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Molecular Characterization of a *thiJ*-like Gene in Chinese Cabbage

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A cDNA clone for a salicylic acid-induced gene in Chinese cabbage (*Brassica rapa* subsp. *pekinensis*) was isolated and characterized. The cabbage gene encoding a protein of 392 amino acids contained a tandem array of two *thiJ*-like sequences. ThiJ is a thiamin biosynthesis enzyme that catalyzes the phosphorylation of hydroxymethylpyrimidine (HMP) to HMP monophosphate. Although the cabbage gene shows a similarity to bacterial *thiJ* genes, it also shares a similarity with the human DJ-1, a multifunctional protein that is involved in transcription regulation, male fertility, and parkinsonism. The cabbage *thiJ*-like gene is strongly induced by salicylic acid and a nonhost pathogen, *Pseudomonas syringae* pv. *tomato*, which elicits a hypersensitive response in Chinese cabbage. Treatment of the cabbage leaves with BTH, methyl jasmonate, or ethephon showed that the cabbage *thiJ*-like gene expression is also strongly induced by BTH, but not by methyl jasmonate or ethylene. This indicates that the cabbage gene is activated *via* a salicylic acid-dependent signaling pathway. Examination of the tissue-specific expression revealed that the induction of the cabbage gene expression by BTH occurs in the leaf, stem, and floral tissues but not in the root.

Keywords: *Brassica rapa*, Chinese cabbage, Defense-related, *thiJ*-like

Introduction

Like animals, plants also have defense mechanisms against various pathogens and pests, although the underlying mechanisms differ from vertebrate immune responses. Salicylic acid is a messenger molecule in the activation pathway of one of the disease-resistance responses, known as systemic acquired resistance (SAR). SAR is locally induced by pathogen or pest attack and spreads systemically, resulting in protection of the whole plant. SAR is also broad-spectrum and long-lasting in effect (Ryals *et al.*, 1996). Defense responses are triggered upon the 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. Many of the signaling components, such as EDS1, NDR1 and NPR1, have been studied by mutant analysis (Delaney, 1997; Feys and Parker, 2000). Biochemical studies have shown that reactive oxygen species and nitric oxide are important signaling molecules in the activation of disease resistance. These studies have also shown that MAP kinase cascades play a critical role in plant defense responses, as in animals (McDowell and Dangl, 2000; Nuerenberger and Scheel, 2001).

Now, a picture of defense mechanisms in plants is emerging. However, much research is still necessary in order to clarify uncertainties and ambiguities. Moreover, studies on defense mechanisms have focused on a limited number of model plants, such as *Arabidopsis thaliana*, tomato, and tobacco. Although the information obtained from these model plants has advanced our understanding of defense responses in plants as a whole, certain plants may have unique resistance mechanisms. 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), studies on defense mechanisms in *Brassica* have not been very active. Progress on identifying defense mechanisms in Chinese cabbage (*Brassica rapa* subsp. *pekinensis*), an important vegetable crop in Asia, has also been very slow, and few defense-related genes in Chinese cabbage have been characterized.

Previously, we isolated a partial cDNA clone that contained a Chinese cabbage *thiJ*-like gene exhibiting strong induction by salicylic acid and a pathogen (Park *et al.*, 2003). Here, we report the cDNA sequence of the full coding region and more

The nucleotide sequence data that is reported in this paper was submitted to the GenBank nucleotide sequence database, and assigned the accession number AY335489.

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detailed expression profiles of the Chinese cabbage *thiJ*-like gene.

Materials and Methods

Plant materials and chemical treatments *Brassica rapa* subsp. *pekinensis* (cultivar Norang) seedlings were grown on potting compost after germination. Unless stated otherwise, the experiments were performed with cabbage seedlings at the seven- or eight-leaf stage. For the salicylic acid treatment, fully developed and healthy leaves from the plants were cut into 1 × 1 cm pieces, and floated onto a 20 mM MOPS buffer (pH 7.5) containing either 5 mM or no salicylic acid (Sigma Chemical Co., St. Louis, USA) in a 10 cm or 15 cm petri dish. The leaf samples were then transferred to a growth chamber and incubated at 25ºC under continuous fluorescent light. After 1-3 days of treatment, the leaf squares were harvested, weighed, and frozen immediately in liquid nitrogen. Next, 0.3 mM benzothiadiazole (BTH), 1 mM methyl jasmonate (Me-JA) in 0.1% [v/v] ethanol, 1 mM ethephon, or 0.1% ethanol were sprayed on leaves of the intact plants. BTH (5% active ingredient in wettable powder) was a kind gift from Novartis, Korea. Me-JA and ethephon were purchased from Aldrich Chemical Co. (Milwaukee, USA) or Sigma Chemical Co. (St. Louis, USA). For an analysis of the tissue-specific expression, the seeds were vernalized for three weeks at 4ºC after sowing. Each germinated seed was then transferred to a vinyl pot (9 cm in diameter) and grown until flowering. The aerial parts of the plants were treated with 0.3 mM BTH by spraying. The roots were treated with BTH by pouring 200 ml of 0.3 mM BTH solution into each pot. The control plants were similarly treated with sterile distilled water.

Pathogen treatment *Pseudomonas syringae* pv. *tomato* (*Pst*) 259 was a kind gift from Dr. Cha at the Phytopathogenic Bacteriology Laboratory at Chungbuk National University, Korea. The *Pseudomonas* bacteria for inoculation were freshly grown on a NA agar medium (5 g of peptone, 3 g of beef extract, 2 g of yeast extract, and 15 g of Bacto-agar per liter) for one or two days at 30ºC. 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 $OD_{600} = 0.1$. Cabbage leaves were inoculated with the bacterial suspension by syringe infiltration. *Pst*-treated cabbage plants were then transferred to a growth chamber and incubated at 25ºC under continuous light. Visible hypersensitive response (HR) usually developed 24 to 36 h after inoculation. The control plants were similarly treated with sterile water.

cDNA library screening The *thiJ*-like gene was isolated from a Chinese cabbage cDNA library that was constructed with the Lambda ZapII vector using mRNA from cabbage leaves treated with salicylic acid. Aliquots of the library were grown on ten LB agar plates at the density of 2.5×10^4 pfu per plate and the phages were eluted with a SM buffer. The phage suspension from each plate was then examined by PCR for the presence of the *thiJ*-like clone. One of the PCR primers was a *thiJ*-like gene-specific primer: 5' CTTACCCTTGAGTAAACCATG 3'. The other primer was a vector sequence near the cloning site: 5' AACAGCTATGACCATG ATTACGCC 3'. The phages in the sub-library fraction yielding positive DNA bands were then diluted and grown on 24 plates at the density of 2,000 pfu per plate. Eluted phages from the plates were again examined by PCR.

To confirm whether the PCR-amplified DNA contained the *thiJ*-like gene sequence, DNA blot hybridization was performed using the partial cDNA as a probe. The preparation of the alkaline phosphatase-conjugated DNA probe and chemiluminescent detection were carried out as described by Min *et al*. (2001) using the AlkPhos Direct labeling and detection kit from Amersham Pharmacia Biotech (Buckinghamshire, UK). Finally, the phage sample containing the largest insert was then screened by *in situ* plaque hybridization. Subcloning of the cDNA insert was done by *in vivo* excision of the pBluescript SK phagemid DNA with ExAssist helper phage, as described in Stratagenes protocol. The obtained plasmid clone was designated pBrThi23.

DNA sequence analysis and database search The nucleotide sequence of the cDNA insert was determined with an Applied Biosystems automatic sequencer at the Macrogen Co. (Seoul, Korea) Conventional M13-forward and M13-reverse primers were initially used to determine the sequence. Based on the sequence information, two oligonucleotide primers for each strand of the cDNA insert were synthesized and used for further sequence analysis. The gene-specific sequencing primers were spaced at about 400-bp intervals. The database search and computation were performed at NCBI (National Center for Biotechnology Information) using the BLAST network service. Motif searches were made using the PROSITE database at http://expasy.hcuge.ch/sprot/prosite.html. Signal peptide prediction was performed using TargetP (Emanuelsson *et al.*, 2000) and http://www.cbs.dtu.dk/services/TargetP/. A multiple sequence alignment was done with the CLUSTAL W software (Thompson *et al.*, 1994) at http://www.ebi.ac.uk.

RNA extraction and Northern analysis Total RNA was prepared from frozen plant materials using the "hot phenol" method of De Vries *et al.* (1988). For the 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, the blots were hybridized using the DNA probe that was labeled with digoxigenin (DIG). Chemiluminescent detection of the hybridized probe was carried out as described by Kim *et al.* (2002). The probe DNA for detecting the cabbage *thiJ*-like mRNA was prepared by PCR amplification of the cDNA insert of pBrThi23. The amplified DNA was labeled with DIG by random priming reaction. The DNA probe for glyceraldehyde 3-phosphate dehydrogenase (GAPD) was obtained using a GAPD cDNA clone (GenBank accession no. AF536826). The DNA probes for the cabbage PR1a (GenBank accession no. AF528177) and CYP83B1 (GenBank accession no. AF528175) were prepared using the cDNA clones (CPL1 and CPE-T9, respectively) that were isolated in our laboratory (Ryang *et al*., 2002). The DIG-labeling, hybridization, and chemiluminescent immunodetection were performed using kits from Roche Molecular Biochemicals (Mannheim, Germany).

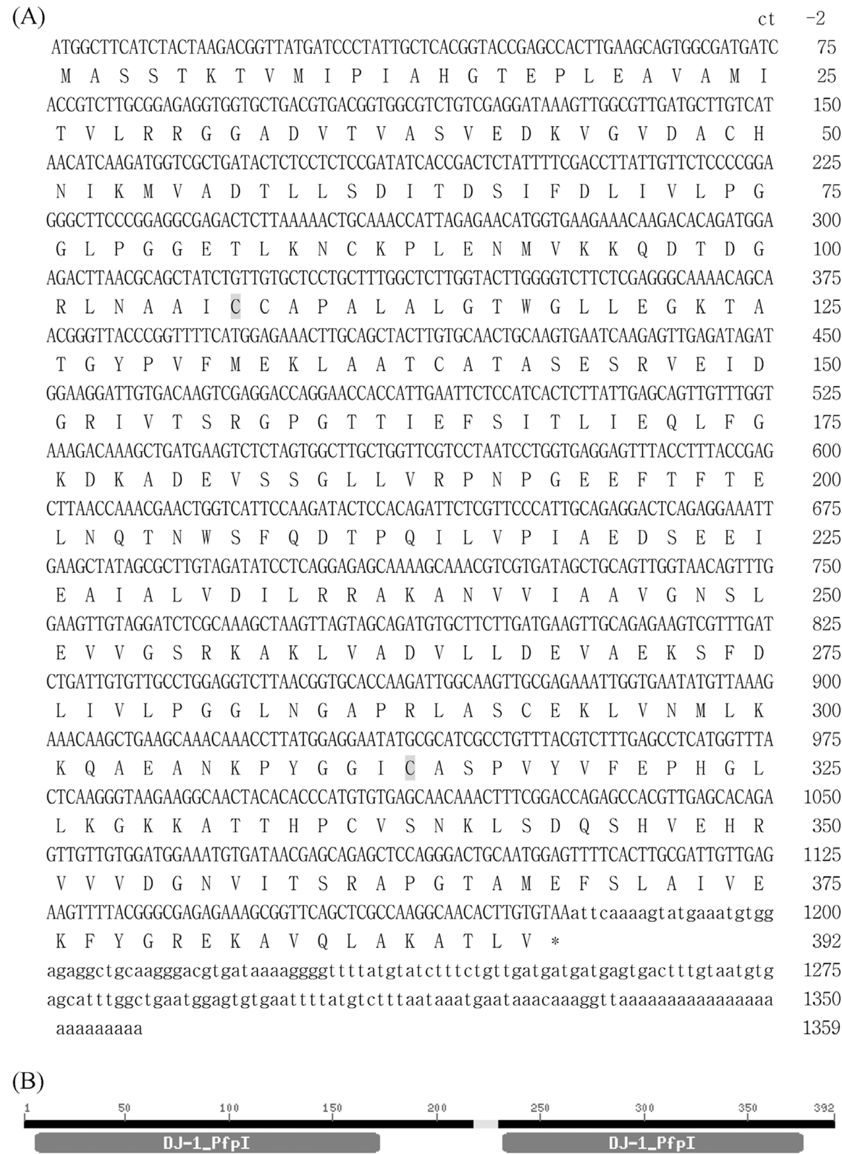

Fig. 1. A *thiJ*-like gene in Chinese cabbage. (A) Nucleotide and deduced amino acid sequences. The 5' and 3' noncoding cDNA sequences are represented by lowercase letters. The putative catalytic cysteine residues (C107 and C313) are shaded. (B) Schematic representation of the gene structure. DJ-1 is a multifunctional human protein involved in transcription activation, male fertility, and parkinsonism. PfpI represents an intracellular cysteine protease from *Pyrococcus furiosus*. The Pfam accession number of the DJ-1/PfpI family is PF01965. The gray region (#218-229 in the sequence) represents a low-complexity segment that was filtered out in the BLASTP conserved domain search.

Results

Isolation and characterization of the Chinese cabbage *thiJ*-like gene To obtain a cDNA clone for the salicylic acid-induced *thiJ*-like gene in Chinese cabbage, a cDNA library was screened by PCR and *in situ* plaque hybridization, as described in Materials and Methods. The cDNA clone, designated pBrThi23, contained a 1,176-bp ORF with 5' and 3' noncoding sequences of 2-bp and 155-bp, respectively (Fig. 1A). The Chinese cabbage gene encoded a protein of 392 amino acids. Scanning the PROSITE database revealed no significant motif in the cabbage protein. A BLAST search showed that the cabbage protein was 92% identical to an *Arabidopsis* ThiJ-like protein of unknown function, which was encoded by AT3g14990. ThiJ was a thiamin biosynthesis enzyme that catalyzed the phosphorylation of hydroxymethylpyrimidine (HMP) to HMP monophosphate. The enzyme was primarily studied in *E. coli* (Mizote *et al.,* 1999), but has not been characterized in plants. There are two other *Arabidopsis* proteins that are highly homologous to the cabbage protein (BLASTP value $E < 10^{-70}$): these two *Arabidopsis* proteins that are encoded by At1g53280 and

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BrThi-N            TKTVMIPIAHGTEPLEAVAMITVLRRGGADVTVASVEDKVG--VDACHNI   52
BrThi-C            TPQILVPIAEDSEEIEAIALVDILRRAKANVVIAAVGNSLE--VVGSRKA  258
EcThiJ          MNMSASALVCLAPGSEETEAVTTIDLLVRGGIKVTTASVASDGNLAITCSRGV   53
hDJ-1            MASKRALVILAKGAEEMETVIPVDVMRRAGIKVTVAGLAGKD--PVQCSRDV   50
PH1704              MKVLFLTANEFEDVELIYPYHRLKEEGHEVYIASFERGT---ITGKHGY   46

                                                                  *
BrThi-N   KMVADTLLSDIT-DSIFDLIVLPGGLPGGETLKNCKPLENMVKKQDTDGRLNAAICCAPA  111
BrThi-C   KLVADVLLDEVA-EKSFDLIVLPGGLNGAPRLASCEKLVNMLKKQAEANKPYGGICASPV  317
EcThiJ    KLLADAPLVEVA-DGEYDVIVLPGGIKGAECFRDSTLLVETVKQFHRSGRIVAAICAAPA  112
hDJ-1     VICPDASLEDAKKEGPYDVVVLPGGNLGAQNLSESAAVKEILKEQENRKGLIAAICAGP-  109
PH1704    SVKVDLTFDKVN-PEEFDALVLPGG-RAPERVRLNEKAVSIARKMFSEGKPVASICHGP-  103

BrThi-N   LALGTWGLLEGKTATGYPVFMEKLAAT-CATASESRVEIDG--RIVTSRGPGTTIEFSIT  168
BrThi-C   YVFEPHGLLKGKKATTHPCVSNKLS---DQSHVEHRVVVDG--NVITSRAPGTAMEFSLA  372
EcThiJ    TVLVPHDIFPIGNMTGFPTLKDKIP---AEQWLDKRVVWDARVKLLTSQGPGTAIDFGLK  169
hDJ-1     TALLAHEIGFGSKVTTHPLAKDKMMNGGHYTYSENRVEKDG--LILTSRGPGTSFEFALA  167
PH1704    QILISAGVLRGRKGTSYPGIKDDMIN-AGVEWVDAEVVVDG--NWVSSRVPADLYAWMRE  160

                                               % Id
BrThi-N   LIEQLFGKDKADEVSSGLLV          188
BrThi-C   IVEKFYGREKAVQLAKATLV          392     40%
EcThiJ    IIDLLVGREKAHEVASQLVMAAGIYNYYE 198     36%
hDJ-1     IVEALNGKEVAAQVKAPLVLKD        189     35%
PH1704    FVKLLK                        166     25%
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Fig. 2. Multiple sequence alignment of the two ThiJ-like domains of the cabbage protein and other homologous proteins. Identical amino acids are boxed, and conserved amino acids or amino acids with similar properties are shaded. An asterisk indicates the catalytic cysteine residue. The catalytic histidine residues in DJ-1 (H126) and PH1704 (H101) are underlined. BrThi-N, N-terminal ThiJ-like domain of the cabbage protein (amino acids #5-188); Br-Thi-C, C-terminal domain of the cabbage protein (amino acids #211-392); hDJ-1, human DJ-1 (Accession no. NP_009193); EcThiJ, ThiJ from *E. coli* (Accession no. Q46948); PH1704, protease I from *Pyrococcus horikoshii* (Accession no. NP_143548).

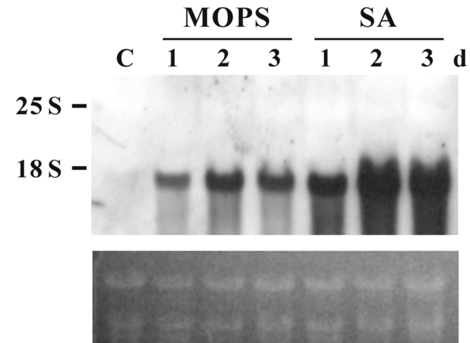

Fig. 3. Induction of the cabbage *thiJ*-like gene expression by salicylic acid. Chinese cabbage leaves were cut into 1 × 1 cm squares and floated onto a 20 mM MOPS buffer (pH 7.5) containing 5 mM salicylic acid. Control samples were treated with the MOPS buffer without salicylic acid. C, control leaf sample with no treatment; MOPS, 1 × 1 cm leaf squares treated with MOPS buffer; SA, leaf squares treated with salicylic acid. 1, 2, and 3 represent the duration of the treatment in days. After the indicated time, the leaf samples were collected and analyzed by Northern blot hybridization. Next, 10 μg of the total RNA from each sample was size-fractionated on a 1% formaldehyde agarose gel. The agarose gel, stained with ethidium bromide, is represented in the lower panel. The fractionated RNA was blotted onto a nylon membrane and hybridized with a DIG-labeled DNA probe. Chemiluminescent detection was done using an alkaline phosphatase-conjugated anti-DIG antibody and CSPD, according to the protocol provided by Roche Molecular Biochemicals.

At4g34020 display a 79% and 43% identity, respectively, with the cabbage protein. The function of these *Arabidopsis* proteins has not yet been determined.

A search for conserved domains revealed the presence of two putative domains of a DJ-1/PfpI family, formerly known as a ThiJ/PfpI family. This structural feature is schematically represented in Fig. 1B. The two predicted ThiJ-like domains of the cabbage protein and other homologous proteins are compared by a CLUSTAL W analysis in Fig. 2. The N-terminal ThiJ-like domain (amino acids #5-188) showed a 40% identity and a 63% similarity with the C-terminal domain (#211-392). Compared to the *E. coli* ThiJ, the N-terminal ThiJ-like domain of the cabbage protein displayed a 36% identity and a 57% similarity. The C-terminal domain showed a 35% identity and a 54% similarity with the bacterial enzyme.

As shown in Fig. 2, the cabbage protein also displayed a comparable similarity to the human protein DJ-1, which was reported to be involved in male fertility (Wagenfeld *et al.*, 1998; Takahashi *et al.*, 2001) and Parkinson's disease (Bonifati *et al.*, 2003). The crystal structure of the DJ-1 was recently elucidated (Honbou *et al.*, 2003). The structure analysis showed that DJ-1 was structurally most similar to the monomer unit of protease I (PH1704) from the hyperthermophilic archaebacterium *Pyrococcus horikoshii* (Du *et al.*, 2000). PH1704 is a homolog of the novel intracellular cysteine protease PfpI that is found in *P. furiosus* (Halio *et al.*, 1996). As shown in Fig. 2, cabbage protein also shows a considerable similarity to the PH1704 protease. The putative catalytic cysteine residue that is conserved in DJ-1 and PH1704 is also present in the two ThiJ-like domains of the cabbage protein (C107 and C313, respectively). In papain, an archetype of the cysteine protease family, the thiolate ion acts as an attacking nucleophile and is stabilized through the formation of an ion pair with neighboring imidazolium group of a histidine residue (Dardenne *et al.*, 2003). The putative histidine residue in the active site (H334) is also conserved in the C-terminal ThiJ-like domain, as in DJ-1. The catalytic histidine residue, however, is not evident in the N-terminal domain.

Expression patterns of the cabbage *thiJ*-like gene To examine whether the expression of the Chinese cabbage *thiJ*-like gene is induced by salicylic acid, a Chinese cabbage leaf was cut into 1 × 1 cm pieces and treated with a MOPS buffer containing 5 mM salicylic acid. As shown in Fig. 3, the salicylic acid treatment strongly induced the expression of the cabbage *thiJ*-like gene. The mock treatment without salicylic acid also induced the gene expression, indicating that the gene is inducible by wounding.

Our research then examined whether the gene would be induced by the stimuli that accompany hypersensitive

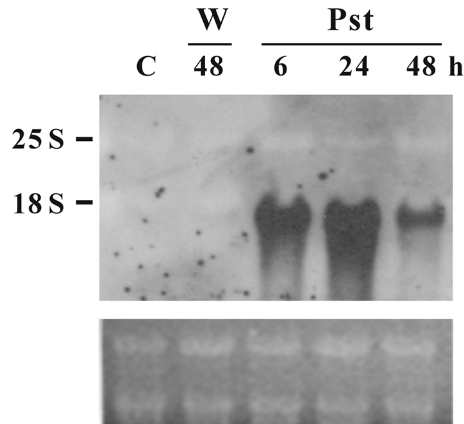

Fig. 4. Induction of the cabbage *thiJ*-like gene expression by pathogen. Chinese cabbage leaves were infiltrated with either sterile water (W) or *Pseudomonas syringae* pv. *tomato* (Pst). C and W48 represent the control leaf sample with no treatment and the leaf sample harvested 48 h after infiltration with sterile water, respectively. After the indicated time, the leaf samples were collected and analyzed by Northern blot hybridization (Fig. 3.)

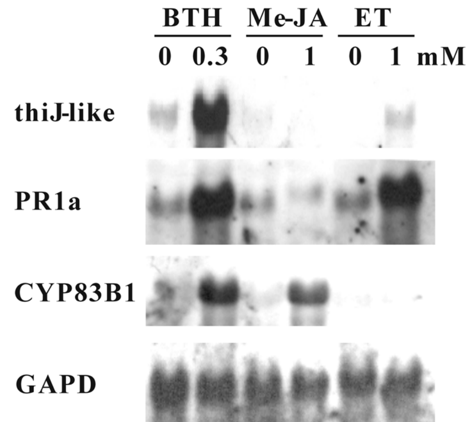

Fig. 5. Induction by BTH, methyl jasmonate, and ethylene. 0.3 mM of BTH, 1 mM methyl jasmonate (Me-JA) in 0.1% ethanol, 1 mM ethephon solution, or 0.1% ethanol and sterile water (control) was sprayed onto cabbage leaves. After 24 h, the leaf samples were collected and analyzed by Northern blot hybridization using DIG-labeled probes (Fig. 3). The transcript levels of the cabbage PR1a (CPL1) and CYP83B1 (CPE-T9) genes were assayed as positive controls for the induction by ethylene and Me-JA, respectively. As a loading control, the samples were also hybridized with the cDNA specific for the cabbage glyceraldehyde-3-phosphate dehydrogenase (GAPD).

response (HR), since HR is often a prerequisite for induction of disease resistance in plants. We used *Pseudomonas syringae* pv. *tomato* (*Pst*) to induce HR in Chinese cabbage. *Pst* causes bacterial speck disease in tomatoes and *Arabidopsis* (Bashan *et al.*, 1981; Whalen *et al.*, 1991), but not in Chinese cabbage (Ryang *et al.*, 2002). In the *Pst*-infiltrated cabbage leaves, visible necrosis was evident 24-36 h after inoculation. As shown in Fig. 4, the Northern analysis showed that the gene was strongly induced in the cabbage leaves that were infiltrated with *Pst*. In contrast, the induction of the gene expression was not observed in the control leaves that were infiltrated with water.

Chemicals other than salicylic acid can also activate defense reactions. One of the most potent SAR-inducing chemicals is benzothiadiazole (Goerlach *et al.*, 1996). To see if the cabbage *thiJ*-like gene is also induced by benzothiadiazole (BTH), the cabbage leaves were sprayed with 0.3 mM BTH and analyzed by Northern blot hybridization. The result showed that the cabbage gene was also strongly induced by BTH (Fig. 5). This confirms the result that was obtained with salicylic acid, since BTH is known to be a salicylic acid-mimicking chemical. We then examined the effect of other defense response activators, jasmonate and ethylene, since plant defense responses can also be activated *via* salicylic acid-independent signaling pathways that are mediated by jasmonates and ethylene (Piterse and van Loon, 1999). To see how the cabbage gene responds to ethylene or jasmonates, the cabbage leaves were treated with methyl jasmonate (Me-JA) or with ethephon, an ethylene-releasing compound. As shown in Fig. 5, the cabbage *thiJ*-like gene was not induced either by ethylene or by Me-JA.

To verify that appropriate induction conditions were used, we included as positive controls the expression of the Me-JA-inducible Chinese cabbage gene CPE-T9 and the ethylene-inducible gene CPL1, which were isolated in our laboratory as *Pst*-induced genes (Ryang *et al.*, 2002). The CPE-T9 gene encodes a putative CYP83B1, an oxime-metabolizing enzyme in the glucosinolate biosynthesis (Hansen *et al.*, 2001). The cabbage cytochrome P450 gene was induced by both BTH and Me-JA (Fig. 5). The CPL24-1 that was used as a positive control for induction by ethylene encodes a PR1a. Figure 5 shows that the cabbage PR1a gene is induced by both BTH and ethylene. These results, therefore, clearly show that the cabbage *thiJ*-like gene is induced by salicylic acid or BTH, but not by Me-JA or ethylene.

We then examined the tissue specificity of the gene expression. To this end, cabbage plants were induced to bolt. After flowering, the whole plants were treated with BTH by spraying (aerial parts) and soaking (roots) with a 0.3 mM BTH solution. As shown in Fig. 6, the gene was weakly expressed in all of the tissues that were examined without the BTH treatment. The result also showed that the strong induction by BTH occurred in the leaf, stem, and mature flower, but not in the root and flower bud.

Discussion

There have only been a few reports on thiamin biosynthesis in plants, except the report by Kim *et al.* (1998) that described a *Brassica napus* gene, which encodes a bifunctional enzyme

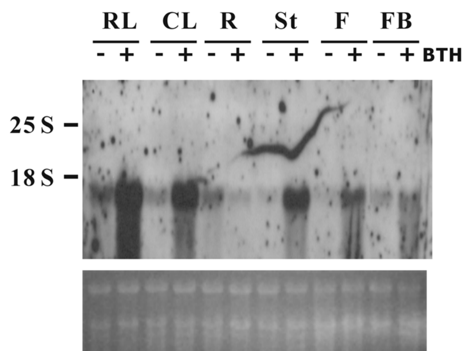

Fig. 6. Tissue-specific expression of the cabbage *thiJ*-like gene. Chinese cabbage plants were induced to bolt by vernalizing the seeds. The cabbage plants bearing flowers were treated with BTH by spraying (aerial parts) and soaking (roots) the plants with a 0.3 mM BTH solution. The control plants were similarly treated with sterile water. Rosette leaf (RL), cauline leaf (CL), root (R), stem (ST), mature flower (F), and flower bud (FB) tissues were collected from the plants and analyzed by Northern blot hybridization using DIG-labeled DNA probe (Fig. 3).

that has 2-methyl-4-amino-5-hydroxypyrimidine-monophosphate kinase (HMP-P kinase) and thiamine-phosphate pyrophosphorylase (TMP-PPase) activities. There has been no report on the HMP kinase (ThiJ) enzyme in plants. A BLAST search with the *E. coli* ThiJ as a query showed that the most homologous *Arabidopsis* protein to the *E. coli* ThiJ was a putative protein that was encoded by AT1g53280, which is 40% identical to the bacterial enzyme. This *Arabidopsis* protein is the same protein that shows a 79% identity with the cabbage ThiJ-like protein. The second most homologous *Arabidopsis* protein to the *E. coli* ThiJ was encoded by AT3g14990, which also displayed a 92% identity with the cabbage *thiJ*-like gene at the amino acid level. Therefore, there seems to be a good possibility that the cabbage gene that was isolated in this study represents the *thiJ* gene that encodes a HMP kinase.

Thiamin biosynthesis may occur in plastid. Consistent with this, the bifunctional protein that has HMP-P kinase and TMP-PPase activities has a transit peptide that directs it to chloroplast. To examine whether the cabbage ThiJ-like protein has the N-terminal transit peptide, a signal peptide prediction was performed using TargetP (Emanuelsson *et al.*, 2000). However, a transit peptide was not evident in the cabbage protein. The protein was predicted to have neither the signal peptide for secretory pathway nor the signal for mitochondrial transport. If this prediction is correct, then the result suggests that the cabbage protein is cytoplasmic, which is inconsistent with the role of the protein in thiamin biosynthesis. Thus, although the cabbage protein is homologous to the bacterial ThiJ, it is still unclear whether it is indeed a HMP kinase.

The cabbage protein shows similarities to the DJ-1 and the cysteine protease, PH1704. The fact may indicate a function that is different from the HMP kinase. Although the exact function is currently unknown, the information on structure and function of the multifunctional DJ-1 is intriguing. DJ-1 was originally reported to show cooperative transforming activity with H-ras (Nagakubo *et al.*, 1997). Later, DJ-1 was shown to be a multifunctional protein that plays essential roles in the testis and brain. DJ-1 was reported to be related to male fertility, and its level was reduced in rat sperm that was treated with sperm toxicants that cause infertility in rats (Wagenfeld *et al.*, 1998). It was also found that DJ-1 positively regulates the androgen receptor by impairing the binding of PIASxα to the receptor (Takahashi *et al.*, 2001). Additionally, a recent report showed that a mutation of DJ-1 is responsible for familial Parkinson's disease (Bonifati *et al.*, 2003). A recent structural analysis by Honbou *et al.* (2003) revealed that DJ-1 contains an additional α-helix at the C-terminal region, which blocks the putative catalytic site of DJ-1 and appears to regulate enzymatic activity. Considering that the DJ-1 expression is induced by oxidative stress, such as H_2O_2 or paraquat, the authors suggest that DJ-1 may induce conformational changes to acquire protease activity in response to oxidative stress.

It is interesting that DJ-1 is induced by oxidative stress (such as H_2O_2), since H_2O_2 is rapidly generated by NADPH oxidase at the site of pathogen infection in plants (Hammond-Kosack and Jones, 1996). Subsequently, salicylic acid and nitric oxide accumulate and synergistically act to induce localized cell death (hypersensitive response) and other defense responses (McDowell and Dangl, 2000). Thus, although it is still unclear whether the cabbage protein is functionally and structurally related to DJ-1, it is tempting to speculate that the protease activity of the cabbage ThiJ-like protein is activated by pathogen infection, possibly via salicylic acid, and regulates downstream defense responses. Recently, reports on the role of cysteine proteases in plant defense have accumulated. A cysteine protease was reported to be required for Cf-2-dependent disease resistance (Krueger *et al.*, 2002). Cysteine proteases and their proteinaceous inhibitors were also reported to regulate hypersensitive cell death (Belenghi *et al.*, 2003).

DJ-1 protein exists as a dimer (Honbou *et al.*, 2003). This dimer formation appears to be essential for the DJ-1 protein to properly function, since a DJ-1 mutation that is associated with human parkinsonism (Bonifati *et al.*, 2003) disrupts the hydrophobic interactions between monomers. Likewise, the PH1704 protease activity is exhibited only in the oligomeric forms of the protein (Du *et al.*, 2000). The dimeric or oligomeric nature of these proteins may answer the questions of why the cabbage gene has sequence duplication. It remains to be elucidated whether the two ThiJ-like domains are functionally related to DH-1 or ThiJ. Whatever the function is, it will be interesting to see how the N-terminal and C-terminal domains of the cabbage protein interact with each other to form an active protein.

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