plants at 7 d after inoculation with *H. parasitica* (5×10^4 spores mL^{-1}). F, Numbers of sporangiophores per cotyledon of wild-type and *sdr1* mutant plants at 7 d after inoculation with *H. parasitica*. Diseased cotyledons were classified as follows: no sporulation (no sporangiophores per cotyledon), light sporulation (approximately 1–10 sporangiopores per cotyledon), medium sporulation (approximately 11–20 sporangiophores per cotyledon), or heavy sporulation (more than 20 sporangiophores per cotyledon). Sporangiophores were counted on more than 50 cotyledons per genotype. Average numbers of sporangiophores on the cotyledons of wild-type and mutant lines are shown at bottom for each of the lines tested. Statistically significant differences between means were determined employing the LSD ($P = 0.05$).

penes (Breitmaier, 2007), was induced in *Xcv*-infected pepper leaves. This may be because these monoterpenes are present at extremely low levels or are absent in pepper plants. Plants elaborate a diverse array of secondary metabolites, many of which have evolved to confer resistance against pathogen attack (Grayer and Harborne, 1994; Pichersky and Gang, 2000; Dixon, 2001; Kliebenstein, 2004). However, little is known about the terpenoid pathways of members of the Solanaceae family, including pepper plants. The extremely low levels or the absence of these monoterpenes is probably why it has been difficult to analyze monoterpene pathways in pepper plants. Further detection of menthone metabolites in pepper plants will be required to gain insights into the in vivo function of CaMNR1 in defense responses to pathogen invasion.

To determine the expression patterns of *CaMNR1* in pepper plants, we performed RNA and protein gel-blot analyses. *CaMNR1* transcripts and proteins were strongly induced in pepper leaves inoculated with an avirulent strain of *Xcv* but not with a virulent strain. Avirulent strains of *Xcv* trigger resistance responses, including the oxidative burst, the HR, and *PR* gene induction in pepper plants (Lee and Hwang, 2005). Therefore, the increased expression of *CaMNR1* suggests that it has a cellular function in the resistance response of pepper plants infected by avirulent strains of *Xcv*. To examine the effect of a loss of function of *CaMNR1* in the defense response, we used the TRV-based VIGS system in pepper plants (Liu et al., 2002; Chung et al., 2004). Plants have evolved a two-branched defense system: pathogen-associated molecular patterns-

triggered immunity (PTI) and effector-triggered immunity (ETI; Jones and Dangl, 2006). Recognition of pathogen-associated molecular patterns by plant receptor-like kinases triggers PTI responses, including the oxidative burst, callose deposition, and defense gene induction (Felix et al., 1999; Zipfel et al., 2004). To overcome PTI, pathogens deliver effectors to plant cells. Successful recognition of effectors by R proteins activates ETI responses, including the HR. However, unsuccessful recognition of effectors may enhance disease susceptibility. Interestingly, *CaMNR1* gene-silenced pepper plants exhibited significantly enhanced susceptibility to virulent and avirulent strains of *Xcv*. These findings suggest that CaMNR1 may act as a common signaling factor for both PTI and ETI. However, its signal intensities were stronger in ETI than in PTI.

RT-PCR and protein gel-blot analyses showed that silencing of the *CaMNR1* gene significantly reduced *CaMNR1* transcript and protein levels in pepper leaves inoculated with virulent and avirulent strains of *Xcv*. Furthermore, expression levels of defense-related genes, including *CaBPR1*, *CaPR4*, *CaPR10*, *CaDEF1*, and *CaSAR8.2*, were significantly lower in *CaMNR1*-silenced leaves compared with unsilenced leaves after infection with *Xcv*. Local and systemic induction of some defense-related genes has been reported for pepper plants (Lee and Hwang, 2005). Defense-related proteins such as CaSAR8.2 and defensin exhibit antimicrobial activity (Broekaert et al., 1995; Lee and Hwang, 2006). The lowered expression of defense-related genes in *CaMNR1*-silenced leaves indicates that *CaMNR1* expression is important for the induction of downstream defense-related genes. Consistent with these results, *CaMNR1*-silenced pepper plants exhibited enhanced susceptibility against the hemibiotrophic bacterial pathogen *Xcv* and the necrotrophic fungal pathogen *C. coccodes*.

To address the biological function of *CaMNR1*, we attempted to generate transgenic pepper plants that constitutively expressed the *CaMNR1* gene. Unfortunately, this approach was unsuccessful due to extremely low efficiencies of transformation and regeneration. Therefore, we established transgenic Arabidopsis plants that overexpressed *CaMNR1* from the cauliflower mosaic virus 35S promoter. Interestingly, ectopic overexpression of *CaMNR1* induced the constitutive expression of *PR* genes in uninfected Arabidopsis plants. The expression levels of Arabidopsis *AtPR1* and *AtPDF1.2* correlated with *CaMNR1* expression levels in transgenic Arabidopsis. The induction of defense responses is known to be activated by various signal transduction pathways, which are regulated by signaling molecules, such as SA, JA, and ethylene (Glazebrook, 1999; Lee and Hwang, 2005). In Arabidopsis, induction of *AtPR1* occurs via a SA-dependent pathway (Ukness et al., 1992), whereas the induction of *AtPDF1.2* (defensin) depends on the JA-dependent pathway (Penninckx et al., 1998; Thomma et al., 1998). This suggests that CaMNR1 plays a critical role in the regulation of *PR* genes through both SA- and JA-dependent pathways. Moreover, ectopic overexpression of *CaMNR1* significantly enhanced basal resistance against infection by *Pseudomonas syringae* and *H. parasitica*. In contrast, T-DNA insertion mutations in the *AtSDR1* gene, a putative ortholog of *CaMNR1* in Arabidopsis plants, significantly enhanced the susceptibility of Arabidopsis plants to these pathogens, followed by lowered expression of *AtPR1*. These findings suggest that CaMNR1 and AtSDR1 may play roles as signaling factors that may increase the basal resistance of plants. In mammalian cells, carbonyl reductase (CR), a well-characterized SDR family enzyme, shows homology with CaMNR1. Reactive carbonyl groups, which are frequently found in endogenous or xenobiotic compounds, can covalently modify DNA or amino acids and initiate degenerative diseases or cancers (Oppermann, 2007). CRs detoxify these reactive carbonyl groups. Moreover, CRs are also important for the metabolic conversion of carbonyl groups of endogenous hormones, such as glucocorticoids, androgens, estrogens, and eicosanoids. Therefore, we can propose some possible mechanisms for increased and decreased basal resistance in the *CaMNR1* transgenic lines and *sdr1* mutants, respectively. CaMNR1 or AtSDR1 may catalyze the conversion of inactive endogenous or xenobiotic compounds into active forms that can exhibit antimicrobial activities or activate defense responses in plants. Alternatively, CaMNR1 may also detoxify xenobiotic effector molecules derived from pathogens to reduce the virulence of pathogens.

Taking all of the available evidence together, this study provides clues for the elucidation of the cellular functions of pepper *CaMNR1* and its putative ortholog Arabidopsis *AtSDR1* in plant defense responses. VIGS of *CaMNR1* in pepper and the T-DNA insertion mutation of *AtSDR1* in Arabidopsis led to significantly enhanced susceptibility to both bacterial and fungal pathogens. In contrast, ectopic overexpression of *CaMNR1* significantly enhanced disease resistance in Arabidopsis. Interestingly, the expression levels of *CaMNR1* correlated with those of SA- and JA-responsive *PR* genes in both pepper and Arabidopsis plants, suggesting the functional involvement of CaMNR1 in a broad spectrum of defense responses through the regulation of downstream defense-related genes.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

Pepper (*Capsicum annuum* 'Nockwang') and Arabidopsis (*Arabidopsis thaliana* ecotype Col-0) plants were used in this study. Pepper plants were grown in a plastic tray (55 × 35 × 15 cm) containing a steam-sterilized soil mix (peat moss, perlite, and vermiculite, 5:3:2, v/v/v) and loam soil (1:1, v/v) at 28°C with a daylength of 16 h at a light intensity of 70 μmol photons m^{-2} s^{-1}. Pepper plants at the six-leaf stage were used for pathogen infection and abiotic elicitor and environmental stress treatments. Arabidopsis plants were grown at 24°C with a photosynthetic flux of 130 μmol photons m^{-2} s^{-1} for 8 h of light and 60% relative humidity in a controlled-environment chamber. Plants were

raised in pots containing peat moss, perlite, and vermiculite (1:0.5:1, v/v/v). Prior to sowing, wild-type (Col-0) and *CaMNR1*-OX mutant seeds were sterilized with 2% (v/v) sodium hypochlorite, followed by imbibition at 4°C for 3 d to overcome dormancy.

Pathogens and Inoculation Procedures

Pepper plants were inoculated with virulent (Ds1) and avirulent (Bv5-4a) strains of *Xanthomonas campestris* pv *vesicatoria* (*Xcv*). Bacteria were cultured overnight in yeast-nutrient broth (5 g of yeast extract and 8 g of nutrient broth per L) at 28°C. Pepper plants at the six-leaf stage were inoculated by infiltrating bacterial suspensions into the abaxial side of fully expanded leaves using a syringe without a needle. Infected plants were incubated in a controlled moist chamber at 28°C with 100% relative humidity for 16 h, and infected leaves were harvested at various time points for bacterial growth and RNA and protein gel-blot analyses (Kim et al., 2007).

Virulent (empty vector) and avirulent (*avrRpm1* and *avrRpt2*) strains of *Pseudomonas syringae* pv *tomato* (*Pst*) DC3000 were used for infection of Arabidopsis. *Pst* DC3000 was grown overnight in King's B medium containing 50 μg mL^{-1} rifampicin and 50 μg mL^{-1} kanamycin. To measure bacterial growth, leaves of 4-week-old wild-type and *CaMNR1* transgenic T2 plants were infiltrated with 10^5 cfu mL^{-1} (optical density at 600 nm [OD_{600}] = 0.001) *Pst* DC3000 in 10 mM $MgCl_2$ using a syringe without a needle. The infected plants were incubated in a moist chamber at 28°C for 18 h, and infected leaves were harvested at various time points for bacterial growth and RNA gel-blot analyses. The oomycete pathogen *Hyaloperonospora parasitica* isolate Noco2 was propagated at weekly intervals on susceptible Col-0 plants (Reignault et al., 1996). One-week-old seedlings of wild-type (Col-0) and *CaMNR1*-OX transgenic plants were spray inoculated using freshly harvested conidiospores (5×10^4 spores mL^{-1}). The infected plants were incubated at 17°C in a controlled-environment chamber. Sporangiophores were counted at 7 d after inoculation. Cotyledons were stained with trypan blue to visualize host cell death and oomycete structures.

Antimicrobial Activity of Monoterpenes

The antimicrobial activities of the monoterpenes (−)-menthone, (+)-(3*S*)-neomenthol, and (−)-(3*R*)-menthol (Sigma-Aldrich) were analyzed in the vapor phase by the microatmosphere method (Arfa et al., 2006). To prepare bacterial inocula for evaluation of antimicrobial activity of these monoterpenes, *Pst* DC3000 and *Xcv* were cultured in nutrient broth for 18 h at 28°C. Bacterial cells were collected by centrifugation and resuspended in 10 mM $MgCl_2$ solution to 10^7 cfu mL^{-1}. The bacterial suspensions were uniformly spread on nutrient agar medium. *Alternaria brassicicola* and *Colletotrichum coccodes* grown in potato dextrose agar medium were sporulated on oatmeal agar medium at 28°C. After harvesting spores in sterile tap water, the concentrations of the spore suspensions were adjusted to 10^5 spores mL^{-1}. These spore suspensions were then uniformly spread on 1% water agar. Different amounts of the monoterpenes (0.1, 0.5, 1.5, 10, and 50 mg per plate [9 cm in diameter]) used to inhibit the growth of plant pathogens were dissolved in 1 mL of ethyl acetate, impregnated homogeneously on sterilized filter paper (63 cm^2), and dried for 1 min to evaporate ethyl acetate. Filter papers were placed on the lids of petri plates containing 20 mL of agar medium whose surfaces had been uniformly covered with the tested bacterial and fungal pathogens. Controls were made with papers impregnated with 1 mL of ethyl acetate alone. The petri plates were incubated for 48 h at 28°C for bacterial growth or for 12 and 24 h at 28°C for spore germination and hyphal growth of fungi, respectively.

Isolation and Sequence Analysis of Pathogen-Induced *CaMNR1* cDNAs

For the construction of a pathogen-induced cDNA library, the *Xcv* avirulent strain Bv5-4a was inoculated into pepper leaves. A pepper cDNA library was constructed using 5 μg of poly(A)$^+$ mRNA extracted from inoculated pepper leaves (Kim and Hwang, 2000). To isolate pathogen-inducible cDNAs from the pepper cDNA library, macroarray-based differential screening was performed according to the method of Jung and Hwang (2000). The *CaMNR1* cDNA sequence was analyzed using BLAST programs provided by the National Center for Biotechnology Information databases (Altschul et al., 1997). Amino acid sequences were aligned using the ClustalW program (http://www.ebi.ac.uk/clustalw). The phylogenetic tree was generated using ClustalW and displayed with Treeview 1.6.6.

Subcloning of *CaMNR1* and *AtSDR1*

Site-directed mutagenesis was utilized to remove 34 amino acid residues between the thrombin cleavage and *Eco*RI restriction sites of the vector pET-32a (Novagen). Forward (5′-CTGTCGCCACGCGGTTCTGAATTCAAAGAAACCGCTGC-3′) and reverse (5′-GCAGCGGTTTCTTTGAATTCAGAACCGGCGTGGCACCAG-3′) primers containing *Eco*RI restriction sites (underlined) were used for QuikChange site-directed mutagenesis (Stratagene). The products were amplified by the addition of 10 units of LA *Taq* DNA polymerase (TaKaRa) with approximately 10 ng of template DNA, 2.5 mM deoxynucleoside triphosphates, 10 mM of each primer, and 2× GC buffer in 50 μL using a Thermal Cycler 2720 (Applied Biosystems). The mutated plasmids were digested with *Eco*RI and religated with T4 DNA ligase (TaKaRa). Resultant DNAs were transformed into *Escherichia coli* BL21(DE3).

The *AtSDR1* gene was cloned from the Arabidopsis cDNA library by PCR amplification using the forward (5′-GGATCCATGGCAGAGGAAACTCCAAGATATG-3′) and reverse (5′-CTCGAGTCAGAATTCTGAAACTTGCTGCG-3′) primers, which contain the *Bam*HI and *Xho*I restriction sites for cloning. The resultant PCR product was cloned into modified pGEX-4T vector, which has an N-terminal glutathione *S*-transferase (GST) followed by a tobacco etch virus protease cleavage site. The cloned vector was transformed into *E. coli* Rosetta(DE3) cells.

Overexpression and Purification of CaMNR1 and AtSDR1

E. coli containing the *CaMNR1*- or *AtSDR1*-expressing plasmid was incubated with vigorous shaking at 37°C until the OD_{600} reached approximately 0.7. CaMNR1 and AtSDR1 were induced by the addition of 1 mM isopropylthio-β-galactoside at 25°C for 12 h. The cells were pelleted by centrifugation for 30 min (6,000 rpm; Beckman JA-10 rotor), resuspended in 50 mM Tris-HCl buffer (pH 8.5; CaMNR1) or 1× PBS buffer (pH 7.4; AtSDR1), and then disrupted by sonication. The lysate was centrifuged for 1 h (15,000 rpm; Beckman JA-20 rotor), and the supernatant was collected. To purify His-tagged thioredoxin-CaMNR1 protein, the soluble fraction was loaded onto a His-Trap HP column (GE Healthcare) preequilibrated with 50 mM Tris-HCl, pH 8.5, and 100 mM NaCl, and the target enzyme was eluted with 150 mM imidazole. To purify GST-tagged AtSDR1 protein, the soluble fraction was loaded onto the column packed with GST-agarose beads preequilibrated with 1× PBS buffer, pH 7.4, and GST-tagged target protein was eluted with 50 mM Tris-HCl, pH 8.0, 10 mM NaCl, and 20 mM reduced glutathione.

Fractions containing the His-tagged thioredoxin-CaMNR1 or GST-tagged AtSDR1 fusion protein were incubated with 120 units of human α-thrombin or tobacco etch virus protease at 22°C for 12 h (1:1,000 or 1:50 molar ratio, respectively). The thioredoxin- and GST-free target proteins were further purified by gel filtration chromatography (Superose 12) followed by anion-exchange chromatography (Resource Q or Mono Q column, respectively). The concentrations of purified CaMNR1 and AtSDR1 were determined by the Bradford assay (Bradford, 1976).

Activity Assay of CaMNR1 and AtSDR1

Preparatory identification and quantification were done with the enzyme preparation (400 μg of CaMNR1 and AtSDR1) in 1 mL of assay buffer (50 mM HEPES, pH 7.5, with 100 mM NaCl and 5 mM β-mercaptoethanol) in the presence of 1 mM menthone and 500 μM NADPH. Following incubation at 31°C for 12 h, monoterpene products were extracted with 0.5 mL of pentane as described (Davis et al., 2005). Reaction products were analyzed by GC and GC-MS (Hewlett-Packard model 6890 series gas chromatograph) with a 25-m × 0.2-mm × 0.33-μm film of polyethylene glycol coating (HP-FFAP 19091F-102; Agilent Technologies) as described. For quantification of the reaction products, 100 μM (+)-camphor was added as an internal standard. The enzymatic activity depending on pH was measured by the same procedure described above, except with an assay buffer of a different pH range (pH 6–10, with intervals of 0.5 pH units). The enzymatic activity for the reverse (oxidation) reaction was also analyzed in the presence of 1 mM neomenthol as a substrate and 500 μM $NADP^+$ as a cofactor. All experimental procedures

were essentially the same as for the forward (reduction) reaction, except for a buffer for optimal pH (50 mM Bicine, pH 9.0).

Analysis of CaMNR1 Enzyme Kinetics

Enzyme kinetics were analyzed as described with minor modifications (Davis et al., 2005). Assay mixtures containing 1 mL of 50 mM HEPES, pH 7.5, with 100 mM NaCl and 5 mM β-mercaptoethanol for the forward reaction and 1 mL of 50 mM Bicine, pH 9.0, with 100 mM NaCl and 5 mM β-mercaptoethanol for the reverse reaction were combined with 500 μM cofactor (NADPH or $NADP^+$) and various concentrations (2.5–500 μM) of a monoterpene substrate (menthone or neomenthol) and preincubated at 31°C before initiation of the reaction by enzyme addition (15 μg of CaMNR1). The reactions were stopped after 15 min by the addition of 0.5 mL of pentane containing camphor as an internal standard, followed by vigorous mixing and cooling on ice. The reaction products in the pentane extract were quantitated by GC using the conditions described above with peak area integration in the chromatograph. The kinetic parameters for the cofactor were determined spectrophotometrically (ΔA_{340}) based on the disappearance or appearance of NADPH. The same assay mixtures were combined with 100 μM monoterpene substrate and various concentrations (2.5–500 μM) of the cofactor (NADPH or $NADP^+$). Kinetic parameters were determined by analyzing Lineweaver-Burk plots from four independent experiments.

Extraction of Metabolic Compounds from Pepper Leaves

The liquid-liquid extraction method was used to extract metabolic compounds from pepper leaves. Five grams of uninoculated and *Xcv*-inoculated pepper leaves was sampled, ground to a fine powder in liquid nitrogen, and mixed with 10 mL of 70% ethanol. Fifty micrograms of camphor was added to each sample as an internal standard. The mixture was stored for 1 d at room temperature. Then, 5 mL of the supernatant of the leaf extract was mixed with 5 mL of distilled water and 1.2 mL of organic solvent A (ethyl acetate:hexane: methylene chloride, 5:1:1, v/v/v; Gherman et al., 2000). The mixture was agitated for 1 h, and 1 mL of the supernatant was dried by nitrogen gas. The residual pellet was dissolved in 100 μL of solvent A, and 5 μL of the extract was used for GC-MS analysis.

GC-MS Analysis

An Agilent 6890N gas chromatograph (Agilent Technologies) coupled with an Agilent 5975A mass spectrometer (Agilent Technologies) was used in this study. The compound mixture was separated on a DB Wax-fused silica capillary column (60 m length, 0.25 mm diameter, and 0.25 μm film thickness; J&W Science) with a temperature program of 75°C (kept for 8 min) to 200°C (kept for 5 min) at a rate of 2°C min^{-1}. The injector temperature was 270°C, and the flow rate of the carrier gas, helium, was 1 mL min^{-1}. The Agilent mass spectrometer had an electron energy of 70 eV, electron emission of 300 μA, and ion source temperature of 100°C. The resulting chromatograms were analyzed using the National Institute of Standards and Technology mass spectral library version 2.0 d.

Treatment with Abiotic Elicitors and Environmental Stresses

Leaves from pepper plants at the six-leaf stage were sprayed with 5 mM SA, 100 μM MeJA, or 10 mM H_2O_2. Pepper plants treated with MeJA were tightly sealed in a plastic bag. For ethylene treatment, whole pepper plants were removed from soil and then placed in a water-containing small glass chamber, followed by injection of ethylene gas (5 μL L^{-1}). For drought stress treatment, whole pepper plants were removed from soil, washed to remove soil particles attached to roots, and then dried. Mechanical wounding stress was performed by injuring leaves with needles. Plants were placed at 4°C for low-temperature treatment. Leaves treated with various elicitors or subjected to environmental stresses were harvested at various time points and stored at −70°C until used for RNA isolation.

RNA Isolation and RNA Gel-Blot Analysis

Total RNA was extracted from pepper leaves, stems, roots, flowers, and fruits using the guanidine isothiocyanate method (Chomczynski and Sacchi, 1987). Total cellular RNA of *CaMNR1*-OX Arabidopsis T2 plants was also extracted from the aerial portions using TRIzol Reagent (Invitrogen) according to the manufacturer's instructions. For probe generation, the coding region of the *CaMNR1* gene was amplified using gene-specific primers (forward, 5′-ATGGCAGAGAAAACCACCAGC A-3′; reverse, 5′-TCAAAA-AAAAGTGACCTCCTTTCTGT-3′). The amplified PCR product was labeled with ^{32}P using a random priming kit (Boehringer Mannheim). Agarose gel electrophoresis, RNA transfer, and hybridization with the *CaMNR1* fragment were performed using standard procedures.

Protein Preparation and Protein Gel-Blot Analysis

The His-tagged CaMNR1 fusion proteins were subjected to SDS-PAGE and purified from the excised bands before being injected into rabbits to generate immune sera against CaMNR1 (LabFrontier). Specific binding of immune sera to CaMNR1 was confirmed, and the antibodies were then used for western-blot analysis at a 1:2,000 dilution.

Total protein extracts were prepared by grinding 200 mg of leaf tissue of pepper and Arabidopsis in 1 mL of grinding buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% SDS, 5 mM dithiothreitol, and Complete Protease Inhibitor Cocktail [Roche]), followed by pelleting the insoluble debris by centrifugation at 20,000*g* for 15 min at 4°C. The concentrations of proteins in the supernatants were determined by the Bradford protein assay (Bradford, 1976). About 20 μg of total protein was separated on 10% SDS-PAGE gels and transferred to polyvinylidene difluoride membranes. Western blotting was done by the standard method. Equal loading of proteins was confirmed by comparing the band intensities of the proteins on a Coomassie blue-stained SDS-PAGE gel.

VIGS

The TRV-based VIGS system was used for gene silencing in pepper plants as described by Liu et al. (2002). The full-length *CaMNR1* open reading frame was amplified by PCR using gene-specific primers (forward, 5′-ATGGCAGA-GAAAACCACCAGCA-3′; reverse, 5′-TCAAAAAAAAGTGACCTCCTTTC-TGT-3′). The pepper *CaMNR1* gene was cloned into the vector pTRV2 to yield pTRV2:*CaMNR1*. *Agrobacterium tumefaciens* strain GV3101 carrying pTRV1 or pTRV2:*CaMNR1* was coinfiltrated into the fully expended cotyledons of pepper plants (OD_{600} = 0.2). Plants were placed in a growth room at 25°C with a 16-h-light/8-h-dark photoperiod for growth and viral spread.

Identification and Isolation of *CaMNR1*-OX Transgenic Lines and *sdr1* Mutants

Transgenic Arabidopsis plants expressing the *CaMNR1* gene were generated using the floral dipping method (Clough and Bent, 1998). Three lines of putative transgenic Arabidopsis plants (T1) harboring *35S:CaMNR1* were selected by plating seeds on Murashige and Skoog medium (Duchefa) containing 50 mg L^{-1} kanamycin.

T-DNA insertion mutants (Salk_147665 and Salk_031785) of the *AtSDR1* gene were obtained from the ABRC (Ohio State University). The kanamycin-resistant plants were selected on Murashige and Skoog agar medium containing 50 mg L^{-1} kanamycin. To identify the homozygous mutant lines, PCR was performed with genomic DNA of *sdr1-1* and *sdr1-2* mutants using the following gene-specific primers: *sdr1-1* LP (5′-GTGAACCGATGGATT-GAATTG-3′), *sdr1-1* RP (5′-TTGGGTTTTCACAAACTCAGC-3′), *sdr1-2* LP (5′-CAAGACTAAAACAACGGCGTC-3′), *sdr1-2* RP (5′-CCCATGGAGGAT-GATACATTG-3′), and left-border-specific primer LBb1 (5′-GCGTGGACC-GCTTGCTGCAACT-3′).

RT-PCR Analysis

Total RNA was extracted from pepper and Arabidopsis leaves as described above. RT reactions were performed with total RNA (2 μg) and oligo $p(dT)_{15}$ primer (Roche) at 42°C using avian myeloblastosis virus reverse transcriptase (Roche) in a 20-μL reaction volume. Aliquots (1 μL) of RT reaction products were used for RT-PCR analysis with the following gene-specific primers: *CaBPR1*F (5′-CAGGATGCAACACTCTGGTGG-3′) and *CaBPR1*R (5′-ATCAAAGG-CCGGTTGGTC-3′) for *CaBPR1* (accession no. AF053343); *CaPR4*F (5′-GCGGT-AGATGCTTGAGGGT-3′) and *CaPR4*R (5′-CAATCTCGACAATAGTATGAA-ATCA-3′) for *CaPR4* (accession no. AF244122); *CaPR10*F (5′-TGTCGAAGGTGGTC-

CAATAAA-3′) and *CaPR10*R (5′-TAGACAGAAGGATTGGCGAGG-3′) for *CaPR10* (accession no. AF244121); *CaPOA1*F (5′-ATCTGTACCAGCTTG-CACGTGT-3′) and *CaPOA1*R (5′-CCCTCACTGTGGCCTTGG-3′) for *CaPOA1* (accession no. AF442387); *CaDEF1*F (5′-CAAGGGAGTATGTGCTAGTGA-GAC-3′) and *CaDEF1*R (5′-TGCACAGCACTATCATTGCATAC-3′) for *CaDEF1* (accession no. AF442388); *CaOSM1*F (5′-ACATTTCAGTAATCGATGGA-TTCA-3′) and *CaOSM1*R (5′-TAGTCCAACTTTGGCAAGTAAAT-3′) for *CaOSM1* (accession no. AY262059); *CaSAR8.2*F (5′-CAGGGAGATGAATTCT-GAGGC-3′) and *CaSAR8.2*R (5′-CATATGAACCTCTATGGATTTCG-3′) for *CaSAR8.2* (accession no. AF313766); *AtPR1*F (5′-ATGAATTTTACTGGCTT-CTCG-3′) and *AtPR1*R (5′-TTAGTATGGCTTCTCGTTCACAT-3′) for *AtPR1* (accession no. At2G14610); *AtPDF1.2*F (5′-ATGGCTAAGTTTGCTTCCATC-3′) and *AtPDF1.2*R (5′-TTAACATGGGACGTAAGTAA-3′) for *AtPDF1.2* (accession no. At5G44420); *AtRD29A*F (5′-GGTAGTGAATCAGGAGCTGAGC-3′) and *AtRD29A*R (5′-TCCACCTCCGGAGATAGGTA-3′) for *AtRD29A* (accession no. D13044); and *AtUBQ*F (5′-GTAGTGCTAAGAAGAGCAAGA-3′) and *AtUBQ*R (5′-TCAAGCTTATTCTT-3′) for *AtUBQ* (accession no. At3g62250). RT-PCR conditions were 95°C for 10 min and 30 cycles of 95°C for 30 s, 52°C for 30 s, and 72°C for 1.5 min. Single bands for PCR products were confirmed on an agarose gel.

Sequence data of *CaMNR1* from this article have been deposited in the GenBank data library under accession number EF576664.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Nucleotide sequence and deduced amino acid sequence of the *CaMNR1* cDNA encoding the MNR protein.

Supplemental Figure S2. RNA gel-blot analysis of the expression of *CaMNR1* in pepper plants.

Supplemental Figure S3. Comparison of GC-MS total ion chromatograms of pepper leaf extracts with those of standard mixtures.

Supplemental Figure S4. GC-MS total ion chromatograms of extracts from pepper leaves infected with virulent (Ds1) or avirulent (Bv5-4a) *Xcv.*

Supplemental Figure S5. Mass spectra of various metabolic compounds obtained from leaf extracts of pepper plants.

Supplemental Figure S6. Purification and enzyme activity of AtSDR1.

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