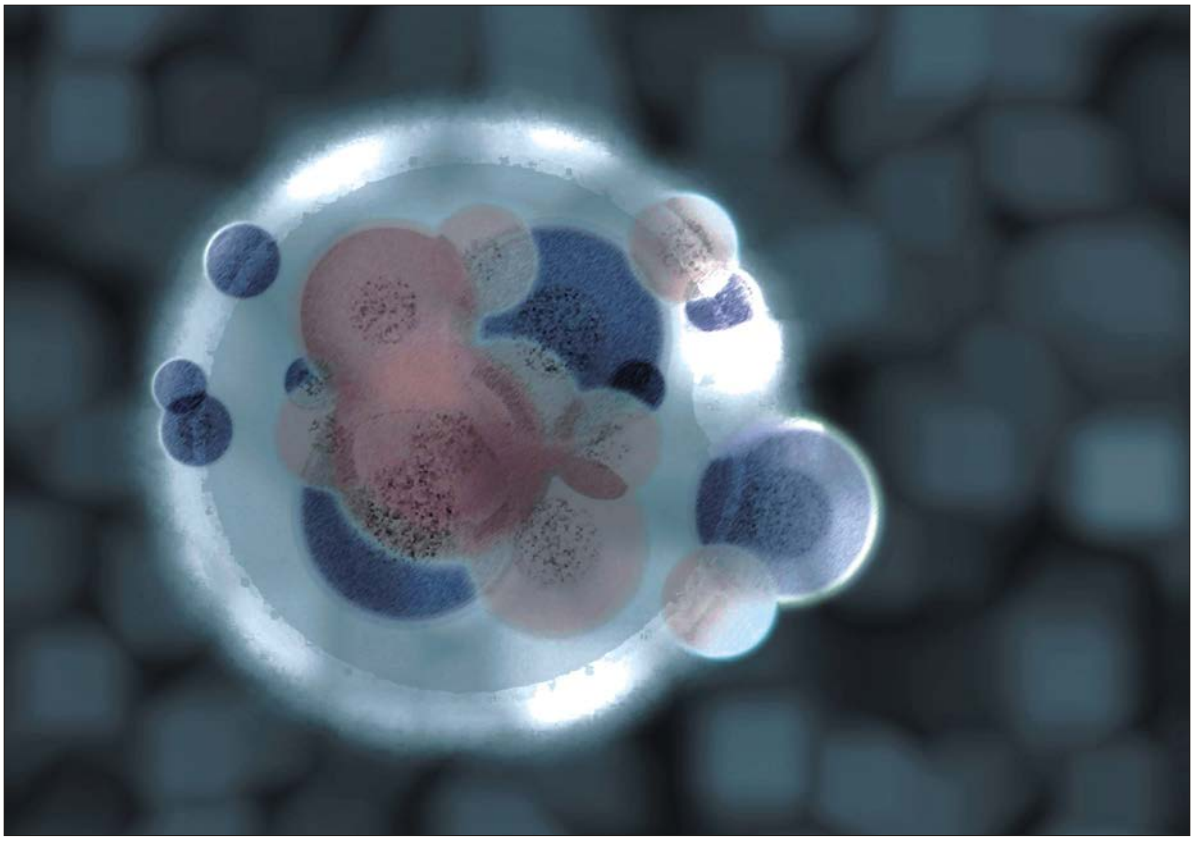

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Progress in the Modulation of the Polyamine Biosynthetic Pathway in Transgenic Rice

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Abstract: Present study have focused on the polyamine biosynthetic pathway as a model to unravel those key factors that still present bottlenecks in metabolic pathway engineering in plants. 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. We suggested also that a key element in facilitating changes in polyamine levels in transgenic tissues is the strength of the promoter used to drive expression of particular 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. Present experiments also demonstrated that seed rather than vegetative tissue is the preferred organ for polyamine accumulation and storage. Present studies shed further light on the complexity of polyamine biosynthesis in intact plants and tissues and provide a basis for their further manipulation using additional genes of the polyamine pathway.

Key words: Arginine decarboxylase, direct DNA transfer, maize ubiquitin 1 promoter, *Oryza sativa*, polyamines

INTRODUCTION

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[1,2]. While metabolic engineering in plants has been carried out predominantly to enhance the production of industrial or pharmaceutical metabolites[3,4], there have been several recent examples where metabolic engineering has been used to improve agronomic or nutritional characteristics in plants[5]. In this review we focus on the manipulation of the polyamine biosynthesis pathway in cereals. 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 simultaneously to improve the nutritional properties of plants and to protect them from environmental stresses, thus enhancing yields.

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[6,7]. In animals, polyamine-supplemented diets have been shown to provide instant energy for use in the small intestine[8], to promote weight gain and feeding efficiency by countering the effect of anti-nutritional factors[9-12] and to promote the maturation of glycan chains on proteins synthesized by intestinal cells[13].

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 (Fig. 1). 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)[14]. As in animals, polyamines in plants are thought to be involved in many different physiological processes[15,16] and are thought to be particularly important in stress responses[17]. Therefore, the

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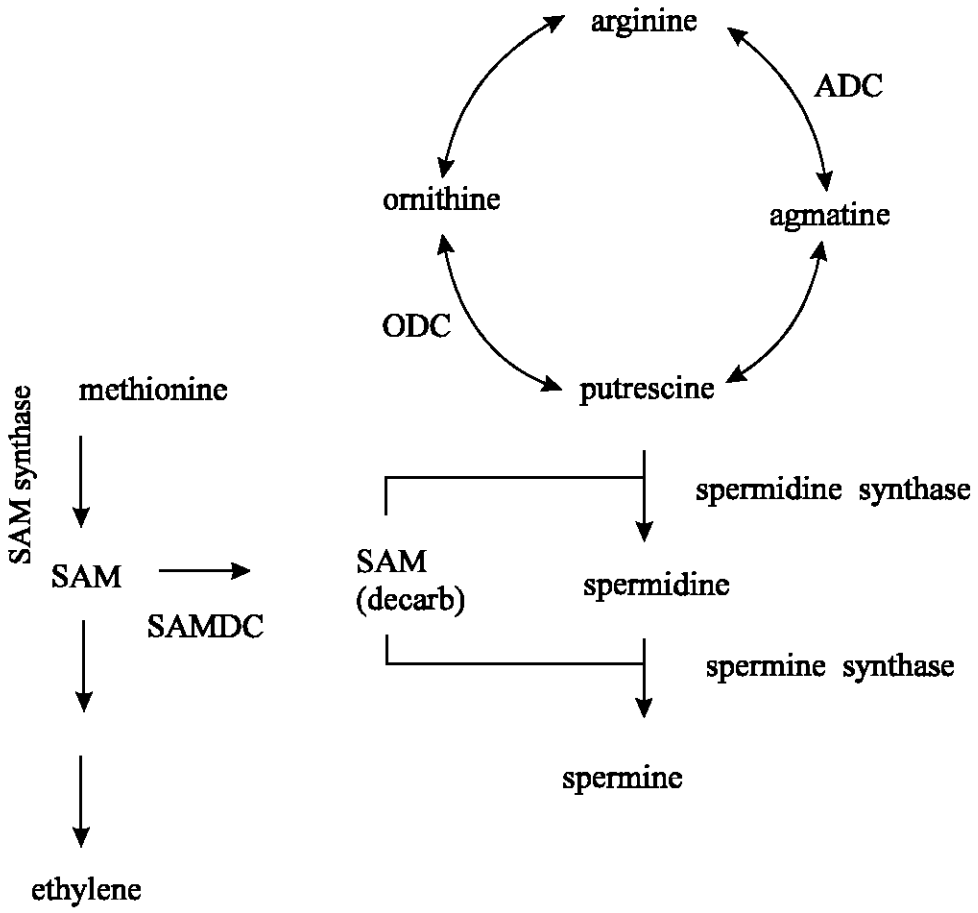

Fig. 1: Polyamine biosynthesis pathway in plants. ADC = arginine decarboxylase, ODC = ornithine decarboxylase, SAM = S-adenosylmethionine, SAMDC = SAM decarboxylase

manipulation of polyamine metabolism in plants promises dual benefits, i.e. enhanced nutritional properties and improved stress responses, allowing 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[16]. All enzymes involved in the pathway have been characterized and corresponding genes/cDNAs have been cloned from different sources[18]. As a result the pathway represents an ideal model to test hypotheses and answer fundamental biological questions in metabolic pathway manipulation using transgenesis.

Studies on the polyamine biosynthesis pathway in plants have been carried out predominantly in tobacco and rice, with many enzymatic steps being perturbed either by over-expression or antisense suppression[16,19,20]. The main aim of these studies was to investigate the effect of increased (heterologous) enzyme activity or suppressed endogenous enzyme activity on the activities of other enzymes in the pathway, the levels of the three major polyamines and the overall impact on phenotype.

Expression of the oat *adc* cDNA in transgenic rice alters ability of dedifferentiated tissue to undergo morphogenesis *in vitro*: In 1998 the recovery of fertile transgenic rice plants expressing oat *adc* cDNA driven by the 35S CaMV promoter was reported. This was the first time that the polyamine biosynthetic pathway was manipulated in cereals using genetic engineering[21]. We described dramatic changes in the development of transgenic callus. The results provided new evidence for the existence of a direct correlation between polyamine levels, plant differentiation and plant morphogenic responses *in vitro*, particularly in cereals. Such are classified responses in different behaviour patterns resulting from differences in *adc* transcript level, ADC enzyme activity and putrescine accumulation. In order to confirm that the phenotype of callus and shoots we

observed was due to the changes in *adc* expression, activity and accumulation of end product (putrescine) we performed a detailed molecular and biochemical characterization of all transgenic tissues. We found that increased transcript levels, elevated ADC enzyme activity and changes in polyamine levels strongly influenced the phenotype and morphogenic response of the engineered tissue. The relationship between *adc* transcript level and ADC activity was not directly proportional. However, in general, higher ADC activity was observed in callus lines which expressed transcript at the higher level. Normal morphogenic development and differentiation of callus lines which expressed mRNA at the highest levels was blocked by exposure to light. In such cases, a significant increase in putrescine levels (up to 4 fold) was observed. We concluded that if the maximum threshold of product accumulation was exceeded, this would result in inability of the tissue to differentiate into plants, suggesting an inhibitory role for putrescine in developmental processes *in vitro*.

In a subsequent series of experiments we generated 30 transgenic rice lines harbouring the oat *adc* cDNA driven by the stronger maize ubiquitin 1 promoter (*Ubi-1*) and its first intron in co-transformation experiments with a plasmid containing the selectable *hpt* gene[21]. We did not observe any direct correlation between integrated transgene copy number and levels of expression consistent with previous results from our laboratory[22-24]. In accordance with other studies involving expression of different transgenes in rice, it was confirmed that the *Ubi-1* promoter confers higher levels of expression *adc* for *adc* compared to the CaMV 35S promoter[25,26]. We confirmed this to be the case at the mRNA level and also for enzyme activity. We measured steady state mRNA in callus lines containing pUbi*adcs*. This was correlated to an increase in enzyme activity. In the previous experiment we had measured mRNA in transgenic rice callus containing the *adc* gene driven by the CaMV 35S promoter. Only a very small number of transgenic lines showed messenger accumulation in the later case. The steady-state transcript in these tissues (p35S*adc*) was only detectable in lines which were terminally differentiated, i.e. not able to regenerate plants[21]. In contrast, messenger accumulation in rice callus transformed with pUbi*adcs* was detected in all lines. This translated to functional protein with enzyme activity increases. For p35S*adc* we measured up to 8 fold increases in ADC activity. For the pUbi*adcs* construct we recovered lines with up to 50 fold increases in ADC but no changes in ODC activity. These results indicated that the polyamine biosynthetic machinery at the DNA, RNA and enzyme levels was functional.

Over-expression of the oat *adc* cDNA in transgenic rice driven by the strong constitutive *Ubi-1* promoter resulted in lines with altered levels of polyamines. These changes were correlated with the morphogenic state of the tissue. We have shown that levels of individual polyamines may vary significantly amongst the different lines and also depending on the developmental stage of the tissues. There is an interesting divergence in biochemical terms between p35S*adc* and pUbi*adcs*. In the former case, in which the weaker 35S promoter drives expression, we observed a significant increase in putrescine levels in dedifferentiated callus. This did not result in any measurable variation in spermidine, however a significant decrease in spermine was detected in 8 out of 20 lines. For pUbi*adcs*, under the same conditions, we observed a decrease in putrescine in 50% of the lines we analysed; 45% lines did not show any variation and only one line showed a significant increase. During callus dedifferentiation all lines showed significant decreases in spermidine, but 85% of the lines had a significant increase in spermine suggesting that over-expression of *adc* driven by the strong *Ubi-1* promoter can activate subsequent steps in the pathway resulting in spermine accumulation. When we calculated total polyamine levels in dedifferentiated callus, approximately one third of the lines showed significant variation (increase or decrease) despite the fact that all transgenic lines had a significant increase in ADC enzyme activity. We postulate that as putrescine and spermidine are intermediate compounds in the polyamine pathway, their levels can fluctuate, unlike spermine, which is the end product of the pathway and as such may accumulate, even though catabolizing enzymes such as diamine-oxidase (DAO) and polyamine-oxidase (PAO) may operate on all three polyamines.

Rice dedifferentiated tissue undergoes morphogenesis when auxin is withdrawn from the culture medium. When we measured polyamines during regeneration, we observed that putrescine and spermidine levels were significantly higher compared to htp-control values in all lines. Most of these values were higher than values from samples taken at the dedifferentiated stage. Spermine showed the reverse trend; values were significantly lower compared to *hpt*-controls and almost all lines exhibited a reduction in endogenous levels compared to dedifferentiated callus. This significant reduction in spermine in all lines compensates the increase of putrescine and spermidine, resulting in only one third of the lines showing an increase in total polyamines during regeneration. This may indicate that induction of morphogenesis requires an increase in ADC enzyme activity and subsequent accumulation of putrescine and spermidine. Catabolizing enzymes such

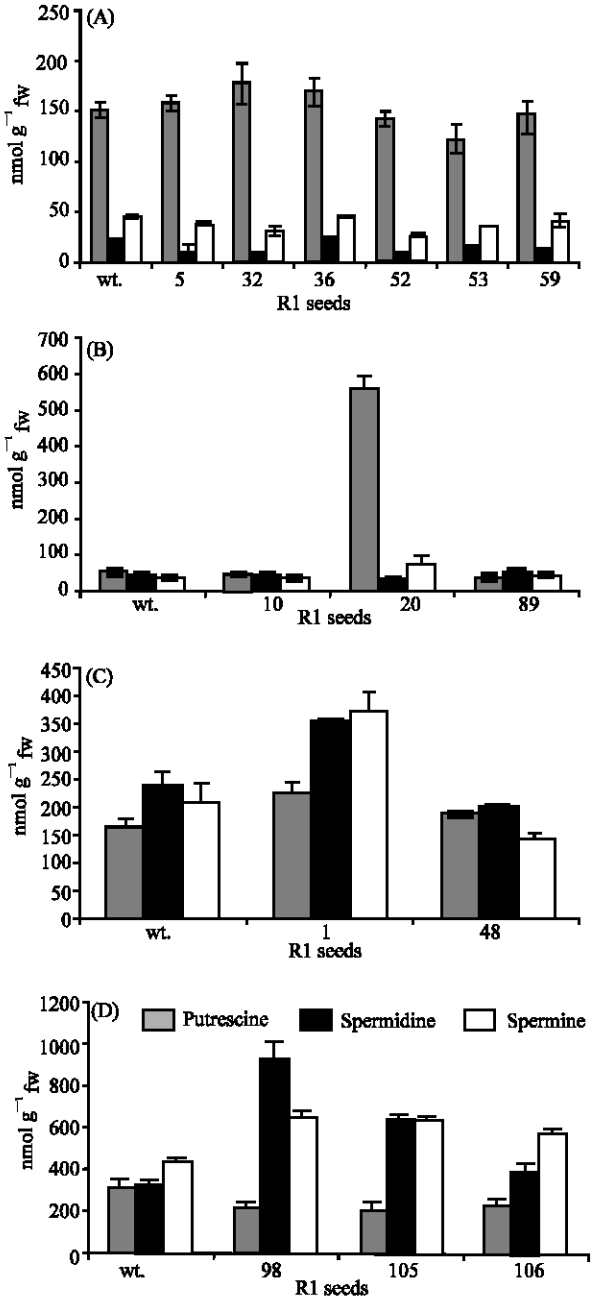

Fig.2: Polyamine levels in R1 seeds. Values are mean"SE for three replicates for every transgenic plant. Control values were determined by analysing 4 different plants. A) Plants transformed with p35SO*adc*; B) Plants transformed with pUbiO*adc*; C) Plants transformed with pUbiH*odc*; D) Plants transformed with p*UbiDsamdc*

PAO are activated to keep spermine at low levels possibly to compensate for the accumulation of putrescine and spermidine

After the onset of morphogenesis and appearance of regenerating shoots in the absence of auxin, we observed a significant increase in putrescine. For spermidine, 50% of the shoots showed a significant increase whereas the remaining tissues showed no variation compared to *hpt*-controls grown under the same conditions. However, spermine levels were increased significantly. In some of the lines we measured increases in spermine levels up to 6 fold compared to *hpt*-controls at the differentiated callus stage. Present results suggest that spermine does not influence morphogenesis because all lines were able to differentiate shoots.

It is generally believed that the polyamine pathway is tightly regulated at the end-product level. Consequently, there is a general notion that it is unlikely that any changes in polyamine accumulation can be achieved. It appears that a key element in facilitating changes in polyamine levels in transgenic tissues is the strength of the promoter used to drive expression of transgenes. We had previously developed a model, which stipulates a minimum threshold in putrescine concentration prior to further conversion into the higher polyamines spermidine and spermine. The CaMV 35S promoter being a moderately strong promoter for driving heterologous transgene expression in rice does not appear to be sufficient to reach the minimum threshold in putrescine accumulation. Consequently, the putrescine pool is not large enough to permit further flux to spermidine and spermine, leading to developmental abnormalities in dedifferentiated rice tissue[21]. In contrast, *Ubi-1* is a much stronger promoter and this manifests in the significantly higher levels of enzyme activity we observed for ADC (50 fold increase compared to an 8 fold increase for the 35S construct). We postulate that this results in increased putrescine production, which, however, does not accumulate; rather it gets rapidly converted to spermidine and spermine. Present findings are therefore consistent with a threshold model which postulates that high *adc* expression leading to production of putrescine above a basal level is necessary to build a big enough metabolic pool to trigger polyamine flux through the pathway to increase levels of spermidine and spermine. This can be best accomplished by a strong constitutive promoter driving *adc*.

Rice seeds are more amenable to alteration of the polyamine content than leaf tissue in transgenic plants expressing the oat *adc* cDNA: We reported previously the recovery of fertile transgenic rice plants expressing oat *adc* cDNA driven by the CaMV 35S promoter and we detected a 2 fold increase in putrescine levels in vegetative tissues of regenerated transgenic plants, but no changes in seed polyamine levels were observed

(Fig. 2A)[21]. In a subsequent series of experiments we generated transgenic rice lines with the pUbi*adcs* gene[27]. We analysed sixteen independent transgenic plant lines by measuring polyamine levels in primary transformants. Even though we observed significant increases in mRNA levels, ADC enzyme activity and polyamine accumulation in transgenic callus, these increases were not maintained in vegetative tissue or seeds in regenerated plants, with the exception of one lineage. This particular lineage showed very significant increases in putrescine levels in seeds (up to 10 times compared to wild type and controls transformed with the *hpt* selectable marker alone, Fig. 2B). These increases were also maintained in the R2 generation thus confirming the heritable nature of putrescine accumulation in seeds. Even though only one transgenic plant lineage exhibited significant increases in polyamine accumulation in seeds, our approach demonstrated that upon screening of adequate numbers of independently-derived transgenic plants, it is possible to identify desirable traits in sub-populations of plants originating from a given transformation experiment.

Putrescine levels in leaves were not significantly different from *hpt* controls. Spermidine and spermine levels were also unchanged. This was the case even for the one individual clone that was shown to accumulate polyamines in seeds. Putrescine accumulation in leaf tissue from 1 month old R1 seedlings from this clone, exhibited a small but statistically significant increase (up to 1.8 fold) compared to *hpt*-controls. These seedlings also exhibited a significant increase in spermidine (1.5 fold) compared to *hpt*-controls. Spermine levels were significantly increased (2 fold) in 40% of the plants, compared to *hpt*-controls. We confirmed that a different polyamine profile was characteristic for different seedlings in this lineage. Transgenic rice plants with enhanced vitamin A content also showed similar behaviour. Transgenic seeds accumulating vitamin A within the same lineage exhibited a wide variation in vitamin A accumulation[28]. This wide variation in end product accumulation for different genes cannot be explained on the basis of hemi- versus homo-zygous sub-populations. It is likely that environmental factors may play an important role as well. Such factors may be more prevalent in the polyamine pathway as these compounds are known to be involved in stress responses in plants.

Enhanced drought tolerance in transgenic rice by manipulation of the polyamine biosynthetic pathway: Many reports link polyamines and abiotic stresses in plants, but 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. We created transgenic plants over-expressing the *Datura adc* gene under the control of the strong monocot maize *Ubi-1* promoter[29] and we investigated 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[30,31]. 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[32]. We found that wild type rice plants subjected to PEG-induced drought stress responded by increasing cellular putrescine levels significantly (Fig. 3A), without any changes in the steady-state rice *adc* mRNA levels (Fig. 3D). In agreement with[33], 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[34] 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[33]. Putrescine accumulation in tissues under stress is also a consequence of the reduction in the rate of spermidine and spermine synthesis[34]. 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[35]. DiTomasso *et al.*[36] suggested that the basis of putrescine toxicity is the presence of an apoplastic DAO that catalyzes the formation of oxidation products, which most probably damage plasma membranes. 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

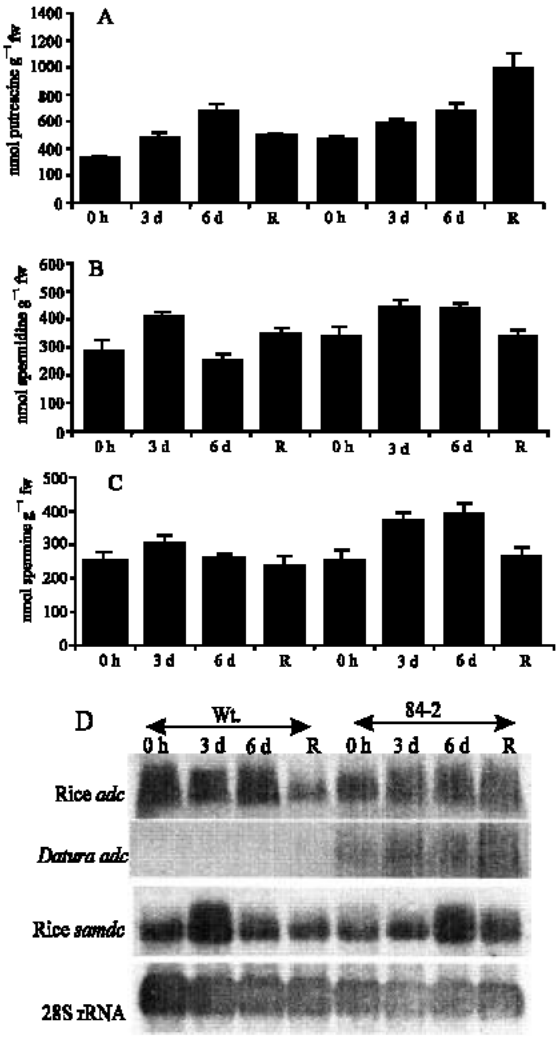

Fig. 3: Molecular and biochemical characterization of transgenic and wild type plants under drought stress.

show a very different response. Putrescine levels are reduced, while the levels of spermidine and spermine increase significantly[34]. 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 possesses a number of stress responsive elements[37] that boost transgene expression under drought stress[38,39]. 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[40]. 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 PAO 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[40]. We observed a remarkably similar reduction in the content of cellular spermidine and spermine in wild type plants after 6 days of drought stress compared to the increases we observed after 3 days. This correlated extremely well with the wilting and rolling of leaves. Transgenic plants that exhibited drought tolerance behaved similarly to wild type plants up to 3 days 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 (Fig. 3B and C). This correlated perfectly with the drought tolerant phenotype of transgenic plants compared to wild type.

In both transgenic and wild type plants under drought stress, the spermidine and spermine content correlated with rice *samdc* steady-state mRNA profile (Fig. 3B-D). When Li and Chen[41] exposed rice seedlings to drought stress using 15% PEG, this resulted in up-regulation of rice *samdc* mRNA after 3 days of stress. However, these investigators were not able to correlate *samdc* mRNA levels with the polyamine content of these plants. The accumulation of spermidine and spermine in leaf segments of mustard subjected to osmotic stress was correlated with an increase in the steady-state mustard *samdc* mRNA. However, the phenotype of these mustard plants was not described[42]. 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.

The two *Arabidopsis thaliana adc* genes [*adc*1 and *adc*2][33,43] were among the genes down-regulated by re-hydration after dehydration, in experiments using full-length cDNA microarrays to monitor profiles of Arabidopsis gene expression under drought stress[44]. In present experiments, steady state mRNA levels of the endogenous *adc* gene declined to very low levels in wild type plants two days after the stress was removed (Fig. 3D) and correlated well with a

significant reduction in putrescine. However putrescine levels in wild type plants did not return to the normal physiological levels measured at t = 0, i.e. before subjecting these plants to drought stress (Fig. 3A). In contrast, putrescine levels peaked at this time point in the *Dadc*-expressing plants, while endogenous levels of the rice *adc* mRNA in the transgenic plants also remained at high levels, as did the *Dadc* mRNA (Fig. 3A, D). This striking differential behavior in the transcript profiles of the rice and *Datura adc* mRNAs in wild type and transgenic plants during their transition from drought stress conditions to recovery and concomitant changes in putrescine levels, perhaps reflects the differential rates at which sensitive and tolerant plants return to their ground state after the stress is removed. As putrescine levels are at their maximum in wild type plants six days after stress induction, *adc* transcript levels need to be reduced immediately after the stress is removed, most likely through a feedback inhibition mechanism, to allow the plants to reduce their putrescine levels and attain a physiologically normal state. As the *Dadc* gene is constantly active, transgenic plants are not able to respond in a similar manner to their wild type counterparts and this results in high *adc* transcript and putrescine levels in these plants. The fact that the rice *adc* transcript levels in transgenic plants is not reduced is most likely a consequence of the complete saturation of the system as a result of *Dadc* expression.

Steady-state mRNA levels for the rice *samdc* gene remained relatively unchanged in the wild type plants two days after the stress was removed. Spermidine and spermine levels in these plants were not significantly different to the levels at t = 0 (Fig. 4B-D). In contrast, the steady-state rice *samdc* mRNA levels in transgenic plants remained substantially higher compared to those at t = 0 (in either transgenic plants or wild type; Fig. 3D). This differential behavior of the rice *samdc* transcript can be explained through a mechanism similar to that discussed earlier for *adc*.

Galston *et al.*[45] 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[46, 47]. 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[40]. In the model proposed by Galston *et al.*[45], 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[40]. 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[38,39], 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[27]. 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 days 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, are not able to raise their spermidine and spermine levels after 6 days of drought stress and consequently exhibit the classical drought-stress response.

Present results are thus 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. Again, 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.

ODC rather then ADC is responsible for the regulation of putrescine synthesis in cereals under normal physiological conditions: Having access to transgenic germplasm over-expressing ADC or ODC is essential in order to carry out detailed biochemical studies that are required to elucidate the relative contribution of each of the two branches to the main pathway. Constitutive expression of the heterologous human *odc* cDNA resulted in mRNA expression in leaf and root tissues. This indicated that the human transgene was transcribed efficiently in rice. Significant increases in ODC activity were also detected in leaf and root tissues. This increase in enzyme activity in turn resulted in substantial increases

in putrescine levels in leaf and root tissues. When the mouse *odc* cDNA was over-expressed in tobacco, transgenic plants regenerated from these lines showed increased putrescine levels in vegetative tissue. Significant changes in putrescine concentration were also observed in dedifferentiated tissues generated from these plants[48]. Hamill *et al.*[49] reported a 2 fold increase in putrescine levels in the transgenic roots of *Nicotiana rustica* over-expressing a yeast *odc* cDNA, but no variation in spermidine or spermine levels were detected.

When the heterologous *adc* gene was previously expressed in rice[50] or tobacco[51] no changes in polyamine levels were detected in vegetative tissue regardless of the promoter used. Modest increases in putrescine levels were detected in tobacco leaves when *adc* expression was driven by an inducible promoter[52]. This body of literature suggests strongly that *odc* rather than *adc* influences putrescine biosynthesis in plants. The ability to modulate levels of enzyme activity and end-product accumulation by heterologous transgene expression suggests that the polyamine pathway can and indeed does exhibit plasticity. Increases in ODC activity appear to modulate polyamine levels whereas changes in ADC activity result in less pronounced effects. Our results thus indicate that ODC rather than ADC is predominantly responsible for putrescine synthesis in plants. In storage tissue such as seeds, polyamine levels were significantly higher in most of the lines we analyzed. All lines showed significant increases in putrescine levels (Fig. 2C). Some lines also had increased levels of spermidine (Fig. 2C). Increases in polyamine levels in seeds occurred as a result of *odc* expression irrespective of the promoter used. Similar results were described in wheat plants in which the *gusA* marker gene was expressed using the same promoter[53].

Through the comparison of ADC and ODC expression profiles and the polyamine levels in transgenic rice populations expressing *adc* or *odc*, we found evidence that ODC is likely the predominant enzyme regulating the formation of putrescine in plants. It has been suggested that the polyamine pathway in plants is so rigidly controlled that the alteration of polyamine levels cannot be achieved by over-expressing key enzymes in the pathway[51]. However, this hypothesis may need to be carefully re-evaluated in view of our results. We have demonstrated that, by determining which of the two alternative enzymes leading to putrescine formation in plants contributes mostly to the polyamine pool, it is possible to generate germplasm with altered polyamine levels. Another key element in this study is the recognition that such changes may occur in a spatially-restricted manner. It is also clear that the polyamine pathway is subject to complex regulation, since no linear correlation between increases in ODC activity and end product accumulation was observed in leaves, roots or seeds.

Levels of the higher polyamines spermidine and spermine can be modulated by expressing enzymes involved in later steps in the pathway: We posed the question whether levels of the higher polyamines spermidine and spermine could be modulated in plants by over-expressing SAMDC, without any involvement of SPD or SPM, which are also involved in their biosynthesis. We had previously demonstrated that levels of these higher polyamines could be altered by expressing early enzymes involved in the pathway and also by down-regulating DAO[54].

We introduced the p*UbiDsamdc* into rice and we recovered transgenic plants that integrated the transgene stably. Transcription of the *Datura samdc* was confirmed by RNA gel blot analysis. Leaf extracts from transgenic rice plants, exhibited significant increases in SAMDC activity. As a result of this increase in enzyme activity, we measured a 1.5 to 2.5 fold increase in the levels of spermidine in leaves, confirming that the *Datura* SAMDC enzyme was functional and that the dicotyledonous enzyme was correctly processed in monocotyledonous plants. RNA gel blot analysis indicated no changes in the steady-state rice *samdc* mRNA in transgenic plants expressing the *Datura* gene. This demonstrates clearly that the heterologous transgene operates independently of its rice orthologue. Increases in spermidine levels in leaves were due to expression of the *Datura samdc* alone, as we did not detect any changes in the endogenous *SPD SYN* transcript. We did not detect any increases in spermine in leaves[55]. The question arises then as to why expression of SAMDC affects levels of spermidine but not spermine in leaves of the transgenc rice plants we generated, as the same enzyme is responsible for the generation of spermine from spermidine by a second transfer of an aminopropyl group from dcSAM. Noh and Minocha[56] over-expressed the human *samdc* cDNA in transgenic tobacco plants resulting in a 2-3 fold increase in spermidine but no significant variation in spermine levels. Similar results were observed when the homologous *samdc* cDNA was re-introduced into potato driven by the tuber-specific patatin promoter[57]. Spermidine concentration was significantly higher in tubers, while no variation was observed in spemine levels. This pattern indicates a tighter regulation of cellular spermine metabolism, compared to putrescine[50] or spermidine[27]. Although spermine is ubiquitous in eukaryotic cells at high levels, the physiological roles of

spermine are unclear[58]. It is possible that the reason we did not observe any changes in spermine levels in leaves of these plants was because the spermidine pool was not large enough to permit conversion of excess spermidine to spermine. We had previously proposed a similar threshold model in terms of the size of the putrescine pool to explain why rice tissues expressing the oat *adc* cDNA driven by a very strong constitutive promoter were able to accumulate higher levels of spermidine and spermine[27], compared to plants engineered with the same transgene driven by a weaker promoter that did not show any changes in the levels of the higher polyamines[21].

We had previously observed a hierarchical accumulation of polyamines in different tissues/organs[59,60]. The general picture that emerges from these studies strongly demonstrates that less metabolically active tissues, such as seeds, accumulate higher levels of polyamines. This was the case in transgenic rice plants expressing the human *odc* or the oat *adc* cDNAs[50,59]. There are no reports in any other transgenic plant system describing the accumulation of any polyamines in storage tissues. In transgenic rice expressing the p*UbiDsamdc*, spermidine as well as spermine levels were significantly increased, while putrescine levels remained unchanged (Fig. 2D). Present results are in line with experiments in which metabolites such as vitamin A and pharmaceutical antibodies accumulate at high levels in seeds of rice[28,61], wheat[62] and pea[63]. It is reasonable to assume that dormant, or less metabolically active tissue provides a conducive environment for the accumulation of such transgenic products. In extreme cases, the formation of high level recombinant proteins in the form of paracrystalline structures in cereal endosperm was observed by optical microscopy[64].

Manipulation of a particular enzyme involved in a metabolic pathway may result in pleotropic changes in other enzymes in the pathway. This may be the result of a compensation mechanism through which plants adjust their metabolism to maintain steady-state pools of key metabolites. Changes in the concentration of metabolites or end products may also affect other enzyme activities, as certain compounds appear to feed-back inhibit or regulate enzymes in different ways. When spermidine and spermine were applied to tobacco cell cultures, a significant reduction in ADC and SAMDC activity was measured. These polyamines did not affect ODC activity[65]. In mammalian systems, an increase in the intracellular content of polyamines reduces the activity of ODC[66]. This reduction occurs as a result of the loss of protein[67]. The decline in protein occurs partly by means of an increased degradation rate[68] and partly by a reduced rate of synthesis[69].

In this study a major metabolic pathway (polyamine biosynthesis) has been discussed, whose genetic manipulation exemplifies the way in which metabolic engineering could contribute to worldwide food and nutritional security in the future. The polyamine case shows how different problems can be addressed by tackling the same pathway in different ways. By modifying single steps in the pathway and controlling when and where the genes are expressed, we have succeeded in producing nutritionally enhanced transgenic rice grains with increased levels of all three major polyamines. We have also produced rice plants constitutively expressing one of the genes in the polyamine pathway that confers protection against osmotic stress, allowing growth in soils that are too dry to support the growth of crops. These plants are currently being studied in the field in two diverse agro-climatic environments.

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