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ORIGINAL PAPER

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Over-expression of a cDNA for human ornithine decarboxylase in transgenic rice plants alters the polyamine pool in a tissue-specific manner

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Abstract We investigated how over-expression of a cDNA for human ornithine decarboxylase (*odc*) affects the polyamine pools in transgenic rice. We further investigated tissue-specific expression patterns and product accumulation levels of the transgene driven by either constitutive or seed-specific promoters. Our results indicate that: (1) whereas the expression of a heterologous arginine decarboxylase (*adc*) cDNA in rice resulted in increased putrescine and spermine levels only in seeds, plants engineered to express *odc* cDNA exhibited significant changes in the levels of all three major polyamines in seeds and also in vegetative tissues (leaves and roots); (2) there was no linear correlation between *odc* mRNA levels, ODC enzyme activity and polyamine accumulation, suggesting that control of the polyamine pathway in plants is more complex than in mammalian systems; (3) ODC activity and polyamine changes varied in different tissues, indicating that the pathway is regulated in a tissue-specific manner. Our results suggest that ODC rather than ADC is responsible for the regulation of putrescine synthesis in plants.

Keywords Arginine decarboxylase · Ornithine decarboxylase · Polyamines · Putrescine · Rice

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Introduction

In animals and fungi, putrescine (the precursor of the higher polyamines spermidine and spermine) is synthesized directly from ornithine by the enzyme ornithine decarboxylase (ODC; EC 4.1.1.17). This is a rate-limiting step in the polyamine biosynthetic pathway (Pegg 1986). Ornithine decarboxylase activity is closely associated with cellular proliferation and is essential for normal cell growth (Pegg 1986). Increased levels of ODC activity are found in cultured mammalian cells that have been subjected to hypo-osmotic stress (Lundgren 1992). In plants, *odc* expression is induced by 2,4-D, GA_3 and by pollination (Alabadi et al. 1998). It is also interesting to note that Bailey et al. (2000) have demonstrated a requirement for ODC in the development of fungal disease in cereals. A knockout strain of *Stagonospora nodorum* was created by targeted gene replacement. Studies on the pathogenicity of these mutants showed that they were greatly reduced in virulence towards wheat as compared with wild-type strains (Bailey et al. 2000).

Plants provide an interesting eukaryotic system for investigating the physiological and biochemical roles of ODC and polyamines, because they can use an alternative route to putrescine catalyzed by arginine decarboxylase (ADC; EC 4.1.1.19; Smith 1985). This involves production of the intermediates agmatine and N-carbamoylputrescine (Michael et al. 1996). In *Escherichia coli*, where both pathways are present, the biosynthetic *odc* and *adc* genes have been cloned and sequenced (Boyle et al. 1994). Several vertebrate, invertebrate, fungal and plant *odc* genes have also been cloned (Burtin and Michael 1997). Heterologous expression of various *odc* genes has been used to complement ODC-deficient bacteria, as well as mutant mammalian cell lines (Fonzi ans Sypherd 1985; Ghoda et al. 1989). Transgenic mice have been generated that over-express the human *odc* gene (Halmekyto et al. 1991). Even though these animals displayed levels of

ODC activity up to 100 times higher than those in the corresponding tissues of their non-transgenic littermates, surprisingly few phenotypic changes were observed. In most tissues, the over-expression of ODC was accompanied by a grossly elevated putrescine content, with no concomitant changes in spermidine and spermine concentrations (Halmekyto et al. 1991, 1993). When the human *odc* gene was expressed in mice, under the control of the mouse metallothionein I promoter, a 150-fold increase in putrescine levels was detected in liver tissue following treatment with zinc (Alhonen et al. 1996). In plants, Hamill et al. (1990) demonstrated that over-expression of yeast *odc* in *Nicotiana rustica* increased the levels of putrescine and nicotine, an alkaloid derived from *N*-methylputrescine via putrescine *N*-methyltransferase (E.C. 2.1.1.53). DeScenzo and Minocha (1993) over-expressed a mouse *odc* cDNA in tobacco plants. This resulted in a 4- to 12-fold increase in putrescine accumulation in callus lines derived from transformed plant tissues. Somatic embryogenesis was promoted when the mouse *odc* cDNA was over-expressed in carrot cells. A 10- to 20-fold increase in putrescine was observed in the transformed carrot cell lines. No variation in spermidine and spermine concentrations was observed (Bastola and Minocha 1995).

We previously reported the recovery of fertile transgenic rice plants that constitutively express an oat *adc* cDNA driven by the CaMV 35S promoter (Capell et al. 1998). No significant increase in putrescine, spermidine or spermine levels could be detected in seeds produced from these plants. When the oat *adc* cDNA was introduced into rice under the control of the stronger maize ubiquitin 1 (Ubi-1) promoter, no significant variation in polyamine levels was observed in vegetative tissue or seeds (Bassie et al. 2000), with the exception of one specific transgenic line (Noury et al. 2000). This particular lineage showed very significant increases in putrescine levels in seeds (up to 10 times higher than those in the seeds of wild-type plants and controls transformed with the selectable marker alone), and a 1.5-fold increase in spermine (Noury et al. 2000).

In the present investigation we generated transgenic rice (*Oryza sativa* L.) lines containing a human *odc* cDNA driven by either the constitutive maize Ubi-1 promoter or the wheat seed-specific low-molecular-weight glutenin promoter (Lm). We observed a significant increase in putrescine, spermidine and spermine levels in rice seeds from transgenic lines expressing the transgene either constitutively or in a seed-specific manner. Results from experiments in which transgenic rice harbouring the oat *adc* cDNA (which produces putrescine through the alternative pathway) allowed us to draw conclusions concerning how these two alternative branches in the pathway determine levels of end-product accumulation. Our results demonstrate that the profile of polyamines in storage tissues such as seeds is determined by the ODC rather than the ADC branch of the pathway, and that this occurs in a tissue/organ-specific manner.

Materials and methods

Plasmids

The 1.8-kb human *odc* cDNA (Auvinen et al. 1992) was excised as an *Eco*RI fragment from the poly vector pLTR (Mäkelä et al. 1992), and subcloned in the *Eco*RI site of pAL76 (Christensen et al. 1992), which contains the maize Ubi-1 promoter, first intron and the *nos* terminator. This plasmid is referred to as pUbi*hodc*s. The same fragment was subcloned in the *Eco*RI site of a pUC19-based plasmid carrying *gusA* and a short version of the wheat low-molecular-weight glutenin promoter (Colot et al. 1987). This plasmid is referred to as pLm*hodc*s.

Recovery of transformed rice plants

Rice transformation, selection and regeneration procedures were as described previously (Sudhakar et al. 1998; Valdez et al. 1998). Rice tissue was co-transformed with the human *odc* cDNA and a plasmid containing the hygromycin phosphotransferase (*hpt*) selectable marker gene (Capell et al. 1998).

PCR and RT-PCR analyses

Genomic PCR amplifications were carried out in a total volume of 50 µl, containing 100 ng of genomic DNA, 1× Roche PCR buffer (50 mM KCl, 10 mM TRIS-HCl pH 9.0, 1.5 mM $MgCl_2$, 0.1% Triton X-100), 400 µM of each deoxynucleoside triphosphate, 100 nM of each primer (forward primer 5′-GGTTTACTGC-CAAGGACATTCTG-3′, Oligo 1; reverse primer 5′-CTCA-GATCCAGGAAAGCCACCGCC-3′, Oligo 3) and 2.5 U of *Taq* DNA polymerase (Roche). We carried out 35 cycles of denaturation (96°C, 40 s), annealing (60°C, 30 s) and extension (72°C, 2 min). The 921-bp product was visualized on a 1% TAE agarose gel.

Total RNA was extracted from plant tissue using the RNeasy Plant Mini Kit (Qiagen). Aliquots (100 ng) of total RNA from leaf tissue, roots and seeds (20 days after pollination) were used in each RT-PCR. Reverse transcription was performed using the Access RT-PCR System (Promega). The resulting cDNA was amplified as described above, using the same primers and amplification conditions. The resulting product was visualized on a 1% TAE agarose gel.

Southern hybridization

DNA was isolated from plant tissue according to the procedure of Creissen and Mullineaux (1995). DNA was digested with *Eco*RI, fractionated by electrophoresis on a 1% agarose gel in 1×TAE (Sambrook et al. 1989) and transferred to a positively charged nitrocellulose membrane (Roche). Nucleic acids were fixed by baking at 80°C for 2 h. Filters were washed in 2×SSC for 30 min and subsequently pre-hybridized at 42°C for 1 h using the DIG-easy hybridization solution (Roche). The 1.8-kb *Eco*RI *odc* fragment from pUbi*hodc*s was labelled using the PCR DIG probe synthesis kit (Roche). 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 PCR buffer (50 mM KCl, 10 mM Tris-HCl pH 9.0, 0.1% Triton X-100); 2.5 U of *Taq* DNA polymerase (Roche), 0.1 mM each of the forward and reverse sequence primers (as above) and 100 ng of the 1.8-kb *Eco*RI *odc* fragment. 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 30 s, and extension at 72°C for 2 min. The 921-bp labeled probe was purified using the QIAquick Gel Extraction Kit (Qiagen) and denatured at 68°C for 10 min prior to use. Hybridization was performed at 42°C overnight. The membranes were washed twice for 5 min in 2×SSC, 0.1% SDS at room temperature, and then twice (15 min) in 0.5×SSC, 0.1% 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) and subsequently exposed to X-ray film (Fuji Photofilm) for 20 min at 37°C.

Determination of ODC and ADC activities

Leaf and root tissues were used for ODC and ADC activity measurements. Tissue was extracted in buffer (100 mM HEPES pH 7.5, 2 mM DTT and 1 mM EDTA) at a ratio of 300 mg/ml buffer. Polyvinylpyrrolidone (100 mg) was added during grinding. Following centrifugation at 12 000×*g* for 10 min, the supernatant was used directly for enzyme activity assays. Tissue was always processed immediately after harvest and all assays were performed using fresh extracts. Enzyme assays were carried out in 1.5-ml Eppendorff tubes. A 6-mm diameter filter paper disc impregnated with 50 µl of 2 N KOH and transfixed with a 3 cm needle was used to trap the $^{14}CO_2$ liberated during the reaction.

For ADC activity measurements the reaction mixture contained 20 µl of extraction buffer (pH 8.0), 160 µl of crude enzyme and 20 µl of the substrate mix [20 µl of L-(U-^{14}C)arginine (specific activity 297 mCi/mmol, Amersham International) diluted with 20 µl of non-radioactive arginine (500 mM) and 60 µl of distilled water] to give a final concentration of 10 mM arginine.

The reaction mixture used to determine ODC activity contained 20 µl of extraction buffer (pH 7.0), 160 µl of crude enzyme and 20 µl of the substrate mix [20 µl of DL-(1-^{14}C)ornithine (specific activity 55 mCi/mmol, Amersham International plc) diluted with 20 µl non-radioactive ornithine (2.5 M) and 60 µl of distilled water] to give a final concentration of 50 mM ornithine.

Assays were carried out at 37°C for 45 min. Then 200 µl of 10% (v/v) perchloric acid was added to stop the reaction. After a further 45-min incubation, the filter paper was placed in scintillation minivials with 2 ml of scintillation liquid (OptiPhase Hisafe II, Fisons Chemicals) and radioactivity was measured in a Wallac 1219 Rackbeta liquid scintillation counter. Enzyme activity was expressed as µmoles of $^{14}CO_2$ released per min per g fresh weight (fw).

Polyamine analysis

Crude extracts from leaves, roots and seeds were recovered, dansylated and fractionated by TLC (thin layer chromatography) as described by Capell et al. (1998). The dansyl-polyamine bands were identified on the basis of their R_f values after visualisation under UV light (312 nm) and by comparison with the dansylated polyamine standards. The image of the chromatogram was captured and analysed with Quantity One (Quantification Software; Bio-Rad). The relative amount of dansyl-polyamine in each sample was determined by calculating the integrated optical density of the bands compared to the integrated optical density of the appropriate dilution of the dansylated control samples. Results were expressed as nmol per g fw.

Statistical analysis

The data were analyzed by one-way analysis of variance followed by the t-test using the Residual Mean Square in the ANOVA as the estimate of variability.

Results

Generation of transgenic rice plants

Immature rice embryos (cv. M12) and mature seed-derived callus (cvs. Bengal and EYI 105) were co-bombarded as described previously with either pUbi*hodc*s or pLm*hodc*s and a plasmid containing *hpt* as a selectable marker (Capell et al. 1998). We analysed in detail fifteen independent transgenic rice lines.

For molecular analysis we used *hpt*-transformed lines as controls, since we have shown that the expression of the selectable marker *hpt* used in the selection process for the recovery of transgenic rice plants does not affect expression of the endogenous polyamine genes (O. Lepri et al., submitted). For biochemical analyses (enzyme activity and polyamine content) we used *hpt*-transformed plants in addition to wild-type controls (average of three samples from six independent lines each; n = 36). Hygromycin-resistant transformants and wild-type controls were not significantly different (at the 5% level) in terms of enzyme activity and polyamine levels.

Molecular characterization of transgenic lines

We carried out PCR analysis of all transgenic rice plants that were resistant to the selectable marker (hygromycin) to confirm the presence of the human *odc* cDNA in all independently derived transformants. The expected PCR product was a 921-bp fragment derived from the *odc* coding region. To confirm stable integration of the human *odc* cDNA in transgenic rice, genomic DNA was digested with *Eco*RI, which releases a 1.8-kb fragment comprising the entire coding sequence of the *odc* cDNA. This confirmed that all rice lines contained the full-length human *odc* cDNA (Fig. 1A). Integration patterns for the transgene in primary transformants were maintained in R1 progeny. Figure 1B illustrates a representative example (clone 11; data from other lines were similar). Transgenes were passed on to R1 progeny in a Mendelian fashion.

RT-PCR analysis of rice lines transformed with *odc* driven by the constitutive Ubi-1 promoter confirmed expression of *odc* mRNA in leaf (Figs. 2A, 3A) and root (Fig. 4A) tissues. When RT-PCR analysis was performed on total RNA extracted from seeds 20 days after pollination, expression of *odc* mRNA was detected in lines transformed either with the Ubi-1 or the Lm promoters (Figs. 3A, 5A and C).

ODC and ADC enzyme activities in leaf and root tissues

In leaf tissue isolated from all three rice varieties transformed with pLmh*odc*s there was no significant variation between transformed plants and controls with respect to ODC activity. When ODC activity was measured in transgenic plants constitutively expressing *odc*, a significant increase was observed only in lines from the EYI genotype (Fig. 2B), which has lower endogenous levels of enzyme activity than other genotypes we have studied. Up to a 25-fold increase was detected in lines 2 and 48 (250 µmol $^{14}CO_2$ evolved per min per g fw; $P < 0.001$) when compared to controls (10 µmol $^{14}CO_2$

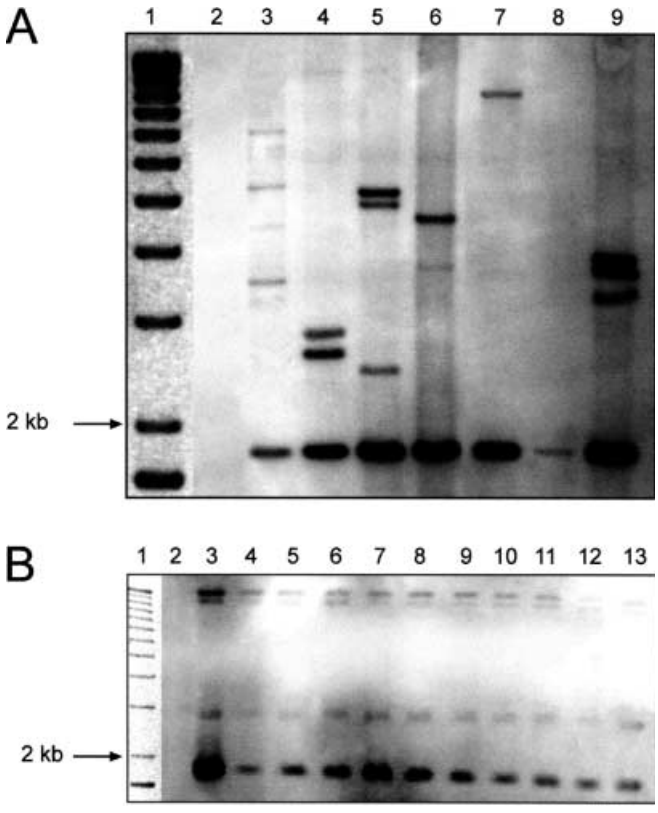


Fig. 1A, B Southern analysis of transgenic rice plants. Ten micrograms of genomic DNA was digested with *Eco*RI, which excises the full length of the *odc* cDNA from the transformation vector, and probed with a 921-bp DIG-labelled PCR product from pUbi*hodc*s. Exposure time was 20 min. **A** Southern analysis of transgenic R0 plants. Lane 1: molecular size marker (1-kb DNA ladder, Gibco-BRL). Lane 2: *hpt*-transformed control. Lanes 3–9: representative transgenic clones. **B** Southern analysis of transgenic R1 plants from clone 11. Only positive lines are shown. Lane 1: molecular size marker (1-kb DNA ladder). Lane 2: *hpt*- transformed control. Lane 3: R0 clone 11. Lanes 4–13: individual R1 progeny derived from clone 11

per min per g fw). Root tissue from the EYI genotype harboring pUbi*hodc*s was analyzed to determine ODC activity. A nine-fold increase in activity was detected in clone 2 ($P < 0.001$) and a two-fold increase was detected in clone 48 ($P < 0.05$); however, no significant variation (at the 5% level) was detected in any of the other lines (Fig. 4B).

No significant variation in ADC activity was detected in leaf or root tissues in any of the lines (results not shown).

Analysis of polyamines in leaf tissue

Polyamine levels were analysed in transgenic plants simultaneously with ADC and ODC enzyme activity measurements. Putrescine, spermidine, and spermine levels were measured in vegetative tissue from fifteen independent transgenic rice plants. As expected there was no significant difference between transformed lines and controls for any of the individual polyamines in rice leaf tissue harbouring the pLm*hodc*s when compared to appropriate controls. When polyamine levels were measured in transformed plants in which *odc* was driven by the Ubi-1 promoter (Figs. 2C and 3B) a 1.5- to 2-fold increase in putrescine content was detected in clones 2 (1600 nmol/g fw; $P < 0.01$; Fig. 2C) and 48 (1100 nmol/g fw; $P < 0.05$; Fig. 2C) compared to control levels (760 nmol/g fw). A significant increase in spermidine (480 nmol/g fw; $P < 0.05$; Fig. 2C) and spermine levels (234 nmol/g fw; $P < 0.05$: Fig. 2C) was detected in clone 2, compared to controls (spermidine, 220 and spermine, 160 nmol/g fw). No significant variation ($P > 0.05$) was detected in clone 48 for spermidine and spermine levels.

Polyamine analysis in seeds

Putrescine levels in transgenic rice seeds were significantly higher than controls, irrespective of whether *odc* expression was controlled by the Ubi-1 or the Lm promoter. A three-fold increase in putrescine levels was observed in clone 21 (M12 genotype; 151 nmol/g fw, compared to 50 nmol/g fw in the control; $P < 0.05$; Fig. 5B). Clones 11 (Fig. 5D) and 40 (Fig. 3C) exhibited a three-fold increase (113 nmol/g fw; $P < 0.05$), while clone 55 showed a 3.6-fold (134.5 nmol/g fw; $P < 0.05$; Fig. 5D) increase in putrescine levels, compared to the controls (Bengal genotype, 37 nmol/g fw). In transgenic EYI 105 plants all lines showed a significant increase in putrescine levels. The highest putrescine level was measured in clone 2 (700 nmol/g fw; $P < 0.001$; Fig. 5B). Putrescine levels in the corresponding controls were 160 nmol/g fw.

Spermidine levels were significantly higher in clones 11 ($P < 0.05$), 40 ($P < 0.05$) and 55 ($P < 0.01$) compared to control values. These ranged from three- to seven-fold higher (154–331 nmol/g fw) compared to the equivalent Bengal control (50 nmol/g fw; Figs. 5D and 3C). Spermidine levels in clone 1 were 1.5-fold higher (360 nmol/g fw; $P < 0.01$; Fig. 5B) compared to the equivalent control (EYI 105; 230 nmol/g fw; Fig. 5B).

Significant increases in spermine levels (1.5-fold) were detected in clone 11 (200 nmol/g fw; $P < 0.05$) compared to the control (Bengal, 115 nmol/g fw; Fig. 5D) and also clone 1 (375 nmol/g fw; $P < 0.05$; Fig. 5B) compared to the appropriate control (EYI 105; 200 nmol/g fw; Fig. 5B).

Polyamine analysis in root tissue

Polyamine levels were analyzed in root tissue from Bengal and EYI 105 plants transformed with *odc* driven by the Ubi-1 promoter. A two-fold increase in putrescine levels was detected in clones 77 (330 nmol/g fw; $P < 0.05$; Fig. 3D) and 48 (450 nmol/g fw; $P < 0.01$; Fig. 4C) when compared to controls (150 nmol/g fw; Fig. 3D;

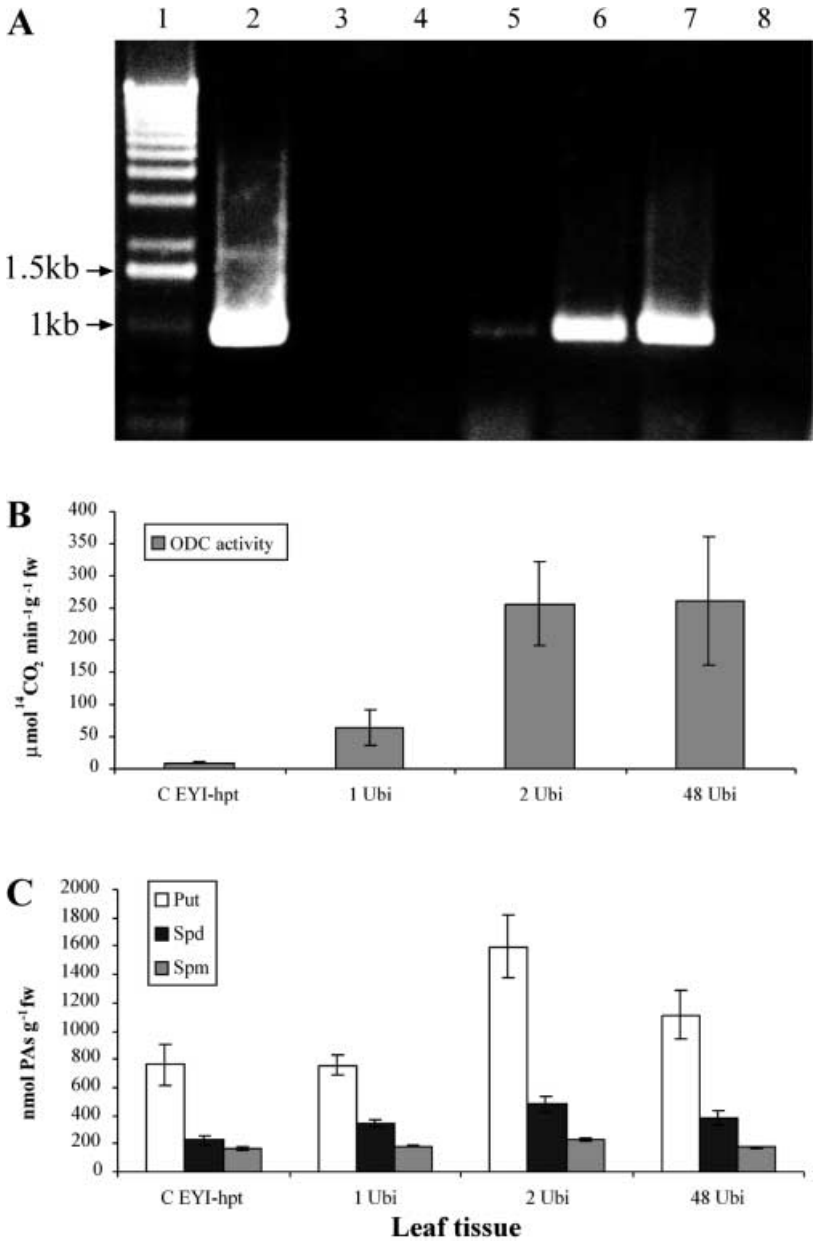


Fig. 2A–C Analysis of leaf tissue of EYI plants transformed with pUbi*hodc*s. For enzyme activity and polyamine content, control values (C EYI-*hpt*) are mean ± SE for three replicates from six wild-type and six *hpt*-transformed plants (n = 36). The values for transgenic lines are mean ± SE (n = 3). **A** RT-PCR analysis of total mRNA. Lane 1: molecular size marker (1-kb DNA ladder). Lane 2: pUbi*hodc*s positive control (plasmid). Lane 3: water control. Lane 4: *hpt*-transformant control. Lanes 5–7: transgenic lines 1 Ubi, 2 Ubi and 48 Ubi. Lane 8: 48 Ubi without reverse transcriptase. **B** ODC activity was significantly different from control at $P < 0.05$ for clone 1 and at $P < 0.001$ for clones 2 and 48. **C** Putrescine values were significantly different from control at $P < 0.01$ for clone 2 and $P < 0.05$ for clone 48. Spermidine and spermine values were significantly different from control at $P < 0.05$ for clone 2. The remaining values were not significantly different from controls ($P > 0.05$)

260 nmol/g fw; Fig. 4C respectively) but no significant variation ($P > 0.05$) was detected in the remaining lines (Fig. 3D). A 1.5-fold increase in spermidine and spermine levels were detected in clone 77 (340 nmol/g fw; $P < 0.05$; and 100 nmol/g fw; $P < 0.01$; Fig. 3D) when compared to respective controls (240 nmol/g fw; and 60 nmol/g fw; Fig. 3D). No significant variation ($P > 0.05$) in spermidine or spermine levels was detected in any of the remaining lines (Fig. 3D) or in the EYI 105 genotype (Fig. 4C).

Polyamine levels in R2 seeds

We studied in detail the two clones (1 and 11) that exhibited the most dramatic changes in all polyamine levels. R2 seeds for both clones showed identical polyamine levels to those measured in the R1 generation (Fig. 5D).

Discussion

The polyamine biosynthetic pathway has been the subject of intensive study for several decades (Smith 1985; Pegg 1986). Polyamines are ubiquitous low-molecular-weight polycationic compounds that are found in all living organisms and are involved in crucial developmental, physiological and metabolic processes (Tiburcio et al. 1997; Kumar and Minocha 1998; Malmberg et al. 1998). In mammalian systems, ornithine is converted into putrescine by the action of ODC. Putrescine is

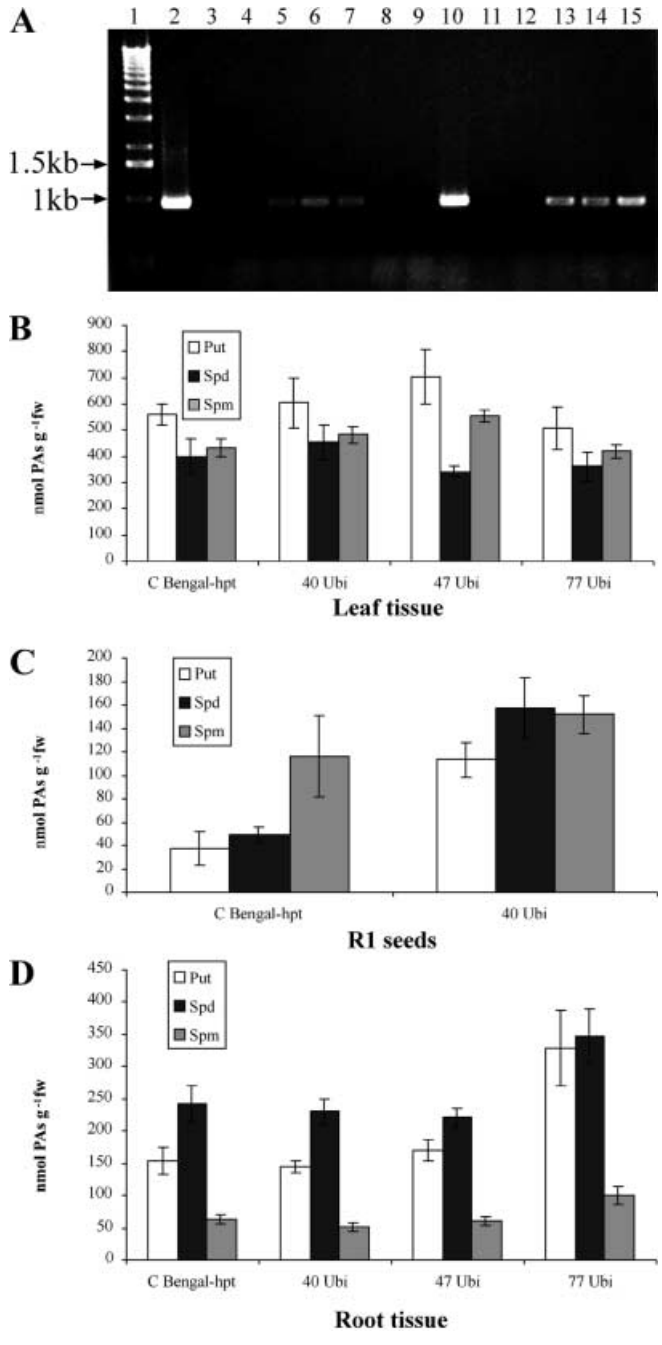


Fig. 3A–D Analysis of various tissues from Bengal plants transformed with pUbi*hodc*s. For enzyme activity and polyamine content, control values (C Bengal-*hpt*) are mean ± SE for three replicates from six wild-type and six *hpt*-transformed plants (n = 36). Values for transgenic lines are mean ± SE (n = 3). **A** RT-PCR analysis of total mRNA extracted from leaves, seeds (20 days after pollination) and roots. Lane 1: molecular size marker (1-kb DNA ladder). Lane 2: pUbi*hodc*s positive control. Lane 3: water control. Lane 4: *hpt*-transformed control leaf tissue. Lanes 5–7: transgenic lines 40 Ubi, 47 Ubi and 77 Ubi (leaf tissue). Lane 8: 77 Ubi without reverse transcriptase. Lane 9: *hpt*-transformed control (seed). Lane 10: 40 Ubi seed. Lane 11: 40 Ubi seed without reverse transcriptase. Lane 12: *hpt*-transformed control (root tissue). Lanes 13–15: transgenic lines 40 Ubi, 47 Ubi and 77 Ubi (root tissue). **B** Cellular polyamine content in leaf tissue. None of the values of these lines was significantly different from control ($P > 0.05$). **C** Cellular polyamine content in seeds. Putrescine and spermidine levels were significantly different from controls at $P < 0.05$ for line 40. Spermine was not significantly different from control ($P > 0.05$). **D** Cellular polyamine content in root tissue. Line 77 had putrescine and spermidine levels that were significantly different from control at $P < 0.05$ and spermine levels were significantly different from control at $P < 0.01$. Putrescine, spermidine and spermine values from the remaining lines were not significantly different from control ($P > 0.05$)

converted into the higher polyamines by the sequential action of two different aminopropyltransferases, spermidine synthase and spermine synthase. The source of these propylamine groups is dcSAM, which is produced by the action of SAMDC on SAM. The second component of the aminopropyltransferase reactions is 5′-methylthioadenosine (Pegg 1986). Polyamine biosynthesis in plants and bacteria differs from that in mammals because the pathway is ramified at an early step. Two alternative enzymes, ODC and ADC, may lead to putrescine formation in plants and bacteria. The ODC reaction converts ornithine to putrescine directly, whereas ADC decarboxylates arginine to agmatine, which is subsequently converted to N-carbamoylputrescine by agmatine iminohydrolase. N-carbamoylputrescine amidohydrolase converts this product to putrescine (Malmberg et al. 1998). Plants provide an interesting eukaryotic system for investigating the physiological and biochemical role of the two alternative branches of the pathway leading to putrescine. A fundamental question in this regard is why plants and bacteria show such potential redundancy in the pathway.

We have previously demonstrated that by introducing a heterologous *adc* cDNA into transgenic rice, it was possible to increase the putrescine pool in seeds but not in leaves or roots (Noury et al. 2000). Small increases in spermine were also detected in seeds. In the current study, we generated transgenic rice plants containing the human *odc* cDNA driven by either a constitutive or a seed-specific promoter. Having access to transgenic germplasm overexpressing ADC or ODC is essential in order to carry out the detailed biochemical measurements required to elucidate the relative contribution of each of the two branches to the main pathway. Molecular analysis confirmed stable integration of the transgene (Fig. 1A), and Mendelian inheritance in progeny (Fig. 1B). Constitutive expression of the heterologous *odc* cDNA resulted in mRNA expression in leaf (Figs. 2A and 3A) and root tissues (Figs. 3A and 4A). This indicated that the human transgene was transcribed efficiently in rice. Expression of human genes in plants is of course now becoming routine. Thus, the human gene for cytochrome P450 2E1 had previously been expressed in tobacco plants. These plants were able to metabolize trichloroethylene (a highly toxic halogenated hydrocarbon) much more efficiently (up to 640-fold) than wild-type plants (Doty et al. 2000). Frese et al. (2000) investigated the role of the interferon-induced human MxA protein in resistance against a range of RNA viruses, using transgenic tobacco plants.

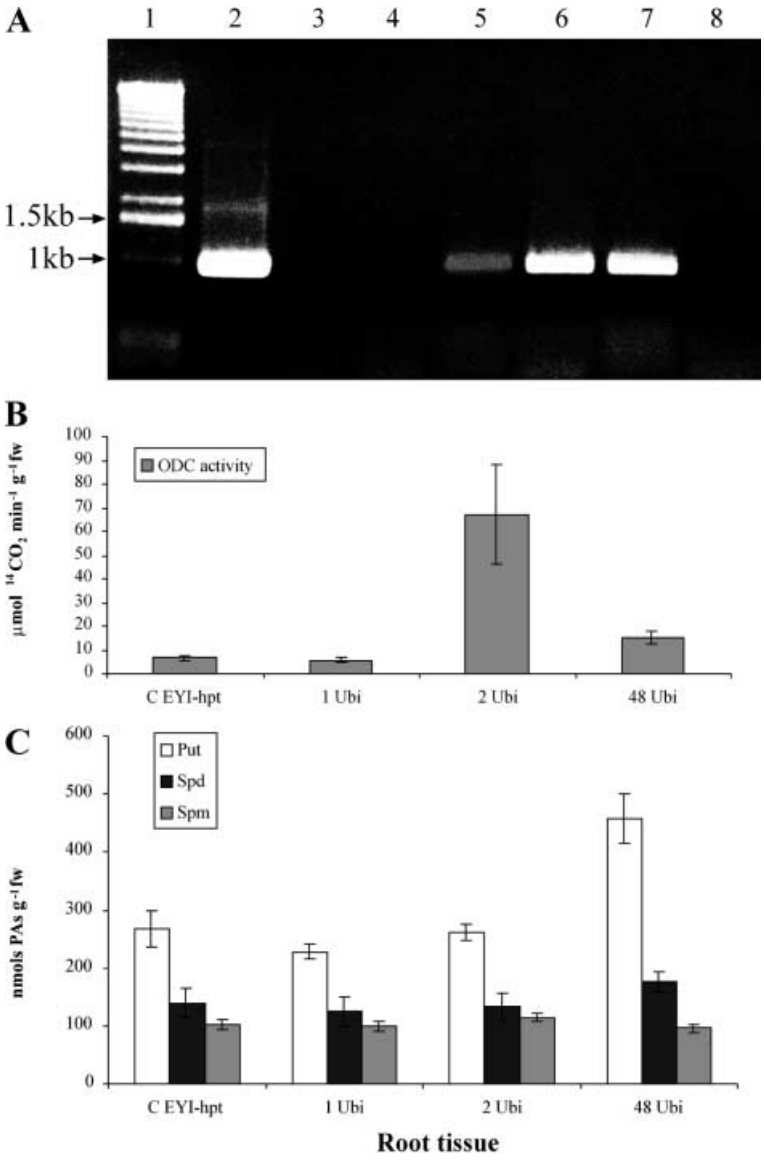
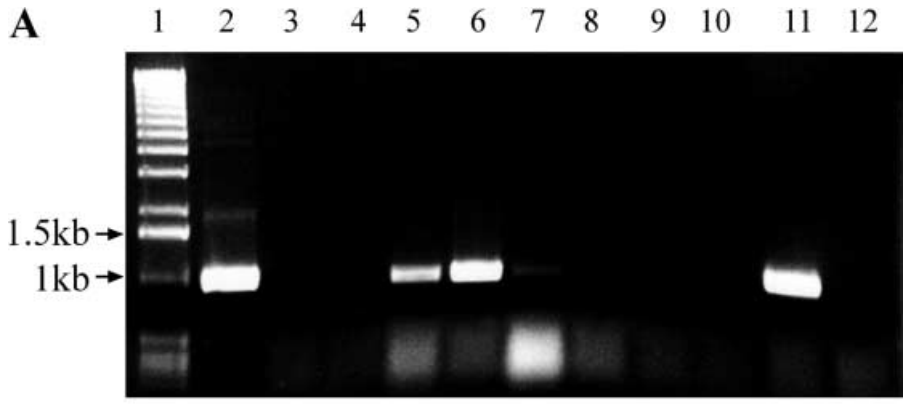


Fig. 4A–C Analysis of root tissue from EYI plants transformed with pUbi*hodc*s. For enzyme activity and polyamine content, control values (C EYI-*hpt*) are mean ± SE for three replicates from six wild-type and six *hpt*-transformed plants (n = 36). Values for transgenic lines are mean ± SE (n = 3). **A** RT-PCR analysis of total mRNA. Lane 1: molecular size marker (1-kb DNA ladder). Lane 2: pUbi*hodc*s positive control. Lane 3: water control. Lane 4: *hpt*-transformed control. Lanes 5–7: transgenic lines 1 Ubi, 2 Ubi and 48 Ubi. Lane 8: 48 Ubi without reverse transcriptase. **B** ODC activity in root tissue was significantly different from control at $P < 0.05$ for line 48 and at $P < 0.01$ for line 2. Line 1 was not significantly different from controls ($P > 0.05$). **C** Putrescine levels in clone 48 were significantly different from control at $P < 0.01$. Remaining putrescine, spermidine and spermine values were not significantly different from control ($P > 0.05$)

Constitutive over-expression of the human *odc* cDNA resulted in significant increases in ODC activity in leaf and root tissues (clones 2 and 48; Figs. 2B and 4B). This increase in enzyme activity in turn resulted in substantial increases in putrescine levels in leaf (Fig. 2C) and root tissues (Fig. 4C). Line 2 also exhibited a small but significant increase in spermidine and spermine levels in leaves (clone 2; Fig. 2C) and line 77 exhibited significant increases in spermidine and spermine levels in root tissues (Fig. 3D). 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 (DeScenzo and Minocha 1993). Hamill et al. (1990) reported a two-fold increase in putrescine levels in the transgenic roots of *N. rustica* over-expressing a yeast *odc* cDNA, but no variations in spermidine or spermine levels were detected.

When a heterologous *adc* was previously expressed in rice (Noury et al. 2000) or tobacco (Burtin and Michael 1997) 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 (Masgrau et al. 1997). This body of literature suggests strongly that *odc* rather than *adc* influences putrescine biosynthesis in plants. Morris and Pardee (1965) rationalized the existence of the two alternative pathways for putrescine biosynthesis in bacteria by addressing the regulatory problem encountered in branched biosynthetic pathways. The pathway for arginine biosynthesis in *E. coli* K-12 is branched, the branch point occurring at ornithine. The most economical route for putrescine synthesis, in terms of energy use, is the direct decarboxylation of ornithine. Interestingly, when repressible strains of *E. coli* are grown in the presence of arginine, the synthesis of the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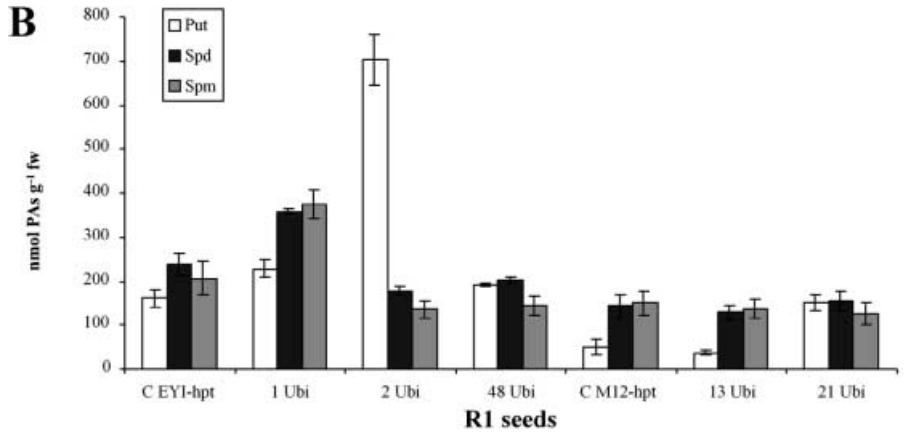
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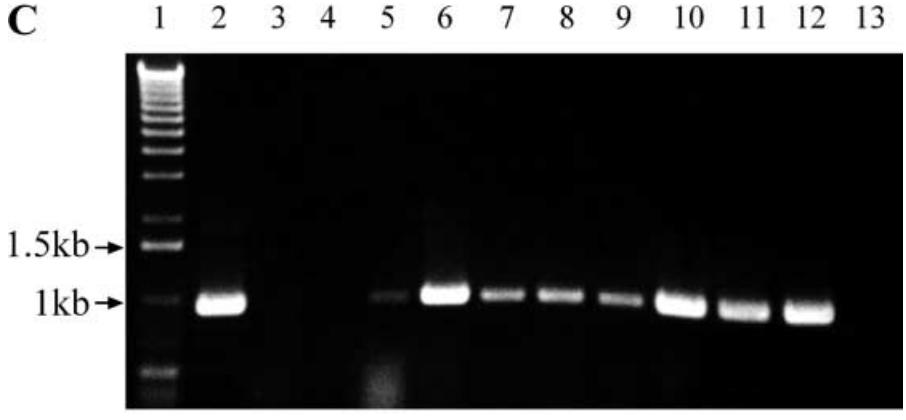
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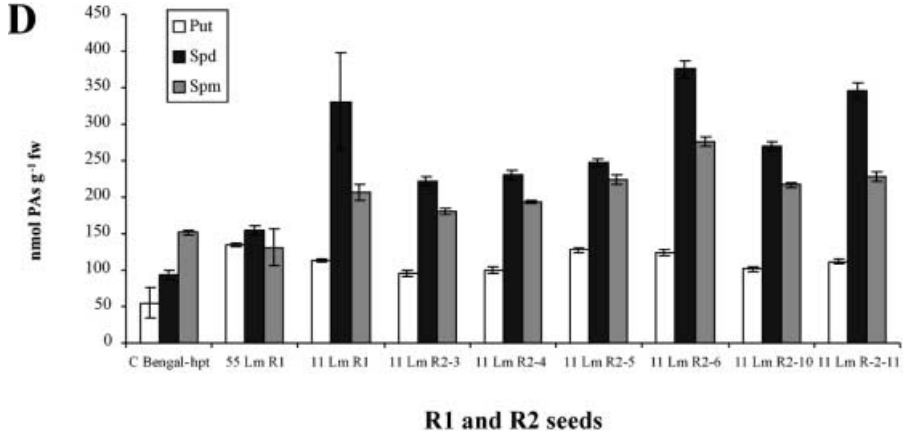
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R1 and R2 seeds

Fig. 5A–D Analysis of rice seeds from EYI 105, M12 and Bengal genotypes. **A** RT-PCR analysis of total mRNA extracted from seeds 20 days after pollination from plants transformed with pUbi*hodc*s. Lane 1: molecular size marker (1-kb DNA ladder). Lane 2: pUbi*hodc*s positive control. Lane 3: water control. Lane 4: *hpt*-transformed EYI control. Lanes 5–7: transgenic lines 1 Ubi, 2 Ubi and 48 Ubi. Lane 8: 48 Ubi without reverse transcriptase. Lane 9: *hpt*-transformed M12 control. Lanes 10 and 11: 13 Ubi and 21 Ubi. Lane 12: 21 Ubi without reverse transcriptase. **B** Cellular polyamine levels in seeds of plants transformed with pUbi*hodc*s. Putrescine levels were significantly different from control at $P < 0.05$ for line 21 (M12 variety) and at $P < 0.001$ for line 2 (EYI variety). Spermidine levels were significantly different from control at $P < 0.01$ and spermine levels were significantly different from control at $P < 0.05$ for line 1. Remaining putrescine, spermidine and spermine values were not significantly different from control ($P > 0.05$). Values are mean ± SE for three replicates from six wild-type and six *hpt*-transformed plants (n = 36). Values for transgenic lines are means ± SE (n = 3). **C** RT-PCR analysis of total mRNA extracted from seeds of plants transformed with pLm*hodc*s. Lane 1: molecular size marker (1-kb DNA ladder, GIBCO BRL, UK). Lane 2: pUbi*hodc*s positive control. Lane 3: water control. Lane 4: *hpt*-transformed Bengal control. Lanes 5–12: 55 Lm R1, 11 Lm R1, 11 Lm R2–3, 11 Lm R2-4, 11 Lm R2-5, 11 Lm R2-6, 11 Lm R2-10, 11 Lm R2-11. Lane 13: 11 Lm R2-11 without reverse transcriptase. **D** Cellular polyamine levels in rice seeds from plants containing the pLm*hodc*s. Putrescine values were significantly different from control at $P < 0.05$ in lines 11 R1, all 11 R2 and 55 R1. Spermidine values were significantly different from control at $P < 0.05$ for line 11 R1, all 11 R2 and at $P < 0.01$ for line 55 R1. Spermine levels were significantly different from control at $P < 0.05$ for line 11 R1 and all 11 R2. The spermine value from line 55 R1 was not significantly different from control ($P > 0.05$). Control values are mean ± SE for three replicates from six wild-type and six *hpt*-transformed plants (n = 36). Values for transgenic lines are mean ± SE (n = 3)

enzymes for arginine biosynthesis is inhibited. Since the formation of ornithine is blocked during growth on arginine, the organism can no longer synthesize putrescine from this source, but uses the now energetically favorable route from preformed arginine. Under these conditions, the conversion of arginine to putrescine is the only route for polyamine biosynthesis. This provides a reasonable explanation for the existence of the two pathways of putrescine biosynthesis. A similar situation may occur in plants; however, the existence of the alternative pathway to putrescine in plants and bacteria may reflect the evolutionary divergence between these taxa and mammals.

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 tissues such as seeds, polyamine levels were significantly higher in six of the ten lines we analyzed (Fig. 3C, and Figs. 5B and D). All lines showed significant increases in putrescine levels. Two lines also had increased levels of spermidine (clone 55, Fig. 5D, and clone 40, Fig. 3C). The levels of all three polyamines were increased significantly in two lines (clone 11, Fig. 5D, and clone 1, Fig. 5B). 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 (Stöger et al. 1999).

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 to be the enzyme predominantly responsible for regulating the formation of putrescine in plants. It has been suggested that the polyamine pathway in plants is so rigidly controlled that an alteration in polyamine levels cannot be achieved by over-expressing key enzymes in the pathway (Burtin and Michael 1997). 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 most 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.

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