Expression of a Heterologous *S-Adenosylmethionine Decarboxylase* cDNA in Plants Demonstrates That Changes in *S*-Adenosyl-L-Methionine Decarboxylase Activity Determine Levels of the Higher Polyamines Spermidine and Spermine[1]

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We posed the question of whether steady-state levels of the higher polyamines spermidine and spermine in plants can be influenced by overexpression of a heterologous cDNA involved in the later steps of the pathway, in the absence of any further manipulation of the two synthases that are also involved in their biosynthesis. Transgenic rice (*Oryza sativa*) plants engineered with the heterologous *Datura stramonium S-adenosylmethionine decarboxylase* (*samdc*) cDNA exhibited accumulation of the transgene steady-state mRNA. Transgene expression did not affect expression of the orthologous *samdc* gene. Significant increases in SAMDC activity translated to a direct increase in the level of spermidine, but not spermine, in leaves. Seeds recovered from a number of plants exhibited significant increases in spermidine and spermine levels. We demonstrate that overexpression of the *D. stramonium samdc* cDNA in transgenic rice is sufficient for accumulation of spermidine in leaves and spermidine and spermine in seeds. These findings suggest that increases in enzyme activity in one of the two components of the later parts of the pathway leading to the higher polyamines is sufficient to alter their levels mostly in seeds and, to some extent, in vegetative tissue such as leaves. Implications of our results on the design of rational approaches for the modulation of the polyamine pathway in plants are discussed in the general framework of metabolic pathway engineering.

Relatively few pathways have been elucidated molecularly and biochemically in plants, and an even smaller number are amenable to modulation by molecular approaches. This is because of the complex nature of metabolic networks that are often regulated at different levels, spatially and temporally. In our ongoing efforts to implement rational molecular approaches to modulate plant metabolism, we chose the polyamine pathway as a model to unravel those key factors that still present bottlenecks in pathway engineering. The polyamine pathway is ubiquitous in living organisms (Bagni, 1989). 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 (Kumar and Minocha, 1998). As a result, the pathway represents an ideal model to test hypotheses and to answer fundamental biological questions in pathway manipulation using transgenesis.

The polyamine pathway comprises an anabolic phase leading to the elaboration of spermidine and spermine from putrescine. Orn decarboxylase (ODC; EC 4.1.1.19) catalyzes the removal of the carboxyl group from Orn to yield putrescine, whereas *S*-adenosyl-L-Met (SAM) decarboxylase (SAMDC; EC 4.1.1.50), introduces SAM into the pathway, which is then used in its decarboxylated form (dcSAM) as an aminopropyl donor in the conversion of putrescine to spermidine and subsequently to spermine (Tiburcio et al., 1997). The actual transfer of the aminopropyl moiety is catalyzed by two separate and distinct enzymes, spermidine synthase (SPD SYN; EC. 2.5.1.16) and spermine synthase (EC 2.5.1.16). In bacteria and also in plants, two alternative pathways lead to putrescine formation. In addition to the ODC pathway, decarboxylation of Arg by Arg decarboxylase (ADC; EC 4.1.1.19) also results in putrescine formation via two intermediate steps (Malmberg et al., 1998). The pathway also comprises a

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catabolic phase. This involves oxidative deamination of putrescine, spermidine, and spermine by the action of amine oxidases, these include the copper diamine oxidase (DAO; EC 1.4.3.6); these enzymes are characterized by their substrate specificity toward diamines. The flavoprotein polyamine oxidases (PAO; EC 1.5.3.3) oxidize spermidine and spermine at their secondary amino groups (Tiburcio et al., 1997). DAO oxidizes the primary amino group of putrescine and spermidine with the formation of pyrroline (from putrescine) and aminopropylpyrroline (from spermidine) along with ammonia and hydrogen peroxide (Smith, 1988). PAO yields pyrroline and aminopropylpyrroline, from spermidine and spermine, respectively, along with 1,3-diaminopropane and hydrogen peroxide. Thus, the pathway ensures the recycling of carbon and nitrogen from putrescine (Flores and Filner, 1985).

SAM is used by plants for the biosynthesis of polyamines and ethylene (Even-Chen et al., 1982). Ethylene is produced from SAM via 1-amino-cyclopropane-1-carboxylic acid by the actions of 1-aminocyclopropane-1-carboxylic synthase and 1-aminocyclopropane-1-carboxylic oxidase (Hedden and Philips, 2000). Interestingly, the functions of polyamines and ethylene in higher plant metabolism differ diametrically. Whereas ethylene is a plant-aging hormone leading to retardation of growth and promotion of senescence (Abeles, 1973), polyamines have been documented to delay senescence (Capell et al., 1993), and they can also inhibit ethylene biosynthesis in several plant tissues (Apelbaum et al., 1981). The mechanism that control these processes have not been elucidated.

SAMDC is a highly regulated enzyme whose levels can fluctuate severalfold depending on the growth state and intracellular polyamine concentration of the cell (Stanley, 1995). Enzyme regulation in vivo can be achieved at the gene expression level, and also post-transcriptionally by polyamines themselves (Pegg, 1986). Transgenic mice harboring a rat *samdc* gene were generated to study implications of overexpression of polyamine-synthesizing enzymes and their regulation (Heljasvaara et al., 1997). A 2- to 4-fold increase in SAMDC activity was detected in liver and brain tissues of transgenic mice expressing *samdc*. However, neither these nor hybrid mice overexpressing simultaneously *odc* and *samdc* displayed any significant changes in spermidine and spermine levels, but putrescine depletion was measured in these animals. When the human *samdc* cDNA driven by the cauliflower mosaic virus 35S promoter was transferred into tobacco (*Nicotiana tabacum*), transgenic plants showed a significant reduction in putrescine levels, whereas spermidine was increased 2- to 3-fold (Noh and Minocha, 1994). Transgenic tissues failed to regenerate when a homologous *samdc* cDNA driven by the cauliflower mosaic virus 35S promoter was re-introduced into potato (*Solanum tuberosum*). Using the same cDNA in antisense orientation, plants could be regenerated from transformed tissues with difficulty, although severe phenotypic abnormalities were observed, including stunted phenotypes (Kumar et al., 1996).

Using rice (*Oryza sativa*) as a model system, we reported previously overexpression or down-regulation of several genes involved in the polyamine pathway (Capell et al., 1998; Bassie et al., 2000a, 2000b; Capell et al., 2000; Noury et al., 2000; Lepri et al., 2001). To investigate later steps in the pathway in terms of how modulation of enzyme activities affect levels of putrescine, spermidine, and spermine, we introduced a heterologous *Datura stramonium samdc* cDNA (GenBank accession no. Y07768) into regenerable rice tissues.

In the current investigation, we describe and characterize transgenic rice germplasm expressing the *D. stramonium samdc* cDNA and discuss how changes in the activity of this key enzyme influence (a) steady-state levels of polyamines in vegetative (leaf) and storage (seeds) tissues; (b) activities of other enzymes involved in the pathway; (c) whether transcription and/or translation of the rice ortholog is affected; and (d) whether transcription of the rice *spd syn* gene is affected.

RESULTS

Recovery of Primary Transformants

The transformation vector containing the *D. stramonium samdc* cDNA was constructed as described in "Materials and Methods." Mature rice (var. EYI 105) embryos were cobombarded with plasmid *Ubi::Dsamdc* (Fig. 1A) containing the *D. stramonium samdc* cDNA driven by the Ubi-1 promoter and a plasmid containing the hygromycin phosphotransferase (*hpt*) gene as a selectable marker (Valdez et al., 1998; Sudhakar et al., 1998). We analyzed 20 independently derived transgenic rice plants.

Molecular Characterization of Transgenic Rice Plants

All regenerated plants were screened by PCR (see "Materials and Methods") to amplify a 0.9-kb fragment of the *Ubi::Dsamdc*. Genomic DNA gel-blot analysis further confirmed integration of the transgene in the genome of these plants. Genomic DNA-blot analysis of a representative sample of plants is shown in Figure 1B. Digestion was carried out with *Kpn*I, which cuts once within the backbone sequence of the transforming plasmid (Fig. 1A). A 0.9-kb PCR-labeled probe was used to detect the transgene in 19 of the 20 lines we analyzed. The remaining line only had the *hpt*-selectable marker. Transformed lines exhibited unique integration patterns confirming their independent origin. Twelve of the 19 transgenic lines expressed the *D. stramonium samdc* mRNA. Reverse transcription (RT)-PCR analysis of total RNA was performed using the pair of primers pDsamdc-1/pDsamdc-3 (Fig. 1C). We studied ex-

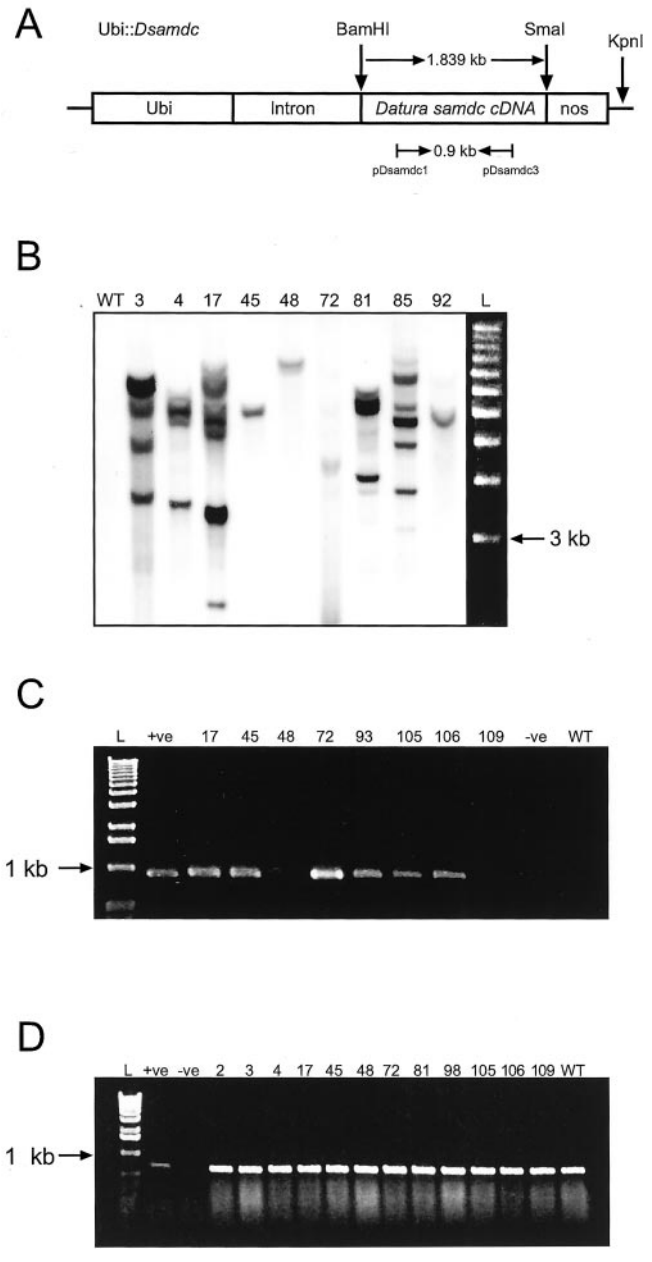

Figure 1. Generation and molecular characterization of transgenic rice plants expressing the *D. stramonium samdc* cDNA. A, Map of *Ubi::Dsamdc* showing transcription unit, relevant restriction sites, and primers used for PCR and RT-PCR analyses. The *D. stramonium samdc* cDNA is 1.839 kb in size. *Kpn*I has a single restriction site in the plasmid. Nos, Nopaline synthase. Arrows represent primers and length of amplified fragment. B, DNA gel-blot analysis of transgenic rice plants. Genomic DNA (10 μg) was digested with *Kpn*I and probed with the 0.9-kb DIG-labeled PCR product from *Ubi::Dsamdc*. Exposure time was 10 min; wt, wild type; numbers represent putative transgenic plants; L, molecular size marker (1-kb DNA ladder, Invitrogen, Carlsbad, CA). C, RT-PCR analysis of *D. stramonium samdc* cDNA (0.9 kb) from total RNA extracted from controls and plants transformed with *Ubi::Dsamdc*. L, Molecular size marker (1-kb DNA ladder, Invitrogen); +ve, positive control, plasmid *Ubi::Dsamdc*; −ve, negative control (water); numbers indicate independent transgenic plants; wt, wild type. D, RT-PCR analysis of rice *samdc* from total RNA extracted from controls and plants transformed with *Ubi::Dsamdc*. L, Molecular size marker (1-kb DNA ladder, Invitrogen); +ve, positive control, plasmid *Ubi::Dsamdc*; −ve, negative control (water); numbers represent indicate independent transgenic plants; wt, wild type.

pression of the rice *samdc* ortholog using the pair of primers pRsamdc-1/pRsamdc-2. We detected no differences in the level of expression of the endogenous rice *samdc* in *Ubi::Dsamdc*-transformed lines, *hpt*-transformants, and wild-type controls (Fig. 1D). RNA gel-blot analysis demonstrated expression of steady-state *D. stramonium samdc* mRNA in 10 of the 12 lines that expressed the gene by RT-PCR (Figs. 1C and 2, A and B). The remaining two lines (45 and 105) that did not show expression of the *D. stramonium samdc* mRNA in northern blots, expressed the RNA at a low level, which was only detectable by RT-PCR. When the same membrane was reprobed using the rice *samdc* and the rice *spd syt* DIG-labeled probes, we observed comparable levels of steady-state rice *samdc* mRNA in all lines (Fig. 2C). Similar results were observed when the membrane was reprobed with the rice *spd syt* DIG-labeled probe (Fig. 2, C and D).

Transgene Transcript Accumulation Results in Increases in SAMDC Activity

SAMDC activity was analyzed in leaf tissue simultaneously with mRNA and polyamine measurements. Background SAMDC activity in control plants was on the order of 0.40 to 0.50 nKat mg^{-1} protein. Six of the 12 transgenic lines that expressed the *D. stramonium samdc* at the mRNA level (3, 4, 72, 81, 98, and 105) had a significant increase in SAMDC activity (Fig. 3A). Plant 3 had a maximum 3-fold increase in SAMDC activity (1.42 nKat mg^{-1} protein; $P < 0.001$) when compared with control lines (Fig. 3A). This plant also accumulated the *D. stramonium samdc* transcript at the highest level (Fig. 2, A and B). The *D. stramonium samdc* transcript levels in plant 105 were significantly lower compared with the other expressing plants (Fig. 1C). However, SAMDC activity in this clone was of the same order as in the other clones (Fig. 3A).

The Triamine Spermidine Accumulates in Leaf Tissue as a Result of Increases in SAMDC Activity

Spermidine and spermine levels were analyzed in transgenic leaf tissue simultaneously with enzyme activity measurements (ADC, ODC, SAMDC, DAO, and PAO). Spermidine levels were increased significantly in all six lines that showed changes in SAMDC activity in leaves. Increases varied from 1.5-fold in plant 3 (370 nmol g^{-1} fresh weight; $P < 0.05$) to 2.5-fold in plant 98 (540 nmol g^{-1} fresh weight; $P < 0.05$) compared with wild-type or *hpt*-

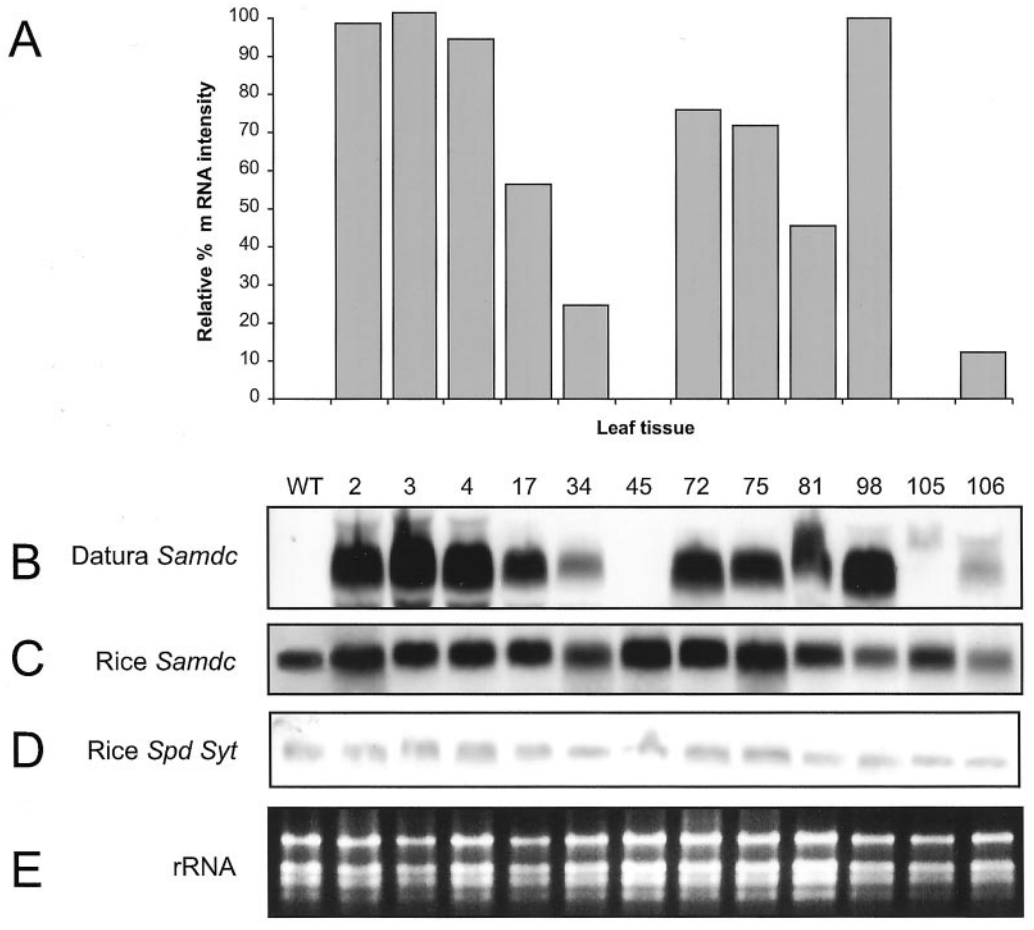

Figure 2. Transcript accumulation in rice leaves. A, Normalization of hybridization signals in leaf tissue after densitometric analysis of autoradiographs. *D. stramonium samdc* mRNA levels were quantified, and the resulting values were normalized using values obtained from RNA loading levels. Column size represents the relative *D. stramonium samdc* mRNA level generated by comparing the normalized values of each lane with that of the highest expressing sample. B, Gel-blot analyses of total RNA from transgenic leaf tissue (WT, wild type 2, 3, 4, 17, 34, 45, 72, 75, 81, 98, 105, and 106). A 0.9-kb DIG-labeled PCR probe from *D. stramonium samdc* cDNA was used. Exposure time was 10 min. C, Gel-blot analyses of total RNA from transgenic leaf tissue (WT, wild type 2, 3, 4, 17, 34, 45, 72, 75, 81, 98, 105, and 106). A 0.7-kb DIG-labeled PCR probe from rice *samdc* cDNA was used. Exposure time was 20 min. D, Gel-blot analyses of total RNA from transgenic leaf tissue (WT, wild type 2, 3, 4, 17, 34, 45, 72, 75, 81, 98, 105, and 106). A 0.9-kb DIG-labeled PCR probe from rice *spd syn* cDNA was used. Exposure time was 30 min. E, UV fluorescence of ethidium bromide-stained gel showing equal amount of total RNA loading from plants used for the hybridization shown above.

transformed controls (175–250 nmol g^{-1} fresh weight; Fig. 3B). No significant variation ($P > 0.05$) in spermine levels in leaves was observed in any of the plants analyzed when compared with controls (Fig. 3B).

Putrescine Accumulation in Leaf Tissue Is Related to Increases in ADC and ODC Activities

Two of the 12 lines analyzed had a significant increase in putrescine levels. A 2-fold increase was detected in plants 98 and 105 [1,220 ($P < 0.01$) and 1,169 ($P < 0.05$) nmol g^{-1} fresh weight, respectively] when compared with wild-type and *hpt*-transformed controls (470–560 nmol g^{-1} fresh weight, Fig. 3B). When we measured activities of early enzymes in the pathway, ADC and ODC, a surprising result was observed in plants 98 and 105. A significant increase in ADC and ODC activity was detected. A maximum 1.6-fold increase in ADC activity (5.4 nKat mg^{-1} protein; $P < 0.01$) and a 5.7-fold increase in ODC activity (6.9 nKat mg^{-1} protein; $P < 0.001$) were detected in plant 98 when compared with controls (3.27 nKat mg^{-1} protein and 1.21 nKat mg^{-1} protein for ADC and ODC activities, respectively; Fig. 4). We then measured activity of the two enzymes involved in the catabolism of polyamines. We did not detect any significant variation in DAO or PAO activities in any of the plants we analyzed (Fig. 4).

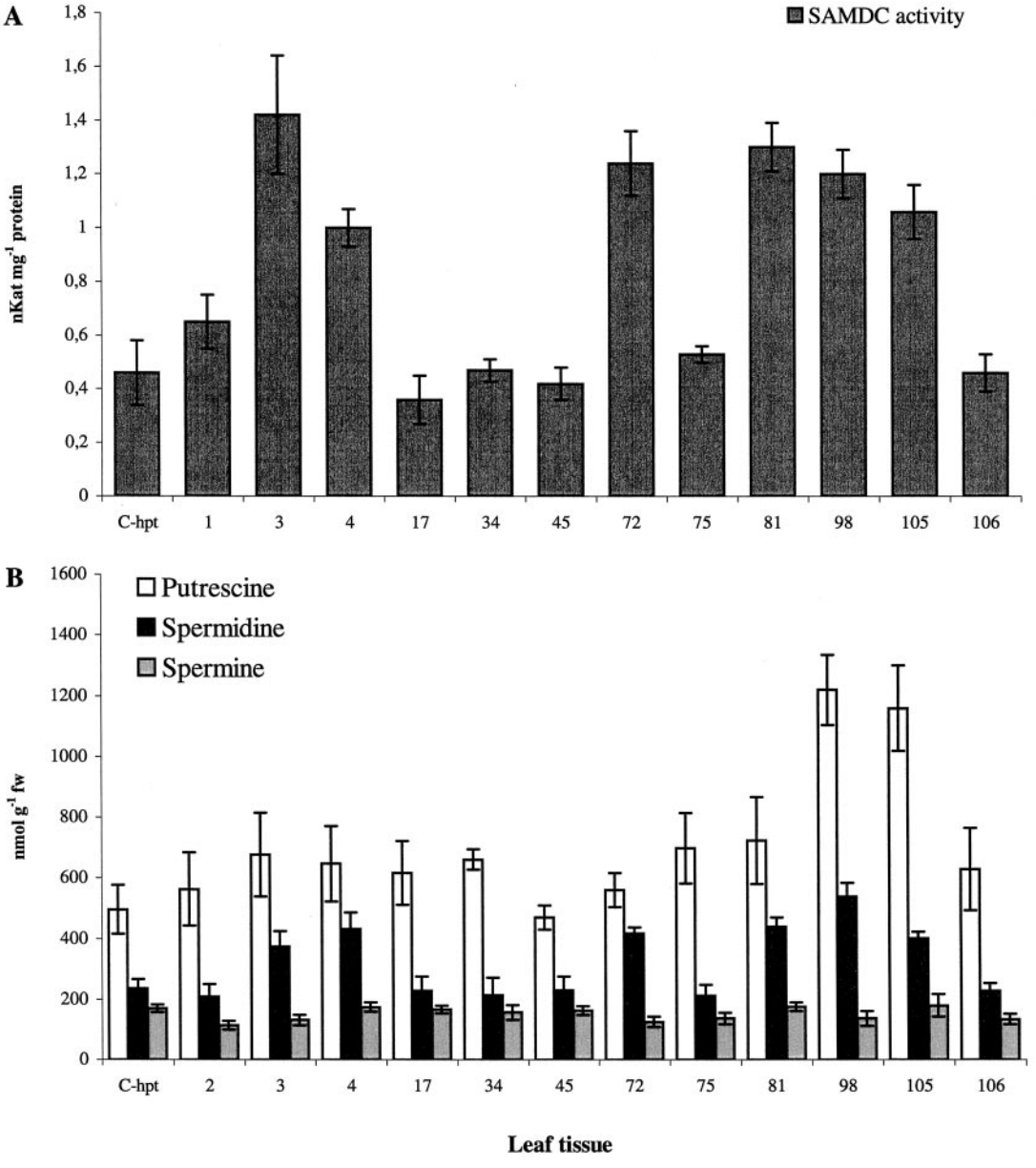

Figure 3. Biochemical characterization of transgenic rice plants expressing *Ubi::Dsamdc*. A, SAMDC enzyme activity in different transgenic lines compared with appropriated controls. Values are means ± SE for control lines ($n = 6$) and means ± SE in transgenic lines ($n = 4$). SAMDC activity in clones 4, 72, 81, 98, and 105 was significantly different from controls at $P < 0.01$; for clone 3 at $P < 0.001$. Remaining values were not significantly different from control levels at $P > 0.05$. B, Cellular polyamine levels in controls and 12 representative transgenic plants. Values are means ± SE in control lines ($n = 36$) and means ± SE in transgenic lines ($n = 9$). Putrescine levels were significantly different from controls at $P < 0.01$ for clone 98 and $P < 0.05$ for clone 105. Spermidine levels were significantly different from controls at $P < 0.05$ for all clones. Spermine levels were not significantly different from controls at $P > 0.05$.

Spermidine and Spermine Accumulate in R1 Seeds of Plants Expressing *Ubi::Dsamdc*

The polyamine content of seeds harvested from primary transformants was determined after collection of mature seeds and desiccation. Seeds from plants 98, 105, and 106 showed a significant increase in spermidine (2.5-fold increase, 700–900 nmol g^{-1} fresh weight; $P < 0.01$) and spermine levels (2-fold increase, 600 nmol g^{-1} fresh weight; $P < 0.05$) when compared with wild-type and *hpt* controls (300 and 30 nmol spermine g^{-1} fresh weight; Fig. 5). No significant variation was detected among the remaining lines and controls in spermidine and spermine levels. No significant variation in putrescine levels was detected in any of the lines (Fig. 5).

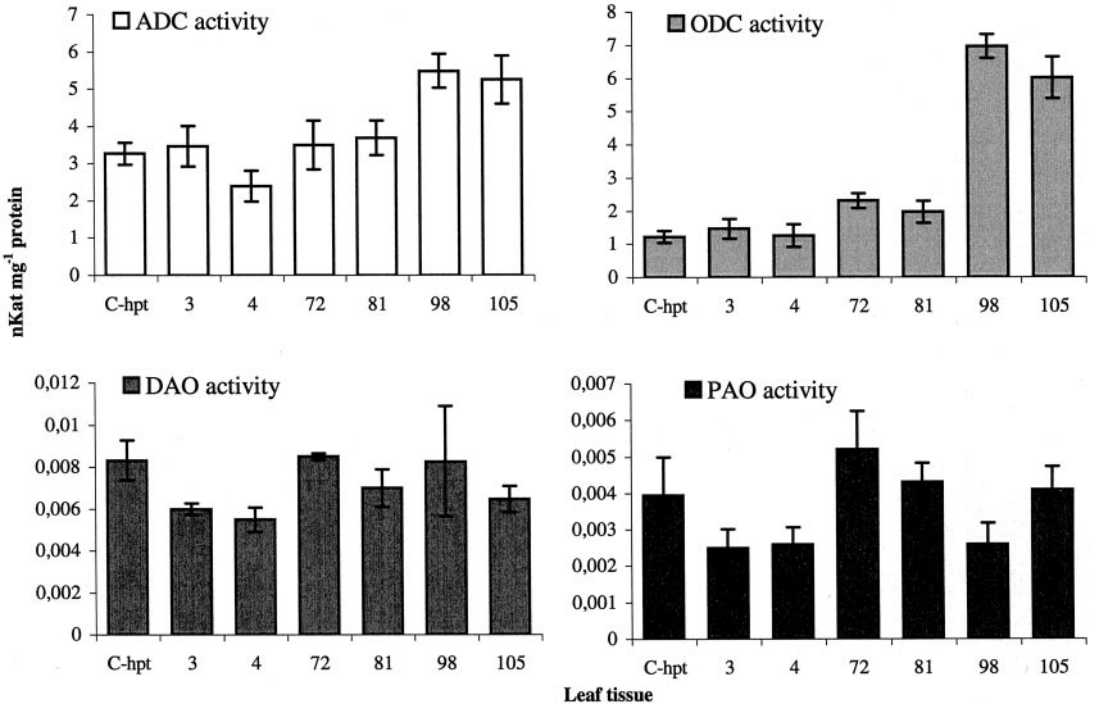

Figure 4. Rice ADC, ODC, DAO, and PAO activities in leaf tissue. Values are means ± SE in control lines ($n = 4$) and means ± SE in transgenic lines ($n = 4$). ADC activity was significantly different from control at $P < 0.01$ for clone 98 and at $P < 0.05$ for clone 105. ODC activity was significantly different from control at $P < 0.01$ for clones 98 and 105. ADC, ODC, DAO, and PAO activities were not significantly different from controls at $P > 0.05$ in any of the remaining lines.

DISCUSSION

A limited number of plant metabolic pathways have been studied in depth, primarily because of the complexity of metabolic networks and how these are regulated. An even smaller number of pathways have been manipulated using molecular, genetic, or biochemical tools. Examples of such pathways include flavonoid biosynthesis (Van der Krol et al., 1990), lignins (Guo et al., 2001), carotenoids (Römer et al., 2000; Ye et al., 2000), fatty acids (Kinney, 1998), and some secondary metabolic pathways (Yun and Hashimoto, 1992; Nessler, 1994). Different approaches have been used to understand metabolic processes in plants. These include the use of inhibitors (Malmberg and McIndoo, 1983; Hiatt et al., 1986), mutants that lack particular enzymatic steps in a given pathway (Somerville and Browse, 1991; Watson et al., 1998), and homologous or heterologous genes in molecular studies (Capell et al., 2000; Halpin et al., 2001). As our appreciation of the complexity of biosynthetic pathways in plants increases, it becomes necessary to develop a knowledge base in molecular and biochemical terms to understand how such pathways control vital physiological, developmental, and metabolic processes (Capell et al., 2000). A reductionist approach to simplify the complexity of biosynthetic pathways in plants will help unravel biochemical components that play a crucial role in determining levels of end-products and intermediates. Such an approach needs to be validated on a well-characterized pathway in terms of enzymology and biochemistry.

The polyamine biosynthetic pathway in higher plants provides such an example. The pathway comprises anabolic and catabolic components (Malmberg et al., 1998). The two higher polyamines spermidine and spermine are synthesized from the diamine putrescine by a sequential addition of aminopropyl moieties from dcSAM by SAMDC (Pegg, 1986). There are two alternative pathways for the biosynthesis of putrescine in plants. All enzymes in the polyamine pathway have been characterized, and corresponding cDNAs have been cloned from different organisms (Kumar and Minocha, 1998). As a consequence, we have in place all components that are necessary to test and validate such an approach using a relatively short, yet complex pathway. The pathway is remarkable for its biochemical diversity, and for the number of regulatory and physiological processes in which it has been implicated (Malmberg and Rose, 1987). A transgenic approach to answer such fundamental biological questions has distinct advantages. By introducing appropriate transgenes into plants and analyzing the effects transgene products have on end-product accumulation, we may begin to understand how individual components of the pathway(s) contribute toward their concerted regulation (Kumar and Minocha, 1998).

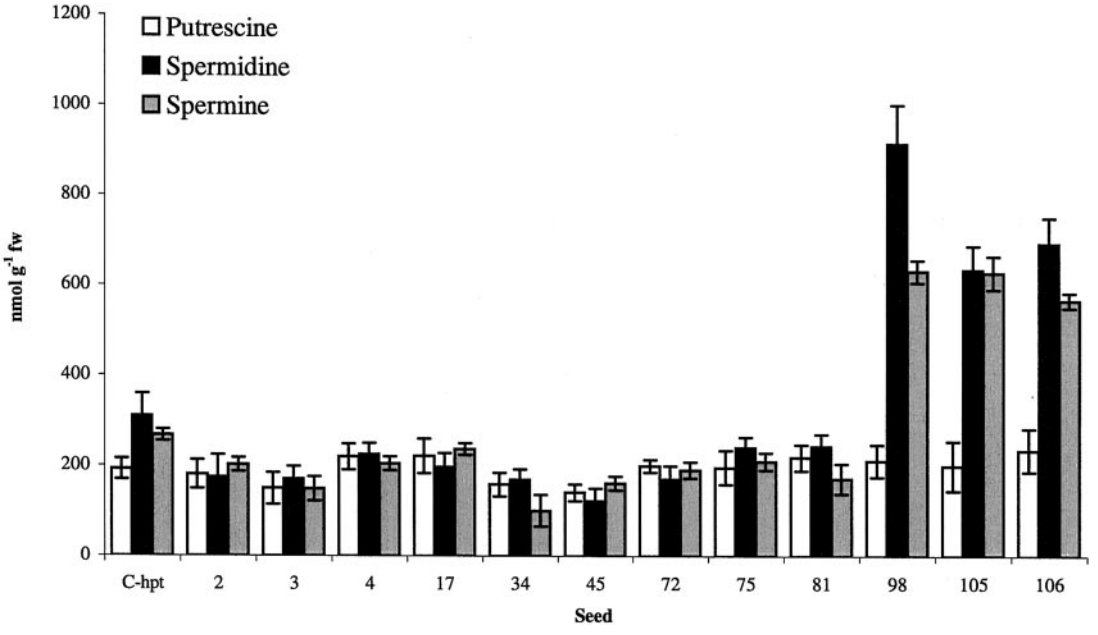

Figure 5. Polyamine levels in controls and *Ubi::Dsamdc*-containing seeds. Values are means ± SE in control lines ($n = 36$) and means ± SE in transgenic lines ($n = 3$). Putrescine levels were significantly different from controls at $P < 0.05$ for clones 98 and 106. Spermidine levels were significantly different from controls at $P < 0.01$ and $P < 0.05$ for clones 98 and 106, respectively. Spermine levels were significantly different from control at $P < 0.05$ for clones 98 and 106. Remaining values were not significantly different from control levels at $P > 0.05$.

For the past several years, we have been studying molecular, biochemical, and genetic components of polyamine metabolism in plants (Capell et al., 1998; Bassie et al., 2000a, 2000b; Capell et al., 2000; Noury et al., 2000; Lepri et al., 2001). Through comparison of ADC and ODC activities and the corresponding polyamine profiles in transgenic rice overexpressing the oat (*Avena sativa*) *adc* or the human (*Homo sapiens*) *odc* cDNAs, we found strong evidence that ODC is most likely the enzyme responsible for regulating the formation of putrescine in plants (Lepri et al., 2001). We showed that the tight regulation of the polyamine pathway at the end-product level can be overcome by overexpressing key enzymes involved in the pathway. Thus, by screening transgenic plants expressing ADC or ODC, we were able to identify populations with substantial changes in polyamine levels (Capell et al., 1998; Noury et al., 2000; Lepri et al., 2001). We also investigated the effect of shutting down ADC by antisense approaches (Capell et al., 2000) and the consequences of down-regulating DAO (Bassie et al., 2000b). Such experiments allowed us to develop an understanding of how early steps in the polyamine pathway control levels of the parent polyamine, putrescine, in plants and how this compound is further converted into the higher polyamines spermidine and spermine. Having investigated the role of early enzymes in the pathway, we wished to elucidate the contribution of enzymes involved in later parts of the pathway, particularly the role of SAMDC.

We posed the question of whether levels of the higher polyamines spermidine and spermine could be modulated in plants by overexpressing SAMDC without any involvement from SPD SYN or spermine synthase, 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 (Bassie et al., 2000b).

Accumulation of the *D. stramonium samdc* Transcript Results in an Increase in SAMDC Activity and Spermidine Accumulation in Leaves

We introduced the *Ubi::Dsamdc* (Fig. 1A) into rice, and we recovered transgenic plants that integrated the transgene stably. DNA gel blots demonstrated the independent origin of all transgenic plants we recovered (Fig. 1B). Transcription of the *D. stramonium samdc* was confirmed by RNA gel-blot analysis (Figs. 1C and 2, A and B). Leaf extracts from transgenic rice plants, exhibited significant increases in SAMDC activity (Fig. 3A). 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 *D. stramonium* 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 (Fig. 2C) in transgenic plants expressing the *D. stramonium* gene. This demonstrates clearly that the heterologous transgene operates independently of its rice ortholog. Increases in spermidine levels in leaves were attributable to ex-

pression of the *D. stramonium samdc* alone, because we did not detect any changes in the endogenous *spd syn* transcript (Fig. 2C). We did not detect any increases in spermine in leaves (Fig. 3). The question arises then as to why expression of SAMDC affects levels of spermidine but not spermine in leaves of the transgenic rice plants we generated, because 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 (1994) overexpressed the human *samdc* cDNA in transgenic tobacco plants resulting in a 2- to 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 (Rafart-Pedros et al., 1999). Spermidine concentration was significantly higher in tubers, whereas no variation was observed in spermine levels. This pattern indicates a tighter regulation of cellular spermine metabolism, compared with putrescine (Noury et al., 2000) or spermidine (Bassie et al., 2000a). Although spermine is ubiquitous in eukaryotic cells at high levels, the physiological roles of spermine are unclear (Hamasaki-Katagiri et al., 1998). It is possible that the reason we do not see any changes in spermine levels in leaves of these plants is because the spermidine pool is 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 (Bassie et al., 2000a) compared with plants engineered with the same transgene driven by a weaker promoter that did not show any changes in the levels of the higher polyamines (Capell et al., 1998).

SAMDC Expression Results in Spermidine and Spermine Accumulation in Storage Tissue

We had previously observed a hierarchical accumulation of polyamines in different tissues/organs (Lepri et al., 2001; P. Trung-Nghia et al., personal communication). 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 (Noury et al., 2000; Lepri et al., 2001). There are no reports in any other transgenic plant system describing the accumulation of any polyamines in storage tissues. In transgenic rice expressing the *Ubi::Dsamdc*, spermidine and spermine levels were significantly increased, whereas putrescine levels remained unchanged. Our results are in line with experiments in which metabolites such as vitamin A and pharmaceutical antibodies accumulate at high levels in seeds of rice (Ye et al., 2000; Torres et al., 2001), wheat (*Triticum aestivum*; Stöger et al., 2000), and pea (*Pisum sativum*; Perrin et al., 2000). It is reasonable to assume that dormant or less metabolically active tissue provides a conducive environment for the accumulation of transgenic products. In extreme cases, the formation of recombinant proteins in the form of paracrystalline structures in cereal endosperm, is easily observed by optical microscopy (Stöger et al., 2001).

Activities of Early Enzymes in the Pathway and Those Responsible for Polyamine Catabolism Are Rarely Altered in Transgenic Plants Expressing *Ubi::Dsamdc*

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, because certain compounds appear to feedback 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 (Hiatt et al., 1986). In mammalian systems, an increase in the intracellular content of polyamines reduces the activity of ODC (Kameji and Pegg, 1987). This reduction occurs as a result of the loss of enzyme protein (Persson et al., 1984). The decline in enzyme protein occurs partly by means of an increased degradation rate (Murakami et al., 1985) and partly by a reduced rate of synthesis (Höltta and Pohjanpelto, 1986).

A majority of the plants that we generated that expressed the *Ubi::Dsamdc* did not have any changes in the activities of the rice ADC, ODC, DAO, or PAO in leaf tissue (Fig. 4). However, two transgenic plants that accumulated high levels of spermidine (up to 2.5-fold) also exhibited increases in putrescine levels (2-fold) as a result of increase in ADC and ODC activity (Figs. 3B and 4). These two plants (98 and 105) exhibited the most dramatic increases in rice ODC activity (up to 5.7-fold) compared with all of the clones we recovered (Fig. 4). Rice ADC activity was increased a maximum of 1.6-fold in these lines as well. These results are indeed consistent with data we published previously that demonstrated that ODC rather than ADC is responsible for changes in putrescine levels in plants (Lepri et al., 2001). Our results suggest that application of exogenous polyamines and their generation in situ in plant cells as a result of heterologous transgene expression appear to result in different responses at the biochemical level. This may be explained by the fact that the two systems are physiologically very different and as such,

endogenous enzyme activities respond in different ways to what appears to be the same stimulus. It may be that changes in the endogenous enzyme activities in some of these transgenic plants is an exception that may be a result of differential regulation of enzymes in different transformants.

CONCLUSION

We have demonstrated that expression of a heterologous *samdc* cDNA in plants is adequate to increase enzyme activity and end-product levels in a tissue-dependent manner and is uncoupled from the endogenous polyamine biosynthetic machinery. Our results further suggest that it is possible to modulate complex pathways in plants by overexpression of appropriate heterologous transgenes, even in situations in which a particular product or intermediate requires input from additional components of the pathway. We can, thus, envisage strategies for the manipulation of other pathways in plants by applying findings we obtained as a result of experiments involving the polyamine pathway.

MATERIALS AND METHODS

Plasmid Construction, Rice (*Oryza sativa*) Transformation, and Plant Regeneration

The 1.8-kb *Datura stramonium samdc* cDNA (GenBank accession no. Y07768) containing the 5′-untranslated sequence and the cDNA coding region, was excised as an *Xho*I fragment from pBluescript, blunt ended, and digested again with *Bam*HI. The *Bam*HI/blunt-end fragment was subcloned into the *Bam*HI/*Sma*I site of the plasmid pAL76 (Christensen and Quail, 1996), which contains the maize (*Zea mays*) 1 ubiquitin (Ubi-1) promoter and first intron, and a Nos transcriptional termination. This plasmid was subsequently referred to as *Ubi::Dsamdc*.

Rice transformation, selection, and plant regeneration procedures were as described previously (Sudhakar et al., 1998; Valdez et al., 1998).

PCR and RT-PCR Analyses

Genomic DNA was extracted from leaf tissue according to the method of Edwards et al. (1991). Genomic PCR amplification to detect *D. stramonium samdc* cDNA was carried out in a total volume of 25 μL comprising 50 ng of genomic DNA, 1× PCR buffer (50 mM KCl, 10 mM Tris-HCl, pH 8.3, and 1.5 mM $MgCl_2$, Roche Molecular Biochemicals, Mannheim, Germany), 200 μM each dNTP, 50 nM of each primer (the forward sequence primer started from position 630 in the *D. stramonium samdc* open reading frame and consisted of 5′-CGGACCTGCTGAGTGCACCATTGT-3′, primer pDsamdc-1; reverse primer, 5′-CCAGCAGCCCTTCAGAACGG-3′, primer pDsamdc-3) and 1.25 units of *Taq* DNA polymerase (Roche Molecular Biochemicals). We carried out 35 amplification cycles: denaturation (94°C, 40 s), annealing (64°C, 1 min), and extension (72°C, 2 min). The 0.9-kb product was visualized by agarose gel electrophoresis (0.8% [w/v] Tris-borate/EDTA [TBE]).

Total RNA was extracted from leaves of transgenic plants using the Trizol-Reagent (Invitrogen). RNA samples were treated with RQ1 RNase-free DNase (Promega, Madison, WI) as recommended by manufacturer. RT was carried out using the Access RT-PCR system (Promega) in 25-μL reaction volumes containing 100 ng of total RNA. The primer pairs used for RT-PCR were pDsamdc-1/pDsamdc-3 to study *D. stramonium samdc* cDNA expression and pRsamdc-1/pRsamdc-2 to study rice *samdc* gene expression. The primer sequences for rice *samdc* cDNA were as follows: the forward sequence primer started from position 1,000 in the rice *samdc* open reading frame and consisted of 5′-GGAGATCCAGCAAAGCCTGGCC-3′ (pRsamdc-1), and the reverse sequence consisted of 5′-CCCAGGGGAGAAGATTGCCCAG-3′ (pRsamdc-2). *D. stramonium samdc* cDNA and rice *samdc* cDNA were amplified for 40 cycles: denaturation (94°C, 40 s), annealing (65°C, 1 min), and extension (68°C, 2 min). The 0.9-kb *D. stramonium samdc* and the 0.7-kb rice *samdc* were visualized on a 1% (w/v) TBE agarose gel.

DNA and RNA Gel-Blot Analysis

Genomic DNA from leaf tissue was extracted as described by Edwards et al. (1991). DNA was digested with *Kpn*I, fractionated by 0.8% (w/v) TBE agarose gel electrophoresis (Sambrook et al., 1989), and transferred to a positively charged nitrocellulose membrane (Roche Molecular Biochemicals). Nucleic acids were fixed by baking at 80°C for 2 h. Filters were washed in 2× SSC for 30 min and subsequently prehybridized at 42°C for 1 h using the DIG-easy hybridization solution (Roche Molecular Biochemicals). The 0.9-kb *D. stramonium samdc*, the 0.7-kb rice *samdc*, and the 0.9-kb rice *spd syn* were labeled using the PCR DIG probe synthesis kit (Roche Molecular Biochemicals). The primer sequences for rice *spd syt* cDNA were as follows: The forward sequence primer started from position 196 bp in the rice *spd syn* open reading frame and consisted of 5′-GGATGGTTCTCCGAGATTAG-3′ (pRspdsyn-1), and the reverse sequence consisted of 5′-GATCTAGTTGGCCTTGGATC-3′ (pRspdsyn-2). Alkali-labile DIG-11-dUTP was incorporated into the probe in a final volume of 50 μL comprising 4 μM dATP, 4 μM dCTP, 4 μM dGTP, 3.2 μM dTTP, 0.8 μM DIG-11-dUTP, 1× Roche Molecular Biochemicals PCR buffer (50 mM KCl, 10 mM Tris-HCl, pH 9.0, and 0.1% [w/v] Triton X-100), 2.5 units of *Taq* DNA polymerase (Roche Molecular Biochemicals), 0.1 mM each of the forward and reverse sequence primers (as PCR above), and 100 ng of the plasmids. After an initial denaturation step for 2 min at 96°C, 35 amplification cycles were carried out, each comprising denaturation at 96°C for 10 s, annealing at 60°C for 10 s, and extension at 72°C for 1 min. The 0.9-, 0.7-, and 0.9-kb-labeled probes were purified using the QIAquick Gel Extraction Kit (QIAGEN, Dorking, Surrey, UK) and denatured at 68°C for 10 min before use. Hybridization was performed at 42°C overnight. The membranes were washed twice for 5 min in 2× SSC, 0.1% (w/v) SDS at room temperature and then twice (15 min) in 0.5× SSC, 0.1% (w/v) SDS at 68°C. Chemiluminescent detection was carried out according to the manufacturer's instructions using the DIG Luminescent Detection Kit. After washing, the membranes were incubated with CSPD Chemiluminescent Substrate (Roche Molecular Biochemicals) and subsequently exposed to x-ray film (Fuji Photo Film, Kanawa, Japan) for 20 min at 37°C.

Total RNA was extracted from leaf tissue using RNeasy Plant Mini Kit (QIAGEN). Denatured RNA (30 μg) was subjected to electrophoresis on 1.2% (w/v) agarose-formaldehyde gel using 1× MOPS buffer (Sambrook et al., 1989). Transfer and hybridization were carried out as described above for DNA procedures. Membranes were exposed to x-ray film for 30 min at 37°C. Stripping and reprobing the membrane with the 0.7-kb rice *samdc* and 0.9-kb rice *spd syn* was performed as described by Hloch et al. (2001).

Determination of SAMDC, ADC, ODC, DAO, and PAO Activities

Leaf tissue was used for SAMDC, ADC, ODC, DAO, and PAO activity measurements. Tissue was extracted in buffer (0.1 M Tris, pH 7.5, and 2 mM dithiothreitol) at a ratio of 300 mg ml^{-1}. Polyvinylpyrrolidone (100 mg) was added during grinding. After centrifugation at 12,000*g* for 20 min, the supernatant was used directly in enzyme activity assays. Enzyme assays were carried out as described in detail in Lepri et al. (2002). Enzyme activity was expressed as nanokatals per milligram of protein.

Polyamine Analysis

Crude extracts from leaves and seeds were dansylated and separated by thin layer chromatography as described (Capell et al., 1998). The dansyl-polyamine bands were identified on the basis of their R_F values after visualization under UV light (312 nm) and comparison with dansylated polyamine standards. The image of the chromatogram was captured and analyzed by Quantity One (Quantification Software; Bio-Rad, Hercules, CA). The relative amount of dansyl-polyamine in each sample was determined by calculating the integrated optical density of the bands compared with the integrated optical density of the appropriate dilution of the dan-

sylated control samples. Results were expressed as nanomoles per gram fresh weight.

Statistical Analysis

For molecular and biochemical analyses (enzyme activity and polyamine content) we used *hpt*-transformed and wild-type controls. The average control for the biochemical analyses was determined by taking three samples from 12 independent lines (six wild type and six *hpt*-transformants; $n = 36$). Hygromycin-resistant transformants and wild-type controls were not significantly different ($P > 0.05$) in terms of enzyme activity and polyamine levels in any of the tissues analyzed (Lepri et al., 2002). Data was analyzed by one-way ANOVA followed by Student's t test using the residual mean square in the ANOVA as the estimate of variability.

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