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Molecular strategies for improving waterlogging tolerance in plants

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Abstract

Plants, like animals, are obligate aerobes, but due to their inability to move, have evolved adaptation mechanisms that enable them to survive short periods of low oxygen supply, such as those occurring after heavy rain or flooding. Crop plants are often grown on soils subject to waterlogging and many are sensitive to waterlogging of the root zone. The combination of unfavourable weather conditions and suboptimal soil and irrigation techniques can result in severe yield losses. The molecular basis of the adaptation to transient low oxygen conditions has not been completely characterized, but progress has been made towards identifying genes and gene products induced during low oxygen conditions. Promoter elements and transcription factors involved in the regulation of anaerobically induced genes have been characterized. In this paper an account is presented of the molecular strategies that have been used in an attempt to increase flooding tolerance of crop plants.

Key words: Waterlogging, anaerobic conditions, flooding tolerance, molecular strategies, crop plants.

Introduction

The inability of crops to withstand low oxygen conditions in the root zone results in substantial yield losses. In Australia, the most common situation is that crop yield is limited by lack of water, but there are large areas that are subject to waterlogging. Much of the Australian wheat crop is grown on duplex soils, which have a layer of sandy soil over a relatively impermeable clay base, so that rainfall events can lead to rising water tables in the root zone. In Western Australia, two million hectares of the wheatbelt are subject to waterlogging and, depending on the rainfall, crop losses can vary from 10–15% to more than 50%. Because of the winter rainfall pattern, most waterlogging occurs in the transition period from winter to spring (July), when stem elongation has started. Another two million hectares in the eastern Australian wheatbelt also experience waterlogging and consequent crop losses. In the Western District of Victoria, expensive raised-bed cropping systems are being investigated to overcome waterlogging. In Australia, yield losses of wheat due to waterlogging are in the order of 300 million Australian dollars per annum—which means that the problem is serious. The problem is not restricted to Australia, but waterlogging can be a problem for wheat growers world-wide. In Asia, especially in China, rice crops can be followed by wheat, which is particularly sensitive to the heavy wet soils. Waterlogging also causes problems in regions with heavy textured soils in North America and Central Europe.

In Australia, 90% of cotton is grown as a furrow irrigated crop. Imperfect land planing or difficult soils cause setbacks following irrigation and if rainfall occurs during or after irrigation, waterlogging can be severe. This problem is compounded by the fact that most Australian cotton is grown in cracking grey clays with slow drainage (Hodgson and Chan, 1982). On average, one bale of cotton per hectare is lost due to waterlogging (Constable, unpublished data), or approximately 11% of the cotton yield. In severe cases yield losses can reach

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40% (Hodgson and Chan, 1982). With a crop of 400 000 hectares, this results in an annual loss of approximately A$240 million to farmers.

Rice, the second food crop in the world, can tolerate some submergence as paddy rice or deep-water rice. It is well adapted to flooding of the roots because of its ability to transport oxygen efficiently from the aerial parts of the plant to the roots, but problems can occur when the rice plant is totally submerged. This occurs in tropical regions prone to monsoonal flooding, including some of the poorest rice-growing regions of the world such as eastern India. In these regions, lack of tolerance to submergence ranks as the third greatest constraint to rice production (Widawsky and O'Toole, 1990). Changes in methods of crop establishment from transplanting seedlings to broadcasting seed from the air have also created new flooding problems for the rice industry. This method requires that rice, due to uneven waterlevels in the field, be able to germinate and grow under transiently submerged conditions.

Other crops such as canola and barley, are very sensitive to waterlogging and can experience significant yield losses. For all these crop plants it would be important to improve waterlogging and flooding tolerance. In order to do this, it will be critical to understand the physiology of flooding tolerance/sensitivity and to identify genes important in mounting a response.

Waterlogging causes low oxygen stress

Waterlogging and submergence lead to reduced gas exchange between the plant tissue and the atmosphere, because gases (in particular oxygen) diffuse 10 000 times more slowly in water than in air (Armstrong, 1979). This leads to hypoxic or anoxic conditions around the roots, which are major determinants of the adverse effects of flooding. Oxygen is vital in the central energy-providing pathway of the cell, and the presence or absence of oxygen determines metabolic activity and energy production. Oxygen serves as an electron acceptor in the oxidative phosphorylation pathway, which generates ATP, the prime source of energy for cellular metabolism, by regenerating essential NAD^+ cofactor from NADH, the reducing power to sustain biochemical pathways (e.g. glycolysis).

Other biochemical pathways that involve cytochromes, oxidases and desaturases, for example, in haem, sterol and fatty acid biosynthesis, are oxygen-dependent. Many microorganisms react to low oxygen tensions by inducing genes encoding alternative enzymes with a higher affinity for oxygen, and are therefore able to utilize limiting oxygen concentrations more efficiently (Zitomer and Lowry, 1992). Some bacteria are able to switch from enzymes using oxygen as a terminal electron acceptor to enzymes using alternative electron acceptors (Bunn and Poyton, 1996). In yeast cytochrome oxidase-*c*, the terminal respiratory catalyst, changes both in holoenzyme levels and subunit structure during low oxygen conditions, resulting in changes in oxygen affinity (Bunn and Poyton, 1996). The oxygen-dependent haem biosynthesis pathway is the main oxygen-sensor in yeast and regulates expression of low-oxygen-induced genes (Zitomer and Lowry, 1992).

It is not known whether plants have any of these adaptive responses to make better use of low oxygen concentrations. But the induction by low oxygen stress of pyrophosphate-dependent phosphofructokinase (Botha and Botha, 1991; Mertens, 1991) and a vacuolar H^+-translocating pyrophosphatase replacing H^+-ATPase (Carystinos *et al.*, 1995; Table 1), suggest that plants might have adaptations to cope with limited availability of ATP.

Plants have fermentation pathways

In plants, research has mainly focused on the presence and function of fermentation pathways as a metabolic rescue mechanism when respiration is arrested. Three main fermentation pathways are active in plants during flooding: ethanol, lactic acid, and a plant-specific pathway which produces alanine from glutamate and pyruvate, involving alanine aminotransferase (Fig. 1). Animals carry out only lactic acid fermentation. Alcohol and lactic acid fermentation pathways are widespread in facultative anaerobic bacteria and yeasts.

In brewers yeast, alcohol fermentation enzymes are present all the time; availability of oxygen and sugar are responsible for the regulation of glycolytic flux, and differences in affinity for pyruvate of pyruvate dehydrogenase and pyruvate decarboxylase, differentiate between respiration or fermentation (Gancedo and Serrano, 1989). In plants, the fermentation pathways are not present under conditions of normal oxygen supply, but their quick *de novo* induction by low oxygen conditions suggests a role in the low oxygen survival mechanism. Exactly how and to what extent these pathways contribute to low oxygen stress tolerance and how the three pathways are interrelated, is not known.

The pH-stat model suggests that the choice between alcohol fermentation and lactic acid fermentation is driven by cytosolic pH: lactic acid fermentation produces lactic acid, thereby decreasing pH and inhibiting lactate dehydrogenase (LDH), but activating pyruvate decarboxylase and alcohol dehydrogenase (Davies *et al.*, 1974; Davies, 1980). Nuclear magnetic resonance (NMR) studies of cytosolic pH have shown that cytosolic pH acidifies during waterlogging of maize roots and that, after prolonged treatment, alcohol is the main fermentation product (Roberts *et al.*, 1984*a*; Fox *et al.*, 1995). Plants which are more flooding tolerant have a more active alcohol fermentation pathway, and ADH null mutants are more flooding sensitive (Kennedy *et al.*, 1992; Roberts *et al.*, 1984*b*). However, more recent evidence suggests that this

Table 1. *List of all known proteins that are actively synthesized (protein level) and genes which are induced (RNA level) by low oxygen conditions*

The fact that anaerobic proteins are involved in a wide variety of cellular processes, will certainly complicate genetic engineering approaches for flooding tolerance in plants.

ANP	Detection Level	Function	Reference
Sucrose synthase	RNA, Protein	Sucrose breakdown	Springer *et al*., 1986; Martin *et al*., 1993
α-Amylase	RNA, Protein	Sucrose breakdown	Perata *et al*., 1993
Glucose-6-phosphate isomerase	RNA, Protein	Glycolysis	Kelley and Freeling, 1984*a*; Sachs *et al*., 1996
Pyrophosphate-dependent phosphofructokinase	Protein	Glycolysis	Botha and Botha, 1991; Mertens, 1991
Hexokinase	Protein	Glycolysis	Bouny and Saglio, 1996; Fox *et al*., 1998
Fructose-1,6-bisphosphate aldolase	RNA, Protein	Glycolysis	Kelley and Freeling, 1984*b*; Dennis *et al*., 1988
Glyceraldehyde-3-phosphate dehydrogenase	RNA, Protein	Glycolysis	Sachs *et al*., 1996
Enolase	RNA, protein	Glycolysis	Sachs *et al*., 1996
Alcohol dehydrogenase	RNA, Protein	Alcohol fermentation	Freeling and Bennett, 1985
Pyruvate decarboxylase	RNA, Protein	Alcohol fermentation	Kelley, 1989
Lactate dehydrogenase	RNA, Protein	Lactic acid fermentation	Hoffman *et al*., 1986; Germain *et al*., 1997
Alanine aminotransferase	RNA, Protein	Alanine fermentation	Good and Crosby, 1989
Glutamine synthase	Protein	Nitrogen metabolism	Mattana *et al*., 1994*a*
Nitrate reductase	Protein	Nitrogen metabolism	Mattana *et al*., 1994*b*
Nitrite reductase	Protein	Nitrogen metabolism	Mattana *et al*., 1994*b*
Formate dehydrogenase	RNA, Protein	C1 metabolism	Hourton-Cabassa *et al*., 1998; Suzuki *et al*., 1998
Xyloglucan Endotransglycosylase	RNA	Cell wall loosening	Sachs *et al*., 1996
1-aminocyclopropane-1-carboxylic acid synthase	RNA	Ethylene biosynthesis	Olson *et al*., 1994
Haemoglobin, AtHgb1	RNA	?; Oxygen storage	Trevaskis *et al., 1998*
Vacuolar H^+-translocating pyrophosphatase	RNA	?; Cytosolic acidosis	Carystinos *et al*., 1995
Cytosolic pyruvate orthophosphate dikinase	Protein, RNA	?; Anoxic CO_2 fixation	Moons *et al*., 1998
AtMYB2	RNA	Transcription factor	Hoeren *et al*., 1998
Myb7 (rice; X89605)	RNA	Transcription factor	Menguzzato *et al*., 1995
G-Box binding factor Gbf1	RNA	Transcription factor	De Vetten and Ferl, 1995
Calcium-dependent protein kinase (CDPK)	Protein	Protein kinase	Morello *et al*., 1994

description of the role of lactic acid fermentation in causing cytosolic acidosis is simplistic. Some new evidence suggests that the pH drop is not only caused by metabolic activity (lactic acid fermentation), but an H^+-ATPase proton pump could also be involved (Ratcliffe, 1997; Germain *et al*., 1997). It has been shown that maize roots die under low oxygen conditions before cellular energy levels (ATP) are depleted (Xia *et al*., 1995), suggesting that energy levels are not the main determinant of survival. Although keeping cytosolic pH under control may be an important factor in determining survival under flooding conditions, the mechanisms contributing to the regulation of cytosolic pH remain largely unknown and could contain the key for a possible genetic engineering approach to obtain flooding-tolerant plants.

The molecular response of plants to low oxygen stress

Our knowledge about the physiology of the response of plants to flooding conditions has benefited from sensitive and non-invasive NMR techniques. Progress has also been made using an approach based on reverse genetics. By studying which genes are expressed in low-oxygen-

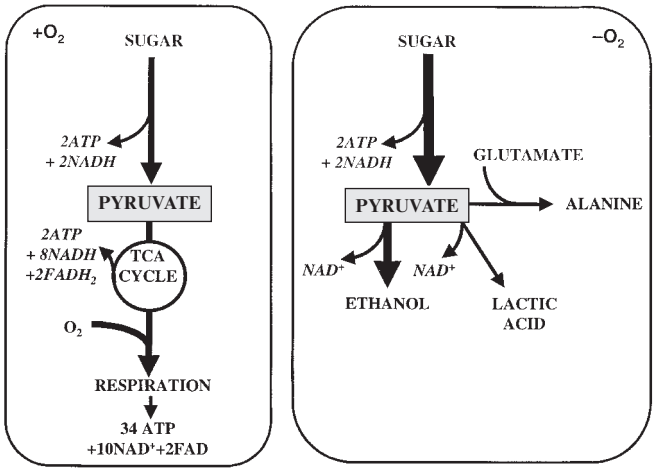

Fig. 1. Shift in metabolism occurring in plants treated by low oxygen conditions.

treated plant roots, it is possible to identify the gene products. Sense/antisense over-expression of these genes in transgenic plants can then be used to identify any contribution they may make to flooding tolerance.

Early studies using ^{35}S-methionine pulse labelling of maize roots, demonstrated a complete switch in the protein synthesis pattern on two-dimensional polyacrylamide gels, from an aerobic to an anaerobic pattern (Sachs *et al*., 1980), and approximately 20 newly synthesized polypeptides are seen (the anaerobic polypeptides, ANPs; Sachs *et al*., 1980). A number of ANPs have been identified via cDNA cloning (Table 1). The ANPs include, as expected, enzymes involved in the three fermentation pathways (PDC, ADH, LDH, AlaAT). Enzymes of the glycolytic pathway and sugar degradation pathways are also amongst the ANPs: fructose-1,6-bisphosphate aldolase, enolase, glyceraldehyde-3-phosphate dehydrogenase, glucose phosphate isomerase, and sucrose synthase (Table 1). Fermentation of carbohydrate enables the plant to maintain ATP production in the absence of oxygen, albeit with a reduced energy yield (fermentation produces only two moles of ATP per mole of glucose instead of the 36 moles produced by oxidative metabolism). It is possible that inducing the enzymes of the glycolytic pathway and sugar metabolism amplifies the flux through this pathway, in order to compensate for this reduction in energy yield. But so far it has not been demonstrated how these changes in gene expression under low oxygen conditions affect the flux through the glycolytic pathway.

As more ANPs become identified (Table 1), it has become obvious that the response of plants to oxygen deficit is more complicated than originally thought. The entire response can be conceptually divided into three stages (Fig. 2). The first stage (0–4 h) consists of the rapid induction or activation of signal transduction components (Table 1). This initial signal reception response in turn activates the second stage (4–24 h), a metabolic adaptation, including the induction of glycolytic and fermentation pathway genes which are necessary to safeguard a continued energy production. This metabolic response is more complex than originally anticipated, as it also involves changes in nitrogen metabolism and other seemingly unrelated metabolic pathways (Table 1). Aminocyclopropane carboxylic acid synthase (ACC synthase), a critical enzyme in ethylene biosynthesis, is also induced in this second stage (Olson *et al*., 1995; Table 1), and presumably results in increased ethylene biosynthesis. The third stage (24–48 h), which is important for survival of prolonged exposures to low oxygen tension, involves the formation of aerenchyma in the roots. It has been demonstrated that ethylene is involved in this response (He *et al*., 1996). A more recently discovered ANP with homology to xyloglucan endotransglycosylase (XET; Table 1), a putative cell wall loosening enzyme, is induced maximally after 48–72 h (Sachs *et al*., 1996) and could play a role in stage 3. Formation of aerenchyma is not a direct consequence of oxygen deficiency, but is presumably triggered by stage 1 and/or stage 2 genes and the accumulation of the hormone ethylene (Drew, 1997). Ethylene was shown not to affect expression of the fermentation pathway genes (Morrell and Greenway, 1989; de Bruxelles and R Dolferus, personal observation).

The molecular approach, in which gene products expressed under low oxygen conditions are identified, has been useful so far in shedding light on the molecular and biochemical changes that take place in the cell. Obviously, there are many more ANPs to be identified and the potential of the molecular approach has not been fully exploited. A large scale sequencing of random clones from a cDNA library, constructed from mRNA of anaerobically induced roots from *Arabidopsis*, has recently been started. The authors were interested in the early response to low oxygen stress (4 h treatment at 5% oxygen/95% N_2), in order to identify key genes in the switch from normal to low oxygen metabolism. The gene products detected so far can be classified into four groups: diverse metabolic genes, including those previously identified as ANPs; signal transduction components, including kinases and transcription factors; defence-related proteins; proteins involved in DNA structure and post-transcriptional regulation. It is hoped that, as this approach progresses, it will be possible to determine the function of genes that are involved in mounting the initial response to low oxygen conditions.

Manipulation of gene expression under low oxygen stress

Induction by oxygen deficit of the maize and *Arabidopsis ADH1* genes involves changes in chromatin structure (Paul and Ferl, 1997) and a dramatic transcriptional

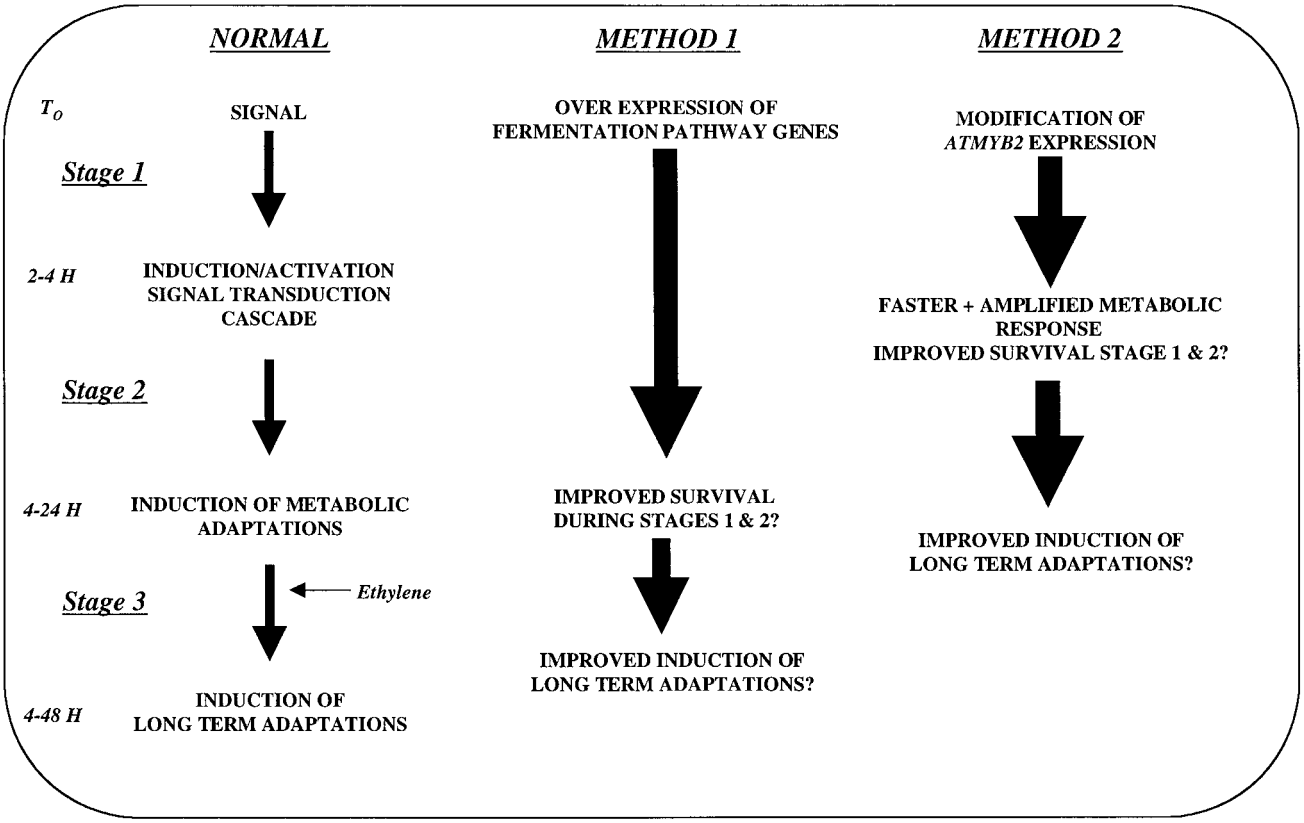

Fig. 2. Strategies to manipulate the anaerobic response. Under normal conditions, the anaerobic response consists of three sequential stages. T_o = start of anaerobic treatment. Two strategies are proposed to manipulate this response. Firstly, by constitutively overexpressing the fermentation pathway genes. This will improve survival chances from T_o (method 1). Secondly, by manipulating the signal transduction cascade using transcription factor AtMYB2 (method 2). This method will make it possible to amplify the entire stage 2 response from T_o.

activation of the gene (Dennis *et al.*, 1984, 1985; Dolferus *et al.*, 1994). However, post-transcriptional regulation mechanisms are also important. Anoxia causes polysomes, translating the aerobic proteins, to dissociate, and anaerobic mRNAs are translated from polysomes with a much lower average size (Rowland and Strommer, 1986; Bailey-Serres and Freeling, 1990; Fennoy and Bailey-Serres, 1995). This suggests that apart from transcriptional activation of the genes encoding ANPs, dramatic changes are occurring in the translational machinery. Sequences in both the 5′ and 3′ end of the maize *Adh1* message are required for efficient translation under anoxia (Bailey-Serres and Dawe, 1996).

At the DNA level, low oxygen-induced genes are characterized by the presence of an anaerobic response element (ARE) in the promoter (Walker *et al.*, 1987). The ARE has been identified in the promoters of maize *Adh1*, *Adh2* and aldolase, and *Arabidopsis ADH1*, *LDH1*, *PDC1* (Olive *et al.*, 1990; Dolferus *et al.*, 1994; Hoeren *et al.*, 1998). Disruption of this motif in either maize or *Arabidopsis* abolished expression and anaerobic induction of the *ADH1* gene (Walker *et al.*, 1987; Dolferus *et al.*, 1994). The ARE consists of a GC-motif and a GT-motif, both being critical for gene activity and induction by low oxygen (Olive *et al.*, 1990, 1991*a*, *b*). A protein binding to the GC-motif was identified (Olive *et al.*, 1991*b*), but it was not possible to identify a specific transcription factor binding to the GT-motif. It was recently recognized that the GT-motif resembles a Myb transcription factor binding site with a 5′-AAC-3′ central motif (=5′-GTT-3′; the GT-motif occurs in opposite orientations in maize and *Arabidopsis ADH1*). It has now been shown that the *Arabidopsis* Myb transcription factor AtMYB2 binds specifically to the GT-motif of *ADH1*. The consensus AtMYB2 binding site, compiled from the GT-motifs in all known anaerobically induced genes is 5′-AAACC(G/A) (G/A)-3′ (Hoeren *et al.*, 1998). Mutations in the GT-motif abolished both binding of the AtMYB2 factor and *ADH1* promoter activity in transient assays and transgenic *Arabidopsis* plants. AtMYB2 has previously been shown to be induced by dehydration, salt stress and exogenous ABA (Urao *et al.*, 1993). It was shown that *AtMYB2* is also induced by hypoxia, with mRNA levels peaking after 2–4 h of hypoxia and its

induction precedes or coincides with *ADH1* mRNA induction under hypoxic conditions. Like *ADH1*, *AtMYB2* expression is restricted to root tissue.

The availability of both a promoter sequence, present in all anaerobically induced genes, and a transcription factor which binds to it, raises the possibility of modulating the expression of the *AtMYB2* transcription factor and regulating all of the ANPs. The question was whether AtMYB2 is the limiting factor in induction of the anaerobic polypeptides. A functional assay using biolistic bombardment of pea leaves or co-transfection of *Nicotiana plumbaginifolia* or *Arabidopsis* protoplasts with *ADH1*-GUS reporter and 35S-AtMYB2 effector constructs, results in transactivation of *ADH1*-GUS in both systems in the absence of anaerobic conditions (Hoeren *et al*., 1998). These results support a role for *AtMYB2* in the early response to low oxygen stress (stage 1), with the induction of *ADH1* and, presumably, of many of the metabolic genes of the second stage in the anaerobic response stage being dependent on AtMYB2.

Strategies to engineer flooding tolerance in plants

Despite having an anaerobic response, many plants suffer severe growth setbacks when flooded or irrigated and do not survive prolonged exposure to low oxygen. The aim of our work has been to understand the metabolic changes during hypoxia and to attempt to improve the agricultural performance of plants during this stress by enhancing the anaerobic response (Fig. 2). The first approach has been to focus on the fermentation pathways. By amplifying the expression of genes encoding the fermentation pathway enzymes, or by making the expression of these pathways constitutive, it may be possible to prepare the plant better to survive the initial stages of anaerobiosis, before longer term adaptation mechanisms are established. The enzymes of the fermentation pathways are induced *de novo* under low oxygen conditions, suggesting they play an important physiological role in survival. But as Table 1 suggests, survival is determined by a complex network of biochemical activities and manipulating only the fermentation pathway genes may have little effect on survival. However, this approach combined with metabolite measurements, will provide information about the role and function of plant fermentation pathways under flooding conditions. The second approach is to modify the expression or function of AtMYB2, thereby amplifying the expression of all of the fermentation pathway genes and possibly the other metabolic genes (stage 2; Fig. 2). It is anticipated that both approaches may have a beneficial effect in switching on the longer term adaptation response to low oxygen stress (stage 3; Fig. 2).

This study has focused on three experimental systems (Table 2); *Arabidopsis*, as a model system, and cotton and rice as examples of flooding-sensitive and -tolerant species respectively (Table 2).

Arabidopsis

To test whether hypoxic tolerance had been improved in transgenic plants, methods were developed for assaying hypoxic performance. A seedling survival test was developed in *Arabidopsis* (Ellis *et al*., 1999). Three-week-old seedlings were subjected to hypoxic stress (0.1% O_2 for 24–48 h), transferred to agar plates, and the position of the root tip marked. Plants were then incubated in aerobic conditions, and their root growth scored. In addition, survival of shoot meristems was measured both by chlorophyll content and detection of new shoot formation.

In order to test whether increasing the levels of ADH and/or PDC or early induction of these enzymes would protect the plants from hypoxic stress, the seedling assay was used to test the effect of mild hypoxic pretreatment (5% O_2) for 24 h on the ability of *Arabidopsis* to survive a subsequent hypoxic stress (0.1% O_2). The mild hypoxic pretreatment induces *ADH1*, *PDC1* and other anaerobic polypeptides without affecting survival of the plant. It was found that such hypoxic pretreatment greatly improves survival of subsequent extremely low oxygen conditions (0.1% oxygen) in both roots and shoot. Similar benefits of low oxygen acclimation have been reported in other species. This suggests that prior induction of the ANPs may enhance survival. Consistent with a role for *ADH1* in hypoxic survival, an *Arabidopsis ADH1* null mutant (ROO2, Jacobs *et al*., 1988) showed reduced root survival relative to the wild type (Ellis *et al*., 1999). However, in the null mutant shoot meristem survival

Table 2. *Summary of transgenic plant material obtained in* Arabidopsis*, cotton and rice: S, sense constructs; AS, antisense constructs*

Species	Construct transformed	Gene used
Arabidopsis	35S-PDC1/S	*Arabidopsis PDC1* (U71121)
	35S-PDC1/AS	*Arabidopsis PDC1* (U71121)
	35S-PDC2/S	*Arabidopsis PDC2* (U71122)
	35S-PDC2/AS	*Arabidopsis PDC2* (U71122)
	35S-ADH1	*Arabidopsis ADH1* (M12196)
	R002	EMS-induced null mutant
	35S-LDH1/S	*Arabidopsis LDH1* (AF043130)
	35S-LDH1/AS	*Arabidopsis LDH1* (AF043130)
	35S-AlaAT/S	Barley *AlaAT*
	35S-AlaAT/AS	*Arabidopsis AlaAT1*
Cotton	35S-ADH2/S	Cotton *ADH2* cDNA (U07339)
	35S-PDC1/S	Rice *PDC1* cDNA (U07339)
Rice	Ubiq-ADH2/S	Cotton *ADH2* cDNA (U07339)
	Ubiq-ADH1/AS	Rice *ADH1* cDNA (U07339)
	6XARE-PDC1/S	Rice *PDC1* cDNA (U07339)
	6XARE-PDC1/AS	Rice *PDC1* cDNA (U07339)

remained the same as the wild type. Another treatment that induces *ADH1* in the roots (abscisic acid, ABA), was found to increase root but not shoot survival. These experiments also demonstrated that *Arabidopsis* has different mechanisms for acclimation of shoots and roots. Even when roots were removed before the pretreatment with hypoxia, shoots were able to acclimate. Both shoot and root acclimation mechanisms were shown to depend on protein synthesis, since treatment with cycloheximide prevents acclimation in both roots and shoots (Ellis *et al*., 1999).

Arabidopsis was used as a model system to study overexpression and underexpression of the three fermentation pathways. The *Arabidopsis* genes encoding ADH (*ADH1*), PDC (*PDC1* and *PDC2*), LDH (*LDH1*), and AlaAT (*AlaAT1*) were cloned and expressed in sense and antisense orientation under the control of the 35S promoter (Table 2). Except for a suitable *LDH1* antisense line, transgenic lines with significantly higher or lower activities for all these genes were obtained. So far only the hypoxic stress survival of transgenic *Arabidopsis* plants carrying additional constitutively expressed *Arabidopsis ADH1*, *PDC1*, *PDC2*, and *LDH1* genes have been analysed. Some of our preliminary results are encouraging.

Currently genetic crosses are being made between these transgenic lines, in order to study the effect of over- and under-expressing the complete alcohol fermentation pathway. This analysis is also coupled to a study of the fluxes of pyruvate through the fermentation pathways, in order to examine to what extent metabolism has been changed in the transgenic lines.

Cotton

In cotton this study focused on the alcohol fermentation pathway, and obtained lines overexpressing *ADH* and *PDC* (Table 2). A system was devised for imposing, and evaluating tolerance to low oxygen stress on cotton roots, based on the system of Leonard and Pinckard (Leonard and Pinckard, 1946). Cotton is grown hydroponically in clear plastic cylinders ('perspex didgeridoos'). The cylinders are fitted with a scintered air-stone at the bottom through which air or gas mixes can be flushed though the culture solution. Root growth is observed daily through the transparent plastic tubes.

Cotton plants containing the cotton ADH cDNA driven by a constitutive 35S promoter showed a 10–30-fold increased ADH activity and a significant increase in the rate of ethanol fermentation (measured from ethanol production by excised roots under anaerobic conditions). Cotton plants with the rice *PDC1* cDNA driven by a constitutive 35S promoter produced more PDC protein, but had only marginally more PDC activity. Neither *PDC* or *ADH* transgenic cotton plants, nor plants containing both constructs showed increased tolerance of hypoxic stress in the 'digeridoo assay system'. Field trials of the plants are currently underway.

Rice

Transgenic rice containing the cotton *ADH* cDNA driven by the ubiquitin promoter, and the rice *PDC* gene driven by a $6\times$ARE promoter (strong anaerobic induction in maize protoplasts; Olive *et al*., 1990; Table 2), are being tested for tolerance to oxygen deficiency under submergence (Ellis and Setter, 1999). Preliminary results suggest that rice plants overexpressing *PDC* do not show increased tolerance to submergence. But the increase in PDC activity obtained in rice was much less than in transgenic *Arabidopsis*. As was the case for *Arabidopsis ADH1* null mutants (R Dolferus, unpublished results), it was found that reducing ADH activity with antisense constructs in rice led to the inability of seeds to germinate under low oxygen conditions. This suggests that ADH is essential for germination and growth of rice seeds under submerged conditions.

The glasshouse and tissue culture testing systems have not yielded encouraging results for any of these enzymes. However, the parameters tested may not be important in the field and, in the end, ability to survive field conditions may be the only real measure of success.

Can AtMYB2 be used to manipulate expression of ANPs?

An attempt to use the transcription factor AtMYB2 to affect levels of expression of the entire ANP gene battery is being made. With the *AtMYB2* gene it may be possible to provide either earlier recognition of anaerobic conditions and anaerobic polypeptide synthesis, or greater tolerance of the plant to these conditions where it would be beneficial. In an effort to enhance hypoxic tolerance by modifying the entire anaerobic response, *Arabidopsis* was transformed with *AtMYB2* under the control of the 35S promoter in both sense and antisense orientation. No transformants expressing either construct were recovered. This suggests that if expression of the *AtMYB2* gene is altered drastically (constitutive high or low levels of expression in all tissues and cell types), the plant does not survive. At present, similar constructs under the control of a dexamethazone inducible promoter are being introduced and whether induction of *AtMYB2* expression at a specific time in sense or antisense orientation can induce or repress *ADH1* and other anaerobic genes will be tested. Overexpressing the transcription factor AtMYB2 only at the appropriate time may enhance tolerance to hypoxia.

Conclusion

Manipulating hypoxia tolerance in plants may still be hampered by inadequate knowledge of the molecular and

physiological basis of the problem. Transgenic plants may clarify the physiological role of the fermentation pathways, and their contribution to flooding tolerance. Sequencing of the *Arabidopsis* anaerobically-induced root cDNA library may identify novel genes concerned with the low oxygen response. The authors are keen to get their first results with the inducible AtMYB2 system, but even using this approach it may fail to enhance hypoxia tolerance because of the complexity of signalling pathways and the difficulties of manipulating a metabolic pathway which has been under evolutionary selection for millions of years.

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