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Transgenic cereals with enhanced resistance to abiotic stress through metabolic engineering

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SUMMARY – A number of current studies have focused on the polyamine biosynthetic pathway as a model to unravel particular key factors that still represent bottlenecks in metabolic pathway engineering in plants. Engineering of metabolic pathways in plants has been carried out in some cases, with the aim of enhancing production of a natural product that can improve the nutritional properties of plants or protect plants from environmental stresses. By engineering rice plants with the oat *adc* cDNA under the control of two different promoters we demonstrated a correlation between polyamine accumulation and the ability of dedifferentiated tissue to undergo morphogenesis. Our studies also suggested that a key element in facilitating changes in polyamine levels in transgenic tissues is the strength of the promoter used to drive expression of individual transgenes. Based on these results we developed a model which stipulates a minimum threshold in putrescine concentration prior to its further conversion into the higher polyamines spermidine and spermine. Our experiments also demonstrated that seed, rather than vegetative tissue, is the preferred organ for polyamine accumulation and storage. Our studies shed further light on the complexity of polyamine biosynthesis in intact plants and tissues and provide a basis for further manipulations using additional genes in the polyamine pathway. We will use this pathway as an example to illustrate one of the strategies used in metabolic engineering, the enhancement of an existing metabolic pathway using a single metabolic engineering approach to improve simultaneously the nutritional properties of plants and also to afford protection against environmental stresses, thus improving crop yields.

Metabolic engineering and polyamine metabolism

Metabolic engineering in plants has been carried out predominantly to enhance the production of industrial or pharmaceutical metabolites (Stoger *et al*., 2002). However, there have been several recent examples where metabolic engineering has been used to improve agronomic or nutritional characteristics in plants. Metabolic engineering in plants involves the modification of endogenous pathways to increase the flux towards particular desirable molecules. In some cases the aim is to enhance the production of a natural product, whereas in others it is to synthesize a novel compound or macromolecule (Capell and Christou, 2004).

Polyamines are small, polycationic compounds that are found in all living organisms and are thought to be involved in a wide range of physiological functions, including the control of growth and cell division. Although humans can synthesize polyamines from the amino acid ornithine, this is an insufficient source and further polyamines must be obtained in the diet. The nutritional benefits of polyamines have been widely studied and are particularly noted for their impact on cell regeneration and growth (Bardocz, 1995). In plants, the simplest polyamine (putrescine) can be synthesized from either ornithine or arginine through the activities of the enzymes ornithine decarboxylase (ODC) and arginine decarboxylase (ADC). The other major polyamines, spermidine and spermine, are synthesized from putrescine. Spermidine is formed by the addition of an aminopropyl group donated by decarboxylated S-adenosylmethionine in a reaction catalyzed by spermidine synthase (SDE). Spermine is formed by the addition of a second aminopropyl moiety to spermidine, a reaction catalyzed by spermine synthase (SME; Capell and Bassie, 2005). As in animals, polyamines in plants are thought to be involved in many different physiological processes and are thought to be particularly important in stress responses (Bouchereau *et al*., 1999). Therefore, the manipulation of polyamine metabolism in plants promises dual benefits, i.e. enhanced nutritional properties and improved stress responses, allowing plant survival in more extreme environments and promoting higher yields. In addition, it is a relatively short pathway in terms of the number of enzymes involved, however it is rather complex because of its impact on crucial physiological, developmental, and regulatory processes in which polyamines are implicated (Malmberg *et al*., 1998). All enzymes involved in the pathway have been characterized and corresponding genes/cDNAs have been cloned from different

sources (Kakkar and Sawhney, 2002). As a result the pathway represents an ideal model to test hypotheses and answer fundamental biological questions in pathway manipulation using transgenesis.

Modulation of the polyamine biosynthetic pathway to enhance tolerance to abiotic stress

Many reports link polyamines and abiotic stress in plants; however, they do not provide unequivocal evidence for the involvement of polyamines in abiotic stress responses. They do provide strong circumstantial evidence that polyamines protect plants from abiotic stress, but they do not establish a cause-and-effect relationship. For the past several years we have been investigating molecular, biochemical and physiological aspects of the polyamine biosynthetic pathway in plants, using rice as a model. We created transgenic plants over-expressing the *Datura adc* gene under the control of the strong monocot maize *Ubi-1* promoter (Capell *et al*., 2004). These plants were generated in order to investigate the role of polyamines in the response to abiotic stress, in particular drought stress, which is a major constraint in rice productivity, mostly in rain-fed agro-ecosystems.

Many plants accumulate specific amino acids or their derivatives in response to environmental stresses (Bohnert and Jensen, 1996). The accumulation of putrescine has been widely reported in monocotyledonous and dicotyledonous plants but is most pronounced in cereals where the putrescine pool represents a major sink for carbon and nitrogen (Slocum and Weinstein 1990). We found that wild type rice plants subjected to PEG-induced drought stress responded by increasing cellular putrescine levels significantly, without any changes in the steady-state rice *adc* mRNA levels (Capell *et al*., 2004). In agreement with Watson and Malmberg (1996), putrescine accumulation as a result of increased ADC enzyme activity did not appear to involve a substantial net change in the steady state levels of *adc* mRNA. Flores and Galston (1984) suggested that the primary event in this stress-induced phenomenon occurs very rapidly, and requires *de novo* protein synthesis. This was attributed to translational or post-translational regulation of ADC, a mechanism that would not involve a net change in steady state *adc* mRNA levels. They suggested a role for putrescine in plants under stress, which extends beyond its involvement as a simple precursor for the higher polyamines along the pathway (Watson and Malmberg, 1996). Putrescine accumulation in tissues under stress is also a consequence of the reduction in the rate of spermidine and spermine synthesis (Flores and Galston, 1984). Such accumulation can be toxic to certain cells. Whether toxicity is a direct result of putrescine accumulation or an indirect response to changes in the kinetics and/or products of its catabolism remains to be investigated.

In our experiments, the physiological stress responses of wild type, negative segregants and transformants that did not exhibit significant accumulation of putrescine in their leaves manifested as progressive wilting and rolling of leaves. Detached leaves from plants subjected to high osmoticum showed a massive accumulation of putrescine, but conversion to spermidine and spermine was very slow and mesophyll protoplasts isolated from such leaves were incapable of cell division. In contrast, dicotyledonous plants that readily regenerate from mesophyll protoplasts show a very different response. Putrescine levels are reduced, while the levels of spermidine and spermine increase significantly (Flores and Galston, 1984). Putrescine accumulation in the *Dadc*-transgenic plants is also a consequence of transgene expression. The *Ubi-1* promoter, driving *Dadc* expression in our transgenic plants, is known to possess a number of stress responsive elements (Christensen and Quail 1996) that boost transgene expression under drought stress (Takimoto *et al*., 1994). Transgene expression under such conditions would provide a constant supply of putrescine, thus maintaining a near constant steady-state pool of this compound in the transgenic plants.

In previous experiments, endogenous levels of spermidine and spermine in detached oat leaf segments under osmotic stress declined sharply 6 h after stress induction (Capell *et al*., 1993). This was attributed, at least in part, to activation of the polyamine catabolic pathway. The decline in spermidine and spermine levels resulted in chlorophyll loss and leaf senescence. When guazatine, an inhibitor of polyamine oxidase activity, was added to the incubation buffer, endogenous spermidine and spermine levels increased significantly and prevented chlorophyll loss in osmotically treated oat leaf segments, thus delaying senescence (Capell *et al*., 1993). We observed a remarkably similar reduction in the content of cellular spermidine and spermine in wild type plants after 6 d of drought stress compared to the increases we observed after 3 d (Fig. 1). This correlated well with the wilting and rolling of leaves (Fig. 2). Transgenic plants that exhibited drought tolerance behaved similarly to wild type plants up to 3 d after the onset of stress, with spermidine and spermine levels increasing in a similar fashion under the same conditions.

However, in contrast to the dramatic reduction in the levels of these two polyamines seen in wild type after day 3, the transgenic plants maintained high spermidine and spermine levels for the duration of the experiment (Figs 1 and 2). This correlated perfectly with the drought tolerant phenotype of transgenic plants compared to wild type (Figs 1 and 2).

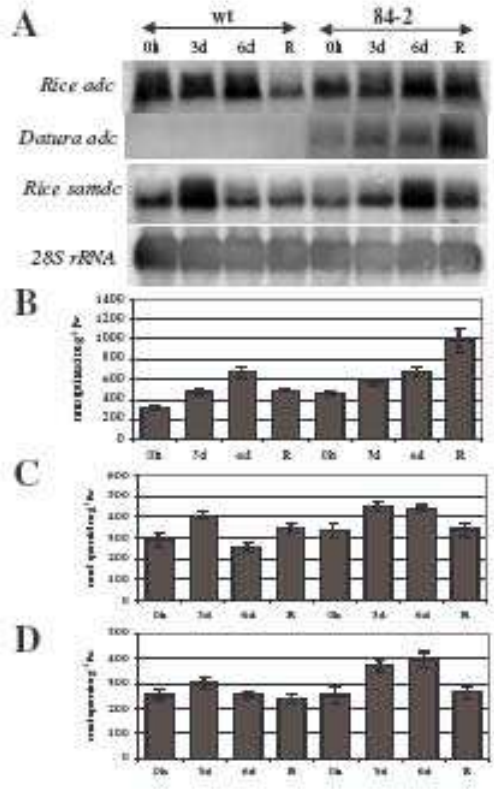

Fig. 1. Molecular and biochemical characterization of wild type (wt) and transgenic plants under drought stress.

In both transgenic and wild type plants under drought stress, the spermidine and spermine content correlated with rice *samdc* steady-state mRNA profile (Fig. 1). Activation of the rice *samdc* gene pushes the pathway forward by utilizing the steady-state putrescine pool that is generated by transgene expression. The net result is an increase in the levels of spermidine and spermine in the transgenic plants.

Galston *et al*. (1997) proposed a model that attempts to explain how ADC activity is regulated by spermine under osmotic stress. Using a detached oat leaf system, they postulated that upon the onset of osmotic stress, a signal activates transcription of the oat *adc* gene. The translation product of the *adc* mRNA is an inactive precursor protein with a molecular weight of ~60 kD. This is cleaved to produce an N-terminal fragment and a 24-kD C-terminal fragment containing the ADC active site (Watson *et al*., 1997). This active ADC form catalyzes the decarboxylation of arginine leading to the accumulation of putrescine. The physiological response to increased putrescine levels includes chlorophyll loss and accelerated senescence. In the model proposed by Galston *et al*. (1997), exogenously applied spermine can inhibit the post-translational processing of the inactive ADC precursor molecule. The consequence of this is a decrease in ADC activity and a concomitant prevention of excess putrescine accumulation. Oat leaf segments exposed to spermine were able to retain chlorophyll after 72 h under osmotic stress (Capell *et al*., 1993). In the rice whole plant system, we showed that endogenous spermidine and spermine accumulation resulting from *adc* transgene expression has a similar effect. Expression of the heterologous *adc* transgene driven by the maize *Ubi-1* promoter, which is known to be activated by stress (Takimoto *et al*., 1994), would augment the putrescine pool to levels that extend beyond the critical threshold required to initiate the conversion of excess putrescine to spermidine and spermine (Fig. 3). Their *de novo* synthesis in transgenic plants under drought stress is corroborated by the activation of the rice *samdc* gene. Transcript levels for rice *samdc* reach a maximum 6 d after stress induction. Such increases in the

endogenous spermidine and spermine pools of transgenic plants not only regulate the putrescine response, but also exert an anti-senescence effect at the whole plant level, resulting in phenotypically normal plants. Wild type plants, however, were not able to raise their spermidine and spermine levels after 6 days of drought stress and consequently exhibited the classical drought-stress response (Fig. 2).

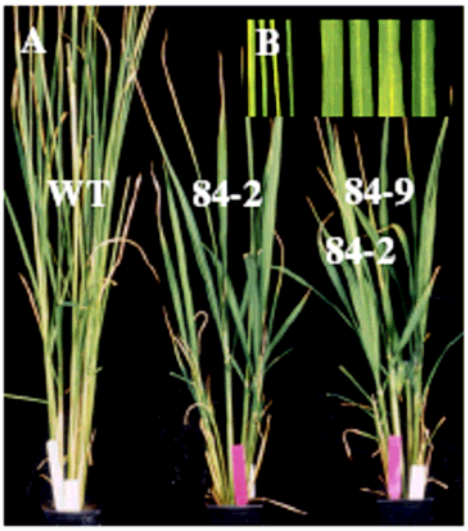

Fig. 2. Response of rice plants to drought stress. (A) Phenotype of 2-month-old wild-type (wt) and transgenic plants (84-2 and 84-9 lineages) growing on soil after drought stress (6 days). (B) Close-up of rice leaves (wt on the left and 84-2 on the right).

Fig. 3. Unified model explaining the role of polyamines in the response of wild type and transgenic plants expressing *Dadc* under abiotic stress conditions. Histograms show relative polyamine levels measured in each type of plant. Once putrescine levels exceed the threshold shown, the synthesis of spermidine and spermine is triggered,

resulting in protection against drought and other forms of stress.

Conclusion

Our results are consistent with an emerging picture in which the temporal profile of transcripts and corresponding polyamines are implicated in the response of wild type and transgenic plants to drought stress. In turn, this supports a threshold model in which a sufficient level of putrescine, perhaps acting as a stress-warning signal, must accumulate before higher polyamines with protective effects are synthesized. Such plants have a great potential to address food insecurity by providing drought tolerance and allowing the cultivation of marginal soils to grow food crops.

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