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SHORT COMMUNICATION

Transcriptional regulation of ABA core signaling component genes in sorghum (*Sorghum bicolor* L. Moench)

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Abstract Abscisic acid (ABA) plays an important role in growth, development and adaptation of plants to environmental stresses. The mechanism of ABA signal transduction involves three core components namely ABA receptors [pyrabactin resistance 1 (PYR1)/PYR1-like (PYL)/regulatory component of ABA receptor (RCAR)], clade A PP2Cs and Class III SnRK2 family proteins. In the present study, we identified and analyzed the core components of ABA signaling in sorghum, which is known for its drought tolerance. Genome wide *in silico* analysis led to the identification of eight PYL ABA receptors, nine clade A PP2Cs and three class III SnRK2 family members. Abiotic stresses and exogenous ABA-mediated transcriptional changes of the genes encoding ABA core signaling components were analyzed at seedling stage. All the members of *SbPYL* gene family were downregulated, except *SbPYL1* and *SbPYL7* which showed significant upregulation in leaf under drought stress. *SbPYL1* and *SbPYL5* were upregulated in response to ABA, cold, high salt and PEG-induced osmotic stress, while *SbPYL4* showed significant upregulation only under cold stress. Expression levels of the *SbPP2C* genes were higher or unaffected in response to exogenous ABA and abiotic stresses in leaf except *SbPP2C5*, which decreased under cold stress. *SbPP2C4*, *SbPP2C5* and *SbPP2C6* were highly induced (up to 56-fold–99-fold increase) under different stresses. Expression of class III *SbSnRK2* genes was either unaffected or downregulated under abiotic stresses and exogenous ABA. Heat stress downregulated the expression of all the ABA core signaling component genes except that of *SbPP2C6* which was upregulated under heat stress. In general, abiotic stresses upregulated the expression of *PP2Cs* but downregulated the expression of *SnRK2* in sorghum seedlings. Differential stress-responsive expression and less number of PYLs in sorghum as compared with *Arabidopsis* suggest that *SbPYL* family members might have acquired distinct functions during evolution.

Keywords ABA signaling · Osmotic stress · PP2C · PYL · SnRK2 · Sorghum

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Introduction

The plant stress hormone abscisic acid (ABA) orchestrates several important physiological functions and adaptive responses of plants to environmental stresses. Shortly after the discovery of ABA as abscisin, dormin and inhibitor-β in early 1960s, its role in stress response was established (Addicott and Lyon 1969). This triggered intensive research in deciphering various biochemical and molecular genetic mechanisms of ABA signaling that led to the identification of protein phosphatase 2C (PP2C) and subclass III sucrose non-fermenting-1 (SNF1)-related protein kinase 2 (SnRK2), ABF/AREB transcription factors, effector genes and secondary messengers involved in ABA responses (Cutler et al. 2010). PP2Cs belong to Mg^{2+}- or Mn^{2+}-dependent protein phosphatase (PPM) family of protein serine/threonine phosphatases that require Mg^{2+}/Mn^{2+} as cofactor (Schweighofer et al. 2004). *Arabidopsis* genome encodes 80 PP2C genes which are categorized into 13 subfamilies namely A–L (Schweighofer et al. 2004; Xue et al. 2008). Clade A subfamily contains nine proteins, of which six are negative regulators of ABA signal transduction (Cutler et al. 2010). SnRK2 proteins belong to family of plant-specific serine/threonine kinases that are involved in abiotic stress and ABA responses (Boudsocq et al. 2004; Fujii et al. 2007). The ten members of SnRK2 family are divided into three subclasses viz. I, II and III in *Arabidopsis* (Kobayashi et al. 2004). Members of SnRK2 subclass I are activated by osmotic stress, while subclass II and III kinases are activated by both ABA and osmotic stress. Subclass III SnRK2s show higher activation by ABA than subclass II (Boudsocq et al. 2004) and are positive regulators of ABA signal transduction (Mustilli et al.2002; Yoshida et al. 2002). The quest for ABA receptor started about three decades ago, and many candidate proteins were proposed as ABA receptors. Yet none of these proteins could be proved as bona fide ABA receptor (McCourt and Creelman 2008). The enigmatic search for the ABA receptor culminated in 2009, when two research groups reported the discovery of soluble receptors of ABA namely pyrabactin resistance (PYR)/PYR1-like (PYL)/regulatory component of ABA receptor (RCAR). These scientists demonstrated ABA-dependent inhibition of clade A PP2Cs (ABI1 or ABI2) by PYR/PYL/RCARs (Ma et al. 2009; Park et al. 2009). Based on these results, it was proposed that ABA-bound receptors (PYR/PYLs/RCARs) inhibit PP2Cs, which relieves repression of SnRK2s. This proposed pathway for signal perception and transduction was successfully reconstituted in vitro demonstrating that ABA receptors (here after collectively referred as PYL), PP2Cs and SnRK2s constitute the core components of ABA signaling for stress-responsive gene expression (Fujii et al. 2009). The structural and functional aspects of ABA core signaling components have been reviewed in Santiago et al. (2012). Since the establishment of core components of ABA signaling in *Arabidopsis*, the orthologs of PYL, PP2C and SnRK2 family were identified in rice, tomato, grape and strawberry (Chai et al. 2011; Sun et al. 2011; Kim et al. 2012; Boneh et al. 2012a, b).

Sorghum is the fifth most important crop of the world. It is grown in arid and semiarid areas of the world and is a source of food, feed, fodder, fiber and fuel. Its diverse germplasm and genomic resources, along with its capability to grow under low input and environmentally adverse conditions, make it an attractive model for functional genomics. Moreover, it provides a link for translational research between model systems such as *Arabidopsis* and rice with the larger and polyploid genomes such as maize and sugarcane. Despite larger variation in genome size, there is syntenic relationship among these species. Studies on the orthologous genes across species may help identification of evolutionarily conserved and distinct functions that are acquired during the course of evolution. Identification and analysis of such distinct functions in turn might reveal the species-specific differences in stress adaptation. Therefore, to understand the mechanism of ABA perception and signal transduction in sorghum, we carried out genome-wide analysis to identify core components of ABA signaling and their transcriptional regulation in response to exogenous ABA and abiotic stresses.

The protein sequences of the *Arabidopsis* ABA core signaling components namely ABA receptors (*PYR/PYL/RCAR*), clade A *PP2C* and *SnRK2* family genes were obtained from TAIR database (http://www.arabidopsis.org). The protein sequences were subjected to a tblastn search against the sorghum genome (www.phytozome.net.in) with default settings. After eliminating redundant sequences, the obtained protein sequences were screened for the existence of specific domains (http://pfam.sanger.ac.uk/) with *E* value cutoff of 0.01 and default setting of

SMART database (http://smart.embl-heidelberg.de/). Conserved motifs were identified through prosite (www.prosite.expasy.org). Rice sequences were obtained from MSU database (www.rice.plantbiology.msu.edu/). Multiple alignment of amino acid sequences were performed using the ClustalW2 algorithm (http://www.ebi.ac.uk/Tools/msa/clustalw2/) with default settings. Phylogenetic trees were constructed using neighborhood-joining method in MEGA 5.2.1 software (http://megasoftware.net/). Bootstrap analysis was performed with 1,000 replicates to evaluate the reliability of different phylogenetic groups.

The expression of *PYL*, *PP2C* and *SnRK2* family genes was analyzed in Sorghum [*Sorghum bicolor* (L.) Moench] genotype BTx623 at seedling stage. The seedlings were raised in pots filled with soil under natural environmental conditions. Fourteen-day-old seedlings were subjected to moisture-deficit stress (drought) by withholding irrigation, and sampling was done after 7 and 14 days when the leaf relative water content (RWC) was 85.54 and 46.36 %, respectively. The samples of 14 days stress with 46 % RWC were used for analysis. Plants that were watered optimally were taken as control (RWC 94.90 %). For RWC measurement, the leaf samples from control and water-stressed plants were cut into small pieces ($\sim$1 cm) and fresh weights were recorded. These leaves were immersed in water for 6 h at room temperature, and then, turgid weight of the leaves was recorded. These leaves were dried in an oven at 80 °C for 2 days, and dry weight was recorded. The RWC was calculated using the formula RWC (%) = (fresh weight − dry weight)/(turgid weight − dry weight) × 100.

For imposing other stresses, the 14-day-old sorghum seedlings were carefully removed from pots and the soil was washed away from roots. The whole seedlings were used for abiotic stress treatments by immersing the roots in water (control), 10 % PEG6000 (−1.48 bars) or 150 mM NaCl solution (15 dS m^{-1}; −7.4 bars). For imposing cold stress, seedling roots were immersed in ice-cold water and placed at 4 °C, while high temperature stress was imposed by keeping the seedlings at 42 °C. ABA treatment was given by applying 100 µM ABA on the leaves. The stress was given for 3 h. For all these treatments, seedlings kept in water at 24 °C served as control. After the stress treatments, leaf and root (for drought stress) samples were collected from the control and stressed seedlings, immediately frozen in liquid nitrogen and stored at −80 °C till further use.

Total RNA was isolated from control and stressed leaf and root tissues using RNeasy plant Mini Kit (QIAGEN). Genomic DNA contamination in RNA was eliminated by on-column DNAse digestion (RNase free DNase set, QIAGEN) according to the manufacturer's protocol. Further, the absence of DNA in total RNA was confirmed by using RNA as template in PCR. The primers used for RT-PCR analysis are given in Supplementary Table S1. All primer pairs amplified expected amplicon in PCR with DNA and cDNA as template. For RT-PCR expression analysis, the first-strand cDNAs were synthesized using superscript III following the manufacturer's protocol (Invitrogen). The cDNA was synthesized from pooled biological replicates for each treatment separately. The reactions were carried out in three technical replicates each. The relative expression of genes was analyzed by real-time PCR using Mastercycler ep Realplex (Eppendorf, AG, Hamburg) with SYBR Premix (KAPA SYBR FAST qPCR kit). Each 10-µL reaction contained 0.4 µL (0.4 µM of forward and reverse primer mix), 2 µL (1:20 diluted) of cDNA template, 5 µL of SYBR master mix. Reaction conditions were as follows: one cycle of 95 °C for 2 min, 40 cycles of 95 °C for 3 s and 60 °C for 45 s followed by melt curve analysis to rule out non-specific amplification. GAPDH (Sb07g002220) was used as house keeping gene for normalization of cDNA concentration. The normalized control (non-stressed) C_t values (ΔC_t) were used as calibrator. Relative fold change in expression in stress samples over control were calculated using the relative $2^{-\Delta\Delta C_t}$ method (Livak and Schmittgen 2001). The fold change values were directly plotted in log scale.

Results and discussion

Arabidopsis genome codes for 14 ABA receptor (ABARs) family genes. Based on the amino acid sequences of these *Arabidopsis* ABARs, eight candidate genes of PYL family were identified in sorghum genome. All these candidate PYL proteins contain polyketide cylase2 domain (PF10604). Polyketide cylase is a subfamily of Bet v 1-like superfamily (PfamC:CL0209). The 14 members of PYR/PYL/

RCAR family in *Arabidopsis* belong to this subfamily of polyketide cyclase-like proteins (Klingler et al. 2010). The Bet v 1-like superfamily is characterized by presence of a hydrophobic cavity that acts as ligand binding site. In PYR/PYL protein, ABA binds to this ligand binding pocket. Binding of ABA brings changes in the surrounding β sheet loops called 'GATE' and 'LATCH' loops (hence referred as gate and latch mechanism of ABA signaling) (Melcher et al. 2009). These conformational changes in turn facilitate ABA-mediated interaction of PYL with PP2C. The gate and latch loops are represented by highly conserved amino acid residues, SGLPA and HRL, respectively (Fig. 1a). Among the eight candidate *PYL* genes in sorghum, six have perfect gate and latch motif. The remaining two genes, that is Sb03g038600 and Sb09g006700, have one mismatch (S–T) making the gate motif as TGLPA instead of SGLPA. The TGLPA motif was also found in some of the PYLs of maize, *Brachypodium*,rice (He et al. 2014) (Supplementary Fig. S1), strawberry and grape (Chai et al. 2011; Bohen et al. 2012b). This suggests S89T is relatively a common feature in gate motif. Therefore, these eight candidate genes were considered as members of PYL family in sorghum and were designated as *SbPYL1–SbPYL8* (Supplementary Table S2). All the SbPYLs conform to the sequence and domain conservation as identified in AtPYLs (Melcher et al. 2009) (Fig. 1a; Supplementary Fig. S2). Phylogenetic analysis also revealed three subfamily of SbPYLs similar to that of *Arabidopsis* PYL family (Ma et al. 2009) (Fig. 1a). Among the eight members of SbPYL family, two of the intron-containing genes, *SbPYL7* and *SbPYL8* (Sb09g006700 and Sb04g009280), clustered with intron-containing *AtPYLs* (Fig. 1a).

The *Arabidopsis* genome encodes 80 *PP2C* genes that are divided into 13 subfamilies (A–L) (Schweighofer et al. 2004; Xue et al. 2008). The subfamily 'clade A' consists of 9 *PP2C* members, of which six are well-characterized negative regulators of ABA signaling (Cutler et al. 2010). Based on Pfam domain search (PF 00481), we identified 80 PP2C genes in sorghum genome. Phylogenetic analysis revealed 12 SbPP2C proteins that clustered with clade A PP2Cs of *Arabidopsis*. These 12 candidate PP2C A sequences were further analyzed for motifs and residues that are highly conserved and necessary for phosphatase activity and interaction of PP2Cs with ABA-PYL and SnRK2 (Santiago et al. 2012; Soon et al. 2012). Among the 12 SbPP2Cs, three genes appear to be non-functional as they encode a protein with N-terminal truncation (Sb09g026860), C-terminal truncation (Sb02g038100) or 34 amino acid deletion (Sb03g32740) corresponding to *HAB1* residues 367-400 (Supplementary Fig. S3). The 34 amino acid deletion encompasses tryptophan residue (Trp385 in HAB1), which is crucial for interaction with PYL and locking mechanism (Melcher et al. 2009). Hence, sorghum genome encodes at least 9 functional clade A PP2C genes (Supplementary Table S3). The multiple alignments of these PP2C protein sequences revealed that most of the functional residues or domains were well conserved within this clade of proteins (Fig. 1b; Supplementary Fig. S3). The clade A PP2C genes from *Arabidopsis* and rice make 4 and 3 paralogous gene pairs, respectively (Xue et al. 2008). In sorghum, also three paralogous gene pairs were found which further supports diversion by gene duplication in both monocots and eudicots (Xue et al. 2008). Furthermore, each gene of the paralogous gene pairs in sorghum had an orthologous gene in rice, and most of these were supported by high bootstrap value (96–100) (Supplementary Fig. S4). Existence of such orthologous gene pairs between species supports their close evolutionary relationship.

The ten members of SnRK2 family from *Arabidopsis* were used as query for BLAST search in sorghum genome database. Initially, 96 putative genes were identified, and later based on SnRK-specific Pfam domain (PF0069), 94 *SnRK* genes were identified in sorghum genome. Phylogenetic analysis revealed that out of 94 sorghum *SnRK* proteins, 10 *SbSnRK* proteins clustered with 10 *At*SnRK2 proteins (Supplementary Table S4). Our study identified more number of SnRK genes than previous report of 41 SnRKs in sorghum (Li et al. 2010). However, the number of SnRK2 family members identified was same as found in our study. There were three *SbSnRK2* proteins (Sb01g007120, Sb01g014720 and Sb08g019700) that clustered with *Arabidopsis* ABA-activated SnRK2 subclass III proteins (SnRK2.2, SnRK2.6 and SnRK2.3). These three SbSnRK2 proteins contain highly conserved ATP-binding loop, activation loop, PP2C interface residues, SnRK2 box (domain I) and highly acidic ABA box (domain II) (Yoshida et al. 2006; Soon et al. 2012; Ng et al. 2011) (Fig. 1c; Supplementary Fig. S5). The domain I

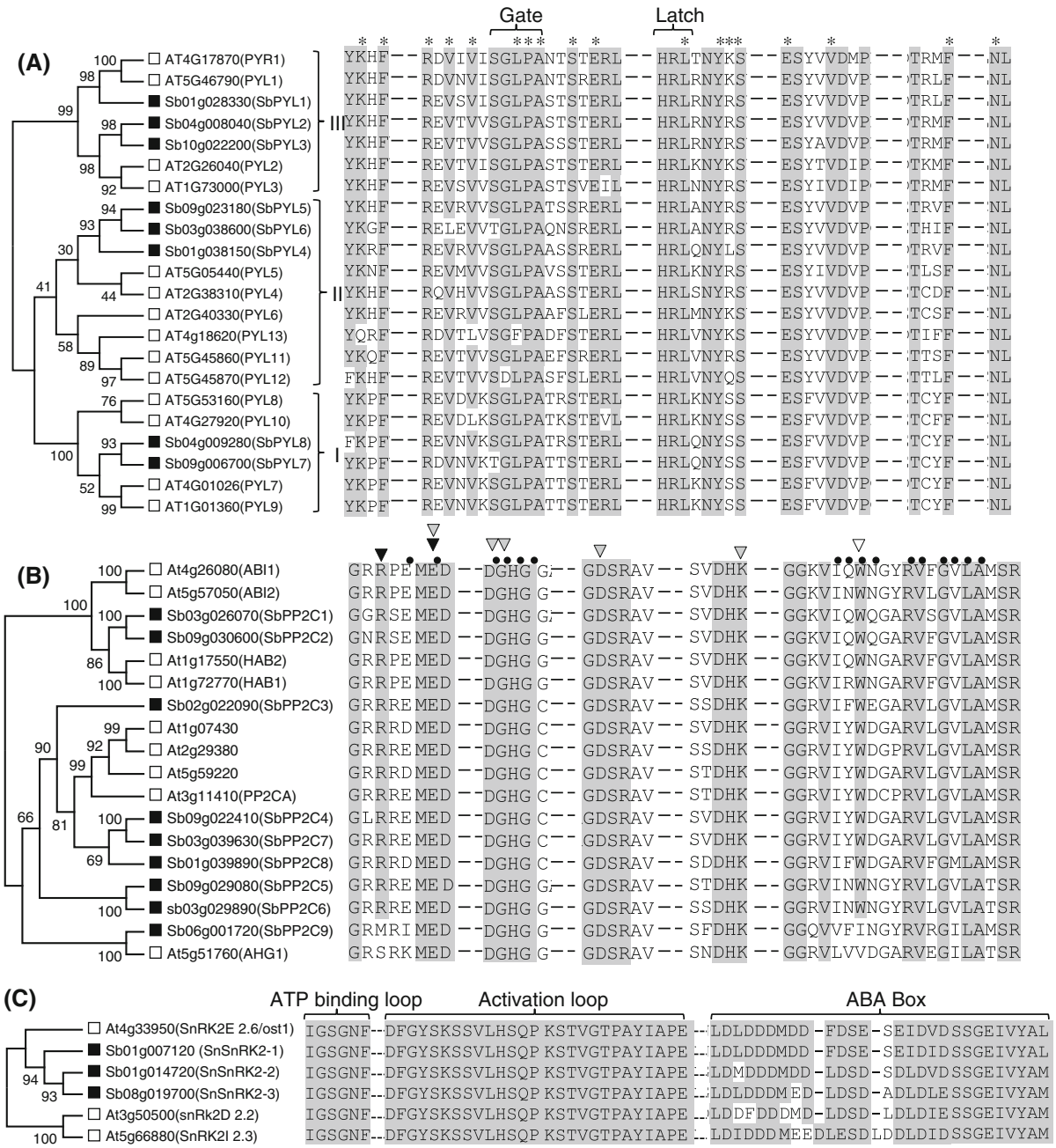

Fig. 1 Phylogenetic analysis of members of ABA core signaling components from sorghum and *Arabidopsis*. Trees were generated by neighbor-joining method (Mega 5.2) with bootstrap value 1,000. The alignment was performed with the CLUSTALW2 program (http://www.ebi.ac.uk/Tools/clustalw2/index.html) using the default settings. Conserved residues are shaded in *gray*. **a** Conserved residues and residues involved in ligand binding (*stars*) in ABA receptors (PYLs) are denoted according to Melcher et al. (2009), **b** residues involved in interaction of PP2C with ABA, PYLs, Mn^{2+} ions are marked based on Santiago et al. (2012), contact points for *inverted triangle* ABA, *filled inverted triangle* phosphate, *filled circle* PYL interaction and *shaded inverted triangle* Mn^{2+}, **c** functional residues and domains in SnRK2 are reported based on Ng et al. (2011) and Soon et al. (2012). Protein sequences of ABA core signaling components of sorghum and *Arabidopsis* were obtained from www.phytozome.net and www.arabidopsis.org, respectively

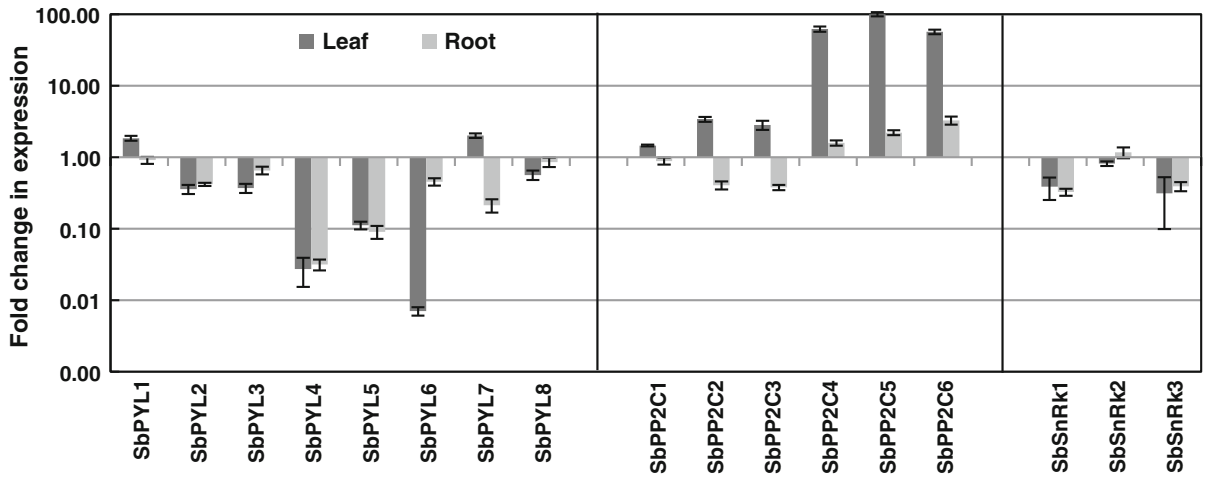

Fig. 2 Real-time RT-PCR expression analysis of *SbPYL*, *SbPP2C* and *SbSnRK2* genes in leaves and roots under moisture-deficit stress. Relative fold change in expression was calculated using the $2^{-\Delta\Delta C_t}$ method (Livak and Schmittgen 2001) and shown in *log scale*. *Error bars* indicate SEM ($n = 3$)

(SnRK2 box) is highly conserved in the ten SnRK2 proteins, while domain II has been shown to be required for ABA-mediated activation of SnRK2 (Yoshida et al. 2006).

Real-time RT-PCR analysis showed that all the members of *SbPYL* family except *SbPYL1* and *SbPYL7* were downregulated in leaf under drought stress (Fig. 2). Expression of *SbPYL1* and *SbPYL7* was almost twofold higher in drought-stressed leaves as compared to that of unstressed leaves. In root tissue, all the *SbPYL* genes were downregulated under drought stress (Fig. 2). Downregulation of *PYL* genes under drought stress has also been reported in tomato and grape. Of the eight *SlPYL* genes in tomato, except *SlPYL1* and *SlPYL8*, rest of the *SlPYL* genes were downregulated by dehydration stress in leaf (Sun et al. 2011). In grape (*Vitis vinifera*) *PYL* expression decreased in roots under abiotic stress condition (Bohen et al. 2012b). High temperature downregulated the expression of all the *SbPYL* genes in leaf (Fig. 3a). *SbPYL1* showed upregulation in response to ABA and osmotic stresses such as PEG and high salt. *SbPYL5* expression was higher under osmotic stresses, and a significantly higher (7.5-fold) increase in expression was recorded with ABA treatment (Fig. 3a). *SbPYL4* was found to be very specific for cold stress and showed highest increase in expression (19-fold) among all the *SbPYL* genes in leaf (Fig. 3a). Sorghum genome encodes at least 9 clade A PP2Cs. From these, six *SbPP2C* genes were selected for expression analysis. All the six *SbPP2C* genes were expressed at different levels in leaf under drought stress condition (Fig. 2). *SbPP2C1*, *SbPP2C2* and *SbPP2C3* showed 1.5-fold–3.4-fold increase, while *SbPP2C4*, *SbPP2C5* and *SbPP2C6* exhibited 56-fold–99-fold increase in expression in leaves under drought stress. Conversely to *SbPYL* genes, which showed downregulation in roots under drought stress, three out of six *SbPP2C* analyzed (*SbPP2C4*, *SbPP2C 5* and *SbPP2C6*) showed 1.5-fold–3.2-fold upregulation in roots under drought stress (Fig. 2). Among these, *SbPP2C6* also showed high temperature responsiveness by exhibiting 3.6-fold increase in expression, while rest of the *SbPP2C* genes were downregulated by high temperature stress in leaf (Fig. 3b). Expression levels of the *SbPP2Cs* were in general higher or unaffected in response to osmotic stresses and exogenous ABA in leaf except *SbPP2C5*, which decreased under cold stress (Fig. 3b). Expression of *SbPP2C6* was predominantly higher under all the abiotic stresses and exogenous ABA treatment in sorghum. The results from different plant species including *Arabidopsis*, rice, barley, tomato, sweet orange and grape also revealed a highly inductive nature of clade A PP2C genes under different abiotic stress conditions (Xue et al. 2008; Singh et al. 2010; Sun et al. 2011; Bohen et al. 2012b; Romero et al. 2012; Seiler et al. 2014). However, the level of induction and stress specificity might differ among these genes. In Grape, *VvPP2C4/8/9* were highly induced in response to

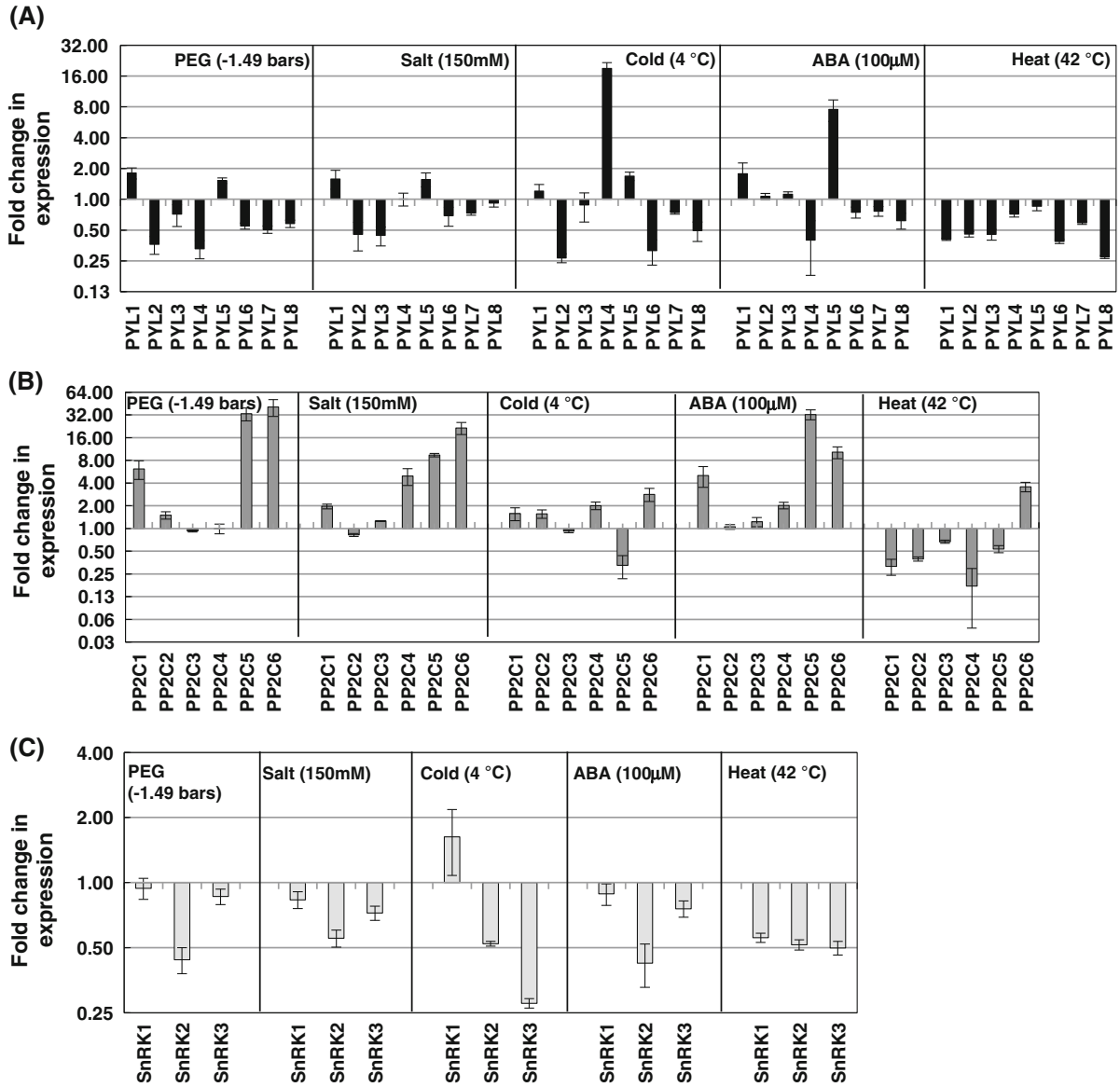

Fig. 3 Real-time RT-PCR expression analysis of **a** *SbPYL*, **b** *SbPP2C* and **c** *SbSnRK2* genes in leaves of 14-day-old sorghum seedlings under osmotic and temperature stresses. Relative fold change in expression was calculated using the $2^{-\Delta\Delta C_t}$ method (Livak and Schmittgen 2001) and shown in *log scale*. *Error bars* indicate SEM ($n = 3$)

exogenous ABA, drought, salt and cold stresses, while *VvPP2C2*, *VvPP2C1* and *VvPP2C3* were specifically induced in response to ABA and drought, cold stress and salt stress, respectively, (Bohen et al. 2012b). Similar to *SbPP2C*s, *VvPP2C*s also showed induction in roots under stress conditions (Bohen et al. 2012b).

Analysis of expression levels of three class III *SbSnRK2* genes that clustered with Class III *AtSnRK2* members revealed that these genes were either unaffected or downregulated under different abiotic stresses and exogenous ABA (Figs. 2, 3c). Expression analysis of class III *SnRK2* genes in rice and grape also revealed that these genes were downregulated by ABA and osmotic stress (Kobayashi et al. 2004; Boneh et al. 2012a). However, at protein level, these genes show activation by osmotic stress as well as ABA (Kobayashi et al. 2004). *ZmSnRK2.8* and *ZmSnRK2.10* from class III genes also showed downregulation under

ABA/heat and cold/heat stress, respectively (Huai et al. 2008). However, in tomato leaf, expression of all the class III *SnRK2* genes increased under dehydration conditions (Sun et al. 2011). These results suggest species-specific variation in stress-responsive expression pattern of *SnRK2* genes.

In general, the expression pattern of genes for core components of ABA signaling has been found to be conserved across many species with some species-specific variations. Exogenous ABA and abiotic stresses positively regulate the transcription of *PP2Cs* but negatively regulate the expression of *SnRK2s*, while *PYL* family members show differential regulation. However, these species differ considerably in their tolerance to abiotic stresses. During the course of evolution, genes have been known to acquire additional or different physiological functions depending on the species. In *Arabidopsis*, *AtPYR1*/*PYL1*/*PYL2*/*PYL4* have been associated with seed germination, root growth and ABA-induced gene expression (Park et al. 2009), whereas the strawberry homolog of *AtPYR1*, *FaPYR1* plays important role in fruit ripening (Chai et al. 2011). Moreover, given the multiple combinations possible with multigene family of *PYL*/*PP2CA*/*SnRK2*, there can be many species or stress-specific combinations of core signaling components. It is likely that *SbPYL* genes might have reduced redundancy as compared to that of *Arabidopsis* and might have acquired distinct functions to instill abiotic stress tolerance in sorghum. Therefore, a detailed species-specific characterization is required for understanding the complexity of ABA signalome. Despite the complexity of stress response, there have been studies where overexpression of single gene such as *AaPYL9*, from *Artemisia annua*, *AtPYL13* and *AtPYL5* has been shown to increase drought tolerance (Santiago et al. 2009; Zhang et al. 2013; Zhao et al. 2013). In our study, we could identify stress-specific ABA receptor genes (*SbPYL*) such as cold stress-specific *SbPYL4* and *SbPYL5,* which showed osmotic stress and ABA-induced expression. Among core component genes, only *SbPP2C6* showed upregulation under heat stress. These genes appear to be potential candidates for detailed characterization and exploitation for improvement of stress tolerance in sorghum.

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