Characterization of *PP2A-A3* mRNA expression and growth patterns in *Arabidopsis thaliana* under drought stress and abscisic acid

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Abstract Phosphoprotein phosphatase 2A (PP2A) plays a crucial role in cellular processes via reversible dephosphorylation of proteins. The activity of this enzyme depends on its subunits. There is little information about mRNA expression of each subunit and the relationship between these gene expressions and the growth patterns under stress conditions and hormones. Here, mRNA expression of subunit A3 of PP2A and its relationship with growth patterns under different levels of drought stress and abscisic acid (ABA) concentration were analyzed in *Arabidopsis thaliana*. The mRNA expression profiles showed different levels of the up- and down-regulation of *PP2AA3* in roots and shoots of *A. thaliana* under drought conditions and ABA treatments. The results demonstrated that the regulation of *PP2AA3* expression under the mentioned conditions could indirectly modulate growth patterns such that seedlings grown under severe drought stress and those grown under 4 μ M ABA had the maximum number of lateral roots and the shortest primary roots. In contrast, the minimum number of lateral roots and the longest primary roots were observed under mild drought stress and 0.5 μ M

ABA. Differences in *PP2AA3* mRNA expression showed that mechanisms involved in the regulation of this gene under drought conditions would probably be different from those that regulate the *PP2AA3* expression under ABA. Co-expression of *PP2AA3* with each of *PIN1-4,7* (*PP2A* activity targets) depends on the organ type and different levels of drought stress and ABA concentration. Furthermore, fluctuations in the *PP2AA3* expression proved that this gene cannot be suitable as a reference gene although *PP2AA3* is widely used as a reference gene.

Keywords Abscisic acid · *Arabidopsis thaliana* · In vitro drought stress · mRNA expression · Subunit A3 of phosphoprotein phosphatase 2A

Abbreviations

ABA	Abscisic acid
MS medium	Murashige and Skoog nutrient medium
PP2A	Phosphoprotein phosphatase 2A
qRT-PCR	Quantitative reverse transcription polymerase chain reaction

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Introduction

Phosphorylation and dephosphorylation of proteins are common phenomena in life, which control most biological processes (Hunter 1995). Protein kinases cause the binding of phosphate groups to specific proteins and the separation of the phosphate groups is performed by protein phosphatases (Stone and Walker 1995). Unlike kinases that belong to one superfamily (Taylor and Kornev 2011), phosphatases are classified into serine/threonine phosphatases, phosphotyrosine phosphatases, and dual specificity phosphatases

based on the substrate type (País et al. 2009). Serine/threonine protein phosphatases are a group of phosphatases which catalyze a reversible dephosphorylation of proteins in the position of serine or threonine residue (Terol et al. 2002). Phosphatases are associated with protein kinases and promote regulation of a lot of cell processes in all organisms (Uhrig et al. 2013). For example, they participate in processes such as signal transduction, cell division and metabolism (Smith and Walker 1996). Serine/threonine phosphatases are categorized into two main families including phosphoprotein phosphatases and metal-dependent protein phosphatases. The phosphoprotein phosphatase family is comprised of PP1, PP2A, PP2B, PP4, PP5, PP6, and PP7. Phosphoprotein phosphatases are composed of two types of subunits, which are catalytic and regulatory subunits. Regulatory subunits determine the specificity of catalytic subunit (Uhrig et al. 2013).

In plants, PP2A contributes to some processes such as the regulation of auxin transport through the control of polar delivery of PIN proteins (auxin efflux carriers) to basal membrane, regulation of cytoskeletal structure, signaling for ethylene and abscisic acid (ABA), plant growth/development, and response to biotic/abiotic stresses (Luan 1998; Delong 2006; Michniewicz et al. 2007; País et al. 2009; Uhrig et al. 2013). Drought stress, as one of the main stresses limiting plant growth and development, threatens life (Mahajan and Tuteja 2005). Plants can be tolerant of or show resistant against the drought stress via various changes in a wide range from morphological to molecular levels (Bray 2004; Kalefetoglu and Ekmekci 2005). In the molecular level, gene regulation under different conditions of drought stress is a crucial step which can modulate plant responses to drought (Bray 2004). ABA, as a plant growth regulator, mediates a lot of plant responses to different stresses such as drought stress (Finkelstein 2013). ABA also plays a key role in the regulation of many genes which are involved in response to drought stress (Zhang et al. 2006). Regarding the role of PP2A in response to environmental factors, drought stress and ABA may affect PP2A function in a manner dependent on the gene regulation.

As a trimeric enzyme, PP2A is composed of a catalytic subunit C, a scaffolding/regulatory subunit A and regulatory subunits B. (Blakeslee et al. 2008; País et al. 2009). When plants respond to drought conditions and ABA treatment, drought stress and ABA may target genes encoding PP2A subunits to regulate the processes that are dependent on the phosphorylation/dephosphorylation status of key molecules. However, information about the effects of drought stress and ABA on PP2A subunits is limited to some genes encoding PP2A subunits. Previous studies showed that overexpression of subunit C of PP2A in tobacco caused an increase in the drought tolerance of transgenic tobacco (Xu et al. 2007). In addition, a mutation

in gene encoding subunit C of PP2A in *Arabidopsis thaliana* created ABA-hyper sensitive mutant seedlings (Pernas et al. 2007). These two examples indicate that drought stress and ABA can regulate the gene encoding subunit C of PP2A.

In this study, it was attempted to evaluate how gene encoding subunit A3 of PP2A (PP2A-A3) is regulated in mRNA level in *Arabidopsis thaliana* grown under in vitro drought stress and ABA treatment. This effort was made in order to determine the involvement of gene encoding 65-kD PP2A-A3 in response to different levels of drought stress/ABA concentration as well as the dependency of the regulation of this gene upon the type of organ and duration of treatment. Moreover, this study was performed to find a relationship between drought and ABA in the regulation of gene encoding PP2A-A3. To achieve these objectives, the effects of different water potentials and ABA concentrations were analyzed on mRNA expression of PP2A-A3 in roots and shoots of *A. thaliana*. In addition, because of the significance of PP2A in the regulation of plant growth and developmental processes, some growth parameters in *A. thaliana* seedlings were determined after drought stress and ABA treatment in order to find out a relationship between PP2AA3 mRNA expression and growth patterns. Furthermore, to understand the co-expression status of PP2A-A3 with each of PIN genes which are well-known targets of PP2A activity (Michniewicz et al. 2007), mRNA expression of PIN genes was analyzed simultaneously in roots and shoots of *A. thaliana* seedlings grown under drought stress and ABA treatment. Based on these results, the correlation between expression of PP2AA3 and genes encoding plasma membrane PIN proteins (PIN1-4, 7) was evaluated under drought stress and ABA treatment.

Materials and methods

Plant material, in vitro growth condition

Sterilized seeds of *A. thaliana* L. (Col-0) were placed on the surface of MS basal medium (pH 5.8) (Murashige and Skoog 1962) containing 0.8% agar (Carl Roth, Karlsruhe, Germany). Stratification step was performed for 24 h at 4 °C. Afterwards, the seeds were allowed to grow vertically at 22 ± 2 °C under 16 h light and 8 h dark. Four-day-old seedlings obtained from this step were used for inducing in vitro drought stress and ABA treatment.

In vitro drought stress induction and ABA treatment

For in vitro drought stress induction, MS media with the same concentrations of nutrients (pH 5.8) were solidified

Table 1 The sequences of forward and reverse primers for *ACTIN2*, *PP2A-A3* and *PIN1-4,7*

Gene	Forward (5'–3')	Reverse (5'–3')
<i>ACTIN2</i> (AT3G18780)	GTATCGCTGACCGTATGAG	CTGCTGGAATGTGCTGAG
<i>PP2AA3</i> (AT1G13320)	GCAATCTCTCATTCGATAGTC	CGAAATACCGAACATCAACATC
<i>PIN1</i> (AT1G73590)	CTCAAGGCTTATCTGCGACAC	AGTTAGAGTTCCGACCACCAC
<i>PIN2</i> (AT5G57090)	CTCGTCACGGTTACTAATAG	TCATACTTCTGCCTCCTCTTC
<i>PIN3</i> (AT1G70940)	AGTGGAGATTTTCGGAGGAGAAC	GGAGCAAGTTTGTGTTAGACCATTC
<i>PIN4</i> (AT2G01420)	CGAAAGAGTGGTGGTGATG	ATGTGTTCCGTTGTTGCC
<i>PIN7</i> (AT1G23080.1)	AACAAAGCTGGTCCGATGAAC	TGTAGTCCGTTAGGCACTTCC

with 0.8, 2 and 4% agar for preparation of MS media with different water potentials of -0.2 mega pascal (MPa) (control condition), -0.5 MPa (mild drought stress), and -0.9 MPa (severe drought stress) respectively. Water potential of each MS media was measured by 15 bar pressure plate extractor. The levels of drought stress were determined based on a primary experiment.

For ABA treatment, MS media containing the same concentrations of nutrients (pH 5.8) including 0.8% agar were supplemented with 0.5 and 4 μ M ABA (Sigma-Aldrich, St. Louis, MO, USA). The ABA solution was added to the autoclaved MS media. The applied concentrations of ABA were determined based on a primary experiment.

Four-day-old seedlings were transferred to the prepared MS media and were grown vertically in a growth chamber under long day conditions (16 h light/8 h dark) at 22 ± 2 °C. Seedlings grown under different water potentials (– 0.2, – 0.5, and – 0.9 MPa) and on media containing 0.5 and 4 μ M ABA, were used for the evaluation of mRNA expression of gene encoding a PP2A-A3 at 24, 48, 120, and 192 h. The evaluation was separately performed on roots and shoots. The seedlings grown under -0.2 MPa were considered as control seedlings for both the drought stress and ABA treatment. Growth parameters including primary root length and the number of lateral roots/rosette leaves were measured after 192 h (8 days) of drought stress and ABA treatment.

Analysis of mRNA expression by real time quantitative reverse transcription polymerase chain reaction

RNeasy mini plant kit (Qiagen, Hilden, Germany) was used for isolation of total RNA from shoots and roots of *A. thaliana* seedlings. After treatment of extracted RNA with DNaseI (Thermo Fisher Scientific, Waltham, MA, USA), cDNA was synthesized according to instruction of Revert Aid First Strand cDNA Synthesis kit (Thermo Fisher Scientific). Quantitative reverse transcription polymerase chain reaction (qRT-PCR) was done in three technical replicates based on instruction of SYBR Premix EX Taq,

TliRNaseH Plus kit (Takara, Kyoto, Japan) for StepOne-Plus applied biosystem thermal cycler (Applied Biosystem, Foster City, CA, USA) with three -step PCR program (5 s at 95 °C, 30 s at 54 °C and 30 s at 72 °C). Method by Pfaffl (2001) was used for calculation of gene expression. *ACTIN2* gene was selected as the reference gene for the normalization of gene expression. Primers for gene encoding *PP2AA3*, *PIN1-4,7* and *ACTIN2* were designed by Beacon designer 7.5 (PREMIER BioSoft International, Palo Alto, CA, USA). cDNA sequences of these genes were obtained from TAIR database (<http://www.arabidopsis.org/>). The sequences of forward and reverse primers for *ACTIN2*, *PP2AA3* and *PIN1-4,7* were summarized in Table 1. Primers were purchased from Bioneer (Bioneer, Alameda, CA, USA).

Statistical analysis

All experiments were performed in a completely random design with 15 replications for the analysis of growth parameters and 3 replications for the analysis of the gene expression. For the gene analysis, each replication was obtained from roots and shoots of 150 seedlings. Minitab 16 software was used for data analysis. Normality of the data and equality of variances for data obtained from each experiment were checked. Only data of primary root length under different water potentials and ABA concentrations were normal and one-way ANOVA and Duncan multiple tests were used to compare the mean values at 5% significant level. Data obtained from other growth parameters and gene expression under different water potentials and ABA concentrations were not normal; therefore, nonparametric tests (Kruskal–Wallis test and Mann–Whitney test) were used for analyzing data. Information was grouped using Tukey method. All graphics were provided by Microsoft Excel 2010. Moreover, Pearson's correlation coefficients (r) were calculated separately by data of gene expression for *PP2AA3* with each of *PINs* (*PIN1-4,7*) under different levels of water potential and ABA concentration by SPSS21 software (IBM SPSS Statistics for Windows, Version 21.0. Armonk, NY: IBM Corp, 2012.)

Results

The mRNA expression of subunit A3 of PP2A under in vitro drought stress

The qRT-PCR results of roots and shoots show different levels of the mRNA expression of gene encoding subunit A3 of PP2A under control and drought conditions (-0.2 , -0.5 , and -0.9 MPa) at 0, 24, 48, 120, and 192 h after drought induction (Fig. 1). In roots under mild drought

Fig. 1 The mRNA expression of subunit A3 of PP2A under in vitro drought stress. The expression of gene encoding regulatory subunit A3 of PP2A was shown in the roots (a) and shoots (b) of the seedlings grown under the control condition ($\Psi_w = -0.2$ MPa), mild ($\Psi_w = -0.5$ MPa), and severe ($\Psi_w = -0.9$ MPa) drought stresses at 0, 24, 48, 120 and 192 h after drought induction. qRT-PCR was performed using cDNA synthesized from total RNA of roots and shoots. Fold change in the gene expression of *PP2AA3* was calculated by Pfaffl's method. *ACTIN2* gene was used as the reference gene for the normalization of the gene expression. Values are mean $\pm$ SD ($n = 3$). Each replication (n) was obtained from roots and shoots of 150 seedlings. Normality of the data and equality of variances for data obtained from each experiment were analyzed. The data did not have the normal distribution. Thus, nonparametric tests (Kruskal–Wallis test) were used for analyzing data. Information was grouped using Tukey method. Dissimilar letters represent the statistical significant difference among different levels of water potentials and time ($P < 0.05$)

stress (-0.5 MPa), mRNA expression of PP2A-A3 during 192 h was almost constant, while there were fluctuations in the mRNA expression of PP2A-A3 under control (-0.2 MPa) and severe drought (-0.9 MPa) conditions (Fig. 1a). In addition, the highest level of the transcripts of subunit A3 of PP2A (about 25-fold) was observed under severe drought conditions at 120 h, whereas the lowest mRNA expression of PP2A-A3 (about 0.4 fold) was related to control conditions at 24 h.

In shoots, although the various levels of the up-regulation of *PP2AA3* at 24 h were observed under all water potentials (-0.2 , -0.5 , and -0.9 MPa), after this time, the alterations in the mRNA expression of PP2A-A3 under all water potentials did not follow a similar pattern (Fig. 1b). However, the fluctuations under severe drought stress were more evident. Moreover, the maximum level of *PP2AA3* (about 90 fold) was determined at 24 h under severe drought stress.

The mRNA expression of subunit A3 of PP2A under in vitro ABA treatment

Expression profiles of *PP2AA3* in roots and shoots of *A. thaliana* seedlings grown under 0, 0.5, and 4 μ M ABA at 0, 24, 48, 120, and 192 h showed fluctuations in level of PP2A-A3 transcripts during 192 h (Fig. 2). In roots of the seedlings treated with 4 μ M ABA, there was a sudden increase in the mRNA expression of PP2A-A3 during first 24 h, while the up-regulation of gene encoding PP2A-A3 for the seedlings treated with 0.5 μ M ABA was gradual and occurred during 48 h (Fig. 2a). The highest mRNA level of gene encoding PP2A-A3 (about 18 fold) was related to roots of the seedlings treated with 4 μ M ABA at 24 h. In shoots, the similar patterns of the mRNA expression of gene encoding PP2A-A3 were observed in the seedlings treated with 0.5 and 4 μ M ABA (Fig. 2b). In addition, the mRNA level of PP2A-A3 in the control seedlings during 192 h was significantly higher than the ones treated with ABA.

Outcome of the fluctuations in *PP2AA3* mRNA expression is toward the up-regulation of this gene

Algebraic sum of consecutive ratios of fold changes in the *PP2AA3* expression to each other showed that the *PP2AA3* expression increased, in total, in comparison with zero time. For these calculations, the plus and minus signs were considered for the up-regulated and down-regulated ratios, respectively.

In roots and under severe drought stress and 4 μ M ABA, *PP2AA3* was up-regulated higher than tenfold, while the up-regulation of this gene under mild drought stress and 0.5 μ M ABA was 0.3 fold. Under control conditions, *PP2AA3* expression increased lower than tenfold.

Fig. 2 The mRNA expression of subunit A3 of PP2A under in vitro ABA treatment. The expression of gene encoding PP2A-A3 was shown in the roots (**a**) and shoots (**b**) of the seedlings grown under 0, 0.5, and 4 μ M ABA at 0, 24, 48, 120 and 192 h after ABA treatment. qRT-PCR was performed using cDNA synthesized from total RNA of roots and shoots. Fold change in the gene expression of *PP2AA3* was calculated by Pfaffl's method. *ACTIN2* gene was used as the reference gene for the normalization of the gene expression. Values are mean $\pm$ SD ($n = 3$). Each replication (n) was obtained from roots and shoots of 150 seedlings. Normality of the data and equality of variances for data obtained from each experiment were analyzed. The data did not have the normal distribution. Therefore, nonparametric tests (Kruskal–Wallis test) were used for analyzing data. Information was grouped using Tukey method. Dissimilar letters represent the statistical significant difference among different levels of ABA concentration and time ($P < 0.05$). The values of the controls at each point time in this ABA-experiment are the same 3 replicates that are used as controls in Fig. 3a, b (drought stress experiment), respectively

In shoots, an up-regulation of 2.7 fold for *PP2AA3* was observed under both concentrations of ABA. However, the *PP2AA3* up-regulation was higher than 20 fold under control and drought conditions.

Growth parameters under in vitro drought stress

The results of evaluation of growth parameters show the effects of water potentials of -0.2 , -0.5 , and -0.9 MPa on the growth parameters including primary root length

(Fig. 3a) and the number of lateral roots (Fig. 3b)/rosette leaves (Fig. 3c) in *A. thaliana* seedlings after 192 h (8 days) of drought induction. Severe drought stress (water potential of -0.9 MPa) resulted in the shortest primary root, the maximum number of lateral roots, and the minimum number of rosette leaves. Despite severe drought stress, mild drought stress (water potential of -0.5 MPa) caused the primary root elongation and promoted leaf formation like the control seedlings and all seedlings grown under mild drought stress had six leaves. However, the number of lateral roots in the seedlings grown under mild drought stress was significantly lower than those grown under severe drought stress. In addition, in the control seedlings, there were no lateral roots (Fig. 3).

Growth parameters under in vitro ABA treatment

Growth parameters including primary root length (Fig. 4a) and the number of lateral roots (Fig. 4b)/rosette leaves (Fig. 4c) in *A. thaliana* seedlings were affected, under 0, 0.5, and 4 μ M ABA for 192 h (8 days). In the seedlings treated with ABA, the effects of 0.5 and 4 μ M ABA on roots were opposing. High concentration of ABA (4 μ M) limited the elongation of primary roots and significantly increased the formation of lateral roots, whereas under low concentration of ABA (0.5 μ M), an increase in primary root length was accompanied with a smaller number of lateral roots. Moreover, the number of rosette leaves in the seedlings treated with both concentrations of ABA was significantly less than the control seedlings.

Co-expression status of *PP2AA3* with each of *PINs* (*PIN1-4*, 7) at mRNA level in roots and shoots under drought stress conditions and ABA treatment

Expression profiles of *PP2AA3* mRNA were compared with transcription pattern of each of *PINs* in roots and shoots grown under different levels of water potential and ABA concentration at 0, 24, 48, 120 and 192 h (Figs. 1, 2). These comparisons showed that in the roots grown under control conditions, the trend of change in the *PP2AA3* expression was similar to the trends of all *PINs* (Fig. 5a–e). In addition, under drought stress or ABA treatment, the highest level of similarities between the trend of changes in the *PP2AA3* expression and *PINs* expression were observed for *PIN1*, 4, 7 in the roots and for *PIN1*, 2 in the shoots grown under severe drought stress (water potential of -0.9 MPa) and for *PIN3* in the roots grown under 0.5 μ M ABA (Fig. 6a–f). A reverse trend of the *PP2AA3* expression was related to *PIN4* and *PIN7* in the shoots under 0.5 μ M ABA (Fig. 6g, h). However, there were differences in the trend of changes under other conditions. In addition to the comparison of the trends of change in the

Fig. 3 Growth parameters under in vitro drought stress. The effect of different drought conditions was shown on primary root length (a), the number of lateral root (b), and the number of rosette leaves (c) in the seedlings grown under the control condition ($\Psi_w = -0.2$ MPa), mild ($\Psi_w = -0.5$ MPa) and severe ($\Psi_w = -0.9$ MPa) drought stresses at 192 h (day8) after drought induction. MS media with different water potentials of -0.2 , -0.5 and -0.9 MPa were prepared by adding 0.8, 2 and 4% agar respectively to MS media with the same concentrations of nutrients. Values are mean $\pm$ SD ($n = 15$). Normality of the data and equality of variances for data obtained from each experiment were analyzed. Only the data of primary root length under different water potentials were normal and one-way ANOVA and Duncan multiple tests were used to compare the mean values at 5% significant level. Data obtained from other growth parameters (the number of lateral root and the number of rosette leaves) were not normal; therefore, nonparametric tests (Kruskal–Wallis test and Mann–Whitney test) were used for analyzing these data. Dissimilar letters represent the statistical significant difference among levels of water potential

Correlation. <http://libguides.library.kent.edu/SPSS/PearsonCorr>). The correlation coefficients more than 0.850 for *PP2AA3* with *PINs* were observed in roots with all *PINs* under control conditions, with *PIN1*, 4,7 under severe drought stress, with *PIN3* under 0.5 μ M ABA and also in shoots with *PIN1*, 2 under severe drought stress. There was no significant correlation between *PP2AA3* with any *PINs* in the roots under mild drought stress and in the shoots grown under control conditions and 0.4 μ M ABA.

Discussion

Effect of in vitro drought stress on the mRNA expression of subunit A3 of PP2A

Transcriptional patterns of gene encoding PP2A-A3 in roots and shoots of the seedlings grown under different water potentials (-0.2 , -0.5 , and -0.9 MPa) showed that different levels of drought stress could regulate the *PP2AA3* expression. With the exception of *PP2AA3* expression under mild drought stress, alterations in the mRNA level of gene encoding PP2A-A3 in roots at 0, 24, 48, 120 and 192 h indicated that the expression of this gene during developmental stages was dynamic under control conditions and severe drought stress. A previous study introduced gene encoding PP2A-A3 as a reference gene because of the stability in its expression under drought, salt, and cold stress (Czechowski et al. 2005). However, the present study showed that the gene expression of *PP2AA3* is not always constant and the mRNA expression of PP2A-A3 could be changed under severe drought stress or non-stress conditions under the natural mechanisms of growth and development.

expression of *PP2AA3* and *PINs*, Pearson's correlation coefficients (r) for *PP2AA3* with each of *PINs* (*PIN1*–4,7) under different conditions showed the correlation between *PP2AA3* with some *PINs* under specific conditions (Table 2). The Pearson's correlation coefficient (r) close to 1 or -1 shows that there is a strong positive or negative relationship between the expression of a gene pair of interest, whereas Pearson's correlation coefficients (r) close to 0 indicates a weak relationship between the expression of two genes (Usadel et al. 2009; SPSS Tutorials: Pearson

Fig. 4 Growth parameters under in vitro ABA treatment. The effect of different concentrations of ABA was shown on primary root length (a), the number of lateral root (b), and the number of rosette leaves (c) in the seedlings grown under 0, 0.5, and 4 μM ABA at 192 h (day 8) after ABA treatment. Autoclaved MS media containing the same concentrations of nutrients and 0.8% agar were supplemented with 0.5 and 4 μM ABA. Values are mean ± SD (n = 15). Normality of the data and equality of variances for data obtained from each experiment were analyzed. Only the data of primary root length under different ABA concentrations were normal and one-way ANOVA and Duncan multiple tests were used to compare the mean values at 5% significant level. Data obtained from other growth parameters (the number of lateral root and the number of rosette leaves) were not normal; therefore, nonparametric tests (Kruskal–Wallis test and Mann–Whitney test) were used for analyzing data. Dissimilar letters represent the statistical significant difference among levels of ABA concentration

drought stress was an essential factor in controlling the growth and development under water deficit stress. For the confirmation of this idea, in previous studies, defects in the root elongation were observed in the seedlings with *rcn1* mutation (Garbers et al. 1996; Derrière et al. 1999). In addition, because plants require a proper root system to respond to water deficit stress (Sharp et al. 2004), the expression of gene encoding PP2A-A3 is likely involved in providing a suitable root system in response to drought stress.

Higher levels of the mRNA expression of gene encoding PP2A-A3 in shoots in comparison with roots might have two possible reasons: (1) the number of processes and mechanisms required to the PP2A activity in shoots was more than that in roots; and (2) the activity level of processes required to PP2A in shoots was higher than roots. Therefore, the *PP2AA3* mRNA expression depends probably on the organ/tissue type.

Effect of in vitro ABA treatment on the mRNA expression of subunit A3 of PP2A

The mRNA expression profiles of gene encoding PP2A-A3 in roots and shoots of the seedlings grown on MS media containing 0.5 and 4 μM ABA indicated that ABA affected the mRNA expression of PP2A-A3 and the intensity of the gene expression depended on ABA concentration. In addition, the expression patterns of gene encoding PP2A-A3 under concentrations of 0.5 and 4 μM ABA were almost similar. The changes in the mRNA expression of gene encoding PP2A-A3 were related to the organ type. However, in shoots, concentration of ABA did not have any effects on the mRNA expression of gene encoding PP2A-A3. It is suggested that the existence of ABA in shoot is a necessary factor for the regulation of gene encoding PP2A-A3 and ABA concentrations may not be

It was reported that the gene encoding catalytic subunit (*PP2Ac-1*) of *Triticum aestivum* was up-regulated in response to drought stress (Xu et al. 2007). In addition, Blakeslee et al. (2008) showed that the expression of *RCN1*, which is a gene encoding subunit A1 of PP2A, in *A. thaliana*, was up-regulated under water deficit. With regard to the regulatory role of subunit A of PP2A and the significance of PP2A in response to developmental and environmental signals, it seems that the increase in the mRNA expression of PP2A-A3 in response to severe

Fig. 5 The trend of change in the expression of *PP2AA3* and *PINs* (*PIN1-4,7*) in the roots of the seedlings grown under the control condition (a–e). qRT-PCR was performed using cDNA synthesized from total RNA of roots. Fold change in the gene expression of *PP2AA3* and *PINs* was calculated by Pfaffl's method. *ACTIN2* gene was used as the reference gene for the normalization of the gene

expression. Values are mean of three replications. Each replication was obtained from roots of 150 seedlings. In the comparison between the trend of changes in the *PP2AA3* and *PINs* expression, the same *PP2AA3* control sample expression values (of the same three replicates) as shown in Fig. 1a and Fig. 2a were used

significant. Other studies showed that *PP2A-A3* may be essential for ABA-dependent stomatal closure and a mutation in *RCN1*, which is a gene encoding *PP2A-A1*, could cause ABA insensitivity in *A. thaliana* seedlings and the inhibition of stomatal closure in response to ABA (Kwak et al. 2002; Saito et al. 2008). Regarding the role of *PP2C* in ABA signaling pathway (Raghavendra et al. 2010), it is proposed that *PP2A* might also be a component of the ABA signaling pathway, which causes different responses to ABA in plants via dephosphorylation of many

proteins. These results with previous findings suggest that the alterations in gene expression of *PP2A-A3* under ABA treatment may indicate the regulatory role of this subunit in dephosphorylation of proteins required in ABA signaling.

Effects of in vitro drought stress on growth parameters in *A. thaliana*

The results of this study showed that in vitro drought stress induced by 2 and 4% agar provided different growth

Fig. 6 The trend of change in the expression of *PP2AA3* and *PINs* (*PIN1*–4,7) in the roots and shoots grown under different drought conditions or ABA treatments. **a–c** In the roots under severe drought stress ($\Psi_w = -0.9$ MPa). **d, e** In the shoots under severe drought stress. **f** In the roots under $0.5 \mu\text{M}$ ABA. **g, h** In the shoots under $0.5 \mu\text{M}$ ABA. qRT-PCR was performed using cDNA synthesized from total RNA of roots and shoots. Fold change in the gene expression of *PP2AA3* and *PINs* were calculated by Pfaffl's method. *ACTIN2* gene was used as the reference gene for the normalization of the gene expression. Values are mean of three replications. Each replication

was obtained from roots and shoots of 150 seedlings. The same *PP2AA3* “severe drought”- root sample expression values (of the same three replicates) as in Fig. 1a were used in this figure **a–c**. The same *PP2AA3* “severe drought”- shoot sample expression values (of the same three replicates) as in Fig. 1b were used in this figure **d, e**. The same *PP2AA3* “ $0.5 \mu\text{M}$ ABA”-root sample expression values (of the same three replicates) as in Fig. 2a were used in this figure **f**. The same *PP2AA3* “ $0.5 \mu\text{M}$ ABA”- shoot sample expression values (of the same three replicates) as in Fig. 2b were used in this figure **g, h**

Table 2 Pearson's correlation coefficients (r) for PP2A-A3 with each of PINs (PIN1–4,7)

	PP2AA3	PIN1	PIN2	PIN3	PIN4	PIN7
<i>Control condition/root</i>						
PP2A-A3	1	0.989**	0.935**	0.993**	0.966**	0.863**
<i>Mild drought stress ($\Psi_w = -0.5$ MPa)/root</i>						
PP2A-A3	1	0.326	0.147	0.377	0.371	– 0.169
<i>Severe drought stress ($\Psi_w = -0.9$ MPa)/root</i>						
PP2A-A3	1	0.950**	0.763**	0.009	0.987**	0.957**
<i>0.5 μM ABA/root</i>						
PP2A-A3	1	0.718**	– 0.345	0.875**	0.645**	0.294
<i>4 μM ABA/root</i>						
PP2A-A3	1	– 0.320	– 0.391	– 0.091	– 0.316	– 0.593*
<i>Control condition/shoot</i>						
PP2A-A3	1	0.059	– 0.033	0.017	0.038	– 0.210
<i>Mild drought stress ($\Psi_w = -0.5$ MPa)/shoot</i>						
PP2A-A3	1	0.624*	0.615*	0.470	0.045	– 0.024
<i>Severe drought stress ($\Psi_w = -0.9$ MPa)/shoot</i>						
PP2A-A3	1	0.874**	0.984**	0.298	– 0.185	– 0.310
<i>0.5 μM ABA/shoot</i>						
PP2A-A3	1	– 0.355	– 0.171	– 0.329	– 0.826**	– 0.599*
<i>4 μM ABA/shoot</i>						
PP2A-A3	1	– 0.297	– 0.443	– 0.300	– 0.387	– 0.513

**Correlation is significant at 0.01 level (2-tailed)

*Correlation is significant at 0.05 level (2-tailed)

changes in *A. thaliana* seedlings (Fig. 3). These growth alterations in response to drought stress depended on the levels of water potential (– 0.2, – 0.5 and – 0.9 MPa). Changes in growth parameters of potato plantlets grown on media with different amount of agar (water potential of MS media from – 0.70 to – 0.98 MPa) were reported (Gopal et al. 2008). Therefore, different levels of drought stress have a determinant role in optimizing the seedling morphology through the induction of growth changes in roots and shoots to maximize the utilization of available water under water deficit conditions by seedlings. Various studies showed different degrees of increasing in the root system volume or reducing the number of leaves in response to different levels of drought stress (Dubrovsky and Gómez-Lomelí 2003; Zhang et al. 2009; Farooqa et al. 2010; Zokaee-Khosroshahi et al. 2014).

Growth changes in the seedlings grown under mild drought stress (– 0.5 MPa) indicated that mild drought stress could have a positive effect on growth of *A. thaliana* seedlings. This effect could be related to adaptive mechanisms to accelerate the life cycle before the intensification of drought stress. Previous studies show that some plants are able to avoid drought stress via completing their life cycle before the beginning of water deficit period (Kalefetoglu and Ekmekci 2005; Claeys and Inzé 2013).

Growth patterns of the seedlings under severe drought stress (– 0.9 MPa) indicated that under severe drought stress, seedlings had likely the higher tendency for the lateral root formation in order to increase water uptake surface. There are some reports about enhancement of the lateral root formation under severe drought stress (Dubrovsky and Gómez-Lomelí 2003; Zhang et al. 2009). In addition, a rise in the percentage of rhizogenesis with increased quantity of PEG as a drought stress inducer was observed in calli of different rice varieties (Biswas et al. 2002).

The reduction of the number of rosette leaves under severe drought stress was a proper mechanism to reduce water loss. Hummel et al. (2010) reported a decrease in the leaf number in *A. thaliana* grown in soils with water potentials of – 0.6 and – 1.1 MPa. In the data presented here, the reduction of leaf number and increasing the number of lateral roots instead of primary root elongation to provide a stable balance between uptake and loss of water are probably also related to different effects of severe drought stress on cell division and cell differentiation at the meristematic zones in the roots and shoots (Sacks et al. 1997; Schuppler et al. 1998).

Changes in the amount and distribution of phytohormones such as auxin are likely another reason for the

induction of such growth patterns under severe drought stress. Shojaie et al. (2015) reported that various levels of drought stress resulted in changes in the mRNA expression of PIN proteins, which are auxin efflux carriers, in roots and shoots of *A. thaliana*. They suggested that different patterns of the PINs mRNA expression in the roots and shoots may cause different patterns in auxin distribution, which finally affect the root and shoot growth.

Effects of in vitro ABA treatment on growth parameters in *A. thaliana*

The results of the present study showed that ABA affected the lateral root formation positively. This effect was intensified with increasing ABA concentrations. Lateral root development in cauliflower treated with ABA and the enhancement of the root density by higher concentrations of ABA was another case of ABA effects on plants (Biddington and Dearman 1982). In addition, some studies showed that less lateral roots were formed in ABA-insensitive (*abi1-1*) and ABA-deficient (*aba*) mutant seedlings (Vartanian et al. 1994).

The limitation in primary root growth under the high concentration of ABA (4 μ M) and the induction of primary root elongation under low concentration of ABA (0.5 μ M) demonstrated that primary root growth is sensitive to concentration of ABA. This might be related to the inhibitory effect of high concentrations of ABA on cell division and cell expansion (Xu et al. 2010). However, Cheng et al. (2002) pointed to the stimulating effect of low concentration of ABA on root growth. Both ABA concentrations prevented the formation of rosette leaves. The negative effect of ABA on the meristematic cells at the shoot apical meristem (Wang et al. 1998) could be a reason for lower number of leaves in the seedlings treated with ABA in comparison with untreated seedlings.

Relationship between the *PP2AA3* mRNA expression and growth patterns under drought stress and ABA treatment

In plants, PP2A is a phosphoprotein phosphatase that can regulate plant growth and development (Uhrig et al. 2013). Changes in the expression of PP2A subunits may affect the PP2A activity, and consequently PP2A-dependent processes such as growth and development. For example, *Arabidopsis* seedlings with mutation in *RCN1*, a gene encoding one of the subunit A isoforms, showed a decrease in growth of hypocotyls and roots under ionic, osmotic and oxidative stresses (Blakeslee et al. 2008). In another example, double mutant seedlings *rcn1 pp2aa2-1* and *rcn1 pp2aa3-1* were dwarf in comparison with wild type seedlings (Zhou et al. 2004). In the present experiment, changes in growth

patterns in response to different levels of drought conditions and ABA concentrations could be the result of the *PP2AA3* mRNA expression, though these changes may not be directly dependent on the *PP2AA3* expression. As shown under severe drought stress (water potential of -0.9 MPa) and 4 μ M ABA, severe fluctuations in the *PP2AA3* mRNA expression and, in total, an up-regulation higher than tenfold of *PP2AA3* during 192 h in comparison with the gene expression at time 0 (Figs. 1a, 2a) might stimulate the lateral root formation rather than primary root elongation (Figs. 3a, b, 4a, b). In contrast, almost the constant expression of *PP2AA3* and an up-regulation lower than one fold (about 0.3 fold) (Figs. 1a, 2a) could promote primary root elongation under mild drought stress (water potential of -0.5 MPa) and 0.5 μ M ABA (Figs. 3a, b, 4a, b). A regular fluctuation in the *PP2AA3* mRNA expression and, an up-regulation lower than tenfold of *PP2AA3* (Figs. 1a, 2a) might lead to that growth pattern which was observed for roots of the control seedlings (Figs. 3a, b, 4a, b). In shoots, an insignificant expression of *PP2AA3* (up-regulation about 2.7 fold in total) under 0.5 and 4 μ M ABA (Fig. 2b) might result in preventing the formation of rosette leaves (Fig. 4c), while higher levels of up-regulation of *PP2AA3* (higher than 20 fold in total) under control and drought conditions (Fig. 1b) could stimulate it (Fig. 3c). Thus, there might be a relationship between the *PP2AA3* mRNA expression and growth patterns under drought conditions and ABA treatment.

Relationship between in vitro drought stress and ABA treatment in the mRNA expression of subunit A3 of PP2A

ABA is introduced as a main phytohormone, which plays a key role in plant responses to drought stress (Leung and Giraudt 1998; Zhang et al. 2006; Cutler et al. 2010). In this study, the treatment of seedlings with 0.5 and 4 μ M ABA could respectively mimic the effect of water potentials of -0.5 and -0.9 MPa on growth parameters of *A. thaliana* grown under in vitro conditions (Figs. 1, 2). However, with exception of shoots, there was no significant correlation in the mRNA expression profiles of PP2A-A3 between roots of the seedlings treated with 0.5 and 4 μ M ABA and roots of the seedlings grown on MS medium with water potentials of -0.5 and -0.9 MPa (Figs. 3, 4). Therefore, it is suggested that the gene regulation of PP2A-A3 in roots might happen in different ways in response to drought stress and ABA treatment. Its regulation might be under the control of different mechanisms though subunit A3 is a key factor for PP2A. In other words, at least in roots, there might be no relationship between the drought stress and ABA treatment in the regulation of gene encoding PP2A-A3.

Co-expression of *PP2AA3* with each of *PINs* (*PIN1*–*4*, *7*) under drought stress and ABA treatment

PP2A mediates dephosphorylation of some proteins in cells (Uhrig et al. 2013). PIN proteins are one of the targets of the PP2A activity (Michniewicz et al. 2007). The phosphorylation and dephosphorylation status of PINs determines the direction of PIN insertion to apical or basal membrane (Michniewicz et al. 2007). This means that phosphorylated PINs are localized in apical membrane, while dephosphorylated PINs are localized in basal membrane. This can regulate polar auxin transport in plants (Michniewicz et al. 2007). Regarding the regulatory role of PP2A-A3 in PP2A activity, it is expected that *PP2AA3* has an expression profile similar to the expression profiles of *PINs*. The Pearson's correlation coefficient (*r*) is a good index for evaluating similarity or complementarity between two variables, which is also used for computing co-expression of genes (Geisler-Lee et al. 2007; Horan et al. 2008; Almeida-de-Macedo et al. 2013). Therefore, according to the data of Table 2, a strong positive correlation between the expression profiles of *PP2AA3* and each *PIN* in the roots and shoots grown under different levels of water potential and ABA concentration indicated that changes in expression of *PP2AA3* strongly correlated with the changes in the expression of each of *PIN1*–*4*, *7*. In addition, a negative and significant correlation coefficient for the expression profile of *PP2AA3* with expression profiles of *PIN4* and *PIN7* in shoots under 0.5 μ M ABA showed a strong inverse relationship between *PP2AA3* and *PIN4*/*PIN7*. Thus, the data of Pearson's correlation coefficients (*r*) represented that co-expression of *PP2AA3* with each of *PINs* at mRNA level could be regulated by the type of tissue/organ, intensity of drought stress and ABA concentrations.

Conclusion

The results of this study showed differences in growth parameters which are primary root length and the number of lateral roots/rosette leaves of *A. thaliana* seedlings at 192 h after growing on MS media with various levels of water potential (– 0.2, – 0.5, and – 0.9 MPa as control, mild, and severe drought conditions, respectively) or different concentrations of ABA (0, 0.5, and 4 μ M). In addition, the alterations in the mRNAs levels of gene encoding PP2A-A3 in the seedlings grown under the above-mentioned water potentials and ABA concentrations at 0, 24, 48, 120, and 192 h after drought stress or ABA treatment were observed. The results indicated that the developmental mechanism, duration/intensity of drought stress, ABA concentrations, duration of ABA treatment, and sensitivity of organ/tissue

to ABA could have a regulatory role on the growth and mRNA expression of gene encoding PP2A-A3. The gene encoding PP2A-A3 was introduced as a reference gene and is widely used in the gene expression experiments, whereas fluctuations in the mRNA expression of this gene under different water potentials and ABA concentrations proved that the gene encoding PP2A-A3 cannot be a proper gene for normalizing the gene expression in qPCR experiments. Furthermore, no relationship was observed between the expression of *PP2AA3* in roots treated with ABA and those grown under drought stress. It is suggested that in roots, mechanisms involved in the regulation of the *PP2AA3* expression under drought stress might be different from those involved under ABA treatment. The *PP2AA3* regulation by drought stress and ABA could affect growth patterns of seedlings and there might be a relationship between the *PP2AA3* mRNA expression and growth patterns under drought conditions and ABA treatment. The co-expression analysis of *PP2AA3* with each of *PINs* (*PIN1*–*4*, *7*) showed the co-regulation of *PP2AA3* with some *PINs* under specific conditions, suggesting that the co-expressions depend on the type of organ, different levels of drought stress and ABA concentration.

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