Engineering Polyamines For Abiotic Stress Tolerance

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ABSTRACT *Crop plants are known to be affected by various types of stresses both natural and manmade. Abiotic stresses like drought and salinity have already been pronounced worldwide causing significant crop losses. Plants adapt to environmental stresses by accumulation of low molecular weight osmolytes such as proline and polyamines (PAs). In past two decades, polyamines have received considerable attention due to its wide range of functions in cellular metabolism, especially in response to wide variety of environmental stresses. Several mutant studies have shown positive changes in activities of PA biosynthetic enzymes for mitigation of stress condition. Studies with exogenous application of PAs have shown the successful alleviation of stress-induced damaging effects on plants. Genes encoding PA biosynthetic enzymes has been well characterised and being used in transgenic approach to get more insights into the role PAs under stress condition. This review focuses on recent developments in transgenic approaches that are being followed with PAs for enhanced stress tolerance in crop plants. Lastly, the novel transgenic approaches for improved stress tolerance have been depicted.*

Introduction

Crop plants are known to be affected by various types of stresses both natural and manmade. Abiotic stress represents the most devastating phenomenon, causing several amplitudes of losses worldwide. Drought and salinity are already widespread in many regions and expected to cause significant losses to crop plants. Generally plants respond to dehydration-stress conditions like salinity and drought stress by a variety of physiological and biochemical changes, most notably by accumulation of low-molecular-weight osmolytes such as proline and polyamines (PAs) (Roy & Wu, 2001). In recent years, much attention has been focused on the involvement of PAs in response to wide variety of stresses especially abiotic stresses.

Polyamines- compounds with diverse functions

PAs are are known to be essential for growth and development in prokaryotes and eukaryotes especially in plants where diamine putrescine (Put), triamine spermidine (Spd) and tetraamine spermine constitute the major PAs (Amooaghaie, 2011). These PAs may be present in free, soluble conjugated (covalently conjugated to small molecules such as phenolic) and insoluble bond (covalently bound to macromolecules such as nucleic acid and proteins) forms (Duan et al., 2008). PAs have found implicated in wide range of physiological processes of plant growth and development which includes cell division, embryogenesis, root formation and growth, floral initiation, floral development, pollen development, fruit development, seed formation, vegetative growth, senescence etc. (reviewed in detail, Bais & Ravishankar, 2002). None the less, PA are known to carry out diverse cellular functions like maintenance of conformation and protection of DNA (D'Agostino et al., 2005), RNA stabilization (Marton & Feuerestein, 1986) and acts as a key factor in protein synthesis (Park et al., 1996) and cell cycle progression (Thomas & Thomas, 1994). PAs and related

compounds are regarded as a type of growth promoters and secondary messengers (Galston, 1983; Davies, 2004).

PA being cationic in nature can associate with anionic components of membrane such as phospholipids thereby stabilizing the bilayer surface and retarding membrane deterioration (Amooghaie & Moghym, 2011). PAs as an antioxidant may protect against oxidative degradation and membrane damage, resulting in maintenance of homeostasis in plant cells (Rodríguez-Kessler et al., 2006). Under weak stress conditions, PAs were shown to function mainly as a scavenger of free superoxide radicals, whereas under strong stress conditions PAs were shown to act as positive regulators of antioxidant genes (Tkachenko & Nesterova, 2004; Liu et al., 2007). PAs are also known to act as cellular protectors by binding to cations like Cu(II) and Fe(II) (Lovaas, 1997).

Studies with exogenous application of PAs has resulted in alleviation of stress-induced growth inhibition (Basra et al., 1997; Wang et al., 1993), and the response was mainly attributed to the protection of membrane and minimization of oxidative stress damage due to the exogenous PA application (Nayyar et al., 2005; Amooghaie & Moghym, 2011; Amooghaie, 2011) which clearly shows the stress adaptive role of PAs. The inhibitors of PA biosynthetic pathway such as difluoromethyl ornithine (DFMO) and difluoromethyl arginine, the suicide inhibitors of ODC and ADC respectively, are very well known to cause significant damage to crop plants which includes increase in stress injury, severely impaired plant growth and loss of thermotolerance (Amooghaie & Moghym, 2011), reduced shoot and root growth in seedlings (Amooghaie, 2011). Spd and Spm are the agents that are believed to protect plants under stress and increased levels of Spd and Spm are generally associated with enhanced plant tolerance to unfavourable

conditions (Jiménez-Bremont et al., 2007). It has been found that wild type plants responded to the onset of drought stress where increase in the endogenous Put levels was observed, but this was not sufficient to trigger the conversion of Put into Spd and Spm (Capell et al., 2004). Spm is reported to show beneficial effect through inhibition of NADPH peroxidase (Kitada et al., 1979), which is known to cause oxidation of NADPH leading to generation of superoxide radical (O_2^-) (Kawano et al., 2001). Regarding the enzymes, Put synthesis enzyme arginine decarboxylase (ADC) has been found to be induced which shows the increased activity during stress condition and is being suggested as 'general stress enzyme' in cereals (Flores et al., 1989; Galston & Kaur Sahwney, 1990; Rajam, 1997).

Abiotic stress tolerance by PA may be due to its involvement in signal transduction pathways that are associated with abiotic stress tolerance, through the activation of protein kinases and transcription factors (Kasukabe et al., 2004; Prabhavathi & Rajam, 2007). Several studies have suggested that hydrogen peroxide (H_2O_2) plays a central role in the orchestration of HR by mediating signal transduction in the activation of defence related genes and H_2O_2 is known to be produced during the polyamine degradation pathway mediated by either flavine-containing polyamine oxidases (PAO) or copper-containing amine oxidases (CuAO) (Fu et al., 2011).

Polyamine related genes and their expression during stress condition
Availability of complete genome sequence has enabled the identification, isolation and further characterization of the genes involved in PA biosynthesis for the improvement of crop plants to combat various stresses. Genes involved in PA biosynthesis have been revealed in different plant species, especially in Arabidopsis. With exception of ODC, genes encoding all of the enzymes have been characterized in Arabidopsis which has no detectable ODC activity (Hanfrey et al., 2001). In Arabidopsis, two genes encode ADC (*ADC1* and *ADC2*) (Janowitz et al., 2003; Piotrowski et al., 2003). There are duplicated genes which code for SPDS (*SPDS1* and *SPDS2*) (Panicot et al., 2002), whereas SAMDC is encoded by at least four genes (*SAMDC1*, *SAMDC2*, *SAMDC3* and *SAMDC4*) (Urano et al., 2004). ODC genes coding for ornithine decarboxylase have been found in several species such as thorn-apple (*Datura stramonium*; Michael et al., 1996), tomato (*Lycopersicon esculentum*; Alabadi & Carbonell, 1998) and tobacco (*Nicotiana tabacum*; Malik et al., 1996). Apart from this there are enzymes which are encoded by single genes. These enzymes include Spm synthase, thermospermine synthase, agmatine iminohydrolase and N-carbamoylputrescine amidohydrolase (Hanzawa et al., 2000; Panicot et al., 2002; Janowitz et al., 2003; Piotrowski et al., 2003; Knott et al., 2007).

Rice cultivars resistant to salinity stress showed an increase in ADC activity under NaCl stress and there was higher accumulation of ADC transcripts as compared to susceptible cultivars which did not show any increase in ADC activity (Chattopadhyay et al., 1997). In Arabidopsis, an increase in expression of arginine decarboxylase 2 (*ADC2*) gene was observed in response to mechanical wounding and methyl jasmonate (JA) where level of Put and mRNA level of *ADC2* was found to be increased, while no induction of *ADC1* gene or other genes coding for Spd and Spm synthase was observed (Sasaki et al., 2001; Perez-Amador et al., 2002). In Arabidopsis *ADC2* expression was strongly induced by dehydration, wounding, high salinity and K+

deficiency (Perez-Amador et al., 2002; Urano et al., 2003; Armengaud et al., 2004; Alcazar et al., 2006), whereas *ADC1* seems to be mainly induced by cold (Hummel et al., 2004). In addition to this, number of studies have reported the increase in Put levels with elevated ADC activity upon experiencing K+ deficiency (Smith, 1970; Murthy et al., 1971; Klein et al., 1979; Young & Galston, 1984; Watson & Malmberg, 1996), acid feeding (Smith & Sinclair, 1967; Young & Galston, 1983), exposure to SO_2 (Priebe et al., 1978) or NH+ nutrition as well as different ionic stresses (Smith, 1963, 1984). In an another study, PA biosynthetic enzyme ODC was also found to be induced upon exposure to ozone and hydrogen fluoride leading to elevated PA levels as a mechanism to minimize the damaging effect in wheat (An & Wang, 1997). Similarly SPMS was also found to be expressed under dehydration and high salinity (Urano et al., 2003; Alcazar et al., 2006). In case of gene coding for SPDS, *SPDS1* was having constitutive expression under different stresses, whereas gene *SPDS2* was having increased expression under dehydration. In case of SAMDC genes, *SAMDC1* and *SAMDC2* have been positively induced by cold (Vergnolle et al., 2005).

Cloning of polyamine related genes and modulation of polyamine biosynthesis
In an attempt to clone SAMDC cDNA from *Saccharomyces cerevisiae*, transgenic tomato produced 1.7 to 2.4 fold higher levels of Spd and Spm than wild-type plants under high temperature stress and contributed to better stress tolerance (Cheng et al., 2009). Transgenic tobacco plants with human *SAMDC* showed 2 to 4 fold increase in SAMDC activity, 2 to 3 fold increase in Spd levels and significant reduction in Put levels (Noh & Minocha, 1994). Kumar et al. (1996) carried out studies in potato with potato *SAMDC* gene in sense and antisense orientation. In case of sense *SAMDC*, transgenic plants showed increased levels of *SAMDC* transcripts and changed PA biosynthesis, whereas decreased *levels of SAMDC* transcripts and PA, abnormal phenotypes were observed with antisense *SAMDC*.

Polyamines and plant response to abiotic stress
Several studies have shown the involvement of polyamines in plant response to abiotic stresses like drought (Yamaguchi et al., 2007; Yang et al., 2007), salinity (Kuznetsov & Shevyakova, 2007), high temperature (Cheng et al., 2009), low temperature (Imai et al., 2004; Nayyar, 2005), oxidative stress (Rider et al., 2007) and metal toxicity (Wang et al., 2007; Shevyakova et al., 2010). Several reviews have pointed out that PA accumulation was found to occur under various abiotic stresses regimes (Liu et al., 2007; Kuznetsov & Shevyakova, 2007; Groppa & Benavides, 2008; Gill & Tuteja, 2010; Hussain et al., 2011).

4.1 Abiotic stress tolerance through polyamine engineering
As depicted above, PAs have been found to confer tolerance to various stresses. However, the exact role of PA and their mode of action at the molecular level is not clearly understood. 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 the use of specific inhibitors of PA biosynthetic enzymes (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 (Prabhavathi & Rajam, 2007). Genetic engineering with PA biosynthesis

genes provide a powerful strategy to identify possible role of PA in stress response (Groppa & Benavides, 2008). Most of the genes involved in PA biosynthesis have been cloned and subsequently overexpressed to confer enhanced tolerance to environmental stresses. These include arginine decarboxylase (ADC) (Masgrau et al., 1997; Roy & Wu, 2001; Capell et al., 2004), ornithine decarboxylase (ODC) (Kumria & Rajam, 2002), S-adenosylmethionine decarboxylase (Roy & Wu, 2002; Waie & Rajam, 2003; Torrigiani et al., 2005; Wi et al., 2006; Cheng et al., 2009) and spermidine synthase (SPDS) (Franceschetti et al., 2004; Kasukabe et al., 2004, 2006) in rice, tobacco, Arabidopsis, tomato and sweet potato.

Polyamine engineering can be done with single biosynthetic gene for specific stress tolerance as well as for multiple stress tolerance. In case of single biosynthetic gene for specific stress tolerance, Capell et al. (2004) have showed that transgenic rice expressing *Datura stramonium adc* gene has high levels of PA under drought than wild type plants which was taken as improved drought tolerance. In another study, Roy & Wu (2002) have showed introduction of *Tritordeum SAMDC* gene into rice resulted in 3-4 fold increase in Spd and Spm levels and also showed normal growth and development under NaCl stress. In case of single PA biosynthetic gene for multiple stress tolerance, over-expression of SPDS gene from *Cucurbita ficifolia* and carnation in Arabidopsis, sweet potato and tobacco have showed broad spectrum tolerance to abiotic stresses like drought, chilling, freezing, salinity and oxidative stress (Kasukabe et al., 2004, 2006; Wi et al., 2006). Similarly Prabhavathi & Rajam (2007) have reported that transgenic eggplant harboring oat *adc* showed increased tolerance to drought, salinity, low and high temp and heavy metals.

Arginine decarboxylase (ADC), the first enzyme in PA biosynthesis pathway has been cloned from oat, tomato, pea, Arabidopsis and soybean and tried for stress tolerance in heterologous plant species. Capell et al. (1998) reported the drought tolerance in transgenic rice plants overexpressing oat (*Avena sativa* L.) *adc* gene. Similarly, transgenic rice plants expressing *adc* transgene under the control of an ABA responsive element showed enhanced tolerance to abiotic stresses (Roy & Wu, 2001). Oat *ADC* gene introduced into rice (*Oryza sativa* L.) under the control of an ABA-inducible promoter led to stress-induced upregulation of ADC activity with increased Put and total polyamine accumulation in transgenic plants, and showed increased biomass under salinity stress conditions (Roy & Wu, 2001). Overexpression of *PtADC* gene in tobacco and tomato showed significantly higher accumulation of Put and total polyamines with reduction in reactive oxygen species (ROS) accumulation and rendered transgenic plants more tolerant to dehydration and drought stress (Wang et al., 2011). Decrease in ROS levels was attributed to the higher levels of free polyamines. More importantly transgenic plants showed elevated transcript levels of three stress-responsive genes (APX, DREB, ERF5) further strengthening the stress tolerance response. Reduction in ROS due to higher levels of PAs may be because of the fact that PAs form a ternary complex through the interaction with metal ions and the phospholipid polar head, leading to the inhibition of metal ion auto-oxidation and subsequent supply of electron for ROS generation. This represents a direct role of PA in reducing ROS generation under stress. secondly, as an indirect role, PAs activate the antioxidant enzymes. In this regard, the stress-derived ROS may be subjected to more robust detoxification, leading to a lower level of ROS accumulation (Wang et al., 2011). Transgenic eggplant expressing oat ADC gene exhibited increased PA content and enhanced level of tolerance to multiple abiotic stresses such as salinity, drought, low and high temperature and heavy metal stress, as well as to biotic stress agent *Fusarium oxisporium*, known to cause wilt disease in plants (Prabhavathi & Rajam, 2007). Transgenic Arabidopsis plants over-expressing the homologous Arginine decarboxylase 2 (*ADC2*) gene showed much higher accumulation of Put and reduction in water loss by transpiration, and conferred drought tolerance. In transgenic lines there was no change in Spd and Spm content even under drought stress, and Put accumulation was correlated with different degree of resistance by plant to dehydration while transgenic plants showed reduction in transpiration rate and stomata conductance (Alcázar et al., 2010). Transgenic rice plants expressing *adc* from *Datura*, which is known for its better water stress tolerance, were found to accumulate PAs to much higher degree than its wild-type counterpart, thus achieving higher level tolerance to water stress than wild-type plants (Capell et al., 2004). Relatively less work has been carried out on transgenic expression of ornithine decarboxylase gene. The over-expression of *odc* gene in tobacco (Kumria & Rajam, 2002) and eggplant (Singh, 2005) has resulted in a significant increase in Put and Spd and conferred salt tolerance.

Genes for SAMDC have been cloned from potato, spinach, periwinkle, carnation and *Tritordeum*. It has been reported that, overexpression of *SAMDC* in tobacco could induce higher mRNA levels of antioxidant enzymes like ascorbate peroxidase, superoxide dismutase and glutathione S-transferase in transgenic plants and could help to alleviate stress induced damaging effects (Wi et al., 2006).

Overexpression of heterologous *SAMDC* gene in plants generally results in improving tolerance to abiotic stresses such as salt (Roy & Wu, 2002), drought (Waie & Rajam, 2003), acidic and oxidant stress (Wi et al., 2006) as well as high temperature stress (Cheng et al., 2009). The primary sequences of *SAMDC* gene are similar in higher plants, therefore so as to overcome the homologous depression, the *SAMDC* gene from heterologous source (*Saccharomyces cerevisiae*) was selected and introduced in tomato plants for high temperature tolerance (Cheng et al., 2009). Transgenic tomato plants expressing *SAMDC* cDNA from *S. cerevisiae* produced 1.7 to 2.4 fold higher levels of Spd and Spm than wild-type plants under high temperature stress, along with enhanced antioxidant enzyme activities and protection of membrane lipid peroxidation, ultimately resulting in enhanced protection against high temperature stress. Transgenic plants also showed improved efficiency of CO_2 assimilation. Leaf electrolyte leakage (EL) measurement showed significantly lowered EL in transgenic plants which suggests that overexpression of *SAMDC* in tomato plants resulted in enhanced protection of cell membrane permeability. Transgenic rice (Roy & Wu, 2002) and tobacco (Waie & Rajam, 2003) over-expressing *samdc* gene have showed increased PA levels and tolerance to salinity and drought.

In some reports, Spd has been found associated more closely with stress tolerance in plants than its other counterparts, Put and Spm (Shen et al., 2000; Li and Chen, 2000; He et al., 2002; Martinez-Tellez et al., 2002; Kasukabe et al., 2004). Recently, Transgenic tomato overexpressing apple spermidine synthase 1 (*MdSPDS1*) showed accumulation of significantly more polyamines, namely Spm and Spd and were capable to maintain higher expres-

sion of *SlApx* genes which resulted in higher APX activity leading to salt tolerance (Neily et al., 2011). In conclusion it was suggested that elevated APX activity might contribute largely to the protection against oxidative stress generated by NaCl stress and thus confer salinity tolerance in tomato overexpressing *MdSPDS1*. Apart from this, there are reports that 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. Overexpression of SPDS gene, *FSPD1* from *Cucurbita facifolia* in Arabidopsis exhibited significant increase in SPDS activity and Spd content along with enhanced tolerance to various stresses including chilling, freezing, salinity, hyperosmosis, drought and paraquat toxicity (Kasukabe et al., 2004). More importantly, genes for stress responsive transcription factors like DREB and stress protective proteins like rd29A have been found to be transcribed in transgenic plants compared to wild-type plants under chilling stress. Particularly SPDS has been suggested as a reliable target for genetic manipulation of PA biosynthesis due to its controlled synthesis to avoid toxic effects caused by excessive PA accumulation (Kasukabe et al., 2004).

Though transgenic approaches, particularly overexpression of PA biosynthesis genes, towards achieving environmental stress tolerance has shown much promise, still it is not devoid of bottlenecks which include complex regulation of PA biosynthesis in relation to endogenous substrate availability, developmental stage and tissue specific expression (Duan et al., 2008; Bhatnagar et al., 2001).

4.2 Novel approaches for engineering polyamines for abiotic stress tolerance

Genes that show the association with and have ability to confer stress tolerance can be introduced into the crop species, which seems to be a promising approach. At some point of time, the introduction of single gene would not confer the expected stress tolerance. This will necessitate the introduction of multiple transgenes into the plants, the approach commonly known as gene pyramiding. In another approach, a totally new or an uncommon metabolic pathway(s) can be introduced into the plants through genetic engineering which will come up with innovative way for stress tolerance (Rajam et al., 1998).

Until now, PA engineering for enhanced stress tolerance in plant has been mainly concentrated on using constitutive promoters with a view to obtain higher expression of genes leading to higher accumulation of PA. Excessive accumulation of PAs, than the required levels may lead to toxic effect on plants, as well as constitutive expression,

even under non-stress condition may impose unnecessary burden on plant metabolism. Yang et al. (2010) proposed the controlled overexpression of PA biosynthetic genes instead of constitutive overexpression to avoid over-accumulation of PA which is toxic to plants. Roy & Wu (2001) stressed the necessity to use a stress-inducible promoter to drive the expression of PA biosynthetic genes to study stress tolerance.

A novel approach would be to express the transgene with such a promoter, possessing stress-responsive element(s) that will boost the transgene expression under stress condition. In accordance with this, Parez-Amador et al. (2002) analysed the promoter sequences of both ADC genes (*ADC1* and *ADC2*) from Arabidopsis and revealed the presence of an ABA response element (ABRE) core sequence in *ADC2* gene promoter, which was absent in *ADC1* gene promoter. Later it was proposed that presence of ABRE core sequence in *ADC2* gene promoter would have led to the induction of *ADC2* and not in case of *ADC1*. Hummel et al. (2004) and Alcazar et al. (2006) also suggested the differential expression due to different promoter context of ADC genes. In a mutant study with Arabidopsis mutants defective in Put biosynthesis genes *adc1* and *adc2*, mutants displayed reduced expression of *NCED3*, a key gene involved in ABA biosynthesis, and down-regulation of ABA regulated genes, ultimately showing reduced freezing tolerance compared to wild-type plants (Cuevas et al., 2008). This clearly suggests the role of Put in freezing tolerance by modulating ABA biosynthesis and gene expression. But this was not the case with increase in Spd levels which did not show the increase in ABA content and this underlines the importance of presence of ABRE element in promoter region of *ADC2* gene. Similarly, maize Ubi-1 promoter which is known to be activated by stress condition has been utilized for transgene expression (Cornejo et al., 1993; Takimoto et al., 1994).

Another approach, Tetracycline-inducible promoter has been proposed for the controlled expression of transgene. This was aroused from the concern that high levels of transgene expression might be lethal to plant cells undergoing regeneration. To overcome this problem, Kumar et al. (1996) used Tetracycline-inducible promoter to regulate the expression of potato *SAMDC* gene in sense and antisense orientation. Leaf explants containing sense *SAMDC/Tet* construct showed 2 to 6 fold increase in SAMDC activity, which then led to an increase in Spd and Spm titres, and intriguingly Put upon Tet induction. Still much work is need to be done to get clear insights into the role of PA in stress tolerance.

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