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REVIEW

Molecular and Cellular Adaptations of Maize to Flooding Stress

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Anaerobic treatment dramatically alters the patterns of gene expression in maize (*Zea mays* L.) seedlings. During anaerobiosis there is an immediate repression of pre-existing protein synthesis, with the concurrent initiation of a selective synthesis of approx. 20 proteins. Among these anaerobic proteins are enzymes involved in glycolysis and related processes. However, inducible genes that have different functions were also found; these may function in other, perhaps more long-term, processes of adaptations to flooding, such as aerenchyma formation and root-tip death. In this article we review our recent work on maize responses to flooding stress, which has addressed two questions: how are these gene expression changes initiated and how do they lead to adaptation to flooding stress? Our results indicate that an early rise in cytosolic Ca^{2+}, as well as a quick establishment of ionic homeostasis, may be essential for the induction of adaptive changes at the cellular as well as organismal level.
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INTRODUCTION

Oxygen limitation is the primary plant stress in flooded soils. The sudden excess of water due to flooding not only threatens the food supply of human populations but also affects the vegetation in river plains. During 1993, approx. 20 million acres of corn (*Zea mays* L.) and soybean (*Glycine max* L.) were inundated in the mid-western United States leading to heavy economic losses, as estimated by the United States Department of Agriculture, National Agricultural Statistics Service (Suszkiw, 1994).

Anaerobic treatment of maize seedlings drastically alters the profile of total protein synthesis. In an anaerobic environment, 20 proteins, which account for more than 70 % of the total translation, are selectively synthesized (Sachs *et al*., 1980). Most of the anaerobic proteins (ANPs) identified were found to be enzymes of glycolysis or sugar-phosphate metabolism, such as aldolase (Kelley and Tolan, 1986), pyruvate decarboxylase (Laszlo and St Lawrence, 1983; Kelley, 1989), enolase (Lal *et al*., 1998), glucose-6-phosphate isomerase (Kelley and Freeling, 1984), glyceraldehyde-3-phosphate dehydrogenase (Russell and Sachs, 1991), sucrose synthase (Springer *et al*., 1986) and alcohol dehydrogenase (Freeling, 1973). Additionally, three genes that are not involved in glucose-phosphate metabolism (Vogel and Freeling, 1992; Peschke and Sachs, 1994; Saab and Sachs, 1995, 1996) have been found to be induced by anoxia, and may also encode ANPs.

Anaerobiosis results in alterations of gene expression in plants leading to the accumulation of ANPs. These alterations occur at transcriptional, translational and post-translational levels (Ferl *et al*., 1980; Sachs *et al*., 1980; Rowland and Strommer, 1986; Bailey-Serres *et al*., 1988; Dennis *et al*., 1989; Russell and Sachs, 1989, 1992; Webster *et al*., 1991; de Vetten and Ferl, 1995; Manjunath and Sachs, 1997; Manjunath *et al*., 1999; Subbaiah and Sachs, 2001). At the level of translation, anaerobic treatment of maize seedlings disrupts polysomes (Bailey-Serres and Freeling, 1990) and leads to a redirection of protein synthesis (Sachs *et al*., 1980; Russell and Sachs, 1992). In the first 5 h of anaerobic treatment (transition period) there is a rapid increase in the synthesis of a class of polypeptides (approx. 33 kDa; the transition polypeptides). After 90 min of anoxia, the synthesis of ANPs is induced. After 72 h, protein synthesis decreases concurrently with the start of seedling death (Sachs *et al*., 1980). The molecular basis of this selective translation is not yet fully understood. Post-translational regulation of initiation factors and ribosomal proteins by reversible phosphorylation appears to play a role (Webster *et al*., 1991; Perez-Mendez *et al*., 1993; Manjunath *et al*., 1999) in addition to the structural determinants in the untranslated regions of mRNA (Bailey-Serres and Dawe, 1996). Other post-translational changes of proteins have also been reported to occur under anoxia (e.g. Subbaiah and Sachs, 2001). From 2-D electrophoresis/MS analysis of proteins synthesized in the root tip under different oxygen regimes, Chang *et al*. (2000) also inferred that several proteins undergo post-translational modifications under conditions of O_2 deprivation. Besides this reprogramming of gene expression, metabolic (e.g. switch to a fermentative pathway; Kennedy *et al*., 1992) and structural (e.g. aerenchyma formation; Drew *et al*., 1979, 1985) changes occur during flooding. The maize anaerobic response has been extensively reviewed previously (Sachs, 1993, 1994; Sachs *et al*., 1996).

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Previous work has focused on genes encoding enzymes involved in glucose-phosphate metabolism, and their expression during oxygen deprivation. Our earlier work also identified a Ca^{2+}-mediated pathway of anoxic gene induction as well as post-anoxic seedling survival. The emphasis of this review will be on our more recent work, i.e. the elaboration of early responses and how they relate to the long-term anoxic tolerance of maize seedlings. Our results indicate that cytosolic Ca^{2+} changes are used by cells to establish ionic homeostasis, besides triggering the downstream signalling pathway. A few novel genes/loci that appear to play a role in plant responses and tolerance to flooding are also discussed. In the final part of the review, we surmise how these cellular and molecular responses are integrated at the organismal level to maximize survival under flooding stress.

Our discussion largely centres around work in our laboratory carried out on maize during the past few years. In most of these studies (with the exception of metabolic labelling experiments conducted in an anaerobic chamber), anoxia was imposed by completely submerging 3-d-old maize seedlings in flooding buffer; this treatment represents a progressive depletion of oxygen rather than a shock. Anoxia tolerance refers to the ability of seedlings to resume growth upon reoxygenation (e.g. Lemke-Keyes and Sachs, 1989*b*; Subbaiah *et al*., 1994*b*; Subbaiah and Sachs, 2001). Hypoxic treatment was given by 'partial submergence' (i.e. submerging the root portion only, leaving the shoot exposed to air; Saab and Sachs, 1996).

IONIC HOMEOSTASIS AS AN INTEGRAL PART OF THE ANOXIA SIGNALLING PATHWAY

Genes encoding the ANPs (e.g. *adh1*) are rapidly turned on even by mild hypoxia and are rapidly turned off upon reoxygenation (Freeling, 1973; Wignarajah and Greenway, 1976; Paul and Ferl, 1991; Chang *et al*., 2000). Such a response implicates a fast and precise O_2-sensing system operating in plant cells. However, until recently, the pathway leading to the perception and transduction of low O_2 signals remained a 'black box'.

The transient changes in Ca^{2+} and H^+ that follow cell stimulation are immediately recognized even at sub-micromolar levels, amplified and finally translated into long-lasting biochemical and physiological responses by plant cells (e.g. Knight *et al*., 1996; Fasano *et al*., 2001; reviewed in Bush, 1995; Sanders *et al*., 1999). Deprivation of O_2 leads to disturbances in ionic balance of plant cells, reflecting energy depletion and membrane depolarization (e.g. Roberts *et al*., 1984; Buwalda *et al*., 1988). We have shown that gene expression and physiological changes in response to O_2 deprivation are preceded and signalled by an elevation of cytosolic Ca^{2+} in maize seedlings and cultured cells (Subbaiah *et al*., 1994*a*, *b*). We developed a single cell system to monitor cytosolic Ca^{2+} changes and an assay to measure anoxic responses in terms of expression of marker genes and seedling (or cell) survival (Subbaiah *et al*., 1994*a*, *b*). Using calcium channel antagonists and analysing cytosolic free calcium ($[Ca]_i$) changes, we demonstrated that calcium acts as a transducer of low O_2 signals both in suspension-cultured cells and in intact seedlings (Subbaiah *et al*., 1994*a*, *b*). Ruthenium red (RR), a Ca^{2+} channel blocker, repressed the activation of the anoxia-inducible genes and impaired the post-anoxic survival of seedlings and cells (Subbaiah *et al*., 1994*a*, *b*). Ca^{2+}, when supplied along with RR, allowed both anoxic gene expression and survival, showing that Ca^{2+} acted very early in the adaptive response to anoxia. In maize suspension-cultured cells, O_2 depletion caused an immediate (within minutes) increase in $[Ca]_i$ and this was reversible within a few seconds of reoxygenation. RR decreased the resting levels of $[Ca]_i$ and blocked the anoxic Ca^{2+} elevation. Caffeine, which induced an elevation of $[Ca]_i$ under aerobic conditions, caused an increase in ADH activity under normoxia.

Furthermore, we showed that Ca^{2+} influx was not necessary for the anoxia-induced $[Ca]_i$ elevation or early anoxic responses, indicating that the Ca^{2+}-rise observed under anoxia was due to mobilization of the ion from intracellular stores (Subbaiah *et al*., 1994*a*). The origin of the calcium signal was traced as part of our attempt to elucidate the nature and intracellular location of the oxygen sensor. Being the primary site of oxygen consumption and also an important target of RR action, it was thought the mitochondrion might serve as a Ca^{2+} store in response to anoxia in maize cells. Confocal analysis using compartment-specific Ca^{2+} probes showed that the Ca^{2+} signal probably originates in mitochondria (Subbaiah *et al*., 1998). The release of Ca^{2+} from mitochondria during early anoxia is probably not due to passive leakage of the ion, since it was not preceded by the depolarization of mitochondria. However, prolonged anoxia (longer than 30 min) leads to a loss of mitochondrial membrane potential and thus may be responsible for further Ca^{2+} release. Furthermore, individual mitochondria within single maize cells responded differently to O_2 deprivation (Subbaiah *et al*., 1998). While the role of plasma membrane redox systems and associated second messengers also needs to be examined, our findings placed mitochondria at the centre of oxygen sensing.

The elucidation of how O_2 deprivation initiates Ca^{2+} release from mitochondria may indicate exactly where the changes in O_2 levels are sensed in the cell. Since oxygen is more diffusive than any potential signal molecule that has to traverse the cellular membranes, anoxia may first be sensed at the mitochondrial electron transport chain, where O_2 would no longer be available as an electron acceptor. However, in view of the sensitivity of gene expression changes even to mild alterations in O_2 availability (Paul and Ferl, 1991), i.e. the genes are induced at much higher concentrations than the K_m (O_2) of cytochrome a_3, a low affinity system could be a more appropriate sensor (such as a component of the plasma membrane redox system). The Ca^{2+} released from mitochondria may communicate the metabolic changes occurring in the cytosol (and mitochondria) under O_2 deprivation to the nucleus. Consistent with this, our preliminary observations indicate that anoxia induces large changes in the nuclear localized Ca^{2+} levels (Subbaiah *et al*., 1998).

Exigent adaptations

The metabolic and structural adaptations to O_2 deprivation are known to precede transcriptional activation/repression of genes, translation of specific mRNA species and post-translational modification of proteins. Our aim is to understand how Ca^{2+} participates or leads to these events. In addition, we believe that the perturbations in cytosolic Ca^{2+} may also mediate immediate adaptations needed for cell survival in the short term. Under energy-deprived conditions, an imminent danger to cells (the organism, as well) is an unregulated traffic of ions. For example, a continued elevation of Ca^{2+} or decline in pH, if unattenuated, is not only detrimental in the long run but may also impair the capacity of cells to mount the adaptive responses. Therefore, ionic homeostasis is probably a key component of the cellular adaptive pathway under stress. We examined the Ca^{2+}-regulation of this process in O_2-deprived cells. In maize root tip cells, cytosolic pH decreases sharply, from pH 7·5 to 6·9, in response to anoxia within the first 10 min, but then quickly stabilizes at 7·1 over the next 10–15 min (Saint-Ges *et al*., 1991; Fox *et al*., 1995). One of the mechanisms proposed to revert the pH decline is the activation of proton-consuming enzymes, such as malic enzyme or glutamate decarboxylase (Roberts *et al*., 1992; Sachs *et al*., 1996; Ratcliffe, 1997), although the actual players have not yet been clearly worked out. Our contention that $[Ca]_i$ changes could be important in the establishment of the pH-stat was reinforced by the discovery that plant glutamate decarboxylases (GADs) have a distinct ability of interacting with calmodulin (CAM) (Snedden *et al*., 1995; Shelp *et al*., 1999). Furthermore, in many species GAD activity exhibits a sharp pH optimum of 5·8 with little activity at or near neutral pH in the absence of CAM (Shelp *et al*., 1999). Therefore, a pH- and/or CAM-dependent activation and its significant ability to consume protons (Snedden *et al*., 1995) make GAD a candidate regulator of cytosolic pH under anoxia. GAD activity was in fact shown to be induced in carrot protoplasts by limiting the supply of O_2 for 2 h (Carroll *et al*., 1994). Hence, we focused our studies on the activation of GAD as an adaptive response and a focal point of interaction between pH and calcium changes in maize roots. A rapid induction of GAD activity, as well as an increased association of this activity with CAM-containing protein complexes, was observed in maize roots within minutes of anoxic treatment. Furthermore, Ca^{2+}/CAM antagonists abolished the activity *in vitro*, indicating that CAM-association may be needed for the activation of GAD under anoxia. The kinetics of GAD activation soon after the onset of anoxia coincided with the time course of pH stabilization in maize root tips reported previously (e.g. Fox *et al*., 1995). Genes encoding GAD have already been cloned from a number of species (Snedden *et al*., 1995). Several maize clones that are similar to GAD from other plant species were identified in a maize EST database (http://www.zmdb.iastate.edu/), indicating that a gene family may encode this enzyme in maize. These clones were obtained, and after confirmatory sequencing analysis were identified as putative maize GAD cDNAs. Sequence comparison indicated that GAD is encoded by at least three genes in maize (Subbaiah and Sachs, unpubl. res.). RNA gel blot analysis showed that only one of them is inducible by anoxia in maize roots and the other two are actually repressed. Furthermore, the transcript levels for the non-inducible clones showed a greater abundance in the axis portion (1 cm away from the tip) of the primary root in 3-d-old seedlings (Subbaiah and Sachs, unpubl. res.). We have also examined the distribution of ESTs among various maize libraries that were made use of. The majority of hits was found in libraries that were made from young and meristematic tissues (e.g. leaf and tassel primordia, early embryo, and anthers) that are expected to be rich in mitochondria and that show intense respiratory activity (Subbaiah and Sachs, unpubl. res.).

Fine-tuning the Ca^{2+} signal

Efflux transporters (transporters that remove Ca^{2+} from the cytoplasm) play an equally important role as influx transporters (transporters that allow Ca^{2+} into the cytoplasm) in Ca^{2+} signalling. High-affinity transporters, such as Ca^{2+}-ATPases, attenuate as well as modulate stimulus-induced Ca^{2+} rise. In view of the versatility of Ca^{2+} to transduce a number of stimuli, it is imperative that Ca^{2+} signals are patterned in a stimulus-specific manner. In coordination with the influx transporters, Ca^{2+} pumps not only orchestrate stimulus-specificity to Ca^{2+} signals but fine-tune and propagate them (e.g. Camacho and Lechleiter, 1993). One objective of our research is to unravel Ca^{2+}-transporters involved in the regulation of anoxic Ca^{2+} signal. To this end, we have isolated a cDNA clone, CAP1, from anoxic maize roots. CAP1 encodes a Ca^{2+}-ATPase and is induced during the first 4–6 h of anoxia (Subbaiah and Sachs, 2000). This clone shares sequence identity with the animal Ca^{2+}-ATPases located on the endoplasmic reticulum (SERCAs or ER-type calcium pumps). However, maize CAP1 differs from other ER-type Ca^{2+}-pumps in that it has a calmodulin-binding domain at its carboxy-terminus. The CAP1 cDNA complemented yeast mutants defective in Ca^{2+} pumps. The CAP1 protein from yeast membranes formed a phosphorylated intermediate characteristic of Ca^{2+}-ATPases and supported CaM-stimulated Ca^{2+} transport (Subbaiah and Sachs, 2000). Therefore, CAP1 represents a novel calcium pump that may be involved in Ca^{2+}/CaM-mediated signalling pathways in maize roots. The *cap1* gene in maize encodes a low abundance mRNA that is induced only during early anoxia, among other abiotic stresses tested (Subbaiah and Sachs, 2000). This indicated the potential involvement of CAP1 in imparting anoxia-specificity to the Ca^{2+}-signal. The low abundance of CAP1 transcripts coincided with the scarce amounts of cognate protein in maize microsomes, indicating a tight regulation of CAP1 expression. Furthermore, calmodulin regulation of Ca^{2+} transport capacity suggests the involvement of CAP1 product in attenuating cytosolic Ca^{2+} rise in a feedback manner during cell stimulation. A stringent regulation of Ca^{2+} efflux should allow the Ca^{2+}-dependent signalling processes to continue without the cell attaining cytotoxic levels of free Ca^{2+}. Induction of CAP1 transcripts in maize roots only during the first few hours of anoxia indicates such a regulation of Ca^{2+}

Table 1. *Chromosomal locations of maize genes involved in the anaerobic response*

Gene name or clone	Symbol	Location
Alcohol dehydrogenase 1	*adh1*	1L
Alcohol dehydrogenase 2	*adh2*	4S
Aldolase 1	*ald1*	8L
Enolase 1	*eno1*	9S
Enolase 2 (c)	*eno2*	1S
Glyceraldehyde-3-phosphate dehydrogenase 1 (c)	*gpc1*	4S
Glyceraldehyde-3-phosphate dehydrogenase 2 (c)	*gpc2*	6S
Glyceraldehyde-3-phosphate dehydrogenase 3	*gpc3*	4S
Glyceraldehyde-3-phosphate dehydrogenase 4	*gpc4*	5L
Phospho-hexose isomerase 1	*phi1*	1L
Pyruvate decarboxylase 1	*pdc1*	8L
Pyruvate decarboxylase 2	*pdc2*	8
Pyruvate decarboxylase 3	*pdc3*	1S
Shrunken 1	*sh1*	9S
Sucrose synthase 1	*sus1*	9L
Xyloglucan endotranglycosylase 1	*xet1*	5S
1032	*wusl1032*= *umc217*	1S

(c) indicates constitutive member of a gene family; other members are anaerobically inducible.

Chromosomal locations are from the maize genome database (URL http://www.agron.missouri.edu).

homeostasis in the O_2-deprived maize cells (Subbaiah and Sachs, 2000).

NOVEL GENES INVOLVED IN THE ANAEROBIC RESPONSE OR FLOODING TOLERANCE

Advances have been made in molecular-level analyses of several cDNAs and genes involved in the anaerobic response (cf. Sachs, 1994; Sachs *et al*., 1996). Table 1 shows the chromosomal locations for maize genes involved in the anaerobic response, for which DNA clones were available to carry out restriction fragment length polymorphism mapping analysis (Peschke and Sachs, 1994). These genes are distributed throughout the genome (Table 1). Until recently, the only genes described in plants that are induced by oxygen deprivation (anoxia or hypoxia) encoded enzymes of glucose-phosphate metabolism (mostly glycolysis and fermentation), and thus apparently function to allow limited energy production in the face of limited oxygen supply (Sachs, 1993, 1994; Sachs *et al*., 1996). We present here three newly described gene systems that appear to function outside the glycolytic pathway.

Maize aie2 *homologue*

Recently, Huq and Hodges (2000) reported early activation of a rice (*Oryza sativa* L.) gene by anoxia (its transcript peaked after 1–3 h of anaerobic treatment); the authors named it the *aie* (anaerobically inducible early) gene. Using a differential display technique to identify cDNA fragments representing mRNAs that are induced within 90 min of anoxia, two cDNA fragments and one full-length cDNA were isolated. These cDNAs represented a small gene family of two or three genes. The full-length cDNA (belonging to the *aie2* gene) encodes a novel protein (127 amino acids long) that has no similarity to any known protein in the GenBank and SwissProt databases. We searched a public database of maize ESTs (http://www.zmdb.iastate.edu/) for a homologue of rice *aie2* gene and found that there are at least 11 ESTs comprising two putative contigs (TUC05-31-1810·1 and TUC05-31-10062·1). They are approx. 80 % identical to each other and >70 % similar to the rice gene at the nucleotide sequence level. However, both the maize TUCs are longer than their rice homologue. Since the initiation and termination codons are further apart in these two cDNAs, they also appear to encode a larger protein. Although confirmatory sequencing needs to be carried out, the predicted protein product is not extensively similar to any known protein in the database. Nevertheless, the putative protein shows short stretches of similarities to functionally interesting proteins (e.g. DNA-binding proteins and nitric oxide synthase), indicating its putative involvement in signalling (Subbaiah and Sachs, unpubl. res.).

Anoxia-tolerance gene(s) identified from the analysis of natural variation in maize

Anaerobic stress can significantly reduce survival or growth of germinating maize seedlings in waterlogged soils. The majority of maize genotypes survive up to 3 d of anaerobic treatment at 27 °C. On the other hand, mutants that are null for ADH activity only survive a few hours of anoxia. We screened several hundred inbred and exotic maize lines for their tolerance to anoxia. Nine exotic accessions showed greater tolerance to anaerobic stress, as they survived 5–6 d of anaerobic treatment at 27 °C. In addition, three inbred lines were found to be significantly less tolerant to anaerobic conditions, surviving for only 2 d (Lemke-Keyes and Sachs, 1989*b*). Results of crosses between the anaerobic-tolerant accessions with one anoxia-sensitive inbred line (Mo20W) and a ‘normal’ inbred line (B73Ht) show that the anoxia-tolerance trait(s) is dominant and shows very simple segregation. This indicated that only one or two genes are involved in each accession. We are presently creating anaerobic-tolerant inbreds in order to analyse the gene(s) involved in this trait.

Xyloglucan endotransglycosylase (XET)

Flooded maize plants, like those of many species, undergo structural changes leading to cell lysis and the formation of cortical air spaces (often referred to as aerenchyma). These spaces may facilitate gas diffusion from aerated plant parts, and as such are considered advantageous for prolonged survival in flooded soils (Drew *et al*., 2000). There is strong evidence indicating that formation of intercellular air spaces is promoted by accumulation of endogenous ethylene in submerged tissues (reviewed in Drew *et al*., 2000). Formation of aerenchyma can also be induced in well-aerated plants that are starved of nitrogen or phosphorus, and this response is proposed to be triggered by increased sensitivity to endogenous ethylene

(He *et al*., 1992). The formation of aerenchyma was found to be associated with increased activity of cellulase, which presumably contributes to cell lysis (Drew, 1992; Grineva and Bragina, 1993; He *et al*., 1994). Additional cell wall and cytoplasmic degradation enzymes are also likely to be involved in the process (Campbell and Drew, 1983; He *et al*., 1994). However, there is little information to date on the molecular regulation of aerenchyma formation or the identity of other enzymes involved. In maize, a flooding-induced gene (*xet1*) encodes a xyloglucan *endo*transglycosylase (XET1), a putative cell wall loosening enzyme (Peschke and Sachs, 1994; Saab and Sachs, 1995, 1996). O_2 deprivation induces expression of XET1 in the primary root, mesocotyl, and coleoptile of maize seedlings. The induction of *xet1* appears to be specific to O_2 deprivation, since other stresses do not induce the gene (Peschke and Sachs, 1994). The *xet1* gene appears to be a member of a large multigene family in which only *xet1 is* inducible by oxygen deprivation. The induction of *xet1* by hypoxia was associated with aerenchyma development and, like aerenchyma, XET1 transcript was induced by ethylene. Hypoxic induction of XET mRNA is repressed by ethylene antagonists [(amino-oxy)acetic acid, 2-aminoethoxyvinyl-glycine, $AgNO_3$]. XET1 is also induced under aerobic conditions by exogenous ethylene, as is aerenchyma. This differs from ADH1, which is also induced by hypoxia but in an ethylene-independent manner in maize (Saab and Sachs, 1996). Taken together, *xet1* appears to be involved in flooding-induced cell wall metabolism leading to aerenchyma development.

We are presently exploring the potential role of maize *xet1 in* flooding-induced cell structural changes, such as tissue deformation, cell lysis, and aerenchyma formation. XET1 cDNA was expressed in *Esherichia coli* and the recombinant protein was tested for XET activity. The results show that *xet1* does encode a functional XET enzyme. However, when total extractable XET levels are measured in maize roots under aerobic and hypoxic conditions, no significant difference is detected (Saab and Sachs, unpubl. res.). This can be explained by a differential induction or repression of individual members of the *xet* gene family under O_2 deprivation, resulting in no net increase in XET activity. Our current focus is to determine if the XET1 gene product specifically co-localizes to those cortical cell layers where aerenchyma is formed.

LOCATION, LOCATION, LOCATION: POST-TRANSLATIONAL REGULATION OF SUCROSE SYNTHASE UNDER ANOXIA

One common mechanism that cells use to rapidly decipher and amplify the $[Ca]_i$ changes is reversible protein phosphorylation (for a review, see Stone and Walker, 1995). Addition or removal of phosphate can lead to changes in the activation status, catalytic activity, or cellular localization of effector proteins (e.g. Kim *et al*., 1994; Huber and Huber, 1996). These changes can, in turn, lead to transient alterations in gene expression and metabolism or even long-lasting modifications in the plant form and function (e.g. Ferreira *et al*., 1993; Maurel *et al*., 1995; Stone *et al*., 1998; Fankhauser *et al*., 1999).

We have recently shown that an anaerobically induced polypeptide, sucrose synthase 1 (SH1), is post-translationally regulated by phosphorylation, and this regulation is among the early responses that culminate in the death of the primary root tip in anoxic maize seedlings (Subbaiah and Sachs, 2001). Sucrose synthase (SS) is a unique enzyme with an ability to mobilize sucrose into diverse pathways that are critical in structural (e.g. cellulose or callose biosynthesis), storage (starch synthesis) and metabolic (e.g. glycolysis) functions of plant cells (e.g. Ruan *et al*., 1997). It is encoded by two genes in maize, *sh1 (*encoding SH1) and *sus1 (*encoding SUS1). The *sh1* gene is expressed mostly in the developing endosperm, while *sus1 is* expressed in many plant parts including the aleurone and basal part of the developing endosperm. The *sh1* gene is induced by anoxia both at transcriptional and translational levels (ANP87; Springer *et al*., 1986). The *sus1* gene is only mildly induced by anoxia. Although the double mutants in SS have been shown to be less tolerant to anoxia (Ricard *et al*., 1998), the contribution of SH1, i.e. the anoxia-inducible isoform, to anoxia tolerance had not previously been examined. Our results indicate that the differential regulation of the two genes at transcriptional and translational levels extends into the post-translational level, with potent effects on adaptation to anoxia and endosperm development (Subbaiah and Sachs, 2001).

Analysis of Ca^{2+}-dependent changes in protein phosphorylation under anoxia indicated that the SH1 isoform of sucrose synthase was phosphorylated at increased rates in maize roots subjected to 2 h of anoxia. In contrast, during prolonged anoxia the protein was under-phosphorylated, and by 48 h most of the protein existed in an unphosphorylated form. In seedlings submerged for 2 h or more, a part of the SH1 became associated with the microsomal fraction (Subbaiah and Sachs, 2001). The membrane localization of SH1 increased with the duration of anoxia, but was confined only to the root tip. This preceded an extensive induction of callose and other symptoms of root tip death (e.g. induction of nuclear DNA breakage; Subbaiah and Sachs, unpubl. res.). Consistent with the Ca^{2+} dependence of SS phosphorylation (e.g. Huber *et al*., 1996), addition of EGTA (ethylene glycol tetra acetic acid) to the submergence buffer led to an increased dephosphorylation as well as membrane localization of SH1 and greater callose accumulation. On the other hand, Ca^{2+} addition decreased the proportion of membrane-bound SH1 and callose deposits (Subbaiah and Sachs, 2001). Thus, these results corroborated two earlier observations: (1) that sucrose synthase is functionally associated with glucan synthases in the plasma membrane (Amor *et al*., 1995); and (2) the phosphorylation status of SS may determine its partitioning between soluble and membrane fractions (Winter *et al*., 1997). Furthermore, our genetic analysis suggested that this response was isoform-specific in that *sh1* mutants, maintained SS phosphorylation and had low amounts of callose deposits in the root tip even under prolonged anoxia. This correlated with the superior anoxia tolerance of *sh1* mutants to that of the non-mutants

(Subbaiah and Sachs, 2001). Our studies thus indicate a functional divergence of SS isoforms due to a differential post-translational regulation, in that SUS1, existing mostly as a soluble form, may supply hexoses to glycolysis, while SH1, being distributed in both soluble and membrane fractions, contributes to the biosynthesis of cell wall polymers as well. Such a dichotomy is also consistent with the proposed roles of SS isoforms in the developing maize endosperm (see Chourey *et al*., 1998).

HYPOXIA-INDUCED AERENCHYMA OR ANOXIA-INDUCED ROOT TIP DEATH: REGULATED CELL DEATH AS A MEANS OF SURVIVAL UNDER OXYGEN DEPRIVATION

The essence of stress adaptation is redirecting scarce resources to the maintenance of essential sinks as well as activation of adaptive pathways, while disinvesting in non-essential sinks and pathways. Being endowed with multiple growing points, plants have a unique ability to eliminate superfluous tissues/organs under stress and regenerate them if favourable conditions demand. O_2-deprived maize roots exhibit two such regulated cell or tissue-death pathways. These two pathways are clearly distinct in their regulation as well as in the location of their occurrence in the root.

As alluded to in the previous section, inner cortical cell layers of the primary or nodal roots are selectively killed under hypoxia (i.e. under partial submergence when only the roots were submerged), leading to aerenchyma formation. This selective cell death not only reduces the demand for O_2 but, more importantly, enhances root porosity and facilitates oxygen diffusion from the exposed plant parts into the submerged ones. Aerenchyma formation requires the presence of oxygen and occurs 3–4 cm behind the tip (He *et al*., 1992). This allows the root tip to adapt to the localized anoxia (Gibbs *et al*., 1995), and prolongs the survival of seedlings. The nature and regulation of cell death during aerenchyma formation have been the subject of recent studies (He *et al*., 1992; Drew *et al*., 2000; Gunavardena *et al*., 2001). These studies indicate that aerenchyma formation occurs by a genetically programmed cell death (PCD) (reviewed in Drew *et al*., 2000). Cytohistological data, however, indicate that the hypoxically induced PCD does not entirely follow the canonical apoptic pathway of animal cells, but partly resembles the cytoplasmic or necrotic death (Gunavardena *et al*., 2001).

Root-tip death

Under complete submergence, maize seedlings exhibit another cell death process that also appears to have an adaptive significance. Although prolonged anoxia ultimately kills the entire seedling, different tissues of an individual plant differ in their tolerance (Johnson *et al*., 1989; Ellis *et al*., 1999). Maize root tips that are not hypoxically acclimated are very sensitive to anoxia and die within a few hours (Roberts *et al*., 1984; Johnson *et al*., 1989). Root tips are composed of tightly packed tissues with few, if any, intercellular spaces, and hence suffer from restricted gaseous diffusion. Consequently, in flooded seedlings, root tip death may be a natural consequence of oxygen starvation and the attendant repression of substrate transport. Considerable attention has been paid to strategies/mechanisms that prolong the anoxia tolerance of the primary root tip in young maize seedlings, as the tip of the primary root is considered to be very important for seedling establishment (for a review, see Drew *et al*., 1994). On the other hand, we proposed that under severe anoxia, when energy generation is extremely limiting, the loss of metabolically intensive tissues such as the root tip might prolong the survival of the shoot and the root axis. The facilitated survival of these two organs during submergence may increase the chances of seedling recovery after reoxygenation. We have recently tested this proposal, and results indicate that the root tip does indeed act as a dispensable and non-functional sink in anoxic seedlings (Subbaiah *et al*, 2000; Subbaiah and Sachs, 2001).

The time course of primary root tip death in submerged maize seedlings was followed using the post-anoxic development of visible necrotic symptoms and uptake of Evans Blue as criteria. Anoxia for 48 h or more led to the death of the root tip (Subbaiah *et al*., 2000). If the seedlings were reaerated prior to 48 h, root tip death did not occur. However, cell death indicators such as callose development, DNA nicking, and induction of hydrolytic activities were observed to occur much earlier than 48 h (Subbaiah and Sachs, 2001; unpubl. res.). These observations indicated that the death process, although initiated before 24 h, became irreversible at 48 h of anoxia. The necrosis extended into the root axis during post-anoxic recovery, leading to the mortality of 30–50 % of the seedlings. Excision of the root tip (de-tipping) before anoxia led to a 30–40 % greater recovery of seedlings from stress injury. In contrast to the slow and progressive death of root tips in intact seedlings, de-tipped seedlings showed less shoot and root damage, resulting in a rapid emergence of leaves as well as lateral roots after reaeration (Subbaiah *et al*., 2000). Our data also indicate that the dying root tip of intact seedlings releases diffusible death-inducing factors into the submergence buffer, as indicated by the acceleration of seedling death when submergence buffer was reused. Preliminary analysis indicates that these factors are proteinaceous in nature (Subbaiah *et al*., 1999; unpubl. res.). Therefore, a reprogramming of root tip death so that it occurs early during anoxia may provide a definite adaptive advantage to maize seedlings to anoxic stress. In arabidopsis, the whole root system is dispensable for hypoxic tolerance of the seedlings; in fact, de-rooted seedlings fared better under O_2 deprivation (Ellis *et al*., 1999). In maize, the primary root axis is helpful (in quickly generating a functional root system), if not essential, for the post-anoxic recovery of seedlings. However, the survival of the shoot meristem is critical for the post-anoxic regrowth and autotrophic life of the seedling.

Anoxia-induced protease (AIP) in root tip death

To identify potential regulators of the cell death process, changes in protease activities were analysed in the root tissues. Cysteine and serine proteases have been implicated

in the cell death/injury induced by abiotic, biotic or developmental signals in plants (e.g. Williams *et al*., 1994; Stroeher *et al*., 1997; Solomon *et al*., 1999; for a review see Hadfield and Bennett, 1997).

Different species of proteases, both soluble and membrane-bound, are induced or suppressed during different durations of anoxic stress and reoxygenation in roots of 3-d-old dark-grown maize seedlings (Subbaiah *et al*., 2000). The major aerobic proteases are suppressed after 6 h of anoxia and new enzymes are detected both in soluble and membrane fractions. Upon reoxygenation, the aerobic activities reappear and the anoxic enzymes persist for at least 24 h after seedlings are reaerated. We observed a soluble enzyme that became detectable after 12 h of anoxia. This enzyme increases with time and accounts for the major proteolytic activity in roots of seedlings submerged for 48 h (Subbaiah *et al*., 2000). Protein synthesis inhibitor studies show that this is a newly synthesized enzyme under anoxia (anoxia-induced protease: AIP). AIP activity runs as a 22–25 kDa complex in SDS–PAGE. Ca^{2+} is required for the renaturation and proteolytic activity of the enzyme, and inhibitor sensitivities indicated that AIP is a cysteine protease. De-tipping caused a decrease in AIP activity. Thus, the appearance of AIP activity in the root tip before 24 h of submergence was spatially and temporally associated with the initiation of the root tissue death (Subbaiah *et al*., 2000).

In addition to AIP activity, XET1 mRNA is also induced in maize roots by anoxia (apparently by a different mechanism than its hypoxic induction; Saab and Sachs, 1996) and may be involved in the root-tip death process. Besides its proposed role in cell wall loosening in growing tissues, XET is associated with cell wall hydrolysis and cell lysis. For example, increased XET activity was shown to have a temporal correlation with ethylene-induced fruit ripening and softening (Redgwell and Fry, 1993).

Root tip death under anoxia: programmed cell death or necrosis?

Cell death is a basic biological process important in the regulated development of multicellular organisms and in their responses to stress. Animal cells show two fundamentally different modes of death, namely apoptosis (or PCD) and necrosis. The most relevant distinction between the two types of death is the early preservation of membrane integrity in apoptosis, whereas a rapid release of intracellular constituents occurs in the case of necrosis. Therefore, necrosis can presumably be dangerous, while an apoptic response is an adaptive mechanism to dispose of cells without compromising the rest of the organism. Nevertheless, there is increasing evidence that apoptosis and necrosis just represent extreme ends of a wide range of possible morphological and biochemical deaths. Root-tip death is preceded by SH1 relocation, DNA nicking, and induction of AIP and callose, indicating that the process, to some extent, is autonomous (and a programmed event; Subbaiah *et al*., 2000; Subbaiah and Sachs, 2001; unpubl. res.). On the other hand, the death of root tip cells is accompanied by acidification of the cytosol (Roberts *et al*., 1984) as well as the external medium, and an extracellular release of diffusible cytotoxins (Subbaiah *et al*., 1999; unpubl. res.). Therefore, in nature, root tip death may be a less cell-autonomous process but more of a necrotic process. Our de-tipping experiments suggest that an acceleration of the process as well as making it more cell-autonomous (i.e. pushing the process more towards PCD) would provide a definite advantage during post-anoxic recovery of maize seedlings. Indeed, some maize genotypes appear to have evolved an accelerated root tip death as a genetically controlled flooding tolerance mechanism (Zeng *et al*., 1999).

CONCLUSIONS

Anoxia is one of the most important abiotic stresses encountered by most higher organisms. The anaerobic stress-response of maize offers an opportunity to characterize the regulatory components of a family of 20 genes that are coordinately expressed. The anaerobically induced proteins appear to be encoded by a set of genes whose expression is stimulated by a deprivation of oxygen, a condition that would occur in nature during flooding. Regulation of protein synthesis under anaerobiosis appears to occur at multiple levels. We have characterized several genes involved in the anaerobic response and provided some insight into a few components of the signal transduction pathway. Our goal has been to understand how maize perceives the changes in external O_2 concentration and adapts its growth and metabolism over the short- and long-term. To this end, we have demonstrated that Ca^{2+} acts as a key transducer of changes in O_2 availability. Additionally, we aim to characterize the promotor elements of the anaerobically induced genes as well as the signalling components down-stream to calcium that trigger gene induction.

Our goal is also to isolate and characterize the genes involved in tolerance to anaerobic stress and to determine the molecular mechanisms. We have begun analysing genes that confer increased flooding and anaerobic tolerance in maize. This trait was found in some exotic maize accessions (Lemke-Keyes and Sachs, 1989*a*). Genetic analysis indicates that tolerance is a fairly simple dominant trait. Additionally, we found a recessive factor that increases anaerobic tolerance in plants that are null for ADH activity (Lemke-Keyes and Sachs, 1989*b*). We have also demonstrated how a simple post-translational modification of sucrose synthase by the addition/removal of phosphate can lead to potent changes in the tolerance of seedlings to anoxia. Our discovery of genes and proteins likely to be involved in structural modifications (aerenchyma formation and root tip death) indicate further that these mechanisms are multi-pronged and multi-component, perhaps tailored to adapt to different levels of stress. We will continue our analyses of maize responses to anaerobiosis at several levels using a variety of approaches. Our long-term objective is to elucidate the mechanisms of plant adaptation to abiotic stresses and also to pave the way for the development of stress-tolerant crops.

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