Tomato CDI as a Strong Chymotrypsin Inhibitor

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Research area: Plants interacting with other organisms
A Novel Function for the Cathepsin D Inhibitor in Tomato

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This work has been supported by grant#BMC2003-07837 from Comisión Interministerial de Ciencia y Tecnología, Spanish Ministry of Science and Technology.

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Abstract

Proteinaceous aspartic proteinase inhibitors are rare in nature and are described in only a few plant species. One of them corresponds to a family of cathepsin D inhibitors described in potato, involving up to 15 isoforms with a high sequence similarity. In this work, we describe a tomato wound-inducible protein called JIP21 (Jasmonic Induced Protein, 21 kDa). The sequence analysis of its cDNA predicted a putative function as a cathepsin D inhibitor. The JIP21 gene, whose protein has been demonstrated to be glycosylated, is constitutively expressed in flowers, stem and fruit, and is inducible to high levels by wounding and methyl-jasmonate in leaves of tomato plants. The genomic sequence of JIP21 shows that the gene is intronless and reveals the presence of both an MeJ box (TGACT) and G-box (CACGT) in the promoter. In contrast to the presumed role of JIP21 based on sequence analysis, a detailed biochemical characterization of the purified protein uncovers a different function as a strong chymotrypsin inhibitor, which questions the previously predicted inhibitory activity against aspartic proteinases. Moreover, Spodoptera littoralis larvae fed on transgenic tomato plants overexpressing JIP21, present an increase in mortality and a delay in growth when compared with larvae fed on wild-type plants. These larvae belong to the Lepidoptera family whose main digestive enzymes have been described as being serine proteases. All these results support that tomato JIP21 should be considered as a chymotrypsin inhibitor belonging to the serine proteinase inhibitors rather than a cathepsin D inhibitor. Therefore, we propose to name this protein as TCI21 (Tomato Chymotrypsin Inhibitor, 21 kDa).
Plants respond to insect attack or wounding by a transcriptional activation of a large number of genes. Proteins encoded by these wound-inducible genes perform different functions such as repairing the damaged tissues, participating in the activation of the wound signaling pathway, adjusting the plant metabolism to the imposed nutritional demands or inhibiting the growth of the predator insect (Reymond et al., 2000; Ryan, 2000; León et al., 2001). Among these wound-induced antinutritional proteins, proteinase inhibitors (PIs) are main components since they interfere with the digestive systems of the attacking herbivores, limiting their growth and development. The activation of PI genes is mediated by jasmonic acid (JA) as a result of the activation of the octadecanoic pathway by insect attack. This activation not only occurs in the wounded leaves, but also in the distal ones (Farmer and Ryan, 1990; Ryan, 1990; Schaller, 2001).

Proteinase inhibitors have been classified into four groups according to the protease they inhibit: serine protease, cysteine protease, aspartic protease or metallocarboxy protease inhibitors. Serine proteinases are divided into two superfamilies: subtilisin and chymotrypsin families. The latter includes digestive enzymes such as trypsin, chymotrypsin and elastase. Inhibitors of these serine proteinases have been described in many plant species and are widespread throughout the plant kingdom. The best studied is the soybean trypsin inhibitor (STI), a representative member of the Kunitz-type serine proteinase inhibitor family whose characterization has provided a basic understanding of the mechanism of action of the remaining inhibitors (Laskowski and Qasim, 2000). Other families of serine proteinase inhibitors are represented by the soybean Bowman-Birk inhibitor (BBI) and the proteinase inhibitor I and II of potato (Birk, 1996; Sánchez-Serrano et al., 1986; Cleveland et al., 1987).

In contrast to this broad distribution of the serine proteinase inhibitor family, proteinaceous inhibitors of aspartic proteinases are less numerous and have been described in only a few plant species: potato (Keilova and Tomasek, 1976a and b), tomato (Werner et al., 1993), wheat (Galleschi et al., 1993), Vicia sativa (Roszkowska-Jakimiec and Bankowska, 1998), Anchusa strigosa (Abuereish, 1998) and squash (Christeller et al., 1998). The biochemical characterization of the potato and Vicia sativa members showed that they were both cathepsin D inhibitors, whereas the proteins from...
Anchusa strigosa and squash inhibit pepsin, a digestive aspartic proteinase. In potato, a large family of cathepsin D inhibitors (CDIs) has been described. The two first isoforms purified (PDI and NDI) were biochemically characterized, showing an inhibitory activity against cathepsin D and serine proteinases (Keilova and Tomasek, 1976a, Mares et al., 1989; Ritonja et al., 1990), and displaying sequence similarities with the STI. Since then, up to 15 isoforms have been found in potato and they have been classified as cathepsin D inhibitors, exclusively on the basis of sequence analysis. Potato CDIs constitutively accumulate in tubers and flower buds, and are also induced by wounding in potato leaves (Strukelj et al., 1990; Maganja et al., 1992; Hildmann et al., 1992; Hannapel, 1993; Herbers et al., 1994; Ishikawa et al., 1994a; Kreft et al., 1997).

The use of PIs to transform crop plants for resistance to insect pests has been well documented (for reviews, see Jouanin et al., 1998; Schuler et al., 1998; Lawrence and Koundal, 2002). Selecting the appropriate PIs to generate this resistance implies taking into account the main digestive enzymes in the midgut of the target insect. In this sense, serine proteinase inhibitors are effective against Lepidoptera (Hilder et al., 1987; Johnson et al., 1989; McManus et al., 1994; Duan et al., 1996; Li et al., 1998; De Leo et al., 2001). On the other hand, aspartic and cysteine proteinase inhibitors have been used to generate resistance against coleopteran species (Orr et al., 1994; Leplé et al., 1995; Girard et al., 1998; Lecardonnel et al., 1999).

In this work, we describe the characterization of JIP21, a tomato wound- and jasmonate-inducible protein. Sequence analysis predicted a putative function as a cathepsin D inhibitor. A thorough biochemical study has been performed with the purified plant protein which revealed a lack of activity against cathepsin D or other aspartic proteinases. Instead, JIP21 shows a powerful activity as a chymotrypsin inhibitor. Tomato plants overexpressing JIP21 have been generated and resistance assays against larvae of the lepidopteran Spodoptera littoralis have been carried out, confirming the new proposed function.
RESULTS

Purification of the JIP21 protein and cloning of the cDNA and the genomic sequences

Comparing the electrophoretic profiles of protein extracts from control and wounded tomato leaves reveals the outstanding presence of a 21-kDa polypeptide that accumulates upon wounding. The accumulation of this polypeptide is even higher when leaves are treated with 2 mM methyl-jasmonate (MeJ) (Figure 1A). We purified this protein, named JIP21 (Jasmonic Induced Protein, 21 kDa), from acidic protein extracts of tomato leaves treated with MeJ by ammonium sulfate fractionating, by two serial chromatographies on a SP-Sephadex C25 and a final FPLC step through a Mono S HR 5/5 (Figure 1B). Once purified, specific polyclonal antibodies were obtained and used to immunoscreen a cDNA library constructed from mRNAs of jasmonate-treated tomato leaves. Several identical clones were isolated and the longest one was used as a probe to obtain the complete cDNA. This sequence proved to be very abundant in our library since around 7% of the cDNA clones corresponded to JIP21 cDNA. In addition, its sequence analysis revealed that it could be assumed as a possible cathepsin D inhibitor (Werner et al., 1993).

With the complete cDNA sequence, a genomic library constructed in the λ-EMBL vector was screened and the corresponding genomic sequence was isolated (Genbank accession number AJ295638). JIP21 gene is intronless as are the genomic sequences described for the potato cathepsin D inhibitors (Herbers et al., 1994; Ishikawa et al., 1994 a and b). The tomato genomic sequence contains a promoter of 1.4 kbp which is highly similar to the potato promoters described, and a 3’ region spanning more than 700 bp downstream from the ORF. An analysis of conserved motifs in the 5’ promoter sequence revealed the presence of a MeJ box (TGACT) between positions –384 and –380 and a putative inverted G-box (CACGT) at position –963. The G-box has been previously described in promoters of wound-inducible genes such as the potato pin II (Sánchez-Serrano et al., 1987; Thornburg et al., 1987), the soybean vspB (Mason et al., 1993), the tomato threonine deaminase (Samach et al., 1995) or the potato sporamin (Wang et al., 2002). In addition, an in silico analysis of the 5’ promoter regions of Arabidopsis thaliana genes, identified as wound-inducible by two-step microarray analysis, revealed G-box-related motifs in a significant proportion of the promoters.
(Delessert et al., 2004). All this supports the idea that JIP21 gene is endowed with the structural and functional characteristics of a wound-inducible gene.

**Pattern of expression**

The expression of proteinase inhibitors in tomato is well known to be wound-inducible (Graham et al., 1985a and b; Martineau et al., 1991; Díez-Díaz et al., 2004). To follow the time course of JIP21 mRNA accumulation upon wounding, we performed a Northern blot analysis from wounded and systemic leaves harvested 0, 1, 6, 12, 24, 48 and 72 hours after wounding. In wounded leaves, the mRNA accumulation can be detected as early as 6 hours, whereas the induction is not detected until 24 hours after wounding in the immediately upper (distal) leaves. In the local response, the maximum accumulation takes place at 48 hours, and at 36 hours in the distal one where the mRNA levels decline faster (Figures 2A and 2B). The pattern of induction by MeJ is similar to that of wounding but, in general terms, is much stronger and remains longer, even for 7 days after treatment (Figure 2C). Similarly to the rest of proteinase inhibitors, JIP21 was not found to be induced by salicylic acid, ethylene, or pathogenic (citrus exocortis viroid and *Pseudomonas syringae pv. tomato*) infections (data not shown).

Regarding the JIP21 levels in other tissues, we could detect the constitutive presence of JIP21 in flowers by Northern blot analysis. Moreover, we detected constitutive levels of JIP21 not only in flowers, but also in stem and fruits by RT-PCR using specific primers against the cDNA sequence (Figure 3). All the PCR products have been cloned and sequenced and they all correspond to the original cDNA. These results appear to indicate that constitutive levels are not due to the expression of JIP21 isoforms in these tissues.

**N-glycosylation of JIP21**

The sequence analysis of the JIP21 protein shows the presence of a putative N-glycosylation site at Asn 51. This site has also been described in some of the cathepsin D inhibitors characterized in potato, but no glycosylation studies had been performed to date. Using the periodic acid/Schiff staining technique associated with Western blot analysis (Strömqvist and Gruffamn, 1992), we found that JIP21 is effectively a glycoprotein (Figure 4). In this assay, ovoalbumin protein has been used as a positive
control of the periodic acid/Schiff staining, and bovine serum albumin as a negative one.

Biochemical characterization of the purified protein

To characterize the predicted cathepsin D inhibitor activity of tomato JIP21, we performed an inhibition assay using hemoglobin labelled with fluorescein as a substrate for the proteinase. Endopeptidase inhibitors directly interact with the active center of the protease at a 1:1 molar ratio (Laskowski et al., 2000). Unexpectedly, the purified JIP21 protein showed no inhibitory activity against cathepsin D at pH 2.8, even at inhibitor:protease weight ratio (w/w) of 10 to 1 (equivalent to 20:1 in the molar ratio). As shown in Figure 5A, an excess of JIP21 does not seem to affect the cathepsin D activity whilst pepstatin, used as a positive control, totally blocked the activity of this protease. To discard the possibility that the lack of activity might be due to a shift in the optimal pH for inhibition, the same studies were performed at a pH range from 2 to 8 and no inhibition was observed (data not shown). Since the cathepsin D is a lysosomal protein and is not, therefore, exactly related to digestive processes, we decided to test JIP21 activity against a digestive aspartic proteinase such as pepsin. As seen in Figure 5B, excess amounts of purified JIP21 displayed no activity against pepsin, unlike the complete inhibition exerted by pepstatin. It has been described (Cater et al., 2002) that cathepsin D inhibitors are also active against proteinase A, a yeast aspartic proteinase. We decided to check the JIP21 inhibitory activity against the yeast enzyme even though it is not a herbivore digestive peptidase. Once again, JIP21 did not inhibit this proteinase (Figure 5C). These results appear to indicate that tomato JIP21 might not be an aspartic proteinase inhibitor, which prompted us to search for other possibilities.

In this regard, we went on to test the activity of the purified protein against digestive proteinases belonging to the serine protease family. We used the soybean trypsin inhibitor (STI), and the bifunctional trypsin and chymotrypsin Bowman-Birk inhibitor (BBI) (Birk, 1996) as controls for these assays. Figure 6B shows that JIP21 is a potent inhibitor of chymotrypsin, where this inhibitory activity is comparable with the BBI inhibitor itself. On the other hand, the inhibition against trypsin was negligible (Figure 6A) when compared with its antichymotrypsin activity. Other serine proteinases such as elastase, proteinase K or subtilisin were also unaffected by JIP21 (data not shown). The same negative results were obtained when assayed against chymosin (an
aspartic proteinase), papain (a cysteine proteinase) or metallo-carboxypeptidase A. Altogether, these results suggest that JIP21 appears to be a serine protease inhibitor with a high specificity against chymotrypsin, which is in contrast to the previously presumed function.

The chymotrypsin inhibitory activity described herein contrasts with the expected CDI activity deduced from sequence analysis. Apart from the obvious similarity to potato CDI, the JIP21 amino acid sequence diverges in relation to the rest of the aspartic proteinase inhibitors described. On the contrary, JIP21 is homologous to the Kunitz-type serine proteinase inhibitors. Figure 7 shows a comparative analysis between JIP21 and two members of this family: the STI and the winged bean chymotrypsin inhibitor (WCI) (Shibata et al., 1998). The putative inhibitory sites are highlighted in this Figure. JIP21 contains the residues Leu-Ser, previously proposed as the binding site to chymotrypsin of the WCI, unlike the STI, with Arg-Ile as active residues for trypsin (Song and Suh, 1998). In fact, it has been described that a single mutation (Leu → Arg) in the reactive site converted WCI into a strong inhibitor of trypsin (Khamrui et al., 2005). Interestingly, the inhibitory site for chymotrypsin in the BBI also contains the residues Leu-Ser (Werner and Wemmer, 1991). All these data further explain the observed activity of JIP21 against chymotrypsin.

**Generation and characterization of tomato plants overexpressing JIP21**

To study the biological function of JIP21, we generated tomato plants overexpressing the protein. For this purpose, a DNA cassette consisting of the *JIP21* cDNA, driven by a double cauliflower mosaic virus 35S promotor and a *nos* terminator, was ligated into a pBin19 plasmid. The resulting construction was used to generate tomato transgenic plants via *Agrobacterium*-mediated transformation. Insertion of the transgene was detected by Southern blot hybridization of genomic DNA digested with Bam HI, a restriction enzyme that does not cut the cDNA sequence. The transgenic lines obtained displayed additional bands to the single endogenous one found in the wild-type plants. Levels of expression of the transgene were detected by Northern and Western blot, and both analysis showed a good correlation (Figures 8A and 8B). Transgenic line number 2 shows a very low level of the *JIP21* transcript, and no detectable protein. Transgenic lines 10 and 13, with high constitutive levels of transcript, accumulate the JIP21 protein at levels that are comparable with a wounded control leaf, as shown in
Figure 8B. These levels are not due to any wound or pest on the transgenic plants, as indicated by the absence of pin I (Graham et al., 1985a) in non wounded leaves (Figure 8B, lower panel). Moreover, when transgenic plants were wounded, the constitutive levels of JIP21 did not appear to be altered, whilst the pin I accumulation was as apparent as in the wounded wild-type plants. This indicates that the CAMV-mediated overexpression of JIP21 does not affect the pattern of expression of other proteinase inhibitors.

Homozygous plants were obtained for line 10, which integrated 1 single copy of the transgene. JIP21 represents around 3% of the total soluble protein in crude protein extracts from this line, as estimated by gel densitometry. These crude extracts displayed a strong antichymotrypsin activity, unlike the wild-type control plants (data not shown). Consequently, this line was used for insect feeding bioassays.

**Effect of the overexpression of JIP21 on *Spodoptera littoralis* larvae**

Since JIP21 is a powerful serine proteinase inhibitor, we decided to test its biological effect on insects whose main digestive enzymes belong to this family of proteases. Thus we performed insect feeding assays with *Spodoptera littoralis* larvae, which belong to the Lepidoptera family. Neonate larvae were placed on detached transgenic or control leaves. Leaves were replaced daily for fresh ones to avoid the accumulation of the endogenous PIs caused by the larval feeding, as pointed out by Abdeen et al. (2005). At the end of the assay (7 days), surviving larvae were counted and weighed. As Table 1 indicates, larvae fed with JIP21 overexpressing tomato leaves presented a percentage of mortality of 20%, while only 6% mortality was observed in the larvae fed on wild-type plants. Besides, we observed a mean weight reduction of approximately 40% in larvae fed on transgenic tomato leaves (Table 1). Our results clearly indicate that JIP21 exerts an antinutritional effect on *Spodoptera littoralis* larvae, which demonstrate its defensive function and support the novel role of JIP21 as a chymotrypsin inhibitor against insects and herbivores.
DISCUSSION

In this work, we have purified and characterized JIP21, a defensive protein with a novel function as a chymotrypsin inhibitor in tomato. This is in contrast to its role as a cathepsin D inhibitor previously predicted on the grounds of its sequence analysis.

JIP21 strongly accumulates after wounding and treatment with MeJ at levels perfectly detectable by Coomassie blue staining. The purification of the protein allowed us to obtain its corresponding antibodies and the cDNA sequence by immunoscreening. The sequence analysis revealed that the cDNA corresponds to a possible cathepsin D inhibitor (Werner et al., 1993). In fact, the JIP21-deduced amino acid sequence presents an elevated identity with the family of cathepsin D inhibitors described in potato (Mares et al., 1989; Ritonja et al., 1990; Strukelj et al., 1990; Maganja et al., 1992; Hildmann et al., 1992; Hannapel, 1993; Herbers et al., 1994; Ishikawa et al., 1994a; Kreft et al., 1997). Apart from this large family in potato, proteinaceous aspartic proteinase inhibitors are uncommon and are described in only a few plant species, yeast (Schu and Wolf, 1991) and the nematode Ascaris lumbricoides (Abu-Erreisch and Peanasky, 1974). None of these inhibitors seem to be related to the potato cathepsin D inhibitors.

The deduced amino acid sequence of the JIP21 cDNA reveals the presence of a putative N-glycosylation site, which is also described in some of the cathepsin D inhibitors characterized in potato. We herein demonstrate that such glycosylation actually occurs. It is well established, at least in animal glycoproteins, that the glycosylation participates in important processes such as the maintenance of protein conformation and solubility, the stabilization of the polypeptide against uncontrolled proteolysis, the intracellular sorting and externalization of glycoproteins or the mediation of its biological activity (Olden et al., 1985; Dwek, 1995). In silico folding simulation indicates that the N-glycosilation site falls close to the putative inhibition site, thus deglycosylation of the purified protein or mutagenesis of the Asn-Ser-Ser site might yield information about the role of the glycan on either the stability or activity of JIP21.

JIP21 has a pattern of expression similar to the first identified tomato proteinase inhibitors, pin I and pin II (Graham et al., 1985a and b). That is, it is locally and systemically induced by both wounding and MeJ treatment. When compared to wounding, MeJ induction is stronger, more rapid and long-lasting. JIP21 is not induced by pathogens (citrus exocortis viroid and Pseudomonas syringae pv. tomato). However,
the cDNA described by Werner et al. (1993) was obtained from a library from RNAs of tomato leaves infected with potato spindle tuber viroid (PSTVd). This might be due to any injury on the material used for the PSTVd library.

We have detected constitutive levels of JIP21 in tomato plant flowers. The accumulation in these organs has also been described for other proteinase inhibitors such as the potato pin II (Peña-Cortés et al., 1991), the cathepsin D inhibitor (Hildmann et al., 1992; Ishikawa et al., 1994a), the tomato MCPI (Martineau et al., 1991) and a Bowman-Birk inhibitor from pea (Domoney et al., 2002). A possible role of this localization could be the protection of vulnerable tissues of flowers. Moreover, we have detected an accumulation of JIP21 mRNA in stem and fruit of tomato control plants by RT-PCR. The fact that all the PCR products have been shown to correspond to the original cDNA suggests that the constitutive levels are not due to the expression of isoforms of JIP21 in these tissues, unlike potato, where a family of 15 members has been described to date. Hence, while the promoter of one isoform of the cathepsin D inhibitor in potato has been shown to direct the expression of the reporter gene GUS in tubers alone and not in leaves upon wounding (Herbers et al., 1994), the JIP21 promoter sequence, that we have isolated by screening a genomic library, could be responsible for the whole pattern of expression observed.

The detailed biochemical characterization of JIP21 has allowed us to uncover a novel function that differs from that predicted by its sequence comparative analysis. Although the JIP21-deduced amino acid sequence presents an elevated identity with the family of cathepsin D inhibitors of potato, we have not detected any inhibitory activity either against this protease or other proteases of the aspartic family assayed. It is also interesting to note that among the large number of putative cathepsin D inhibitors described in potato over recent years, the biochemical characterization was only performed in early studies when the two first potato isoforms (PDI and NDI) were purified. In those studies, a weak affinity of PDI for cathepsin D was described when compared with the affinity shown by soybean and trypsin inhibitors to their proteinase targets (Keilova and Tomasek, 1976a). Since then, potato isoforms have been identified only by means of sequence analysis. In a more recent study using a recombinant protein obtained in Pichia pastoris from a tomato cDNA clone with a high identity to that described by Werner et al. (1993), a revision of the name is proposed given the weak activity detected against human cathepsin D (Cater et al., 2002).
In the present work, the biochemical activity of the purified tomato protein is tested against a number of proteinases. By doing so, we observed a strong inhibitory activity of JIP21 against chymotrypsin, which showed no effect against other proteinases. Thus, we propose JIP21 to be a member of the serine proteinase inhibitors family, acting specifically against chymotrypsin. This is consistent with the fact that the first study of the primary structure of the cathepsin D inhibitor from potato considered its structure homologous to that of the STI, which belongs to the Kunitz-type serine proteinase inhibitors family (Mares et al., 1989). Tomato JIP21, along with all the potato cathepsin D inhibitors, presents a strong sequence similarity to the potato Kunitz-type serine proteinase inhibitors (Walsh and Twitchell, 1991; Valueva et al., 1998; Heibges et al., 2003). Besides, the comparative sequence analysis of JIP21 identifies 3 domains in JIP21 according to functional and structural protein domains using the PRODOM database (http://www.toulouse.inra.fr/prodom.html). The main central domain is Kunitz-like, whose member of reference is precisely the STI. In fact, we have conducted in silico structural studies using the SWISS-MODEL server (http://www.expasy.ch/swissmod/SWISS-MODEL.html), and we observed a good folding compatibility between JIP21 and the STI. All this points to the Kunitz-like nature of JIP21 and accounts for its inhibitory activity to serine proteinases.

All our data question the formerly proposed activity of JIP21 against cathepsin D. Moreover, cathepsin D is a lysosomal aspartic protease implicated in cancer, apoptosis and Alzheimer’s disease (for reviews, see Callahan et al., 1998; Liaudet-Coopman et al., 2005). It is synthesized in the endoplasmic reticulum as pre-procathepsin D, and once in the lysosome, the single-stranded procathepsin (52 kDa) is activated to cathepsin D to finally constitute the mature double-stranded cathepsin D (31 and 14 kDa, respectively) (Yamamoto, 1995). Its functions are related with the degradation or activation of proteins inside the lysosome. Therefore, it is not an extracellular protein and it is unlikely to have a digestive role.

If JIP21 effectively is a serine proteinase inhibitor, it should have a biological effect on insects of which the main digestive proteases belong to this family. To verify this point, we have generated tomato plants overexpressing JIP21 at levels comparable to a wounded tomato plant, and we have evaluated the effect of this overexpression on the mortality and growth of Spodoptera littoralis larvae. Spodoptera littoralis belongs to the Lepidoptera family whose main digestive enzymes are serine proteases (Houseman et al., 1989, reviewed in Terra and Ferreira, 1994). The biochemical
characterization of our protein and the antinutritional effect of the overexpressed protein on these larvae strongly supports our proposal that JIP21 is a serine protease inhibitor instead of the function assumed to date. All our data suggest that the formerly called tomato CDI (Cathepsin D Inhibitor) could be referred to henceforth as TCI21 (Tomato Chymotrypsin Inhibitor, 21 kDa).

MATERIALS AND METHODS

Plant material and treatments

Tomato plants (Solanum lycopersicum L. cv. ‘Rutgers’) were grown under standard greenhouse conditions (20-25°C and 16 h light / 8 h dark).

Wounding and MeJ treatments were performed with 3- to 4-week old plants. Wounding was performed by crushing one compound leaf per plant with forceps. To study the local response, wounded leaves were harvested at different times, and the immediate upper leaves were used to analyze the systemic response. MeJ was applied by spraying a 2 mM solution, and treated leaves were harvested at different times. Plant material was used immediately or stored frozen at –80°C.

Protein analysis

Protein extracts of tomato leaves were performed by homogeneization in acidic extraction buffer (84 mM citric acid, 32 mM sodium phosphate, pH 2.8) as described in Rodrigo et al. (1993). Proteins were separated by SDS polyacrylamide electrophoresis and stained with Coomassie brilliant blue R-250 following the method described by Conejero and Semancik (1977).

Purification of the JIP21 protein

Crude extracts from tomato leaves, sprayed with 2 mM MeJ and harvested after 48 hours, were subjected to fractionated precipitation using ammonium sulfate. Proteins precipitating between 20 to 30% (w/v) saturation were sedimented, dialyzed against 50 mM sodium acetate buffer (pH 5.5) and chromatographed in a SP-Sephadex C25 (Pharmacia) column using a linear salt gradient (0 to 0.5 M NaCl in acetate buffer).
Fractions enriched in JIP21 protein (eluted around 0.2 M NaCl) were collected, concentrated by lyophilization and re-chromatographed in a SP-Sephadex C25 under the same conditions. Finally, fractions containing JIP21 were applied to a FPLC system (Pharmacia) using a Mono S HR 5/5 column and eluted with a linear NaCl gradient (0 to 0.5 M NaCl in acetate buffer). The protein peak corresponding to JIP21 was collected, concentrated and equilibrated in 50 mM Tris-HCl (pH 7.5) to be stored at –20°C.

**Antibodies and Immunoblots**

Anti-JIP21 serum was obtained by injecting female New Zealand rabbits with purified preparations of JIP21 following standard procedures. For Western blot immunoassay, proteins were separated by SDS-PAGE, transferred onto nitrocellulose membranes using a semidry electrotransfer equipment and immunodetected using a 1:5000 dilution of the anti-JIP21 serum or a 1:500 dilution of the anti-pin I serum previously obtained in our laboratory from the recombinant protein (Graham et al., 1985a). Membranes were incubated with a goat anti-rabbit IgG conjugated to alkaline phosphatase (Promega) as a secondary antibody. Immunodetection was carried out with nitroblue tetrazolium and 5-bromo, 4-chloro, 3-indolyl phosphate according to standard protocols.

**N-glycosylation assay**

For the N-glycosylation assay, we followed the method described by Strömqvist and Gruffman (1992) which combines the protein transfer to polyvinyl difluoride membranes with the periodic acid/Schiff sugar staining. Samples containing purified JIP21, ovoalbumin as a positive control and the bovine seroalbumin as a negative control, were separated in a SDS/PAGE and electrotransferred onto Immobilon membranes (Millipore). The homogeneous transfer was checked by using reversible Ponceau staining. After washing with distilled water, membranes were treated with 1% (w/v) periodic acid in 3% acetic acid for 5 minutes, then washed with distilled water for 15 minutes. The Schiff reagent (Sigma) was added and membranes were kept in darkness for 15 minutes. Successive washings were performed, firstly with sodium bisulphite 0.5% (w/v) for 5 minutes and later with distilled water again. Finally,
membranes were rinsed in methanol for a few seconds to eliminate the background and enhance the contrast of the bands.

**Proteinase inhibitor assay**

Proteinase activity was assayed using hemoglobin labelled with fluorescein as a substrate, based on the method described by Twining (1984) for casein. Proteolytic activity was determined as soluble fluorescence in trichloroacetic acid, originating from the hydrolysis of the hemoglobin. Reaction buffers were citrate-phosphate buffer (84 mM citric acid, 32 mM sodium phosphate, pH 2.8) for the aspartic proteinases and 50 mM Tris-HCl (pH 7.5) buffer for the rest of proteinases assayed. To perform inhibition analyses through a pH range from 2 to 8, McIlvaine buffers were used. These buffers were prepared by mixing the proper volumes of 0.1 M citric acid and 0.2 M disodium phosphate to achieve the desired pH values. Reactions were performed in Eppendorf tubes containing 50 µl of the enzyme to a final concentration of 1 to 10 µg/ml, and increasing amounts of the different inhibitors. Control reactions contained no inhibitor. Reactions were preincubated for 15 min at 4°C and then 10 µl of fluoresceinated haemoglobin (0.5% w/v) were added. After 1 hour at 37°C, digestions were stopped by adding one volume of 20% (w/v) trichloroacetic acid, and the precipitate was removed by centrifugation. Supernatants were added to 2.5 ml 0.5 M Tris-HCl (pH 8.5) and the fluorescence at 525 nm was measured using an excitation wavelength of 490 nm in a Perkin-Elmer LS 50 B luminescence spectrophotometer. Enzymatic activity is expressed in relative terms as the net emitted fluorescence (without the background) in relation to the control reaction. Three independent assays were performed for each protease experiment. Cathepsin D was purchased from Calbiochem. Trypsin and Chymotrypsin were obtained from Roche. The rest of proteinases (pepsin, proteinase A, chymosin, elastase, subtilisin, papain and carboxypeptidase A), as well as the proteinase inhibitors used (soybean trypsin inhibitor, Bowman-Birk inhibitor and pepstatin), were obtained from Sigma.

**cDNA library, screenings and DNA sequence analysis**

A cDNA library was constructed from mRNAs of tomato leaves harvested after 48 hours of a 2 mM methyl-jasmonate treatment in a Uni-ZAP XR vector (Stratagene),
following the manufacturer’s instructions. Phagemid-infected E. coli cells were grown in the presence of 10 mM isopropyl-beta-D-thiogalactopyranoside to induce the synthesis of the β-galacturonase fusion protein, and upon plaque formation, proteins were transferred to nitrocellulose membranes. Clones expressing the JIP21 fused to β-galacturonase were revealed by immunostaining as indicated above. As a control, membranes containing protein extracts of tomato leaves, which were either treated or not with 2 mM Methyl-jasmonate and separated by SDS-PAGE, were processed simultaneously.

The cDNA obtained in the immunoscreening was used as a probe to screen a tomato genomic DNA library constructed in λ-EMBL (Clontech), and the positive clones were isolated, purified and characterized, as described in Sambrook et al. (1989).

DNA sequencing was performed on an ABI PRISM DNA sequencer 377 (Perkin-Elmer, Foster City, CA, USA). Computer-assisted analyses of DNA sequences were carried out using the University of Wisconsin Genetics Computer Group (GCG) package (Genetics Computer Group, INC., Madison, WI, USA), and the online services available at the National Center of Biotechnology Information (http://www.ncbi.nlm.nih.gov).

**Nucleic acids**

Total RNA was prepared by using the TRIzol reagent (Invitrogen) following the manufacturer’s instructions. For Northern analysis, 30 µg of RNA were separated in formaldehyde-agarose gels and transferred onto Nytran (Schleicher & Schuell) membranes. 32P-labelled probes were prepared using the Rediprime labelling kit (Amersham) as recommended by the manufacturer. Hybridization and washing conditions were performed as described in Church and Gilbert (1984).

**RT-PCR and cloning of the PCR products**

For reverse transcriptase reactions, we used 5 µg of total RNA obtained from different tomato tissues, and the M-MLV reverse transcriptase (Promega). 5 µl of the reverse transcriptase reaction were used for PCR, employing the following oligonucleotide primers to specifically amplify JIP21: JIP21F (5’-
CCGAATT CATATG AATGTTTATTT-3') and JIP21R (5'-CCAATTTTATTAAGAAAGACATGC-3'). The primers used to amplify RPL2 (Fleming et al. 1993) were RPL2F (5'-GGTGACC GTGTTTGGTCCTTGCG-3') and RPL2R (5'-ACCAACGTTTTGTCCAGGAGGT-3'). PCR reactions were performed in a Perkin-Elmer thermocycler under the following conditions: 30 cycles of 94ºC for 30 s, 50ºC for 1 min and 72ºC for 1 min, followed by a final extension of 72ºC for 15 min.

JIP21 PCR products were purified by elution from agarose gels, cloned into the vector pGEM-T Easy (Promega) and sequenced on both strands.

Generation of transgenic plants

To generate the overexpression construct, the JIP21 cDNA was prepared by a digestion of the plasmid pBlue-JIP21cDNA, obtained in the immunoscreening, with the enzymes Eco RI and Xho I. The cDNA insert was blunt-end ligated between a double cauliflower mosaic virus 35S promotor and the nos terminator signal in a modified pBlueScript vector. The correct sense orientation of the cDNA was checked, then the cassette CaMV35S 2X:JIP21cDNA: nos was digested with Hind III and finally cloned into the vector pBIN19 to give the plasmid called pBin19-JIP21sense.

The pBin19-JIP21sense construct was introduced into the Agrobacterium tumefaciens strain LBA 4404 and used for tomato (Solanum lycopersicum cv ‘Rutgers’) transformation as described by Ellul et al. (2003). Transformants were selected in kanamycin-containing medium and propagated in soil for subsequent analysis.

Feeding bioassays

Neonate larvae of the Egyptian cotton worm (Spodoptera littoralis) were kindly provided by Koppert Biological Systems (Almería, Spain). These larvae were placed in 140 mm diameter Petri dishes containing freshly detached tomato leaves. Plates were kept at 22ºC with a photoperiod of 8 h light/16 h dark. Damp absorbing paper provided sufficient humidity to the plates. Leaves were replaced daily and surviving larvae were weighed every day throughout the assay. At the end of the test (7 days), mortality was evaluated and surviving insects were weighed.
ACKNOWLEDGEMENTS

We thank Asunció̱n Saurí and Dr. Joaquín Fayos for all their assistance with the purification of JIP21 and the obtaining of the anti-JIP21 and the anti-pin I polyclonal antibodies. We also thank Dr. Fernando García-Marí (Universidad Politécnica de Valencia) for all his technical advise and Koppert Biological Systems for providing the Spodoptera littoralis larvae. We would like to thank Prof. C.A. Ryan (Washington University, Pullman) for kindly providing us the tomato pin I cDNA.

LITERATURE CITED


FIGURE LEGENDS

Figure 1. SDS-polyacrylamide (14%) electrophoresis. A) Detection of tomato JIP21. Crude protein extracts of: untreated control tomato leaves (Control); wounded tomato leaves harvested upon 48 hours (Wounding); tomato leaves harvested after 48 hours of a 2 mM MeJ treatment (MeJ). B) Purification of JIP21 protein. Crude: crude extract of MeJ treated tomato leaves; 20-35% AS: proteins present after 20 to 35% ammonium sulfate fractionating; SP-Seph I and II: enrichment achieved by two consecutive chromatographies on SP-Sephadex C25; MonoS: final FPLC step using a Mono S HR 5/5 column. Lane M: molecular weight standards. Proteins were stained with Coomassie brilliant blue R-250. Bands corresponding to JIP21 are indicated with an arrow.

Figure 2. Time course analysis of JIP21 mRNA accumulation in tomato leaves, in response to wounding or methyl jasmonate treatment by Northern blot. A) Local response to wounding. mRNAs from tomato wounded leaves, harvested 0, 1, 6, 12, 24, 48, 72 hours after wounding. B) Distal response to wounding. mRNAs from immediately upper tomato leaves, harvested 0, 1, 6, 12, 24, 48, 72 hours after wounding. C) Response to methyl jasmonate treatment. mRNAs from tomato leaves, harvested 0, 6, 12, 24, 36, 48, 72 hours, 4 days and 7 days after spraying plants with a 2 mM solution.

Figure 3. Constitutive mRNA levels of JIP21 in different tissues (roots, stem, leaves, flowers and fruits) of tomato, as analyzed by RT-PCR. mRNA from tomato leaves harvested 24 hours after a 2 mM methyl jasmonate treatment is included as a positive control. Upper panel: PCR performed with specific JIP21 primers. Lower panel: PCR performed with specific primers of the RPL2 gene (Fleming et al., 1993), used as a control for the reverse transcription.

Figure 4. N-glycosylation of JIP21. 2 µg of JIP21 purified protein, ovoalbumin (OA), used as positive control, and bovine seroalbumin (BSA), used as negative control, were separated in 14% SDS polyacrylamide electrophoresis. A) Proteins stained with Coomassie brilliant blue R-250. B) Proteins transferred onto Immobilon membrane and
stained with Ponceau-S. C) Proteins transferred onto Immobilon membrane and stained with periodic acid/Schiff reagent. Lane M: molecular weight standards.

**Figure 5.** Inhibition assay of aspartic proteinases by JIP21. Reactions containing 50 ng of cathepsin D (A), pepsin (B) or proteinase A (C) were preincubated with increasing amounts of JIP21 in a final volume of 50 µl, then 10 µl of fluoresceinated haemoglobin (0.5% w/v) were added as a substrate. The proteolytic activity was determined as soluble fluorescence measured at 525 nm. Enzymatic activity is expressed in relative terms as the net emitted fluorescence with respect to the control reaction. 10 ng pepstatine was used as a positive inhibition control in all the assays. Results are the means of three independent assays.

**Figure 6.** Inhibitory activity of JIP21 on trypsin and chymotrypsin. Reactions containing 50 ng of trypsin (A) or chymotrypsin (B), and increasing amounts of JIP21 (continuous line), STI (Soybean Trypsin Inhibitor, dotted line) or BBI (Bowman-Birk inhibitor, dashed line) were preincubated in a final volume of 50 µl, and then 10 µl of fluoresceinated haemoglobin (0.5% w/v) were added as a substrate. The proteolytic activity was determined as soluble fluorescence measured at 525 nm. Enzymatic activity is expressed in relative terms as the net emitted fluorescence with respect to the control reaction. Three independent assays were performed for each protease experiment.

**Figure 7.** Comparison of the deduced amino acid sequence of JIP21 with the soybean trypsin inhibitor (STI, Genbank accession number S45092) and the winged bean trypsin inhibitor (WCI, Genbank accession number D13976). Identical or closely related amino acids are shaded in gray. Residues corresponding to the active sites of both STI and WCI, and the putative active site of JIP21, are boxed and highlighted in boldface.

**Figure 8.** Characterization of different JIP21-overexpressing tomato lines (2, 13 and 10). A) Northern blot analysis. Total RNA was extracted from transgenic lines and control plants, separated in formaldehyde-agarose gels and transferred onto Nytran membranes. Hybridization was performed at 65°C using JIP21 cDNA as a probe. B) Western blot analysis. Crude protein extracts were obtained, separated in 14% SDS polyacrylamide electrophoresis and transferred onto nitrocellulose membranes. The membranes were then incubated with JIP21 antibody (upper pannel) or with the tomato

TABLES

Table 1. Effect of the overexpression of JIP21 on the mortality and growth of Spodoptera littoralis larvae. Neonate larvae were placed on daily renewed detached tomato control or transgenic leaves. Sufficient humidity was provided by damp absorbing paper. Seven days later, the surviving larvae were counted and weighed. Bioassays were repeated 3 times.

<table>
<thead>
<tr>
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<th>Ratio of Mortality</th>
<th>Weight (mg)</th>
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<tbody>
<tr>
<td>Control</td>
<td>6 %</td>
<td>51.5 ± 5.1</td>
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<tr>
<td>Line 10</td>
<td>20 %</td>
<td>30.1 ± 4.6</td>
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Figure 1
Figure 2
Figure 3
Figure 4
Figure 5

A. Cathepsin D

B. Pepsin

C. Proteinase A
Figure 6

A

Trypsin

Remaining activity (%)

Inhibitor-to-protease molar ratio (fold)

B

Chymotrypsin

Remaining activity (%)

Inhibitor-to-protease molar ratio (fold)
Figure 7
Figure 8