Theor Appl Genet (2002) 105:594–603
DOI 10.1007/s00122-002-0922-4

O. Lepri · L. Bassie · P. Thu-Hang · P. Christou
T. Capell

Endogenous enzyme activities and polyamine levels in diverse rice cultivars depend on the genetic background and are not affected by the presence of the hygromycin phosphotransferase selectable marker

Received: 9 July 2001 / Accepted: 6 August 2001 / Published online: 17 July 2002
© Springer-Verlag 2002

Abstract We used the polyamine biosynthetic pathway and rice as a relevant model to understand the genetic basis of variation in endogenous levels of metabolites and key enzymes involved in the pathway. Wild-type tissues and also tissues containing a commonly used selectable marker gene were employed. We detected a wide variation in levels of arginine decarboxylase activity and in the three polyamines, putrescine, spermidine and spermine, in different tissues and varieties, but this was not dependent on the presence of the selectable marker. A more-extensive profile of enzyme activities (ADC, ODC, SAMDC, DAO and PAO) and polyamine levels in different tissues was generated in two different varieties. Our results indicate that genetic background is important in terms of the basal levels of metabolites and enzyme activity, particularly in situations in which we aim to engineer metabolic pathways that are also encoded by homologous endogenous genes. We did not find any evidence that the presence of a selectable marker in any way influences enzyme activity or metabolite levels.

Keywords Putrescine · Spermidine · Spermine · Hygromycin resistance · *Oryza sativa*

Introduction

Engineering value-added traits and ascertaining the effects of genetic manipulation in transgenic plants frequently require the accurate determination of the basal levels of enzyme activities and corresponding metabolites. Such measurements need to be carried out in diverse germplasm to ensure the general applicability of results obtained by manipulating specific cultivars (Christou 1994). Furthermore, it is important to evaluate the effects of selectable markers accompanying introduced transgenes of interest into plants, in order to establish unequivocally that observed changes in biochemical and physiological parameters are solely a result of expressing particular genes of interest (Christou 1994). A sound statistical treatment needs to accompany such an analysis. These measurements are extremely important in the manipulation of metabolic pathways.

The antibiotic hygromycin B in conjunction with the *hygromycin phosphotransferase* (*hpt*) gene is the most-widely used selectable marker system in rice transformation (Tyagi and Mohanty 2000). Many different genes encoding mono- or multi-genic traits have been introduced into plants such as rice in conjunction with the *hpt* gene. These include traits for pest and disease resistance (Maqbool et al. 1998; Vain et al. 1998; Zhang et al. 1998; Maqbool and Christou 1999), resistances to abiotic stresses (Xu et al. 1996; Cheng and Wu 1997; Tanaka et al. 1999), value-added traits such as pharmaceutical antibodies (Torres et al. 1999, Stöger et al. 2000), micronutrient enhancement (Gotto et al. 1999; Drakakaki et al. 2000; Lucca et al. 2001) and metabolic pathways (Ye et al. 2000).

A major focus in our laboratory is the study of metabolic pathways using transgenic tools. We have employed the polyamine pathway as a model to understand how metabolic networks are controlled and regulated since the pathway is ubiquitous in plants (Malmberg et al. 1998). The simplest polyamine, putrescine, is derived from ornithine by ornithine decarboxylase (ODC, EC 4.1.1.19). Putrescine may then be converted into the longer aliphatic polyamines spermidine and spermine by spermidine and spermine synthases, respectively (EC 2.5.1.16 and EC 2.5.1.22), which add propylamino groups generated from S-adenosylmethionine (SAM) by SAMdecarboxylase (SAMDC; EC 4.1.1.50; Malmberg et al. 1998). In plants, putrescine may be synthesised from either ornithine or arginine, the former through the ODC pathway as found in animals, and the latter through the alternative arginine decarboxylase (ADC, 4.1.1.17) path-

Communicated by G. Wenzel

O. Lepri · L. Bassie · P. Thu-Hang · P. Christou · T. Capell (✉)
Molecular Biotechnology Unit, B35, John Innes Centre,
Norwich Research Park, Colney Lane, Norwich, NR4 7UH,
United Kingdom
e-mail: Teresa.Capell@ime.fraunhofer.de
Tel.: +49-2972-302304, Fax: +49-2972-302328

way, involving two intermediates, agmatine and N-carbamoylputrescine (Malmberg et al. 1998). Polyamines are oxidatively deaminated by the action of amine oxidases. Amine oxidases include the copper diamine oxidase (DAO, EC 1.4.3.6); these enzymes are characterized by their substrate specificity towards diamines. The flavoprotein polyamine oxidases (PAO, EC 1.5.3.3), oxidise spermidine and spermine at their secondary amino groups (Tiburcio et al. 1997).

The cellular content of soluble polyamines in plants is regulated by their biosynthesis, conjugation with phenolics acids, binding to cellular macromolecules, conversion into secondary metabolites (at least in some plants), and their degradation by di- and poly-amine oxidases (Smith 1985). Endogenous enzymes and polyamine levels vary extensively in different plant species (Matilla 1996), and also within a species for different varieties or cultivars (Bonneau et al. 1994). Consequently, it is essential to establish an accurate basal level of enzyme activity and metabolites, particularly if one is to use exogenous transgenes to modulate gene expression. The consequence of the presence of selectable marker genes, required for plant transformation, needs also to be evaluated to confirm that such moieties do not have any direct or indirect effect on enzyme activities and levels of polyamines.

The objective of the present study was to investigate variation of enzyme activities and metabolites in the polyamine pathway in different rice varieties, and also to ascertain whether plants engineered with the *hpt* selectable marker alone exhibited any different characteristics from wild-type plants in terms of enzyme activity and polyamine levels. Furthermore, we wished to establish basal levels of polyamines in a wide range of tissues in a particular rice cultivar in order to investigate spatial differences in end-product accumulation. This information is invaluable if we are to make general statements in terms of the effects of introduced transgenes in plants accompanied by selectable markers, such as the *hpt* gene, in a range of diverse cultivars.

Materials and methods

Transformation vector

The 1.341-kb *hpt* cDNA *Hin*dIII/*Sma*I fragment (Van den Elzen et al. 1985) was inserted between the 35SCaMV promoter (Gardner et al. 1981) and the *nos* transcription terminator site (Depicker et al. 1982) in a pUC19 backbone (Yanisch-Perron et al. 1985). The plasmid was linearized by digestion with *Kpn*I, and was named p35S*hpt*.

Transformation, callus induction and recovery of wild-type and transgenic plants

Rice varieties used in this particular study were: M12, ITA 212, ISDA 6, IR64, Bengal and EYI 105. Immature rice embryos (cvs M12, ITA 212, ISDA6 and IR64) and mature seed-derived callus (cvs Bengal and EYI 105) were bombarded as described previously with p35S*hpt* or with naked gold (Capell et al. 1998; Sudhakar et al. 1998; Valdez et al. 1998). Callus induction and plant regeneration was performed as described elsewhere (Capell et al. 1998; Sudhakar et al. 1998; Valdez et al. 1998).

Molecular characterisation of transgenic rice callus, and plants containing the hpt selectable marker

DNA was isolated from callus and plant tissues according to the procedure of Creissen and Mullineaux (1995). Genomic PCR amplification from callus to detect *hpt* cDNA was carried out in a total volume of 50 µl, comprising 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′-ACTCACCGCGACGTCTGTCG-3′, Oligo 1; reverse primer, 5′-GATTCTCCAATCTGCGGGATC-3′, Oligo 2) and 0.5 units of *Taq* DNA polymerase (Roche UK). After an initial denaturation step for 2 min at 96 °C, 35 amplification cycles were carried out, each comprising denaturation at 96 °C for 40 s, annealing at 60 °C for 30 s and extension at 72 °C for 2 min. The forward sequence primer started from position 221 in the *hpt* cDNA open reading frame. The 1.1-kb final product was visualized on a 1% TAE agarose gel.

DNA from leaf tissue was digested with *Kpn*I, fractionated by 1% TAE agarose-gel electrophoresis (Sambrook et al. 1989) and transferred to a positively charged nitrocellulose membrane (Roche, UK). 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, UK). The 1.3-kb *Hin*dIII/*Sma*I *hpt* fragment from p35S*hpt* was labeled using the PCR DIG probe synthesis kit (Roche, UK). Alkali-labile DIG-11-dUTP was incorporated into the probe in a final volume of 50 µl comprising 4 µM of dATP, 4 µM of dCTP, 4 µM of dGTP, 3.2 µM of dTTP, 0.8 µM of DIG-11-dUTP, 1 × Roche PCR buffer (50 mM KCl, 10 mM Tris-HCl pH 9.0, 0.1% Triton X-100); 2.5 units of *Taq* DNA polymerase (Roche, UK), 0.1 mM each of the forward and reverse sequence primers (as PCR above) and 100 ng of the 1.3-kb *Hin*dIII/*Sma*I *hpt* 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 10 s, and extension at 72 °C for 1 min. The 1.1-kb labeled probe was purified using the QIAquick Gel Extraction Kit (Qiagen, UK) 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, UK) and subsequently exposed to X-ray film (Fuji Photofilm Co., Ltd, Kanawa, Japan) for 20 min at 37 °C.

Total RNA was extracted from the callus using the RNeasy Plant Mini Kit (Qiagen). Denatured RNA (30 µg) was subjected to electrophoresis on a 1.2% agarose-formaldehyde gel using 1 × MOPS buffer (Sambrook et al. 1989). The primer sequences for the rice *samdc* cDNA probe were as follows: forward primer 5′-GGAGATCCAGCAAAGCCTGGCC-3′ (rsamdc 1) and reverse primer, 5′-CCCAGGGGAGAAGATTGCCCAG-3′ (rsamdc 2). Rice *samdc* cDNA was amplified for 35 cycles: denaturation (94 °C, 40 s), annealing (65 °C, 30 s) and extension (72 °C, 2 min). The 0.7-kb rice *samdc* probe was purified as described earlier. 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.

Arginine decarboxylase, ODC and SAMDC activity measurements

Callus (samples collected as described in Bassie et al. 2000), leaves (from 3 month-old plants) and roots (also from 3-month-old plants) were used for ADC, ODC and SAMDC activity measurements. Tissue was extracted in buffer (1 M Tris pH 7.5, 2 mM DTT and 1 mM EDTA) at a ratio of 300 mg ml^{-1} of buffer. Polyvinylpyrrolidone (100 mg) was added during grinding. Following centrifugation at 12,000 *g* for 20 min, the supernatant was used directly in 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 2N KOH and transfixed with a 3-cm needle was used to trap the $^{14}CO_2$ liberated during the reaction. The reaction mixture for ADC activity contained 20 ml 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^{-1}, Amersham International plc, UK) diluted with 20 µl of non-radioactive arginine (500 mM) and 60 µl of distilled water] to give a final concentration of 10 mM of arginine. The reaction mixture to determine ODC activity contained 20 µl of extraction buffer (pH 8.0), 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^{-1}, Amersham International plc, UK) diluted with 20 µl of non-radioactive ornithine (2.5 M) and 60 µl of distilled water] to give a final concentration of 50 mM of ornithine. The reaction mixture to determine SAMDC activity contains 20 µl of extraction buffer (pH 7.5), 160 µl of crude enzyme and 20 µl of the substrate, 200 µl of S-adenosylcarboxy[^{14}C]methionine (specific activity 62 mCi mmol^{-1}, Amersham International plc, UK; radioactive concentration 925 kBq/ml) diluted with 200 µl of non-radioactive SAM (2.5 M) and 600 µl of distilled water to give a final concentration of 5 mM of SAM. Assays were carried out at 37 °C for 45 min. Two hundred microliters of 10% (v/v) perchloric acid were 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, UK) and radioactivity was measured in a Wallac 1219 Rackbeta liquid scintillation counter. One nKat of ADC, ODC and SAMDC activity was defined as the amount (µmol) of $^{14}CO_2$ released per min and per g fresh weight (fw).

Determination of DAO and PAO activities

For DAO and PAO enzyme activity measurements, leaves (from 3-month-old plants) were extracted in buffer (0.1 M K-phosphate buffer pH 7.5 and 2 mM DTT) at a ratio of 1 g per 3 ml of buffer. Polyvinylpyrrolidone (100 mg) was added during grinding. Following centrifugation at 27,000 *g* for 20 min, the supernatant was used directly in enzyme activity assays.

For DAO activity measurements the reaction mixture contained 250 µl of crude enzyme, 12 µl of 0.1 M putrescine and up to 1 ml of 0.1 M K-phosphate buffer (pH 7.5). After incubation at 37 °C for 2 h, the reaction was stopped by adding 0.5 ml of 10% (w/v) trichloroacetic acid followed by 25 µl (10 mg ml^{-1}) of o-aminobenzaldehyde in ethanol (Hausman et al. 1997). Absorbance at 430 nm was measured after removal of proteins by centrifugation ($E_0 = 1.86 \times 10^3$ mol^{-1} cm^{-1}; Federico et al. 1985). Enzyme activity was expressed as nKat g^{-1} fw.

Polyamine oxidase measurements were performed by modification of the method described by Storer and Ferrante (1989). Tris 0.5 M (pH 7.5) and horseradish peroxidase (1 mg ml^{-1}; SIGMA) made up to 700 µl with MilliQ water, were added to 300 µl of extract and incubated at 37 °C for 10 min. Five microliters of homovanillic acid (1 mg ml^{-1}; SIGMA) and 10 µl of a mixture of spermidine/spermine, 0.5 M each, made up to 150 µl with MilliQ water were subsequently added. After 30-min incubation at 37 °C the reaction was stopped by adding 100 µl of 1N NaOH. The amount of hydrogen peroxide released was measured in a Kontron SFM 25 spectrofluorophotometer at an excitation wavelength of 350 nm and an emission wavelength of 495 nm (Storer and Ferrante 1989). Enzyme activity was expressed as nKat g^{-1} fw.

Polyamine analysis

Crude extracts from callus (samples collected as described in Bassie et al. 2000), leaves (from 1- and 3-month-old plants), roots (from 1- and 3-month-old plants), husk, immature embryos (20 days after pollination), immature endosperm (20 days after pollination) and dry seeds were recovered, dansylated and run on thin layer chromatography plates as described (Capell et al. 1998). The dansyl-polyamine bands were identified on the basis of their Rf values after visualization under UV light (312 nm) and comparison with dansylated polyamine standards. The image of the chromatogram was captured and analysed by Quantity One (Quantification Software; Bio-Rad). The relative amount of dansyl-polyamines 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 g^{-1} fw.

Statistical analysis

All measurements were based on three replicate samples from six independent control plants (wild-type, $n = 18$) and six *hpt*-transformed transgenic plants ($n = 18$) for each genotype. Sampling was carried out by the same individual on the same day. The data were analyzed by one-way analysis of variance followed by a *t*-test using the Residual Mean Square in the ANOVA as the estimate of variability.

Results

Wild-type and hpt-containing callus and plants – molecular characterisation

We generated rice callus and plants containing the *hpt* selectable marker and also wild-type material from six important diverse cultivars representing indica, japonica and javanica ecotypes. De-differentiated tissues and transgenic plants harbouring the *hpt* gene were recovered using previously published procedures (Capell et al. 1998; Sudhakar et al. 1998; Valdez et al. 1998). Callus lines from all genotypes that were resistant to hygromycin were screened by PCR to amplify a 1.1-kb fragment of the *hpt* cDNA. We selected six lines from each genotype for detailed Southern-blot analysis. Genomic DNA was extracted from leaves of plants regenerated from callus. Digestion with *Kpn*I (a single-cut in plasmid p35S*hpt*) and hybridisation with the 1.1-kb DIG-labelled PCR product (see Materials and methods) confirmed integration of the gene of interest and the independent origin of the transgenic lines studied (Fig. 1A). In order to determine whether the *hpt* gene product influenced levels of expression of endogenous genes involved in polyamine biosynthesis, we analysed mRNA expression of the rice *samdc* and *spermidine synthase* genes in de-differentiated tissues. Northern blots hybridised with probes made with the rice *samdc or spermidine synthase* cDNAs, did not show any variation in the expression of the endogenous rice genes. Figure 1B shows Northern blots for the rice *samdc* mRNA in three different genotypes. Results for *spermidine synthase* were very similar (data not shown). This indicates that the expression of the selectable marker does not affect the expression of the endogenous rice genes at the mRNA level.

ADC activity in wild-type and hpt-containing tissues

We measured enzyme activity for ADC in de-differentiated callus from wild-type and *hpt*-transgenic lines

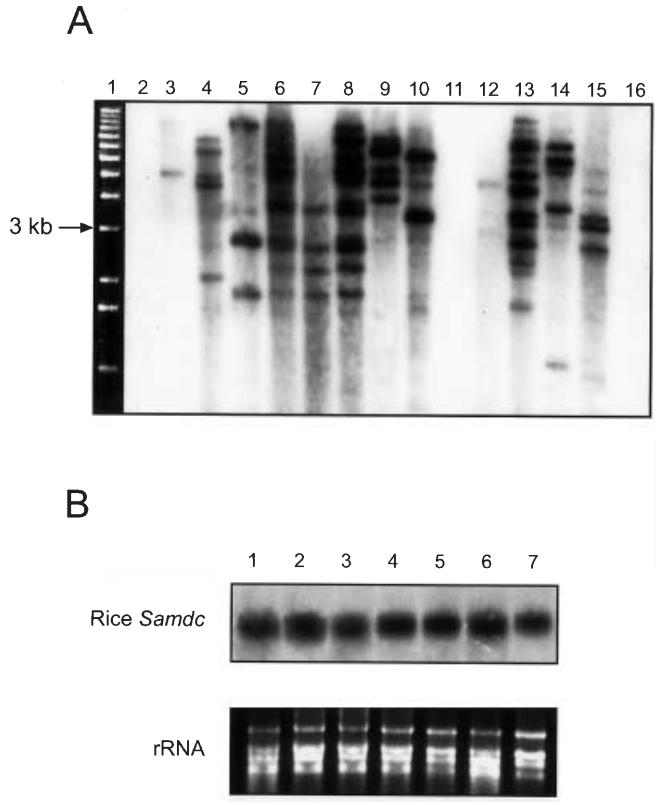

Fig. 1 **A** Southern-blot analysis of wild-type and *hpt*-containing plants. Ten micrograms of genomic DNA were digested with *Kpn*I (single cut) and probed with a 1.1-kb DIG-labelled PCR product from p35S*hpt*. Exposure time was 15 min. *Lane 1* molecular size marker (1-kb DNA Ladder; GIBCOBRL, UK). *Lane 2* IR64 wild-type. *Lane 3 to 6* representative IR64 *hpt*-transformed clones. *Lane 7 to 10* representative M12 *hpt*-transformed clones. *Lane 11* M12 wild-type. *Lane 12 to 15* representative ITA 212 *hpt*-transformed clones. *Lane 16* ITA 212 wild-type. **B** Northern-blot analysis of total RNA extracted from wild-type and *hpt*-transformed callus under selection pressure. Each lane was loaded with 30 µg of RNA and probed with a 0.7-kb DIG-labelled PCR product from rice *samdc* cDNA. Exposure time was 30 min. *Lane 1, 3 and 5* IR64, M12 and ITA 212 wild-type, respectively. *Lane 2 and 4* IR64 and M12 *hpt*-transformed clones, respectively. *Lane 6 and 7* two different clones of ITA 212 transformed with *hpt*

(Fig. 2A). We also measured activities of this enzyme in leaf and root tissues from the corresponding regenerated plants (Fig. 2B and C). ADC activity was measured in callus, leaves and roots at the same time as polyamine determinations. No significant variation ($P > 0.05$) was found between wild-type and *hpt*-transformants in ADC activity from the same genotype, in any of the lines we analysed. Enzyme activity levels in the six cultivars varied significantly amongst the population we analysed. Callus for both *hpt* and wild-type material exhibited the highest level of activity. This ranged from approximately 25 nKat g^{-1} fw in EYI 105 to 1,100 nKat g^{-1} fw in ISDA6 (Fig. 2A). Vegetative tissues such as leaves and roots from 3-month-old plants, showed less variation in ADC levels compared to callus tissue. M12 and IR64 showed the highest activity in leaves (50–60 nKat g^{-1} fw) whereas the four remaining genotypes exhibited lower activity, with Bengal showing the lowest level (30 nKat g^{-1} fw; Fig. 2B). ADC activity in roots from wild-type or *hpt*-transformed plants was not significantly different ($P > 0.05$) among genotypes (35–45 nKat g^{-1} fw). We thus infer that even though different genotypes contain different basal levels of ADC activity, this does not depend on whether the *hpt* gene is present or not.

Polyamine levels in wild-type and hpt-containing tissues

Large variations in individual polyamine concentrations were measured it the six different rice cultivars we studied. Figure 4 shows polyamine levels in wild-type and *hpt*-containing transgenic tissues. The profile of individual polyamines varied significantly amongst different tissues and genotypes, irrespective of whether the *hpt* selectable marker was present. No significant variation ($P > 0.05$) was found between wild-type and *hpt*-transformants in terms of polyamine levels in any given genotype in any of the tissues we analyzed (callus, leaf, roots and seeds). Putrescine was the most-abundant polyamine in callus tissue from all genotypes (Fig. 3A). Samples were taken 12 days following transfer of the callus to proliferation medium (Bassie et al. 2000). Putrescine levels were between two- to four-fold higher compared to levels of spermidine and spermine, ranging from 200 to 2,300 nmol g^{-1} fw. Spermidine and spermine levels were very similar for ITA212 and M12, ranging between 400 and 600 nmol g^{-1} fw. In Bengal, spermidine levels (50 nmol g^{-1} fw) were lower than spermine (100 nmol g^{-1} fw). EYI 105 had the maximum concentration of spermidine (1,200 nmol g^{-1} fw). ISDA6 had no detectable levels of spermidine and IR64 had no detectable levels of spermidine or spermine (Fig. 3A).

Polyamine content in leaves from 3-month-old plants showed two different patterns (Fig. 3B). Spermidine levels in IR64 and ISDA6 were higher than putrescine and spermine, which were present in equal amounts (120–130 nmol g^{-1} fw for spermidine and 60 nmol g^{-1} fw for putrescine and spermine). Putrescine was the predominant polyamine in Bengal, M12, ITA212 and EYI 105 (500 nmol g^{-1} fw). Within this second group, Bengal and M12 had equivalent relative amounts of spermidine and spermine, with absolute levels differing by 10-fold in the two cultivars (i.e. 400 nmol g^{-1} fw of spermidine and spermine in Bengal, and 40 nmol g^{-1} fw in M12). Putrescine was also the major polyamine in ITA212 and EYI 105; however, spermidine levels were twice those of spermine (300 nmol g^{-1} fw and 140–170 nmol g^{-1} fw, respectively, for the two genotypes; Fig. 3B).

The polyamine pattern in roots was similar to that in leaves in some of the genotypes, e.g. ITA212 and EYI 105 (Fig. 3B and C). IR64 and ISDA6 had a lower content of putrescine (30–60 nmol g^{-1} fw). IR64 had no detectable levels of spermidine or spermine while in ISDA6 spermidine levels (60 nmol g^{-1} fw) were the same as putrescine (60 nmol g^{-1} fw). No spermine could be detected in ISDA6. Bengal showed the opposite profile; spermidine levels were higher (240 nmol g^{-1} fw)

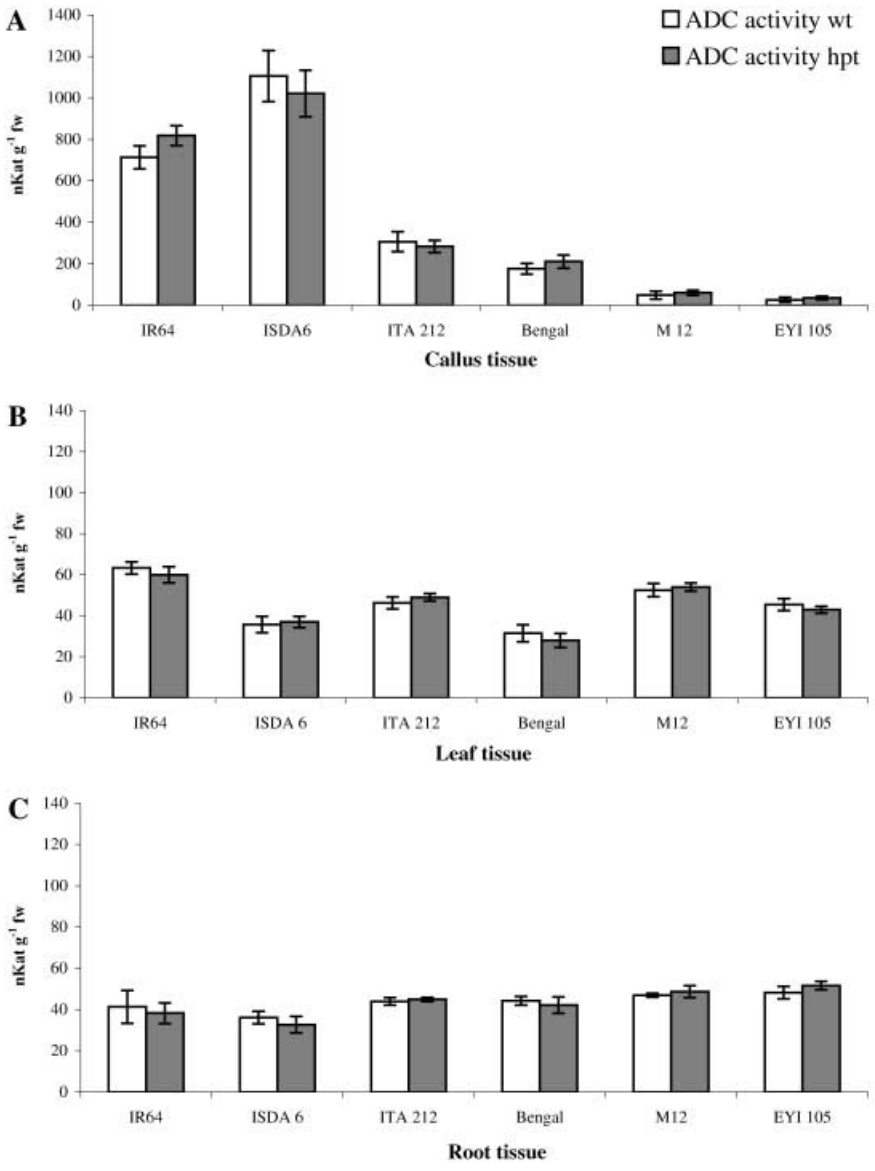

Fig. 2 Arginine decarboxylase activity in wild-type and *hpt*-containing tissues. Enzyme activities in *hpt*-transformants were not significantly different from wild-type values ($P > 0.05$). **A** Callus; **B** leaf; **C** root (N.B. scale in **B** and **C** is 10 × that of **A**)

than putrescine (130 nmol g^{-1} fw) with spermine levels in the order of 60 nmol g^{-1} fw (Fig. 3C).

Polyamine measurements in mature seeds were performed after desiccation of the seeds for 3 days. All six genotypes analysed had detectable levels of putrescine, spermidine and spermine. The polyamine pattern observed in seeds (Fig. 3D) exhibited the reverse trend to that observed in leaves (Fig. 3B) and roots (Fig. 3C). Spermidine and spermine were the two major polyamines in seeds in all but the ITA212 variety. EYI 105 had the highest levels of the three polyamines and IR64 and ISDA6 had the lowest levels (Fig. 3D).

Polyamine enzyme activities in wild-type and hpt-containing leaf tissue

We measured the activities of three anabolic and two catabolic enzymes involved in polyamine metabolism. Arginine decarboxylase activity was in the range of 45 to 55 nKat g^{-1} fw; ODC activity was between 25 and 30 nKat g^{-1} fw and SAMDC activity was between 7.5 and 8.5 nKat g^{-1} fw (Fig. 4A). Activities of enzymes involved in polyamine catabolism were many fold lower to those of enzymes involved in anabolism. Polyamine oxidase activity was in the order of 0.150 nKat g^{-1} fw and PAO activity was 0.070 nKat g^{-1} fw (Fig. 4B). No significant variation ($P > 0.05$) was found in any of the enzyme activities between wild-type and *hpt*-transformants for the same genotype.

Polyamine levels in different tissues in ISDA6

ISDA6 and IR64 did not contain measurable levels of particular polyamines in different tissues. We therefore investigated the distribution of the three main polyamines in different tissues in *hpt*-containing plants from

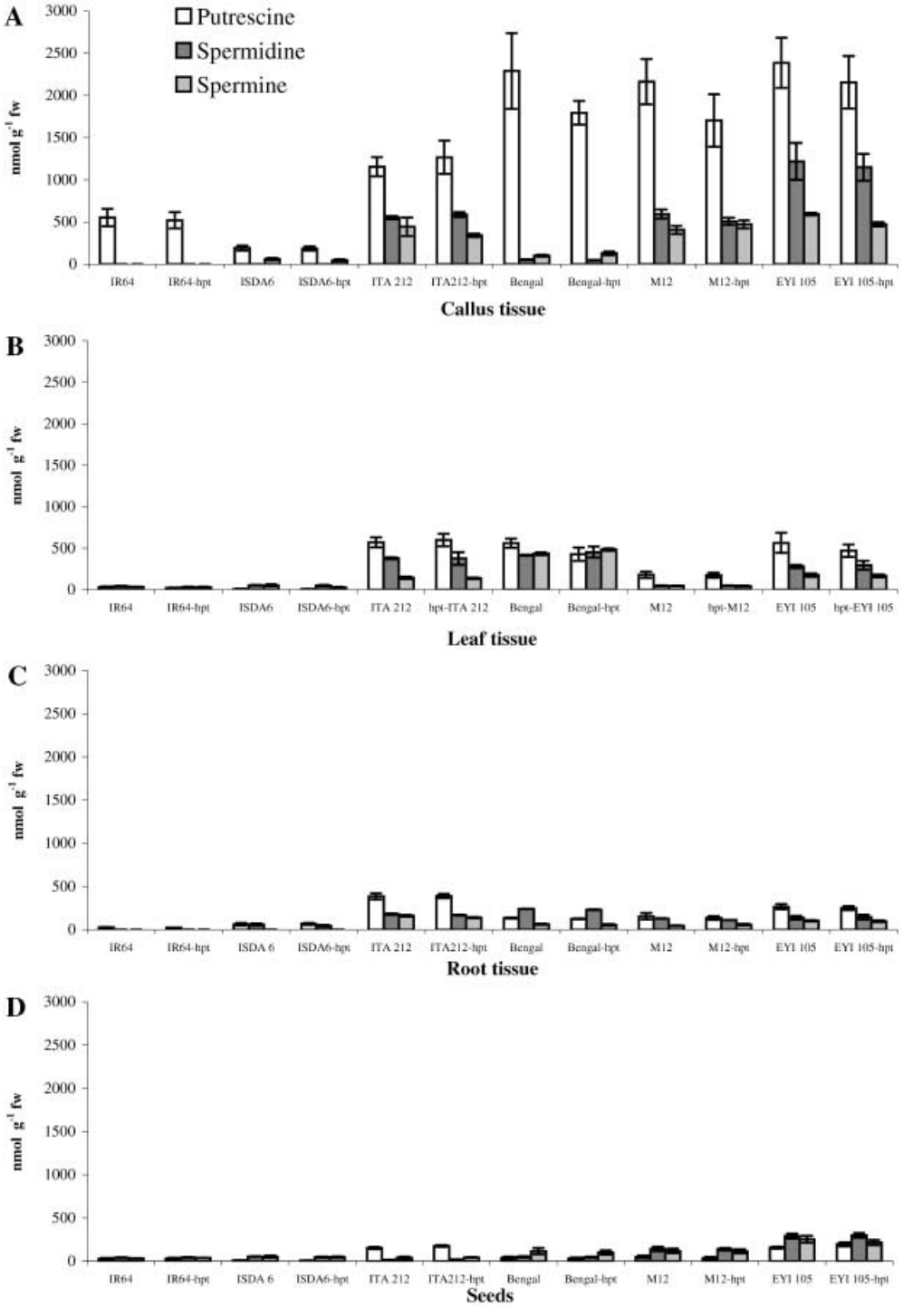

Fig. 3 Cellular polyamine levels in wild-type and *hpt*-transformants. Polyamine values from *hpt*-transformants were not significantly different from wild-type values for any of the polyamines ($P > 0.05$) in any of the tissues. **A** Callus; **B** leaf; **C** root; **D** seeds. (N.B. identical scales for **A**–**D**)

the ISDA6 genotype (Fig. 5). We measured polyamine concentration in seeds at different developmental stages. Putrescine (350 nmol g^{-1} fw) and spermidine (280 nmol g^{-1} fw) were the major polyamines in immature endosperm (20 days after pollination), with levels of spermine being 9-fold lower (30 nmol g^{-1} fw). Putrescine was not detectable in immature embryos (20 days after pollination), while spermidine and spermine were detectable at similar levels (70 nmol g^{-1} fw). The concentrations of spermidine and spermine were lower in immature embryos compared to endosperm tissue. In mature seeds, polyamine content was significantly reduced (7 nmol g^{-1} fw of putrescine; 50 nmol g^{-1} fw of spermidine and 50 nmol g^{-1} fw of spermine, $P < 0.001$). Husk removed from mature seeds had the highest putrescine content (600 nmol g^{-1} fw). One- and three-month-old leaves had the same polyamine pattern, i.e. the total polyamine content remained constant, with changes only in the relative levels of particular polyamines. No significant differences in polyamine levels ($P > 0.05$)

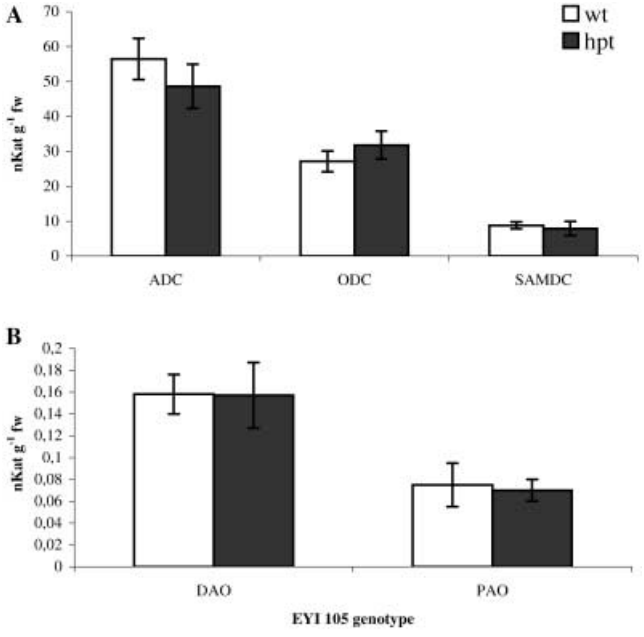

Fig. 4 Arginine decarboxylase, ODC, SAMDC, DAO and PAO activities in wild-type and *hpt*-containing tissues from the EYI 105 genotype. Enzyme activities in *hpt*-transformants were not significantly different from wild-type values for any of the enzymes ($P > 0.05$)

were observed in tissues from young or old roots. Putrescine was the major polyamine (620 nmol g^{-1} fw), with levels of spermidine in the order of 60 nmol g^{-1} fw. No measurable levels of spermine were detected (Fig. 5A). No significant variation ($P > 0.05$) was found in any tissue between polyamine levels from wild-type and *hpt*-transformants for the same genotype.

Discussion

As we embark on crop-improvement programs to engineer more-complex traits, such as metabolic pathways, it becomes apparent that a key prerequisite for the success of such experiments is the establishment of basal levels of endogenous enzyme activities and metabolites. This is essential if we are to interpret correctly results from experiments involving over-expression or down-regulation of homologous or heterologous transgenes introduced into plants already containing homologues of such genes. It is important to ascertain how genetic variation may affect enzyme and metabolite levels, as this may influence the choice of genetic background for carrying out transformation experiments that have specific targets in terms of deployment of particular improved varieties in specific geographical locations under different environmental conditions. It is likely that certain cultivars within a species are more-suitable than others in terms of engineering complex traits merely because of endogenous levels of such enzymes and metabolites. This is not trivial because such experiments may provide an explanation as to why different investigators often report apparently conflicting results from experiments involving the manipulation of metabolic traits in different species and different genetic backgrounds.

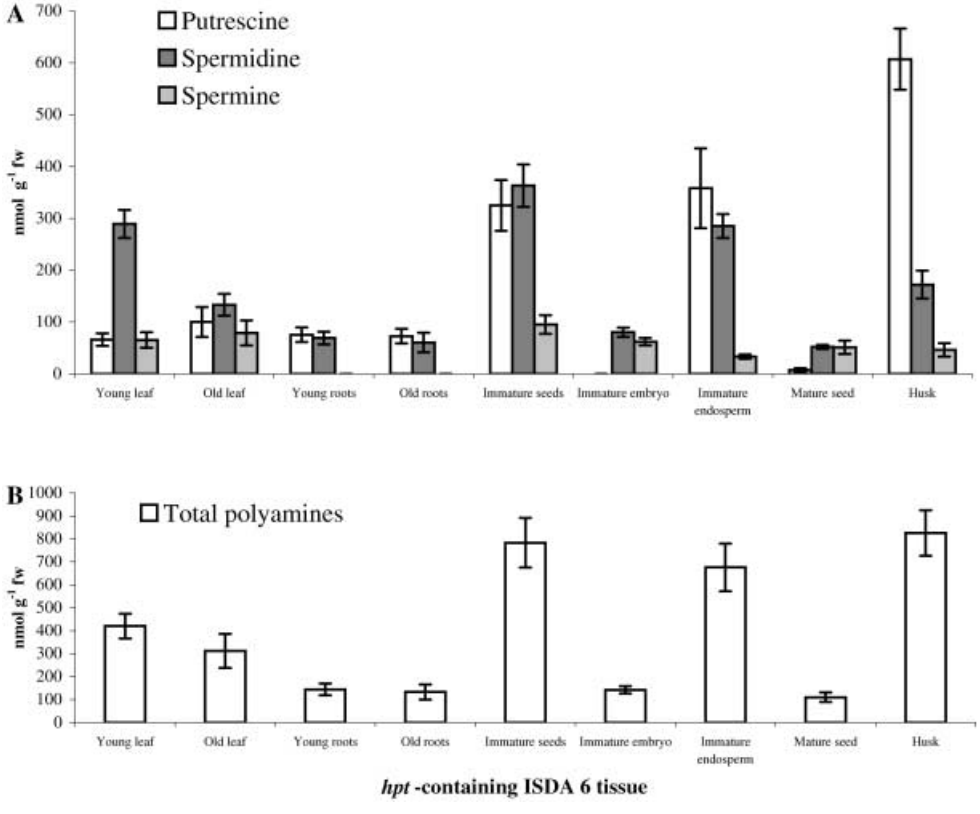

Fig. 5 Cellular polyamine levels in wild-type and *hpt*-transformants for the ISDA6 genotype. Polyamine values from *hpt*-transformants were not significantly different from wild-type values for any of the polyamines ($P > 0.05$). **A** Individual polyamines; **B** total amount of polyamines

Selectable marker genes are required in transformation experiments in order to facilitate recovery of transgenic plants expressing useful genes that do not have an easily identifiable phenotype (Christou 1994). Normally, such markers, comprising antibiotic or herbicide resistance genes or metabolic inhibitors, are introduced into plants with single or multiple genes of interest in a co-transformation experiment. Usually, selectable markers and genes of interest co-integrate into the genome of transgenic plants at the same location, which of course is random from plant to plant, and they do not segregate in subsequent generations (Chen et al. 1998; Kohli et al. 1998; Abranches et al. 2001). Consequently, by selecting for the marker, one will recover plants containing also the gene(s) of interest (Christou 1994). Even though selectable markers are not expected to have any pleiotropic effects, on occasion this may happen. One such example involves the use of the *bar* gene which is the marker of choice for the transformation of cereals (mostly maize, wheat and barley). When transgenic plants containing the *bar* gene were sprayed with Basta or Bialaphos (the commercial herbicide containing PPT, which is the compound that the *bar* gene de-toxifies) these plants performed normally and they were completely resistant in the laboratory and in the GH. However, transgenic plants deployed in the field were severely damaged after spraying with the herbicide when the ambient temperature exceeded a certain threshold (Broer 1996; Neumann 1997). Results from such experiments suggest that it may be prudent to re-visit the issue of selectable markers in terms of the likelihood to confer unexpected properties to transgenic plants. This assumes extreme importance in the context of engineering complex pathways, as such manipulations may result frequently in saddle differences in enzyme activities and metabolite levels that often are slightly over background levels. It is important, therefore, to ascertain whether any biochemical or physiological effects of introduced transgenes are solely a result of the expression of the introduced transgenes rather than other factors including the presence of selectable markers.

In an effort to address the influence of the genetic background and the effect, if any, of an important selectable marker commonly used in the creation of transgenic rice plants, we generated a *hpt*-expressing population comprising six diverse rice cultivars representing popular japonica, indica and javanica ecotypes. The polyamine pathway is involved in many essential biochemical, physiological and developmental processes, and is ubiquitous in plants (Malmberg et al. 1998). Consequently, all plants have endogenous polyamine enzyme activities and accumulate the three key metabolites, putrescine, spermidine and spermine. We therefore chose the polyamine pathway as a relevant model for our studies. We measured ADC activity and polyamine concentration in callus and vegetative tissues from these different genotypes, and we compared statistically all results from *hpt*-transformants and wild-type plants. No significant variation ($P > 0.05$) between wild-type and *hpt*-transformants was found in any of the tissues for the same genotype. ADC activity in callus (Fig. 2A) was higher than in vegetative tissues (Fig. 2B). Leaves and roots had similar levels of activity in all genotypes (Fig. 2B and C). A likely reason for the higher metabolic rate in de-differentiated tissues might be the rapid cell division that takes place in such tissues. Polyamine levels reached maximum levels also in de-differentiated tissues; however, we observed an interesting trend which essentially demonstrated an inverse relationship between ADC activity levels and polyamine accumulation in specific cultivars. For example, ADC activity in ISDA6 callus (Fig. 2A) was the highest we measured. Levels of polyamines in this cultivar were very low (Fig. 3A) compared to varieties such as EYI105 which exhibited very low levels of enzyme activity. This cultivar accumulated polyamines at the highest levels. Such results provide some evidence supporting a likely feedback-inhibition of the enzyme by the end-product.

ADC activity in leaves and roots in the six cultivars we studied were similar (Fig. 2B and C), but significantly lower to the levels we measured in callus (Fig. 2A). Polyamine levels followed the same trend (Fig. 3B and C). This hierarchical pattern in polyamine accumulation was also observed in seeds (Fig. 3D). Thus, the most metabolically active tissue, i.e. de-differentiated callus, accumulated the highest levels of polyamines, and the least metabolically active tissue, i.e. seed, accumulated the lowest levels. This again, provides evidence of a link between polyamine levels and the metabolic state of the cell. It is not surprising that such a trend exists as it is reasonable to expect that highly specialized tissues and organs, such as leaves, roots and seeds, contain cells which are committed to a number of different processes. It is not surprising that polyamine pools in highly differentiated tissues and organs will be committed to a variety of tasks, consistent with the involvement of polyamines in a multitude of biochemical, developmental and physiological processes. This will lead to a rapid and significant depletion of polyamine pools consistent with our observations. Chattopadhyay et al. (1997) reported differential responses of two rice cultivars to salt stress. They demonstrated that a salt-tolerant and a salt-sensitive rice variety had different basal levels of ADC activity in shoots and roots in each cultivar. Bonneau et al. (1994) reported a two-fold difference in the levels of polyamines in rice seeds exhibiting full germination potential compared to those which were severely compromised in their ability to germinate. These two examples also indicate that genetic background influences levels of polyamines and links these to a physiological response which is genotype dependent.

We also investigated how polyamine pools, in this case in leaves from EYI 105, are regulated in terms of enzyme activities. Figure 4 summarizes the activities for ODC, SAMDC, PAO and DAO in leaf tissue of EYI 105 (wild-type and *hpt*-containing plants). We chose EYI 105 to carry out measurements for these enzymes because this variety exhibited the highest levels of polyamines in

all tissues we analyzed. We observed that, similarly to measurements for ADC activity previously, there was no difference between wild-type and *hpt*-containing plants. This analysis indicated that in leaf tissue, activity of the anabolic component of the pathway was significantly higher compared to activities of enzymes involved in polyamine catabolism. ADC is the predominant activity in leaf tissues, followed by ODC. Only a fraction of putrescine, formed by ADC, is converted to the higher polyamines spermidine and spermine, as putrescine also is the precursor of polyamine conjugates and other compounds. This perhaps provides an explanation as to why SAMDC activity is lower compared to ADC and ODC. To the best of our knowledge, there is no prior report describing the simultaneous measurement of all five activities in plants. Cohen and Kende (1986) measured ADC, ODC and SAMDC in deepwater rice internodes. Consistent with our results, they observed that ADC was the main enzyme involved in the production of putrescine, while levels of ODC activity were significantly lower. Levels of SAMDC were significantly higher than ODC but lower than ADC. The genotype under investigation in this study was unusually high in spermidine. This is consistent with the higher levels of SAMDC activity these authors observed compared to our studies, and supports the argument that it is important to determine basal levels of polyamines and enzyme activities in different genotypes.

We then investigated the spatial distribution of individual and total polyamines in ISDA6 in a wide range of tissues and organs (Fig. 5). ISDA6 is representative of a group of genotypes (including IR64) in that particular tissues/organs do not contain measurable levels of particular polyamines. These two varieties had similar levels of polyamines in leaves, roots and seeds, in contrast to other varieties such as ITA212, Bengal, EYI 105 and M12 that exhibited a hierarchical distribution of polyamines in different tissues/organs. In ISDA6, we observed a direct relationship between polyamine levels and the metabolic state of the tissue, with young leaves, roots and immature seeds containing significantly higher levels of polyamines compared to tissues/organs sampled at later stages of development.

Our study confirms that: (1) there are no differences in enzyme activities and polyamine levels between wild-type and plants containing the selectable marker *hpt*, and (2) polyamine enzyme activities and the profile of polyamines is dependent on the genetic background and varies significantly in different tissues. The genetic basis of such variation is important in any study involving enzyme-activity measurements and the determination of levels of metabolites.

Acknowledgements We thank J. Dix for art design and E. Aguado for assistance with maintaining plants in the greenhouse. T.P.H. is supported by a Rockefeller Foundation fellowship.

References

Abranches R, Santos AP, Williams S, Castilho A, Christou P, Shaw P, Stoger E (2001) Widely-separated multiple transgene integration sites in wheat chromosomes are brought together at interphase. Plant J 24:713–723

Bassie L, Noury M, Lepri O, Lahaye T, Christou P, Capell T (2000) Promoter strength influences polyamine metabolism and morphogenic capacity in transgenic rice tissues expressing the oat arginine decarboxylase cDNA constitutively. Transgenic Res 9:33–42

Broer I (1996) Stress inactivation of foreign genes in transgenic plants. Field Crops Res 45:19–25

Bonneau L, Carre M, Martin-Tanguy J (1994) Polyamines and related enzymes in rice seeds differing in germination potential. Plant Growth Reg 15:75–82

Capell T, Escobar C, Lui H, Burtin D, Lepri O, Christou P (1998) Over-expression of the oat arginine decarboxylase cDNA in transgenic rice (*Oryza sativa* L.) affects normal development patterns in vitro and results in putrescine accumulation in transgenic plants. Theor Appl Genet 97:246–254

Chattopadhyay MK, Suchiranjan G, Sengupta NS, Ghosh B (1997) Expression of arginine decarboxylase in seedlings of indica rice (*Oryza sativa* L.) cultivars as affected by salinity stress. Plant Mol Biol 34:477–483

Chen L, Marmey P, Taylor NJ, Brizard J-P, Espinoza CD, Cruz P, Huet H, Zhang S, Kochko A, Beachy RN, Fauquet CM (1998) Expression and inheritance of multiple transgenes in rice plants. Nature Biotechnol 16:1060–1064

Cheng W, Wu R (1997) Rice plants over-expressing *Hva 1* and *cor 47* transgenes showed tolerance to drought, cold and salt. In: Abstracts, General Meeting of the International Programme on Rice Biotechnology, Malacca, Malaysia, pp 356–357

Christou P (1994) Rice biotechnology and genetic engineering. Biotechnology of food crops. Technomic Publishing Company, Lancaster, Pennsylvania, USA

Cohen E, Kende H (1986) The effect of submergence, ethylene and gibberellin on polyamines and their biosynthetic enzymes in deepwater-rice internodes. Planta 169:489–504

Creissen GP, Mullineaux PM (1995) Cloning and characterisation of glutathione reductase cDNAs and identification of two genes encoding the tobacco enzyme. Planta 197:422–425

Depicker A, Stachel S, Dhaese P, Zambryski P, Goodman HM (1982) Nonpaline synthase: transcript mapping and DNA sequence. J Mol Appl Genet 1:499–512

Drakakaki G, Christou P, Stoger E (2000) Constitutive expression of soybean ferritin cDNA in transgenic wheat and rice results in increased iron levels in vegetative tissues but not in seeds. Transgenic Res 9:445–452

Federico R, Angelini R, Cesta A, Pini C (1985) Determination of diamine oxidase in lentil seedlings by enzymatic activity and inmunoreactivity. Plant Physiol 79:62–64

Gardner R, Howarth A, Hahn P, Brown-Luedi M, Shepherd R, Messing J (1981) The complete nucleotide sequence of an infectious clone of cauliflower mosaic virus by M13mp7 shotgun sequencing. Nucleic Acids Res 9:2871–2888

Gotto F, Yoshihara T, Shigemoto N, Toki S, Takaiwa F (1999) Iron fortification of rice seeds by the soybean ferritin gene. Nature Biotechnol 17:282–286

Hausman JF, Kevers C, Evers D, Gaspar T (1997) Conversion of putrescine into γ-aminobutyric acid an essential pathway for root formation by poplar shoots in vitro. In: Altman A. Waisel Y (eds) Biology of root formation. Plenum Press, New York London, pp 37–44

Kohli A, Leech M, Vain P, Laurie DA, Christou P (1998) Transgene organization in rice engineered through direct DNA transfer supports a two-phase integration mechanism mediated by the establishment of integration hot-spots. Proc Natl Acad Sci USA 95:7203–7208

Lucca P, Hurrell R, Potrykus I (2001) Genetic engineering approaches to improve the bioavailability and the level of iron in rice grains. Theor Appl Genet 102:392–397

Malmberg RL, Watson MB, Galloway GL, Yu W (1998) Molecular genetic analysis of plant polyamines. Crit Rev Plant Sci 17:199–224

Maqbool SB, Christou P (1999) Multiple traits of agronomic importance in transgenic indica rice plants: analysis of transgene integration patterns, expression levels and stability. Mol Breed 5:471–480

Maqbool SB, Husnain T, Riazuddin S, Masson L, Christou P (1998) Effective control of yellow stem borer and rice leaf folder in transgenic rice indica varieties Basmati 370 and M7 using a novel δ-endotoxin cry2A *Bacillus thuringensis* gene. Mol Breed 4:501–507

Matilla AJ (1996) Polyamines and seed germination. Seed Sci Res 6:81–93

Neumann K, Droge-Laser W, Kohne S, Broer I (1997) Heat treatment results in a loss of transgene-encoded activities in several tobacco lines. Plant Physiol 115:939–947

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning; a laboratory manual, 2nd edn. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York

Smith T (1985) Polyamines. Annu Rev Plant Physiol 36:117–143

Stöger E, Vaquero C, Torres E, Sack M, Nicholson L, Drossard J, Williams S, Keen D, Perrin Y, Christou P, Ficher R (2000) Cereal crops as viable production and storage systems for pharmaceutical scFv antibodies. Plant Mol Biol 42:583–590

Storer RJ, Ferrante A (1989) Hydrogen peroxide assay for amine oxidase activity. In: Morgan D (ed) Polyamine protocols. Methods in molecular biology vol. 79: Humana Press Inc, Totowa, New Jersey, pp 81–89

Sudhakar D, Fu X, Stöger E, Williams S, Spence J, Brown DP, Bharathi M, Gatehouse JA, Christou P (1998) Expression and immunolocalisation of the snowdrop lectin, GNA, in transgenic rice plants. Transgenic Res 7:371–378

Tanaka Y, Hibino T, Hasashi Y, Tanaka A, Kishitani S, Takabe T, Yokota S, Takabe T (1999) Salt tolerance of transgenic rice over-expressing yeast mitochondrial Mn-SOD in chloroplasts. Plant Sci 148:131–138

Tiburcio AF, Altabella T, Borrel A, Masgrau C (1997) Polyamine metabolism and its regulation. Physiol Plant 100:664–674

Torres E, Vaquero C, Nicholson L, Sack M, Stöger E, Drossard J, Christou P, Ficher R, Perrin Y (1999) Rice cell culture as an alternative production system for functional diagnostic and therapeutic antibodies. Transgenic Res 8:441–449

Tyagi AK, Mohanty A (2000) Rice transformation for crop improvement and functional genomics. Plant Sci 158:1–18

Vain P, Worland B, Clarke MC, Richard G, Beavis M, Liu H, Kohli A, Leech M, Snape J, Atkinson H (1998) Expression of an engineered cysteine proteinase inhibitor (Oryzacystatin-IΔD86) for nematode resistance in transgenic rice plants. Theor Appl Genet 96:266–271

Valdez M, Cabrera-Ponce JL, Sudhakhar D, Herrera-Estrella L, Christou P (1998) Transgenic Central American, West African and Asian elite rice varieties resulting from particle bombardment of foreign DNA into mature seed-derived explants utilizing three different bombardment devices. Ann Bot 82:795–801

Van den Elzen PJM, Townsend J, Lee KY, Bedbrook JR (1985) A chimeric hygromicin resistance gene as a selectable marker in plant cells. Plant Mol Biol 5:299–302

Xu D, Duan X, Wang B, Hong B, Ho THD, Wu R (1996) Expression of a late embryogenesis abundant protein gene, *Hva 1*, from barley confers tolerance to water deficit and salt stress in transgenic rice. Plant Physiol 110:249–257

Yanisch-Perron C, Vieria J, Messing J (1985) Improved M13 phage cloning vectors and host strains: nucleotide sequence of the M13mp18 and pUC19 vectors. Gene 33:103–119

Ye X, Al-Babili S, Kloti A, Zhang J, Lucca P, Beyer P, Potrykus I (2000) Engineering the provitamin A (β-carotene) biosynthetic pathway into (carotenoid-free) rice endosperm. Science 287:303–305

Zhang S, Song W-Y, Chen L, Ruan D, Taylor N, Ronald P, Beachy R, Fauquet C (1998) Transgenic elite indica rice varieties resistant to *Xanthomonas oryzae* pv *Oryzae*. Mol Breed 4:551–558