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Cloning and abiotic stress responsive expression analysis of Arginine decarboxylase genes in contrasting rice genotypes

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Abstract

Arginine decarboxylase (ADC) is the first enzyme of polyamine biosynthesis in plants, an important mediator of abiotic stress tolerance. Two genes *OsADC1* and *OsADC2* were found to be differentially expressed under various abiotic stresses namely salinity, drought, low temperature and high temperature. Significant differences in gene expression were found among contrasting rice genotypes Nerica-L-44 (NL44; tolerant) and Pusa Sugandh 2 (PS2; sensitive). Among the homologs, *OsADC2* was induced frequently in abiotic stresses with a higher transcript level than *OsADC1*. When the stress dependent gene expression was estimated relative to control conditions, PS2 showed a significant and higher level of induction. The estimation of relative gene expression between genotypes for each stress in all shoot tissues showed significantly higher level of expression in NL44 than PS2. In roots, the stress induced expression was higher in the sensitive genotype PS2. Construction of phylogenetic tree provided an insight on the evolution of *OsADC* gene from lower to higher organisms. The *OsADC2* gene was found to be highly diverged from *OsADC1* as well as from the counterparts of related and distant taxa. The analysis of amino acid sequence identified the conserved substrate binding, cofactor binding and dimerisation domains essential for enzyme activity.

Key words: Polyamines, drought, heat, low temperature, salinity, cloning

Introduction

Genetic improvement for grain yield under abiotic stresses is a challenging problem in agriculture research. The interaction between various physiological traits and genes/QTLs controlling them are being revealed by the advancements in stress biology research (Mishra et al. 2017; Barik et al. 2018). In this regard, polyamines are a unique category of biomolecules involved in various stress signalling pathways in plants. They are ubiquitous organic polycations found in all living organisms playing major roles in cell development (Igarashi and Kashiwagi 2015). The three major polyamines having physiological relevance in plants are putrescine (Put), spermine (Spm) and spermidine (Spd) (Berberich et al. 2015). The diamine Put, is the smallest polyamine and precursor to Spm, thermo, Spd, etc. (Valdes-Santiago et al. 2012). Put was shown to provide tolerance to various abiotic stresses such as drought (Gupta et al. 2012), low osmotic (Kotakis et al. 2014), high temperature (Hassanein et al. 2013) salinity (Shi et al. 2013) and water deficit (Yang et al. 2017). Polyamines are speculated to play in complex signalling pathways to impart stress tolerance (Pál et al. 2015). Studies of Liu et al. (2016) shown that Put aggravated the effect of drought on wheat grain filling by enhancing ethylene evolution and accumulation of ABA on grains. Arginine decarboxylase (ADC; E.C. 4.1.1.19) is the key enzyme in Put biosynthesis pathway of plants (Hanfrey et al. 2001). Due to the prominent role of *ADC* in abiotic stress tolerance, several researchers attempted to impart tolerance in various plant systems by transgenic approach. Overexpression of *D. stramonium ADC* genes in *O. sativa* rendered drought stress tolerance (Capell et al. 2004). Similar results were obtained in *L. tenuis* using oat *ADC* gene (Espasandin et al. 2014). Studies on *Arabidopsis adc* mutants showed chilling susceptibility, which upon Put spray rescued them from stress sensitivity (Cuevas et al. 2008).

ADC protein was found to be localized in chloroplast of photosynthesising tissues, nucleus and cytosol of non-photosynthesising tissues (Bortolotti

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et al. 2004). This indicates its role in normal chloroplast functioning and thereby photosynthesis. ADC belongs to type III - PLP dependent superfamily of enzymes and like other members it requires PLP, as cofactor for the catalytic property (Kidron et al. 2007). As it is an important protein for growth and abiotic stress tolerance of higher plants including cereals, its cloning and mechanism of action require special attention. Here we report isolation and characterization of the rice *ADC* gene homologs which is differentially expressed in various plant tissues when subjected to different abiotic stresses.

Materials and methods

Plant materials and stress treatments

Experiments were carried out using two *Indica* rice cultivars Nerica-L-44 (NL44) and Pusa Sugandh 2 (PS2). Plants were raised in the pot culture facility of Division of Plant Physiology, ICAR-IARI, New Delhi. Three weeks old seedlings were transplanted to pots and continued to grow till reproductive stage (pre-anthesis). Irrigation and nutrients were applied by following recommended practices.

The abiotic stress treatments were imposed at 50 % anthesis stage. Salinity stress was imposed by irrigating with 100 mM NaCl; drought stress (soil matric potential; SMP ~ –50 kPa) by withholding irrigation; low temperature (10^{o}C) in growth chamber and high temperature (~45^{o}C) in temperature gradient tunnel. Plants kept at 28±2^{o}C temperature at SMP higher than 10 kPa served as unstressed control. One week after treatments, samples were collected from root, stem, leaf and panicle, and used for further molecular analysis.

Selection of OSADC genes from database

The *ADC* gene sequences were obtained from NCBI database. Two homologs *viz*., *OsADC1* (GenBank accession no. EU220429) and *OsADC2* (GenBank accession no. FJ746894) were selected, and primers for gene expression profiling and cloning were designed manually.

Reverse transcription PCR

Total RNA was extracted from young leaves using the RNeasy Plant Mini Kit (Qiagen, Germany); cDNA was synthesised using SuperScript® III First-Strand cDNA Synthesis System (Thermo Fisher Scientific, USA), and iQ™ SYBR® Green Supermix Kit (Bio-Rad, USA) was used to perform qRT-PCR. The Stratagene Mx3005P (Agilent Technologies, USA) was used to perform real-time PCR. The primers used were as follows ADC1_F: GACGACGAGTGGGAGTTCATG and ADC1_R: GACAACGACCATGACACGATAC for *OsADC1*; ADC2_F: AGCGATGTGATCGAGAA GGTTG and ADC2_R: CATGGATCATGGTCGA GGCACTC for *OsADC2*. The Ubiquitin gene (GenBank accession no. X15865) (Ubi_F: GAAGCACAAGCAC AAGAAGGTG and Ubi_R: CTGGTTGTAGACGTA GGTGAG) was used as internal reference. Calculation on relative gene expression was done by $2^{-\Delta\Delta Ct}$ method as per Livak and Schmittgen (2001).

Cloning of ADC genes

The ADC1 and 2 were isolated from the cDNA of genotype NL44. For PCR, proof reading enzyme of KAPA HiFi PCR Kit (Roche, Switzerland) and the Applied Biosystems Veriti Thermal Cycler (Thermo Fisher Scientific, USA) were used. The primers used to flank complete CDS were as follows ADC1_F: GTTCTCCGGTTTGTGACGAGATG and ADC1_R: CTCAGTGCGACAGGTACAATGGAG for ADC1; ADC2_F: CAATGGCGAAGAAGAACTACGGTC and ADC2_R: CATGGATCATGGTCGAGGCACTC for ADC2. The cloning vector used was pTZ57R/T from InsTAclone PCR Cloning Kit (Thermo Fisher Scientific, USA) and sequencing was performed by Sanger sequencing (Xcelris Genomics, India). The sequencing results were aligned and confirmed using Bioedit software (Hall 1999). The cloned sequences were submitted to NCBI with the GenBank accession numbers KT748757 and KT748758 respectively for *OsADC1* and *2*.

Construction of phylogenetic tree

To elucidate phylogenetic relationships of ADC genes among model species, the protein sequences were retrieved from NCBI to generate a phylogenetic tree using MEGA7 (Kumar et al. 2016). The evolutionary history was inferred by using the Maximum Likelihood method based on the Poisson correction model (Zuckerkandl and Pauling 1965). Initial tree(s) for the heuristic search were obtained automatically by applying the Maximum Parsimony method. A discrete Gamma distribution (+G) was used to model evolutionary rate differences among sites in 5 categories. The rate variation model allowed for some sites to be evolutionarily invariable (+I). All positions containing gaps and missing data were eliminated. A total of 1000 bootstrap replications (Felsenstein 1985) were used for evolutionary analyses.

Prediction of conserved motifs

The deduced aminoacid sequence of the cloned sequences were performed by ExPaSy Translate tool (https://www.expasy.org/tools/). Additionally the protein sequences from *Arabidopsis thaliana*, ADC of *Physcomitrella patens*, *Chlorella variabilis*, and *Escherichia coli* were retrieved from NCBI for identifying the conserved domains and functional motifs. The protein sequences were aligned in BioEdit software (Hall 1999) and the output was represented using BoxShade server. The subcellular localisation was predicted using CELLO v.2.5 (Yu et al. 2006) and WoLF PSORT (Horton et al. 2007) tools. The chloroplast transit peptides were predicted using the chlorop v1.1 tool (Emanuelsson et al. 1999); and prediction of conserved motifs was performed using the ScanPROSITE program (De Castro et al. 2006), the CD-Search tool (Marchler-Bauer and Bryant 2004) and literature survey.

Results and discussion

Tissue specific inducibility of OsADC1 and 2 under different abiotic stresses

The transcript level of *OsADC1* was significantly up-regulated in stem, leaf and panicle of NL44. It was down-regulated in leaf of NL44 and up-regulated (6 folds) in leaf of PS2 under salinity stress. This was concomitant to the report in rice, where salt sensitive genotypes showed higher Put content in leaves compared to tolerant ones (Do et al. 2014). Exposure to drought stress caused up regulation of *OsADC1* in root, leaf and panicle of PS2, and root and panicle of NL44. In leaf under drought stress, down-regulated expression of both *OsADC1* and *2* was observed in NL44, and up-regulated in PS2. These findings are consistent with Do et al. (2013), where *OsADC1* was down-regulated in all genotypes of rice studied and *OsADC2* in a few under drought stress. Exogenous application of polyamines showed an increased grain filling rate even under drought stress in rice (Chen et al. 2013). Under low temperature stress *OsADC1* was up-regulated in leaf and panicle of PS2, and in panicle of NL44. Transcript levels of *OsADC1* got down-regulated in root and leaf, and up-regulated in panicle of NL44 under high temperature stress. In PS2, it was induced in root, stem and panicle (Fig. 1.A and B).

Gene expression of *OsADC2* was highly induced in roots of PS2 under all treatments as compared to control. Do et al. (2013; 2014) also reported that *OsADC2* is a general stress induced gene. In both the

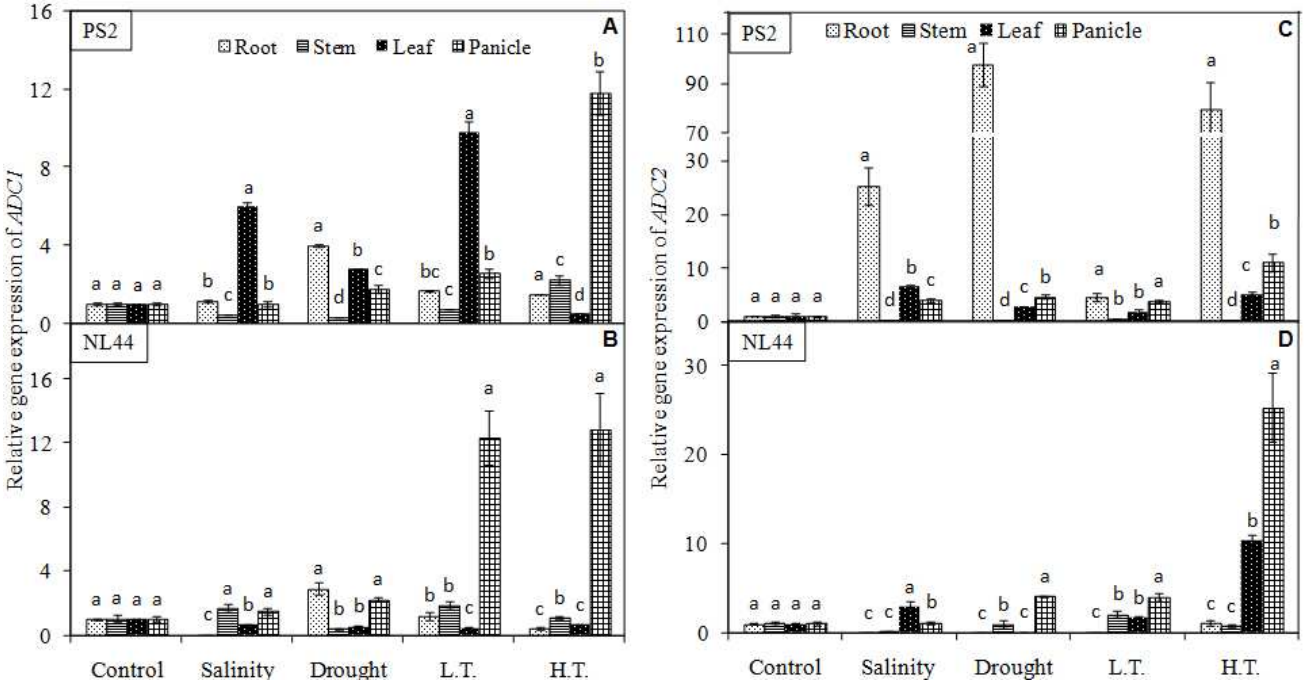

Fig. 1. Relative gene expression of *OsADC1* and *2* in different plant tissues under salinity, drought, low and high temperature stress conditions. A and B represent data for *OsADC1*, and C and D show data for *OsADC2*, respectively in PS2 and NL44. Vertical bars indicate mean±SE. Bars with same letter are not statistically significant at $P \leq 0.05$. Abbreviations: L.T. = Low temperature; H.T. = High temperature; PS2 = Pusa Sugandh 2; NL44 = NERICA-L-44

genotypes, the stress dependent expression of *OsADC2* was down-regulated or non-significant in stem. High temperature stress showed significant induction in gene expression in leaf and panicle of both the genotypes. We observed that *OsADC1* expression was enhanced under cold stress (Fig. 1C and D). The *Arabidopsis adc* mutants were shown to be chilling sensitive (Cuevas et al. 2008) that clearly indicates its essentiality for chilling tolerance in rice. Our results were affirmative to the studies in *Arabidopsis*, where the two *ADC* isoforms showed contrasting expression depending on the nature of stress (Alcázar et al. 2010).

Genotypic variability in OsADC1 and 2 gene expression

When the gene expression was compared between PS2 and NL44 under various stresses in corresponding tissues, a general trend in induced expression of *OsADC1* and *2* was found in aerial tissues of NL44, and in root tissues of PS2. The expression of *OsADC2* in roots was higher under salinity and drought in PS2 than NL44 (Table 1). Supporting evidence in rice showed that salinity sensitive genotypes accumulated more agmatine than tolerant ones in roots (Katiyar and Dubey 1990). *OsADC1* transcript was higher in stem, leaf and panicle of NL44 than PS2 in all the treatments. It was significantly higher in stem under salinity and low temperature, leaf under drought, salinity and high temperature, and in panicle under low temperature of NL44. In freezing tolerant chromosome 5A substitution line of wheat, the Put content was higher at cold stress (Kovács et al. 2010). Under salt stress there is an induction in transcript level of *OsADC* (Do et al. 2014). When the response to salinity was studied in rice using Pokkali and M-1-48 where the salt tolerant genotype showed higher expression of *OsADC*s (Chattopadhyay et al. 1997). Such an increase was also found at metabolic level in Pokkali, where Put level was increased in roots and shoots than sensitive genotype IKP (Lefevre et al. 2001). In case of *OsADC2*, a constitutive nature of expression was observed in all non-stressed tissues of the tolerant genotype NL44. In stem, leaf and panicle, the general expression in NL44 was higher than PS2 under all treatments that probably promote grain filling and yield stability under various abiotic stresses (Table 1).

Table 1: Gene expression of *OsADC1* and 2 genes in NL44 relative to PS2 under salinity, drought, low and high temperature stresses. Data shown as mean±SE. Abbreviations: L.T. = Low temperature; H.T. = High temperature; PS2 = Pusa Sugandh 2; NL44 = NERICA-L-44

		OsADC1		*OsADC2*	
Tissue	Treatments	PS2	NL44	PS2	NL44
Root	Control	1.01±0.10	0.32±0.02	1.00±0.07	167.63±10.77
	Saline	1.01±0.08	0.01±0.00	1.02±0.14	0.20±0.00
	Drought	1.00±0.01	0.23±0.03	1.01±0.09	0.09±0.02
	L.T.	1.00±0.02	0.24±0.05	1.03±0.18	3.39±0.00
	H.T.	1.03±0.18	0.02±0.00	1.02±0.14	2.22±0.67
Stem	Control	1.00±0.06	3.78±0.73	1.04±0.21	66.97±12.57
	Saline	1.00±0.03	13.20±1.68	1.01±0.08	30.30±0.88
	Drought	1.00±0.02	4.83±0.64	1.09±0.28	349.77±78.78
	L.T.	1.00±0.02	9.07±1.20	1.00±0.05	274.44±41.11
	H.T.	1.01±0.11	1.79±0.12	1.02±0.13	180.29±37.61
Leaf	Control	1.00±0.02	33.46±0.83	1.10±0.33	31.33±1.34
	Saline	1.00±0.03	3.67±0.20	1.00±0.05	14.24±2.62
	Drought	1.00±0.01	6.41±0.30	1.00±0.01	0.91±0.20
	L.T.	1.00±0.06	1.54±0.11	1.04±0.20	29.68±1.67
	H.T.	1.00±0.03	43.86±1.40	1.02±0.14	65.48±4.28
Panicle	Control	1.01±0.10	2.15±0.27	1.01±0.12	2.99±0.56
	Saline	1.02±0.13	3.11±0.51	1.01±0.08	0.85±0.11
	Drought	1.01±0.12	2.74±0.16	1.01±0.08	2.56±0.07
	L.T.	1.01±0.09	10.26±1.41	1.00±0.06	3.10±0.34
	H.T.	1.01±0.09	2.32±0.41	1.02±0.15	6.73±1.02

In silico analysis of ADC protein homologs

The cloned sequence of ADC1 and 2 of rice showed a nucleotide sequence length of 2216 and 2109 bp, respectively. They encompass intron-less coding sequences that encoded a proteins of 702 and 623 bp, respectively for ADC1 and 2. Analysis of the deduced ADC1 protein in ExPASy Protparam tool (Gasteiger

et al. 2005) predicted a molecular mass of 74.08 kDa having a theoretical isoelectric point of 5.07 and extinction coefficient of 52675 $M^{-1}cm^{-1}$ while OsADC2 had a calculated molecular mass of 67.35 kDa with theoretical isoelectric point of 6.45 and extinction coefficient of 61240 $M^{-1}cm^{-1}$.

Results of phylogenetic analysis revealed that ADC gene originated very early in evolution (Fig. 2).

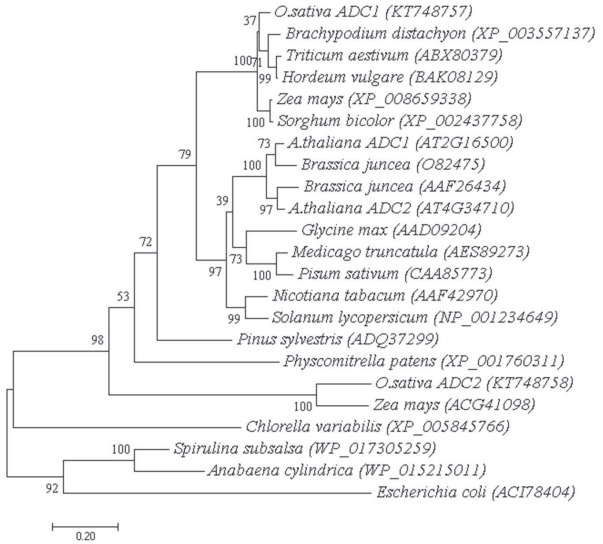

Fig. 2. Phylogeny tree constructed from amino acid sequences of *OsADC1* and 2 with various taxa. The rooted tree was constructed using maximum likelihood method with 1000 bootstrap replicates in Mega7. The corresponding GenBank accession numbers are provided in parentheses

Ancestors of this gene family were found in archaebacteria, eubacteria and green algae. Further this gene went forth into primitive plants such as bryophytes and pteridophytes in the course of evolution. They further got transceded into higher plants- gymnosperms and angiosperms. Within angiosperms, monocot-dicot divergence was observed into three families of dicots *viz*., brassicaceae, fabaceae and solanaceae; and a single cluster in monocots. Interestingly, by gene duplication, deletion and mutation events, *ADC* gene got diverged significantly to adapt with environmental conditions. These events might be responsible for the development of a new homolog of ADC gene (*OsADC2*) in rice and maize (GenBank Accession no. ACG41098).

The multiple protein sequence alignment with orthologous proteins from model plant species *viz. A. thaliana*, *P. patens*, *C. variabilis*, and *E .coli* showed significant homology with our cloned ADC genes. Interestingly, the identity between OsADC1 and OsADC2 was found to be 45.96% as ADC2 showed major deletions at N and C termini, and some minor deletions internally (Fig. 3). OsADC1, a monocot protein exhibited significant identity with angiosperm dicot *A. thaliana* (AtADC1 by 63.20%), bryophyte (*P. patens*; PpADC by 50.94%), green algae (*C. variabilis*; CvADC by 49.69%) and prokaryote (*E. coli*; EcADC by 34.94%). OsADC2 exhibited significant identity with AtADC2 (44.16%), PpADC (39.67%), CvADC (36.81%) and EcADC (31.70%).

OsADC1 encodes a 74 kDa protein which was reported to be localized in chloroplast, whereas OsADC2 a 67 kDa protein in cytoplasm (Peremarti et al. 2010). The analysis of deduced amino acid sequence of the cloned genes by WoLF PSORT and CELLO prediction tools also corroborated this report. The transit peptide was predicted to be encoded by 33 amino acids at the N-terminus of *OsADC1* by chlorop v1.1 analysis. The OsADC2 failed to contain any transit peptides and was hence inferred to function in cytoplasm itself (Fig. 3).

Mechanistic analysis of ADC protein action

ADC enzyme catalyzes the decarboxylation of L-arginine to agmatine in both PLP and Mg^{2+}-dependent manner (Sun et al. 2015). To functionally characterize OsADC1 protein which belongs to type III-PLP dependent superfamily of enzymes (Kidron et al. 2007), the conserved domains were analyzed along with literature search. ADCs encode three domains namely PLP binding site, substrate binding site and dimer interface.

Protein sequence analysis by ScanProsite tool of ExPASy (De-Castro et al. 2006) showed two signature motifs for pyridoxal phosphate (PLP) dependent Orn/DAP/Arg decarboxylase family II. They were motif I (aa position 148-166 for OsADC1; 106-124 for OsADC2) and motif II (327-343 for OsADC1; 288-302 for OsADC2). One key residue involved in PLP binding site is Lys present in PLP attachment site (Moore and Boyle 1990; Peremarti et al. 2010). Our cloned sequence had conserved Lys residue at position 151 and 109 respectively for OsADC1 and 2 in Motif I (Fig. 3). Studies on the mutated version of *Arabidopsis ADC1* (Lys Ala) resulted in 97 % loss of enzyme activity (Hanfrey et al. 2001). In the Orn/DAP/Arg decarboxylases family

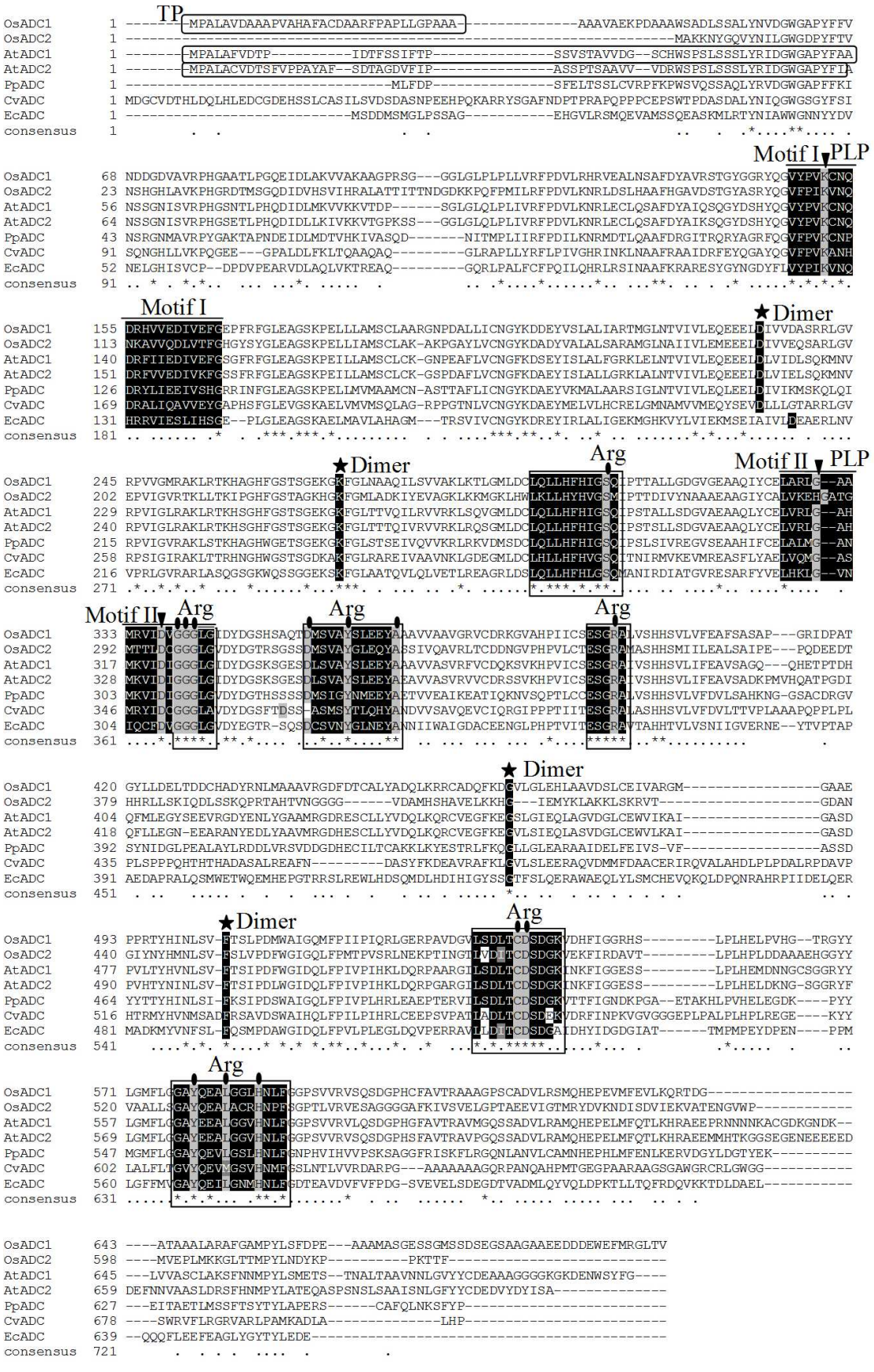

Fig. 3. Multiple sequence alignment of cloned *OsADC1* and 2 with distant taxa. The species used for comparison are *Arabidopsis thaliana* (NP_179243, NP_195197), *Physcomitrella patens* (XP_001760311), *Chlorella variabilis* (XP_005845766), and *Escherichia coli* (ACI78404). The sequences were aligned in Bioedit and represented using BoxShade server. The conserved motifs are indicated along with core residues. Abbreviation: TP = Chloroplast transit peptide; PLP = Pyridoxal PO_4 binding; Arg = Substrate (Arginine) binding; Dimer = Dimerisation residues. The core residues are represented as –triangle (▼) at PLP binding, ellipse (.) at Arg binding, and star (*) at dimerisation sites

2 signature 2 two conserved moifs in tandem were found. First a PLP binding motif (having Glu and Asp core residues) followed by a substrate binding motif (with three core residues GGG) as reported by Moore and Boyle (1990) was found. In our sequence the motif was present at positions 326-343 for OsADC1 and 283-302 for OsADC2 (Fig. 3).

Comparing the studies by Jackson et al. (2000), we were able to identify several conserved residues *viz*., S304, R393, C540, D541, Y579, L583 and H587 for OsADC1, and S261, R352, C488, D489, Y528, L532 and H536 for OsADC2 involved in substrate (Arg) binding. The $C540^{OsADC1}$ and $C488^{OsADC2}$ residue was reported to play a significant role as the mutant AtADC1 protein for this residue resulted in 91% reduction in enzyme activity (Hanfrey et al. 2001). Studies on *E. coli* ADC by X-ray crystallography revealed inter-convertible forms of the enzyme through an inactive homodimer to an active decamer composed of five homodimers at acidic pH (Andréll et al. 2009). Based on the dimer structures of ornithine (Almrud et al. 2000) and diaminopimelate decarboxylases (Ray et al. 2002), we demarcated the residues involved in dimerization and decamerisation to be mediated by D233, K271, G467 and F504 of OsADC1, and D190, K228, G420 and F451 of OsADC2 (Fig. 3).

The ADC enzyme consumes a proton in the decarboxylation of arginine to agmatine (Andréll et al. 2009). Here, PLP act as an electron sink to stabilize decarboxylated substrate. It forms an internal aldimine bond (Schiff base linkage) with the catalytic residue lysine ($K151^{OsADC1}$ and $K109^{OsADC2}$). During the catalytic reaction, substrate displaces the PLP and makes an external almidine bond. The second catalytic residue, a cysteine ($C541^{OsADC1}$ and $C488^{OsADC2}$) from the other subunit of the dimer, is involved in the protonation of decarboxylated substrate during catalysis (Jackson et al. 2000; Gokulan et al. 2003).

The present study demonstrates the role of polyamines through abiotic stress dependent gene expression of *OsADCs* in rice. It also suggest the application of *OsADC*s for generating transgenics for multiple abiotic stress tolerance. These genes can be targeted as candidate gene during association mapping for identification of SNPs (Krishna et al. 2018) to be used as markers for crop improvement in rice.

Authors' contribution

Conceptualization of research (ST, MP); Designing of the experiments (ST, GKK, MP); Contribution of experimental materials (ST, MP); Execution of field/ lab experiments and data collection (ST, GKK, PY); Analysis of data and interpretation (ST, GKK, MP); Preparation of manuscript (ST, GKK, MP).

Declaration

The authors declare no conflict of interest.

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