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Isolation and Characterization of Methyl Jasmonate-Inducible Genes in Chinese Cabbage

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Methyl jasmonate (MeJA) is a signal molecule in the activation of defense responses in plants. In this study, we isolated 15 MeJA-inducible genes by subtractive hybridization. These genes encode two myrosinase-binding proteins, five lipase-like proteins, a polygalacturonase inhibitor, a putative chlorophyll-associated protein, a terpene synthase, a dehydroascorbate reductase, an ascorbate oxidase, a cysteine protease, an O-methyltransferase, and an epithiospecifier protein. Northern analysis showed that most of the Chinese cabbage genes are barely expressed in healthy leaves, but are strongly induced by MeJA treatment. We also examined whether these MeJA-inducible genes were activated by ethethon, BTH, and *Pseudomonas syringae* pv. *tomato* (*Pst*), a nonhost pathogen of Chinese cabbage. The results showed that none of the MeJA-inducible genes was strongly induced by ethephon or by BTH. The genes encoding lipase-like proteins and a myrosinase-binding protein were weakly induced by *Pst*. Other MeJA-inducible genes were not activated at all by the pathogen.

Like animals, plants also have defense mechanisms against various pathogens and pests, although the underlying mechanisms differ from vertebrate immune responses. Defense responses are triggered upon perception of invading pathogens by specific receptors that are encoded by disease resistance genes (Bent et al., 1996; Dangl and Jones, 2001). This recognition in turn activates a complex array of signaling pathways in plant cells (McDowell and Dangl, 2000; Nuernberger and Scheel, 2001).

One of the well-characterized disease-resistance responses is systemic acquired resistance (SAR), which is induced locally by pathogen or pest attack and spreads systemically, resulting in protection of the whole plant. SAR is broad-spectrum and long-lasting in effect (Ryals et al., 1996). Salicylic acid has long been known to act as a messenger molecule in the activation of SAR. Recently, it has been reported that systemic resistance is also mediated by jasmonate or ethylene (Piterse and van Loon, 1999). Thus, two major signaling pathways are operating in plant defense responses. One is the salicylic acid-dependent pathway. The other is the salicylic acid-independent pathway, where jasmonates and ethylene act as signaling molecules.

The genus *Brassica* includes many important vegetable crops, such as broccoli, cabbage, Chinese cabbage, cauliflower, mustard, rape, kale, and turnip. Although these *Brassica* species have served as good model plants to study self-incompatibility (Takasaki et al., 2000), very little is known about the defense mechanisms in *Brassica*. Progress on identifying the defense mechanisms in Chinese cabbage (*Brassica rapa* subsp. *pekinensis*), an important vegetable crop in Asia, has also been very slow.

Previously, we have identified Chinese cabbage genes induced by *Pseudomonas syringae* pv. *tomato* (*Pst*), which elicits a hypersensitive response in Chinese cabbage (Ryang et al., 2002). The *Pst*-inducible genes were found to be primarily activated via a salicylate-dependent signaling pathway. We have also identified salicylic acid-inducible genes in Chinese cabbage (Park et al., 2003; Lee and Cho, 2003). In this study, we have sought methyl jasmonate-inducible genes in Chinese cabbage as a step toward understanding the salicylate-independent defense signaling pathway in this plant. Here we report isolation of 15 methyl jasmonate-inducible genes, the majority of which have not been characterized before as MeJA-inducible genes.

Materials and Methods

Plant materials

Brassica rapa subsp. *pekinensis* (cultivar Norang) seedlings were grown on potting compost after germination. Unless otherwise stated, experiments were performed with Chinese cabbage seedlings at the seven- or eight-leaf stage. Methyl jasmonate (1 mM in 0.1% [v/v] ethanol),

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ethephon (1 mM), BTH (0.3 mM) and the control of 0.1% ethanol were applied by spraying them on leaves. After 24 h of treatment, the leaf samples were harvested, weighed, and frozen immediately in liquid nitrogen. Methyl jasmonate (Me-JA) and ethephon were purchased from Aldrich Chemical Co. (Milwaukee, USA) or Sigma Chemical Co. (St. Louis, USA). BTH (5% active ingredient in wettable powder) was a kind gift from Novartis, Korea.

Pseudomonas syringae pv. *tomato* (*Pst*) strain 259 was freshly grown on NA agar medium (5 g peptone, 3 g beef extract, 2 g yeast extract, and 15 g Bacto-agar per liter) for one or two days at 30°C. The bacteria were collected by gently scraping the colonies from the agar medium with a sterile glass rod after adding 2 ml of sterile water per 85-mm plate. After centrifugation at 5,000×*g*, the bacterial pellet was resuspended in sterile water and adjusted to an OD_{600}=0.1. Chinese cabbage leaves were inoculated with *Pst* by infiltration of the bacterial suspension using a 1 ml syringe without a needle. The syringe infiltration was done at more than ten places per leaf so that about half of the leaf area was infected with the bacteria. *Pst*-treated Chinese cabbage leaves were then transferred to a growth chamber and incubated for 24 h at 25°C under continuous fluorescent light. Control plants were similarly treated with sterile water. The *Pst*-inoculated and control leaves were then collected, frozen immediately in liquid nitrogen, and used for RNA analysis.

RNA extraction and cDNA synthesis

Total RNA was prepared from frozen plant materials using the "hot phenol" method of De Vries et al. (1988). mRNA was purified from the total RNA using biotin-labeled oligo(dT) and streptavidin-conjugated paramagnetic particles (Promega, Madison, USA). cDNA was synthesized using the cDNA synthesis kit from Promega. In the cDNA synthesis reaction, a set of oligo(dT) anchor primers was used so that cDNA synthesis started where the poly(A) tail began. The oligo(dT) primers used were as follows: 5'-CGGGGTAC$(T)_{15}$A-3' (RsaTA), 5'-CGGGGTAC$(T)_{15}$C-3' (RsaTC) and 5'-CGGGGTAC$(T)_{15}$G-3' (RsaTG). After the reaction, the cDNA was purified with a QIAprep spin column (QIAGEN, Hilden, Germany) according to the manufacturer's protocol for DNA cleanup.

Isolation and sequence analysis of methyl jasmonate-inducible genes

Subtractive hybridization was performed as described by Min et al. (2001). The cDNA prepared from methyl jasmonate-treated leaves was used as a tester, and the cDNA from mock-treated samples was used as a driver in subtractive hybridization. Both tester and driver DNAs were digested with *Rsa*I, ligated with the linker DNA containing a *Kpn*I recognition site and amplified by PCR. The tester DNA samples were divided into two aliquots, each digested with either *Rsa*I or *Kpn*I. The two DNA samples were then combined and hybridized with an excess of the driver DNA retaining the linker. After hybridization, the DNA mixture was ligated to a new adaptor compatible only with double-stranded tester/tester DNAs. The DNA with the new adaptor sequence was then amplified by PCR, and the amplified DNA was used as a new tester after adjusting the DNA concentration to the initial condition.

PCR products from the final subtractive hybridization round were purified with a QIAprep spin column. The DNA was digested with *Kpn*I and cloned into a pBluescript SK vector. The nucleotide sequence of the cDNA insert was determined with an automatic sequencer (Applied Biosystems) at the Macrogen Inc., Seoul. Conventional M13-forward and M13-reverse primers were used to determine the sequence. The database search and computation were performed at the NCBI (National Center for Biotechnology Information) using the BLAST network service. Multiple sequence alignment was done with the CLUSTAL W software (Thompson et al., 1994) at http://www.ebi.ac.uk.

Northern blot analysis

For Northern analysis, 10 μg of total RNA was separated on a 1.0% formaldehyde-agarose gel and blotted onto a Hybond-N^+ nylon membrane (Amersham Pharmacia Biotech, Buckinghamshire, UK) using the standard capillary transfer method. After UV-crosslinking at 125 mJ, blots were hybridized using the DNA probe labeled with digoxigenin (DIG). Chemiluminescent detection of the hybridized probe was carried out as described by Kim et al. (2002). The probe DNA was prepared by PCR amplification of the insert DNA of the cDNA clones. The DNA probe for glyceraldehyde 3-phosphate dehydrogenase (GAPD) was obtained using a Chinese cabbage GAPD cDNA clone isolated in our laboratory (GenBank accession no. AF536826). The DNA probe for the ethylene- and *Pst*-inducible PR4 gene was prepared using the cDNA clone CPL30 (GenBank accession no. AF528181; Ryang et al., 2002). The DIG-labeling, hybridization, and chemiluminescent immunodetection were performed using kits from Roche Molecular Biochemicals (Mannheim, Germany).

The nucleotide sequence data that are reported in this paper have been submitted to the GenBank nucleotide sequence database, and assigned the accession numbers AY337002-AY337016.

Results and Discussion

Isolation of MeJA-inducible genes in Chinese cabbage

To isolate MeJA-inducible genes in Chinese cabbage

Table 1. Methyl jasmonate-inducible genes isolated by subtractive hybridization

Clone	GenBank accession No.	Best match in database[a]	Identity[b]
MJ3-1	AY337002	rape polygalacturonase inhibitor (AF529692[c])	96% (61)
MJ9	AY337003	*Arabidopsis* myrosinase-binding protein (NM_129488)	76% (117)
MJ12	AY337004	cauliflower water-soluble chlorophyll protein (AB078330)	92% (112)
MJ18-2	AY337005	*Arabidopsis* terpene synthase (NM_122301)	75% (24)
MJ22-1	AY337006	*Arabidopsis* putative lipase (AY085113)	54% (62)
MJ22-2	AY337007	*Arabidopsis* putative lipase (AY085113)	46% (166)
MJ24	AY337008	rape myrosinase-binding protein 3 (T08146)	59% (191)
MJ25-2	AY337009	*Arabidopsis* dehydroascorbate reductase (NM_101814)	83% (143)
MJ26	AY337010	*Arabidopsis* putative lipase (AY085113)	56% (171)
MJ27	AY337011	*Arabidopsis* putative lipase (AY085113)	59% (161)
MJ32	AY337012	*Arabidopsis* putative cysteine proteinase (AY051062)	90% (149)
MJ33	AY337013	*Arabidopsis* O-methyltransferase protein (BT000302)	85% (189)
MJ38	AY337014	*Brassica juncea* ascorbate oxidase (AF206721)	88% (82)
MJ39	AY337015	*Arabidopsis* putative lipase (AY085113)	52% (62)
MJ43	AY337016	*Arabidopsis* epthiospecifier (AF416790)	76% (112)

[a]Best match from the BLAST analysis. The GenBank accession number of the match is in parentheses. [b]Percent identity at the amino acid level. The number of amino acid residues compared is in parentheses. [c]Protein ID.

(*Brassica rapa* subsp. *pekinensis*), we performed a subtractive enrichment experiment using the method developed in our laboratory (Min et al., 2001). Briefly, the method enriches target sequences by selective adaptor ligation and PCR following subtractive hybridization. The cDNA sample prepared from the leaves treated with MeJA was used as a tester, and the cDNA sample from the mock-treated leaves was used as a driver. After three rounds of subtractive hybridization, the subtracted DNA sample was cloned into a pBluescript vector. A total of 29 clones were randomly chosen for nucleotide sequencing. It turned out that some clones contained two or more inserts and that the 29 clones represented 15 different genes. Northern analysis revealed that these 15 genes were strongly induced by MeJA treatment.

The results of the BLAST analysis of the 15 MeJA-inducible genes are summarized in Table 1. The MJ3-1 clone shows a strong similarity with a rape polygalacturonase inhibitor. Polygalacturonase inhibitor suppresses the activity of the polygalacturonase derived from invading fungal pathogens. By doing this, more oligogalacturonides, which act as elicitors, can be generated. Over-expression of polygalacturonase inhibitor in fact increases resistance to fungal pathogens (Powell et al., 2000). In apple, polygalacturonase inhibitor gene expression is regulated by fruit development and responds to wounding, fungal infection and cold storage (Yao et al., 1999).

Both the MJ9 and MJ24 clones are similar to a putative myrosinase-binding protein in *Arabidopsis*. The MJ9 clone shows 37% identity with the MJ24 at the amino acid level. The physiological function of myrosinase-binding proteins has not been well characterized, although the proteins belong to a large protein family (Taipalensuu et al., 1997) and are necessary for myrosinase complex formation (Eriksson et al., 2002). Myrosinase is an enzyme that degrades glucosinolates, major defense compounds in crucifer plants. Glucosinolates have been considered as feeding deterrents or repellents to herbivores including insects. Glucosinolates were also known to have antifungal activity (Osbourn, 1996). Recently, it was shown that glucosinolates protected *Arabidopsis* against a soft rot pathogen, *Erwinia carotovora* (Brader et al., 2001). The MJ43 clone turned out to encode an epithiospecifier protein. In *Arabidopsis,* the epithiospecifier protein is able to convert glucosinolates both to epithionitriles and to simple nitriles in the presence of myrosinase (Lambrix et al., 2001).

BLAST analysis showed that the MJ12 clone is most homologous to a cauliflower gene encoding a putative chlorophyll-associated protein of unknown function. MJ18-2 clone is similar to a putative *Arabidopsis* δ-cadinene synthase, a terpene synthase. The δ-cadinene synthase is known to catalyze the first step in the conversion of the isoprenoid intermediate, farnesyl diphosphate (FDP), to sesquiterpene phytoalexins (Benedict et al., 2001).

Of the 15 genes, five genes (MJ22-1, MJ22-2, MJ26, MJ27, and MJ39) were found to encode lipase-like proteins that belong to a family II lipase. The most homologous *Arabidopsis* protein (GenBank accession no. AY085113) contains the motif [FT]G[ND]SxxDxG[NG]x(10,20)PYG found in family II lipases (Brick et al., 1995). One of the characteristic features of the family II lipases is that

A

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                                                                              % ID
AY085113   LVNLHNNLLQQVVAKLNNETKQSTFIILDLYNAFLTVFKNKGSNPGSTRFESPLKPCCV
MJ22-1     LVGYHNALLQKSVAKLNKENNHSAFTIIDYYNTFLAVFNNKGDIPGIQMFPNPSILCLS   55%
MJ39       LVEFHNGLLQKSVVKLNKENNHSAFTTIDYYNTFLAVFNNKGDIPGTQMFSNPSIPCLS   52%
MJ22-2     LVEYHNALLQKSVAKLNKENNHSAFAIMDYYNTFLAIF-NTGGIPGIPTFQNISIPCLG   58%
MJ26       LVEYHNALLQKSVAKLNKENNHSAFTIIDYYNTFLAIFNNTGGIPGIPTSQSISIPCLG   59%

AY085113   GVSREYNCGSVDEKGVKKYIVCDNPKTAFFWDGLHPTEEGWRSVYSVLRESLTASLI-KA
MJ22-1     ------------DNG
MJ39       ------------DNG
MJ22-2     ------------DDGK----VCDDRKSAFFWDGIHPTQEGWKSVYKVLRHNITAALMPKA
MJ26       ------------DDGK----VCDDRKSAFFWDGIHPTQEGWKSVYKVLRHNITAALMPKA
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B

	MJ39	MJ22-2	MJ26
MJ22-1	90%	82%	82%
MJ39		75%	78%
MJ22-2			96%

Fig. 1. Multiple alignments of the lipase-like proteins. A, Predicted amino acid sequences of the Chinese cabbage lipase-like proteins and the most homologous *Arabidopsis* protein in the database (AY085113) were compared. The multiple sequence alignments were done using CLUSTAL W. B, The similarities between the Chinese cabbage lipase-like proteins were calculated and represented in per cent identities.

they have a GxSxxxG motif around the active site serine residue rather than the GxSxG found in classical lipases. Among the five clones encoding lipase-like proteins, four clones (MJ22-1, MJ22-2, MJ26, and MJ39) encoded the same C-terminal part of the protein, and therefore could be compared to each other. The amino acid sequences of the four Chinese cabbage lipase-like proteins were compared with the most homologous *Arabidopsis* protein in the database using CLUSTAL W (Fig. 1A). The amino acid sequences of the Chinese cabbage lipase-like proteins were also compared to each other (Fig. 1B). The result showed that the four Chinese cabbage lipase-like proteins are distinct from the *Arabidopsis* protein, but are very similar to each other. Based on the similarities between the Chinese cabbage proteins, they can be divided into two groups: one group comprises MJ22-1 and MJ39, and the other includes MJ22-2 and MJ26. The MJ27 clone encoded an N-terminal part of the lipase-like protein, thus, could not be compared with the other four lipase-like Chinese cabbage proteins.

Previously, we identified a salicylic acid- and pathogen-induced lipase-like protein that showed a family II lipase motif (Lee and Cho, 2003). Previous study also revealed that plant genomes have a large number of genes that contain the family II lipase motif. However, the *in vivo* functions of these genes are mostly unknown. The MeJA-inducible lipase-like genes obtained in this study did not share significant similarities with the salicylic acid-induced lipase-like gene. This raises a possibility that Chinese cabbage plant uses different lipase-like proteins in response to different environmental signals. Recently, pathogen-induced lipase-like proteins were reported in *Arabidopsis*. These proteins show a classical lipase motif around the active site serine residue, and the corresponding genes are induced by salicylic acid, β-aminobutyric acid, and ethylene, but not by jasmonate (Jakab et al., 2003).

MJ25-2 clone is highly homologous to an *Arabidopsis* dehydroascorbate reductase (DHAR), which regenerates ascorbic acid from its oxidized form using glutathione. Although the role of DHAR in plant defense has not been actively studied, it was reported that the DHAR activity was reduced in the TMV-infected region and that the DHAR gene was induced by salicylic acid. With this observation, Fodor et al. (1997) postulated that DHAR serves to suppress uncontrolled spreading of necrotic cell death. Recently, antioxidant enzymes including DHAR were reported to be activated by drought stress (Walz et al., 2002). The MJ38 clone turned out to encode an ascorbate oxidase, which catalyzes oxidation of ascorbate. The function of this enzyme in plant defense is virtually unknown. Recently, the plant over-expressing this enzyme was found to display increased sensitivity to ozone, thereby resulting in more damage to leaf and reduced photosynthesis (Sanmartin et al., 2003). It is not clear why both the two genes (DHAR and ascorbate oxidase genes), whose protein products act against each other, are induced by MeJA. It is also possible the two genes have functions other than the defensive roles, considering that MeJA mediates diverse developmental processes as well as responses to biotic and abiotic stimuli (Cheong and Choi, 2003).

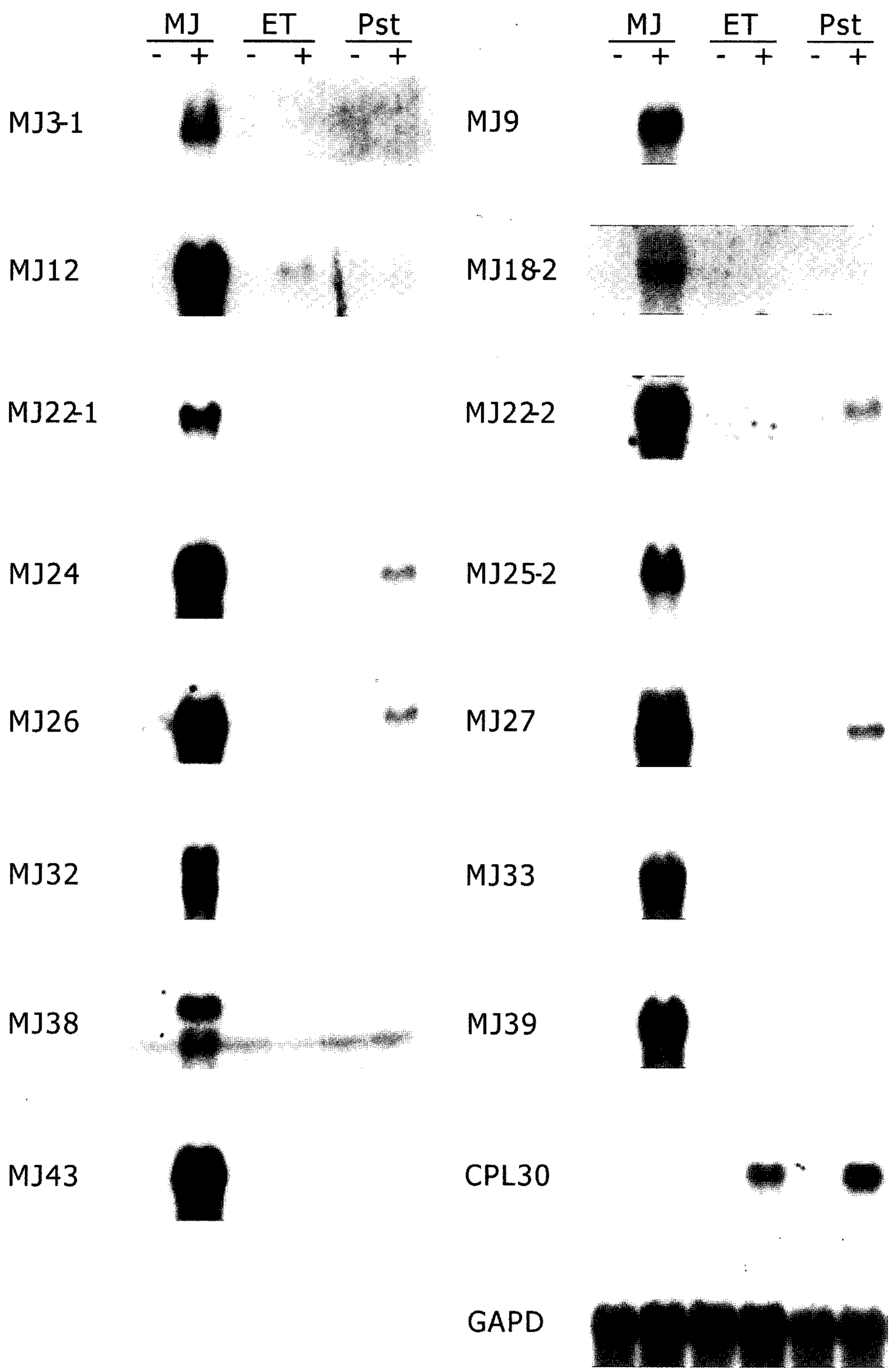

Fig. 2. Northern blot analysis of the Chinese cabbage genes isolated in this study. One mM methyl jasmonate (MeJA), 1 mM ethephon (ET) solution, 0.1% ethanol (control for MeJA treatment) or sterile water (control for ET treatment) was sprayed onto Chinese cabbage leaves. For pathogen treatment, the Chinese cabbage leaves were infiltrated with *Pseudomonas syringae* pv. *tomato* (*Pst*). Control plants were similarly infiltrated with sterile water. After 24 h the leaf samples were collected and analyzed by Northern blot analysis. Ten μg of total RNA was size-fractionated on a 1% formaldehyde agarose gel, blotted onto a nylon membrane, and hybridized with DIG-labeled DNA probe. Chemiluminescent detection of the DIG-labeled DNA was done with an alkaline phosphatase-conjugated anti-DIG antibody and CSPD following the protocol provided by Roche Molecular Biochemicals. CPL30 and GAPD represent the Chinese cabbage PR4 and glyceraldehyde-3-phosphate dehydrogenase genes, respectively.

MJ33 clone is homologous to an *Arabidopsis* O-methyltranferase. A S-adenosyl-L-methionine-dependent O-methyltransferase catalyzes the methylation of hydroxycinnamic acid derivatives for the synthesis of methylated plant polyphenolics, including lignin (Li et al., 1997). Recently, it was reported that the O-methyltransferase is also involved in the synthesis of volatile phenolics, many of which act as defense compounds (Lavid et al., 2002). The MJ32 clone shows a strong identity with a putative cysteine protease in *Arabidopsis*. Although the function of the *Arabidopsis* protein is not known, it is worth noting that protein degradation is involved in the activation of defense signaling. For example, the bacterial virulence effector from *Pseudomonas syringae*, AvrRpt2, targets the *Arabidopsis* RIN4 protein and induces its post-transcriptional disappearance. This elimination of the RIN4 is essential in the induction of disease resistance mediated by the RPS2 R protein (Axtell and Staskawicz, 2003; Mackey et al., 2003). In Cf2-dependent disease resistance to a fungal pathogen *Cladosporium fulvum*, Rcr3, which is specifically required for the function of Cf2, turned out to encode a papain-like cysteine protease (Kueger et al., 2002).

Expression profiles of MeJA-inducible genes

We examined whether the MeJA-inducible Chinese cabbage genes were activated by ethephon, an ethylene-releasing compound. The result showed that most MeJA-inducible Chinese cabbage genes were not induced by ethephon, except that the clone MJ12 is weakly induced by the chemical (Fig. 2). We then examined how the MeJA-inducible genes responded to benzothiadiazole (BTH), one of the most potent SAR-inducing chemicals (Goerlach et al., 1996). But, again, none of the MeJA-inducible genes was found to be activated by BTH (data not shown). Previously, we observed that hypersensitive response (HR) could be induced by *Pst*, a nonhost pathogen of Chinese cabbage (Ryang et al., 2002). When Chinese cabbage leaves were infiltrated with *Pst* and the expression of the MeJA-inducible genes was examined, it was found that the genes encoding lipase-like proteins (MJ22-2, MJ26, MJ27, and MJ39) and a myrosinase-binding protein (MJ24) were weakly induced by *Pst* (Fig. 2).

To verify that appropriate induction conditions were used, we included an ethylene-inducible CPL30 gene as positive controls in the Northern blot analysis. The CPL30, which was obtained while isolating *Pst*-induced genes in Chinese cabbage, encodes a PR4 protein (Ryang et al., 2002). Northern analysis showed that the Chinese cabbage PR4 gene was strongly induced by both ethephon and *Pst*, indicating that the induction conditions used in this study were appropriate.

Weak induction of the MeJA-inducible Chinese cabbage genes by *Pst* was not unexpected, since it was reported that resistance to *Pst* depends on salicylate-dependent signaling in *Arabidopsis* (Delaney et al., 1994). Genetic studies with *Arabidopsis* signaling mutants have shown that the salicylate-dependent response is deployed against a biotrophic pathogen that obtains nutrients from living cells, whereas the ethylene- or jasmonate-dependent response is important for induced resistance to a necrotrophic pathogen that kills plant tissue (Piterse and van Loon, 1999; McDowell and Dangl, 2000). Considering that *Pst* is a biotrophic pathogen in *Arabidopsis* and tomato (Bashan et al., 1981; Whalen et al., 1991), therefore, it is likely that the MeJA-inducible Chinese cabbage genes can be activated by pathogens other than *Pst*, possibly by necrotrophic pathogens.

The temperature, 50°C, used in the Northern blot hybridization was a rather stringent temperature that did not allow more than 18% mismatches between probe and target. The two myrosinase-binding protein genes could be distinguished at this temperature, since the homology between the two clones is 51% at the nucleotide level. However, the homologies between lipase-like protein genes, 95% identity between MJ22-1 and MJ39 and 97% identity between MJ22-2 and MJ26 at the nucleotide level, are so high that cross-hybridization could have occurred. Thus, use of gene-specific probes will be necessary to get the information on the specific gene's expression profiles.

In conclusion, we have isolated 15 MeJA-inducible Chinese cabbage genes, most of which have not been previously characterized. Expression of these genes is at low levels in healthy and unstressed leaf tissues, but is strongly induced by MeJA. None of these genes is strongly induced by either ethephon or by BTH. Thus, these genes represent the genes activated specifically by MeJA. The defensive roles of these genes remain to be elucidated.

Acknowledgments

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