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Identification of a Basic Glycoprotein Induced by Ethylene in Primary Leaves of Azuki Bean as a Cationic Peroxidase[1]

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Ethylene causes the accumulation of seven different proteins (each designated AZxx according to its molecular mass, xx in kD) in excised primary leaves of azuki bean (*Vigna angularis*) (F. Ishige, H. Mori, K. Yamazaki, H. Imaseki [1991] Plant Cell Physiol 32: 681–690). A complementary DNA encoding an ethylene-induced basic glycoprotein, AZ42, from azuki bean was cloned and its complete nucleotide sequence was determined. Characterization of the cDNA was accomplished by monitoring expression of an immunoreactive protein in *Escherichia coli* that harbored the cDNA and by the identification of a partial amino acid sequence that was the same as that determined from the purified protein. An open reading frame (1071 base pairs) in the cDNA encoded a protein of 357 amino acids with a molecular mass of 39.3 kD. The amino acid sequence contained three regions that are highly conserved among peroxidases from eight different plants. Purified AZ42 exhibited peroxidase activity. The basic glycoprotein induced by ethylene was identified as a cationic isozyme of peroxidase. The corresponding mRNA was not present in leaves that had not been treated with ethylene, but it appeared after 1 h of treatment with ethylene and its level increased for the next 15 h. Accumulation of the mRNA was also induced after wounding or treatment with salicylate. The wound-induced increase in the level of the mRNA was suppressed by 2,5-norbornadiene, but the salicylate-induced increase was not.

Ethylene elicits many different physiological responses from plants. Some of its effects are the result of synthesis of a large variety of enzymes (Lieberman, 1979). The increase in protein synthesis caused by ethylene is controlled at the transcriptional level (Christofferson and Laties, 1982; Nichols and Laties, 1984; Broglie et al., 1986; Ecker and Davis, 1987; Lincoln et al., 1987). However, the molecular mechanisms by which ethylene induces the expression of multiple genes have not been elucidated. To address this issue, we used a system in which multiple genes are coordinately expressed in response to ethylene. We showed previously that, in response to ethylene, primary leaves of azuki bean (*Vigna angularis*) plants accumulate at least seven different proteins, which include an extracellular acidic chitinase (AZ27), β-1,3-glucanase (AZ32), and an intracellular basic glycoprotein (AZ42) (Ishige et al., 1991). A cDNA for the acidic chitinase has been cloned and sequenced (Ishige et al., 1993). In this report, we demonstrate that the intracellular basic glycoprotein is an isozyme of peroxidase.

MATERIALS AND METHODS

Plant Materials

Seedlings of azuki bean (*Vigna angularis* Ohwi et Ohashi cv Tamba-Dainagon) were grown in the light, and primary leaves excised from 10-d-old plants were treated with ethylene (10 $\mu L\ L^{-1}$) or other chemicals as described previously (Ishige et al., 1991).

Isolation of cDNA

A cDNA library constructed in the expression vector pTTQ18 was screened with antibodies raised against purified and deglycosylated AZ42 (Ishige et al., 1991) as described previously (Nakajima et al., 1990). A cDNA clone of 1.3 kb that directed the synthesis of an immunopositive protein of about 39 kD was obtained and designated pAZE42.

Peroxidase Assay

The reaction buffer was prepared by mixing 50 μL of 1% (w/v) *o*-dianisidine in methanol and 6 mL of 0.003% (v/v) H_2O_2 in 10 mM phosphate buffer, pH 6.0. The reaction was started by addition of 0.1 mL of the enzyme solution to 2.9 mL of the reaction buffer, and the rate of oxidation of *o*-dianisidine was measured at A_{460}. Enzymic activity is defined by the formula: 1 unit = $\Delta A_{460}\ min^{-1}$ [11.3 × mg protein (mL reaction mixture)$^{-1}$].

For activity staining of peroxidase, crude extracts (10 μg of protein per lane) and purified AZ42 (1 μg per lane) in 1% (v/v) PVP were subjected to analytical flat-bed isoelectric focusing on a polyacrylamide gel that contained Ampholine (pH range 3.5–9.5; LKB, Uppsala, Sweden). After electrophoresis at 10°C for 1.5 h at 30 W, the gel was washed with PBS for 40 min to remove the ampholytes and then soaked in a solution of *o*-phenylenediamine (0.6 mg mL^{-1} in PBS) for 5

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Abbreviations: IPTG, isopropylthio-β-D-galactoside; NBD, 2,5-norbornadiene.

min. Peroxidase activity was visualized by soaking the gel in PBS that contained the substrate and 0.16% (v/v) H_2O_2.

The folding of urea-inactivated forms of AZ42 and horseradish peroxidase was performed by the procedures described by Smith et al. (1990). The native protein was solubilized in 20 mM Tris-HCl, pH 8.0, 1 mM EDTA, 8 M urea, and 30 mM DTT and incubated at 30°C for 1 h. Excess DTT was removed by a NAP-5 gel filtration column (Pharmacia Japan, Tokyo) that had been equilibrated with 20 mM Tris-HCl, pH 8.0, 1 mM EDTA, 6 M urea, and 1 mM DTT. The reduced and unfolded protein was refolded in a mixture that contained 5 mM $CaCl_2$, 5 mM bovine hemin (type II, Sigma), and 0.75 mM oxidized GSH at 22°C for 3 h, and then it was assayed for peroxidase activity.

Northern Blot Analysis

Total RNA isolated by the SDS-phenol method from leaves that had been treated with various chemicals was subjected to electrophoresis on a 1.2% (w/v) agarose gel (15 μg of RNA per lane) that contained 0.66 M formaldehyde and was transferred to nylon membranes (Hybond N; Amersham Japan, Tokyo). A 0.95-kb *Hin*dIII fragment from pAZE42 was labeled with [α-^{32}P]dCTP and used as the probe. Hybridization was carried out in 5× SSPE, 5× Denhardt's solution, 50% (v/v) formamide, and 0.5 mg mL^{-1} of denatured salmon sperm DNA as described previously (Nakajima et al., 1990). The membrane was washed with 0.1× SSPE that contained 0.1% SDS at room temperature.

Sequencing of DNA

The entire cDNA (1.3 kb) excised by *Bam*HI and *Sph*I from pAZE42 was filled in by Klenow enzyme and subcloned into the *Sma*I site of pUC118 in both possible orientations. The nucleotide sequence was determined by the dideoxy chain-termination method (Sanger et al., 1977) after a series of deletion mutants had been prepared.

General Methods

Manipulations of DNA and RNA were performed as described by Sambrook et al. (1989). SDS-PAGE immunoblot analysis was performed as described previously (Ishige et al., 1991). Amounts of protein were determined by the method of Bradford (1976).

Enzymes and Chemicals

Restriction endonucleases and other enzymes for manipulation of nucleic acids were obtained from Takara Shuzo Co. (Tokyo, Japan) or Toyobo (Osaka, Japan). Radiochemicals were purchased from New England Nuclear (Boston, MA). Purified peroxidase from horseradish (type VI) was from Sigma. Other chemicals were from Wako Pure Chemicals (Osaka, Japan), Takara Shuzo Co., or Sigma Chemicals.

RESULTS

Isolation of cDNA for AZ42

pAZE42 directed the synthesis of an immunoreactive protein of 38.5 kD in *Escherichia coli* in the presence of IPTG

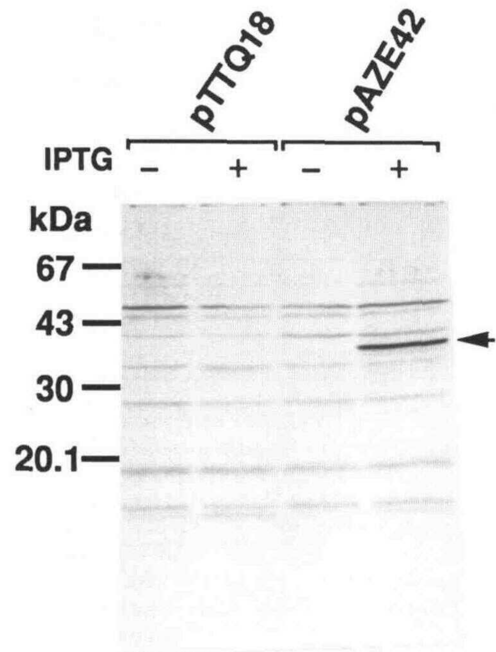

Figure 1. Immunoblot analysis of crude extracts of *E. coli* cells that had been transformed with pAZE42. The bacterial cells were extracted with an SDS-containing buffer, and the extracts were fractionated by SDS-PAGE. Proteins transferred to a nitrocellulose membrane were stained with antibodies against deglycosylated AZ42. –IPTG, Noninduced cells; +IPTG, induced cells. An arrowhead indicates the position of the protein recognized by the antibody.

(Fig. 1). The mass of the protein was in good agreement with the molecular mass of the product of in vitro translation (38–40 kD; Ishige et al., 1991). The cDNA of 1260 bp contained an open reading frame of 1071 bp that encoded a protein of 357 amino acids with a calculated molecular mass of 39.3 kD (Fig. 2). The calculated mass is about 2 kD larger than that of deglycosylated AZ42 (37 kD; Ishige et al., 1991). A sequence of 19 amino acids from Pro[230] to Thr[248] was identical to that determined from peptides obtained after cleavage of the purified protein by cyanogen bromide or weak acid (Ishige et al., 1991). A consensus signal for polyadenylation, AATAAA, was found 37 bases upstream of the poly(A) tail.

Characterization of AZ42 as a Basic Isozyme of Peroxidase

The nucleotide and deduced amino acid sequences exhibited significant similarity to those of other peroxidases. The purified preparation of AZ42 had almost the same enzymic activity as that of horseradish peroxidase (Table I). The isoelectric point of AZ42 was between 8.5 and 8.9 (Ishige et al., 1991). When a crude extract of leaves was fractionated on an isoelectrofocusing polyacrylamide gel and the gel was stained for peroxidase activity, a highly basic isozyme (isoelectric point 8.8–8.9) was detected in the case of leaves that had been treated with ethylene, but not in the case of fresh leaves or those that had been incubated without ethylene (Fig. 3). Two acidic isozymes of peroxidase were present in

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   1 tctttctacttttgatcATGGCTTCTATTTCTTCTAATAAGAATGCTATTTTCAGCTTTC
                      M  A  S  I  S  S  N  K  N  A  I  F  S  F  L    15

  61 TTCTCCTCTCTATCATTCTTTCTGTTTCAGTTATTAAAGTGTGTGAGGCACAAGCCAGGC
      L  L  S  I  I  L  S  V  S  V  I  K  V  C  E  A  Q  A  R  P    35

 121 CTCCTACTGTCAGAGGTCTATCATATACCTTCTATTCCAAAACTTGCCCTACGCTTAAAT
      P  T  V  R  G  L  S  Y  T  F  Y  S  K  T  C  P  T  L  K  S    55

 181 CCATAGTTAGAACTGAGCTCAAAAAGGTCTTCCAGAGCGACATTGCTCAAGCTGCTGGCT
      I  V  R  T  E  L  K  K  V  F  Q  S  D  I  A  Q  A  A  G  L    75

 241 TGCTTCGCCTTCACTTCCATGACTGCTTTGTTCAGGGATGTGATGGGTCAGTTTTATTGG
      L |R  L  H  F  H  D  C  F  V  Q  G  C  D  G  S  V  L  L  D|   95
        |___________________________ I ___________________________|
 301 ATGGATCTGCAAGTGGGCCGAGTGAGAAAGATGCGCCACCAAACTTGACTTTGAGAGCTG
      G  S  A  S  G  P  S  E  K  D  A  P  P  N  L  T  L  R  A  E   115
                                             ▲
 361 AAGCTTTTAGGATCATCGAAAGGATTCGTGGTCTGTTAGAGAAGAGCTGTGGAAGAGTCG
      A  F  R  I  I  E  R  I  R  G  L  L  E  K  S |C  G  R  V  V   135
                                                  |______________
 421 TCTCATGTTCAGACATCACTGCCCTCGCTGCACGTGATGCTGTTTTCCTTTCAGGGGGAC
      S  C  S  D  I  T  A  L  A  A  R  D  A  V| F  L  S  G  G  P   155
     ____________ II _________________________|
 481 CAGACTATGAGATTCCCTTGGGAAGGAGAGATGGGTTAACCTTTGCCTCTAGACAGGTGA
      D  Y  E  I  P  L  G  R  R  D  G  L  T  F  A  S  R  Q  V  T   175

 541 CATTAGACAACCTTCCACCACCCTCAAGCAACACCACCACCATCCTAAACTCCCTCGCCA
      L  D  N  L  P  P  P  S  S  N  T  T  T  I  L  N  S  L  A  T   195
                                 ▲
 601 CCAAAAACCTCGACCCCACCGATGTGGTATCCCTCTCTGGTGGCCACACCATAGGCATAA
      K  N  L  D  P  T |D  V  V  S  L  S  G  G  H  T  I  G| I  S   215
                       |____________ III ______________|
 661 GTCACTGCAGCTCTTTCAACAACAGACTCTACCCTACACAGGACCCTGTCATGGACAAAA
      H  C  S  S  F  N  N  R  L  Y  P  T  Q  D  P  V  M  D  K  T   235

 721 CCTTTGGCAAAAACCTCAGACTCACTTGCCCCACCAACACCACCGACAACACCACAGTCT
      F  G  K  N  L  R  L  T  C  P  T  N  T  T  D  N  T  T  V  L   255
                                       ▲           ▲
 781 TGGACATTCGATCCCCCAATACCTTCGACAACAAATACTACGTTGACCTCATGAACCGAC
      D  I  R  S  P  N  T  F  D  N  K  Y  Y  V  D  L  M  N  R  Q   275

 841 AGGGCCTCTTCACCTCCGACCAAGACCTCTACACCGATAAGAGGACCAGAGGCATTGTCA
      G  L  F  T  S  D  Q  D  L  Y  T  D  K  R  T  R  G  I  V  T   295

 901 CCAGCTTTGCCGTGAACCAGAGTCTCTTCTTTGAGAAGTTTGTGTTCGCCATGCTCAAGA
      S  F  A  V  N  Q  S  L  F  F  E  K  F  V  F  A  M  L  K  M   315
                  ▲
 961 TGGGTCAGCTCAGTGTGCTCACGGGAAATCAAGGGGAGATTCGTGCCAACTGCTCCGTGA
      G  Q  L  S  V  L  T  G  N  Q  G  E  I  R  A  N  C  S  V  R   335
                                                   ▲
1021 GGAATGCCAACAGCAAGGCCTTCTTGAGTTCCGTCGTGGAAAATGTGGCCCAAGAATTCA
      N  A  N  S  K  A  F  L  S  S  V  V  E  N  V  A  Q  E  F  I   355

1081 TAGAAATGtaaccgggtcttctttgttgtatatgttatgaccatgaataatgcgtaaccc
      E  M  *                                                      357

1141 ttgtttctggatgatctaacgtggtagggaaccgttctctaatgttcctagttatatata
1201 catacgtacttgagttgtaataaattttaaaatctgaacaagagcttctcattggcatgt
1261 aaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaa
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Figure 2. Nucleotide and deduced amino-acid sequence of the cDNA for the ethylene-induced basic peroxidase of azuki bean. The coding region is shown in uppercase letters. A portion of the amino acid sequence identical to that determined from the purified enzyme is underlined. Three regions (I, II, and III) that are highly conserved in other known peroxidases are bracketed. A potential polyadenylation signal is marked with double underlining. Potential sites of *N*-glycosylation are marked with closed triangles.

each of the crude extracts, but the relative activities of these isozymes did not change upon treatment of leaves with ethylene. Antibodies against deglycosylated AZ42 also recognized horseradish peroxidase (our unpublished observation).

As described above, pAZE42 directed the synthesis of an immunoreactive protein in *E. coli*, but a soluble extract of the transformed bacteria had no peroxidase activity, nor did it contain the immunoreactive protein. The immunoreactive protein was extracted only by buffers that contained SDS (Fig. 1) or urea at concentrations above 4 M (data not shown). Although horseradish peroxidase synthesized by *E. coli* and extracted from the bacterial cells with 8 M urea could be refolded in the presence of calcium ions and heme, with restoration of enzymic activity (Smith et al., 1990), the basic peroxidase extracted with 8 M urea from the transformed bacterial cells did not regain catalytic activity after refolding (data not shown). When purified AZ42 was dissolved in 8 M urea and subjected to refolding, only 2% of the original enzymic activity was restored, whereas horseradish peroxidase could be fully reactivated by the same treatment (Table I).

Expression of the Gene for the Basic Peroxidase

The mRNA for AZ42 was about 1.4 kb in length. It was not detected in fresh leaves or in leaves incubated without ethylene (Fig. 4). The mRNA appeared within 1 h of treatment with ethylene, and its level increased markedly for the next 13 h (Fig. 4a, lanes 2, 3, 4, 5, 7, and 9). A larger RNA

Table I. *Enzymic activities of AZ42 and horseradish peroxidase (HRP) before and after the treatment with urea or SDS*

Refolding of the urea-treated and SDS-treated enzymes was performed as described in "Materials and Methods." Treatment of the enzymes with SDS was included as a negative control.

Pretreatment	Peroxidase Activity	
	AZ42	HRP
	unit (mg protein)$^{-1}$	
Water	1461	1210
8 M urea	31	1628
1% SDS	ND[a]	ND

[a] ND, Not detected.

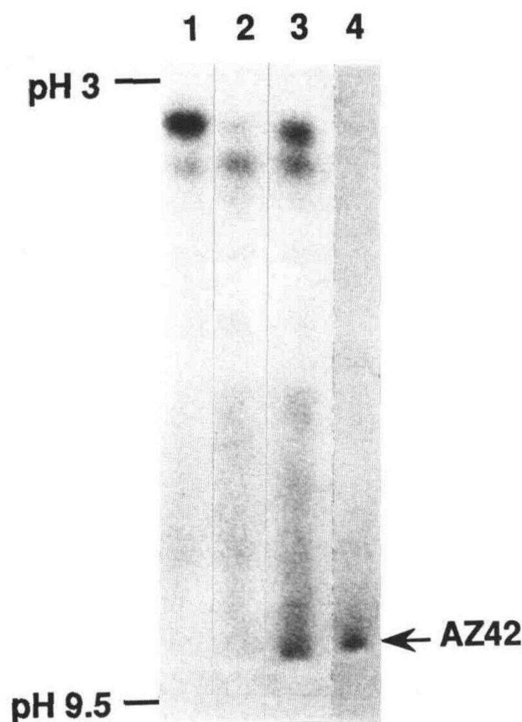

Figure 3. Zymogram of peroxidases from azuki leaves. Crude extracts (10 μg of protein) from fresh leaves (lane 1), leaves incubated without ethylene (lane 2) and with 20 μL L^{-1} of ethylene for 48 h (lane 3), and purified basic peroxidase (1 μg, lane 4) were subjected to isoelectric focusing and activity staining for peroxidase.

(>3 kb in size), which hybridized to AZ42 cDNA, was detected in leaves when the rapid increase in level of the mRNA was taking place. This large RNA was also detected when a 3′ noncoding region of the cDNA was used as a probe (data not shown). The synthesis of the mRNA induced by ethylene ceased rapidly when ethylene was withdrawn from the ambient air, and the level of mRNA fell rapidly during further incubation in air (Fig. 4a, lanes 4, 6, and 8).

Sucrose is known to affect the expression of some plant genes (Rocha-Rosa et al., 1989; Wenzler et al., 1989; Hattori et al., 1990). Although sucrose alone did not induce the expression of the gene for the basic peroxidase, it stimulated the ethylene-induced expression of the gene (Fig. 4b, lanes 2–4).

The ethylene-induced increase in the level of the mRNA was suppressed in the presence of NBD (Fig. 4b, lanes 5 and 6). The expression of the gene also occurred in wounded leaves and in intact leaves that had been treated with salicylate (Fig. 4c, lanes 2 and 4). The effect of wounding was greater than that of salicylate. NBD suppressed the wound-induced expression but not the salicylate-induced expression of the gene (Fig. 4c, lanes 3 and 4).

DISCUSSION

AZ42, a glycoprotein induced by ethylene in leaves of azuki bean, was found to be a basic isozyme of peroxidase by determination of the nucleotide sequence of its cDNA and deduction of the encoded amino acid sequence. The nature of the isolated cDNA clone was confirmed by the presence in the deduced amino acid sequence of a partial amino-acid sequence that had been determined with purified AZ42 and by the expression in the transformed *E. coli* of an immuno-

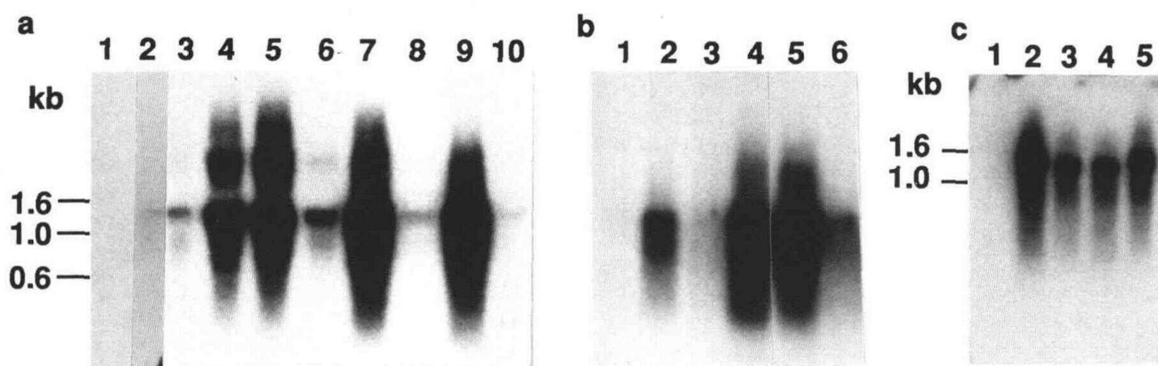

Figure 4. RNA blots probed with the ^{32}P-labeled cDNA for the basic peroxidase from azuki bean. a, Time course. Total RNA extracted from leaves of azuki bean after 0 h (lane 1), 1 h (lane 2), 3 h (lane 3), 6 h (lane 4), 10 h (lane 5), 14 h (lane 7), or 22 h (lane 9) of treatment with ethylene. Lanes 6, 8 and 10, Total RNA from leaves incubated in air for an additional 4, 8, or 16 h, respectively, after a 6-h treatment with 20 μL L^{-1} of ethylene. b, Effects of sucrose and NBD. Lane 1, Total RNA from fresh leaves. Total RNA extracted from leaves incubated for 24 h with 20 μL L^{-1} of ethylene (lane 2), with 30 mM sucrose (lane 3), or with a combination of ethylene and sucrose (lane 4), and from leaves treated for 72 h with ethylene (lane 5) or with a combination of ethylene and NBD (lane 6). c, Effects of wounding and salicylate. Total RNA extracted from fresh leaves (lane 1), from leaves that had been cut into 5-mm-wide strips and incubated for 48 h in air (lane 2) or in 6 mL L^{-1} of NBD (lane 3), and from leaves treated with 0.6 mM salicylate (lane 4), as well as from leaves treated with both salicylate and NBD (lane 5). Fifteen micrograms of RNA per lane were subjected to electrophoresis except in the case of the RNA from salicylate-treated leaves (30 μg; c, lanes 4 and 5).

reactive protein. Moreover, purified AZ42 had specific peroxidase activity similar to that of horseradish peroxidase.

cDNAs or genes encoding peroxidases have been cloned from tobacco (Lagrimini et al., 1987), potato (Roberts et al., 1988), horseradish (Fujiyama et al., 1988), tomato (Roberts and Kolattukudy, 1988), cucumber (Morgens et al., 1990), peanut (Buffard et al., 1990), barley (Rasmussen et al., 1991), and wheat (Rebmann et al., 1991). The cDNAs from potato and wheat encode isozymes of peroxidase that are induced by wounding or viral infection. Morgens et al. (1990) attempted to isolate a cDNA for a basic isozyme of peroxidase that was induced by ethylene in cucumber leaves. However, although the cDNA that they isolated apparently encoded an ethylene-inducible peroxidase, it was different from the basic enzyme that they were hoping to find.

The predicted primary structure of AZ42 showed 35 to 40% similarity to those of other known peroxidases. However, three regions (Fig. 5, boxes I–III) are highly conserved in the primary structure, and two of them (boxes I and III) have been implicated in the catalytic reaction and the binding of heme (Lagrimini et al., 1987). Moreover, the numbers of amino acid residues between adjacent boxes are almost constant among the different peroxidases examined. In addition, eight cysteine residues are located at positions that are nearly identical to those in other known peroxidases. On the basis of structural studies of horseradish peroxidase (Mazza and Welinder, 1980), the presence of four intrachain disulfide bridges is predicted in AZ42: between cysteine residues 50 and 131; 83 and 88; 137 and 332; and 217 and 244. These positional relationships among the three conserved regions and the locations of the cysteine residues in the peroxidases indicate that the three regions may constitute a part of a similar tertiary structure in different peroxidases (Welinder, 1985; Sakurada et al., 1986). However, six potential sites of *N*-glycosylation (Asn-X-The/Ser; at residues 109, 185, 247, 251, 300, and 331) were present in the basic peroxidase from azuki at positions different from those of other peroxidases.

The molecular mass of the protein predicted from the nucleotide sequence is about 2 kD larger than that of the chemically deglycosylated AZ42 (37 kD; Ishige et al., 1991). It is likely that the primary product of translation of the mRNA for the azuki peroxidase is processed to give rise to a smaller mature peroxidase. Although the N-terminal residue of AZ42 could not be determined (Ishige et al., 1991), Gln^{32} was tentatively assigned as the N terminus of AZ42 because the N terminus of many peroxidases is a Gln residue, which is often modified. A region of 31 amino acids prior to the glutamine residue contains several basic amino acids that are

	32	BOX I (x over H)	
AZ42	QARPPTVRGLSYTFYSKTCPTLKSIVRTELKKVFQSDIAQAAGLL	RLHFHDCFVQGCDGSVLLD	-35-
CUC	xxxxTFYDESCPDVSNIVRRVVQQALVSDERAGARLI	.........N........E	-33-
TOM	QLTPEACVFSAVRAVVDSAIDAETRMGASLI	.........D....GI...	-35-
POT	QLTPEACVFSAVRGVVDSAIDAETRMGASLI	.........D....GI...	-35-
HRP	QLTPTFYDNSCPNVSNIVRDTIVNELRSDPRIAASIL	.........N...A.I...	-34-
TUR	ZLTTNFYSTSCPNLLSTVKSGVKSAVSSQPRMGASIL	..F......N.....I...	-34-
TOB	QLSATFYDTTCPNVTSIVRGVMDQRQRTDARAGAKII	.........N.....I...	-33-
PNT	QLSSNFYATKCPNALSTIKSAVNSCVAKEARMGASLL	.............A.....	-34-
WHT	QLSPTFYDTSCPRALAIIKSGVMAAVSSDPRMGASLL	.............A....S	-28-

		BOX II		BOX III (x over H)	
AZ42	-	CGRV-VSCSDITALAARDAV	-53-	DVVSLSGGHTIG	-144
CUC	-	.PG.-...A..L.I.SVGS.	-52-	.L.A...A..F.	-125
TOM	-	.PN.S...A..L.I....S.	-52-	EM.A.A.A..V.	-122
POT	-	.PNIS...A..L.I....S.	-52-	EM.A.A.A..V.	-122
HRP	-	.P-RT...A.LL.I..QQS.	-53-	.L.A......F.	-150
TUR	-	.PG.-...A..L.I....S.	-52-	.M.A...A....	-124
TOB	-	.P..-...A..L...SEIG.	-52-	.L.A...A..F.	-132
PNT	-	.P..-...A..L.V....S.	-52-	EL.T...A....	-122
WHT	-	.NQ-T...A..LTV....S.	-51-	.M.A...A....	-123

Figure 5. Comparison of the amino acid sequences of three regions that are highly conserved among eight different peroxidases, and the relative locations of these regions. Data were compiled from reported sequences of peroxidases from cucumber (CUC; Morgens et al., 1990), tomato (TOM; Roberts and Kolattukudy, 1989), potato (POT; Roberts et al., 1988), horseradish (HRP; Fujiyama et al., 1988), turnip (TUR; Mazza and Welinder, 1980), tobacco (TOB; Lagrimini et al., 1987), and peanut (PNT; Buffard et al., 1990). Amino acid residues different from those of the peroxidase from azuki bean are indicated. Histidine residues that have been implicated in the binding of heme and the catalytic reaction are marked with an X. Numbers between boxes and on the C-terminal side represent numbers of amino acid residues present. The sequences begin at the predicted N terminus of each mature peroxidase.

followed by a highly hydrophobic region, and it appears to consist of the proposed components of a signal peptide (Chrispeels, 1991). AZ42 is an intracellular glycoprotein (Ishige et al., 1991). Although its cellular localization remains to be determined, the basic nature of the protein may indicate the localization to vacuoles (Maeder, 1986). Because the protein binds to Con A, it probably contains glycans of the high-mannose type, and the glycosylation must occur in the lumen of the ER. The leader sequence of the primary product of translation probably plays a role as the signal peptide for transport to the ER.

Because the mRNA for the basic peroxidase was not present at a detectable level in leaves that had not been treated with ethylene, it appears that ethylene specifically regulates transcription of the gene for the basic isozyme of peroxidase. This conclusion is supported by an examination of zymograms of crude extracts. There are at least two isozymes of acidic peroxidase in both control and ethylene-treated leaves, but the level of the basic isozyme increased only in the treated leaves. The azuki gene for the basic peroxidase was also expressed in response to wounding of tissue and treatment with salicylate. In tobacco leaves, wounding induced an increase in the level only of basic isozymes of peroxidase (Lagrimini and Rothstein, 1987). The effect of wounding appears to be mediated by wound-induced ethylene because induction by wounding was suppressed by NBD, but the effect of salicylate may not be mediated by ethylene. We confirmed that production of ethylene increased after wounding of azuki bean leaves, but none was detected for at least 48 h after treatment with salicylate (our unpublished observation), by which time the mRNA for AZ42 had already accumulated in the leaves in response to salicylate. Malamy et al. (1990) reported that endogenous levels of salicylate in tobacco were increased by pathogenic infection, but levels were not altered by wounding. Although changes in levels of endogenous salicylate in response to ethylene remain to be determined, it is likely that ethylene and salicylate act through different signal-transduction systems.

Two RNAs of different sizes were detected in the RNA blots probed with the cloned cDNA. The two RNAs were also detected even when the 3′ noncoding region of the cDNA was used as a probe. Thus, it seems likely that the two RNAs originated from transcription of the same gene, with the larger RNA being a precursor of the mRNA.

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