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Polyamine accumulation in transgenic eggplant enhances tolerance to multiple abiotic stresses and fungal resistance

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Abstract Polyamines (putrescine, spermidine and spermine) have been shown to be important in stress tolerance. Thus, the present study was undertaken with the aim to enhance stress tolerance in eggplant by introduction of a key polyamine biosynthetic gene arginine decarboxylase, *adc* under the control of a constitutive promoter of cauliflower mosaic virus, CaMV35S through *Agrobacterium*-mediated transformation. Several putative transgenic plants were generated and the transgene integration and expression was confirmed by PCR and Southern blot analyses, and RT-PCR analysis, respectively. These transgenic plants have shown an enhanced level of polyamines due to the increase in ADC enzyme activity. The diamine oxidase (an enzyme involved in putrescine and spermidine degradation) activity was also increased in these transgenic plants. Polyamine-accumulating transgenic plants exhibited an increased tolerance levels to multiple abiotic stresses such as salinity, drought, low and high temperature, and heavy-metal and resistance against fungal wilt disease caused by *Fusarium oxysporium*.

Key words: *Agrobacterium*, arginine decarboxylase, eggplant, plant transformation, polyamines, stress tolerance.

Crop plants are affected by a variety of abiotic stresses like salinity, drought, low and high temperature and heavy-metal as well as biotic stresses like pathogens. These stresses result in significant loss of crop yield and quality. Therefore, it is necessary to improve crop plants to withstand both abiotic and biotic stresses. Genetic engineering, especially the engineering of biosynthetic pathways associated with stress responses has emerged as a promising way to improve tolerance in crop plants (Flowers 2004; Chinnusamy et al. 2005; Kumar et al. 2006).

Plants have evolved various mechanisms to cope with stress conditions, and these include the shifts in the physiology of the plant as well as the expression of stress- associated genes leading to the formation of a wide variety of low molecular weight metabolites like mannitol, proline, glycine betaine and polyamines (Rajam et al. 1998; Kumar et al. 2006). Polyamines (PAs), putrescine (Put), spermidine (Spd) and spermine (Spm) play a pivotal role in plant response (or defense) to both abiotic and biotic stresses. They are small polycationic compounds that are present in all living organisms (Rajam 1997). Besides their involvement in stress reactions (Rajam 1997; Bouchereau et al. 1999; Walters 2003; Bhattacharya and Rajam 2006; Kumar et al. 2006), they also play an important role in the regulation of various cellular and molecular processes including growth and development, membrane integrity, macromolecular synthesis and function (Igarashi and Kashiwagi 2000; Thomas and Thomas 2001; Kumar et al. 2006). However, the exact mode of PA action at the molecular level is not clearly understood, and until recently the role of PAs in biological processes has been demonstrated by raising mutants that are deficient in PA metabolism as well as by transient inhibition of their biosynthetic enzymes with the use of specific inhibitors (Kumar et al. 2006). Therefore, transgenic plants are being raised to gain better insight into the role of PA metabolism in various biological processes as this approach is highly specific to a gene and allow the modification of the metabolic flux due to a persistent shift in the PA metabolism. Unfortunately, there are very few reports on the transgenic plants expressing PA metabolic genes, and examining such transgenic plants for stress responses (reviewed by Rajam et al. 1998; Kumar et al. 2006). It has been reported that the over-expression of PA biosynthetic genes like *adc* (Roy and Wu 2001; Capell et al. 1998; 2004), *odc* (Kumria and Rajam 2002), *samdc* (Roy and Wu 2002; Waie and Rajam 2003) and *spd syn* (Franceschetti et al. 2004;

Abbreviations: ADC, arginine decarboxylase; DAO, diamine oxidase; MS, Murashige and Skoog; ODC, ornithine decarboxylase; PAs, polyamines; Put, putrescine; Spd, spermidine; Spm, spermine.
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Kasukabe et al. 2004; 2006) in plants like rice, tobacco, *Arabidopsis* and sweet potato, has resulted in increased tolerance to abiotic stresses. There is only one report on fungal resistance in transgenic tobacco plants over-expressing *samdc* gene (Waie and Rajam 2003).

Therefore, the present work was aimed to generate transgenic eggplants with oat arginine decarboxylase (ADC, EC 4.1.1.19) gene, a key gene involved in PA biosynthesis, and evaluate such transgenic plants for PA levels and tolerance against both abiotic and biotic stresses under *in vitro* and *in vivo* growth conditions. Indeed, these transgenic eggplants have shown elevated PA content that could be correlated with increased tolerance to multiple abiotic stresses and fungal resistance.

Materials and methods

Plant material and plasmid

The seeds of eggplant (*Solanum melongena* L., cv. Pusa Purple Long) were obtained from the National Seeds Corporation, Indian Agricultural Research Institute, New Delhi. The *Agrobacterium tumefaciens* strain LBA4404 containing binary plasmid pMVR3ADC with oat *adc* gene under the control of cauliflower mosaic virus CaMV35S promoter and nopaline synthase terminator, and hygromycin phosphotransferase (*hpt*) as plant selection marker was used for eggplant transformation (Figure 1A).

Eggplant transformation and regeneration

Leaf and cotyledon explants, collected from about thirty days and fifteen days old axenic seedlings respectively, were used for transformation as described earlier (Prabhavathi et al. 2002). The *Agrobacterium* culture grown to an O.D. (A_{600}) of 0.1–0.2 was used for infection (10 min) using pre-cultured explants on shoot regeneration medium (SRM), i.e. MS medium supplemented with 2.5 mg l^{-1} benzylaminopurine (BAP) and 0.5 mg l^{-1} indole-3-acetic acid (IAA) for 2 days. After infection, the explants were co-cultured on SRM for 2 days, and then transferred to selection medium, i.e. SRM containing 10 mg l^{-1} hygromycin (selection agent) and 300 mg l^{-1} augmentin (bacteriostatic agent) and cultured for about a month with one sub-culture. The small shoots obtained on selection medium were subjected for proliferation on MS medium fortified with 0.5 mg l^{-1} BAP. The well-grown shoots were excised and transferred to the rooting medium (half-strenth MS + 250 mg l^{-1} augmentin). The rooted plants were transferred to pots containing vermiculite: soil in a 1 : 1 ratio and plants were covered with polythene bags for 1 week to maintain high humidity for hardening in the tissue culture room and then transferred to green-house.

Polymerase chain reaction

Genomic DNA was isolated from leaf tissue by CTAB method (Doyle and Doyle 1990). The putative transgenic plants were analyzed by PCR for the integration of the transgene. The conditions for PCR were 40 cycles of denaturation at 94°C for 1 min, annealing for 1 min at 52°C for *hpt* and 53°C for *adc* gene and synthesis at 72°C for 2 min, and finally the extension step of 10 min at 72°C. The PCR products were checked on 1% agarose gel with the ladder. The primer pairs for the amplification of a 1-kb fragment of *hpt* gene were -5′CCCATGAAAAAGCCTGGACTCACCGCG3′ and 5′GCAGGCTCCCGTTTCCTTATCGAT3′. The primer pairs specific for the amplification of a 1-kb fragment of *adc* gene were -5′CGGCCACCTCTGCGTCAGAATCTAT3′ and 5′ACGCCGTGATAGTCGCACTTGAGC3′.

Southern blot hybridization

Genomic DNA (10 μg) was restricted with *Eco*RI and *Kpn*I for checking the transgene integration and copy number and probed with *adc* and *hpt* gene respectively. Southern blots were prepared by standard procedure (Sambrook et al. 1989) using Hybond-N Nylon membrane (Pharmacia). The *hpt* and *adc* gene probes were prepared using ^{32}P- labeled dCTP by nick translation as per the manufactures guidelines (Gibco-BRL). Hybridization was carried out for 18–24 h at 65°C. The membrane was washed and then exposed to X-ray film (XK-5 Kodak film).

RNA extraction and RT-PCR

Total RNA was isolated as per the protocol described by Chomczynski and Sacchi (1987). RT-PCR was done to check the level of transcription of the transgene in the developed transgenic plants. Total RNA at the concentration of 1.5 μg (treated with RNase-free DNase) was used as a template and was mixed with 1X buffer, 1X Q solution, 400 μM of dNTP mix, 1.2 μM primers, 5 U of RNase inhibitor and 2 μl of enzyme mix (Reverse Transcriptase and Taq Polymerase). The reaction volume was made to 25 μl. The reaction mixture was incubated at 50°C for 30 min. After reverse transcription, the reaction mixture was heated to 95°C for 15 min (to activate Hot Start Taq DNA polymerase and to simultaneously inactivate the reverse transcriptase), followed by 30 cycles of 1 min denaturation at 94°C, primer annealing at 53°C for 1 min, extension at 72°C for 1 min and final extension for 10 min. The PCR products were analyzed on 1% agarose gel.

Analysis of transgene segregation

The T_1 seeds obtained from primary transgenic plants were analyzed for the segregation of the transgenes. The surface-sterilized seeds were inoculated on to MS basal medium supplemented with 10 mg l^{-1} hygromycin and incubated at 26°C ± 1 and a 16-h photoperiod. The germination was observed up to 10 days and the percentage germination was scored for segregation analysis after one month (Prabhavathi et al. 2002).

Analysis of polyamines

PAs were estimated in seedlings of the wild-type and transgenic lines as per the protocol described by Bajaj and Rajam (1995).

Assay of arginine and ornithine decarboxylases

Leaf tissue of transgenic and wild-type plants were homogenized in extraction buffer containing 200 mM Tris.Cl [pH 8.5 for ADC and 8.2 for ornithine decarboxylase (ODC)], 10 mM DTT, 0.1 mM pyridoxal phosphate and 0.1 mM EDTA and centrifuged at 15000 × *g* at 4°C for 20 min. The homogenates were dialysed and the activity of ADC and ODC

was measured by labelling with ^{14}C arginine (specific activity 300 mCi /mmol) or ^{14}C ornithine (specific activity 55 mCi/mmol, 100 μCi ml^{-1}) respectively. The enzyme activity was measured by the amount of $^{14}CO_2$ released in the reaction using a LKB-1209 Rackbeta liquid scintillation counter and expressed as nmoles of $^{14}CO_2$ per hour per mg of protein (Birecka et al. 1985). The protein amount was estimated by Bradford method (Bradford 1976). The assay was repeated twice with two different clones of each transgenic line.

Assay of diamine oxidase

The diamine oxidase (DAO) was assayed using the protocol developed by Naik et al. (1981). DAO activity was measured by spectrophotometer at a 510 nm wavelength and expressed as μmol of Δ pyrroline formed min^{-1} per mg protein. The assay was repeated twice using leaf tissue from three different clones of each transgenic line.

Salinity and drought tolerance assays

The seeds of T_1 transgenic lines and wild-type plants were tested for tolerance to salinity and drought. The seeds were inoculated on MS basal medium containing 150 and 200 mM NaCl or 7.5 and 10% PEG (MW 20,000) for salt and drought tolerance assays, respectively. Tolerance was based on percent seed germination (Prabhavathi et al. 2002).

The T_1 transgenic and wild-type seedlings were grown in either test tubes (150 mm length and 20 mm diameter) containing 15 g of vermiculite: soil (1 : 1 w/w) mix or 10 ml liquid MS (one-tenth) medium supplemented with 150 or 200 mM NaCl (salt stress), and 7.5 or 10% PEG (drought) for 8–10 days. In the case of *in vitro* assay using the solid substrate, 1 ml of salt (the sub-lethal 150 mM NaCl and the lethal 200 mM NaCl were used) or 1 ml of PEG (7.5 or 10%) was poured into the test tubes (containing vermiculite: soil) twice a day for a total period of 8–10 days. In the case of *in vitro* assay using the liquid substrate, the seedlings were inserted into test tube containing NaCl (200 mM) or PEG (10%) in one-tenth strength liquid MS medium. The tolerance were based on the survival and growth of the seedlings under stressed conditions. Data on shoot height, fresh and dry weight was scored after a period of thirty days for wild-type and transgenic seedlings grown under stress conditions (Prabhavathi et al. 2002).

Desiccation tolerance assay

The detached leaves of the transgenic lines as well as wild-type plants were placed on dry Whatman No. 1 filter paper and incubated for 24 h at 26°C±1, and the leaves were scored for water loss (dehydration) as compared to the controls (Prabhavathi et al. 2002).

High temperature tolerance assay

The seeds of T_1 transgenic lines and wild-type plants were surface-sterilized and inoculated on to the solid MS basal medium in Petriplates and then placed in the incubator maintained at 45°C for different days intervals (1, 2 and 3 days) under light condition. After the stress, the Petriplates were transferred to tissue culture room at 26°C±1 and a 16-h photoperiod in cool white fluorescent light (40 μmol m^{-2} s^{-1}).

The T_1 transgenic seedlings (one-month) were grown in vermiculite: soil (1 : 1 w/w) mix in the plastic egg trays and allowed to establish for one week. The egg trays were placed at 45°C for 3 h and they were transferred to tissue culture room. The tolerance was based on the survival and growth of the seedlings under stressed condition. Data on shoot length, fresh and dry weight were scored after a period of one month for wild-type and transgenic seedlings grown under stress condition.

Low temperature tolerance assay

The seeds of T_1 transgenic lines and wild-type plants were inoculated on to the solid MS basal medium in Petriplates as well as egg trays containing vermiculite: soil (1 : 1 w/w) and placed in the cold room (6–8°C) for 10 days, and then they were transferred to tissue culture room maintained at 26°C±1 and a 16-h photoperiod. The data on percent seed germination, shoot length, fresh and dry weight of seedlings were taken after one month.

Heavy-metal tolerance assay

The seeds of T_1 transgenic lines and wild-type plants were surface-sterilized and inoculated on to the solid MS basal medium in Petriplates containing different concentrations of cadmium chloride ($CdCl_2$ - 0, 0.5, 1, 1.5 and 2 mM) and placed in tissue culture room at 26°C±1 and a 16-h photoperiod. After one-month of incubation, data were scored for percent seed germination.

The T_1 transgenic and wild type seedlings were transferred to test tubes (150 mm length and 20 mm diameter) containing 15 g of vermiculite: soil mix (1 : 1 w/w), and 1 ml one-tenth strength liquid MS medium fortified with 1 mM $CdCl_2$ poured once a day for a total period of 15 days. The tolerance was based on the survival and growth of the seedlings under heavy-metal stressed condition. Data on shoot length, fresh and dry weight of seedlings were scored after a period of one-month treatment.

Fungal resistance assay

One-month-old seedlings of T_1 transgenic and wild-type eggplants were tested for resistance against fungal wilt disease using either the root-dip method or soil mix method (Waie and Rajam 2003). For *in vitro* stress assay, the roots of the seedlings were dipped in the spore suspension (10^8 spores ml^{-1}) of *F. oxysporum* for 15–20 min and then the seedlings were transferred to test tubes containing one-tenth strength liquid MS medium. The tubes were placed under controlled growth conditions with a 16-h photoperiod and temperature of 26°C±1. The wilting symptoms were monitored after 10–15 days of inoculation. For *in vivo* assay, the soil and vermiculite were taken in a 1 : 1 (w/w) ratio and mixed with fungal spore suspension (10^8 spores ml^{-1}) and filled in the egg trays. The seedlings were inserted into the egg tray, and grown under controlled growth conditions with regular watering till the appearance of disease symptoms (i.e. wilting). The disease symptoms were examined after 10–15 days of inoculation. The degree of tolerance was determined by calculating the disease index, based on the extent of disease symptoms (Waie and Rajam 2003). Disease index was calculated by the formula $\Sigma XP_{0-5}/\Sigma n \times 5$, where X is the number of plants per phenotypic class, P, the phenotypic classes: 0—plants showing no aerial symptoms, 1—only bottom leaf necrotic or curled, 2—three leaves developed wilt symptoms, 3—only newest leaves remained healthy, older ones being necrotic and curled, 4—all

leaves fallen out, plants having only newly formed leaves, 5—plant death, and *n* is the total number of plants tested. The plants with disease index of 1 were considered susceptible to the disease, whereas those having an index of less than 1 were considered resistant. The experiments were repeated thrice with 6 seedlings of each transgenic line.

Results and discussion

Transformation and regeneration

The co-cultivated explants started showing shoot regeneration after 7–10 days of culture on the selection medium. The transformation frequency, which was based on the number of explants surviving on selection medium upon the total number of explants co-cultivated was in the range of 16–32%. All the transgenic plants were phenotypically normal and showed normal flowering and seed setting. The pollen viability in the transgenic plants was also similar to that of wild-type plants (data not shown).

In the present study, the over-expression of *adc* gene in eggplant did not affect plant regeneration during transformation and the transgenic plants were normal. Similar results were obtained in *adc* tobacco and rice transgenic plants (Burtin and Michael 1997; Capell et al. 2004) and in rice (Roy and Wu 2001), tobacco (Franceschetti et al. 2004), *Arabidopsis* (Kasukabe et al. 2004) and sweet potato (Kasukabe et al. 2006) transgenic plants over-expressing other PA biosynthetic genes, *samdc* and *Spd synthase*. However, morphological abnormalities such as short internodes and wrinkled leaves have been reported in transgenic tobacco plants over-expressing *odc* (DeScenzo and Minocha 1993; Kumria and Rajam 2002), *adc* (Masgrau et al. 1997; Panicot et al. 2002) and *samdc* (Noh and Minocha 1994; Waie and Rajam 2003) genes. Our results and the published work indicate that the phenotypic abnormalities in transgenic plants over-expressing PA genes might depend upon the transgene expression and cellular of PA levels.

Transgene integration and expression

PCR analysis of the genomic DNA from transformed plants with primers specific to *hpt* (Figure 1B) and *adc* (Figure 1C) gene has revealed the integration of the transgene as the expected 1-kb PCR products were obtained. The PCR positive plants were analysed by Southern hybridization to check the integration and copy number of the transgene. Genomic DNA was restricted with *Eco*RI for integration (Figure 1D) and *Kpn*I for copy number (Figure 1E) and probed with *adc* and *hpt* gene, respectively. About 50% of the transgenic plants had a single copy of the transgene, whereas other lines showed multiple copy insertion. The transgene expression was analyzed in different transgenic lines by RT-PCR using *adc* gene-specific primers. The transgenic lines AD3, AD7 and AD14 showed high transgene expression at the transcript level (Figure 1F).

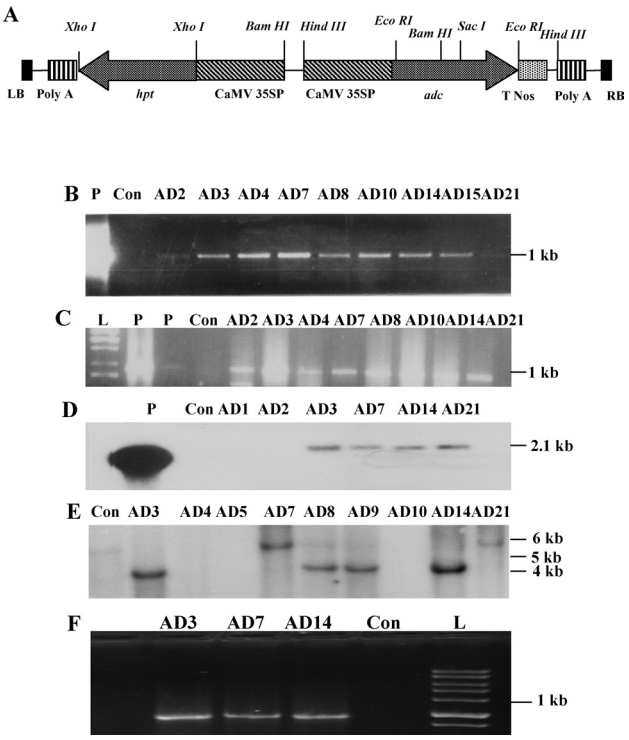

Figure 1. Molecular characterization of transgenic plants. (A) T-DNA map of the binary vector construct pMVR3ADC. Poly A, Cauliflower mosaic virus 35S terminator; *hpt*, hygromycin phosphotransferase; CaMV35SP, Cauliflower mosaic virus 35S promoter; *adc*, arginine decarboxylase; T Nos, nopaline synthase terminator; (B) PCR analysis of putative transgenic plants using primers specific to *hpt* gene: DNA from plasmid (P), wild-type plant (Con) and different putative transgenic plants (AD2, AD3, AD4, AD7, AD8, AD10, AD14, AD15 and AD21); (C) PCR analysis of putative transgenic plants with primers specific to *adc* gene: 1 kb ladder (L), DNA from plasmid (P), wild-type plant (Con) and different transgenic plants (AD2, AD3, AD4, AD7, AD8, AD10, AD14 and AD21); (D) Southern analysis of transgenic plants for checking transgene integration using *adc* gene probe: DNA from plasmid (P), wild-type plant (Con) and different transgenic lines (AD1, AD2, AD3, AD7, AD14 and AD21); (E) Southern analysis of transgenic plants for checking transgene copy number using *hpt* gene as a probe. DNA from wild-type plant (Con) and different transgenic lines (AD3, AD4, AD5, AD7, AD8, AD9, AD10, AD14 and AD21); (F) RT-PCR analysis for transgene expression at transcript level using primers specific to *adc* gene: DNA from different transgenic plants (AD3, AD7 and AD14) and wild-type plant (Con), and 1-kb ladder (L).

Segregation of the transgene

The T_1 seeds obtained from the *adc* primary transgenic plants were analysed for the segregation of the transgene on solid MS basal medium supplemented with 10 mg l^{-1} hygromycin-B. The transgenic seeds germinated within 10 days of inoculation, and seedlings survived on the hygromycin-amended medium and showed segregation for the transgene. The control seeds did not germinate on hygromycin-amended medium. The transgenic lines

Table 1. Transgene segregation analysis in eggplant transgenic lines based on seed germination on MS basal medium containing 10 mg l^{-1} hygromycin

Transgenic line	Number of seeds inoculated	Number of seeds germinated	% Seed germination	Chi-square value
Control	40	3*	—	—
AD3	40	28	70	0.53
AD7	40	35	88	3.33
AD14	40	32	80	0.53

Chi-Square test was performed at $P>0.05$ for 3 : 1 ratio. Data were scored after one month of seed inoculation. * Although a few seeds of wild-type plants germinated, the seedlings remained in cotyledonary stage and did not grow further.

having a single copy of the tranagene segregated in 3 : 1 ratio, while other lines containing multiple transgene integration showed deviation from this ratio (Table 1). Such segregation pattern was also observed in transgenic plants expressing PA or other transgenes (Prasad et al. 2000; Prabhavathi et al. 2002; Singh 2005)

Polyamine metabolism in transgenic lines

The transgenic lines showed increase in the total PA levels. There was a significant increase in the Put (3–7 fold) and Spd (3–5 fold) levels, particularly in the conjugated fraction (Figure 2). In some of the lines, Spm levels were also increased, especially in the free fraction (2-fold). The increase in the conjugated fraction of Put was most pronounced in AD14 line with approximately 8-fold increase, and 6-fold increase was recorded in AD3 and AD7 lines. The transgenic lines AD3, AD7 and AD14 showed 3- to 4-fold increase in the ADC activity with a concomitant increase in the ODC activity (Figure 3A). Further, the activity of the PA catabolic enzyme, DAO was also increased in all the transgenic lines tested and the increase ranged from 3-fold in AD3 to 4-fold in AD7 and AD14 (Figure 3B).

The *adc* eggplant transgenic lines showed PA accumulation, due to the increase in ADC activity. In these transgenic lines, the increase in Put has also led to the increase in higher PAs, Spd and Spm, which could be due to the conversion of excess Put to Spd and Spm (Bhatnagar et al. 2002). Capell et al. (2004) reported that in transgenic rice plants over-expressing *adc* gene under the maize Ubi-1 promoter showed increased levels of not only Put but also Spd and Spm under drought stress. The increased Put pool probably extended beyond the critical threshold required to initiate the conversion of excess Put to Spd and Spm (Bassie et al. 2000). Similar observations were also reported in transgenic plants over-expressing *odc* gene (Kumria and Rajam 2002; Singh 2005). However, in some of the previous reports, although the over-expression of PA genes in transgenic plants has resulted in a significant increase in Put levels, but there was little alteration in Spd and Spm levels

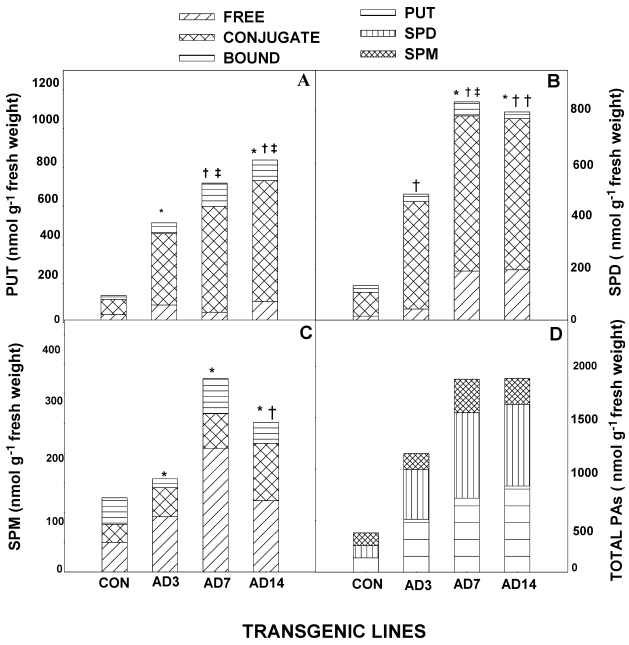

Figure 2. PA analysis in seedlings of wild-type (Con) and different transgenic lines (AD3, AD7 and AD14). A) Put, B) *Spd*, C) Spm and D) Total PAs. *, † and ‡—Significant differences from wild-type plants for free, conjugated and bound forms of PAs at 5% level.

(DeScenzo and Minocha 1993; Bhatnagar et al. 2001). Burtin and Michael (1997) reported that over-expression of *adc* in transgenic tobacco plants resulted in high levels of ADC transcript and ADC activity with the accumulation of agmatine (the direct product of ADC), but there was no increase in PA (Put, Spd and Spm) levels. These results suggest that the levels of Spd and Spm are under strict homeostatic regulation (Bhattacharya and Rajam 2006).

Stress response of transgenic lines

The T_1 seeds of *adc* eggplant transgenic lines obtained from self pollinated primary transformants were checked for tolerance against various abiotic stresses like salinity, drought, high and low temperature, and heavy-metal under both *in vitro* and *in vivo* growth conditions.

Salt tolerance

The surface-sterilized transgenic T_1 and wild-type seeds were inoculated on to solid MS basal medium containing 200 mM NaCl. The transgenic seeds showed germination after 15 days of inoculation and the seedlings grew well on salt-amended medium. The wild-type seeds on the other hand failed to germinate (Table 2).

One-month-old wild-type and T1 transgenic seedlings were transferred to test tubes containing vermiculite: soil and watered with 200 mM NaCl solution. The wild-type seedlings wilted and showed loss of chlorophyll within 15 days of salt stress. On the other hand, the transgenic seedlings survived and grew well. The wild-type and T_1

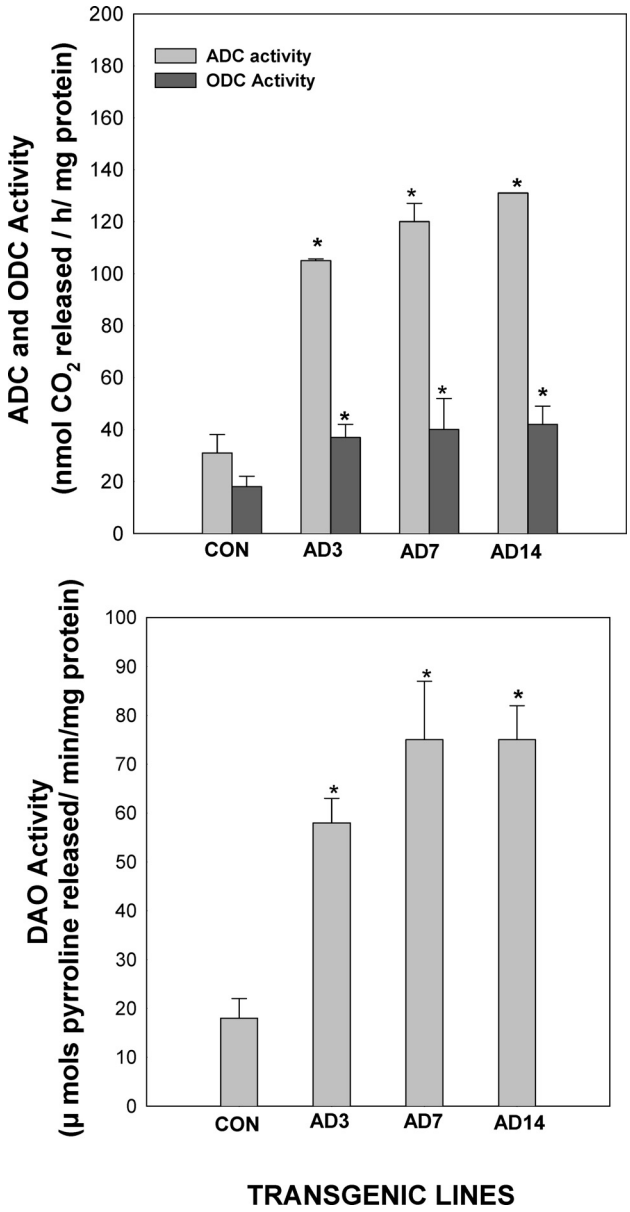

Figure 3. Activity of PA biosynthetic enzymes, ADC and ODC (top panel) and catabolic enzyme, DAO (bottom panel) in seedlings of wild-type (Con) and different transgenic lines (AD3, AD7 and AD14). * Significant differences from respective controls at 5% level.

transgenic seedlings were also used for salt stress assays using one-tenth strength liquid MS medium in the test tubes supplemented with 200 mM NaCl. The wild-type seedlings wilted after 10 days, but the transgenic seedlings survived and grew well under salt stress (Figure 4C). The survival of seedlings was also checked at a sub-lethal concentration of salt, 150 mM NaCl. The transgenic seedlings performed better and their growth in terms of shoot length, fresh and dry weight was greater than the wild-type seedlings (Table 3).

Drought tolerance

The seeds of T_1 transgenic lines and wild-type plants were inoculated in Petriplates containing Whatman No. 1 filter paper moistened with one-tenth strength liquid MS basal medium containing 10% PEG for drought stress

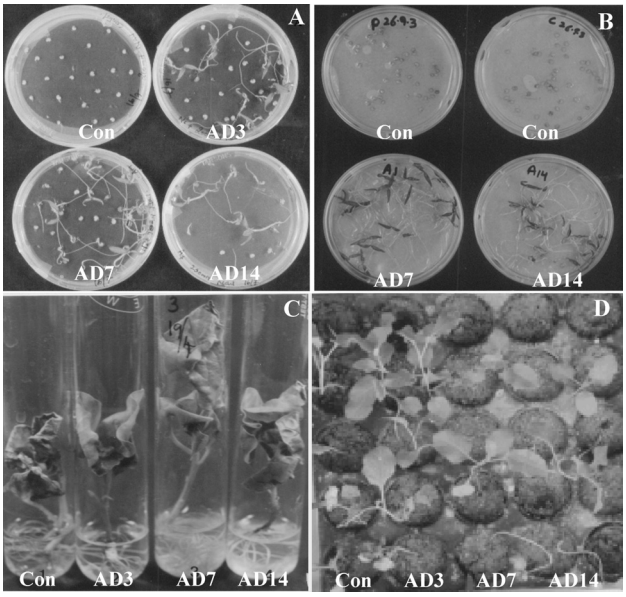

Figure 4. Abiotic stress tolerance and fungal disease resistance in wild-type seedlings (Con) and different transgenic lines (AD3, AD7 and AD14). Seed germination on solid MS basal medium containing 200 mM NaCl (A) or 10% PEG (B). Seedling growth in one-tenth strength liquid MS medium containing 200 mM NaCl (C). Results of *in vivo* fungal resistance assay with *F. oxysporum* (D). The seedlings of wild-type (Con) and different transgenic lines (AD3, AD7 and AD14).

Table 2. Germination of wild type and T_1 transgenic seeds of eggplant on MS basal medium amended with 200 mM NaCl (salt stress), 10% PEG (drought) and under high temperature 45 oC (heat) (after 3 days), low temperature 6–8°C (cold) and 2 mM $CdCl_2$ (heavy-metal) stress conditions

Abiotic stress	Control		Transgenic line					
			AD3		AD7		AD14	
	No. of seeds inoculated	No. of seeds germinated	No. of seeds inoculated	No. of seeds germinated	No. of seeds inoculated	No. of seeds germinated	No. of seeds inoculated	No. of seeds germinated
Salinity	20	0 (0)	20	15 (75)	20	18 (90)	20	16 (80)
Drought	30	0 (0)	30	15 (50)	30	18 (60)	30	19 (63)
Heat	50	0 (0)	50	25 (50)	50	28 (56)	50	21 (42)
Cold	40	10 (25)*	40	28 (70)	40	32 (80)	40	24 (60)
Heavy-metal	40	0 (0)	40	25 (63)	40	26 (65)	40	21 (53)

Values in parentheses represent percent seed germination.
* Seeds were germinated but there was no further growth.
The experiments were repeated twice with 6 seedlings in each transgenic line and control.

Table 3. Results of *in vivo* assay for abiotic stress tolerance of T_1 transgenic and wild-type seedlings grown in egg tray containg soil: vermiculite.

Abiotic stress	Control	Transgenic lines		
		AD3	AD7	AD14
Salt (150 mM NaCl)				
Shoot length (cm)	8.00±0.33 (100)	NT	10.10±0.03 (126)*	10.80±0.020(135)*
Fresh weight (g)	0.110±0.03 (100)	0.400±0.05 (364)*	0.490±0.003 (445)*	
Dry weight (g)	0.001±0.01 (100)	0.003±0.006 (300)*	0.003±0.001 (300)*	
Drought (7.5% PEG)				
Shoot length (cm)	8.20±0.03 (100)	NT	10.60±0.03 (129)*	9.80±0.040 (119)*
Fresh weight (g)	0.17±0.03 (100)	0.42±0.04 (247)*	0.37±0.004 (217)*	
Dry weight (g)	0.01±0.01 (100)	0.02±0.05 (200)	0.03±0.010 (300)	
Heat (45°C)				
Shoot length (cm)	4. 60±0.05 (100)	8.0±0.03 (173)*	9.00±0.03 (196)*	9.40±0.02 (204)*
Fresh weight (g)	0.023±0.05 (100)	0.040±0.03 (173)	0.050±0.03 (250)*	0.070±0.03 (304)*
Dry weight (g)	0.003±0.00 (100)	0.005±0.003 (167)	0.007±0.02 (233)*	0.006±0.00 (200)*
Cold (6–8°C)				
Shoot length (cm)	4. 00±0.06 (100)	9.20±0.03 (230)*	9.80±0.01 (245)*	10.00±0.06 (250)*
Fresh weight (g)	0.050±0.03 (100)	0.100±0.01 (200)*	0.120±0.01 (240)*	0.160±0.08 (320)*
Dry weight (g)	0.005±0.01 (100)	0.007±0.03 (140)	0.007±0.02 (140)	0.006±0.01 (120)
Heavy-metal ($1 mM CdCl_2$)				
Shoot length (cm)	5. 00±0.05 (100)	8.00±0.09 (160)*	9.00±0.06 (180)*	9.00±0.02 (180)*
Fresh weight (g)	0.20±0.01 (100)	0.30±0.01 (150)*	0.39±0.03 (200)*	0.40±0.02 (200)*
Dry weight (g)	0.02±0.00 (100)	0.04±0.01 (200)	0.05±0.01 (250)*	0.06±0.01 (345)*

Values represent the mean±SEM, and the data were scored after a period of one month. The experiments were repeated twice with 6 seedlings in each transgenic line and control.. *Significant differences from control at 5 % level. The percent of control values are in parentheses. NT, not tested.

assay. The transgenic seeds germinated and seedlings grew well, while the wild-type seeds did not germinate (Figure 4B). The percent seed germination in transgenic lines ranged from 50 to 63% (Table 2). The seedlings of T_1 transgenic lines were grown in vermiculite: soil mix and watered with sub-lethal (7.5%) as well as lethal (10%) concentration of PEG. At sub-lethal concentration, the growth of transgenic seedlings was better than that of wild-type seedlings (Table 3). At lethal concentration, the control seedlings became pale yellow and wilted after 15 days, but transgenic seedlings were healthy and continued to grow.

Dessication tolerance

In the dessication tolerance assay, the leaves from wild-type and transgenic plants were checked for their ability to retain water upon detachment from the plant for 24 h on Whatman No. 1 filter paper. The detached leaves from wild-type plants started drying within 24 h and retained less water, while the leaves of transgenic plants remained fresh even after 24 h of drying and retained higher water content (Table 5; Figure 5).

High temperature tolerance

The T_1 transgenic and wild-type seeds were inoculated on to solid MS basal medium and kept at high temperature (45°C) for different days (1, 2 and 3 days) and then incubated in the tissue culture room. Transgenic seeds that were heat-stressed for 1 day germinated within 1 week of inoculation, and those heat-stressed for 2 and 3 days germinated after 10 days of inoculation with the percent germination ranging from 42–56. The wild-type seeds, which were heat-stressed for 1 and 2 days germinated after 10 days of inoculation, but failed to germinate when heat-stressed for 3 days (Table 2). The T_1 transgenic and wild-type seedlings were subjected to heat stress for 3 h at 45°C and the transgenic seedlings showed better growth than the wild-type seedlings. The

Table 4. Disease index of wild-type and transgenic lines of eggplant for resistance against fungal wilt disease caused by *Fusarium oxysporum*

Transgenic line	Disease index
Control	1.00
AD3	0.78
AD7	0.76
AD14	0.72

Data were scored after one month of infection. The experiments were repeated thrice with 6 seedlings in each transgenic line and control.

Table 5. Water content (%) of leaves from wild-type and transgenic plants following dessication

Transgenic line	Water content (%) of leaves (Relative)
Control	24 (100)
AD3	58 (241)
AD7	59 (246)
AD14	56 (233)

Data were scored from six replicates after 24 h of dessication. Water content (%) of leaves was calculated by using formula: leaf weight before dessication—leaf weight after dessication/leaf weight before desiccation×100. The percent of control values are in parentheses.

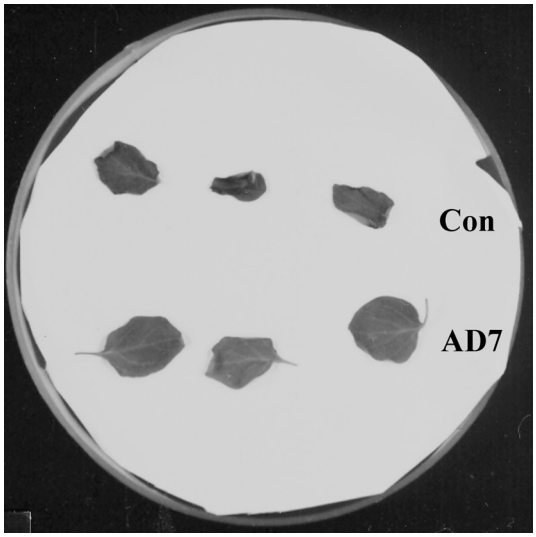

Figure 5. Dessication tolerance of transgenic lines. The leaves of wild-type (Con) and transgenic (AD7) plant.

data were scored after one-month of heat stress (Table 3).

Low temperature tolerance

The T_1 transgenic seeds along with wild-type seeds were inoculated on to solid MS basal medium, as well as in the egg trays containing soil: vermiculite and placed in the cold room (6–8°C) for 10 days, and later they were transferred to tissue culture room. Although, the seeds of both wild-type and transgenic plants germinated on MS basal medium following cold stress, the transgenic seeds germinated early by one week (Table 2). In the vermiculite: soil condition, the transgenic seeds germinated after 3 days of inoculation, while wild-type seeds germinated after one week. The transgenic seedlings also grew faster than the wild-type seedlings. The height, fresh and dry weight of the transgenic seedlings were found to be higher than the wild-type seedlings (Table 3).

Heavy-metal tolerance

The seeds of T_1 transgenic and wild type plants were inoculated on to solid MS basal medium containing different concentration of $CdCl_2$ (0.5, 1, 1.5 and 2 mM). Although, the seeds of transgenic plants germinated on MS basal medium containing all the concentrations (0.5, 1, 1.5 or 2 mM) of $CdCl_2$, no seed germination was observed in case of wild type at 2 mM $CdCl_2$ (Table 2).

The survival and growth of the transgenic seedlings were also checked under heavy-metal stress. The T_1 transgenic and wild-type seedlings, grown in vermiculite: soil were poured with one-tenth strength liquid MS medium containing different concentrations of $CdCl_2$. At 1 mM $CdCl_2$, the growth of the transgenic seedlings was better than that of the wild-type seedlings (Table 3).

The improved performance of eggplant transgenic seeds and seedlings under the various abiotic stress conditions may be attributed to PA accumulation. In fact, PA accumulation due to the increase in ADC activity has been reported in plants under stress condition (Rajam 1997; Kumar et al. 2006). It has also been reported that the abiotic stresses cause increase in the production of reactive oxygen species, ionic imbalance, damage to membranes and macromolecules and changes in osmolarity (Tiburcio et al. 1993; 1994, Zhu 2001) and PAs play an important role in the regulation of these processes (Kumar et al. 2006). Thus, the enhanced abiotic stress tolerance in *adc* eggplant transgenic lines may be due to the involvement of PAs in the scavenging of free radicals (Das and Misra 2004), membrane stabilization (Besford et al. 1993), and protection of macromolecules (Kumar et al. 2006) under stress condition. Further, PAs may be involved in signal transduction pathways associated with abiotic stress tolerance, probably through the activation of protein kinases and trancription factors (Datta et al. 1987; Sudha and Ravishankar 2002; Childs et al. 2003; Kasukabe et al. 2004).

Although there are many reports on transgenic plants expressing PA genes, only few of them contained the data on the response of PA transgenic plants to abiotic stresses (reviewed by Kumar et al. 2006; Bhattacharya and Rajam 2006). One of the early reports is by Capell et al. (1998), in which rice transgenic plants over-expressing oat *adc* gene were found to be drought tolerant. Similarly, transgenic rice plants expressing *adc* transgene under the control of an ABA responsive element showed enhanced tolerance to abiotic stresses (Roy and Wu 2001). The over-expression of *odc* gene in tobacco (Kumria and Rajam 2002) and eggplant (Singh 2005) has resulted in a significant increase in Put and Spd and conferred salt tolerance. The transgenic rice (Roy and Wu 2002) and tobacco (Waie and Rajam 2003) over-expressing *samdc* gene have showed increased PA levels and tolerance to salinity and drought. Recently, the introduction of *Spd syn* gene into tobacco (Franceschetti et al. 2004), *Arabidopsis* (Kasukabe et al. 2004) and sweet potato (Kasukabe et al. 2006) has led to the increased tolerance against multiple abiotic stresses. Our results and the previous data suggest that PAs play a major role in abiotic stress tolerance.

Resistance to a fungal pathogen

The transgenic plants were also tested for resistance against Fusarium wilt under both *in vitro* and *in vivo* growth conditions. In *in vitro* root-dip method, the fungal mycelium was observed within one week of inoculation in the wild-type seedlings, while the mycelial development got delayed by 3 days in case of transgenic lines. The wild-type seedlings completely wilted and

died after 15 days of inoculation. On the other hand, the transgenic lines (AD3, AD7 and AD14) were healthy even after one-month of fungal spore inoculation, although slight wilting of the lower leaves were observed after two weeks. In the soil mix method, the wild-type seedlings showed wilting symptoms in the lower leaves after one week of inoculation and after one month the entire stem had developed necrosis and completely wilted. While the transgenic plants did not show any disease symptoms after one week of inoculation, although slight necrosis and wilting of the lower leaves was observed after 10 days of inoculation. In both the methods, the transgenic lines AD3, AD7 and AD14 showed increased resistance to Fusarium wilt when compared to wild-type seedlings (Figure 4D). However, the severity of infection and resistance varied between transgenic lines (Table 4), which may be due to the difference in transgene expression and cellular PA levels (Waie and Rajam 2003).

The eggplant transgenic seedlings exhibited increased resistance against Fusarium wilt as compared to wild-type seedlings. These results are in agreement with a previous report where the introduction of *samdc* gene in tobacco conferred resistance against Fusarium and Verticillium wilts (Waie and Rajam 2003). It has been shown that PAs play a protective role against fungal and viral infection in plants (Martin-Tanguy 1985; Kumar et al. 2006). The levels of PAs, particularly PA conjugates are known to increase during fungal infections and are implicated in providing resistance against various plant pathogens. In fact, PA conjugates with low molecular weight phenolic compounds like hydroxycinnamic acid (HCA) (known as HCA amides) show anti-microbial property (Martin-Tanguy 1985; Rajam 1997). Moreover, Spm is known to be an endogenous inducer of pathogenesis-related (PR) proteins, PR-1, PR-2, PR-3 and PR-5 (Yamakawa et al. 1998) and also probably involved in inducing the caspase activity and hence hypersensitive response (Walters 2003). Further, increase in PA catabolic enzyme, DAO in transgenic plants over-expressing PA genes (Waie and Rajam 2003), suggests the possible involvement of this hydrogen peroxide-producing enzyme in plant defense against pathogens (Walters 2003). Thus, the enhanced resistance to Fusarium wilt in eggplant transgenic seedlings could be due to an integrated mechanism rather than a single event. However, high titers of PAs, especially conjugated fraction of PAs have been recorded in the eggplant transgenic lines, suggesting their involvement in fungal resistance.

In summary, the introduction of *adc* gene in eggplant resulted in PA accumulation due to the increase in ADC activity and exhibited enhanced tolerance to multiple abiotic stresses and a fungal pathogen. Thus, the PA biosynthesis can be engineered for producing stress-tolerant plants.

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References

Bajaj S, Rajam MV (1995) Efficiant plant regeneration from long-term callus cultures of rice by spermidine. *Plant Cell Rep* 14: 717–720

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 oat *adc* cDNA constitutively. *Transgenic Res* 9: 33–42

Besford RT, Richardson CM, Campos JL, Tiburcio AF (1993) Effect of polyamines on stabilization of molecular complexes in thylakoid membranes of osmotically stressed leaves. *Planta* 189: 201–206

Birecka H, Bitonti AJ, McCann PP (1985) Assaying ornithine and arginine decarboylases in plant species. *Plant Physiol* 79: 509–514

Bhatnagar P, Glasheen BM, Bains SK, Long SL, Minocha R, Walter C, Minocha SC (2001) Transgenic manipulation of polyamine metabolism in poplar (Populus nigra X maximoviczii) cells. *Plant Physiol* 125: 2139–2153

Bhatnagar P, Minocha R, Minocha SC (2002) Genetic manipulation of polyamines in poplar cells. The regulation of putrescine catabolism. *Plant Physiol* 128: 1455–1469

Bhattacharya E, Rajam MV (2006) Polyamine biosynthesis as a novel target for engineering crop plants for abiotic stress tolerance. *J Plant Biol* 33: 1–7

Bouchereau A, Aziz A, Larher F, Martin-Tanguy J (1999) Polyamines and environmental challenges: recent developments. *Plant Sci* 140: 103–125

Bradford MM (1976) A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein dye binding. *Ann Biochem* 72: 248–254

Burtin D, Michael AJ (1997) Over-expression of arginine decarboxylase in transgenic plants. *Biochem J* 325: 331–337

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

Capell T, Bassie L, Christou P (2004) Modulation of the polyamine biosynthetic pathway in transgenic rice confers tolerance to drought stress. *Proc Natl Acad Sci USA* 101: 9909–9914

Childs AC, Mehta DJ, Grener EW (2003) Polyamine-dependent gene expression. *Cell Mol Life Sci* 60: 1394–1406

Chinnusamy V, Jagendorf A, Zhu JK (2005) Understanding and improving salt tolerance in plants. *Crop Sci* 45: 437–438

Chomczynski P, Sacchi N (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem* 162: 156–159

Das KC, Mishra HP (2004) Hydroxyl radical scavenging and

singlet oxygen quenching properties of polyamines. *Mol Cell Biochem* 262: 127–133

Datta N, Schell MB, Roux SJ (1987) Spermine stimulation of a nuclear NII kinase from pea plumules and its role in the phosphorylation of a nuclear polypeptide. *Plant Physiol* 84: 1397–1401

DeScenzo RA, Minocha SC (1993) Modulation of cellular polyamines in tobacco by transfer and expression of mouse ornithine decarboxylase cDNA. *Plant Mol Biol* 22: 113–117

Doyle JJ, Doyle JL (1990) Isolation of plant DNA from fresh tissue. *Focus* 12: 13–15

Flowers TJ (2004) Improving crop salt tolerance. *J Exp Bot* 55: 307–319

Franceschetti M, Fornale S, Tassoni A, Zuccherelli K, Mayer M.J, Bagni N (2004) Effects of spermidine synthase over-expression on polyamine biosynthetic pathway in tobacco plants. *J Plant Physiol* 161: 989–1001

Igarashi K, Kashiwagi K (2000) Polyamines: mysterious modulators of cellular functions. *Biochem Biophys Res Commun* 271: 559–564

Kasukabe Y, He L, Nada K, Misawa S, Ihara I, Tachibana S (2004) Overexpression of spermidine synthase enhances tolerance to multiple environmental stresses and up-regulates the expression of various stress-regulated genes in transgenic *Arabidopsis* thaliana. *Plant Cell Physiol* 45: 712–722

Kasukabe Y, He L, Watakabe Y, Otani M, Shimada T, Tachibana S (2006) Improvement of environmental stress tolerance of sweet potato by introduction of genes for spermidine synthase. *Plant Biotechnol* 23: 75–83

Kumar SV, Sharma ML, Rajam MV (2006) Polyamine biosynthetic pathway as a novel target for potential applications in plant biotechnology. *Physiol Mol Biol Plants* 12: 13–28

Kumria R, Rajam MV (2002) Ornithine decarboxylase transgene in tobacco affects polyamine metabolism, *in vitro* morphogenesis and response to salt stress. *J Plant Physiol* 159: 983–990

Masgrau C, Altabella T, Fareas R, Flores D, Thompson AJ, Besford RT, Tiburcio AF (1997) Inducible overexpression of oat arginine decarboxylase in transgenic tobacco plants. *Plant J* 11: 465–473

Martin-Tanguy J (1985) The occurrence and possible function of hydroxycinnamoyl acid amides in plants. *Plant Growth Regul* 3: 381–399

Naik BI, Goswami RG, Srivastava SK (1981) Rapid and sensitive colorimetric assay of amine oxidase. *Anal Biochem* 101: 146–148

Noh E, Minocha SC (1994) Expression of a human S-adenosylmethionine decarboxylase cDNA in transgenic tobacco and its effects on polyamine biosynthesis. *Transgenic Res* 3: 26–35

Panicot M, Masgrau C, Borrell A, Corderio A, Tiburcio AF, Altabella T (2002) Effects of putrescine accumulation in tobacco transgenic plants with different expression levels of oat arginine decarboxylase. *Physiol Plant* 114: 281–287

Prabhavathi V, Yadav JS, Kumar PA, Rajam MV (2002) Abiotic stress tolerance in transgenic eggplant (*Solanum melongena* L.) by introduction of bacterial mannitol phosphodehydrogenase gene. *Molecular Breed* 98: 137–147

Prasad KVSK, Sharmila P, Kumar PA, Pardha Saradhi P (2000) Transformation of *Brassica juncea* (L.) Czern with bacterial codA gene enhance its tolerance to salt stress. *Molecular Breed* 6: 489–499

Rajam MV (1997) Polyamines. In: Prasad, MNV (ed) *Plant Ecophysiology*, John Wiley & Sons, New York, pp 343–374

Rajam MV, Dagar S, Waie B, Yadav JS, Kumar PA, Shoeb F, Kumria R (1998) Genetic engineering of polyamine and carbohydrate metabolism for osmotic stress tolerance in higher plants. *J Biosci* 23: 473–482

Roy M, Wu R (2001) Arginine decarboxylase transgene expression and analysis of environmental stress tolerance in transgenic rice. *Plant Sci* 160: 189–193

Roy M, Wu R (2002) Over-expression of S-adenosylmethionine decarboxylase gene in rice increases polyamine levels and enhances sodium chloride stress tolerance. *Plant Sci* 163: 987–992

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning: A laboratory manual. 2nd ed. Cold Spring Harbour, New York: Cold Spring Harbor Laboratory Press

Singh S (2005) Development of eggplant transgenic plants for abiotic stress tolerance by metabolic engineering of polyamines. PhD Thesis, University of Delhi. Delhi, India.

Sudha G, Ravishankar GA (2002) Involvement and interaction of various signaling compounds on the plant metabolic events during defense response, resistance to stress factors, formation of secondary metabolites and their molecular aspects. *Plant Cell Tissue Org Cult* 71: 181–212

Thomas T, Thomas TJ (2001) Polyamines in cell growth and cell death: molecular mechanisms and therapeutic applications. *Cell Mol Life Sci* 58: 244–258

Tiburcio AF, Campos JL, Figueras X, Besford RT (1993) Recent advances in the understanding of polyamine functions during plant development. *Plant Growth Regul* 12: 331–340

Tiburcio AF, Besford RT, Capell T, Borell A, Testillano PS, Risueno MC (1994) Mechanisms of polyamine action during senescence responses induced by osmotic stress. *J Exp Bot* 45: 1789–1800

Walters DR (2003) Polyamines and plant disease. *Phytochem* 64: 97–107

Waie B, Rajam MV (2003) Effect of increased polyamine biosynthesis on stress response in transgenic tobacco by introduction of human S-adenosylmethionine gene. *Plant Sci* 164: 727–734

Yamakawa H, Kamada H, Satoh M, Ohashi Y (1998) Spermine is a salicylate-independent endogenous inducer for both tobacco acidic pathogenesis-related proteins and resistance against tobacco mosaic virus infection. *Plant Physiol* 118: 1213–1222

Zhu JK (2001) Plant salt tolerance. *Trends Plant Sci* 6: 66–71