The Heat-Shock Element Is a Functional Component of the Arabidopsis APX1 Gene Promoter

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Ascorbate peroxidases are important enzymes that detoxify hydrogen peroxide within the cytosol and chloroplasts of plant cells. To better understand their role in oxidative stress tolerance, the transcriptional regulation of the apx1 gene from Arabidopsis was studied. The apx1 gene was expressed in all tested organs of Arabidopsis; mRNA levels were low in roots, leaves, and stems and high in flowers. Steady-state mRNA levels in leaves or cell suspensions increased after treatment with methyl viologen, ethephon, high temperature, and illumination of etiolated seedlings. A putative heat-shock cis element found in the apx1 promoter was shown to be recognized by the tomato (Lycopersicon esculentum) heat-shock factor in vitro and to be responsible for the in vivo heat-shock induction of the gene. The heat-shock cis element also contributed partially to the induction of the gene by oxidative stress. By using in vivo dimethyl sulfate footprinting, we showed that proteins interacted with a G/C-rich element found in the apx1 promoter.

AOS such as superoxide radicals, hydrogen peroxide, and hydroxyl radicals are continuously formed in aerobic organisms. AOS cause oxidative damage of cell constituents (Halliwell and Gutteridge, 1989), and their involvement in different kinds of biotic or abiotic stresses such as chilling, drought, environmental pollution (ozone, sulfur dioxide), and pathogen attack is well documented (Bowler et al., 1992).

Plants have both nonenzymatic and enzymatic AOS-detoxification systems. Several small antioxidant molecules, such as ascorbic acid, glutathione, α-tocopherol, carotenoids, and flavonoids, can quench all kinds of AOS (Halliwell and Gutteridge, 1989). Because of the simple chemical nature of the quenching reactions, some of these molecules have to accumulate to very high concentrations within the cells to be effective (Loewus and Loewus, 1987). Several enzymes can efficiently detoxify AOS; whereas superoxide radicals are disproportionately detoxified by superoxide dismutases, hydrogen peroxide is destroyed by catalases and different kinds of peroxidases (Bowler et al., 1992).

A major hydrogen peroxide-detoxifying system in plant chloroplasts and cytosol is the so-called ascorbate-glutathione cycle, in which APXs are the key enzymes (Asada, 1994). So far, APX activity has been found in plants, algae, and some cyanobacteria, and it has also recently been identified in insects (Mathews et al., 1997). APX has been purified and characterized from several plant species. Functionally and structurally distinct from the typical peroxidase superfamily, APX is unique in having a high specificity toward ascorbic acid as an electron donor (for review, see Asada, 1994). The APXs are a fast-growing family of proteins. Several different protein isoforms are known: two soluble cytosolic forms, several cytosolic forms bound to membranes of glyoxisomes and peroxisomes, and two chloroplastic forms, one of which is stromal and the other is thylakoid bound (for review, see Jespersen et al., 1997). Analysis of the expressed sequence tag databases has allowed the distinction of as many as seven different types of APXs in Arabidopsis (Jespersen et al., 1997). Recently, APX activity in pea mitochondria has been reported (Jimenez et al., 1997).

APX is believed to be involved in the detoxification of photoproduced hydrogen peroxide. The activities of the cytosolic and chloroplastic APX increase in carotenoid-less mustard seedlings exposed to light because of the increased rate of AOS photoproduction (Thomsen et al., 1992). Furthermore, APX activity has been shown to increase in response to a number of stress conditions, such as drought (Smirnoff and Colombé, 1988; Tanaka et al., 1990; Mittler and Zilinskas, 1994), air pollution (Tanaka et al., 1985; Mehlihorn et al., 1987; Conklin and Last, 1995; Kubo et al., 1995; Rao et al., 1996), high light intensity combined with chilling (Schöner and Krause, 1990) or deficiency in microelements (Cakmak and Marschner, 1992), iron stress (Vansuyt et al., 1997), excessive light (Karpinski et al., 1997), UV-B light (Rao et al., 1996), and salt stress (Lopez et al., 1996). In some cases posttranslational components are

Abbreviations: AOS, active oxygen species; APX, ascorbate peroxidase; apx1(HSEmut), apx1 promoter-gus fusion mutated in HSE; apx1(HSEwt), wild-type apx1 promoter-gus fusion; DMS, dimethyl sulfate; hHSF, hexahistidine-HSF fusion; HSE, heat-shock cis element; HSF, heat-shock factor; tHSF, T7 immunotag-HSF fusion.
involved in the regulation of the APX activity (Mittler and Zilinskas, 1994; Lopez et al., 1996). However, increases in APX activity are usually accompanied by transcriptional activation of the gene. In spite of this fact, surprisingly little is known about the mechanisms underlying this regulation or about the promoter organization of the APX genes.

At present the sequences of three APX genes are available: two from Arabidopsis (apx1 and apx2; Kubo et al., 1993; Santos et al., 1996) and one from pea (apx1; Mittler and Zilinskas, 1992). All of them code for cytosol-soluble isozymes of APX that are highly homologous to each other (approximately 80% amino acid identity). However, apx2 from Arabidopsis seems to differ from the Arabidopsis apx1 gene as well as from the pea apx1 gene in many respects, particularly in the induction pattern, and represents another isoform of cytosol-soluble APX (Santos et al., 1996; Karpinski et al., 1997).

In pea apx1 gene expression is induced by oxidative stress, heat, and drought stress and by ethylene and ABA (Mittler and Zilinskas, 1992, 1994). In Arabidopsis the expression of the apx1 gene was shown to be induced by ozone, sulfur dioxide, and excessive light (Kubo et al., 1995; Karpinski et al., 1997). Sequence comparison of the promoter of the pea and Arabidopsis apx1 genes has revealed the presence of only one region of high homology that is located around the TATA box and contains several sequence motifs characteristic of the HSE identified in promoters of all heat-shock-inducible genes. The heat-shock response is a general reaction of all organisms after exposure to elevated temperatures and is characterized by a rapid synthesis of a set of specific heat-shock proteins (for review, see Nover, 1991). However, the heat-shock response can be induced by other stresses, particularly by oxidative stress (Morgan et al., 1986; Courgeon et al., 1988; Liu and Thiele, 1996; McDuffee et al., 1997). Conversely, heat shock can result in an oxidative stress, which induces genes involved in the oxidative stress defense (Morgan et al., 1986).

We present an analysis of the Arabidopsis apx1 gene promoter. Data from in vitro interactions of a tomato (Lyccopersicon esculentum) HSF with the apx1 promoter and mutational analysis confirmed that the HSE is responsible for the heat-shock induction of the gene and partially contributes to the induction by oxidative stress. Other putative regulatory cis elements were characterized by in vivo footprinting.

**MATERIALS AND METHODS**

**Plant Material**

Plants of Arabidopsis ecotype Columbia were grown in soil at 20°C under a 16-h light/8-h dark regime. The Arabidopsis suspension culture was grown on a rotary shaker in B5 Gamborg medium (GIBCO-BRL) supplemented with 1 mg L\(^{-1}\) 2,4-D. For the expression analysis, plants were sprayed with 10\(^{-6}\) M methyl viologen or with 15 mM ethephon. For the light-induction experiments, 5- to 6-d-old etiolated seedlings were exposed to light. Protoplasts were prepared from soil-grown plants essentially as described previously (Altman et al., 1992).

**Screening of Arabidopsis cDNA and Genomic Libraries**

Phages (5 \times 10^5\) of an Arabidopsis cDNA library in Agt10 were screened with a probe prepared from a spinach apx1 cDNA (S. Kushnir, unpublished results). Twelve positive plaques were purified to homogeneity and the two longest cDNAs were sequenced on both strands.

To clone the apx1 gene, 3 \times 10^6 plaques of an Arabidopsis ecotype Landsberg erecta genomic library in \(\lambda\) Charon 35 were screened with the Arabidopsis apx1 cDNA probe. After the two overlapping clones, APAG7 and APAG3, were isolated and mapped, the apx1 gene sequence was determined on both strands, including 1.5 kb of the promoter sequence.

**RNA Analysis**

RNA was extracted from Arabidopsis organs according to the method of Shirzadeh et al. (1991). RNA gel-blot analysis of glyoxylated RNA was performed according to standard procedures (Ausubel et al., 1993). To ensure equal loading of RNA, blots were stained with methylene blue (Ausubel et al., 1993). Hybridizations of RNA blots were according to the method of Church and Gilbert (1984). Poly(A\(^+\)) mRNA was prepared using oligo(dT)-beads (Dynabeads, Dynal, Oslo, Norway) as recommended by the manufacturer. The transcription start was mapped by T4 DNA polymerase primer extension mapping (Hu and Davidson, 1986) and by the PCR amplification of 5' ends of the apx1 mRNA (Troutt et al., 1992).

**HSF Expression**

A tomato (Lycopersicon esculentum) HSF cDNA was amplified by PCR from tomato cDNA using Pfu polymerase (Stratagene) and the primers CCAACTTCACCTCAGTATAAACC and GGATCCCATATGTCGCAAAGACGCCGGCC. Primers were designed according to the known cDNA sequence of the tomato HSF B2 (Scharf et al., 1990; Nover et al., 1996). The cloned PCR fragment was sequenced and the encoded protein was identical to HSF B2 except for a few differences in amino acid sequence (data not shown). The tomato HSF B2 cDNA was subcloned in the pQE8 (Qiagen, Chatsworth, CA) and pET11a (Novagen, Madison, WI) expression vectors to produce an in-frame amino-terminal fusion with a stretch of six His residues (the fusion protein referred to as hHSF) and with a T7 immunotag (the fusion protein referred to as tHSF), respectively. The expression of proteins was in Escherichia coli strains M15 (Qiagen) for pQE8 or BL21 for pET11a. The protein synthesis was induced in logarithmically grown E. coli cultures in Luria-Bertani medium (37°C) by the addition of 1 mM isopropyl-b-D-thiogalacto-pyranoside, and the cultures were grown for an additional 4 to 5 h at different temperatures. HSF active in DNA binding was obtained only when E. coli cells were grown at 25°C to 28°C but not at 37°C. Most of the hHSF fusion protein was found.
in inclusion bodies regardless of the temperature at which cells were grown, whereas the tHSF was detected in soluble form and with high activity in DNA binding.

Both HSF proteins were purified under native conditions. The hHSF was purified as recommended by the manufacturer on a nickel-chelating column (Qiagen), dialyzed against TM buffer (Kroeger et al., 1993), and stored at −70°C.

For the tHSF preparation 200 mL of induced E. coli culture was used. The tHSF was purified as follows: after induction, cells were harvested by centrifugation and the pellet was resuspended in TM buffer supplemented with 20 mg mL⁻¹ leupeptin. Cells were disrupted by sonication and the homogenate was cleared by centrifugation in a SW27 rotor (Beckman) for 30 min at 4°C. The supernatant was mixed with 5 mL of CM Sephadex G-50 (Pharmacia, Uppsala, Sweden) equilibrated in the same buffer, and proteins were allowed to absorb for 30 min at 4°C. Sephadex was then packed in a small column and washed with TM buffer. Bound proteins were eluted by TM buffer containing 0.4 M KCl. The eluate was diluted 2-fold with TM buffer, applied to a heparin-Sepharose column (5 mL) equilibrated with TM buffer containing 0.2 M KCl, washed, and subsequently eluted with 0.4 M KCl in TM buffer. At this step, the tHSF was approximately 80% pure and the major contaminating proteins were truncated forms of the recombinant protein that were efficiently removed by gel filtration in TM buffer on an Ultrahydrogel 500 column (7.8 mm × 30 cm [Waters]). The tHSF peak fractions were separated into aliquots and stored at −70°C.

**Gel-Shift Analysis and in Vitro Footprinting**

Two apx1 promoter fragments were amplified by PCR and subcloned in pBluescript KS+ (Stratagene). Fragment A contained sequences from −274 to +42 (primers CCCTC-CACACGAAAGCATGTATCC and CTGGAGAAATGCGA), and fragment B contained sequences from −246 to −499 (primers ATGGAGGATACTTCGGTGG and GGTGAGAAACCTAATAACACTG). The 3' and 5' end-labeled fragments were prepared according to standard procedures (Ausubel et al., 1993).

DNase I footprinting was done essentially as described previously (Kroeger et al., 1993). Labeled probe (20,000-50,000 CPM), 1 μg of poly(dIdC)-poly(dIdC), and tHSF were incubated in binding buffer (50 mM Tris-HCl, pH 7.3, 50 mM NaCl, 5 mM MgCl₂, 1 mM EDTA, 5% Suc, and 5% glycerol) in a total volume of 20 μL. After the sample was incubated for 20 to 30 min at room temperature, 2 μL of a Ca/Mg mixture (10 mM CaCl₂ and 10 mM MgCl₂) and 2 μL of DNase I (Pharmacia) dilution were added to the binding buffer. After 1 min of DNase I digestion, samples were processed as described previously (Kroeger et al., 1993). The same labeled fragment was chemically cleaved at the G and A bases following standard procedures (Ausubel et al., 1993) to provide molecular mass markers.

For the electrophoretic mobility-shift assays, labeled fragments were incubated with tHSF or hHSF under the same conditions as for the DNase I footprinting, and DNA-protein complexes were resolved by native 5% PAGE in 0.5× Tris-borate buffer at room temperature. Variables were the amount of nonspecific competitor DNA, the type of competitor DNA (salmon-sperm), and the presence or absence of specific competitors (fragments A and B).

**apx1 Promoter-gus Fusion Construction, Site-Directed Mutagenesis of HSE, and Plant Transformation**

Fusion at the ATG start codon of the gus reporter gene was obtained after site-directed mutagenesis by PCR. The apx1 gene-specific primer CCATGGTAGCTAAGCTTCTGTCGAACAA was used to introduce a NcoI site at the ATG start codon. The amplified 1.2-kb fragment was digested with NcoI and HindIII and subcloned into NcoI plus HindIII-cleaved pGUSI plasmid (Peleman et al., 1989). apx1 promoter-gus fusions were subcloned as EcoRI-HindIII fragments into the binary vector pGVS6 (Plant Genetic Systems N.V., Gent, Belgium). Binary vectors were transformed into Agrobacterium tumefaciens C58C1Rif¹ (pGV2260) according to the method of De Block et al. (1987). Arabidopsis C24 was transformed according to the method of Valvekens et al. (1988).

To introduce point mutations into the HSE, site-directed mutagenesis was done by PCR. The HSE-specific primer CAGATCTACCATAACATTATCATTAATGAC with two mutated bases was used as a mutagenic PCR primer, whereas the above-mentioned apx1 gene-specific primer generating the NcoI site at the ATG codon was utilized as the second PCR primer. The wild-type sequence in the apx1 promoter-gus fusion was replaced with an amplified genomic fragment with mutated HSE using BglII and NcoI sites.

To study the induction of the gene with mutated HSE, 2-week-old, in vitro-grown Arabidopsis seedlings of four independent transgenic lines transformed with the mutant or wild-type apx1 promoter-gus fusion were pooled and infiltrated with 10⁻⁵ M methyl viologen. To induce heat shock, plants were grown in a chamber (Weiss Climatech GmbH, Reiskirchen, Germany) at 22°C and then subjected to single-step increases in temperature to 37°C.

**In Vivo Footprinting**

The ligation-mediated PCR version of in vivo footprinting was used (Mueller and Wold, 1989; Pfeifer et al., 1989). Arabidopsis protoplast suspensions (10 mL) were treated with 0.2% DMS in W5 salt solution (Altmann et al., 1992) at room temperature for 5 and 10 min. Protoplasts were then pelleted and washed with 10 mL of ice-cold W5 solution. The protoplast pellet was lysed with a lysis buffer and DNA was purified as described above. The DNA samples were processed as described previously (Mueller and Wold, 1989; Pfeifer et al., 1989). Three specific overlapping oligonucleotides (CCTTAGTCCAATTGGGATCTTCGCCGAGACCA at position −415, TTGGGATCTTCTCGTCGACCCG, and CGCCTCGGTCGACCCGCGTCACACCTGCG) were used in the ligation-mediated PCR footprinting of the apx1 promoter. The adaptor for the ligation was prepared by annealing two oligonucleotides (GGGTGACCCCG-
GAGATCTGAATTG and GAATTTCAGGTC (Mueller and Wold, 1989).

**RESULTS**

Previously, a cDNA and a gene encoding the cytosolic APX from Arabidopsis were cloned (Kubo et al., 1992, 1993). The independent isolation, and sequencing of the gene, and mapping of the transcription start confirmed the published data (data not shown).

A comparison of the promoter sequences showed that the Arabidopsis apx1 gene shared only one region (approximately 44 bp) with strong homology to the promoter of the pea apx1 gene (80% identity over 44 bp; Fig. 1B). This region, located around the TATA box, contains a putative HSE. The promoter sequence of another APX gene from Arabidopsis, apx2, had no extended sequence homology with the promoter of apx1. However, two HSE-like sequences were also present in the promoter of the apx2. A first HSE was identified by Santos et al. (1996) at position −293, but careful sequence inspection exposed a second HSE at position −205.

Computer analysis of the Arabidopsis apx1 gene also revealed the presence of two other elements, the H box and the as-1/ocs box, which are possibly recognized by Myb and b-Zip proteins, respectively (Fig. 1A). The as-1/ocs-like motifs were also found in the pea apxl promoter and were similar to the xenobiotic responsive element that is recognized by AP-1-like b-ZIP transcription factors in mammalian cells (Mittler and Zilinskas, 1992).

![Figure 1](image)

**Figure 1.** A, Schematic representation of the apx1 promoter organization. ATG indicates the initiating translation codon. An intron located in the 5′ untranslated region and the transcription start are marked by a black box and an arrowhead, respectively. The positions (in bp) of the putative and identified cis elements as related to the transcription start are indicated above the line. as/ocs, Sequences similar to the cis elements as-1 from the 35S cauliflower mosaic virus promoter (Benfey and Chua, 1990) and ocs from the octopine synthase promoter (Singh et al., 1990), which are recognized by a member of the b-Zip family of DNA-binding proteins; H, sequence similar to the H box, recognized by proteins from the myb family (Sahlofski et al., 1994); G/C, G- and C-rich sequence for which in vivo footprints have been identified in this study. B, Comparison of the heat-shock elements from pea (P.s.) and Arabidopsis (A.th.) apx1. Sequences matching the nGAAn, the basic 5-bp HSE motif, are indicated in uppercase letters. The two central motifs are in reverse orientation and perfectly match requirements for the minimal HSF-binding motif nGAAnTTCn. They are flanked by two other motifs, of which the upstream motif has one tolerated substitution and the downstream motif perfectly fits the nGAAn consensus except that it is shifted on 1 bp and has a direct instead of a reverse orientation to the adjacent 5-bp motif. Orientations of the nGAAn-like motifs are indicated by arrows. The sequence of the TATA box is shown in bold uppercase letters.

**Figure 2.** RNA gel-blot analysis of APX mRNA. A, Size and levels of apx1 mRNA in roots (R), stems (S), flowers (F), and leaves (L) of soil-grown Arabidopsis. Labeled and glyoxylated HindIII fragments were loaded to evaluate the size of the mRNA, which is indicated in bases. B, The effect of methyl viologen (10−6 M), ethephon (15 mM), and light on the apx1 mRNA levels of in vitro-grown cells, mature plants, and etiolated seedlings, respectively. Hours after treatment are given at the top of the lanes.

Analysis of apx1 mRNA Levels in Response to Stress

Expression analysis was undertaken to characterize the apx1 gene. A single transcript of approximately 1080 nucleotides hybridizing to the apx1 probe could be detected by RNA gel-blot analysis of total Arabidopsis RNA. Figure 2A shows that the apx1 mRNA of Arabidopsis was present in all tested organs, with the highest level in flowers and lower levels in leaves, stems, and roots.

Methyl viologen is commonly used in experiments to induce oxidative stress. Apx1 mRNA levels increased within a few hours after Arabidopsis plants were sprayed with this herbicide (Fig. 2B). No visible damage of the leaves was observed 24 h after 10−6 M methyl viologen was sprayed. Elevated apx1 mRNA levels could also be detected after treatment of an Arabidopsis cell-suspension culture with methyl viologen (Fig. 2B).

APX activity was shown to increase in plants after ethylene treatment (Mehlhorn et al., 1987). To determine whether ethylene had an effect on the apx1 mRNA levels, Arabidopsis plants were sprayed with a 15 mM solution of ethephon, which releases ethylene after decomposition. Figure 2B shows that such a treatment led to a severalfold increase of the apx1 mRNA within 3 h. Furthermore, light
had positive effects on the amount of the \textit{apx1} mRNA in etiolated Arabidopsis seedlings (Fig. 2B).

Because the pea \textit{apx1} gene was induced by heat, and the putative HSE was present in the \textit{apx1} gene promoter of both pea and Arabidopsis genes, we analyzed the expression of the \textit{apx1} gene after heat shock and found that the steady-state Arabidopsis \textit{apx1} mRNA level was also induced by heat treatment (see below).

\textbf{In Vitro Interactions of the HSF with the \textit{apx1} promoter}

Heat-shock induction of the gene strongly suggested that the putative HSE might be responsible for the induction. A necessary condition for the heat-shock-activated transcription was a binding of the HSF trimer(s) to the sequence of HSE (for review, see Wu, 1995).

In vitro binding assays were performed to obtain evidence for the possible binding of an HSF to the putative HSE. The full-length cDNA encoding the tomato HSF B2 (Scharf et al., 1990; Nover et al., 1996) was cloned after PCR amplification, and the encoded HSF was expressed, albeit at low amounts, in the soluble fraction as hHSF in \textit{E. coli}. The interaction of the hHSF with \textit{apx1} promoter fragments was analyzed by an electrophoresis mobility-shift assay. As shown in Figure 3A, incubation of the 316-bp fragment containing the HSE of the \textit{apx1} promoter (fragment A) with the hHSF resulted in the formation of DNA-protein complexes that were insensitive to increasing amounts of nonspecific competitor nucleic acids. This hHSF-binding activity could be competed out by only a 50- and 100-fold excess of the same unlabeled fragment but not with fragment B from the \textit{apx1} promoter.

To optimize the expression of the HSF we expressed it as an amino-terminal fusion in the pET11a plasmid in \textit{E. coli} BL21 (see “Materials and Methods”). The expressed protein (tHSF), which was mainly present in the soluble fraction, was purified with near homogeneity by three chromatography steps (Fig. 3B). In a dilution series of tHSF, complete saturation of the binding sites in the \textit{apx1} promoter could be reached (Fig. 3C). Although in vivo HSF binds DNA as a trimer (Scharf et al., 1990; Lis and Wu, 1993), trimerization increases only the affinity of HSF to DNA, and only the monomeric HSF and the DNA-binding domain of the HSF are able to specifically bind DNA (Flick et al., 1994). Retention time of the tHSF peak elution in the HPLC gel-filtration step indicated that the tHSF was in a monomeric form. Because the tHSF protein existed as a monomer in the solution, and several binding sites for the protein were present in a HSE, we observed the formation of several DNA-protein complexes.

To prove that the interaction of tHSF is specific and to delineate the HSE in the \textit{apx1} gene, we performed in vitro DNase I-footprinting analysis. As shown in Figure 4, incubation of the \textit{apx1} promoter fragment with increasing amounts of tHSF resulted in the complete protection of a 30-bp sequence on both strands. This sequence contained two 5-bp core HSE motifs arranged in reverse orientation, a\textsc{GAAcgTTCt}, which perfectly fit a defined minimal unit n\textsc{GAAAnnTTCn} necessary for HSF trimer binding (Perisic et al., 1989). Two other similar 5-bp motifs were found on both sides of the central inverted repeat, which served as additional binding sites for the HSF; it has been shown that at least three such 5-bp basic motifs are necessary for high-affinity binding of the HSF (Perisic et al., 1989; Kroeger et al., 1993).
To prove that the putative HSE is involved in the regulation of apx1 gene expression in vivo, we designed an apx1 promoter-gus translational fusion at the ATG start codon. This construct contained 1.2 kb of apx1 promoter sequence and was used to introduce point mutations into the putative HSE. The HSE mutant version of the apx1 promoter was prepared by introducing two nucleotide replacements into the central inverted repeat aGAAcgTTCt, which then read aTAAcgTTAt (Fig. 5A). The replacement of the G base in the nGAAn 5-bp HSE motif is known to have the most deleterious effect on the HSF binding in vitro (Fernandes et al., 1994) and dramatically affects heat-shock inducibility in vivo (Barros et al., 1992).

The promoter-gus fusions were subcloned into a binary vector and stably integrated into the genome of Arabidopsis C24. The transgenic plants were monitored for their ability to express the apx1(HSEmut) and apx1(HSEwt) mRNAs under normal and stress conditions. Transgenic plants expressing the apx1(HSEmut) were used as a control. Because the size of the gus fusion mRNA was substantially higher than the apx1 messenger, we could easily distinguish both the fusion and endogenous apx1 expression on the same RNA gel-blot membrane by using corresponding mixed probes.

Under normal conditions the basal level of the apx1(HSEmut) and apx1(HSEwt) mRNAs seemed to be similar (Fig. 5B). However, a dramatic difference in expression was found after the heat-shock treatment. The levels of the endogenous apx1 and apx1(HSEwt) mRNAs clearly increased, as discussed above. In contrast, the apx1(HSEmut) was repressed, with mRNA being almost undetectable after 1.5 h of the onset of heat shock. These data confirm that the putative HSE is the functional HSE responsible for the heat-shock induction of the apx1 gene.

We also analyzed the oxidative stress response of apx1(HSEmut). Results of the induction with methyl viologen in the same system are shown in Figure 5C. The promoter containing the mutant HSE was still induced, suggesting that this type of apx1 induction depends mainly on some other cis element(s) to be identified further. However, quantitative analysis of the intensity of the bands using phosphor imaging revealed that the induction by methyl viologen of the apx1(HSEmut) was weaker, ranging...
from 70% of the apx1(HSEmut) level during the first 2 h of the treatment to 30% of the apx1(HSEwt) level by the end of the treatment. The high reproducibility of the induction pattern of the endogenous apx1 in the transgenic plants expressing both the apx1(HSEmut) and apx1(HSEwt) ruled out the possibility that the observed differences were due to variability in the methyl viologen treatments.

**In Vivo Footprinting of the apx1 Promoter**

As a second approach to gaining insight into the promoter of the apx1 gene, we analyzed DNA-protein interactions in the proximal part of the apx1 promoter using ligation-mediated PCR-DMS in vivo footprinting (Mueller and Wold, 1989; Peifer et al., 1989). The G ladder was visualized using two different modifications of this method, blotting with subsequent DNA hybridization (Pfeifer et al., 1989) and extension of 32P-labeled primer (Mueller and Wold, 1989). Figure 6 shows the results obtained with DNA extracted from DMS-treated Arabidopsis leaf protoplasts compared with DMS-treated naked DNA. As confirmed by the two methods and several independent experiments, G at --273, --272, and --271 on the noncoding strand were protected from DMS modification and G at --269 was hypersensitive to DMS. Similar G/C-rich sequences were found in several plant promoters that had homology to the ethylene-inducible bean chalcone synthase and avocado cellulase promoters (Fig. 6). A strong DMS hypersensitivity at G --55 was detected close to the TATA box in the HSE sequence, confirming our results on the functionality of HSE in the apx1 promoter.

**DISCUSSION**

APXs are thought to play an essential role in protecting plants from oxidative stress. It was shown previously that steady-state transcript levels of the pea apx1 gene strongly increase after treatment of plants with ethephon, methyl viologen, heat shock, and drought stress (Mittler and Zilinskas, 1992, 1994). In agreement with this observation, we found that the steady-state mRNA levels of the apx1 from Arabidopsis are up-regulated by methyl viologen, ethephon, and heat shock. The heat-shock response is very fast; a significant increase in transcript levels is seen already after 15 min. The observed changes in apx1 mRNA levels after various environmental stimuli indicate that the transcriptional activation of the apx1 gene might be an important, although not the sole (as shown for the pea apx1 [Mittler and Zilinskas, 1992, 1994]) control step leading to higher APX activity in plants under oxidative stress.

As an initial step in the analysis of the apx1 promoter, we studied a putative HSE in more detail for at least two reasons. First, the putative HSE is the sole conserved sequence between promoters of pea and Arabidopsis apx1, implying its importance for the promoter activity. Moreover, the promoter of the other recently cloned apx gene from Arabidopsis, apx2, also contains the two putative HSEs (Santos et al., 1996; our data). Second, the heat-shock response is known to be induced not only by heat but also by a number of other environmental stimuli including oxidative stress. For example, it was shown that hydrogen peroxide treatment results in the induction of heat-shock proteins in Drosophila melanogaster (Courgeon et al., 1988); and Salmonella typhimurium cells (Morgan et al., 1986).

Moreover, oxidative stress imposed by mandelone induced HSF phosphorylation and an HSF-dependent transcriptional activation of the yeast metallothionein cup1 gene (Liu and Thiele, 1996). Direct evidence has also been presented that proteins containing nonnative disulfate bonds as a result of oxidative stress can serve as a signal for the activation of the heat-shock response (McDuffee et al., 1997). Conversely, oxidative stress has been shown to play a major role in heat-induced cell death in yeast (Davidson et al., 1996). In parsley a small heat-shock protein was found to be induced by ozone and heat shock (Eckey-Kaltenbach et al., 1997). These few examples suggest that

**Figure 5.** Study of the stress responsiveness of the apx1 promoter mutated in HSE. A, Site-directed mutagenesis of the putative HSE. Sequences matching the nGAAAn consensus are indicated in uppercase letters. The arrows point from the nucleotides that have been changed to the nucleotides introduced instead. The BglII site used for cloning is underlined and the TATA box is indicated in bold uppercase letters. B, Study of the apx1 promoter with mutated HSE in response to heat shock. In vitro-grown, 2-week-old transgenic Arabidopsis seedlings expressing apx1(HSEmut) and apx1(HSEwt) were shifted from 22°C to 37°C, and RNA was extracted from the whole seedlings at different times and analyzed by gel-blot hybridization using apx1- and gus-specific probes. The times in minutes are indicated at the top of the lanes. C, Effect of methyl viologen treatment on the apx1 promoter function with mutated HSE. The same plant material as in B was infiltrated with 10–5 M methyl viologen solution. RNA was extracted at different times after the treatment and analyzed by gel-blot hybridization using mixed apx1- and gus-specific probes. The times in hours are indicated at the top of the lanes. The difference between the endogenous apx1 and gus mRNA levels reflects the difference between specific radioactivities of the probes used rather than the levels of real mRNA. Infiltration of the plants with water was also used as a control and no increase in the mRNA levels was observed (data not shown).

**Figure 6.** In vivo footprinting of the apx1 promoter. A, Site-directed mutagenesis of the putative HSE. Sequences matching the nGAAAn consensus are indicated in uppercase letters. The arrows point from the nucleotides that have been changed to the nucleotides introduced instead. The BglII site used for cloning is underlined and the TATA box is indicated in bold uppercase letters. B, Study of the apx1 promoter with mutated HSE in response to heat shock. In vitro-grown, 2-week-old transgenic Arabidopsis seedlings expressing apx1(HSEmut) and apx1(HSEwt) were shifted from 22°C to 37°C, and RNA was extracted from the whole seedlings at different times and analyzed by gel-blot hybridization using apx1- and gus-specific probes. The times in minutes are indicated at the top of the lanes. C, Effect of methyl viologen treatment on the apx1 promoter function with mutated HSE. The same plant material as in B was infiltrated with 10–5 M methyl viologen solution. RNA was extracted at different times after the treatment and analyzed by gel-blot hybridization using mixed apx1- and gus-specific probes. The times in hours are indicated at the top of the lanes. The difference between the endogenous apx1 and gus mRNA levels reflects the difference between specific radioactivities of the probes used rather than the levels of real mRNA. Infiltration of the plants with water was also used as a control and no increase in the mRNA levels was observed (data not shown).

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there is a considerable overlap in cellular processes induced by heat shock and oxidative stress and in the subsets of genes reacting to these stimuli. Therefore, we addressed the question of whether the HSE may play a key role in the regulation of \textit{apx1} gene expression.

In vitro analysis of the interaction between recombinant tomato HSF and the \textit{apx1} promoter confirmed that the \textit{apx1} HSE represents a functional HSF-binding site. Furthermore, the \textit{apx1} promoter with a mutated HSE loses inducibility and even becomes repressed under the heat-shock treatment. Nevertheless, the inducibility by methyl viologen is retained. A careful analysis of the phosphor images, however, demonstrated that the strength of \textit{apx1}(HSE\textsuperscript{mut}) transcription was lower than \textit{apx1}(HSE\textsuperscript{wt}) after the methyl viologen treatment. This finding may not be surprising in view of the indiscriminate chemical reactivity of AOS, which probably leads to the elicitation of a number of redundant and/or overlapping signaling pathways ending on different \textit{cis}-acting elements in the \textit{apx1} promoter.

We carried out an additional in vivo analysis of the \textit{apx1} promoter aiming to find other putative \textit{cis}-acting elements. For several “stress-related” plant genes it has been shown that relevant \textit{cis} elements are located close to the TATA box, usually about 200 to 400 bp upstream from the transcription start. To study this sequence in detail, in vivo footprinting analysis of the \textit{apx1} promoter was started, which particularly focused on the first 300 bp. We found strong differences in DMS reactivity of G residues in a sequence (GTGGCCCTCC) located at approximately −270 bp from the transcription start. This sequence is highly similar to the G/C-rich boxes found in the chlorophyll \textit{a/b}-binding protein and alcohol dehydrogenase genes, although further experiments are necessary to show whether it is recognized either by the GCBP-1 (Olive et al., 1991) or the GC-1 (Schindler and Cashmore, 1990) transcription factors. A similar element is present in a bean chalcone synthase promoter, where it is essential for ethylene induction (Broglie et al., 1989), and a homologous element is also found in promoters of ethylene-regulated cellulase genes in avocado (Cass et al., 1990).

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