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Fruit-Specific Expression of a Defensin-Type Gene Family in Bell Pepper

Upregulation during Ripening and upon Wounding

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We have isolated a 454-bp cDNA that encodes a novel fruit-specific defensin from bell pepper (*Capsicum annuum*). The encoded 75-amino-acid polypeptide contains an N-terminal domain characteristic of a signal peptide and a 48-amino-acid mature domain named J1. The mature protein, from which the N-terminal amino acid sequence was determined, contains eight cysteines that form four intramolecular disulfide bridges, suggesting a monomeric form for J1. In healthy fruits J1 is undetectable at the green stage but high levels accumulate during ripening. In wound areas of the green fruit the accumulation of J1 dramatically increased, suggesting a role for J1 in the plant's defense response. Moreover, we have demonstrated that J1 possesses an antifungal activity. We have isolated and characterized the corresponding two homologous genes (*j1–1* and *j1–2*) that exist in the bell pepper genome. Both genes are interrupted by the insertion, at the same position, of one intron of 853 bp for *j1–1* and 4900 bp for *j1–2*. Northern blot and reverse transcriptase-polymerase chain reaction and restriction fragment length polymorphism analyses revealed that *j1–1* transcripts are present only in fruits, only in trace amounts in mature green fruits, and that they accumulate to high levels in fully ripe fruits, whereas no *j1–2* transcripts were detected in the samples monitored.

Fruit ripening is a genetically programmed process that involves coordinated changes in organelle structures and cellular metabolism. Ripening involves changes in fruit firmness, sweetness, acidity, aroma, and pigmentation. Although ripening was originally thought to be simply degradative in nature, research in recent years has shown that it is a complex, highly controlled process requiring differential gene expression and de novo synthesis of specific proteins (Coombe, 1976; Brady, 1987). Rich in macromolecules, fruits and especially ripe fruits are vulnerable to pathogen infection because plants do not possess an immune system such as that found in higher vertebrates, and their resistance is based mainly on a dynamic defense system composed of physical barriers, chemical responses, and antimicrobial proteins of different classes.

There are now a number of examples that show that small, Cys-rich proteins play a role both in the specificity and pathogenicity of fungal pathogens and in the plant's defense response (for a review, see Templeton et al., 1994). Among them are the thionins, a group of small (5 kD), highly basic, and Cys-rich proteins that seem to be involved in plant resistance to fungal and bacterial pathogens. They are generally located intracellularly in the endosperm of monocotyledonous plants such as wheat (purothionin), barley (hordothionin), rye, maize, and oat (for a review, see Bohlmann and Apel, 1991), and both intracellular and extracellular forms have also been observed in leaves of the same plants (Bohlmann et al., 1988). Thionins were also detected in leaves and seeds of a few dicotyledonous species. They have a compact amphipathic structure stabilized by three or four disulfide bridges that could interact directly with the lipid membrane by forming pores. Similarity has been noted in the molecular structure and function of the plant toxin purothionin and the mammalian pore-forming proteins (Oka et al., 1992).

On the other hand, a new class of Cys-rich extracellular proteins was isolated from barley and wheat endosperm (Colilla et al., 1990; Mendez et al., 1990). These proteins were originally called γ-thionins because they have a similar size (5 kD) and the same number of disulfide bridges as α- and β-thionins, but they do not share significant sequence homologies with the latter. For this reason, and based on structural and functional homologies with pathogen-inducible insect defensins, the term "plant defensin" was proposed to describe this class of plant peptides (Terras et al., 1995). Plant defensins have been found in radish seeds (Terras et al., 1992), tobacco flowers (Gu et al., 1992), potato flowers (Moreno et al., 1994), petunia pistils (Karunanandaa et al., 1994), and sugar beet leaves infected with *Cercospora beticola* (Kragh et al., 1995). These plant defensins present a specific Cys arrangement involving a particular globular structure stabilized by four disulfide bridges. They are essentially toxic to fungi and seem to interact with the membrane by means of electrostatic interactions.

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Abbreviations: CSH motif, Cys-stabilized α-helical motif; RFLP, restriction fragment length polymorphism; RT, reverse transcriptase; SP fraction, soluble protein fraction.

To characterize aspects of gene expression during ripening of bell pepper (*Capsicum annuum*) we have isolated the genes encoding a fruit-specific defensin. In this paper we describe the isolation of a fruit-specific cDNA and the nucleotide sequence and expression of the two homologous bell pepper genes. We also report on the purification and characterization of the corresponding new antifungal protein. To our knowledge, this is the first report that describes a family of genes encoding a plant defensin specifically accumulated in ripe fruits.

MATERIALS AND METHODS

Bell pepper seeds (*Capsicum annuum* var Yolo Wonder) were germinated and the plants were grown under greenhouse conditions.

Screening of Libraries

Differential screening of a bell pepper red fruit cDNA library (Houlné et al., 1994) allowed the selection of a full-length, ripe, fruit-specific cDNA, which was cloned into the *Eco*RI site of pBluescript KS^+ (Stratagene). Screening of a *C. annuum* genomic library was done as previously described (Pozueta-Romero et al., 1995). Positive clones were selected after three rounds of screening with the cDNA previously isolated. Subfragments were cloned into the pBluescript SK^+ vector. In all cases, *sure* (rec A−) (Stratagene) bacteria were used as the recipient strain. Subsequently, plasmid DNA was isolated according to the rapid Qiagen (Chatsworth, CA) method for DNA preparation and used for double-stranded sequencing (Zhang et al., 1988).

Differential Gene Expression Analysis

RNA extraction was carried out as described (Bogorad et al., 1983). For northern blot analyses, the RNAs (10 μg) were separated by formaldehyde agarose gel electrophoresis (Maniatis et al., 1982) and blotted onto Hybond-N^+ membranes (Amersham). For the expression analysis of the two homologous genes, RT-PCR with RFLP was carried out as described (Song and Osborn, 1994). Primer sequences are 5′-GATATGATGGCGGAGGCAAAG-3′ and 5′-AGAGTTAATTAAGCACAGGGCTTC-3′. Since RT-PCR is sensitive to trace amounts of DNA contamination, RNA samples were incubated with DNase. To test the efficiency of the DNase treatment, RT-PCR was conducted, but no RT was added.

Northern and Southern Hybridizations

^{32}P-Labeled probes prepared by random priming were used for hybridizations. Hybridizations were performed in 50% deionized formamide, 5× SSC (1× SSC is 0.15 M NaCl, 0.015 M sodium citrate), 5× Denhardt's solution (1 mg/mL Ficoll, 1 mg/mL PVP, 1 mg/mL BSA), 0.1% SDS, 200 μg/mL sonicated salmon sperm DNA at 42°C for 16 h. Filters were washed with 2× SSC, 0.1% SDS at room temperature for 20 min, and with 0.2× SSC, 0.1% SDS at 60°C for 15 min.

Electrophoresis and Protein Staining

Proteins were extracted from different plant tissues by the phenol method as previously described (Pozueta-Romero et al., 1995). Twenty micrograms of protein were separated by Tricine-SDS-PAGE (Schägger and von Jagow, 1987) and stained with silver (Ausubel et al., 1987).

Western Blotting and Immunodetection

Attempts to overexpress the J1 protein in vivo were unsuccessful. Therefore, a synthetic peptide corresponding to residues 26 to 39 of the cDNA coding region was coupled to ovalbumin and injected in rabbits to produce an antiserum against the protein. This antiserum was used in western blotting experiments after the electrotransfer onto a PVDF membrane (Immobilon, Millipore) of the proteins separated on gel. A peroxidase-conjugated second antibody was used for immunostaining with the enhanced chemoluminescence kit obtained from Amersham.

Purification of the J1 Protein

Protein Extraction

Fifty grams of pepper fruit were ground in an electric grinder in 60 mL of extraction buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl). After filtration through a double layer of cheesecloth, the extract was ultracentrifuged in a SW50 rotor (Beckman) at 100,000*g* for 1.5 h at 4°C. The pellet was discarded.

$(NH_4)_2SO_4$ Fractionation

The supernatant was adjusted to 60% $(NH_4)_2SO_4$ saturation and the precipitate was dissolved in 30 mL of extraction buffer. This fraction was dialyzed overnight against the extraction buffer and concentrated by ultrafiltration through a low-adsorption, hydrophilic, YM-type membrane (Centricon-30, Amicon, Beverly, MA). This concentrated fraction was named the SP fraction.

Gel Filtration

The proteins in the SP fraction were purified further by HPLC gel filtration. The sample solution (about 200 μL) was loaded onto a Superdex 75 HR 10/30 column (Pharmacia LKB Biotechnology, Uppsala, Sweden) equilibrated with 20 mM Tris-HCl, pH 7.5, 200 mM NaCl. Elution was carried out with the same buffer at a flow rate of 0.2 mL/min and 120-μL fractions were collected. The protein content of the fractions collected was analyzed immunologically by dot-blot experiments. Fractions containing the protein of interest were desalted and concentrated for further analyses. For protein microsequencing, purified protein was electroblotted onto a PVDF membrane (Immobilon, Millipore) and the N-terminal amino acid sequence was determined using a protein sequencer (model 473A, Applied Biosystems).

Assay for Inhibition of Fungal Growth

Standard assays were carried out in Petri dishes containing about 20 mL of malt-Glc-agar (1.5:1:2%) medium. For the assay a piece of agar containing frontal mycelia of *Fusarium oxysporum* (isolated from beets, Institut National de la Recherche Agronomique [INRA], Colmar, France) was placed in the center of the plate. The plates were incubated for 24 h at 24°C. After this first incubation period, sterile paper discs (3MM, Whatman) 8 mm in diameter were placed at a distance of 0.5 cm around the frontal mycelia. Twenty microliters of different samples were added to each disc. The plates were incubated at 24°C for approximately 36 h until mycelial growth had enveloped peripheral discs containing control buffer and had formed crescents of inhibition around discs containing an effective concentration of antifungal agents. Assays were performed in a similar manner using *Botrytis cinerea* (BC4, INRA Avignon Montfavet, France).

For proteolytic digestion of the samples, pronase was added to a final concentration of 200 μg/mL and incubated at 37°C for 24 h.

RESULTS

Characterization of a Fruit-Ripening-Specific cDNA Coding for a Plant Defensin

Differential screening of a cDNA library constructed from red fruit allowed us to isolate and characterize a full-length cDNA of 454 bp. The 3′ untranslated region of 197 bp is followed by a poly(A)$^+$ tail, and 90 bp upstream from this tail is an AATAAA sequence corresponding to the consensus polyadenylation signal. Sequence analysis of the DNA fragment indicated that the cDNA contained only one long, open reading frame of 225 nucleotides. The deduced protein sequence, shown in Figure 1, contains 75 amino acids with a calculated molecular mass of about 8.5 kD, a pI of 7.78, and a global net charge of +2. Analysis of the encoded protein sequence indicates that it contains a putative signal peptide with the predicted cleavage site obeying the (−3; −1) rule (von Heijne, 1986), and a mature domain with a calculated molecular mass of about 5 kD, a pI of 8.02, and a global net charge of +3. The signal peptide is 27 amino acids long with the three structurally distinct domains (n, h, and c). The mature domain, named J1, consists of a single polypeptide chain of 48 amino acids including 8 Cys's. Alignment of the J1 amino acid sequence with the sequences of proteins similar to plant defensins revealed a well-distributed homology all along the primary structure (about 57% of sequence similarity) (Fig. 2A). The most remarkable aspect of the homology is that all of these proteins contain 8 Cys residues the alignment of which is strictly conserved. Furthermore, 6 other amino acids, located at positions 7, 10, 12, 27, 32, and 38 on the J1 sequence, are conserved. In the animal kingdom, this consensus Cys alignment is found in scorpion neurotoxins and in insect defensins. All of the reported plant defensins present a typical signal peptide similar to that of J1, indicating that all of these proteins are secreted.

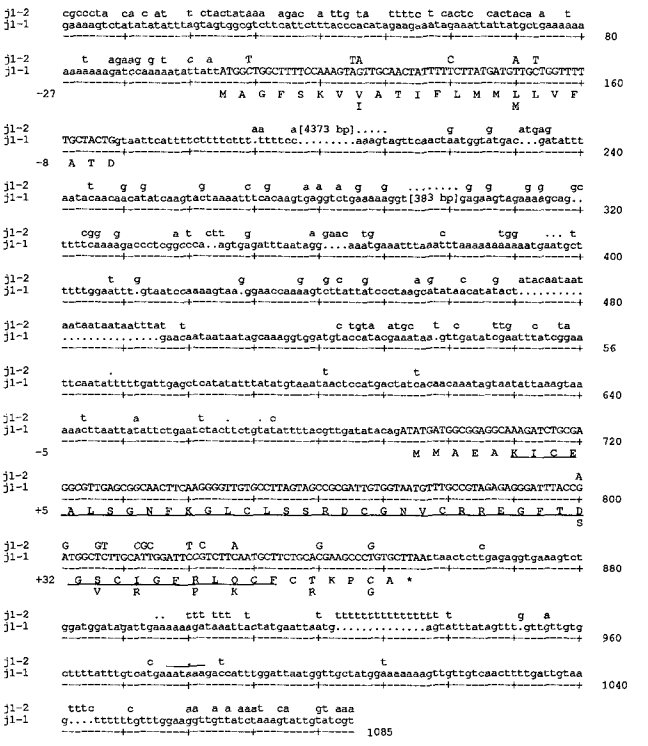

Figure 1. Nucleotide sequences and predicted amino acid sequence of genomic *j1* clones and *j1* cDNA. Only the nucleotides of *j1–2* differing from those of *j1–1* are presented, and only the amino acids of J1–2 differing from those of J1–1 are presented. Gaps in the nucleotide sequence of either *j1–1* or *j1–2* are represented by dots. The cDNA sequence begins and ends at the nucleotide positions 85 and 1073, respectively. The ATG translation start and the TAA termination codons are located at positions 105 and 854 bp, respectively. The open reading frame is indicated in uppercase letters. The experimentally determined N-terminal sequence of J1–1 is underlined. The putative polyadenylation signal is indicated by overlining. The sequence upstream from the translation initiation codon is consistent with Kozak's consensus sequence. The additional sequences present in the two introns are indicated only by their respective sizes, in brackets.

Structure and Genomic Organization of the *j1* Gene Family

Using the *j1* cDNA as a probe under highly stringent conditions, the Southern blot analysis of the restricted bell pepper genomic DNA revealed the existence of more than one copy homologous to the *j1* probe (Fig. 3A). Using this probe we isolated two families of λ phage clones from a previously constructed genomic DNA library. The restriction maps (Fig. 3B) of the different λ genomic clones belonging to the two isolated families are consistent with the pattern obtained by Southern blot analysis of the pepper genomic DNA, confirming the existence of only two copies, designated as *j1–1* and *j1–2*, per haploid genome.

To investigate the structure of the *j1–1* and *j1–2* genes, two fragments of 8500 and 6600 bp, respectively, which hybridize with the cDNA, were subcloned and sequenced (Fig. 1). *j1–1* corresponded exactly to the characterized cDNA, but *j1–2*, which also has a putative coding sequence

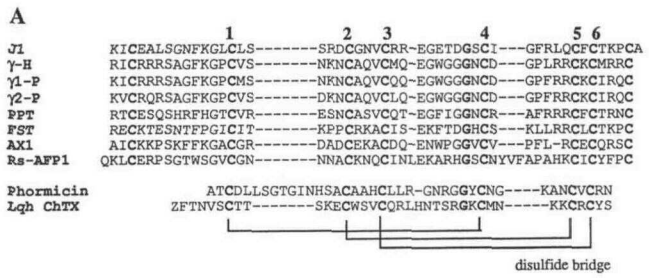

Figure 2. A, Amino acid sequence alignment of the deduced J1–1 protein with similar polypeptides from barley (γ-H) (Mendez et al., 1990), wheat (γ1-P and γ2-P) (Colilla et al., 1990), petunia pistil (PPT) (Karunanandaa et al., 1994), tobacco flowers (FST) (Gu et al., 1992), sugar beet leaves (AX1) (Kragh et al., 1995), radish seeds (Rs-AFP1) (Terras et al., 1992), insect (phormicin or insect defensin A) (Lambert et al., 1989), and scorpion (Lqh ChTX, charybdotoxin from *Leiurus quinquestriatus hebraeus*) (Martins et al., 1990). Boldface letters indicate the conserved residues. The Cys residues involved in the CSH motif are numbered from 1 to 6. DNA sequences were analyzed using the programs of the University of Wisconsin Genetics Computer Group (Madison). B, Secondary structure of J1. Extrapolation from NMR structures of γ-thionins in solution (Bruix et al., 1993). C, Secondary structure of insect defensin A (phormicin) determined by NMR studies (Bonmatin et al., 1992). B and C, Arrows represent β strands, ribbons represent α helix, hatched lines represent disulfide bridges, bold lines represent nondefined secondary structures. The N- and C-terminal parts of the proteins are represented by N and C, respectively.

of 75 codons, was only 92.1% homologous with the cDNA. The main differences between *j1–1* and *j1–2* were found in the 3′ coding region, leading to a deduced protein sequence in which the eighth Cys residue is substituted by a Gly. The conservation of all eight Cys residues seems to be important for the globular structure stabilization of the J1–2 protein. The 200-bp sequences upstream from the start codons of the two genes share only 47.9% homology. The sequences surrounding the putative translation initiation codon (TATTATGGC for *j1–1* and TAATATGGC for *j1–2*) are similar to the consensus sequence for plant genes (Lutcke et al., 1987).

Both *j1–1* and *j1–2* contain a single intron inserted at 64 bp downstream from the translation start codon and localized in the signal peptide between the h and c regions. The sizes of the introns are 853 and 4900 bp for *j1–1* and *j1–2*, respectively. The two introns are highly homologous (84.6%) except for additional insertions of 383 bp (position 294) for *j1–1* and 4373 bp (position 197) for *j1–2*. The introns of both genes have splice junctions obeying the GT. . . . AG rule for eukaryotic introns and have a high AT content.

Expression of the *j1* Genes

Northern blot analysis, using the 454-bp cDNA as a probe, revealed that among various parts of the plant tested, the 450-bp *j1* transcript is detectable only in fruits at the orange and fully ripe stages (Fig. 4). Overexposure allowed us to detect transcripts at the mature, green fruit stage and in very low amounts in young, green fruits (growth stage), (data not shown). The relative amount of *j1* transcripts remains very low during fruit development and dramatically increases during ripening. It was interesting to determine whether the two genes are expressed. The main problem in the RNA analysis is the difficulty of identifying homologous transcripts that have almost iden-

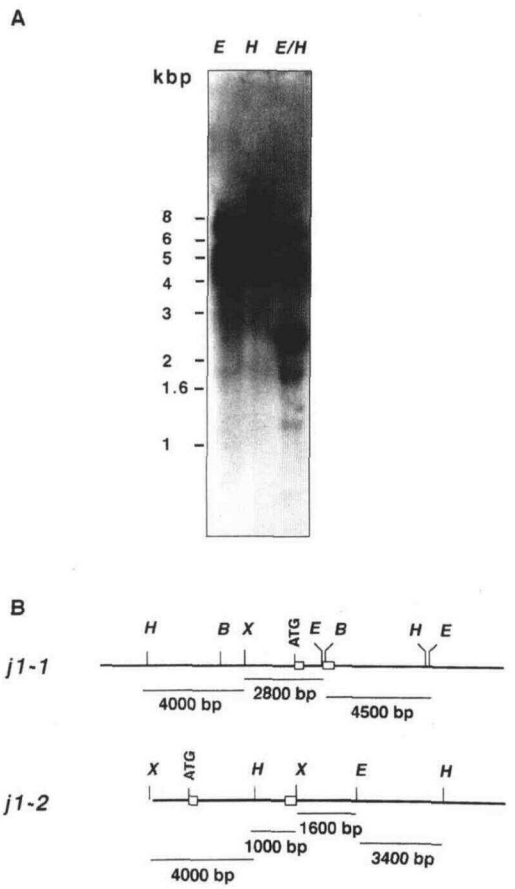

Figure 3. A, Southern blot analysis of the *j1* gene family in the *C. annuum* genome. Pepper genomic DNA (10 μg) was digested with *Eco*RI (lane E), *Hin*dIII (lane H), and with the two enzymes simultaneously (lane E/H). Digested DNA was electrophoresed on a 0.8% agarose gel, transferred to a nylon membrane, and hybridized with the ^{32}P-labeled *Eco*RI insert from the cDNA clone. B, Restriction endonuclease site maps of *j1–1* and *j1–2* genomic clones. Translated sequences are shown by open boxes. Sizes of the restriction fragments are indicated in bp. Restriction sites are as follows: H, *Hin*dIII; E, *Eco*RI; B, *Bgl*II; X, *Xho*I. The faint gray line in *j1–1* represents the lambda arm.

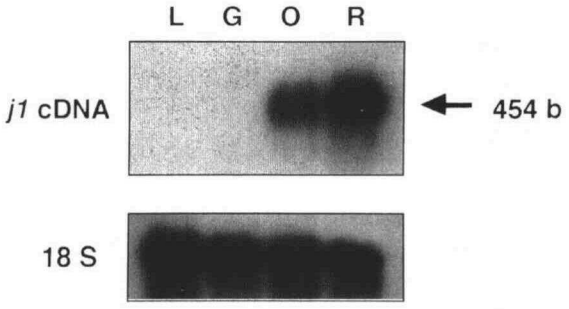

Figure 4. Northern blot analysis of the *j1* transcript in leaves and during fruit development. Ten micrograms of total RNA isolated from leaves (L) and green (G), orange (O), and red, ripe fruit (R) were analyzed with a radiolabeled 454-bp *j1* cDNA or with a 18S rDNA as a control.

tical sequences. For this reason, a combination of RT-PCR with RFLP was used to analyze the expression pattern of the two genes.

Primer sequences for PCR were selected from regions conserved between *j1–1* and *j1–2* in the second exon (Fig. 5A). Total RNAs extracted from red, ripe fruits were used as templates for RT-PCR analysis. The amplification reaction yielded a single band of 170 bp (Fig. 5B), and the presence of transcripts of two different origins was revealed after digestion of the PCR products with appropriate restriction enzymes. Thus, there is a specific and unique

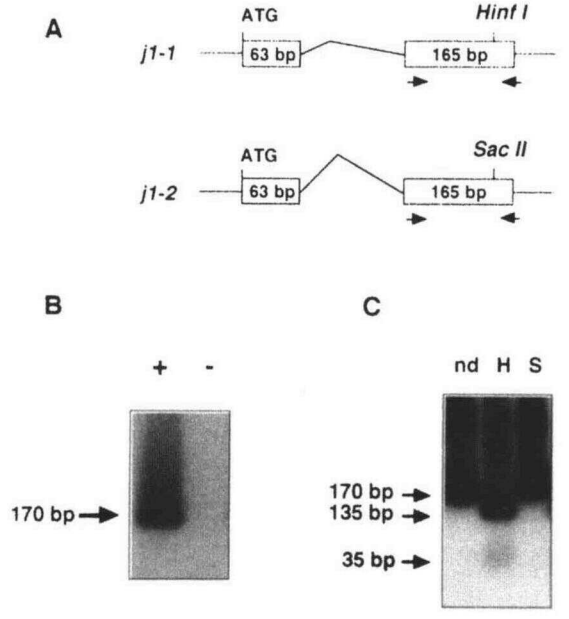

Figure 5. Differential expression of the *j1* genes. A, Schematic representation of the *j1* genes and primers (represented by arrows) used for PCR. Exons are represented by open boxes and introns by lines. *Hin*fI and *Sac*II are two unique and specific restriction sites of *j1–1* and *j1–2*, respectively, used for the RFLP analysis. B, RT-PCR products from pepper RNAs at the fully ripe stage of fruit development. For the experiment the RNA samples were treated with DNase I and the RT was (+) or was not (−) performed. C, RFLP analysis. This panel shows RT-PCR products after endonuclease digestion. nd, Nondigested; H, *Hin*fI; S, *Sac*II.

*Hin*fI restriction site in the *j1–1* coding sequence that divides the PCR product into two fragments of 135 and 35 bp, and a similar specific and unique *Sac*II restriction site in the *j1–2* coding sequence that divides the PCR product into two fragments of 135 and 35 bp. *Hin*fI digestion of the PCR products and electrophoresis yielded two fragments of 135 and 35 bp as expected for the *j1–1* transcript, whereas *Sac*II digestion was ineffective (Fig. 5C). The same result was obtained with total RNAs extracted from green mature and orange fruits (data not shown). These results suggest that only transcripts corresponding to *j1–1* but not to *j1–2* are detectable in fruits.

J1 Accumulates during Ripening and after Wounding

Western blot analysis of proteins extracted from leaves and fruits at different developmental stages, using the antiserum raised against a synthetic peptide of J1 (see "Materials and Methods"), revealed a unique band of 3 to 4 kD in samples taken from fruit (Fig. 6A). As we have noted for the *j1* transcripts, the J1 protein accumulates in large amounts in ripe fruits. The high homology of the J1 protein with plant defensins likely to play a role in host defense prompted us to analyze the effect of wound stress on the expression of *j1* genes. Western blot analysis was performed using samples from mature, green fruits upon wounding (Fig. 6B). J1 accumulated after 3 d of wounding, which further strengthens the view that J1 is likely to be involved in the plant's defense response. It has been shown that γ-hordothionins inhibit protein synthesis in vitro (Mendez et al., 1990) and that the *Raphanus sativus* antifungal proteins are fungistatic toward a broad spectrum of phytopathogenic fungi (Terras et al., 1992). To study the biological activity of J1, we purified the protein and carried out antifungal activity assays.

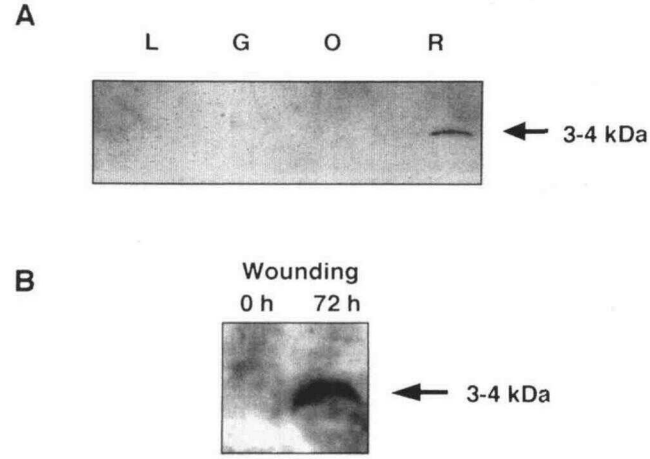

Figure 6. Immunological analysis of samples from leaves (L) and green (G), orange (O), and red (R) fruits (A) and green fruits (B) after 0 and 72 h of wounding. In each case 20 μg of proteins were subjected to Tricine-SDS-PAGE, followed by immunoblotting using antisera raised against the J1 synthetic peptide.

Purification and Characterization of the J1 Protein

An SP fraction was obtained from red pepper fruit extract by initial purification steps consisting of ultracentrifugation at 100,000*g* and $(NH_4)_2SO_4$ fractionation. Analysis of the protein content of this SP fraction by SDS-PAGE and silver staining revealed several bands with a molecular mass higher than 2.5 kD (Fig. 7B, lane SP). Western blot analysis of this SP fraction, using the antiserum raised against J1, showed that the electrophoretic band of about 3 to 4 kD corresponds to the protein J1 (Fig. 7C, lane SP). Subsequently, the SP fraction was resolved by HPLC gel filtration into several peaks (Fig. 7A). The protein content of the collected fractions was analyzed immunologically by dot-blot experiments. Two fractions corresponding to peaks 1 and 2 gave rise to immunological reactions with J1 antibodies (data not shown). Fraction 1 contains several proteins that include a protein of about 3 to 4 kD not detected by the J1 antibodies, and a protein of 20 kD (Fig. 7, B and C, lanes 1) reacting faintly with the antibodies. The N-terminal sequence determination allowed the identification of an osmotin-like protein. On the other hand, fraction 2 seems to be homogenous and contains a single protein reacting strongly with the antibodies (Fig. 7, B and C, lanes 2). Fraction 2 was used to determine the N-terminal sequence of the purified proteins. The sequence obtained corresponds to J1–1 and the first residue is the Lys^{28} (Fig. 1). This result confirms the predicted cleavage site obtained by the (-3; -1) rule according to von Heijne (1986). Moreover, the determination of only one protein sequence corresponding to J1–1 indicates that fraction 2 is homogenous and confirms that the *j1–2* transcripts are not detected.

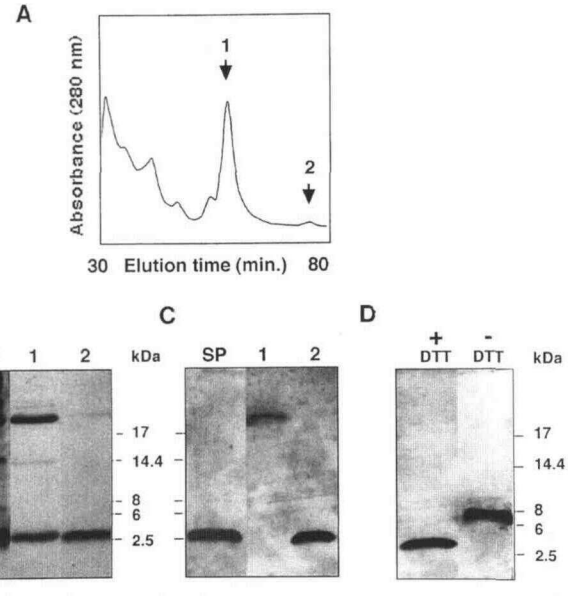

Figure 7. Purification of J1 from fully ripe pepper fruit proteins. A, Separation of the 60% $(NH_4)_2SO_4$ precipitated fraction (SP fraction) by HPLC gel filtration showing peaks 1 and 2 (see "Materials and Methods"). Tricine-SDS-PAGE analysis (B) and immunodetection with anti-J1 antibodies (C) of the SP fraction (lanes SP, with 20 μg of proteins) and the purified fractions 1 and 2 (lanes 1 and 2, respectively, with 1 μg of proteins). D, Immunological analysis of the purified fraction 2 (1 μg of proteins) treated (+) or untreated (−) with DTT.

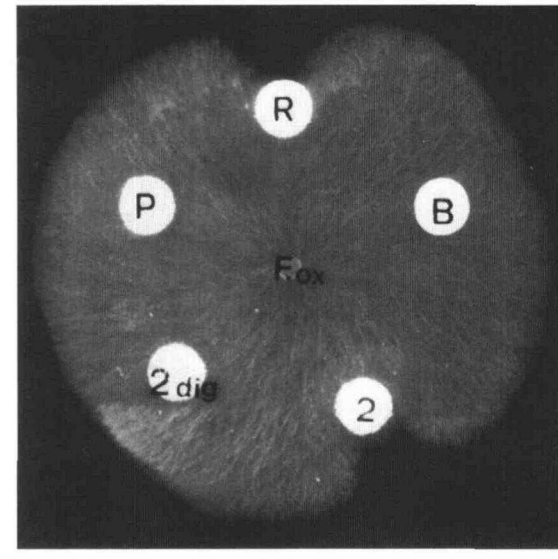

Figure 8. In vitro antifungal activity of the purified J1 against *F. oxysporum*. Ten to 20 μg of purified protein were spotted on discs: 2, fraction 2; 2 dig, pronase digest of fraction 2; R, red fruit SP fraction; B, extraction buffer (50 mM Tris, pH 7.5, 150 mM NaCl); P, pronase (200 μg/mL); F.ox, *F. oxysporum*.

To determine whether J1–1 possesses an oligomeric quaternary configuration stabilized by intermolecular disulfide bridges, electrophoresis was carried out without reducing agent in the loading buffer. The unreduced fraction 2 yielded a band with an apparent molecular mass of 7 to 8 kD (Fig. 7D). In the absence of reducing agent, the lower mobility of the unreduced peptide is probably due to low SDS binding as a consequence of the presence of disulfide bridges rather than because of its oligomeric structure. Thus, J1–1 seems to exist as a monomeric form in the crude extract.

Antifungal Activity Assay

The possible toxicity of the J1 protein isolated from the ripe pepper fruit was tested using the two filamentous fungi, *Fusarium oxysporum* and *Botrytis cinerea*. For the assay, 10 to 20 μg of J1 equivalent were spotted on discs. The red fruit SP fraction was used as a positive test and the extraction buffer (50 mM Tris, pH 7.5, 150 mM NaCl) was used as a control. It is apparent from Figure 8 that the SP fraction (disc R) strongly inhibited the mycelial spread of *F. oxysporum* and formed a crescent of growth inhibition, whereas the extraction buffer (disc B) had no detectable effect on the fungus. Fraction 2 containing the purified J1 (disc 2) was effective in suppressing the growth of the fungus *F. oxysporum*. Upon pronase treatment of the fraction 2 (disc 2 dig) no fungal growth inhibition was observed. We obtained similar results with *B. cinerea*. These results suggest that J1 exhibits an antifungal activity. When the culture of the fungus was maintained for another 3 d, the mycelium completely enveloped each disc. This obser-

vation suggests that J1 exerts a fungistatic rather than a fungicidal effect.

DISCUSSION

We have isolated and characterized a fruit-specific bell pepper cDNA that codes for a J1–1 protein the level of which increases dramatically in the fully ripe fruit. On the basis of the amino acid sequence homology, J1 is a plant defensin-like protein and belongs to a large family of small, basic, Cys-rich polypeptides that include plant defensins, neurotoxins from scorpion venoms, and insect defensin A. All of these peptides present the consensus Cys arrangement -C(. . .)C-X-X-X-C(. . .)G-X-C(. . .)C-X-C-, which forces the alignment of the secondary structural elements into a CSH motif. Two-dimensional computer structure extrapolation of the J1 protein from NMR structures of γ-thionins in solution (Bruix et al., 1993) indicated the presence of the CSH motif, which consists of a small, triple-stranded, antiparallel β sheet linked to an α helix by two disulfide bridges, and to an extended fragment by a third bridge (Fig. 2B). This motif was reported to occur in toxin from scorpion venom (charybdotoxin) (Bontems et al., 1991) and insect defensin A (Bonmatin et al., 1992) (Fig. 2C). Proton NMR analysis indicates that the plant defensins formerly called γ-hordothionins and purothionins exist as monomers with four intramolecular disulfide bridges, the fourth one linking the C- and N-terminal regions of the molecules. The fact that the primary sequences of all of the proteins mentioned are well conserved through the animal and vegetal kingdoms points to a similar three-dimensional organization and a similar biological function. Charybdotoxin blocks membrane K^+ channels via electrostatic interactions. We could imagine a similar electrostatic interaction with unknown receptors in the case of plant defensins and insect defensin A. Obviously, all of these antibiotic peptides constitute the first line of defense against the multitude of pest and pathogen aggressions. The idea of a common ancestral gene leads to the hypothesis that this antibiotic barrier was the first to emerge during evolution and is still the first to act.

Terras et al. (1992) observed that the two antifungal proteins, Rs-AFP1 and Rs-AFP2, that purified to homogeneity from radish seeds are assembled in an oligomeric quaternary configuration stabilized by intermolecular disulfide bridges. However, as is the case for the γ-thionins extracted from wheat and barley endosperms, J1–1 protein was found to exist as a monomer in the crude protein extract with, presumably, four intramolecular bridges. J1–1 is a protein that accumulates during fruit ripening and during adaptation of the mature green fruit to wound stress. This result suggests that the synthesis and accumulation of J1 are developmentally regulated and controlled by environmental signals such as wounding. The J1 wound-sensitive characteristic, fungistatic activity, and its amino acid sequence homology with γ-thionins suggest that J1 is probably a secretory protein involved in the defense system of the fruit. Fruits are especially vulnerable to pathogen infection at the fully ripe stage; therefore, the putative extracellular localization of J1 protein in ripe fruit enhances the chances of the maintenance of fruit integrity and seed maturation.

Using the cDNA as a probe, we isolated and characterized a bell pepper gene family (*j1–1* and *j1–2*) homologous to the probe. Remarkable features of the genes encoding the radish seed- and tobacco flower-specific plant defensins are their large number and their variability (Gu et al., 1992; Terras et al., 1995). High-stringency hybridization of the fruit-specific *j1* cDNA to pepper nDNA revealed the existence of only two genes. This result is supported by the fact that mRNAs homologous to the *j1* probe were detected exclusively in fruits. Low-stringency hybridization revealed a complex array of both strongly and weakly hybridizing fragments, indicating the existence of multiple *j1*-related genes (data not shown). Thus, *j1*-related mRNA are likely to be present in other pepper plant organs. Both *j1–1* and *j1–2* genes are interrupted by one intron inserted in the signal peptide between the h and c regions. It is interesting that the same feature has been observed in the *Petunia inflata* gene *PPT* (Karunanandaa et al., 1994). This observation also confirms that these various genes have developed from a common ancestor gene.

Northern blot and RT-PCR with RFLP analysis have led to the detection of *j1–1* transcripts only in fruits and in very large amounts in fully ripe fruits. On the other hand, we have not detected any *j1–2* transcripts in either of the samples monitored. We were able to purify the J1 protein from the red bell pepper fruits, but the determination of the N-terminal sequence of the purified fraction 2 revealed that this fraction is homogenous and only contains the protein corresponding to the *j1–1* gene. There are two main differences between the two genes. First, the sequences upstream from the translation start codons of *j1–1* and *j1–2* share only 47.9% homology and have no *cis*-acting regions in common. Thus, these two genes could be expected to have a different expression pattern, suggesting a transcriptional activation of *j1–2* under particular conditions. Second, compared with the *j1–1* intron, *j1–2* contains in its intron an additional sequence of 4549 bp. In our laboratory, such a structural difference between two genes has already been characterized in the introns of two fruit-specific genes of a *sn* gene family (*sn-1* and *sn-2*) encoding a PR-related protein (Pozueta-Romero et al., 1995). As with *j1–2*, the *sn-2* transcripts are not detectable in any parts of the plant. We found that *sn-2* could be transcribed in the fruit, but the presence of the additional sequence probably acts as a mRNA destabilizing agent imposing a spatial or temporal pattern of expression different from that of *sn-1* (the functional gene).

Currently, we are examining the expression of *j1–1* and *j1–2* genes in fruits subjected to abiotic and biotic stresses and analyzing the structure of the promoters to characterize the *cis*-acting regions involved in the expression of the *j1* genes.

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