The Abscisic Acid Induction of a Novel Peroxidase Is Antagonized by Cytokinin in Spirodela polyrrhiza L.¹

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The growth regulator abscisic acid (ABA) can be used to induce dormant bud structures (turions) in the duckweed Spirodela polyrrhiza L. In this paper we show that during this process, ABA rapidly induces elevated levels of mRNA transcripts encoding a novel basic peroxidase. In addition, we show that in the presence of the cytokinin kinetin the maintained increase is attenuated. Kinetin not only totally inhibits the induction of turions by ABA but also alleviates ABA-induced growth inhibition. This antagonism of an ABA-induced gene by a cytokinin correlates with an easily observable antagonistic effect of these two hormones on plant morphogenesis. These data contribute to a growing body of evidence linking growth regulators with changes in peroxidase gene expression and to the concept of pairs of hormones playing antagonistic roles during plant development. Finally, we discuss the possible functions that peroxidases could have during ABA-induced turion formation and growth inhibition.

Spirodela polyrrhiza L. is a floating aquatic monocot of the family Lemnaceae. As such, it undergoes vegetative reproduction to produce clonally related groups of plants (fronds) that can colonize large areas of still water (Hillman, 1961; Landolt, 1986). As an overwintering device, S. polyrrhiza produces specialized dormant buds, termed turions, from which new fronds can be produced under appropriate environmental conditions (Jacobs, 1947; Landolt, 1986). Turion development, as opposed to vegetative frond development, involves an apparent reduction in cell expansion and aerenchyma formation as well as an accumulation of starch and anthocyanin (Smart et al., 1987).

S. polyrrhiza can be grown aseptically with ease and rapidity in the laboratory, thereby providing an excellent experimental system for the analysis of factors regulating leaf heterophyllly, of which turion/frond formation is an extreme example (Trewavas and Jones, 1991). Such analysis has revealed that turion formation can be affected by a number of environmental factors, including temperature, nitrate availability, and light (Jacobs, 1947; Landolt, 1986). Moreover, it has been shown that turion formation can be rapidly induced by addition of low physiological concentrations of the growth regulator ABA (Perry and Byrne, 1969; Stewart, 1969; Smart and Trewavas, 1983) and suppressed by the growth regulator cytokinin (Stewart, 1969). In this respect, S. polyrrhiza provides a unique system for the investigation of the antagonistic effects of two growth regulators on morphogenesis in an intact plant.

We are interested in exploiting the unique aspects of the S. polyrrhiza system to investigate the molecular processes by which growth regulators can induce or regulate plant morphogenesis. Toward this end, we have previously synthesized a cDNA library from mRNA extracted from ABA-treated S. polyrrhiza plants and, via a differential screening strategy, identified a number of cDNAs whose respective transcripts are up-regulated early during ABA-induced turion formation (Smart and Fleming, 1993). One of these cDNAs (tur1) we have identified as encoding a protein with homology to d-myo-inositol-3-phosphate synthase an enzyme that plays a key role in inositol metabolism. Based on an analysis of the expression of the tur1 gene in ABA-treated plants, we hypothesized that changes in cell wall inositol derivatives following ABA induction might represent an important mechanism by which morphogenesis is affected in this system (Smart and Fleming, 1993).

In this paper we report the identification and characterization of another cDNA, tur4, whose respective mRNA accumulates upon ABA treatment. Tur4 has been characterized by sequencing and genomic Southern analysis. The induction of the tur4 mRNA by ABA has been studied by northern analysis. Our analysis reveals that tur4 codes for a basic peroxidase that is likely to be localized to the cell wall. The maintained increase in the level of tur4 peroxidase mRNA by ABA can be inhibited by the concomitant addition of cytokinin to the growth medium. This phenomenon correlates with the observed inhibition of ABA-induced turion formation and growth inhibition by this same growth regulator. We discuss the possible function of the ABA-induced peroxidase, in particular its potential effect on cell wall extensibility and morphogenesis, and examine the significance of the antagonistic effects of ABA and cytokinin on the expression of the gene encoding this peroxidase.

MATERIALS AND METHODS

Plant Material

Spirodela polyrrhiza L. was grown aseptically on 100 mL of half-strength Hutner's medium in 250-mL Erlenmeyer flasks as described previously (Smart and Trewavas, 1983). Each

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Abbreviation: pl, calculated isoelectric point.
experimental flask was inoculated with one plantlet consisting of between five and eight fronds and allowed to multiply for 7 d before the start of the experimental manipulations. Each experimental sample consisted at this time of between 50 and 100 plants. Turion formation was induced by adding 250 µL of a 100-µM solution of filter-sterilized (x) cis-trans-ABA in medium to the flask, to give a final concentration of 250 nM ABA. Control cultures were treated with an equal volume of fresh medium. For cytokinin-reversal experiments, aliquots of filter-sterilized 1 mM kinetin dissolved in 10 mM NaOH were added to the medium to give final concentrations of between 1 and 100 µM. In these cases control cultures were treated with an equal volume of 10 mM NaOH. When kinetin and ABA were both added to a culture, they were added simultaneously. The growth of cultures was measured by determining the fresh weight growth constant k as previously described (Smart and Trewavas, 1983), and the t test was used to analyze the significance of the differences between sample means. Tissue for RNA and DNA isolation was frozen in liquid nitrogen before use.

Construction of S. polyrrhiza cDNA Library and Differential Screening

A cDNA library was constructed from poly(A)+ RNA extracted from fronds of S. polyrrhiza treated with 250 nM (±) cis-trans-ABA for 2 h as previously reported (Smart and Fleming, 1993). A portion of the unamplified library on nylon filters was differentially screened for ABA-up-regulated cDNA sequences by hybridization to single-stranded 32P-labeled cDNA probes prepared from poly(A)+ RNA isolated from control fronds (ABA−) and fronds treated for 2 h with 250 nM ABA (ABA+). Filters were washed at high stringency with the known plant peroxidases. Alignment with the other peroxidase sequences similar to tur4 at the nucleotide and amino acid level were done using the FASTA program of the University of Wisconsin Genetics Computer Group sequencing package (Devereaux et al., 1984). The multiple alignment of the amino acid sequence of tur4 to the other peroxidases was done using the CLUSTAL program, whereas individual optimal alignments were done with the GAP program.

DNA Sequencing

A deletion library from both ends of the tur4 insert was generated by exonuclease III digestion according to the manufacturer’s instructions (Pharmacia). In this way, both strands of the insert cDNA of the clone tur4 were sequenced by dideoxynucleotide chain termination using T7 DNA polymerase (Pharmacia). In some cases Sequenase and dITP (United States Biochemical) were used to interpret compressions.

Sequence Analysis

Searches of GenBank/EMBL and SWISS-PROT for sequences similar to tur4 at the nucleotide and amino acid level were done using the FASTA program of the University of Wisconsin Genetics Computer Group sequencing package (Devereaux et al., 1984). The multiple alignment of the amino acid sequence of tur4 to the other peroxidases was done using the CLUSTAL program, whereas individual optimal alignments were done with the GAP program.

Genomic Southern Blot Analysis

S. polyrrhiza genomic DNA was prepared from fronds as described by Rogers and Bendich (1988). The DNA was digested with restriction endonucleases, subjected to electrophoresis in 0.7% (w/v) agarose in 0.5 X TBE (1 X TBE = 89 mM Tris-borate, 2.5 mM EDTA, pH 8.3), and blotted onto Hybond N membranes (Amersham). The blot was probed with the tur4 cDNA insert labeled with [α-32P]dCTP by the random-primer method (Pharmacia). Hybridization and subsequent high-stringency washes (0.1 X SSC [1 X SSC = 150 mM NaCl, 15 mM tri-sodium citrate, pH 7.0], 0.1% [w/v] SDS at 65°C) were performed according to the membrane manufacturer’s protocols. Fragment sizes were calculated by comparison with HindIII-digested λ fragments.

RNA Isolation and Northern Blot Analysis

Total RNA from fronds of S. polyrrhiza was prepared by a double guanidine salt method (Han et al., 1987), size-fractionated on 1.1% (w/v) agarose-formaldehyde gels, transferred to Hybond N membranes (Amersham), and fixed by baking. The blots were stained with methylene blue and destained before hybridization (Herrin and Schmidt, 1988) to detect the amount of RNA loaded onto each lane and to visualize the RNA mol wt markers (Gibco BRL) loaded onto an adjacent lane. The EcoRI-excised tur4 cDNA insert, purified by electroelution from an agarose gel and labeled with [α-32P]dCTP by the random-primer method (Pharmacia), was hybridized to the RNA blots at 65°C according to the filter manufacturer’s instructions (Amersham). The blots were washed to a final stringency of 0.1 X SSPE (1 X SSPE = 180 mM NaCl, 10 mM NaH2PO4, 1 mM EDTA, pH 7.7), 0.1% (w/v) SDS at 65°C to ensure specific hybridization and exposed to Hyperfilm MP (Amersham) at −80°C with an intensifying screen for 3 to 19 d depending on the signal.

RESULTS

Sequence Analysis of tur4

Figure 1 shows the nucleotide and predicted amino acid sequence of tur4. The 1219-bp cDNA contains an open reading frame beginning with the first ATG initiation codon (position 14) and ending with a TAG stop codon (position 1001). It encodes a 329-amino acid polypeptide with an apparent mol wt of 35,586. The deduced amino acid sequence contains a typical eukaryotic signal peptide of 23 amino acids with a basic N terminus followed by many hydrophobic residues (von Heijne, 1990). Ala and Leu constitute over 50% of the residues in the signal peptide. The nucleotide sequence is GC rich (61% in the coding region), and 85% of the codons have a C or G in the third position. The protein is rich in Phe and poor in Trp (only one residue), Tyr, and Met. The sequences 1099 AATAAT and 1174 AAGAAT are possible polyadenylation signals.

tur4 Codes for a Peroxidase

The encoded protein shows significant homology with all the known plant peroxidases. Alignment with the other per-
oxidase sequences makes it clear that processing of the signal peptide at the presumed site of cleavage (shown by a vertical arrow in Fig. 1) would result in a mature protein of 306 amino acids and a mol wt of 33,313.

The TUR4 protein contains the two peroxidase motifs common to most peroxidases (Fig. 1) and shows extensive homology to the three regions (boxes 1, 2, and 3) conserved in all plant peroxidases. The apparent signal peptide cleavage (shown by a vertical arrow (1)). The PROSITE peroxidase motifs are underlined with a dotted line, and three regions that are highly conserved in plant peroxidases are overlaid (boxes 1, 2, and 3). Two potential polyadenylation signals are underlined. Potential sites of N-glycosylation are marked with an asterisk (*). The distal His residue predicted to be involved in acid/base catalysis and the proximal His residue predicted to be the fifth ligand of heme are shown in boldface. The 8 conserved Cys residues involved in the four disulfide bridges characteristic of plant peroxidases are also shown in boldface.

**Figure 1.** Nucleotide and deduced amino acid sequence of the *S. polyrhiza* tur4 cDNA insert, which codes for a peroxidase. Nucleotides are numbered in the 5' to 3' direction beginning with the first nucleotide in the cDNA clone. The amino acid sequence is shown below the sequence of the 5'. The amino acid sequence is shown below the

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ABA-Induced Turion Formation Is Reversed by Cytokinins

Figure 6 shows the effect of kinetin on ABA-induced turion formation and growth inhibition after 9 d of treatment. Kinetin suppresses ABA-induced turion formation at a concentration of 5 μM, and at 10 μM, ABA-induced turion formation was completely reversed. These data also suggest that kinetin can to some extent reverse ABA-induced growth inhibition. That this is the case was shown by a separate statistical analysis of frond growth in the presence of 250 nM ABA with or without kinetin at a concentration of 10 μM. This analysis gave a mean value for the fresh weight growth constant k in the presence of ABA of 0.0494 with an se of 0.0019 (n = 8), whereas in the presence of ABA and kinetin together the growth constant had a value of 0.0691 ± 0.0019 (n = 9). These mean values are significantly different at the level P < 0.00001. Thus, it appears that kinetin does indeed alleviate a proportion of the growth inhibition brought about by ABA. Kinetin on its own has no significant effect on frond growth until 50 μM, at which concentration it inhibits growth. At these high concentrations of kinetin, there is no reversal of ABA-induced growth inhibition, although turion formation is still abolished. However, the growth inhibition seen at high concentrations of kinetin is not additive to the inhibition shown by ABA. BA was also effective at reversing ABA-induced turion formation and alleviating ABA-induced growth inhibition, but a 5-fold higher concentration of BA was required to abolish ABA-induced turion formation compared to kinetin (results not shown), and all further experiments were conducted with kinetin.

Genomic Southern Analysis of the tur4 Gene in S. polyrhiza

Southern hybridization analysis of restriction fragments of genomic DNA from S. polyrhiza resulted in a single hybridization band of a different size with each of the four enzymes tested (Fig. 3). This result indicates that tur4 is encoded by a single gene or a small gene family.

tur4 Transcript Levels Are Induced Transiently by ABA

A northern blot analysis of the time course of tur4 mRNA induction by ABA is shown in Figure 4. tur4 was induced 2 h after adding 250 nM ABA. The transcript level increased thereafter with a broad maximum around 6 h and then slowly began to decline after 12 h (Fig. 4A). The transcript level returned to near-control levels 3 to 4 d after ABA addition and remained at this low level throughout ABA-induced turion formation (Fig. 4B). Although turion formation can also be induced by low-temperature treatment, there was no induction of the tur4 transcript at any time during cold-induced turion formation (results not shown). The induction of the tur4 transcript is therefore specific to ABA. The induction of the tur4 transcript was also very sensitive to ABA. It was found that only 50 nM ABA was necessary to significantly induce the transcript after 6 h. The transcript level increased with increasing ABA concentration until at 2.5 μM ABA the response was saturated (Fig. 5).

Kinetin Suppresses the Maintained ABA Induction of tur4

Because ABA-induced turion formation and growth inhibition were reversed by cytokinins, we decided to test whether the induction of the tur4 transcript by ABA was similarly affected. Cultures were grown with or without the addition of 250 nM ABA in the presence or absence of 20 μM kinetin. At this concentration kinetin has no obvious effects on frond morphology (compare Fig. 7, A and B), has only a small inhibitory effect on growth (26% inhibition), and has no effect on the level of the tur4 transcript level (Fig. 8, lanes 4, 9, and 14). ABA alone has the effect of inducing turions (Fig. 7C) and of increasing the level of the tur4 transcript level (Fig. 8, lanes 5, 10, and 15). The addition of kinetin
Figure 2. Comparison of TUR4 to three other plant peroxidase amino acid sequences. Shown is only part of a multiple alignment of TUR4 performed to all plant peroxidase sequences either in CenBank/EMBL release 34 or published elsewhere (42 sequences). The alignment of S. polyrhiza TUR4 to tomato TPXZ (Valpuesta et al., 1993), tomato TAPl (Roberts and Kolattukudy, 1989), and horseradish HRPC (Fujiyama et al., 1988) is shown. The amino acid sequence of TUR4 is shown in full, identified by the single-letter code. Identical amino acids to the TUR4 sequence are indicated by dots (·) in the lower lines, and only those amino acids that are not identical to the TUR4 sequence are indicated. Gaps in the sequences, which are included to better the alignment, are indicated by dashes. The position numbers above the TUR4 sequence refer to the alignment shown in the figure and not to an individual sequence. Every 10th residue (or gap) is indicated by a dot.

A

B

DISCUSSION

ABA Induces an mRNA Encoding a Novel Basic Peroxidase

Our analysis of an ABA-induced cDNA (tur4) from S. polyrhiza indicates that it encodes a novel basic peroxidase. This identification is based on an extensive sequence analysis of the tur4 cDNA, which shows that the protein encoded contains the conserved sequence elements identified so far in all plant peroxidases, including those elements at and around the conserved active site residues, and the conserved positions of the 8 Cys residues predicted to be of importance for the correct folding of the mature protein (Welinder, 1991, 1992).

The postulated catalytic site residues Arg38, Phe41, His42, Phe45, Glu53, Asn71, His169, and Asp220 (Welinder, 1992; Welinder and Gajhede, 1993), the invariant salt bridge residues Asp55, Gly122, and Arg125 (Welinder et al., 1992), as well as the putative substrate-binding sites Arg42 and Tyr184 (Sakurada et al., 1986) are all present in TUR4. The regions Ala48 to Gln72, Leu138 to Ser142, and Thr181 to Tyr184 are predicted to line the substrate channel (Welinder, 1992). TUR4 also possesses the 8 Cys residues involved in the four intramolecular disulfide bridges characteristic of plant peroxidases (Welinder, 1991). These bridges are predicted between Cys residues 11 and 91, 44 and 49, 97 and 302, and 176 and 211.

Outside of these conserved regions, the predicted amino acid sequence of tur4 shows only limited identity to previously reported sequences for plant peroxidases. Based on the criteria of Welinder (1991), the sequence identity of TUR4 relative to the other reported peroxidases is sufficiently low to warrant the assignment of the protein encoded by the tur4 gene to a new family of peroxidases, along with the tomato sequences TPX1 (pI = 6.2) and TPX2 (pI = 8.5) (Valpuesta et al., 1993).
The origin and the size in kb of the genomic fragments detected by polyrrhiza genomic DNA (3 µg) digested with EcoRI (E), XbaI (X), Clal (C), or BamHI (B) were separated by gel electrophoresis, blotted, and hybridized with 32P-labeled tur4 cDNA (7.8 × 10^6 dpm ml^-1) with total RNA (7.5 µg) from fronds treated with either a turion-inducing concentration of ABA (250 nm) or an equal volume of fresh medium (control) for short periods of time up to 24 h. The autoradiograph was exposed for 6 d. This blot had been stripped of probe from a previous tur1 hybridization, which explains the relatively low level of signal compared with the blot in A above.

Figure 3. Genomic Southern blot analysis of tur4. Fragments of S. polyrrhiza genomic DNA (3 µg) digested with EcoRI (E), XbaI (X), Clal (C), or BamHI (B) were separated by gel electrophoresis, blotted, and hybridized with 32P-labeled tur4 cDNA (7.8 × 10^6 dpm ml^-1). The autoradiograph was exposed for 11 d. The position of the origin and the size in kb of the genomic fragments detected by tur4 cDNA are indicated.

Table 1. Amino acids in the new plant peroxidase family that do not conform to the previous consensus

<table>
<thead>
<tr>
<th>Invariant</th>
<th>Residue in TUR4</th>
<th>Residue in TPX1/TPX2</th>
<th>Residue Variant in Other New Sequences a, b, c</th>
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<tbody>
<tr>
<td>Leu 104</td>
<td>Leu</td>
<td>Val/Val</td>
<td>Thr in No. D11337</td>
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<tr>
<td>Ala 107</td>
<td>Ile</td>
<td>Val/Val</td>
<td>Gly in No. M91372</td>
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<td>Ser in No. L08199 and No. M91373</td>
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<tr>
<td>Cys 215</td>
<td>Cys</td>
<td>Asn/Cys</td>
<td>Gly in rice</td>
</tr>
<tr>
<td>Leu 245</td>
<td>Val</td>
<td>Leu/Val</td>
<td>Ile in No. L08199</td>
</tr>
<tr>
<td>Gln 256</td>
<td>Ala</td>
<td>Ala/Ala</td>
<td>His in No. L08199</td>
</tr>
<tr>
<td>Phe 286</td>
<td>Phe</td>
<td>Phe/Pro</td>
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a, b, c. We are not in a position to explain the Asn at position 215 in the TPX1 sequence (Valpuesta et al., 1993) nor the Gly in the rice sequence (Intapruk et al., 1993), since the Cys normally in this position is a member of the four disulfide bridges found in all plant peroxidases.

Figure 4. Northern blot analysis of the temporal induction of the transcript for tur4 in S. polyrrhiza by ABA. RNA samples were separated by denaturing gel electrophoresis, blotted, and hybridized with 32P-labeled tur4 cDNA. The tur4 transcript size was estimated to be approximately 1.3 kb by comparison with RNA mol wt markers. A, Hybridization of 32P-labeled tur4 cDNA (8.1 × 10^6 dpm ml^-1) with total RNA (7.5 µg) from fronds treated with either a turion-inducing concentration of ABA (250 nm) or an equal volume of fresh medium (control) for short periods of time up to 24 h. The autoradiograph was exposed for 3 d. B, Hybridization of 32P-labeled tur4 cDNA (5.5 × 10^6 dpm ml^-1) with total RNA (10 µg) from fronds treated with a turion-inducing concentration of ABA (250 nm) for up to 5 d to include the whole period of turion induction. The autoradiograph was exposed for 6 d. This blot had been stripped of probe from a previous tur1 hybridization, which explains the relatively low level of signal compared with the blot in A above.

The nucleotide sequence of tur4 is most similar, however, to the rice, wheat, and barley peroxidase sequences, probably reflecting the high GC content of the monocot sequences. This bias in codon usage has been observed for many genes expressed in monocots (Murray et al., 1989) and probably reflects a high translation rate (Fincher, 1989).
sent target genes for regulation by ABA in a number of different plant systems. Peroxidase genes might thus represent a small gene family. It also suggests that the TUR4 protein would function in the cell wall. Thus, the predicted TUR4 amino acid sequence lacks any C-terminal acidic propeptide, which has been postulated as a vacuolar targeting signal in plants (Bednarek et al., 1990; Welinder, 1992). It has been shown that peroxidases directed to intracellular compartments have such a propeptide, which is removed posttranslationally (HRPC, Welinder, 1979; BP1, Johansson et al., 1992; AZ42, Ishige et al., 1993; BP2, Theilade et al., 1993), whereas peroxidases shown to function in the cell wall lack the propeptide (TobAnPOD, Lagrimini et al., 1987; PNPC1, Burfard et al., 1990; HRPA2, Welinder, 1992).

Genomic Southern analysis indicates that tur4 is encoded by a single gene or a small gene family. It also suggests that the number of different genes encoding the S. polyrrhiza tur4 peroxidase is considerably less than for horseradish peroxidases (Fujiiyama et al., 1990). A situation similar to that in Spirodela appears to exist for Arabidopsis (Intapruk et al., 1991).

Our data show that the level of the tur4 transcript is up-regulated by ABA. An induction of anionic peroxidase mRNAs by ABA has been demonstrated in both potato and tomato callus cultures (Roberts and Kolattukudy, 1989), as well as in tomato petioles (Robb et al., 1991; Sherf et al., 1993). More indirect evidence for a role of ABA in regulating the transcription of peroxidase genes comes from the finding that at least two of the promoters of peroxidase genes so far studied contain sequence elements that might lead to regulation of the genes by ABA (Kawaioka et al., 1992; Theilade and Rasmussen, 1992). Peroxidase genes might thus represent target genes for regulation by ABA in a number of different plant systems.

Our analysis of the expression of the tur4, HRPC, and BP1 genes in Spirodela shows that it is expressed at a low level in plants growing under our control conditions, but that the addition of ABA at very low physiological concentrations leads to an increase in the level of the tur4 transcript within 2 h, with a maximum transcript level being reached 6 h after addition of the growth regulator. This peak is followed by a slow decline in mRNA level over the following 4 d. In the presence of kinetin and ABA, there is a similar increase in tur4 transcript. However, the subsequent decline in transcript is more rapid than that observed in the absence of kinetin, returning to low levels within 24 h after hormone treatment.

We have thus identified a novel basic peroxidase gene, tur4, whose transcript level can be rapidly up-regulated by ABA, and whose up-regulation by ABA can, after a transient induction, be attenuated by kinetin. Because it has been shown, both in this study and in previous work (Stewart, 1969), that these two growth regulators have distinct and antagonistic effects on the development of S. polyrrhiza, this raises the question of whether the TUR4 peroxidase has a role in the mechanism by which ABA and cytokinins carry out their functions.

The Function of the tur4-Encoded Peroxidase

Although over 40 peroxidases have now been cloned from plants, assigning a precise role within the plant to any of these proteins has proved problematical (Gaspar et al., 1991). Thus, although various peroxidases have been implicated in a large number of processes (McDougall, 1992; Ros Barceló and Muñoz, 1992), the vast number of potential substrates for these enzymes within the plant, the existence of multiple isoenzymes, and the variety of tissue expression patterns reported make a precise interpretation of their function(s) in vivo rather difficult. However, one general theme that recurs...
throughout the literature on peroxidases is a correlation between high peroxidase activities and a decrease in the rate of cell elongation (Lagrimini, 1992; MacAdam et al., 1992; McDougall, 1992; Ros Barcelo and Munoz, 1992).

Following the addition of ABA to *S. polyrrhiza*, a number of changes at the biochemical and cellular level that accompany the formation of the turion structure can be detected. Among these, a general decrease in cellular expansion is most obvious. Thus, the cells of the turion are distinguished by being smaller and having thicker cell walls than their counterparts in the frond, and the turion itself is attached to its mother frond by a stolon structure that is greatly shortened in comparison to one that normally attaches a daughter vegetative frond to its mother (Smart and Trewavas, 1983; Smart and Fleming, 1993). Moreover, actual measurements of the cell wall plastic extensibility in *S. polyrrhiza* have shown a decrease in the value of this parameter following the addition of ABA (Longland, 1986). Taking these observations on the effects of ABA on *S. polyrrhiza* in conjunction with the proposed functions of many peroxidases in affecting the rate of cell elongation (Fry, 1986; Bradley et al., 1992), it is possible that the ABA-induced TUR4 peroxidase functions at least partly by increasing the extent of cell wall polymer cross-linking and thus decreasing cell wall extensibility.

If the ABA-induced peroxidase does affect cell wall extensibility, then it is pertinent to ask whether there is an intrinsic link between a change in such extensibility and the developmental fate of the tissue in which such changes are occurring. For example, it has been shown that a cationic peroxidase isolated from cultures of carrot somatic embryos has the ability to restore embryogenesis in cultures where it has been blocked by use of the inhibitor tunicamycin (Cordewener et al., 1991). These data have been interpreted as suggesting that an appropriate cell wall extensibility is a prerequisite for embryogenesis to occur, and that peroxidases might modulate such extensibilities in vivo. Our results add correlative evidence to support the notion that peroxidases could act to affect organ differentiation via their effects on cell wall extensibility.
polymer cross-linking. Proof of such an effect awaits further experimentation.

The Antagonistic Effects of ABA and Cytokinins

In many plant experimental systems, an antagonism in the effects of pairs of growth regulators has been reported. These range from the classic role of auxin/cytokinin ratios in regulating root/shoot regeneration in tissue culture (Skooog and Miller, 1957) to the modulation of $\alpha$-amylase transcript levels in the aleurone of barley by GA$_3$ and ABA (Ho, 1989).

Cytokinins and ABA have often been reported to have opposite effects on plant metabolic, physiological, and developmental events. These include effects on nitrate reductase activity and transcript levels (Lu et al., 1992), PEP carboxylase mRNA levels (Thomas et al., 1992), stomatal opening (Incoll and Jewer, 1987), and senescence (Noorden, 1988; Zeevaart and Creelman, 1988; Smart et al., 1991). S. polyrrhiza is an example of an intact plant system in which ABA and cytokinin have antagonistic effects on a morphogenic event (turion formation) (Stewart, 1969). Our results show that cytokinins antagonize both responses of S. polyrrhiza to ABA (growth and morphogenesis), in contrast to the previous suggestion that only ABA-induced turion formation was repressed by cytokinin (Stewart, 1969). We can explain this apparent discrepancy by pointing out that the alleviation of ABA-induced growth inhibition by cytokinins may be observed only at low cytokinin concentrations that alone have little inhibitory effect on frond growth. We presume that the alleviation of part of the ABA-induced growth inhibition is directly due to the reversal of turion formation. Primordia that would normally develop into turions in the presence of ABA develop into small fronds when kinetin as well is present, thus leading to the observed increase in fresh weight.

Our data show that the antagonism at the level of morphogenesis is reflected at the molecular level by an antagonism in the effect of these growth regulators on the expression of the tur4 peroxidase gene. This observation raises two questions. First, is the inhibition of the ABA-up-regulated transcript level for the tur4 peroxidase gene functionally linked to the inhibition of ABA-induced turion formation or growth inhibition? Second, what is the molecular nature of the interaction between the two growth regulators ABA and cytokinin? At present we can only speculate on the answers to these questions.

As to the functional link between the ABA-induced peroxidase and ABA-induced turion formation, we are now carrying out experiments to see whether it is possible to identify a concentration of kinetin at which inhibition of the level of ABA-induced tur4 transcript occurs but at which inhibition of ABA-induced turion formation does not occur. Thus, using the ratio of ABA:cytokinin as a handle, can we dissociate some of the molecular events we observe during ABA-induced turion formation from the morphogenic event?

The pattern of expression of the tur4 transcript with exogenous ABA concentration correlates physiologically with the concentrations of ABA that induce turion formation and inhibit growth (Smart and Trewavas, 1983). Because we find that a similar induction of tur4 gene expression is not observed during low-temperature induction of turion formation, we conclude that the rise in the tur4 transcript level is not a general feature of turion formation per se, and is either specifically linked to ABA's induction of this morphogenic event or is a specific effect of ABA on S. polyrrhiza fronds, unrelated to turion formation.

With respect to the nature of the interaction between ABA and cytokinin, one can envisage a number of scenarios. For example, it is possible that the signal-transduction pathways of the two regulators converge relatively late at the level of gene transcription, thus predicting the presence of distinct ABA- and cytokinin-associated transcription factors and binding sites. In this respect the regulation of tur4 in S. polyrrhiza by ABA and cytokinins would be reminiscent of the regulation of $\alpha$-amylase by GA$_3$ and ABA in the barley aleurone system (see, for example, Lazarus, 1991; Skriver et al., 1991). However, since our analysis of the transcript levels of tur4 is based on northern blots, and therefore reflects steady-state transcript levels only, we cannot as yet pinpoint either the level of the ABA up-regulation of tur4 or of the cytokinin interference with this up-regulation. Because the early (2 h) ABA induction of the tur4 transcript is not affected by the simultaneous addition of kinetin, whereas 22 h later this level is very much reduced by kinetin, one could envisage a scenario whereby kinetin accelerates the degradation of the tur4 transcript level. However, because kinetin appears to have no such effect on the steady-state levels of the tur4 transcript in the absence of ABA, the situation may be far more complex. A nuclear run-on analysis of the transcriptional activity of tur4 in the presence and absence of ABA and kinetin should provide some answers to this question.

Alternatively, the signal-transduction pathways might interact much earlier, even to the point where the two molecules competitively interact for the same binding site on receptors or carriers, as suggested by van Overbeek et al. (1967). Moreover, it has also been suggested that ABA and cytokinins might affect the metabolism of each other, thus modulating the effect of each on the plant's metabolism and development. Cytokinins have been shown to inhibit the biosynthesis of ABA (Cowen and Ralston, 1987) and to promote its conjugation (Even-Chen and Itai, 1975). Likewise, ABA may change cytokinin transport and metabolism (Sondheimer and Tzou, 1971; Back et al., 1972).

Such potential complexity in the mode of action of two growth regulators makes predictions of their interactions rather difficult. However, S. polyrrhiza now affords an intact plant system in which two growth regulators (ABA and cytokinin) interact antagonistically to control an easily observable phenotype (turion formation) and an easily detectable molecular marker (tur4), and that, due to its growth in liquid, is amenable to the analysis of growth regulator metabolism following feeding of labeled precursors.

In conclusion, our identification of a novel ABA-inducible basic peroxidase whose maintained induction is suppressed by cytokinin in a plant system that shows a clear developmental response to these two growth regulators provides an insight into how these regulators might affect plant development and provides an experimental framework for the investigation of how growth regulators might interact.
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