Polyamine and Ethylene pathways crosstalk revisited on a genome wide and gene function scale

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Abstract

Background Polyamine and ethylene biosynthesis pathway genes are widely involved in the regulation of plant abiotic stresses. For their biosynthesis, both pathways require the same precursor, Synthase Adenosyl Methionine (SAM) enzyme. Whether they function as competitors or collaborators to regulate plant abiotic stress tolerance is still an elusive topic. Genome wide analysis of *Cleistogenes songorica* polyamine and ethylene pathway gene families was conducted to study their evolutionary relationship. And, using *Arabidopsis* plants transformed with a polyamine gene *SAMDC2* from *C. songorica*, the expression of key genes from both pathways, and other previously well-studied stress responsive genes was investigated under salt or drought stress. Further, the ABA's role on this interaction salt stress was also studied. Results 17 polyamine, 12 ethylene and 6 SAM biosynthesis related genes were identified at genome wide level in *C. songorica*. Phylogenetic analysis revealed close evolutionary similarities between gene families from both pathways. Also, analysis of cis regulatory elements indicated that SAM family genes promoters were rich into both ABA and ethylene related cis regulatory elements. Transcriptomic analysis, qRT-PCR validation, and confirmation using transgenic *Arabidopsis* showed that polyamine and ethylene key pathway genes can be concurrently expressed during abiotic stresses. *Arabidopsis* plants expressing a polyamine gene *CsSAMDC2* driven by *RD29A* showed an improved drought and salt stress tolerance, and an increased expression of key polyamine and ethylene pathway genes. These plants maintained higher chlorophyll content and photosynthetic capacity. Morphological analysis of transgenic seedlings showed that leaves of these lines exhibited a more compact architecture following salt stress exposure. Application of ABA on transgenic lines under salt stress further improved the expression of polyamine and ethylene pathway genes. Further, lateral and primary root development were found improved during salt stress and ABA treatments. Interestingly, the expression of ethylene pathway genes was not reversed by exogenous ABA during salt stress treatment. Conclusion In silico and gene functional analysis assays revealed potential evolutionary and functional similarities between polyamine and ethylene pathway gene families. Such findings imply a synergetic interaction between polyamine and ethylene pathways, and the significant role of ABA on this crosstalk.

Abstract

Background: Polyamine and ethylene biosynthesis pathway genes are widely involved in the regulation of plant abiotic stresses. For their biosynthesis, both pathways require the same precursor, Synthase Adenosyl Methionine(SAM) enzyme. Whether they function as competitors or collaborators to regulate plant abiotic stress tolerance is still an elusive topic. Genome wide analysis of *Cleistogenes songorica* polyamine and ethylene pathway gene families was conducted to study their evolutionary relationship. And, using *Arabidopsis* plants transformed with a polyamine gene *SAMDC2* from *C. songorica*, the expression of key genes from both pathways, and other previously well-studied stress responsive genes was investigated under salt or drought stress. Further, the ABA's role on this interaction salt stress was also studied.

Results: 17 polyamine, 12 ethylene and 6 *SAM* biosynthesis related genes were identified at genome wide level in *C. songorica*. Phylogenetic analysis revealed close evolutionary similarities between gene families from both pathways. Also, analysis of *cis* regulatory elements indicated that *SAM* family genes promoters were rich into both ABA and ethylene related *cis* regulatory elements.

Transcriptomic analysis, qRT-PCR validation, and confirmation using transgenic Arabidopsis showed that polyamine and ethylene key pathway genes can be concurrently expressed during abiotic stresses. Arabidopsis plants expressing a polyamine gene *CsSAMDC2* driven by *RD29A* showed an improved drought and salt stress tolerance, and an increased expression of key polyamine and ethylene pathway genes. These plants maintained higher chlorophyll content and photosynthetic capacity. Morphological analysis of transgenic seedlings showed that leaves of these lines exhibited a more compact architecture following salt stress exposure.

Application of ABA on transgenic lines under salt stress further improved the expression of polyamine and ethylene pathway genes. Further, lateral and primary root development were found improved during salt stress and ABA treatments. Interestingly, the expression of ethylene pathway genes was not reversed by exogenous ABA during salt stress treatment.

Conclusion: In silico and gene functional analysis assays revealed potential evolutionary and functional similarities between polyamine and ethylene pathway gene families. Such findings imply a synergetic interaction between polyamine and ethylene pathways, and the significant role of ABA on this crosstalk.

Keywords: Polyamine, ethylene, ABA, abiotic stresses, *Cleistogenes songorica*, Arabidopsis

Background

Abiotic stresses such as droughts, heat waves and salt severely burden crop productivity, and are expected to exacerbate as a consequence of climate change [1]. During stress injuries, stress responsive genes play important defensive role by mobilizing diverse functional metabolites to stabilize cellular components, mitigate oxidative stresses, and maintain proper water status.

Polyamine are among these metabolites that alleviate cellular injuries during abiotic stresses [2] by improving the structure and stability of functional proteins and nucleic acid [3]. Also, they are known to control the expression of functional genes during various stresses[4]. The widely studied plant polyamines are putrescine, spermine and spermidine. Expression of genes encoding enzymes involved in the biosynthesis of these polyamines improved polyamine contents and stress tolerance of several crops [5]. For instance, overexpressing *ODC*, *ADC*, *SAMDC* and *SPDS* genes in rice [6], tomato [7] and tobacco [8] conferred a broad spectrum of abiotic stress tolerance in these plants. Moreover, various studies have documented an induction of *SAMDC* gene transcripts in plants exposed to a range of stresses including salt, high temperature, drought, and oxidative stress.

Plant response to drought and/or salt stresses largely depends on how they sense signals from these stresses [2], and signaling crossroads are common in stress responsive pathway [9]. The metabolic pathway for polyamine biosynthesis is interconnected with other stress regulators and phytohormones including ABA [10] and ethylene [11]. These hormones are key regulators of several physiological responses including biotic and abiotic stresses signaling cascades[12]. They also enhance the activity of transcriptional regulators in stress responsive genes[13].

The metabolic pathway for polyamine biosynthesis is interlinked with ethylene pathway through SAM that acts as a common substrate for both metabolic routes[11]. Right downstream of SAM, are SAMDC and ACO enzymes for polyamines and for ethylene biosynthesis pathways, respectively[14]. SAM is converted to ACC by 1-aminocyclopropane-1-carboxylate (ACC) synthase enzyme. Next, ACC is oxidized to ACO that is finally converted to ethylene[15]. The antagonistic nature of both pathways has been well documented [16, 17]; while polyamines may retard fruit, flower and/or leaf senescence and ripening, ethylene would contribute to their occurrence[16]. And silencing *ACC* or *ACO* genes shifted the activity of SAM in favor of polyamine pathway[18]. Yet, a recent study indicated that *SAM* gene can function as a signaling molecule for ABA and H₂O₂, and *Medicago truncatula* plants expressing *MfSAM* gene exhibited an improved cold tolerance while maintaining stable levels of polyamine and ethylene biosynthesis related genes activities[19]. Furthermore, Absciscic Acid Responsive Element (ABRE) *cis*-regulatory elements were found in both polyamine (*SAMDC*) [20] and ethylene (*ACO*) family genes [21], and it was also shown in different plant species that a short term saline shock induces both polyamines and ethylene levels [22].

Drought and salt tolerant plants such as xerophyte and halophytes have evolved with particular adaptation strategies. *Cleistanthus songorica*, a xerophyte C₄ desert plant, is widely distributed in wild areas in the north west part of China where annual precipitations are around 100 mm. It is known to have novel drought stress adaptation strategies and contains rich pools of stress tolerance genes [23]. Earlier studies have shown that transgenic *Arabidopsis* lines expressing *C. songorica* *LEA* genes exhibited improved salt and drought tolerance by reducing CO₂ assimilation levels [24]. Moreover, gene identification and transcriptome analysis in other xerophytes, such as *Populus euphratica* has allowed to functionally identify several stress responsive genes [25, 26].

Polyamine and ethylene pathway have intricate functions in the regulation of plant abiotic stress signaling mechanism [27, 28]. How the two pathways collaborate to answer to the same ultimate functions, and the role played by ABA on their interaction is yet to be investigated by genome wide analysis of *C. songorica*'s polyamine and ethylene pathway and functional analysis of a *C. songorica* polyamine gene into *Arabidopsis*.

Materials And Methods

Identification of *C. songorica* polyamine and ethylene pathways gene families

Arabidopsis (*Arabidopsis thaliana*. L), rice (*Oryza sativa*. L) and maize (*Zea mays*. L) protein sequences of genes involved in polyamine and ethylene metabolic pathways were used as queries to Blast search against *C. songorica* reference genome available at BMK cloud platform <http://www.biocloud.net/>. To obtain protein sequences of Arabidopsis polyamine genes, genes accessions of the previously reported Arabidopsis polyamine genes [29] were used to search against Arabidopsis protein genome data retrieved from plant genomics database (<https://phytozome.jgi.doe.gov/pz/portal.html>)[30]. Protein sequences of genes involved in ethylene pathway were obtained from previously published Arabidopsis *ACO/2OG(Fell)* [31] and *ACS* genes families [32]. *Oryza sativa*, and *Zea mays* polyamine and ethylene *SAM* family gene accessions were obtained by querying gene identifiers against the respective plant genome data in phytozome[30]. The obtained accession numbers were then used to retrieve the respective protein sequences from their corresponding protein full genome sequence [30].

The putative *C. songorica* polyamine and ethylene pathway protein sequences were analyzed according to Pfam family using Hidden Markov Model [23] within Pfam database <https://pfam.sanger.ac.uk/search>[33]. The resulting *C. songorica* protein sequences were then submitted to SMART (<https://smart.emblheidelberg.de/>) [34] and to the conserved domain search within NCBI (<https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi>) databases [35] to confirm the presence of ethylene and polyamine related pathway gene protein domains. The obtained proteins were then named based on their Pfam conserved protein domain families and the appellations adopted for *A. thaliana*, and their sequential order on different *C. songorica* chromosomes and contigs.

Multiple sequence alignment and phylogenetic analysis of *C. songorica* polyamine and ethylene proteins.

To assign polyamine and ethylene pathway proteins to their respective clades, *C. songorica* polyamine and ethylene pathway protein sequences were aligned with Arabidopsis and *O. sativa* polyamine and ethylene protein sequences. All the protein sequences with their respective accession numbers are shown in Additional file 6. The multiple proteins sequence alignment was performed by using ClustalW algorithm (Feng and Doolittle 1987) within MEGA 6 software. The resulting protein sequence alignments were then used to construct phylogenetic tree after pair-wise deletion of gaps with p-distance matrix [36]. The Neighbor-Joining algorithm was used with one thousand bootstrap [36, 37].

cis-regulatory element and motif analysis

C. songorica sequences of 2000 bp of the gene promoter region corresponding with the identified polyamine and ethylene genes were analyzed for putative *cis*-regulatory elements using PlantCARE database (<http://bioinformatics.psb.ugent.be/webtools/plantcare/html/>) [38]. We then recorded and compared the number of stress and hormonal-related *cis*-regulatory elements in individual genes from polyamine and ethylene pathway, and from *SAM* family.

Transcriptomic analyses and qRT-PCR validation

C. songorica seeds were sown in vermiculite medium supplied with 1/4 diluted Hoagland's nutrient solution, pH 5.8. Growth chamber conditions were set at 75–80 % relative humidity, 30 / 28°C (day / night), and 16 / 8 h (day / night) at the light intensity of 200 mmol photons m⁻² s⁻¹. For heat stress treatment, one-month old seedlings were treated at 40 °C for 24 h. Dehydration stress treatment was done by withholding irrigation to 6-10% of soil water content (for mild drought stress), and to 1-3% for severe dehydration stress. For salt stress treatment, different NaCl concentrations (50mM; 100mM; 200 mM) were prepared and applied on seedlings by spraying. In another set of experiment seedlings were treated with 100 µM ABA to investigate the role of ABA on polyamine and ethylene pathway genes expression. For each treatment, root and shoot tissue samples were collected at 0 and 24 h post treatment and kept at -80 °C till RNA extraction. Three samples were collected for each treatment. Total RNA was isolated from root or shoot samples using the Shengong RNA isolation kit as instructed (Shengong Ltd, Shanghai, China). The isolated RNA samples were further pooled, and sequenced following Illumina sequencing guidelines. The root and shoot total RNA samples were also used to synthesize first strand cDNAs using an oligo dT primer and the cDNA synthesis kit (Shengong). The cDNAs samples were individually diluted to 100 ng/µL prior to qPCR using gene specific primers (Additional file 2). For each PCR reaction, three biological replicates with three technical replicates each were used.

qPCR reaction was conducted using 40 cycles of 95 °C 5 s, 60 °C 15 s, and 72 °C 34 s, a SYBR Green Master (Shengong), and gene specific primers. The relative gene expression levels were analyzed using the comparative $\Delta\Delta C_t$ method [39]. The expression level of *C. songorica GADPH* gene was used as an internal control. Two ways analysis of variance and Duncan's multiple range test (DMRT) were used for multiple mean comparisons. SPSS (IBM Corp. 2013, IBM SPSS Statistics for Windows, Version 21.0, Armonk, NY) was used to determine the significant differences between means ($p < 0.005$).

Cloning and transformation of a polyamine gene, *CsSAMDC2* into Arabidopsis

The amplification of cDNA *CsSAMDC2* (EST) was performed using a SMARTTM RACE cDNA Amplification Kit (Clontech, Japan). After sequencing, both 5' and 3' ends primers (Supplementary Table 1) of *CsSAMDC2* were designed and used to amplify *CsSAMDC2* open reading frame. The PCR was conducted according user manual (Clontech, USA). The same primers were used for full length cDNA amplification of 2.1kb of *CsSAMDC2* genomic sequence. The resulting PCR products were ligated into the binary vector pBI121 under the control of a constitutive promoter, *CaMV35S* and stress inducible promoter *RD29A*, and sequenced at Shanghai Shengong Biotechnological Ltd. (Shanghai, China). The vector harbored selectable marker nptII conferring kanamycin resistance. The recombinants plasmids were separately mobilized into Hypervirulent *Agrobacterium tumefaciens*, strain GV3101, before transformation into Arabidopsis using floral dipping method. The expression of *CsSAMDC2* gene was assessed in T3 plants using real-time PCR and *AtActin* (AY096397) as a housekeeping gene.

Stress treatment of Arabidopsis transgenic plants

Wild type and transgenic *Arabidopsis* were cultivated in a mixture of peat and vermiculite soils (2:1). Growth chamber conditions were set at 75–80 % relative humidity, 22°C, and 16 /8 h (day / night) with the light intensity of 200 mmol photons m⁻² s⁻¹. For drought tolerance study, irrigation was withheld until 1-3% of soil water content (SWC) for both three-week old wild-type and transgenic plants.

For salt stress tolerance analysis, germination percentage of transgenic and wild-type was assayed by growing wild-type and transgenic lines on MS plates containing different NaCl concentration (0mM; 100mM; 150 mM). In another set of experiments of salt stress study, plants were grown on MS medium for seven days before being transferred on new MS plates containing 100 or 150 mM NaCl supplemented or not with 100 µM ABA. For each plant, the whole plant was sampled at 0 and 24 h for further physiological assays and gene expression analysis. Phenotypes of both treated and untreated were photographed.

Expression analysis of polyamine and ethylene pathway genes under different stress treatments in transgenic *Arabidopsis*

Drought, salt, and salt +ABA treatments were carried out to compare the expression levels of key polyamines pathway genes (*AtADC2*, AT4G34710; *AtSAMDC1*, AT3G02470; *AtSPMS*, AT5G53120 and ethylene genes (ACC synthase AT5G51690; ACC oxidase, AAC27484) in transgenic and wild-type *Arabidopsis* plants.

Measurement of chlorophyll content

Chlorophyll content was determined according to Arnon[40]. For each sample leaf tissues weighing 100mg were detached and bleached by soaking them in 10 ml of 30 mL 80% (V/V) ethanol. The mixture was incubated under dark for 24 hours then centrifuged for 5 min at 13,000 rpm using micro centrifuge (Eppendorf MiniSpin®) at room temperature to fully remove leaf pigmentation. The optical density of the mixture was measured at 654 nm using a spectrophotometer.

Chlorophyll Fluorescence Fv/Fm

The quantum efficiency of PSII is represented by dark-adapted values given by the ratio Fv/Fm. This parameter is considered as a key indicator of plant photosynthetic performance [41]. Lower values reflect damage to the PS II complexes, and thus certain level of photo inhibition [42]. The values of this parameter were assayed between transgenic and wild-type plants. Three plant samples per line were primarily incubated for 30 minutes under dark conditions, and values were recorded for each individual line considering three replications per treatment using a PAM-2100 (Heinz Walz GmbH). The determination of maximum quantum yield PSII was by using the formula $F_v/F_m = (F_m - F_o) / F_m$ Where: Fm is the maximum fluorescence yield. Fo, the yield of fluorescence in the absence of an actinic (photosynthetic) light; minimum fluorescence yield [43].

Results

Phylogenetic analysis of polyamine and ethylene pathway genes

A phylogeny analysis was conducted to breakdown the evolutionary relationship between gene families from polyamine and ethylene pathways. Results showed that clusters were formed based on gene families not on metabolic pathways (Fig.1). Ethylene pathway clades of genes could be found adjacent to polyamines pathway gene clusters. For example, *SAMDC* family of genes (polyamine pathway) was found grouped in the same cluster with *ACS* gene family (ethylene pathway), while *SAM* family genes were cladded next to both polyamine and ethylene pathway gene clusters

Promoter regions of *SAM* family genes are rich into stress related *cis* regulatory elements

Promoter regions of stress responsive genes are generally rich into *cis* regulatory elements that provide binding sites for transcriptions factors [44, 45]. Herein, we surveyed the type and amount of stress related *cis* regulatory elements in *C. songorica* polyamine and ethylene pathway genes, and in *SAM* family gene promoters to get insights on the contribution of these *cis* elements on the particular biological and physiological functions in each pathway. A plethora of CAAT and TATA enhancers was found in both pathways (Table 1). Strikingly, the average number of MYB and ABRE *cis*-regulatory elements was almost comparable in both polyamine and ethylene pathway gene promoter regions, with a slight difference in favor of polyamine genes. In average, 1.3 ABRE and 2 MYB *cis* acting elements were counted in the promoter regions of polyamine pathway genes, while 1 ABRE and 1.4 MYB were recorded for ethylene pathway genes. Promoters of *SAM* family genes were the most enriched in ABRE *cis* regulatory elements and averaged of 3.1 counts per gene. Also, higher number of G-box, W-box and Jasmonic acid inducible motifs, CGTCA-motif and TGACG-motif was recorded in the promoter regions of *SAM* family genes.

Transcriptomic analysis

Expression analysis revealed that many of *SAM* family genes were constitutively expressed across normal irrigations, mild drought stress and high dehydration stress treatments (Fig. 2A). *CsSAM1* and *CsSAM5* gene expression levels were induced after dehydration treatments in both root and shoot tissues, and consequently their expression was reduced after re-watering. The expression level of *CsSAM6* gene was consistently high across all the treatments, and re-watering remarkably reduced its expression particularly in root.

Conversely, expression levels of *CsADC3*, *CsSAMDC2* and *CsSAMDC3* (polyamine pathway genes) were remarkably induced under severe dehydration stress and sharply reduced after re-watering, in both roots and shoots tissues for *CsADC3* and *CsSAMDC3* genes. The expression level of *CsSAMDC2* gene was the most induced by dehydration stress and its expression declined in shoot after re-watering.

Transcriptomic expression levels of *Cs2OG(Fell)1*, *Cs2OG(Fell)5* and *Cs2OG(Fell)7* (ethylene pathway genes) increased in shoot after strong dehydration stress treatment, and re-watering reduced the expression of *CsCs2OG(Fell)5* and *Cs2OG(Fell)7* genes in shoot, while the expression level of *Cs2OG(Fell)1* gene was reduced in both tissues. The expression level of another ethylene pathway gene

CsACC4 went up steadily with increasing dehydration stress and reduced after re-watering in both tissues (Fig. 2A).

Expression levels of polyamine and ethylene pathway genes were also analyzed under different levels of salt stress, heat stress, low temperature stress, and under ABA treatment (Fig. 2B). For ethylene pathway, the expression level of *Cs2OG(Fell)1* gene increased after applying 200 mM NaCl stress conditions, interestingly, ABA treatment did not reverse its expression in shoot. Similarly, the expression levels of *Cs2OG(Fell)3* gene increased in both tissues following 100 mM NaCl, heat and ABA treatments. Also, the expression levels of another ethylene pathway gene *CsACC1* were induced by 50 mM of NaCl and low temperature treatment in shoot and root tissues, and its expression went up in root after ABA treatment. In the same metabolic pathway, *CsACC3* gene exhibited an increased expression in both tissues after 100 mM NaCl treatment, and its expression was also high in root after 200 mM NaCl stress conditions. Also, ABA treatment induced its expression level in shoot compared to normal treatment conditions.

For polyamine pathway genes, the relative expression level of *CsADC2* gene was slightly induced by 100 mM NaCl stress in roots and by ABA treatment in shoots. For *SAMDC* family genes, *CsSAMDC4* gene expression was induced by all the treatments in shoot tissue. *CsSPMS* genes were constitutively expressed across all treatments in both tissues, and ABA slightly increased the expression levels of *CsSPMS1*, *CsSPMS3* and *CsSPMS4*. The expression level of *CsSPMS2* was highly induced in roots after 100 mM NaCl or 200 mM NaCl and heat treatments in both tissues. On the other hand, expression levels of *SAM* family genes were generally higher compared to other families in both pathways, and from all the analyzed genes, *CsSAM6* gene exhibited the highest expression level in shoot tissues after ABA treatment (Fig. 2B)

qRT-PCR validation

Quantitative RT-PCR validations of these expression profiles were conducted for drought, salt stress and ABA treatments in root and shoot tissues (Fig. 3). For genes involved in ethylene biosynthesis pathway, *CsACC3* was selected as a representative (Fig. 3A). qRT-PCR validated the expression of *CsACC3* as it was induced by 16.87 times in shoot after strong dehydration stress, while its expression in root increased from 2.7 folds after 50 mM NaCl to 5.4 folds under 100 Mm NaCl treatment, and then dropped to 2.6 folds at 200 mM NaCl. Treatment with ABA did not reduce its expression levels relative to control conditions. This trend correlates well with the findings from the transcriptomic analysis.

The expression of *CsSAM6* gene, was induced by all levels of drought and salt treatment applied, more importantly, as observed during transcriptome analysis, its expression level was the highest in shoot after ABA treatment as it soared by 18.3 folds (Fig. 3B). The expression *CsSAMDC2* was induced by all stress treatments and ABA application. The expression level of *CsSAMDC2* gene after strong dehydration stress in shoot was the highest as it increased by 754 times relative to control. Also, its expression was induced to 11.7 and 16.4 times in root and shoot tissues following ABA treatment (Fig. 3C).

Expression of *CsSAMDC2* gene enhanced drought stress tolerance in transgenic Arabidopsis

As *CsSAMDC2* gene was highly induced in *C. songorica* under drought conditions, it was transformed into Arabidopsis under a stress responsive and constitutive promoter, *RD29A* and *35S*, respectively. Using primers of *CsSAMDC2* gene promoters, and transformed Arabidopsis genomic DNA, an amplicon band of around 1200 bp size was detected in 9 Arabidopsis lines (Additional file 1). Similarly, successful transformation of *RD 29A* and *35S* promoters was also confirmed through PCR using Arabidopsis genomic DNA and gene specific primers (Additional file 2). Among these confirmed transgenic plants, two lines transformed with *RD29A::CsSAMDC2* gene construct (TL01 and TL18) and other two lines (TL05 and TL11) transformed with *35S::CsSAMDC2* gene construct were selected for further abiotic stress analysis. The expression level of *CsSAMDC2* gene was induced in the studied transgenic plants after 14 days of drought stress (Fig. 4A). *CsSAMDC2* gene enhanced dehydration tolerance of transgenic Arabidopsis as compared to wild-type plants which showed signs of wilting after 14 days of water withdrawing (Fig 4B). Furthermore, *CsSAMDC2* gene expression was found higher in lines harboring *RD29A::CsSAMDC2* gene construct than in *35Cs::CsSAMDC2* transgenic plants. The expression of *CsSAMDC2* gene was also followed by an improved chlorophyll content (Fig. 5A) and photosynthetic capacity (Fig. 5B) in transgenic Arabidopsis compared to wild-type plants.

CsSAMDC2 gene improved seed germination percentage in transgenic Arabidopsis under salt stress

Seed germination percentage of transgenic plants and wild-type plants was investigated on different concentrations of NaCl. Under 0 mM NaCl, germination rates of 98-100% were reached after 3 days following sowing. There was no significant difference among the studied lines (Additional file 3A and C). Under 100 mM NaCl, germination percentage after nine days of salt stress exposure was 96 % (TL01), 99% (TL18); 97% (TL05), and 98% (TL11), while under the same stress conditions, it was at 84% in wild-type plants (Additional file 3B and D)

Phenotypes of Arabidopsis seedlings after extended exposure on salt stress

Wild-type Arabidopsis and transgenic lines expressing *CsSAMDC2* gene were grown on 150 mM NaCl stress medium. After reaching maximum germination rate (after 28 days) (Additional file 4A), all seedlings were maintained under the same treatment conditions for 18 days (Fig. 6A). The number of cotyledons that maintained green colors after treatment was found higher in transgenic seedlings than in their wild-type plants (Fig. 6B). Moreover, leaves of transgenic Arabidopsis showed a more compact architecture (Fig. 6A), a typical salt avoidance mechanism in stress tolerant plants [46, 47].

Expression of Polyamine and Ethylene pathway genes and other stress responsive genes under different treatments

The expression of different stress responsive genes including polyamine related genes *AtSAMDC2* and *AtADC2*, and ethylene pathway genes, *AtACO2* and *AtACS6*, an Ethylene responsive gene *AtERF1*, and ABA responsive gene *AtRD29A* were more improved in transgenic Arabidopsis after drought stress treatment than in their wild-type counterparts (Fig. 7A).

All studied polyamine and ethylene pathway genes showed positive upregulations after salt stress (Fig. 7B) or salt stress +ABA treatments (Fig. 7C). Analysis of the expression of key polyamine pathway genes *AtSAMDC2* and *AtADC2* under salt stress +ABA treatments revealed that their expression levels increased by 24 (*AtSAMDC2*) and 21 (*AtADC2*) folds in TL01, and 29 (*AtSAMDC2*) and 28.7 (*AtADC2*) folds in TL18. (Fig. 7C). However, the expression of both genes only increased by 4.9 (*AtADC2*) and 4.7 folds (*AtSAMDC2*), respectively, in wild-type plants relative to non-treatment conditions. The activity of *AtRD29A* was also highly induced by salt stress (Fig. 7B), and ABA application enhanced its expression levels to 89 and 87 folds in TL01 and TL018 plants (Fig. 7C) respectively, which were the highest recorded expression levels in all lines analyzed.

Exogenous ABA altered plant architecture of transgenic Arabidopsis under salt tolerance

Analysis of leaves and root morphology in wild-type and transgenic lines grown on MS plates supplemented with 150 mM NaCl indicated no significant phenotypical differences between these lines after 72 h (Fig. 8B). However, application of ABA induced formation of lateral roots and triggered root growth in all lines compared to salt stress treatment alone (Fig. 8D). *RD29A::CsSAMDC2* lines exhibited the most improved lateral root development under these treatments conditions (Fig. 8D). Measurements of root length in *RD29A::CsSAMDC2* lines of 4.3cm (TL01) and 4.2 cm (TL018) were the highest, and were significantly different with those in other lines (Additional file 5B)

Discussion

Cleistogenes songorica is perennial monocots plants highly adapted to arid conditions. This study indicated that *SAMDC2* gene expressed in *Cleistogenes songorica* shoot and root tissues after high dehydration stress, can also enhance abiotic stress tolerance of Arabidopsis by inducing the expression of polyamines, ethylene and ABA stress responsive genes.

One of the strategies for abiotic stress tolerance in perennial monocots plants are the enhancement of photosynthesis and redox metabolisms, and tendency to form molecular chaperons [48]. Transgenic Arabidopsis plants expressing *CsSAMDC2* gene showed an improved tolerance to salt and drought stresses, and higher photosynthetic parameters such a high Fv/Fm and chlorophyll content. Maintaining low chlorophyll degradation and high photosynthetic capacity is an imperative adaptation mechanism in stress tolerant species [49].

Different studies have previously shown that polyamine and ethylene compete for the same precursor S-adenosylmethionine (SAM), a donor of methyl group required by both metabolic pathways[50, 51]. The present study suggests that under stress conditions both pathways may enter a mutually beneficial synergistic relationship instead of merely competing for SAM. And for the first time, this hypothesis was studied on genome wide scale. Results from phylogenetic analysis indicated close evolutionary relationships between some gene families from polyamine and ethylene pathways. These findings may infer functional similarities that could have narrowed divergence of these families during their evolutionary history. A such gene functional evolution concept corroborates with former studies in

Brassica napus LEA gene family [52]. Also, *SAM* genes were found to be rich into *cis* acting elements which provide binding sites for both ethylene, Jasmonic and ABA related transcription factors. This could explain their functional relevance in mediating both polyamine and ethylene pathways during stresses conditions. The above results were further supported by a simultaneous increased expression of both polyamine and ethylene pathway genes, ethylene and ABA responsive gene in Arabidopsis expressing a polyamine gene *CsSAMDC2* during salt or drought stresses.

Other earlier studies suggested that instead of metabolically competing for *SAM*, other molecular mechanisms may play into their role [53, 54]. For instance, it was proposed that the activity of *AtERF1* gene enhanced plant tolerance to a broad spectrum of abiotic stresses by integrating Absciscic acid, ethylene and Jasmonic acid [55]. To investigate this hypothesis, exogenous ABA was applied on salt-stressed transgenic lines and wild type plants. Results showed that during salt stress, application of ABA further enhanced mRNA expression levels for polyamine (*AtSAMDC2* and *AtADC2*) and ethylene pathway genes (*AtACO2* and *AtACS6*), and for ABA (*AtRD29A*) and ethylene (*AtERF1*) responsive genes in transgenic Arabidopsis under salt stress. Interestingly, the expression of *AtERF1* was found higher in *RD29A::CsSAMDC2* lines than in *35S::CsSAMDC2* and wild-type plants.

Furthermore, ABA predominantly promoted the expression of *AtRD29A* and *AtSAMDC2* in *RD29A::CsSAMDC2* Arabidopsis lines; TL01 and TL018. Subsequently, TL01 and TL018 showed improved primary and lateral and root growth. Previous studies showed that ABA induces lateral roots development under salt stress [56, 57] through its signaling that are transduced in cell specific manner and through intricate hormonal interactions [58, 59]. However, some previous studies reported that lateral and primary root growth suppress each other's development [58]. Interestingly, in this study both traits were found enhanced in Arabidopsis plants expressing *RD29A::CsSAMDC2* gene construct. This was possibly due to an enhanced activity polyamines genes, *AtADC2* and *AtSAMDC2*. Polyamines are known to promote cell division and differentiation necessary for root apex and lateral root development [60]. It was previously shown that disrupting the activity of *ADC* genes in Arabidopsis [61] or in *P. vulgaris* [62] correlated with reduced lateral root formation and primary root growth, respectively. Similarly, downregulating *SAMDC* gene in potato plants was associated with limited root growth [63].

This study showed that though *CsSAMDC2* may suppress salt stress under ABA independent manner, exogenous ABA could promote its activity by enhancing the upregulation of polyamine (*AtADC2* and *AtSAMDC2*), ethylene (*AtERF1*, *AtACO2*, *AtACO6*) and ABA (*AtRD29A*) related stress responsive genes. From these findings we speculate that under abiotic stress ABA, ethylene and polyamine could mutually interact to respond to their respective functions in plant defensive mechanism. Moreover, as a results of a rich diversity *cis* acting elements found in the promoter regions of *SAM* family genes, we speculate that *SAM* enzymes may function as mediator for polyamine and ethylene pathways during stress conditions. A network for stress response regulation by polyamine, ethylene pathway genes through *SAM* was proposed in this study (Fig. 9), however further molecular assays are required for confirmation.

Conclusions

Phylogenetic and *cis*-regulatory analyses revealed potential evolutionary and functional similarities between polyamine and ethylene pathway gene families. Subsequently, the expression of genes from both pathways was induced during drought or salt stresses, or salt stress +ABA treatments in Arabidopsis expression a polyamine gene, *CsSAMDC2*. Findings from this study suggest molecular interaction between polyamine and ethylene pathways genes during salt or drought stresses, and the important role of ABA on this crosstalk during salt stress.

Abbreviations

ABA: Absciscic Acid ABRE: Absciscic Acid Responsive Element ACC: 1-aminocyclopropane-1-carboxylate. ACO: 1-aminocyclopropane-1-carboxylate oxidase. ACS: 1-aminocyclopropane-1-carboxylate synthase. ADC: arginine decarboxylase. CaMV: Cauliflower Mosaic Virus. CDS: coding sequence. ERF: Ethylene responsive factor. EST: expressed sequence tag.

ODC: ornithine decarboxylase. MYB:myeloblastosis. 2OG(FeII): 2-oxoglutarate (2OG) and Fe(II). NaCl: Sodium Chloride. RNA: Ribonucleic acid. Put: putrescin. PSII: Photosystem II. SAM: S-adenosylmethionine. *SAMDC*: S-adenosylmethionine decarboxylase. SE: standard error. Spd: spermidine. SPDS: spermidine synthase. Spm: spermine. SPMS: spermine synthase. TL: transgenic line. qRT-PCR: quantitative polymerase chain reaction. uORF: upstream open reading frame. UTR: untranslated region. V/V: volume/volume. WT: wild type.

Declarations

Ethics approval and consent to participate:

Not applicable.

Consent for Publication:

Not applicable.

Availability of data and material:

Most of the supporting data are presented in the additional file and plant materials are available at Lanzhou University in the laboratory of the Corresponding author (zhangjy@lzu.edu.cn). Information on *Cleistogenes songorica* genome sequencing are available at <http://www.biocloud.net/> and can be accessed through the corresponding author.

Competing interests:

The authors declare that they have no competing interests.

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Authors' contributions:

YW, JZ and BPM conceived the study. BPM and ZZ performed Arabidopsis transformation. ZZY and BPM conducted stress treatment analysis. BPM, QY, ZYY and WF contributed on RNA library preparing and sequencing. XM, BPM and GK conducted bioinformatics analysis. KO and TM curated data. BPM and YZ wrote the original draft and J.Z, BPM, KO reviewed and edited the manuscript. YW and JZ administered the project.

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Figures' Legends

Fig. 1 Comparative phylogenetic analysis of genes involved in polyamine and ethylene biosynthesis pathways. Red, blue and green dots represent genes participating in ethylene, polyamine and both (*SAM*

family) pathways, respectively. The phylogenetic evolution of *C. songorica* proteins was determined using the known Arabidopsis and rice polyamine and ethylene pathway related protein sequences.

Fig.2 Expression profiles of *C. songorica* genes involved in polyamine and/or ethylene biosynthesis pathways under drought stress and rewatering treatments (a) and during salt stress, ABA, low temperature, and heat stress treatments (b).

Fig. 3 qRT-PCR validation of expressions of different *C. songorica* genes involved in metabolic pathways genes. Expression of *CsACC3*, representative for ethylene pathway genes (a). Expression of the selected SAM family gene (b) and expression of *CsSAMDC2*, a polyamine gene (c).

Fig. 4 Relative mRNA transcript expression level of *CsSAMDC2* gene in plants upon 14 d of drought stress (a). Growth morphology of *CsSAMDC2* lines and Col-0 under normal (b) and after 14 d of water withdraw (c).

Fig. 5 Morpho-physiology of transgenic Arabidopsis under normal watering conditions and after drought stress. Estimation of chlorophyll content in *CsSAMDC2* Arabidopsis lines and in wild-type plants (a). PSII quantum efficiency ratio Fv/Fm of transgenic Arabidopsis lines overexpressing *CsSAMDC2* genes compared to wild-type plants (b). The quantum efficiency of PSII was assayed during normal and after 14 d of drought stress. All plants were incubated under dark for 25 minutes before each measurement. The data are the means $\pm$ standard errors. For each treatment, three biological samples were used and for each biological sample three technical replicates were used.

Fig. 6 Seedlings morphology was examined based on cotyledon greening rates plant architecture in transgenic and wild type plants grown under 150 mM NaCl stress conditions. After all plants reached the maximum germination on 150 mM NaCl stress medium (on the 28th day), they were transferred on a new medium of similar composition (a) to assess the germination percentage until the 45th day(b).

Fig.7 Relative mRNA transcript expression level of polyamine genes *AtADC2*, *AtASAMDC2*, and ethylene genes *AtACO2* and *AtACO6*, and drought responsive genes *AtRD29A* and *AtERF1* (a). Gene expression levels after 72 h of salt stress of polyamine genes (*AtADC2* and *AtASAMDC2*), ethylene pathway genes (*AtACO2* and *AtACO6*), a stress inducible gene *AtRD29A*, and of *AtERF1* (ethylene responsive gene) (b). Effect of exogenous ABA on the expression of polyamine genes *AtADC2* and *AtASAMDC2*, ethylene genes *AtACO2* and *AtACO6*, stress inducible gene *AtRD29A*, and of an ethylene responsive gene, *AtERF1*(c). Expression levels were measured after 72 h and were relative to the expression before treatment (0h) that was set to 1. Data are the means $\pm$ standard errors. For each treatment, three biological samples were used and for each biological sample three technical replicates were used.

Fig. 8 Effect of salt stress and ABA application on stress responsive gene activity and plant morphology. Morphology of wild type and transgenic plants under 150 mM NaCl at 0 h (a), after 72 h (b). The effect of ABA on root growth and morphology of *CsSAMDC2* lines under 150 mM NaCl stress at 0h (c) and after 72 h (d).

Fig. 9 Proposed network for stress response regulation by polyamine, ethylene pathway genes through SAM.

Additional file1 PCR Confirmation of Arabidopsis transgenic lines. PCR amplification of *RD29A::CsSAMDC2* and *35S::CsSAMDC2* gene constructs (a). Amplification of 35S promoter (b) and RD29A promoter (c).

Additional file2: Supplementary table1, list of primers used in this study.

Additional file 3 Effects of *CsSAMDC2* gene on germination under 0 and 100 mM NaCl stress of transgenic Arabidopsis plants. Photograph taken after 3 days following sowing on 0 mM NaCl medium (a). Photograph taken after 9 days after sowing on 100 mM NaCl medium(b). Germination percentage under 0 mM. d. Germination percentage on100 mM NaCl stress (c). The data are the means $\pm$ standard errors.

Additional file 4 Effect of *CsSAMDC2* gene on germination under 150 and 200 mM NaCl stress of transgenic Arabidopsis plants. Photograph taken after 16 days following sowing on 150 mM NaCl medium(a) percent germination rates under 150 mM(b). The data are the means $\pm$ standard errors.

Additional file 5 Phenotyping transgenic and wild type Arabidopsis roots. Root length under 150 mMNaCl salt stress(a) and under 150 mM NaCl+ABA(b). For each treatment, three biological samples were used.

Additional file 6 Sequences of polyamine and ethylene proteins used in this study.

Table

Due to technical limitations, table 1 is only available as a download in the supplemental files section.

Supplementary Files

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