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ORIGINAL PAPER

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DNA sequence analysis of a cyclophilin gene from maize: developmental expression and regulation by salicylic acid

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Abstract In plants, such as maize, cyclophilin (Cyp) genes are expressed at a basal level in all tissues. Amounts of Cyp mRNA above the basic level are observed in germinating seedlings, in growing tissues/organs such as roots and leaf meristematic tissue of young maize plants, nodes and embryonic female inflorescences of adult plants and also in non-proliferating tissues such as the internodes of adult plants. Salicylic acid (SA) enhances the transcription of maize Cyp genes. The possible involvement of SA in the pathway leading to defense responses induced by abiotic stresses such as mercuric chloride treatment is discussed. A maize Cyp genomic clone isolated using a maize Cyp cDNA probe contains 737 bp of the 5′ upstream and the entire coding region. This Cyp gene is not interrupted by intervening sequences. In the 5′ upstream region, characteristic transcription signals as well as putative regulatory sequences were identified. Two TATA boxes are found at positions –56 bp and –66 bp with respect to the transcription start site. Two putative heat shock elements were identified in the promoter region; a metal regulatory element and a third heat shock element were localized in the 5′ untranslated leader. Several putative polyadenylation signals and (G)T-rich sequence motifs were identified in the 3′ untranslated region.

Key words Cyclophilin · Peptidyl-prolyl *cis-trans* isomerase · Salicylic acid · Mercuric chloride

Introduction

Cyclophilin (Cyp) belongs to the family of immunosuppressant binding proteins termed immunophilins (Heitman et al. 1992; High and Handschumacher 1992; Schreiber and Crabtree 1992; Walsh et al. 1992). This protein was first identified in 1984 from bovine thymocytes on the basis of its affinity for cyclosporin A (CsA) (Handschumacher et al. 1984), a powerful immunosuppressive agent. Indeed, CsA blocks the transcription of cytokine genes, an early event during T-cell activation.

Later in 1989, it was found that Cyp is identical to a previously described enzyme named peptidyl-prolyl *cis-trans* isomerase or rotamase (EC 5.2.1.8) (Fischer et al. 1989; Takahashi et al. 1989). The enzyme catalyzes rotation of X-Pro peptide bonds (where X represents a given amino acid) and facilitates the folding of proteins in vitro and in vivo (for review see Gething and Sambrook 1992). This activity is inhibited by CsA.

A great deal of work, mainly focused on immunosuppression mechanisms by CsA, allowed the discovery of a family of highly conserved Cyp genes, encoding ubiquitous and abundant proteins with a common domain for CsA binding and enzyme activity, surrounded by unique domains involved in organelle and membrane targeting (for reviews see Heitman et al. 1992; Trandinh et al. 1992; Walsh et al. 1992). In view of all these properties, it has been suggested that Cyp may play an important role in protein folding, trafficking and complex formation.

The molecular cloning of plant Cyp genes began only recently and, to date, cDNA clones have been isolated from tomato, *Brassica napus* and maize (Gasser et al. 1990), bean (Marivet et al. 1992), *Arabidopsis thaliana* (Bartling et al. 1992) and tobacco (Marty et al. 1993). We have previously reported the isolation of a Cyp cDNA clone by differential screening of a mercuric chloride-treated maize cDNA library (Marivet et al. 1992). We have shown that Cyp genes are stress-responsive as their expression is enhanced by various abiotic stresses such as mercuric chloride treatment, heat shock, wounding, salt stress and low temperature. These observations suggest a possible involvement of cyclophilin in biological processes during stress conditions. In the present report we studied maize Cyp gene expression during germina-

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tion as well as in young and adult maize tissues. The effect of salicylic acid (SA) on maize Cyp gene expression has also been investigated and the possible involvement of SA in the mercuric chloride response has been examined. To understand the regulation of Cyp gene expression by various external stimuli and to investigate whether such regulatory sequences are associated with this gene, we have isolated a maize genomic clone, and to our knowledge, the sequence presented and analyzed here is the first of a plant Cyp gene.

Materials and methods

Plant material: growth and stress conditions

Maize (*Zea mays* L., var. INRA 258) plants were seeded in pots and grown in a greenhouse under standard conditions. Photoperiod was maintained for 16 h by additional illumination, temperature was 22°C during the day and 18°C at night. When 10–12 days old, maize plants were transferred to a growth chamber and maintained under the controlled greenhouse conditions. To analyze the effects of SA leaves were sprayed or plants watered once with a 10 mM SA solution. Control plants were sprayed with water and maintained under the same conditions as treated plants.

Seedlings were obtained from seeds germinated on Whatman 3MM paper moistened with distilled water and placed at 22°C in the dark. Prior to germination, seeds were sterilized for 10 min in a 2.5% calcium hypochlorite solution and rinsed several times with distilled water.

Leaves and seedlings were harvested at various time periods, frozen in liquid nitrogen and stored at –80°C for subsequent RNA isolations.

Isolation of genomic clones and DNA sequencing

Maize Cyp genomic clones were isolated from a λEMBL3-Sau3A genomic library (Clontech) constructed using total maize genomic DNA isolated from 7-day-old etiolated leaves. Library screening and subcloning were performed according to standard procedures (Sambrook et al. 1989). Filters were hybridized at 42°C with a α-[32P]dCTP-labelled (Feinberg and Vogelstein 1983) maize Cyp cDNA insert (Marivet et al. 1992). Positive clones were isolated by plaque purification and analyzed by digestion with restriction enzymes. A 2.6 kb *Kpn*I fragment from one of the positive clones was subcloned into the pBluescript KS^+ phagemid vector (Stratagene). Sequencing of the phagemid inserts was done by the dideoxy chain-termination method (Sanger et al. 1977). DNA sequence analysis was performed on a VAX computer using the GCG package program (Devereux et al. 1984). The sequence has been deposited in the EMBL data base under the accession number X68678.

Primer extension mapping of maize Cyp mRNA

Primer extension mapping of the maize Cyp transcript was carried out following the procedure of Ausubel et al. (1987) using 30 μg of total RNA extracted from mercuric chloride-treated plants and a synthetic 17-nucleotide oligonucleotide with the following sequence: 5′ ACGGTCATGTCGAAGAA 3′. The primer oligonucleotide complementary to the maize Cyp cDNA clone (Marivet et al. 1992) started 34 nucleotides downstream from the first ATG in the coding region. Primer extension products were analyzed by electrophoresis on a 6% (w/v) polyacrylamide sequencing gel and autoradiography.

RNA isolation and Northern blot analysis

Total RNAs were isolated from plant material by phenol/SDS extraction and LiCl precipitation (Ausubel et al. 1987). According to standard protocols (Sambrook et al. 1989), RNA samples (10 μg) were separated by electrophoresis in 1.2% formaldehyde-agarose gels, transferred onto Hybond N membranes (Amersham) and hybridized to ^{32}P-labelled Cyp cDNA (Marivet et al. 1992) by random priming (Feinberg and Vogelstein 1983). To check integrity and verify even loading of RNA samples, ethidium bromide was added to the samples prior to heating, which leads to markedly enhanced sensitivity of fluorescence detection (Rosen and Villa-Komaroff 1990). This method allows verification of the quantitative transfer onto the membrane by scanning on a Shimadzu CS-9000 scanner. The amount of RNAs transferred onto the membrane was also checked by using a bell pepper 25S RNA probe and scanning the autoradiogram.

Results

Expression of maize cyclophilin genes during germination, and in various tissues of young and adult maize plants

Northern blot analyses of maize Cyp mRNA levels in non-stressed tissues were performed to define the expression pattern of cyclophilin during normal development. Total RNAs were extracted from excised maize embryos collected every 12 h for 5 days after the beginning of germination, electrophoresed in a formaldehyde-agarose gel and transferred onto Hybond N membrane. Northern blots were hybridized to the maize Cyp cDNA probe (Fig. 1A). No maize Cyp mRNA was detected in non-germinating embryos, showing that this mRNA is not stored in dry seeds. Maize Cyp transcripts (about 0.9 kb) were first detected about 2 days after onset of germination and their level increased to reach a maximum during the third and fourth days and thereafter Cyp mRNA remained present in significant amounts.

Total RNAs were also extracted from different tissues/organs of young and adult maize plants and blot-hybridized to the maize Cyp cDNA probe (Fig. 1B–D). The genes encoding maize Cyp are expressed at significant levels in young and adult plants in all tissues examined. However, the expression pattern revealed differences between the various tissues: as compared to the levels observed in young or adult leaves, higher levels of transcripts were observed in leaf meristematic tissue and roots of young plants (Fig. 1B) and in nodes, internodes, female and male inflorescences of adult plants (Fig. 1D).

Regulation of maize cyclophilin gene expression by SA

To determine whether the expression of maize Cyp genes is enhanced by SA, a compound thought to act as an endogenous signal molecule responsible for induction of plant defense responses (Enyedi et al. 1992; Malamy and Klessig 1992), maize plants were treated with exogenously applied SA and time course studies of Cyp mRNA synthesis performed. Total RNAs were extracted from

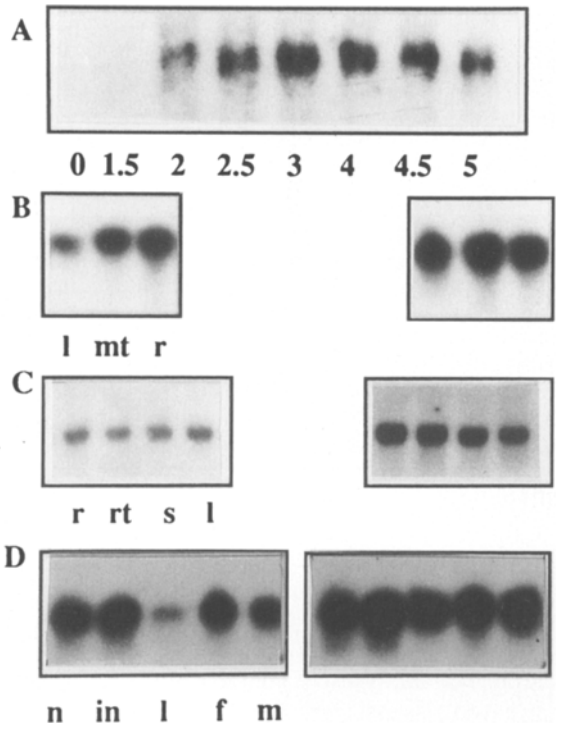

Fig. 1A–D Northern blot analysis showing the levels of maize cyclophilin (Cyp) mRNA during germination and in various young and adult maize tissues. **A** Different stages of germination from 0–5 days as indicated below each lane. **B** Different tissues from young maize plants (10 to 12-day-old). (*r*, root; *mt*, leaf meristematic tissue; *l*, expanded leaves) **C**, **D** Different tissues of adult plants (1.5–2 m high bearing 13–15 nodes). **C** *r* root cut above the tip; *rt*, root tip; *s* sheath; *l*, leaf. **D** *n*, node; *in*, internode; *l*, leaf; *f*, embryonic female inflorescence; *m*, mature male inflorescence. Total RNAs extracted from the indicated tissues were separated on formaldehyde-agarose gels, transferred onto membranes and hybridized with the ^{32}P-labelled insert from the maize Cyp cDNA clone. **B–D** (right side) show the corresponding Northern blots hybridized with the bell pepper RNA probe used as a control for even loading

SA-sprayed leaves harvested at various times after onset of the treatment. Northern blots were hybridized with maize Cyp cDNA used as a probe (Fig. 2A). Spraying with SA resulted in a stimulation of maize Cyp gene expression: two maxima, one at 3–6 h and the second at 72 h after the beginning of treatment, were observed. The same pattern was observed on watering the plants with SA (results not shown).

Effect of temperature on cyclophilin gene expression enhanced by mercuric chloride or SA treatments

A set of experiments was performed to investigate the effect of temperature on SA- or mercuric chloride-stimulated Cyp gene expression. The experiments were carried out on plants treated with either mercuric chloride or SA or simultaneously with both chemicals and then placed at 32°C or maintained at 22°C. Leaves were harvested 6 h after onset of the treatments and RNAs were

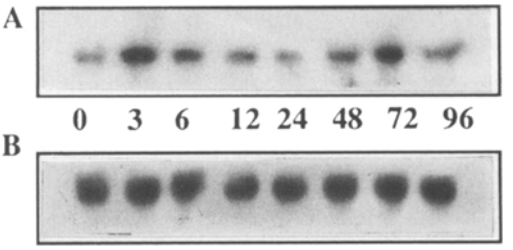

Fig. 2A,B Northern blot analysis showing the time course of Cyp mRNA accumulation in response to salicylic acid (SA) treatment. Gels contained equal amounts of total RNA isolated at various time points (indicated in hours below each lane) from SA-sprayed plants. The blot was hybridized with maize Cyp cDNA (**A**) or bell pepper RNA as a control (**B**)

extracted. The 6 h time point was chosen because a significant increase in Cyp gene expression has taken place in mercuric chloride-treated plants by this time (Marivet et al. 1992) and in SA-treated plants as well (see above). Maize Cyp mRNAs are present in control plants at a basal level, and no effect was observed when the temperature was raised from 22°C to 32°C (Fig. 3A–C, lanes a and b). As expected, at 22°C, higher levels of maize Cyp mRNAs were found in mercuric chloride-treated plants than in control plants (Fig. 3A–C, lane c). However, when mercuric chloride-treated plants were placed at 32°C, no increase in mRNA synthesis was observed (Fig. 3A–C, lane d). SA treatment also resulted in the synthesis of larger amounts of maize Cyp mRNAs (Fig. 3A–C, lane g), but unlike what has been observed in mercuric chloride-treated plants, the higher temperature did not abrogate the effect of SA on mRNA synthesis (Fig. 3A–C, lane h). In plants treated simultaneously with mercuric chloride and SA and maintained at 22°C, the amounts of Cyp mRNA observed were slightly higher than those found in plants treated with mercuric chloride or SA alone (Fig. 3A–C, lane e), thus suggesting a slight additive effect of the two treatments. As in SA-treated plants, elevated temperature had no inhibiting effect on the enhanced gene expression, but significantly strengthened the stimulating effect (Fig. 3A–C, lane f).

Isolation and characterization of a genomic clone encoding maize cyclophilin

A maize genomic library constructed in the bacteriophage λ vector EMBL3 library (Clontech) was screened by plaque hybridization using the maize Cyp cDNA clone (Marivet et al. 1992). Out of 3×10^4 recombinant phages, four Cyp-specific clones were obtained and purified through three rounds of plaque purification. Analysis of one of these clones by digestion with various restriction endonucleases and hybridization of the resulting fragments with the cDNA probe indicated that the Cyp gene is confined to a 2.6 kb *Kpn*I fragment, which was subcloned into the pBluescript KS$^+$ phagemid vector (Stratagene). The genomic nucleotide sequence of

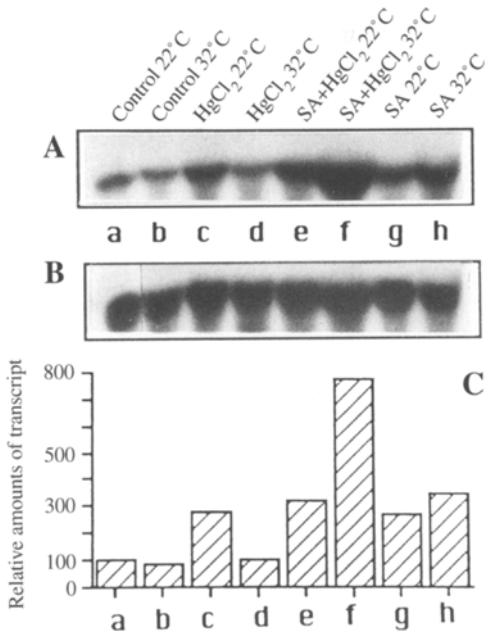

Fig. 3A–C Effects of temperature on Cyp gene expression on mercuric chloride or SA-treated plants. Northern blots containing equal amounts of total RNAs isolated from control plants kept at 22°C (a) or for 6 h at 32°C (b), from mercuric chloride-treated plants at 22°C (c) or at 32°C (d), from plants treated simultaneously with mercuric chloride and SA at 22°C (e) or 32°C (f) and from SA-treated plants at 22°C (g) or 32°C (h) and hybridized with maize Cyp cDNA (**A**) or bell pepper RNA (**B**). **C** The Histogram represents the mean values of three different experiments identical to the one shown in **A** and performed with different RNA preparations. The amount of hybridizing RNA measured in unstressed plants was taken as the 100% value. RNAs were isolated 6 h after onset of the treatments from plants maintained at 22°C or from plants placed at 32°C for 6 h with or without treatment with the chemicals

this fragment is shown in Fig. 4. This sequence contains an uninterrupted open reading frame indicating that the maize Cyp gene does not contain introns. The open reading frame of 516 nucleotides would encode a protein of 172 amino acids with a calculated molecular weight of 18.2 kDa and an isoelectric point of 8.9. Upstream of the translation initiation codon, at position 310 bp, an in-frame termination codon (TAG) has been found, indicating that this clone cannot encode a larger preprotein. The DNA site corresponding to the transcriptional initiation site was determined by primer extension experiments. Only one major extension product was observed (Fig. 5), indicating that the transcription initiation site starts with an A (+1) (Fig. 4) located 324 bp beyond the translational start site.

Flanking sequences

In the 3′ untranslated region, three putative polyadenylation signals (Joshi 1987a), AATAAG, AATTTA and GATAAT are located at positions 78 bp, 136 bp and 220 bp downstream from the stop codon, respectively (Fig. 4). Sequence comparison with maize Cyp cDNA clones (Marivet et al. 1992) did not allow precise definition of the poly(A) addition site because of the presence of three A residues in the genomic sequence (positions 1082, 1083 and 1084) at this site. Downstream, the possible poly(A) addition sites, and three (G)T-rich sequence motifs, GGTTGTG, TGGTTGTT and GTGTTTT (Joshi 1987a), were found.

Analysis of the 737 bp genomic 5′ flanking sequence of the maize Cyp gene led to the identification of structural elements which might be involved in the regulation of Cyp gene expression. For example, two sequence motifs (TATATA and TATA), similar to the TATA box (Joshi 1987b), occur at positions –66 bp and –56 bp, respectively. Heat shock elements (HSE, nGAAnnTTCn) (for reviews see Gurley and Key 1991; Sorger 1991), occur at positions –568 bp and –456 bp, and interestingly, in the 5′ untranslated leader, two motifs were identified: a motif TGGACACA similar to a metal regulatory element (MRE) TGCACACC (6/8 bases) (Evans et al. 1990) found at position 10 bp and a HSE at position 121 bp.

Discussion

Cyclophilin genes are known to be constitutively expressed in a broad range of tissues/organs in vertebrates (Bergsma et al. 1991; Danielson et al. 1988; Hasel and Sutcliffe 1990; Iwai and Inagami 1990; Koletsky et al. 1986; Schumacher et al. 1991) and at a higher level in young tissues (Caroni et al. 1991; Jakubowski et al. 1991) and in mitotic cells (Koletsky et al. 1986; Sarris et al. 1992). The results presented here show that in plants, such as maize, genes encoding cyclophilin are also expressed at a basal level in all tissues investigated; amounts of maize Cyp mRNA above the basal level were observed in germinating seedlings and in growing tissues/organs such as roots and leaf meristematic tissue of young maize plants, nodes and embryonic female inflorescences of adult plants. Our results are consistent with those reported for other plant species. Thus, in tomato and *B. napus*, Cyp genes are highly expressed in a number of young tissues such as young leaves, floral buds, growing shoots, immature stamens and ovaries (Gasser et al. 1990) and in tobacco, higher expression is observed in young leaves, young roots and flower buds and in growing tobacco cells than in mature tissues and resting cells (Marty et al. 1993). An enhanced expression of Cyp genes in growing tissues and in germinating seeds suggests a correlation between Cyp gene expression and the proliferative state of the cells. This supports the hypothesis of a possible involvement of cyclophilin in the process of protein synthesis. It is well known that throughout the growth period of a tissue or during germination, active synthesis of all kinds of proteins takes place, and such proteins must undergo maturation. It is therefore conceivable that proteins involved in maturation processes such as cyclophilins, which are believed to catalyze

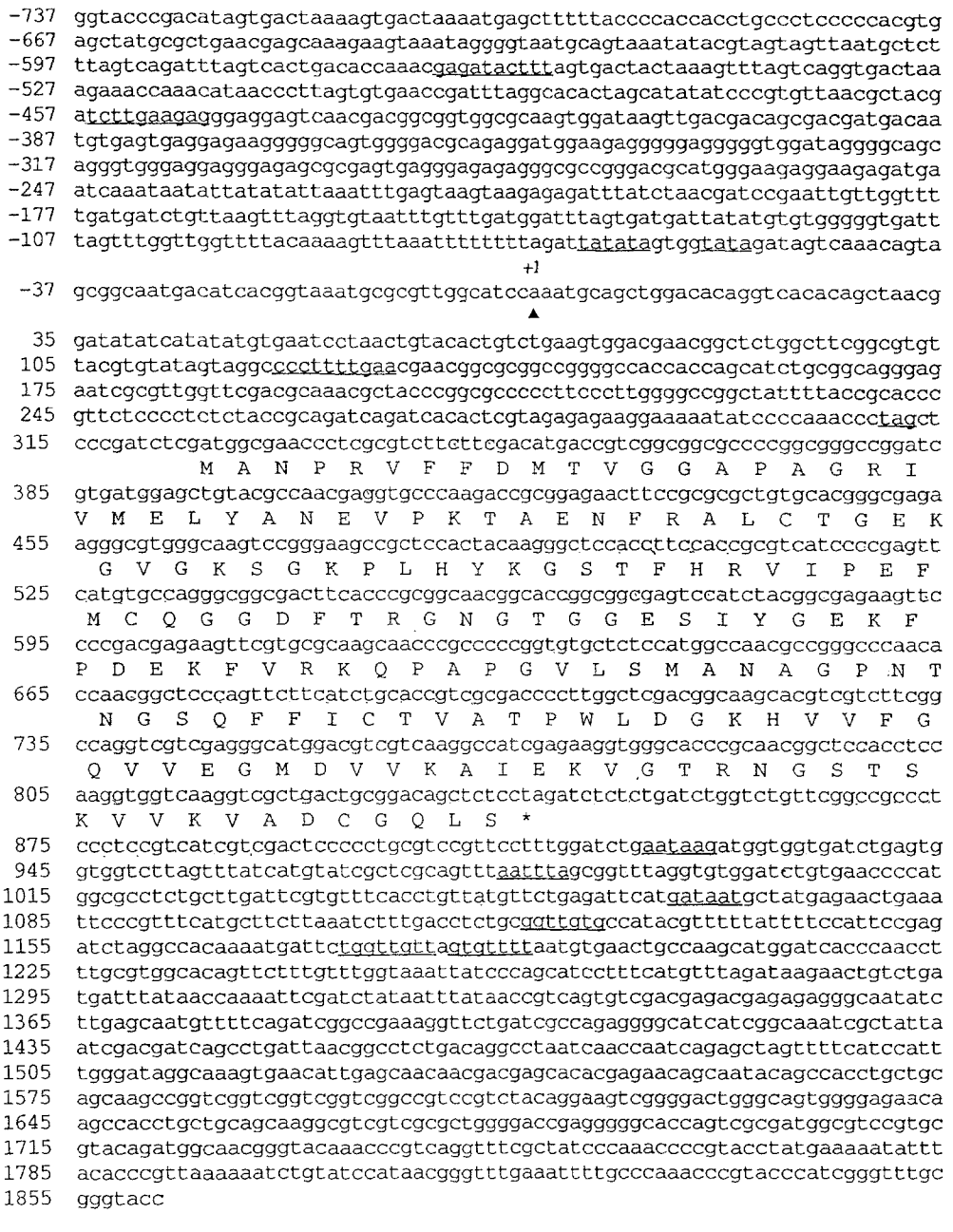

Fig. 4 Nucleotide sequence and deduced amino acid sequence of a maize Cyp gene and 5′ and 3′ flanking regions. The sequence of the coding strand is shown numbered in the left-hand column. The transcription initiation site is marked by a *vertical arrow* (+1). TATA box, potential heat shock elements and possible polyadenylation sites are *underlined*

protein folding, might be needed in higher amounts to accelerate protein maturation. Surprisingly, our experiments show that increased amounts of maize Cyp transcripts are also found in tissues with no known mitotic activity, such as internodes. Whether this finding is of any functional significance remains to be determined.

We demonstrated in a previous report that, in higher plants, Cyp gene expression is regulated in part by various forms of environmental stress, including heat, cold and salt stresses, chemical treatment and wounding (Marivet et al. 1992). To gain more insight into the signalling process, experiments were performed with SA, a potent chemical inducer of pathogenesis-related (PR) proteins, which is believed to be a natural signal in the induction of defense responses, specifically those associated with infection processes (for reviews see Enyedi et al. 1992; Malamy and Klessig 1992). We were curious as to whether exogenously applied SA had any effect on Cyp gene expression. The significant increase in maize Cyp mRNA level observed shows that the effect of SA is not restricted to the induction of PR proteins. The time course of Cyp mRNA synthesis is noteworthy, showing two maxima, upon SA treatment. This bi-phasic pattern has not been observed for other chemical inducers (Marivet et al. 1992). One interpretation is that SA activates a second, different signal transduction pathway after a given period of time. Alternatively, some positive feedback mechanism may operate to potentiate the first signal transduction pathway.

To further analyze Cyp gene expression upon SA and mercuric chloride treatments, we took advantage of the

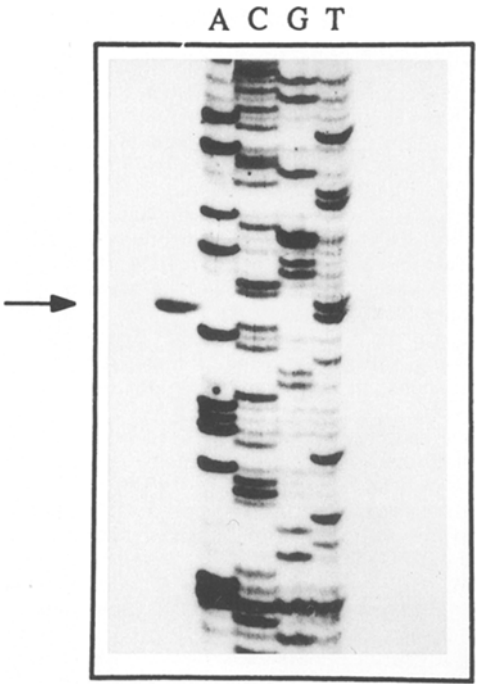

Fig. 5 Primer extension mapping to identify the 5′ end of the maize Cyp gene transcript. Primer extension products, using 30 μg maize total RNA and 0.5 pmol primer (oligonucleotide complementary to the sequence: positions 343–359 in Fig. 4), were separated in a 6% sequencing gel together with unrelated sequencing reactions used as a DNA molecule size ladder. The *arrow* indicates the position of the single primer extension product obtained

temperature sensitivity of part of the defense responses. It has been reported that in virus-infected plants placed at 32°C, SA production is blocked and several PR proteins are not synthesized (Malamy et al. 1992; Yalpani et al. 1991). However, when exogenous SA is applied, plants express PR proteins at elevated temperatures, indicating that the lack of induction of PR protein genes is due to the absence of SA. Similarly, when mercuric chloride-treated maize plants are placed at 32°C, the increase in Cyp mRNA synthesis, usually observed at 22°C, did not occur and the amount of Cyp transcripts remained at the basal level. However, the application of SA to mercuric chloride-treated plants maintained at 32°C resulted in a strikingly enhanced Cyp mRNA synthesis. The similarity in the responses observed in virus-infected plants and in mercuric chloride-treated maize at elevated temperatures in the presence of SA, leads to the conclusion that SA might be involved in the pathway of Cyp mRNA synthesis in response to mercuric chloride treatment.

In order better to understand Cyp gene regulation, a Cyp genomic clone has been isolated and characterized. It is likely that the isolated gene is not the only Cyp gene expressed in maize, as two major DNA fragments and six minor fragments were detected by Southern blot analysis (Marivet et al. 1992). This maize Cyp gene is not interrupted by intervening sequences. The DNA sequence of the gene shows complete identity with the previously isolated cDNA clones of 792 bp (Gasser et al. 1990) and 814 bp (Marivet et al. 1992) encoding a maize cyclophilin. Analysis of the 5′ flanking sequence at the nucleotide level revealed the presence of characteristic transcription signals, such as TATA boxes, as well as putative regulatory sequences described in genes responsive to various environmental stimuli. The proposed TATA boxes are found 56 bp and 66 bp from the start site. The distance often observed for the TATA box in higher plant genes transcribed by RNA polymerase II is approximately 30 bp upstream of the transcription start site, however, larger distances have been observed in maize genes encoding triose isomerase (66 bp) and zein (63 bp) (Joshi 1987b). Among several notable motifs, the presence of heat shock elements is of particular interest; the presence of these elements is consistent with our observation reported earlier that maize Cyp gene expression is stimulated by high temperature (42°C) (Marivet et al. 1992). Another observation with relevance to our finding has recently been made. In yeast the expression of two Cyp genes is induced by heat, and the induction is mediated through a *cis*-acting sequence similar to the well-characterized HSE (Sykes et al. 1993). Two additional motifs (HSE and MRE), have been identified in the 5′ non-coding region. The functional significance of this finding has to be determined; these elements are probably not involved in gene regulation at the level of transcription but might rather have an effect on the stability or translation of the mRNA. Further experiments are needed before all these sequence motifs can be functionally characterized.

The putative polyadenylation signal (GATAAT) found approximately 20 nucleotides upstream from the deduced poly(A) sites (the consensus distance in animal genes is 10–33 nucleotides), closely resembles the most conserved plant polyadenylation signal (AATAAT) (Dean et al. 1986).

That Cyp gene expression is stimulated under various stress conditions, clearly suggests that cyclophilin may participate in plant defense mechanisms. Determination of the precise role of this protein may be approached by specifically inactivating or overproducing cyclophilin within a plant. Pending further information, we suggest that cyclophilin may function as a chaperone-like molecule implicated in the folding and maturation of de novo synthesized proteins during stress conditions.

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