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A Flooding-Induced Xyloglucan *Endo*-Transglycosylase Homolog in Maize Is Responsive to Ethylene and Associated with Aerenchyma[1]

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Development of aerenchyma (soft cortical tissue with large intercellular air spaces) in flooded plants results from cell-wall hydrolysis and eventual cell lysis and is promoted by endogenous ethylene. Despite its adaptive significance, the molecular mechanisms behind aerenchyma development remain unknown. We recently isolated a flooding-induced maize (*Zea mays* L.) gene (*wusl1005[gfu]*; abbreviated as *1005*) encoding a homolog of xyloglucan *endo*-transglycosylase (XET), a putative cell-wall-loosening enzyme active during germination, expansion, and fruit softening. XET and related enzymes may also be involved in cell-wall metabolism during flooding-induced aerenchyma development. Under flooding, 1005 mRNA accumulated in root and mesocotyl locations that subsequently exhibited aerenchyma development and reached maximum levels within 12 h of treatment. Aerenchyma development was observed in the same locations by 48 h of treatment. Treatment with the ethylene synthesis inhibitor (aminooxy)acetic acid (AOA), which prevented cortical air space formation under flooding, almost completely inhibited 1005 mRNA accumulation in both organs. AOA treatment had little effect on the accumulation of mRNA encoded by *adh1*, indicating that it did not cause general suppression of flooding-responsive genes. Additionally, ethylene treatment under aerobic conditions resulted in aerenchyma development as well as induction of *1005* in both organs. These results indicate that *1005* is responsive to ethylene. Treatment with anoxia, which suppresses ethylene accumulation and aerenchyma development, also resulted in *1005* induction. However, in contrast to flooding, AOA treatment under anoxia did not affect 1005 mRNA accumulation, indicating that *1005* is induced via different mechanisms under flooding (hypoxia) and anoxia.

Plants respond to flooding by undergoing changes at the molecular, biochemical, and cell structural levels. To date, the majority of molecular-level research on flooding responses has focused on the enzymology of energy production, namely the induction of genes encoding enzymes of Glc-P metabolism (e.g. ADH, enolase, and glyceraldehyde-3-P dehydrogenase). These enzymes allow limited energy production in the face of limited oxygen supply and thus aid in survival during short-term flooding (reviewed by Sachs et al., 1996). Flooded plants also undergo significant structural modifications leading to visible changes in cell and organ structure (Drew et al., 1979; Grineva et al., 1988). Of these, the development of aerenchyma has been studied extensively at the physiological level (e.g. He et al., 1992, 1994; Brailsford et al., 1993).

Aerenchyma development results from the lysis of cells in cortical tissues and the ensuing formation of air spaces and is proposed to involve a wide range of cell-degrading enzymes (He et al., 1994). The adaptive significance of aerenchyma lies in the fact that the tissue provides a low-resistance pathway for air diffusion into submerged tissues and, therefore, may promote flooding survival (Drew et al., 1979). Despite its significance, however, the molecular mechanisms behind the development of aerenchyma and other flooding-induced structural modifications are largely unknown. It is established that aerenchyma development in flooded tissues is associated with increased activity of cellulase, and that both are promoted by the accumulation of ethylene (Drew et al., 1979; He et al., 1994). Thus, it is likely that genes involved in cell-wall loosening or hydrolysis under flooding are induced in response to ethylene. Besides the observed increase in cellulase activity, however, there is no available information about the enzymes involved in the development of aerenchyma or other flooding-induced structural changes.

We recently cloned a flooding-induced gene from maize (*Zea mays* L.), which encodes a homolog of XET, a putative cell-wall-loosening enzyme (gene *wusl1005(gfu)*, Peschke and Sachs, 1994; Saab and Sachs, 1995; referred to as gene *1005*). The induction of 1005 mRNA was specific to oxygen deprivation among a range of stresses, including heat shock, salt, and cold (Peschke and Sachs, 1994). *1005*, which is one of the first flooding-responsive genes that does not encode an enzyme of Glc-P metabolism, is proposed to be associated with the onset of structural modifications induced by flooding (Saab and Sachs, 1995).

Xyloglucans, the substrates for XET, are composed of β-1,4-linked glucans with xylosyl side chains and can form strong hydrogen bonds with cellulose microfibrils (Valent and Albersheim, 1974). As such, xyloglucans are proposed to act as tethers that cross-link cellulose microfibrils and contribute to cell-wall strength (Fry, 1989; Passioura and

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Abbreviations: ADH, alcohol dehydrogenase; AOA, (aminooxy)acetic acid; XET, xyloglucan *endo*-transglycosylase.

Fry, 1992). The primary activity of XET is transglycosylation, the cutting and rejoining of xyloglucan chains. However, depending on substrate conditions, some XETs may also cause hydrolysis of xyloglucans (Fanutti et al., 1993; S.C. Fry, personal communication). Consistent with its proposed role as a cell-wall-loosening enzyme, increased activity of XET was associated with several cell-wall-loosening and hydrolysis processes, including expansion (Hetherington and Fry, 1993), fruit softening (Redgwell and Fry, 1993; Maclachlan and Brady, 1994), and responses to water deficit (Wu et al., 1994).

The hypothesis behind this work is that gene *1005* encodes a flooding-induced XET or related enzyme that is involved in cell-wall metabolism associated with development of aerenchyma. Our objectives in this study are first, to characterize this gene and its regulation by flooding and anoxia; second, to test its association with flooding-induced air space formation leading to aerenchyma development; and third, to determine if it is responsive to ethylene, a promoter of aerenchyma development.

MATERIALS AND METHODS

Experimental Conditions

All experiments were conducted on the maize (*Zea mays* L.) inbred line B73Ht. Kernels were germinated for 4 d at 28°C in the dark, and 25 seedlings of uniform average length with preemergent shoots (leaves remaining within the coleoptile) were selected. For flooding treatments (hypoxia), seedlings were placed in 2-L containers containing 1.2 L of flooding buffer (5 mM Tris-Cl, pH 8, 100 mg L^{-1} ampicillin). Seedlings were maintained in a vertical orientation and submerged in flooding buffer so that coleoptile tips were just below the liquid surface. Uncovered containers were incubated at 28°C in an aerated chamber for the number of hours indicated. For anoxia treatments, seedlings were submerged in flooding buffer and sealed in a 2-L glass container. A plastic tube connected to an argon gas tank was sealed into the screw cap, and the container was overfilled with buffer before sealing to remove as much air as possible. Seedlings were bubbled continuously with argon gas, which was vented into a water-filled beaker (procedures similar to those of Sachs et al., 1980). The container was incubated at 28°C for the duration of the experiments. For inhibitor treatments, AOA was added to the germination medium and flooding buffer at a concentration of 0.7 mM. Other experimental conditions were the same as those used for flooding and anoxia treatments.

For experiments with exogenous ethylene, 25 seedlings (3 d old) were placed on moist germination paper, which was then rolled loosely. The rolled paper (30 cm high) was placed in 1 cm of water in a 2-L glass container fitted with a cap connected to a tank containing 100 $\mu L\ L^{-1}$ ethylene in air. The seedlings, including root tips, remained above the water level for the duration of the experiments to avoid potential hypoxic conditions. Ethylene gas was bubbled in the water at the bottom of the container to maintain high humidity and was vented into a water-filled beaker to monitor gas flow. All experiments in this study were repeated in their entirety one or two times.

Tissue Sampling and Histology

Ten seedlings were harvested after 48, 72, or 96 h of flooding treatment, and primary roots and mesocotyls were examined for structural modifications as described below. Locations in each organ exhibiting the first signs of modifications (visible cell deformation, lysis, or air space formation) were selected for RNA extraction in subsequent experiments. In the primary root, a 2-cm location starting at 2 cm below the kernel was selected for analysis. In the mesocotyl, a 2-cm location starting at 3 cm below the coleoptile was selected for analysis. These locations were used in all experiments.

For structural analysis, primary roots and mesocotyls were separated from seedlings and sectioned by free-hand at 1-cm intervals along each organ. Sections were stained with 1% toluidine blue, rinsed with water, and mounted on microscope slides. Sections were examined immediately under a microscope with 63× magnification. Photographs were taken using Kodak T 160 film.

RNA Extraction and Northern Analysis

Total RNA was extracted from 20 root or mesocotyl sections as described by Saab et al. (1995), and 30-μg samples were fractionated on 1% formaldehyde gels. RNA was capillary transferred to nylon membranes (MagnaGraph, Micron Separations, Westborough, MA)[2] using 10× SSC (1.5 M NaCl, 0.15 M trisodium citrate) for 20 h. RNA was cross-linked to membranes by exposure to UV light for 1 min (UV Stratalinker, Stratagene). Northern hybridizations were performed under high-stringency conditions as follows: blots were prehybridized in hybridization solution for 3 h at 42°C followed by hybridization in fresh solution (plus probe) for 20 h at 42°C. Hybridization solution composition was 50% formamide, 6× SSC, 20 mM phosphate buffer, pH 6.8, 1% SDS, 1× Denhardt's solution, 5% (w/v) dextran sulfate, and 100 mg/mL sheared salmon sperm DNA. After probing, blots were washed with 2× SSC, 0.1% SDS at room temperature for 15 min, then at 65°C for 30 min, and finally with 1× SSC, 0.1% SDS at 65°C for 20 min. Solutions were prewarmed to the appropriate temperatures before washing. Blots were stripped by soaking in 0.2× SSC, 0.1% SDS at 100°C for 15 min.

Cloning, Sequence Analysis, and Probes

Details of cloning of genomic and full-length cDNA sequences representing *1005* were reported by Saab and Sachs (1995). Homologous sequences in the GenBank were identified using a BLAST search (National Center for Biotechnology Information, Bethesda, MD). Amino acid sequences were aligned with the aid of MacDNASIS Pro software (Hitachi Software Engineering Co., San Bruno, CA). For northern analysis, blots were probed with a 0.8-kb

[2] Names are necessary to report factually on available data; however, the U.S. Department of Agriculture (USDA) neither guarantees nor warrants the standard of the product, and the use of the name by the USDA implies no approval of the product to the exclusion of others that may also be suitable.

partial-length 1005 cDNA (pZmL1005, Peschke and Sachs, 1994) or with a probe containing the 3′UTR of the clone generated using an internal *Sst*I site in the clone (Fig. 1) and an *Xho*I site in the vector. The latter produced a single band on Southern analysis of genomic DNA, suggesting that it is specific to only one gene (Peschke and Sachs, 1994). The other probes used were ADH1 cDNA (clone pZmL793), which served to distinguish treatment effects on *1005* from effects on other flooding-responsive genes, and clone pZmL1055, an unidentified clone the transcript level of which is unaffected by oxygen deprivation (Sachs, 1994) and, as such, served as a loading control. Even loading was also verified by examining ethidium bromide-stained ribosomal bands in the gels and on the membranes. Probes were labeled with [α^{32}P]dATP or [α^{32}P]dCTP (3000 Ci/mmol, ICN) by random priming (Prime-It II, Stratagene).

RESULTS

1005 Is Homologous to XET

Figure 1 shows the transcriptional unit of *1005* and an additional 120 bp 5′ to the ATG start site. Comparison with the full-length cDNA revealed that this gene contains a single intron, 125 bp long, starting with GT at the 5′ end and ending with AG at the 3′ end (the intron is denoted by lowercase letters). At least one XET encoding gene, *TCH4* from *Arabidopsis thaliana*, was also found to include only one intron (Xu et al., 1995). It is interesting that the intron position relative to the translation initiation site in *1005* and *TCH4* differs by only 3 bp, indicating an evolutionary relationship between the two genes.

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   1 CGGCACGAGA TCGCACGCAC CATCGTCCTG CCGATCGATC GCAGCCAGCC AGTCTTGCTG
  61 AGAGACGAAC TGAAGCCTGA AGTGAACCGA TCGATCGATC GATCGAGCGA GCGAGCTCCC
 121 ATGAAGGCGT TGATTCTGGC GGCGGTGCTG CTGCTGCAGC ACGGGGGAGC CGCGAGCGCC
 181 GGCGGCAACT TCTACCAGGA CGTGGACATC ACCTGGGGCG ACGGCCGCGG CAAGATCCTC
 241 GACAACGGCC AGCTCCTGAC GCTGTCCATG GACAGGTCCT CCGGCTCGGG CTTCCAGTCC
 301 AAGGCCCAGT ACCTCTACGG CCGCTTCGAC ATGCAGCTCA AGCTCGTCCC GGGGGACTCC
 361 GCCGGCACCG TCGCCACCTT CTATGTACgt actactacgt acatactaca ccgccgccgc
 421 ctcctcgtcc tagcttcgta tatattcgct gtcgtctctc tctctcgcgc gctgaacaca
 481 tgtcgtcggt cacgcacgca cgcgcacagC TTTCGTCGCA GGGTTCGCAG CACGACGAGA
 541 TCGACTTCGA GTTCCTGGGG AACGCGAGCG GGGAGCCGTA CACGGTGCAC ACGAACGTGT
 601 ACAGCCAGGG GAAGGGCGGG CGGGAGCAGC AGTTCCGGAT GTGGTTCGAC CCCACGGCGG
 661 CCTTCCACGC CTACTCCGTG CTGTGGAACC CCGCCCACGT CGTCTTCTAC GTGGACGGCG
 721 TCCCCATCCG GGAGTTCCGG CGCCGCGGCG ACGGGACCGT GCCGTTCCCG ACGTCGCAGC
 781 CGATGCGGGT GTACGCCAGC GTGTGGGACG CGGAGGAGTG GGCCACGCAG GGGGGGCGCG
 841 TCCGGACCGA CTGGTCCAAG GCGCCCTTCG TCGCGTCGTA CCGCGGGTAC GCCGCCGCCG
 901 GGTGCACCGC GCCGGACGCC GCCGCCTGCG CGCGCTCCAA CGGCGCATGG ATGTCGCAGG
 961 AGCTCGACAG CGCCGGCCAG GAGCAGCTCC GCCGGGCGCA GGCCAGCTAC ATGATCTACA
1021 ACTACTGCAC CGATAAGTAC CGGTTCCCGC AGGGCCCGCC GCCCGAGTGC TCGTCGCCGG
1081 CCAAGTAGTA GATGAGTAGA ATTGATCGGA TACAGAGAGC AATCAATTAA TTAAATCGAC
1141 CCGTCGCTTG GTTTTTGGTT TACACATTGT ACTACGGCAT AAGTTCGGCG GATATATGTA
1201 TACGTAGACG GCTAGACGCC ATGCATGTGT CTTGGCATGT ATCGTATTCG TATATATCGT
1261 AATTCGTATG TACCTCAGTA TACCTGCATC GTACATTTAC ATGCATATAT ATCTCCCTAT
1321 ATATAAATAT ATATGGTTTT ATTA
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Figure 1. The nucleotide sequence of *1005*. Predicted translation initiation site and an *Sst*I restriction site used to generate a 3′ end gene-specific probe are underlined. The intron location, determined by comparison with the complete cDNA, is denoted in lowercase letters.

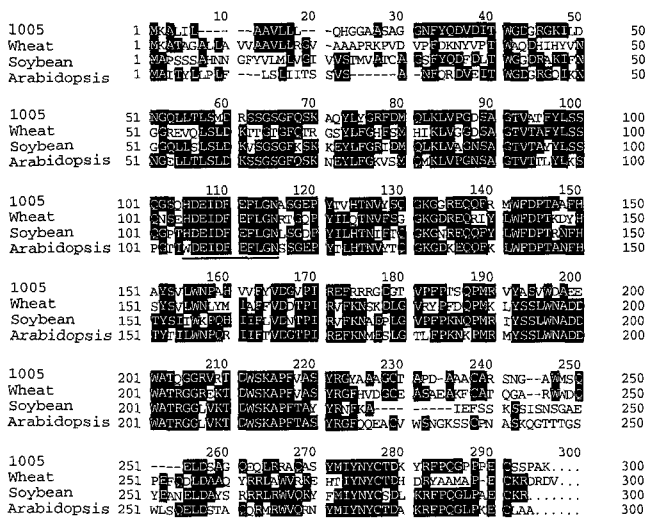

Figure 2. The comparison of the deduced amino acid sequence of *1005* and XET sequences from wheat (Okazawa et al., 1993), soybean (Zurek and Clouse, 1994), and Arabidopsis (Xu et al., 1995) in the GenBank. Dark regions denote identities among two or more of the sequences.

Evidence for the identity of the deduced 1005 protein comes from comparison with XET sequences in the databases, which shows that 1005 is homologous to all known XET sequences. Figure 2 shows a comparison of 1005 with three XET sequences isolated from vegetative tissues. The wheat sequence (Okazawa et al., 1993) is the only known XET homolog from a monocot species in addition to 1005. Furthermore, recombinant proteins produced from the soybean (BRU1, Zurek and Clouse, 1994) and Arabidopsis (TCH4, Xu et al., 1995) sequences were found to possess XET activity in vitro (Xu et al., 1995; S. Clouse, personal communication). Both of these sequences are inducible by treatment with a brassinosteroid growth regulator, and *TCH4* is additionally inducible by heat shock and mechanical stimulation (Xu et al., 1995).

All known XET homologs, including 1005, share an identical or near-identical 11-amino acid stretch (Fig. 2, underlined), which is proposed to contain the active site of β-glucanases from *Bacillus subtilis* (Borriss et al., 1990). As is expected for proteins that are putatively targeted to cross the plasma membrane, 1005 contains a hydrophobic sequence at the N terminus (first 20 residues). Similar sequences have been observed in other XET homologs (e.g. Zurek and Clouse, 1994; Arrowsmith and de Silva, 1995; Xu et al., 1995).

Induction of *1005* and Aerenchyma by Flooding

To begin to address the association of *1005* expression with flooding-induced structural modifications, we first examined the effects of the flooding conditions used in this study on root and mesocotyl structure. Cortical air spaces were generally detected by 48 h of flooding in both organs, and aerenchyma development was clearly discernible in the root (Fig. 3) and the mesocotyl (data not shown) by 72 h. In the primary root, air space formation was first

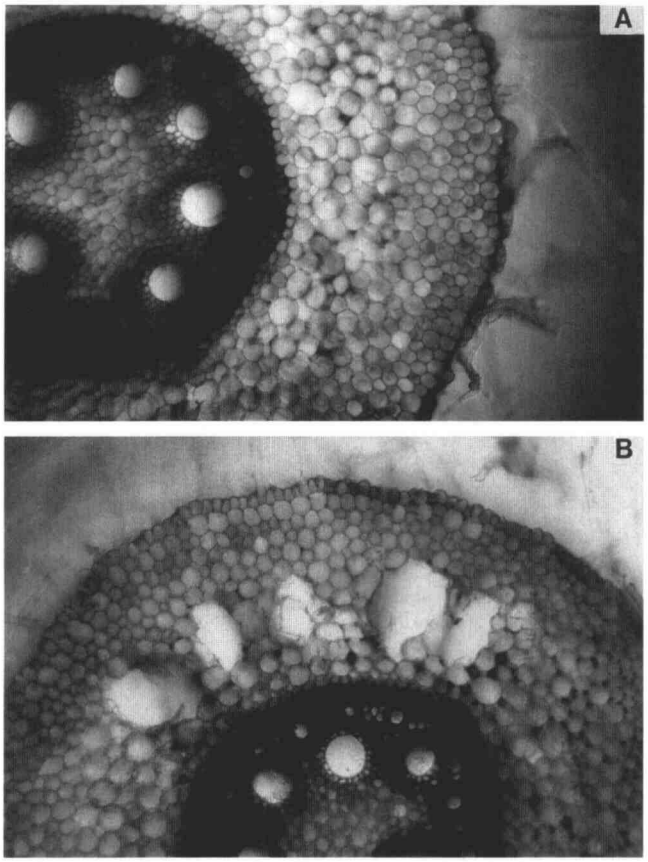

Figure 3. (A) Aerenchyma induction in the primary root by flooding. Seedlings were harvested at 0 (A) and 72 h (B) after flooding, and sections were taken at 3 cm below the kernel. Sampling location was determined from results of preliminary experiments that identified locations exhibiting early signs of aerenchyma development, as described in "Materials and Methods."

observed within a 2-cm location starting at approximately 2 cm below the kernel, whereas in the mesocotyl air space formation was first observed within a 2-cm location starting at approximately 3 cm below the coleoptilar node. By 96 h of flooding, air spaces were prevalent in all locations of both organs except for the apical 2 cm of the primary root. Similarly, Drew et al. (1979) reported no aerenchyma in apical regions of flooded nodal roots.

The accumulation of 1005 mRNA was examined in the above-mentioned locations exhibiting early signs of aerenchyma development. Transcript level was barely detectable in either organ under aerobic conditions. Under flooding, however, 1005 mRNA accumulated in both organs in advance of cortical air space formation and reached a maximum within 12 h of treatment (Fig. 4). Transcript levels remained high through 144 h of flooding (data not shown).

Is *1005* Induction by Flooding a Response to Endogenous Ethylene?

Since aerenchyma development is promoted by endogenous ethylene (Drew et al., 1979; He et al., 1992), the question arises whether endogenous ethylene is a trigger for *1005* induction under flooding. To address this question, we treated seedlings with AOA, an inhibitor of ethylene synthesis (Yang and Hoffman, 1984), and examined its effects on aerenchyma development and accumulation of 1005 mRNA under flooding. As a control, we also examined AOA effects on ADH1 mRNA accumulation to determine if AOA acts indiscriminately on gene expression during flooding. This was a necessary control because, depending on concentration, AOA can act as a general inhibitor of enzymes requiring pyridoxal phosphate, leading to potentially toxic effects (Imaseki, 1991). Preliminary experiments were conducted to determine the appropriate AOA concentration for this study. Seedlings were treated with 0.1, 0.3, or 0.7 mM AOA and examined for cortical air space formation under flooding. Results indicated that AOA at 0.7 mM was most effective in reducing aerenchyma development. Out of 30 seedlings examined, none exhibited air space formation in the mesocotyl and 4 showed reduced air space formation in the root after 48 h of flooding. Thus, this concentration was chosen for analysis of mRNA accumulation.

Treatment with AOA almost completely abolished 1005 mRNA accumulation within 24 h of flooding in root and mesocotyl tissues compared with untreated controls (Fig. 5). On the other hand, the accumulation of ADH1 transcripts was affected to a much lesser extent by this treatment (Fig. 5). This indicated that the AOA treatment did not cause a general inhibition of flooding-induced gene expression, but specifically affected 1005 mRNA accumulation. In maize seedlings of similar age, AOA at 0.7 mM was shown to inhibit ethylene accumulation in roots under water deficit. Also, its effect on root elongation was similar to that obtained with silver thiosulfate, an inhibitor of ethylene action, suggesting that AOA at this concentration affected mainly ethylene synthesis (Spollen and Sharp, 1994; W. Spollen, personal communication). These results suggest that 1005 mRNA accu-

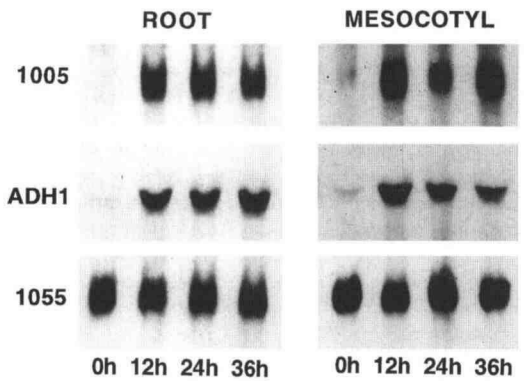

Figure 4. The induction of 1005 and ADH1 in the primary root and mesocotyl by flooding. Total RNA was extracted from locations exhibiting early aerenchyma development in the primary root and mesocotyl (Fig. 3). A 2-cm location starting at 2 cm below the kernel was selected in the primary root, and a 2-cm location starting at 3 cm below the coleoptilar node was selected in the mesocotyl. RNA was fractionated and blotted, and blots were hybridized as described in "Materials and Methods." Blots were hybridized with pZmL1005 (1005 partial cDNA), stripped, and then hybridized with pZmL793 (ADH1 cDNA) and pZmL1055 (loading control).

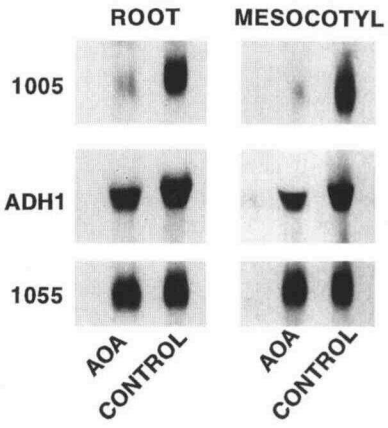

Figure 5. The effect of AOA treatment on induction of 1005 and ADH1 in the primary root and mesocotyl by 24 h of flooding. AOA at 0.7 mM was added to the germination and flooding media; untreated controls were germinated and flooded under identical conditions. Tissue sampling and experimental procedures were the same as those described in the legend of Figure 4.

mulation under flooding is responsive to ethylene accumulation, and thus, induction of *1005* and aerenchyma development appear to share a common trigger. Also, the results suggest that induction of ADH1 by flooding is not responsive to ethylene accumulation.

1005 Is Induced by Ethylene Treatment under Aerobic Conditions

To further test the responsiveness of *1005* to ethylene, we treated seedlings with exogenous ethylene under fully aerobic conditions to determine whether the gene can be induced by the hormone without flooding. mRNA accumulation was examined in the same locations in the primary root and the mesocotyl that were used in the flooding and anoxia experiments, using a *1005* gene-specific probe (Fig. 6). This confirmed that the ethylene-responsive gene is the same as the flooding-responsive gene. Treatment with ethylene for 48 h resulted in the accumulation of 1005 mRNA in both organs (Fig. 6). In addition, this correlated with the induction of aerenchyma in both organs (data not shown). On the other hand, ethylene treatment did not cause the accumulation of ADH1 mRNA in either organ (Fig. 6). This confirmed that the seedlings did not experience hypoxia during the treatment, since *adh1* is induced even by mild hypoxia (Paul and Ferl, 1991). The results also confirmed that, unlike *1005*, *adh1* is not responsive to ethylene.

Differential Regulation of *1005* by Flooding and Anoxia

In an earlier study, *1005* was induced in maize seedlings that were sealed in drowning buffer, a condition of near anoxia (Peschke and Sachs, 1994). Under anoxic conditions, however, ethylene accumulation is inhibited along with aerenchyma development (Drew et al., 1979; Brailsford et al., 1993). This suggested that the induction of *1005* by anoxia occurs in the absence of ethylene accumulation. To investigate this possibility, we compared the effect of AOA treatment on *1005* mRNA accumulation under flooding (hypoxia) versus anoxia (Fig. 7), using a *1005* gene-specific probe to confirm that the same gene is detected under both conditions. Results showed that *1005* was strongly induced by 24 h of anoxia in root and mesocotyl tissues despite treatment with AOA. Under flooding (hypoxic) conditions, however, AOA treatment almost completely abolished *1005* induction in both organs (Fig. 5). This points to differential mechanisms for the regulation of *1005*, depending on the severity of oxygen deprivation; the gene appears to be induced by ethylene accumulation under hypoxia and by an alternative mechanism under anoxia.

DISCUSSION

Although the majority of known flooding-responsive genes encode Glc-P metabolism enzymes, it was not surprising that a flooding-responsive gene encoding a homolog of XET, the putative cell-wall-loosening enzyme, would be isolated. After all, flooded tissues undergo substantial structural modifications, manifested mainly in the development of aerenchyma, and it is plausible that this would involve the activation of genes encoding wall-loosening and degrading enzymes, including XET.

XET has generated considerable interest among investigators of wall properties during growth and development and responses to environmental stress. Until fairly recently, cell-wall mechanical properties pertaining to wall loosening and cell expansion were described mainly using mathematical models (reviewed by Passioura, 1994). However, the identification of XET and putative wall-loosening factors such as expansins (McQueen-Mason et al., 1992) has offered a biochemical explanation, albeit partial, of wall loosening and its interactions with overall wall metabolism. The activity of XET has been observed in many species and found to be correlated with a wide range of processes, including expansive growth (Fry et al., 1992), wall adjustments in response to water deficit (Wu et al., 1994), and fruit ripening and softening (Redgwell and Fry, 1993; Maclachlan and Brady, 1994). These observations

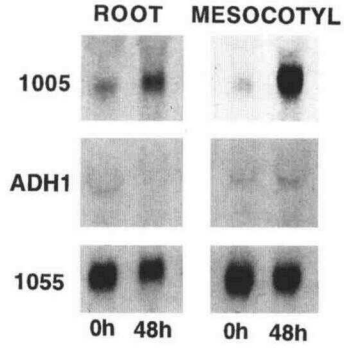

Figure 6. The induction of 1005 by ethylene under aerobic conditions. Seedlings were treated with 100 μL L^{-1} ethylene in air as described in "Materials and Methods." Tissue sampling and experimental procedures were the same as those described in the legend of Figure 4. Blots were hybridized with a 3′ end *1005*-specific probe to confirm that the ethylene-responsive gene is the same as the one induced by hypoxia.

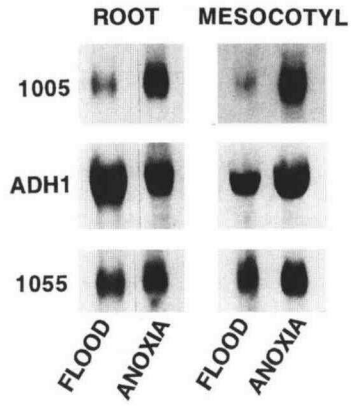

Figure 7. The effect of AOA treatment on 1005 induction under flooding and anoxia. AOA-treated seedlings were submerged in flooding buffer or treated anaerobically as described in "Materials and Methods." Tissue sampling and experimental procedures were the same as those described in the legend of Figure 4. Blots were hybridized with a 3′ end *1005*-specific probe to confirm that the anoxia-induced gene is the same as the one induced by hypoxia.

indicate that XET activity is associated with a broad range of physiological events from seed germination to fruit ripening, probably through the action of multiple forms of the enzyme. In maize cell walls, however, xyloglucan content is relatively low (Carpita and Gibeaut, 1993), which raises questions about the importance of XET and other xyloglucanases in controlling wall metabolism in this species. Nevertheless, Wu et al. (1994) found that XET activity in growing tissues of maize was higher than that in several dicot species, despite the low abundance of xyloglucans in maize tissues. Also, levels of 1005 mRNA were highly abundant under flooding (Fig. 4), suggesting that XET and related enzymes may play a significant role in cell-wall metabolism in flooded maize tissues.

On the molecular level, XET sequences have been isolated from several species, and in some species more than one XET cDNA has been identified (e.g. Okazawa et al., 1993; Arrowsmith and de Silva, 1995). Overall, the current molecular picture of the XET gene expression indicates that the enzyme is encoded by multiple genes (Peschke and Sachs, 1994; Arrowsmith and de Silva, 1995; Xu et al., 1995) and that some genes are induced in response to growth regulators (Zurek and Clouse, 1994; Xu et al., 1995; this work) and/or environmental stimuli (Xu et al., 1995; this work).

The association of *1005* with flooding-induced structural modifications is indicated by the observation that the gene is induced in regions of the primary root and mesocotyl that exhibit early signs of aerenchyma development under flooding. The association is also supported by the apparent responsiveness of *1005* induction under flooding to endogenous ethylene, a promoter of aerenchyma development, and by its responsiveness to exogenous ethylene under aerobic conditions, which also promotes aerenchyma development.

The role of the 1005 protein in flooding responses may depend on whether it is involved in transglycosylation or hydrolysis in vivo. Although most XETs subjected to activity assays in vitro were found to be predominantly transglycosylases, at least one possessed significant hydrolytic activity (Fanutti et al., 1993). 1005 may contribute to wall metabolism directly by hydrolyzing xyloglucans or other matrix polysaccharides. Alternatively, as a transglycosylase, 1005 may contribute to wall degradation indirectly by facilitating access to the dense wall matrix, or by regulating the accumulation of biologically active oligosaccharides generated by hydrolases. An assessment of the enzymatic activity of the recombinant protein is currently underway to address these possibilities.

The 1005 protein may also be involved in early events leading to structural modifications. Indeed, XET encoding *TCH4* from Arabidopsis is rapidly induced by touch and is proposed to be involved in early response to changing environments (Xu et al., 1995). Clearly, a role for the 1005 protein in the development of aerenchyma or other structural modifications would have to be in concert with an array of hydrolytic enzymes to bring about a change of this magnitude. This is also supported by the observation that 1005 mRNA accumulation under anoxia is not sufficient for aerenchyma development, although actual protein levels have not been determined. In addition, the fact that aerenchyma develops only in the inner cortex suggests that cells in that region are specific targets for ethylene action (Baluska et al., 1993) and that genes involved in this process are regulated in a coordinated and tissue-specific manner, leading to a form of programmed cell death.

It is interesting that 1005 mRNA accumulated under anoxia despite treatment with AOA, even though the same treatment almost completely abolished mRNA accumulation under hypoxia (flooding). This raises a question about possible differential mechanisms of *1005* induction under flooding versus anoxia, and suggests that the gene may play different roles under anoxia and hypoxia. In maize roots, anoxia has been shown to suppress ethylene accumulation as well as aerenchyma development (Drew et al., 1979). Although we did not measure ethylene accumulation under our anoxia conditions, we observed a lack of cortical air space formation in root and mesocotyl tissues. This indicates that *1005* induction by anoxia occurred despite an apparent inhibition of ethylene accumulation (or action), and may explain why it was not affected by AOA. This result also suggests that at least two separate mechanisms exist for *1005* induction: a mechanism that is active under hypoxia or flooding, which requires ethylene accumulation, and an additional or alternative mechanism active during anoxia independent of ethylene accumulation. Although they do not develop localized aerenchyma per se, anaerobically treated seedlings develop regions of tissue softening in the primary root and mesocotyl, which eventually spread to the entire organ. This may involve the action of *1005*. It is possible that, in the absence of ethylene production during anoxia, the targeting of programmed cell death specifically to the cortex and the ensuing aerenchyma development are lost. Another possibility is that, in contrast to *1005*, other genes associated with aerenchyma development are suppressed due to the lack of ethylene accumulation during anoxia. Alternatively, the accumu-

lated 1005 mRNA may simply not be translated under anoxia. To begin to address these questions, the activity of XET and its responsiveness to ethylene should be examined under hypoxic and anoxic conditions. These studies and other investigations on the role of *1005* in flooding responses are currently underway.

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