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H. 知的財産権の出願・登録状況

1. 特許取得

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2. 実用新案登録
　なし

3. その他
なし。

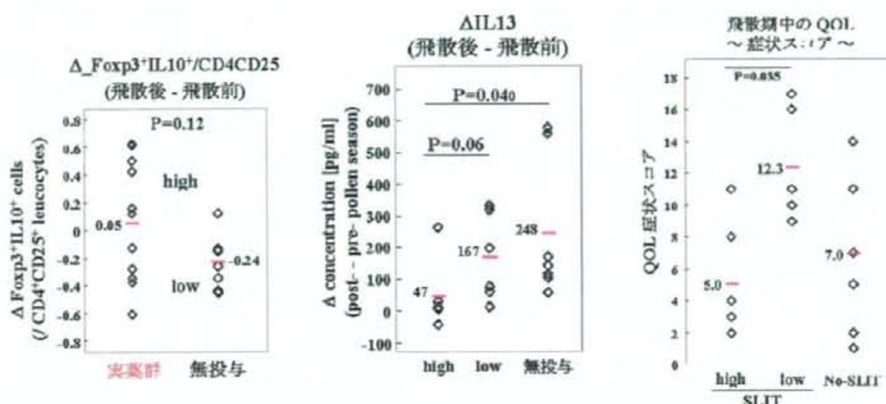

図１　オープン試験、実薬投与群、無投与群由来 PBMC をスギ花粉抗原刺激後の iTreg の花粉飛散前後の割合の差、iTreg により階層化後の IL13 産生量の花粉飛散前後での差、および花粉飛散中における QOL 症状スコア

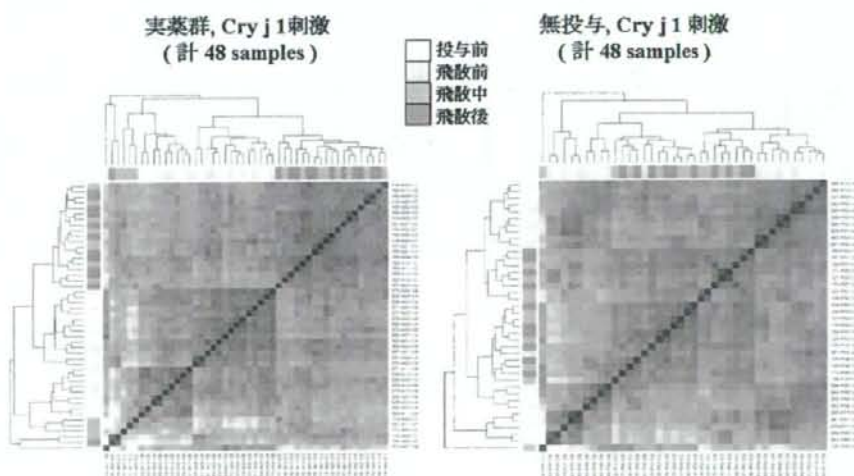

図２ 実薬投与群、無投与群由来の CD4 陽性細胞をスギ花粉抗原刺激後の遺伝子発現
ヒートマップ中、□→■に従い、相関係数が高いことを示している。

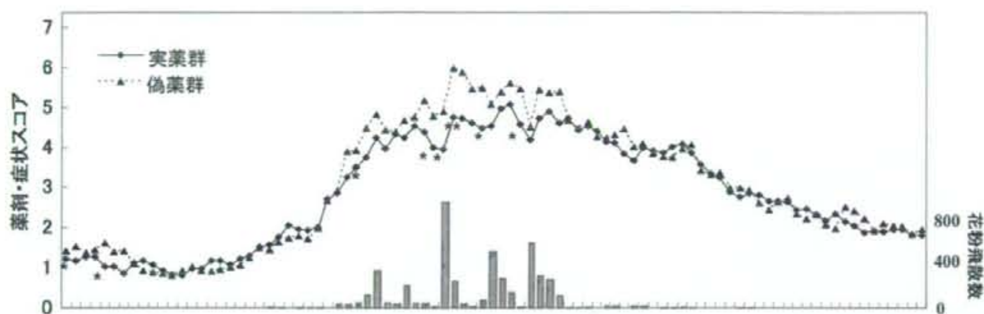

図３　2008年スギ花粉飛散期中の薬剤・症状スコア（Symptom-medication score）
花粉飛散量を棒グラフで示している。　*: P<0.05 vs 偽薬群

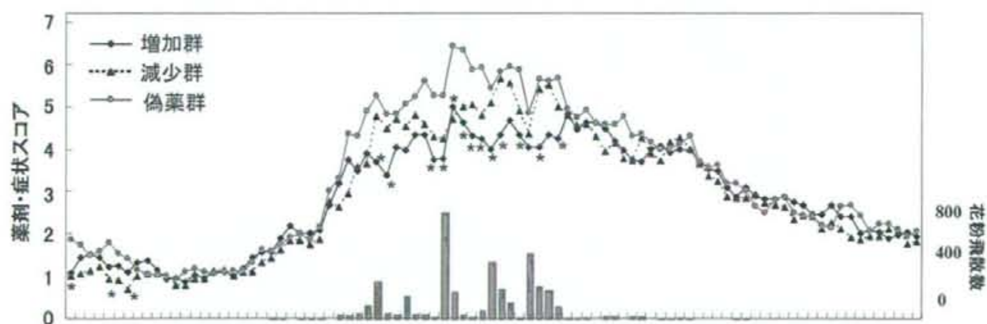

図４　iTregによる階層化後の2008年スギ花粉飛散期中の薬剤・症状スコア
花粉飛散量を棒グラフで示している。　*: P<0.05 vs 偽薬群

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Characterization of genes for novel thaumatin-like proteins in *Cryptomeria japonica*

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Summary Thaumatin-like proteins (TLPs) are induced by a variety of phytopathogens in many plants and several TLPs are allergenic. Previously, we isolated three TLP-encoding cDNAs (*Cry j 3.1*, *Cry j 3.2* and *Cry j 3.3*) from a cDNA library derived from the pollen of *Cryptomeria japonica* D. Don. Here, we describe three new TLP cDNAs (*Cry j 3.4*, *Cry j 3.5* and *Cry j 3.6*). We compared the sequences, the genetic map location and the expression patterns of the *Cry j 3* genes. The amino acid sequence predicted from *Cry j 3.5* exhibits only limited similarity to those predicted from the other *Cry j 3* genes. Linkage analysis showed that the *Cry j 3.1* to *Cry j 3.4* genes are located in the same linkage group, but *Cry j 3.5* is located in a different group. Organ-specificity and induction by stresses and plant hormones differed among the *Cry j 3* mRNAs. In pollen grains, the *Cry j 3.5* mRNA expression level was higher than that of the other *Cry j 3* genes. Exposure to UV-B and salt stress induced expression of *Cry j 3.1*. The ethylene-releasing compound ethephon strongly induced expression of *Cry j 3.4*. Salt stress and salicylic acid also induced expression of *Cry j 3.4*. Abscisic acid weakly induced expression of *Cry j 3.5*. Arachidonic acid strongly induced expression of *Cry j 3.4* and *Cry j 3.6*, and weakly induced that of *Cry j 3.3*, whereas expression of *Cry j 3.1* and *Cry j 3.5* was unaffected. These results suggest that the roles of TLPs and the cascades that regulate their expression differ among the members of the TLP family in *C. japonica*.

Keywords: gene expression, pollen, PR-5 protein, stress response, sugi (Cryptomeria japonica D. Don).

Introduction

Pathogenesis-related (PR) proteins are members of a large group of diverse plant-defense proteins whose expression is induced in response to microbial attack, either through signaling pathways involving diverse receptors or by chemical elicitors such as salicylic acid (SA), methyl jasmonate (MJ) and ethylene (Kitajima and Sato 1999). Synthesis of PR proteins is considered to be part of an active, general defense response in plant systems. The PR proteins have been grouped into families on the basis of amino acid sequence similarity, serological relationships and biochemical properties (van Loon et al. 1994, van Loon and van Strien 1999).

The family of PR-5 proteins contains proteins with diverse functions. Because of the sequence homology between PR-5 proteins and thaumatin, an intensely sweet-tasting protein isolated from the fruit of the West African rainforest shrub *Thaumatococcus daniellii* (Bennett) Benth. (Iyengar et al. 1979), members of this family of proteins are referred to as thaumatin-like proteins (TLPs). This family includes proteins known as osmotins (Singh et al. 1987) and permatins (Vigers et al. 1991). The TLPs play a variety of roles in the development of seed, fruit and flower tissues, and specific TLPs have been shown to protect plants against osmotic stress, pathogen attack and the deleterious effects of freezing (Velazhahan et al. 1999, An lovar and Dermastia 2003). The expression of some TLPs is induced by biotic and abiotic factors, such as microbial infection, osmotic stress, abscisic acid (ABA), ethylene, SA, MJ and elicitors (Kitajima and Sato 1999, Velazhahan et al. 1999).

Purified TLPs from several plant species have in vitro antifungal activity. In addition to inhibiting hyphal growth and spore germination, some TLPs greatly increase the permeability of fungal and oomycete plasma membranes (Kitajima and Sato 1999, Yun et al. 1997). Osmotin subverts signal transduction in fungal cells to enhance its cytotoxic effects (Yun et al. 1998) and induces microbial apoptosis (Narasimhan et al. 2001). Moreover, not only are TLPs effective against a broad range of fungal species, including several plant pathogens, but they also exhibit specificity toward certain fungi (Yun et al. 1997). Overexpression of TLPs appears to enhance resistance to fungal disease (Liu et al. 1994, Zhu et al. 1996, Chen et al.

1999, Datta et al. 1999), but conflicting results have been reported (Anand et al. 2003).

Several TLPs in various plant-derived foods (Hoffmann-Sommergruber 2002) and conifer pollen (Midoro-Horiuti et al. 2000, Cortegano et al. 2004) are allergens. Sugi (*Cryptomeria japonica* D. Don (Taxodiaceae)) is one of the most commercially important conifers in Japan and sugi pollinosis is one of the country's most serious allergic diseases. Two major allergens, the Cry j 1 and Cry j 2 proteins, have been identified in *C. japonica* pollen (Yasueda et al. 1983, Sakaguchi et al. 1990). Previously, we isolated three other TLP cDNAs to identify additional allergens present in *C. japonica* (Futamura et al. 2002*b*); however, their gene expression levels were low in pollen grains, indicating that their role in sugi pollinosis is likely to be minimal. In the present study, we attempted to isolate TLP cDNAs that are expressed at high levels in sugi pollen.

Materials and methods

Plant materials and stress treatments

All plant materials, with the exception of seeds, have been described previously (Futamura et al. 2002*b*). The seeds were collected from *C. japonica* that had been planted in Satomi, Ibaraki prefecture, Japan. The seeds were kept overnight at room temperature between wet paper towels prior to RNA extraction.

All experiments were conducted in a chamber set at 23 °C with a 16-h photoperiod provided by cool white fluorescent lights (80 μmol m^{-2} s^{-1}). The treated seedlings were stored at −80 °C until analyzed. One-month-old seedlings were pretreated for 24 h in dishes of water and then subjected to various stresses for the following 24 h. Seedlings were exposed to UV-B (280–320 nm) at an irradiance of 0.13 μmol m^{-2} s^{-1} during daylight hours. Seedlings exposed to salt stress were treated with an aqueous solution of 200 mM NaCl. For plant hormone and elictor treatments, seedlings were placed in an aqueous solution of each reagent. Control seedlings were treated with water only and were collected at the same times as other samples.

Salicylic acid, MJ, ABA, ethephon and cellulase (derived from *Trichoderma viride* Persoon) were purchased from Wako Pure Chemical Industries (Osaka, Japan); 3-amino-1,2,4- triazole (3-AT) was purchased from Nacalai Tesque (Kyoto, Japan); and arachidonic acid (AA) was purchased from ICN Pharmaceuticals (Costa Mesa, CA).

Isolation of RNA and DNA

Total RNA was isolated from mature pollen grains as described by Sone et al. (1994) and from other plant organs as described by Shinohara and Murakami (1996). Genomic DNA was extracted from current-year needles as described by Wagner et al. (1987).

Isolation of clones and sequence analysis

A cDNA library in λZAPII (Stratagene, La Jolla, CA), consisting of 8.5 × 10^5 recombinants, was constructed from 5 μg of polyadenylated mRNA that had been isolated from *C. japonica* pollen. The cDNAs from recombinant phages were excised in vivo in plasmid form (pBluescript SK(−); Stratagene) by co-infection with ExAssist helper phage, according to the manufacturer's instructions. For identification of novel TLPs, we first identified Jun a 3-like expression sequence tags (ESTs) from *C. japonica* (Ujino-Ihara et al. 2000). Jun a 3 is a TLP allergen in the pollen of *Juniperus ashei* Buchh. (Midoro-Horiuti et al. 2000). Fourteen ESTs that exhibited strong similarities to Jun a 3 were subdivided into six groups. Several primers were constructed from the EST sequences and polymerase chain reaction (PCR) was performed with genomic DNA or cDNA excised from the library as a template. Three primer and template combinations successfully generated amplified products (Table 1). We performed PCR with KOD-plus-DNA polymerase (Toyobo, Osaka, Japan), and cloned the products into pBluescript II SK(−). The identities of the PCR products were determined by DNA sequencing. To obtain cDNA clones that contained entire open reading frames, we screened a cDNA library in λZAPII using the cloned PCR products as probes. The DNA probes were labeled with [α-^{32}P]dCTP with the Megaprime DNA labeling system (Amersham Biosciences, Piscataway, NJ). Hybridization was performed as described previously (Futamura et al. 2000). The cDNAs from recombinant phages were excised in vivo in plasmid form, as described previously. The sequences of both strands of the cDNA insert were determined.

DNA and RNA gel blot analysis

To label full-length cDNA, we labeled each cDNA insert with [α-^{32}P]dCTP using the Megaprime DNA labeling system ac-

Table 1. PCR products and the primers for cDNA cloning.

PCR Product		Sequence of primer (5′ to 3′)	Source of primer	Template for PCR
Cry j 3.4	Forward	TCTAGAACTAGTGGATCCCCCG	pBluescript	cDNA library
	Reverse	TCCCTATTCGACTGCATAAAGAC	AU085152	cDNA library
Cry j 3.5	Forward	TGGGGGCGGACGGACTG	AU084853	cDNA library
	Reverse	CGGCAGAACAAGTGTAAAGACTGG	AU084853	cDNA library
Cry j 3.6	Forward	GATATATGGAGACTACTGCCATTC	BP176369	Genomic DNA
	Reverse	GCCTTGGCAGTTGAGCGTG	BP176369	Genomic DNA

cording to the manufacturer's instructions. To label the 3′-UTR (untranslated region) of *Cry j 3.1*, *Cry j 3.3* and *Cry j 3.4*, we amplified the 3′-UTR of each cDNA by PCR. The sense primer (5′-CTTTTAACTGCATCTCCTTTGTG-3′) and the antisense primer (5′-GTTTCCTGACTTGACAT CAG-3′) were used for amplification of the 3′-UTR of *Cry j 3.1*. The sense primer (5′-CACTCCATTTCCTTGTCTC TATG-3′) and the antisense primer (5′-CATGATGCGGAG CACAGAG-3′) were used for amplification of the 3′-UTR of *Cry j 3.3*. The sense primer (5′-CATAAATTCAAAGTGCCC CGGA-3′) and the antisense primer (5′-AGAAAATGGAT ACAGAGCAATATAC-3′) were used for amplification of the 3′-UTR of *Cry j 3.4*. Each PCR product was cloned into pBluescript II SK(–). The inserted DNA was amplified by PCR with modified SK (5′-TCTAGAACTAGTGGATCCC CG-3′) and T7 (5′-TAATACGACTCACTATAGGGCGA-3′) primers. Each PCR product was labeled with the Megaprime DNA labeling system using two vector-specific 9-mer primers (5′-GA TCCCCCG-3′ and 5′-ACGGTATCG-3′).

For DNA gel blot analysis, genomic DNA (13 μg) was digested with restriction endonucleases, fractionated electrophoretically on a 0.7% agarose gel and transferred to a nylon membrane (Biodyne B, Pall, East Hills, NY) by alkaline transfer. The DNA gel blot hybridization was performed as described previously (Futamura et al. 2002*a*).

For RNA gel blot analysis, total RNA (5 or 10 μg) was fractionated by electrophoresis on a formaldehyde-agarose gel and blotted onto a nylon membrane (Biodyne A; Pall) by capillary transfer. The RNA gel blot hybridization was performed as described previously (Futamura et al. 2002*a*). The membranes were exposed to X-ray film and the autoradiogram signals quantified with a Molecular Imager (Bio-Rad, Hercules, CA).

Linkage analysis

The YI pedigree, namely, 150 full-sibs of F_2 plants derived from sib-crosses of two F_1 plants (YI96 and YI38) from a cross between Yabukuguri and Iwao, was used for linkage analysis (Tani et al. 2003). We used PCR and restriction fragment length polymorphisms (RFLPs) to identify the linkage map positions of *Cry j 3.1* through *Cry j 3.6*. Primers specific for PCR amplification of *Cry j 3.2* were used to identify the gene map position. The sense primer (5′-GACATTAAT TCCAAGT GTCCTTCT-3′) and the antisense primer (5′-CAAAGAAG GGGAGTTAAAGTTAGC-3′) were based on the *Cry j 3.2* cDNA sequence. The linkage map positions of *Cry j 3.1*, *Cry j 3.3*, *Cry j 3.4* and *Cry j 3.5* were identified by RFLP analysis. The 3′-UTR of *Cry j 3.1* and the full-length cDNAs corresponding to *Cry j 3.3*, *Cry j 3.4* and *Cry j 3.5* were used as probes. These probes were used in combination with six restriction enzymes (*Bgl* II, *Dra* I, *Eco*R I, *Eco*R V, *Hae* III and *Hind* III). The *Hae* III-, *Dra* I-, *Dra* I- and *Bgl* II-digested genomic DNA was used for the analysis of *Cry j 3.1*, *Cry j 3.3*, *Cry j 3.4* and *Cry j 3.5*, respectively. The *Cry j 3* genes were genetically mapped on an existing *C. japonica* linkage map (Tani et al. 2003) with JoinMap 3.0 (Van Ooijen and Voorrips 2001).

Results

Isolation and characterization of three novel cDNAs that encode TLPs in C. japonica

We isolated three cDNAs corresponding to genes (*Cry j 3.4*, *Cry j 3.5* and *Cry j 3.6*) from a pollen-specific cDNA library of *C. japonica* with PCR products that encoded TLPs as probes (Table 1). The *Cry j 3.4* and *Cry j 3.5* cDNAs contained full-length coding regions. The polypeptide predicted from the sequence of *Cry j 3.6* cDNA appeared to include only part of the signal peptide, but it included the entire mature protein (Table 2). The predicted amino acid sequences of these three Cry j 3 proteins included thaumatin domains and the 16 Cys residues that are conserved in other TLPs (Ogata et al. 1992, Batalia et al. 1996). Comparison with other TLPs suggested that 25, 22, and 24 amino acids at the amino termini of Cry j 3.4, Cry j 3.5 and Cry j 3.6, respectively, constitute signal peptides (Figure 1). The structures of the signal sequences are consistent with the (–3, –1) rule (von Heijne 1986) and with the pattern of information for a cleavage site (Nielsen et al. 1997). The predicted Cry j 3.4, Cry j 3.5 and Cry j 3.6 peptides also included additional carboxy-terminal regions of 20, 2 and 18 amino acid residues, respectively, unlike Cry j 3.1, Cry j 3.2 and Cry j 3.3 of *C. japonica* (Figure 1). We occasionally found three nucleotide changes in the 3′-UTR of *Cry j 3.4* cDNA; and we found one deletion and one nucleotide change in the 5′-UTR of *Cry j 3.5* cDNA, as well as six nucleotide changes in the coding region and eight nucleotide changes and one deletion in the 3′-UTR. Three of the six nucleotide substitutions in the coding region of *Cry j 3.5* resulted in a change in an amino acid residue. These nucleotide changes are assumed to be polymorphisms among individuals, because we isolated cDNAs from a cDNA library derived from several clones. No substitutions or deletions were detected in *Cry j 3.6* cDNA.

Pairwise comparisons of the predicted amino acid sequences of the Cry j 3 proteins, including previously reported sequences, revealed that the sequences are from 37 to 92% homologous. Three previously reported Cry j 3 proteins (Cry j 3.1, Cry j 3.2 and Cry j 3.3) are more than 85% homologous, whereas Cry j 3.4, Cry j 3.5 and Cry j 3.6 are from 37 to 51% homologous to one another. In particular, the predicted amino acid sequence of Cry j 3.5 exhibits only limited homology (37 to 41%) to those of other Cry j 3 proteins. The phylogenetic tree constructed from an alignment of the thaumatin domains indicated that the Cry j 3 proteins, with the exception of Cry j 3.5, form a group with other PR-5 proteins from gymnosperms. This group is distinct from the group that includes Cry j 3.5 (Figure 2).

Distribution of Cry j 3.4, Cry j 3.5 and Cry j 3.6 genes in the C. japonica genome

We performed genomic DNA gel blot analysis to determine the distribution of *Cry j 3.4*, *Cry j 3.5* and *Cry j 3.6* in the *C. japonica* genome (Figure 3). The hybridization patterns of *Cry j 3.4*, *Cry j 3.5* and *Cry j 3.6* differed from one another. When we used entire cDNAs as probes, we obtained more than four signals corresponding to *Cry j 3.4*, and one to three signals corre-

Table 2. Major cDNA features of thaumatin-like proteins (TLPs) (*Cry j 3.1* through *Cry j 3.6*) derived from *C. japonica*, the cDNA for *Jun a 3* of *Juniperus ashei* and the corresponding deduced amino acid sequences. Abbreviation: ORF = open reading frame.

cDNA	Accession no.	ORF length (bp)	Predicted mature protein length (no. amino acids)	Predicted molecular mass of mature protein (kDa)	Predicted pI of mature protein	Predicted features
Cry j 3.1[1]	AB081303	699	206[4]	21.7	7.95	Signal sequence, thaumatin domain, one site of N-glycosylation
Cry j 3.2[1]	AB081304	702	206[4]	21.9	7.94	Signal sequence, thaumatin domain, one site of N-glycosylation
Cry j 3.3[1]	AB081305	693[3]	206[4]	21.9	4.47	Signal sequence, thaumatin domain
Cry j 3.4	AB186384	747	223[4] (205)[5]	23.9 (22.0)[5]	5.31 (7.12)[5]	Signal sequence, thaumatin domain
Cry j 3.5	AB186385	723	218[4]	22.4	4.38	Signal sequence, thaumatin domain
Cry j 3.6	AB186386	738[3]	221[4] (205)[5]	23.9 (22.0)[5]	4.65 (4.88)[5]	Signal sequence, thaumatin domain, two sites of N-glycosylation
Jun a 3[2]	AF121776	678	199[2]	21.0	4.63	Signal sequence, thaumatin domain, one site of N-glycosylation

[1] Futamura et al. 2002*b*.
[2] Midoro-Horiuti et al. 2000.
[3] The first part of the signal sequence is missing.
[4] Signal peptides were estimated from a comparison to other TLPs.
[5] After cleavage of the carboxy-terminal region, in the case of AP24 (Melchers et al. 1993).

sponding to *Cry j 3.5* and *Cry j 3.6*. These results indicate that the Cry j 3.4 protein is encoded by several genes, whereas the Cry j 3.5 and Cry j 3.6 proteins are encoded by a single gene or a few identical genes. When we used the 3′-UTR of *Cry j 3.4* as the probe, we detected two or three signals. The number and intensity of signals indicate that some members of the *Cry j 3.4* family have strong homology in coded regions, whereas the homology among UTRs is weaker.

Linkage analysis of Cry j 3.1 through Cry j 3.5

To locate the *Cry j 3* genes on the linkage map of *C. japonica*, we tried to detect RFLPs with *Cry j 3.1* through *Cry j 3.6* as probes. *Cry j 3.1*, *Cry j 3.3*, *Cry j 3.4* and *Cry j 3.5* yielded RFLPs between two parental lines after hybridization with genomic DNA that had been digested with *Hae* III, *Dra* I, *Dra* I and *Bgl* II, respectively (Figure 4A). We used the 3′-UTR of *Cry j 3.1* as a probe to prevent cross-hybridization with *Cry j 3.3*. Hybridization of full-length *Cry j 3.3* cDNA to genomic DNA that had been digested with *Hae* III did not yield RFLPs similar to those obtained with the 3′-UTR of *Cry j 3.1* (data not shown). When we used the 3′-UTR of *Cry j 3.4* as a probe, we detected polymorphic *Dra* I fragments similar to those detected with the full-length cDNA (data not shown). These observations indicate that the RFLPs shown in Figure 3A were specific to each gene. Furthermore, PCR performed with primers specific for *Cry j 3.2* yielded a positive band in the case of the YI96 cultivar and no band in the case of the YI38 cultivar (Figure 4B). No RFLPs and no PCR polymorphisms were detected when we used *Cry j 3.6* cDNA.

We exploited the various polymorphisms for mapping, using the YI pedigree (Tani et al. 2003). Figure 5 shows the locations of *Cry j 3.1* through *Cry j 3.5* on the linkage map of *C. japonica*. The markers associated with *Cry j 3.1*, *Cry j 3.2* and *Cry j 3.3* were clustered at the bottom of YI3. A RFLP marker associated with *Cry j 3.4* cDNA was also located on YI3. By contrast, a RFLP marker associated with *Cry j 3.5* was located on YI10.

Organ-specific expression of Cry j 3 genes

Figure 6 shows the results of RNA gel blotting of total RNA

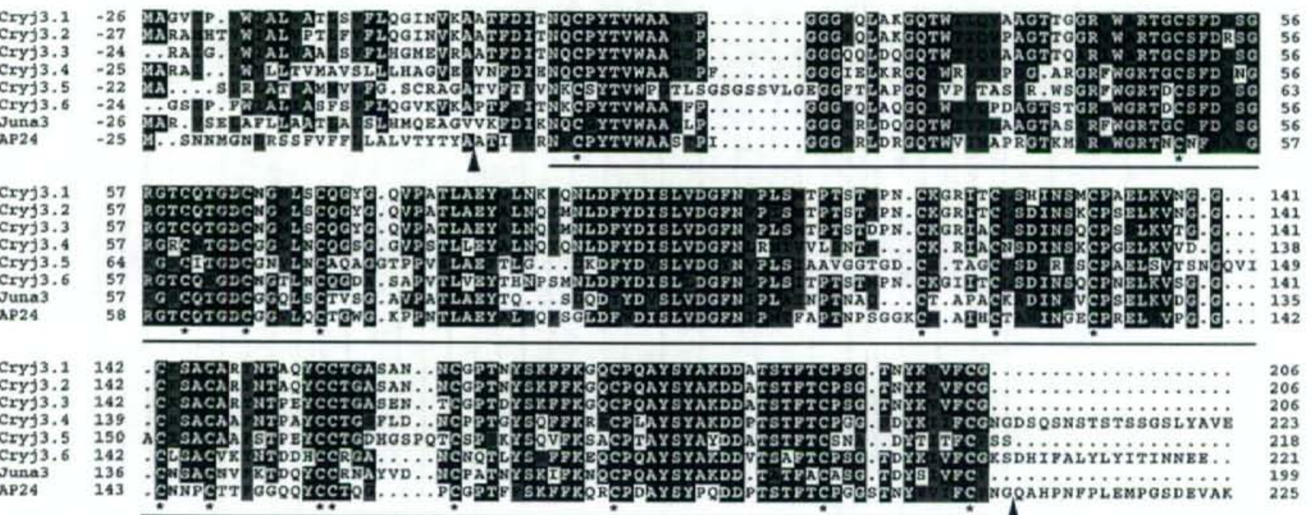

Figure 1. Alignment of the deduced amino acid sequences of Cry j 3.1 through Cry j 3.6 proteins with those of Jun a 3 from *Juniperus ashei* (Midoro-Horiuti et al. 2000) and AP24 from tobacco (Melchers et al. 1993). Sequence alignments were performed with CLUSTAL X (Thompson et al. 1997). Amino acids in black and gray boxes are identical and similar, respectively, in at least five of the sequences. Processing sites in Jun a 3 and AP24 are indicated by arrowheads. Asterisks indicate the 16 Cys residues that are conserved in the pathogenesis-related (PR-5) proteins. The thaumatin domain indicated in the Pfam database (http://www.sanger.ac.uk/Software/Pfam/) is underlined.

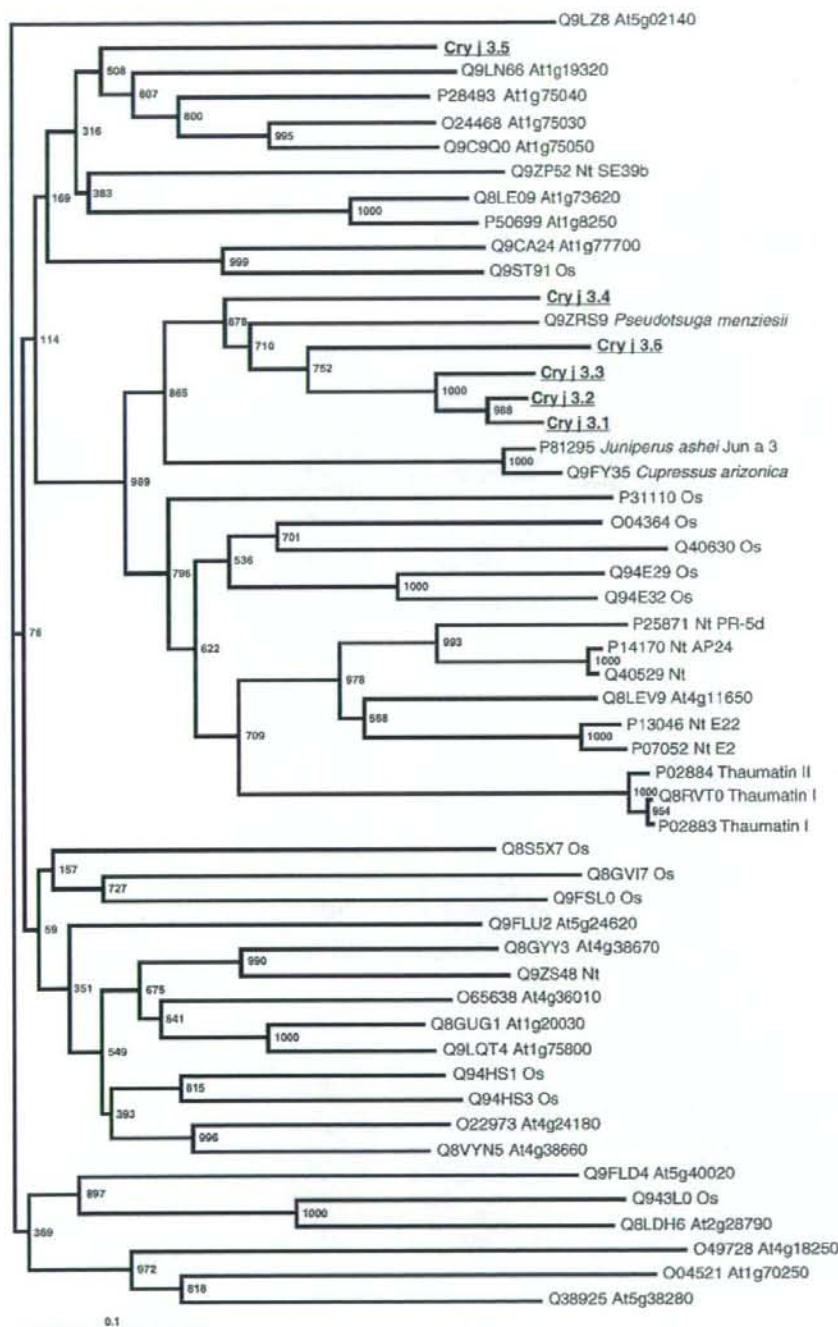

Figure 2. A phylogenetic tree based on thaumatin motifs. The thaumatin domains of thaumatin and thaumatin-like proteins (TLPs) were aligned with Clustal X (Thompson et al. 1997) and gaps were removed from the alignment. The unrooted tree was calculated by the neighbor-joining method (Saitou and Nei 1987) and bootstrap values from 1000 resamplings are indicated at the branches. The bars indicate genetic distances (percent differences) among sequences. The designation in the Swiss-Prot/TrEMBL database is given for all polypeptides except those from *C. japonica*. The TLPs from *Arabidopsis* are indicated by locus names. The TLPs from *Nicotiana tabacum* and *Oryza sativa* are indicated by Nt and Os, respectively. The TLPs from *C. japonica* are underlined and presented in boldface.

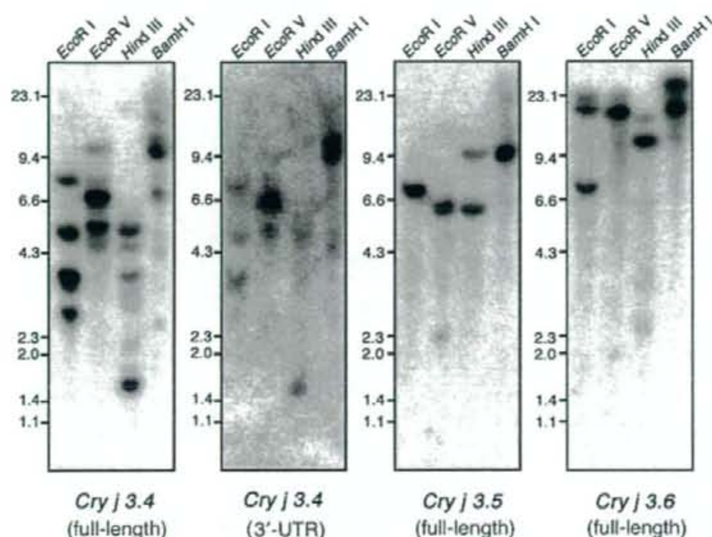

Figure 3. Genomic DNA gel blotting of *Cry j 3.4*, *Cry j 3.5* and *Cry j 3.6* in *C. japonica*. Genomic DNA was digested separately with *Eco*R I, *Eco*R V, *Hin*d III and *Bam*H I and then subjected to electrophoresis on a 0.7% agarose gel, transferred to a nylon membrane and allowed to hybridize with ^{32}P-labeled probes prepared from *Cry j 3.4*, *Cry j 3.5* and *Cry j 3.6* (as indicated below each blot). The positions of size markers are shown on the left of each panel in kilobase pairs. Abbreviation: UTR = untranslated region.

extracted from various organs *C. japonica*. In a previous study, we detected the expression of *Cry j 3* genes using the entire cDNA of the *Cry j 3.1* gene as probe (Futamura et al. 2002*b*); this probe cross-hybridized with the *Cry j 3.1*, *Cry j 3.2* and *Cry j 3.3* genes. In the present study, we used the 3′-UTRs of *Cry j 3.1* and *Cry j 3.3* as specific probes. Because *Cry j 3.2* was not detected in the genome of some cultivars, such as Iwao and YI38, we did not use *Cry j 3.2* cDNA as a probe. Our analysis revealed differences in expression patterns between *Cry j 3.1* and *Cry j 3.3*. The transcript of *Cry j 3.1* was strongly expressed in mature male and female strobili and in developing female strobili, whereas *Cry j 3.3* mRNA was most abundant in mature male strobili, with strong expression in mature female strobili and no expression in developing female strobili. Significant expression of *Cry j 3.1* was detected in the stem, the root and developing male strobili. By contrast, no transcripts of *Cry j 3.3* were detectable in these organs. Low- level expression of *Cry j 3.3* was detected in seeds but no expression of *Cry j 3.1* was detected.

Cry j 3.4 mRNA was most abundant in mature female strobili, with low-level expression in roots of seedlings and saplings, in developing female strobili and in mature male strobili. *Cry j 3.5* mRNA was most abundant in pollen but weak expression was also detected in seedling roots, in sapling stems and in developing male strobili. *Cry j 3.6* mRNA was most abundant in mature male strobili and it was also found at high levels in mature and developing female strobili. Low-level expression of *Cry j 3.6* was also detected in roots of seedlings and saplings.

Effects of various types of stress and plant hormones on the expression of Cry j 3 genes

Expression of genes for TLPs is regulated by many hormonal and environmental signals, including UV light, NaCl, reactive oxygen species (ROS), SA, ABA, ethylene and MJ (Yun et al. 1997, Surplus et al. 1998, Velazhahan et al. 1999). We subjected seedlings of *C. japonica* to these stresses and to plant hormones to determine if they induced expression of *Cry j 3* genes (Figure 7). Ethephon is an ethylene-releasing compound and 3-AT is known as a generator of ROS that induces formation of hydrogen peroxide through the inhibition of catalase (Chen et al. 1993). Exposure to UV light, NaCl stress and an oxidant led to increases in *Cry j 3.1* mRNA levels. The expression of *Cry j 3.4* mRNA was induced by NaCl stress, SA and ethephon. Abscisic acid induced a doubling of *Cry j 3.5* mRNA. Methyl jasmonate did not have a marked effect on the expression of any of the *Cry j 3* genes.

Effects of elicitors on the expression of Cry j 3 genes

In a previous study, AA, which is a fatty-acid fungal elicitor, and a preparation of cellulase from *Aspergillus niger*, which is a protein-type fungal elicitor, induced the expression of the gene for osmotin in tobacco (Chang et al. 1995). To examine the effects of AA and cellulase on the levels of *Cry j 3* transcripts, we exposed seedlings of *C. japonica* to AA, cellulase or a combination of the two (Figure 8). Cellulase (at 250 mg l^{-1}) and 0.3 mM AA did not damage seedlings but seedlings subjected to 3 mM AA were clearly damaged after 24 h. Cellulase by itself had little effect on transcript levels of any *Cry j 3* genes; however, expression of *Cry j 3.1* was induced after seedling exposure to a combination of cellulase and 0.3 mM AA. Expression of *Cry j 3.3*, *Cry j 3.4* and *Cry j 3.6* increased after treatment of seedlings with 3 mM AA or with the 3 mM AA plus cellulase combination.

Discussion

This report describes the structural characteristics of three

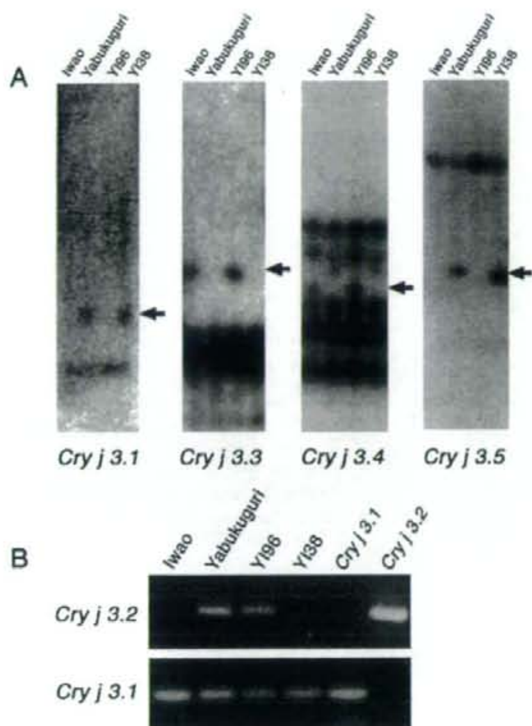

Figure 4. Detection of polymorphisms in *Cry j 3.1*, *Cry j 3.2*, *Cry j 3.3*, *Cry j 3.4* and *Cry j 3.5* genes. (A) Restriction fragment length polymorphisms (RFLPs) of *Cry j 3.1*, *Cry j 3.3*, *Cry j 3.4* and *Cry j 3.5*. Genomic DNA was digested with *Hae* III, *Dra* I, *Dra* I and *Bgl* II to detect RFLPs of *Cry j 3.1*, *Cry j 3.3*, *Cry j 3.4* and *Cry j 3.5*, respectively. The RFLP bands are indicated by arrows. YI96 and YI38 are F_1 plants of crosses between the Yabukuguri and Iwao cultivars. (B) Confirmation of results for *Cry j 3.2* by PCR. Primers specific for *Cry j 3.2* were used to detect *Cry j 3.2*, and primers specific for *Cry j 3.1* were used as controls. pBluescript SK(–) containing *Cry j 3.1* cDNA and pBluescript SK(–) containing *Cry j 3.2* cDNA were used as templates to confirm primer specificity. The YI pedigree, namely, 150 full-sibs of F_2 plants derived from sib-crosses of YI96 and YI38, was used for linkage analysis.

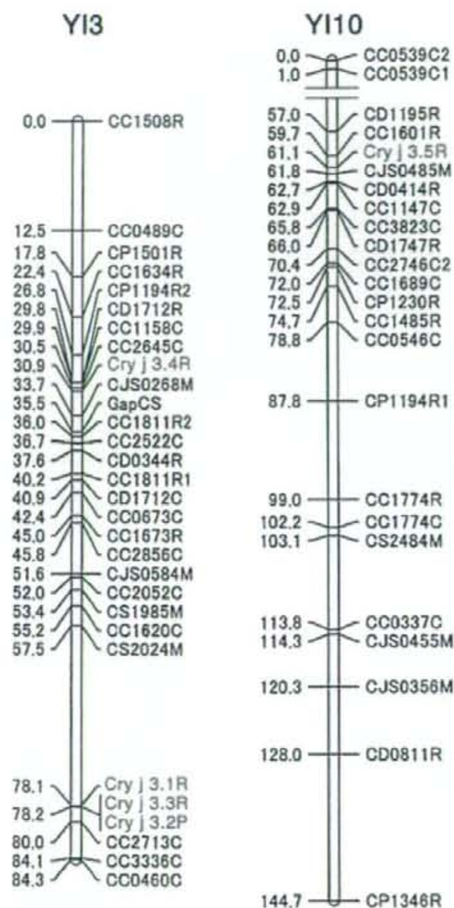

Figure 5. Location of *Cry j 3.1* through *Cry j 3.5* genes on the linkage map of *C. japonica*. The diagrams show the relevant portions of the consensus linkage map of *C. japonica*, as constructed by Tani et al. (2003). The restriction fragment length polymorphism (RFLP) markers (R) associated with *Cry j 3.1*, *Cry j 3.3* and *Cry j 3.4* and the polymerase chain reaction markers (P) associated with *Cry j 3.2* were located on YI3. One RFLP marker associated with *Cry j 3.5* was placed on YI10. The markers are indicated in red.

novel TLPs, namely, Cry j 3.4, Cry j 3.5 and Cry j 3.6, and the patterns of expression of the corresponding genes, as well as those of *Cry j 3.1* and *Cry j 3.3* (Futamura et al. 2002*b*). Alignment of deduced amino acid sequences showed that each Cry j 3 protein includes a thaumatin domain and 16 conserved Cys residues (Figure 1). These residues form eight disulfide bridges that stabilize the respective proteins and contribute to the conformational similarities among TLPs (Ogata et al. 1992, Batalia et al. 1996). The presence of a thaumatin domain and the conserved Cys residues in the Cry j 3 proteins confirms that these proteins are TLPs.

Pathogenesis-related proteins are generally classified in two subclasses. One subclass consists of acidic proteins that are usually secreted into the extracellular space, and the other subclass consists of basic proteins that are usually transported to vacuoles (Kitajima and Sato 1999). However, apoplastic basic TLPs have been identified in barley and chestnut (Trudel et al. 1998, Garcia-Casado et al. 2000), and some vacuolar TLPs have a neutral isoelectric point (Kitajima and Sato 1999). Signal sequences at the carboxy-terminal end are diagnostic for vacuolar TLPs (Melchers et al. 1993). Both Cry j 3.1 and Cry j 3.2 are basic proteins but lack an additional carboxy-terminal sequence. By contrast, Cry j 3.4 and Cry j 3.6 are acidic proteins and have a carboxy-terminal region of similar length to that of AP24 (Melchers et al. 1993). The presence of a carboxy-terminal region suggests that Cry j 3.4 and Cry j 3.6

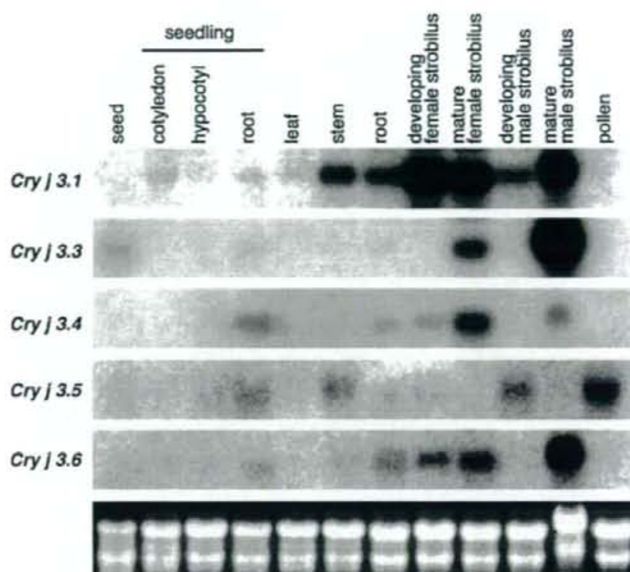

Figure 6. Upper panels: RNA gel blot analysis of transcripts of *Cry j 3* genes in various organs of *C. japonica*. Ten μg of total RNA was loaded in each lane of a 1% agarose formaldehyde-containing gel. After electrophoresis, the RNA bands were transferred to a nylon membrane and allowed to hybridize with ^{32}P-labeled 3′-UTR cDNAs for *Cry j 3.1*, *Cry j 3.3* and *Cry j 3.4* and with entire cDNAs for *Cry j 3.5* and *Cry j 3.6*. Lowest panel: gel stained with ethidium bromide, before blotting, to confirm that amounts of RNA in the various lanes were equal. Abbreviation: UTR = untranslated region.

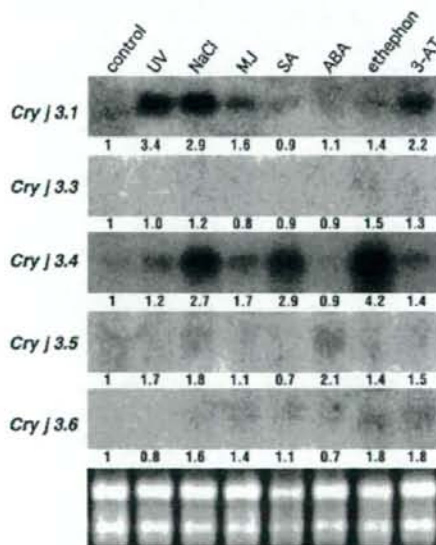

Figure 7. Upper panels: induction of *Cry j 3* gene expression in *C. japonica* seedlings by various stresses and phytohormones. The RNA isolated from seedlings had been exposed for 24 h to UV-B, 200 mM NaCl, 100 μM methyl jasmonate (MJ), 100 μM salicylic acid (SA), 100 μM abscisic acid (ABA), 10 mM ethephon or 8 mM 3-AT. Five μg of total RNA was loaded in each lane of a 1% agarose formaldehyde-containing gel. Transcript levels of each gene were calculated by reference to the intensity of ethidium bromide staining of total RNA and the resultant values were normalized by reference to control values. Values shown are means from three independent experiments. Bottom panel: gel stained with ethidium bromide, before blotting, to confirm that amounts of RNA in the various lanes were equal.

might be localized in vacuoles, whereas the other Cry j 3 proteins might be associated with a secretory pathway. Melchers et al. (1993) suggested that post-translational cleavage of the carboxy-terminal peptide was a requirement for the appropriate vacuolar localization of AP24 that would otherwise be directed to the apoplast. Further analysis is required to identify the cellular localization of each Cry j 3 protein.

A comparison of deduced amino acid sequences revealed that Cry j 3.5 exhibits relatively weak homology to other Cry j 3 proteins (Figures 1 and 2). A phylogenetic analysis of plant TLPs showed that Cry j 3.5 belongs to a group that is distinct from the group of other Cry j 3 proteins (Figure 2). All known TLPs isolated from gymnosperms form a group with all the Cry j 3 proteins except Cry j 3.5, suggesting that a large number of gymnosperm TLPs multiplied after divergence of the angiosperm and gymnosperm lineages. It is likely that the number of gymnosperm TLPs is larger than that of angiosperms. A database search revealed 24 genes in the *Arabidopsis* genome (MAtDB at http://mips.gsf.de/proj/thal/db/) and 30 genes in the *Oryza* genome (TIGR Rice Genome Annotation at http://www.tigr.org/tdb/e2k1/osa1) that contain the thaumatin domain (Pfam profile PF00314). The total number of TLPs in *C. japonica* remains to be determined.

Linkage analysis indicated that the RFLP marker of *Cry j 3.5* is located on linkage group YI10, whereas the markers of *Cry j 3.1* through *Cry j 3.4* are located on YI3 (Figure 5). The sequence similarities among Cry j 3 proteins roughly reflect the distances between them on the linkage map. The *Cry j 3.1*, *Cry j 3.2* and *Cry j 3.3* genes are clustered on the linkage map. Members of gene families organized in tandem arrays of two or more units have been described in *Arabidopsis*, and 17% of all genes in *Arabidopsis* are arranged in tandem arrays (Arabi-

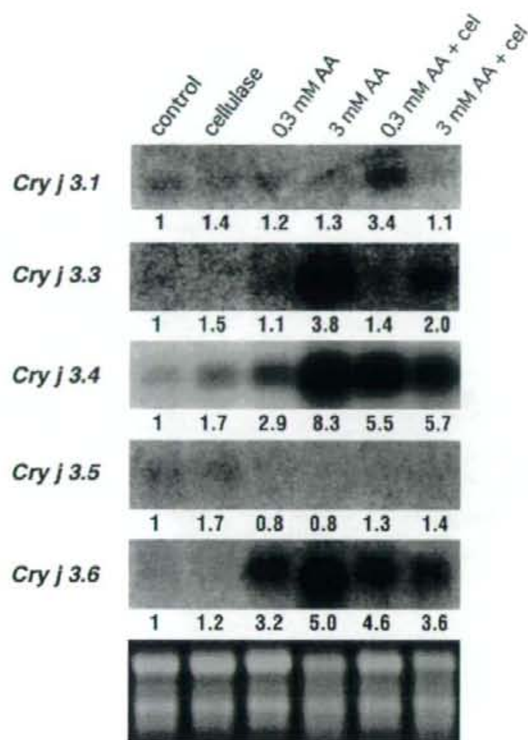

Figure 8. Upper panels: induction of *Cry j 3* gene expression in *C. japonica* seedlings by cellulase (cel), arachidonic acid (AA) or a combination of both. The RNA was isolated from seedlings that had been exposed for 24 h to 250 mg l^{-1} cellulase, 0.3 mM AA or 3 mM AA or a combination of cellulase and AA. Five μg of total RNA was loaded in each lane of a 1% agarose formaldehyde-containing gel. Transcript levels of each gene were calculated by reference to the intensity of ethidium bromide staining of total RNA and the resultant values were then normalized by reference to control values. Values shown are means from three independent experiments. Lowest panel: gel stained with ethidium bromide, before blotting, to confirm that RNA amounts in the various lanes were equal.

dopsis Genome Initiative 2000). Moreover, genes for three TLPs (Accession Nos. O24468, P28493 and Q9C9Q0) are arranged in a tandem array. It is likely that *Cry j 3.1*, *Cry j 3.2* and *Cry j 3.3* are arranged in a similar fashion. We found that primers specific for *Cry j 3.2* recognized sequences from only 11 of 21 *C. japonica* cultivars (data not shown): another set of primers specific for the *Cry j 3.2* gene yielded similar results (data not shown), indicating that some populations of *C. japonica* do not possess the *Cry j 3.2* gene.

Expression patterns of the *Cry j 3* genes differed among plant organs (Figure 6). We detected high expression of all *Cry j 3* genes except *Cry j 3.5* in mature female strobili. Genes for TLPs with stigma- and style-specific patterns of expression have been isolated from tobacco and Japanese pear (Kuboyama 1998, Sassa and Hirano 1998). Transcripts of all *Cry j 3* genes, with the exception of *Cry j 3.5*, were also detected in mature male strobili, although there were considerable differences in expression levels. Expression of TLP in male strobili has also been observed in *Pinus radiata* D. Don. (Walden et al. 1999). Some TLPs have endo-β-1,3-glucanase activity (Grenier et al. 1999), an enzyme that plays an important role in the development of mature pollen grains by degrading the callose wall of tetrads of microspores (Worrall et al. 1992), suggesting that TLPs in male strobili might act as β-1,3-glucanases. The pattern of *Cry j 3.5* expression differed from that of other *Cry j 3* genes: it was the *Cry j 3* gene whose transcript was most strongly expressed in pollen (Figure 6) and it was the only gene inducible by ABA. Pollen grains exhibit various degrees of desiccation at the final stage of maturation (Hoekstra and van Roekel 1988), and many of the changes in gene expression that occur in response to drought require the action of ABA (Bray 1993). Desiccation- and ABA-induced transcripts have been characterized in *Lilium* pollen (Huang et al. 2000, Ko et al. 2002). The Cry j 3.5 protein might play a role in protecting macromolecules from desiccation in *C. japonica* pollen grains.

The effects of various stresses, plant hormones and AA on the expression of the various *Cry j 3* genes differed. Expression of *Cry j 3.1* was induced by UV-B, NaCl and 3-AT, which resulted in the generation of ROS. We speculate that ROS is involved in the induction of *Cry j 3.1* mRNA expression, because UV-B irradiation and salt stress both stimulate the generation of ROS (Hasegawa et al. 2000, Brosché and Strid 2003), and ROS are required for the accumulation of PR-1 in the UV-B signal transduction pathway (Green and Fluhr 1995).

Expression of the *Cry j 3.3*, *Cry j 3.4* and *Cry j 3.6* genes was strongly induced by AA (Figure 8), which is an effective defense response elicitor in potato tubers (Bostock et al. 1981). Chang et al. (1995) showed that AA alone was not an effective inducer of the gene for osmotin in tobacco seedlings but AA plus a preparation of cellulase stimulated expression of this gene. We found that AA alone had a significant effect on gene transcript levels for some TLPs in *C. japonica* seedlings. To our knowledge, this is the first report of AA acting as an elicitor in a gymnosperm. The synergism of the AA and cellulase effects was unclear. The effect of 0.3 mM AA plus cellulase was about equal to the sum of their separate effects, whereas treatment of seedlings with 3 mM AA plus 250 mg l^{-1} of cellulase was less than the effect of 3 mM AA alone. However, because seedlings subjected to 3 mM AA were damaged after 24 h, the combination of cellulase and 3 mM AA might produce severe tissue damage and cause RNA degradation.

The Cry j 3 proteins induced by elicitors are probably involved in pathogen defense responses. However, the respective effects of plant hormones on the expression of the various *Cry j 3* genes differed greatly (Figure 7). The expression of *Cry j 3.4* was induced by SA and ethephon but that of *Cry j 3.3* and *Cry j 3.6* was not. The plant hormones SA and ethylene are associated with the induction of systemic disease resistance (Dong 1998). Perhaps Cry j 3.4 is involved in systemic disease resistance, whereas Cry j 3.3 and Cry j 3.6 are involved in local

defenses.

Recently, TLPs from cedar were characterized as pollen allergens (Cortegano et al. 2004, Midoro-Horiuti et al. 2000). In *Cryptomeria japonica* pollen, the *Cry j 3.5* gene transcript was the most abundant *Cry j 3* gene transcript (Figure 6). Cry j 3.5 might play a role as a pollen allergen if the transcript levels reflect the protein levels. Reports on TLP allergens in *Juniperus ashei* (Midoro-Horiuti et al. 2000) and *Cupressus arizonica* Greene (Cortegano et al. 2004) showed that allergen production varied with environmental conditions. The TLP allergen, Cup a 3, from *C. arizonica* was expressed at elevated levels under polluted conditions (Cortegano et al. 2004). We found that the expression of *Cry j 3.1* was induced by UV-B and oxidative stress, whereas that of *Cry j 3.4* was induced by elicitors, SA and ethylene. The amounts of Cry j 3.1 and Cry j 3.4 proteins in pollen might be affected by such stresses and invading pathogens. Therefore, these two proteins also act as allergens under stressful conditions. Further studies are necessary to determine the allergenicity of Cry j 3 proteins and the relationships between the pollen allergenic potency and various environmental conditions.

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ORIGINAL PAPER

Oral administration of pulverized Konjac glucomannan prevents the increase of plasma immunoglobulin E and immunoglobulin G levels induced by the injection of syngeneic keratinocyte extracts in BALB/c mice

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Clinical and Experimental Allergy

Summary

Background Immunoglobulin (Ig) E plays a key role in the pathogenesis of atopic diseases, such as asthma, atopic dermatitis and allergic rhinitis. Oral administration of pulverized Konjac glucomannan (PKGM) has recently been demonstrated to prevent both plasma IgE elevation and developing dermatitis in NC/Nga mice, a model of atopic dermatitis.

Objective To clarify the direct effect of PKGM on the increase of plasma IgE, we employed the system of BALB/c mouse that increases IgE level without developing dermatitis in response to continuous injection of the extract of syngeneic keratinocytes, PAM 212 cells (PAM extract).

Methods Three weeks after the start of feeding with either control or PKGM diet, mice were injected subcutaneously with PAM extract bi-weekly for 10 weeks. The levels of plasma Igs were measured by enzyme-linked immunosorbent assay every 2 weeks after the injection. The levels of ε germline transcription and the amounts of mRNA for IL-4, IFN-γ, GATA-3 and T bet gene in the spleen were evaluated by real-time RT-PCR at the end of the experiment.

Results On the one hand, PKGM prevented the increase of plasma IgE and IgG (IgG1, IgG2b) induced by PAM extract, and on the other hand, it enhanced the levels of plasma IgG3. However, it did not affect the level of plasma IgM. PKGM also reduced the levels of plasma ovalbumin (OVA)-specific IgE in OVA-sensitized mice. Moreover, PKGM attenuated the induction of ε germline transcription and expression levels of mRNA for IL-4, IFN-γ and GATA-3 in the spleen of PAM extract-injected mice. PKGM also attenuated the induction of ε germline transcription and mRNA for IFN-γ and T bet in the spleen of phosphate-buffered saline-injected control mice.

Conclusions These results suggested that oral administration of PKGM prevents the elevation of plasma IgE by suppressing IgE class switching in B cells and/or the commitment development of naive lymphocytes to both T-helper type 1 (Th1) and Th2.

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Keywords ε germline transcription, konjac glucomannan, oral administration, plasma IgE, Th1/Th2 balance

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Introduction

A high serum immunoglobulin (Ig) E concentration is one of the critical characteristics of atopic diseases, such as atopic dermatitis, asthma and allergic rhinitis [1–5]. Clinical observations have suggested a correlation between serum IgE levels and the extent and/or the severity of a disease, especially with cutaneous lesions [4–6]. The antigen-specific IgE on Fcε receptors in mast cells induce the release of inflammatory mediators, such as histamine, leukotrienes and prostaglandins, in response to the binding of multivalent antigens, which subsequently cross-link the Fcε receptor. In case of skin, these factors cause the immediate hypersensitivity reaction and the late phase inflammatory reaction involving T cells and eosinophils followed by the development of dermatitis [1, 7]. Recent

studies have shown that the ligation of IgE itself on Fcε receptors without antigen can stimulate the release of such inflammatory mediators from mast cells [8, 9]. Hence, the inhibition of IgE production is an important therapeutic target for atopic dermatitis.

IgE production is associated with a predominant T-helper type 2 (Th2) cellular response [10]. Naive CD4$^+$ T cells stimulated with allergen in the presence of IL-4 express GATA-3, a member of the GATA family transcription factors, and develop into Th2 cells that secrete cytokines such as IL-4 and IL-13 [11]. On the other hand, naive CD4$^+$ T cells stimulated with allergen in the presence of IL-12 express T bet, a T-box family transcription factor, and preferentially differentiate into Th1 cells that secrete cytokines such as IL-2 and IFN-γ [12]. Furthermore, either Th1 or Th2 effectors have the ability to down-regulate the other one to each other.

Konjac glucomannan is a highly viscous water-soluble and high molecular weight polysaccharide, consisting of a single chain of D-glucose and D-mannose joined by β-1, 4-linkage with some branches [13], and is rich in Konjac, a Japanese traditional food, obtained from the tubers of the potato-like plant *Amorphophallus konjac*. Recently, Onishi et al. [14] showed that oral administration of pulverized Konjac glucomannan (PKGM), that is reduced to a size of less than 105 μm in average diameter by means of a pulverizer, prevented the elevation of plasma IgE, as well as the development of eczematous lesions and scratching behaviour in NC/Nga mice, a model for atopic dermatitis. However, the mechanisms of PKGM action on the suppression of plasma IgE levels and/or the development of dermatitis remained unclear, especially whether PKGM directly suppresses the production of IgE.

Several reports have proposed the crucial involvement of endogenous substances in the initiation of immunological reactions by dendritic cells and T cells [15, 16]. According to this hypothesis, destruction or stress of the skin may be more important than exposure to exogenous antigens in atopic dermatitis. Indeed, we have demonstrated that continual injection of syngeneic keratinocyte (PAM-212 cells) extracts (PAM extract) without adjuvant induced the increase of serum IgE, IgG1 and IgG2b levels without developing skin lesions in BALB/c mouse [17]. This result suggested that PAM extract might initiate the increase of serum Ig levels by a Th2-dominant mechanism. The Ig-inducing factor(s) in PAM extract has not been identified, but is distinct from known factors such as IL-4, IL-13, IL-18 and prostaglandin E2 (Morimoto et al., manuscript in preparation). In this study, we aimed to clarify the effect of PKGM on the increase of plasma IgE and IgE class switching in B cells by evaluating the expression of ε germline transcription by this system. Moreover, we measured the levels of IL-4, IFN-γ, GATA-3 and T bet gene expression in the spleen to examine the effect of PKGM on the differentiation of naive T cells to Th1 and Th2 effector cells.

Materials and methods

Animals

BALB/c mice were purchased from Charles River Japan (Yokohama, Japan). Mice were 4- or 5-weeks old at the start of the experiments. They were maintained in a specific pathogen-free environment in closed racks with free access to food and water in the Institute of Laboratory Animal Science, Hiroshima University. This study was carried out in accordance with the Guideline for Animal Experiment in Hiroshima University and the Committee of Research Facilities for Laboratory Animal Science, Natural Science Center for Basic Research and Development (N-BARD), Hiroshima University.

Feeding of pulverized Konjac glucomannan diet and injection of keratinocyte extracts into the mice

The mice (male) were fed either with a commercial rodent diet, MF (Oriental Yeast, Tokyo, Japan), or 5% PKGM-containing MF (w/w), which inhibited the development of dermatitis and the increase of plasma IgE levels in NC/Nga mice [14, 18]. PKGM was a gift from Shimizu Chemical (Mihara, Japan), and its mean particle sizes were estimated to be about 77 μm. Keratinocyte cellular extract was prepared as described previously [17]. Briefly, PAM 212 cells, a murine keratinocyte cell line derived from BALB/c mouse [19], were cultured and harvested at subconfluence. Cells were washed with calcium- and magnesium-free phosphate-buffered saline (PBS), centrifuged and finally resuspended in five volumes of ice-cold PBS to the weight of the pellet, followed by sonication and centrifugation at 105 000 *g* for 60 min. The supernatants were collected as cellular extracts (PAM extract) and stored at − 20 °C until use. Three weeks after the start of feeding with either control or PKGM diet, we started to inject mice with either PBS or PAM extract every 2 weeks for six times in total. For each mouse, an aliquot of 250 μL solution was injected subcutaneously into bilateral inguinal regions (total 500 μL/mouse). Blood was obtained by retro-orbital bleeding. Blood from each mouse was placed in a heparinized plastic tube and centrifuged at 10 000 *g* for 10 min. After centrifugation, plasma was collected and stored at − 80 °C for analysis.

The plasma concentrations of IgE, IgM, IgG (total IgG, IgG1, IgG2a, IgG2b, IgG3) and IgA in either plasma or culture media were determined by enzyme-linked immunosorbent assay (ELISA) kits purchased from Bethyl Laboratories (Montgomery, TX, USA), following the manufacturer's instructions.