

SERINE/THREONINE PHOSPHATASE TAPP2Cs MIGHT BE SERVED AS AN EARLY SIGNAL MOLECULE FOR WATER STRESS IN WHEAT

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Abstract

Much progress has been made towards understanding the role of serine/threonine phosphatases type 2C (PP2Cs) in abscisic acid (ABA) signaling transduction. However, how the negative regulator, PP2Cs, responds to plant water loss remains unclear. Here, we used a series of relative soil moisture [RSM: 85% (well watered), 65% (moderate stress), 45% (severe stress)] potted winter wheat (*Triticum aestivum* L.) and the detached leaves to detect ABA levels and transcripts of PP2Cs, including PP2C40, PP2C45, PP2C59 and PP2C6 as well as the core downstream signals of ABA, including ABF, SnRK2.4 and SnRK2.5. The results showed that the continual loss of water led to a consistent increase in ABA levels, and that the mRNA expression levels of PP2Cs were dependent on plant water condition. PP2Cs expression could be induced by a slight loss of water, and inhibited under severe loss of water. These results were further confirmed by the transcripts of ABF, SnRK2.4 and SnRK2.5. Furthermore, in slight loss of water, 100 μ M exogenous ABA could promote PP2Cs expression; in severe loss of water, it inhibited PP2Cs expression. In conclusion, ABA accumulation is controlled by water condition and the PP2C expression is dependent on plant water condition, suggesting that PP2Cs might be served as an early signal molecule for water stress in wheat.

Key words: Wheat, Abscisic acid (ABA), Type 2C protein phosphatase (PP2Cs), Water loss, Gene expression.

Introduction

Drought often occurred in many areas of the world including China and thereby becomes a major limiting factor for crop production. For survival, the sessile plants develop precise and complex regulation mechanisms, by which protein reversible phosphorylation is demonstrated to play an important role in the response and adaptation of plants to water loss (Mizoguchi *et al.*, 1996). In the recent years, much progress has been made toward understanding this mechanism through the core signaling transduction pathway triggered by ABA in response to water stress (Bano & Aziz., 2003; Kang *et al.*, 2010; Khan *et al.*, 2011; Fujii *et al.*, 2009). Nevertheless, as a hub component of ABA signaling transduction, how the negative regulator serine/threonine phosphatases type 2C (PP2Cs) responds to plant water loss remains unclear.

As an important regulatory mechanism in both prokaryotic and eukaryotic organisms, reversibly-phosphorylated proteins is referred to protein kinases and protein phosphatases, including Ca²⁺-dependent protein kinases (CDPKs), SNF1-related protein kinases (SnRKs), mitogen-activated protein kinase (MAPK), and receptor type kinases (RPKs), serine/threonine phosphatases PP1, PP2A, PP2B, and PP2C (Cohen & Cohen, 1989; MacKintosh *et al.*, 1991; Chang & Stewart, 1998; Sopory & Munshi, 1998; Hirayama & Shinozaki, 2007). The Arabidopsis strong ABA-insensitive loci ABI1 and ABI2 are first identified to encode PP2C enzymes, which play a central role in ABA signaling pathway as negative regulators of plant stress response (Leung *et al.*, 1994; Meyer *et al.*, 1994; Schweighofer *et al.*, 2004). In the past years, the negative regulatory role of PP2Cs in ABA signaling were extensively studied in many plants (MacKintosh *et al.*, 1992; Meskiene

et al., 1998; Sheen, 1998; Gosti, *et al.* 1999; González-García *et al.*, 2003; Hu *et al.*, 2010; Jia *et al.*, 2013a).

Notably, in recent years, the ABA-PYR/PYL/RCAR-PP2C core signaling pathway has been established by genetic and structural biology (Fujii *et al.*, 2009; Ma *et al.*, 2009; Melcher *et al.*, 2009; Miyazono *et al.*, 2009). In this model, the early reported PP2Cs, serving as a central and negatively-regulated hub in ABA signaling (Leung *et al.*, 1994; Merlot and Giraudat, 1997; Gosti *et al.*, 1999; Merlot *et al.*, 2001), were first integrated into the canonical ABA signaling network by reversible phosphorylation, namely ABA promotes the interaction of PYR1 with protein phosphatase 2C (PP2C), resulting in the inhibition of PP2C and the activation of SnRK2; the activated SnRK2 turns on ABA signaling via the phosphorylation of the downstream factors such as AREB/ABF (Fujii *et al.*, 2009). However, under different soil moisture, especially under slight loss-of-water condition, how the plant PP2Cs conduct in responsive to ABA remains unclear.

In the present study, winter wheat (*Triticum aestivum* L.), as the important crop in North China, is prone to water stress, which led to a reduction in yield and even plant death. Thus, winter wheat was used as experimental material and cultivated under relative soil moisture (RSM) 85% (well watered), 65% (moderate stress), and 45% (severe stress). The ABA contents and the transcripts of PP2Cs, including PP2C40, PP2C45, PP2C59 and PP2C6 together with its downstream signals, including ABF, SnRK2.4 and SnRK2.5 were detected. Our results demonstrate that ABA accumulation is controlled by plant water condition, and PP2C expression is dependent on plant water condition, suggesting that the serine/threonine phosphatases PP2Cs might be served as an early signal molecular for water stress.

Materials and Methods

Plant materials and growth conditions: The cultivated wheat species 'zhongmai12' used here was provided by the Institute of Crop Sciences of Chinese Academy of Agriculture Sciences. All the plants were cultivated in pots and auto-monitored by Em50 soil moisture data collectors and Volumetric Water Content Sensors EC-5 Lowest Cost VWC (Decagon Devices). The plants were well watered and the temperature was controlled between 22 to 25°C during seedling stage.

Drought stress assay: The potted wheat plants with three true leaves were on drought treatment. Collect the wheat leaves when the relative soil moisture successively reached at 85%, 65%, and 45% and quick-frozed the leaves with liquid nitrogen and finally stored at -80°C for use. The experiment was repeated three times.

ABA treatment analysis using detached leaves: Fifty treated and control leaves were collected from the potted wheat at the same developmental stage and divided them into five groups, each group includes 4-5 leaf blades and was treated by 100µM ABA, then placed them in the 10,000 Lx light incubator (the temperature was controlled at 25°C, relative humidity controlled at 75%) and dried naturally. The leaves were collected respectively after 0 h (control), 0.5 h, 2 h, 4 h, and 8 h, and quick-frozed them in liquid nitrogen and stored at -80°C. The experiment was repeated three times.

ABA treatment analysis using potted wheat: Relative soil moisture of three potted wheat groups were kept at 85% (well watered), 65% (moderate stress), 45% (severe stress), respectively. After spraying 100µM ABA to each group, the leaves were collected after 0.5 h, 2 h, 4 h, and 8 h, respectively, and quick-frozed them in liquid nitrogen and stored at -80°C. The experiment was repeated three times.

Determination of stomatal conductance, leaf temperature, leaf water potential: The stomatal conductances of the leaves was measured under natural conditions using portable SC-1 stable stomatal (Decagon Devices, USA). Infrared thermometer Testo testo845 (Testo ltd, Germany) was used for the detection of leaf temperature. The leaf water potential was measured using WP4-T Dewpoint Potentiometer (Decagon Devices, USA). The experiment has three replications.

Measurement of endogenous ABA content: ABA content was determined by gas chromatography-mass spectroscopy. The frozed leaves were taken out, and 1 g of leaves was used for ABA detection by the previously-reported methods (Asami *et al.*, 1999). The experiment was repeated three times.

Extraction of total RNA: Total RNA was extracted from wheat leaves using TRIzol® Reagent (Invitrogen, USA). Wheat leaves were ground in liquid nitrogen using mortars and pestles and transferred to a plastic tube. The tissue powder was quickly suspended in TRIzol® Reagent (1ml per 50mg of tissue samples) and incubated for 5-10 min at room temperature. The chloroform was added by 0.6 ml per 1 ml of TRIzol® Reagent. Shake tube vigorously by hand for 30 s and incubated them at room temperature for 3 min. Centrifuge the tube at 12,000 g for

10 min at room temperature to remove insoluble materials. The upper aqueous phase was transferred to a new tube, mixed with an equal volume of isopropyl alcohol, and ncubated for 20min at room temperature and centrifuged at 12,000g for 10min at 4°C to removed the supernatant. At least 1 ml of 75% ethanol was used to wash the RNA pellet and after centrifugation at 12,000 g for 5 minutes at 4°C, the wash was discarded completely. The RNA was finally dissolved in 30µl DEPC-treated water and the RNA was finally dissolved in 30µl DEPC-treated water and 2 µl were taken to conduct 1% agarose gel electrophoresis test. The rest RNA solution stored at -80°C for until use.

Synthesis of cDNA: Every 2µg total RNA of wheat leaves was used as a template for synthesis of the first-strand cDNA with the oligo(dT)₁₈ primer and reverse transcriptase M-MLV(RNase H-) (TaKaRa Dalian Liaoning China) and followed the manufacturer's instruction.

Real-time PCR: The sequences of *PP2Cs*, *SnRK2.4*, *ABF*, *Actin* in wheat were obtained from NCBI, and the primers of real-time PCR were designed using Primer Premier 5 and DNAMAN V6. Different cDNA concentration gradients have used to test the specificity of the primers (Table.1). Real-time PCR was carried out using the Quantitative Fast SYBR Green RT-PCR Kit (TaKaRa Dalian Liaoning China) on an Bio-Rad CFX96 thermal cycler (Bio-Rad, Hercules, CA, USA), using *actin* as the endogenous control. Every processed cDNA was used as template to conduct the PCR. Keep the reaction volume at 10 µl including 3.5 µl DEPC-treat water, 0.25 µl forward primer, 0.25 µl reverse primer and 1µl cDNA. The PCR was done as follows: 3 min 95°C and 10 s 94°C, 20 s 58°C, 20s 72°C, for 40 cycles; Melt Curve was controlled from 65°C to 95°C, increment 0.5°C for 30s. The experiment was repeated three times.

Statistical analysis: The standard error compute used excel and all the data of significance levels were analyzed used statistical method by SPSS software.

Results

Changes of physiological parameters and transcripts of TaPP2Cs under different soil relative moisture in potted wheat: To explore the changes of ABA levels and transcripts of *TaPP2Cs* in response to soil water loss, a series of RSM was controlled at 85% (well watered), 65% (moderate stress), 45 % (severe stress) levels. As shown in Fig. 1A, with soil water loss from 85% to 45% RSM, ABA contents increased rapidly and significantly at $p < 0.05$ and $p < 0.01$. The changes of stomatal conductance and leaf water potential showed the same trends, namely with water loss, increasing rapidly then declining continuously (Fig. 1C and D). As shown in Fig. 1B, with RSM from 85% to 65%, the mRNA expression levels of *TaPP2Cs*, including *PP2C6*, *PP2C40*, *PP2C4*, and *PP2C59*, all increased rapidly; hereafter with soil water further loss, namely RSM from 85% to 45%, all reduced.

Taken together, with soil water loss, the complex trends occurred between ABA levels and *PP2Cs* transcripts: similarity from 85% to 65% RSM; reverse from 65% to 45% RSM (Fig. 1). These results suggest that under special soil water conditions, *PP2Cs* expression could be induced by slight loss of water or be inhibited by severe water loss in potted wheat.

Effect of detached leave water loss on TaPP2Cs expression levels in wheat *In vitro*: In order to further explore the response of *PP2Cs* to water loss, the detached wheat leaves were tested at 25°C for 0-8 hr, and the transcripts of *PP2C40*, *PP2C45*, *PP2C59*, *PP2C6* were detected by real-time PCR. The results showed that with the leave water loss, the mRNA expression levels of *PP2C40* and *PP2C6* increased significantly during 0-2 hr, hereafter declined; the mRNA expression levels of *PP2C45* increased significantly during 0-0.5 hr and 2-4 hr, hereafter decreased, respectively; the mRNA expression levels of *PP2C59*, on the whole, changed slightly in 0-8 hr (Fig. 2). These results indicated that *PP2Cs* expression could be induced by slight loss of water or be inhibited by severe water loss in wheat *In vitro*.

Effect of exogenous ABA on TaPP2Cs expression levels in different RSM potted wheat leaves *In vivo*: In order to further determine the response of *PP2Cs* expression to ABA in different soil water condition, the potted 85%, 65%, and 45% RSM wheat leaves were treated with exogenous 100 μ M ABA under 25°C for 0-8 hr. The results showed that

in 85% RSM, transcripts of *PP2C40*, *PP2C45*, *PP2C59*, and *PP2C6*, on the whole, showed an increased trends during 0-8 hr; in 45% RSM, in addition to *PP2C6* in 0.5 or 8 hr point, the transcripts of *PP2C40*, *PP2C45*, *PP2C59*, and *PP2C6*, on the whole, showed a decreased trends during 0-8 hr; in 65% RSM, in addition to *PP2C45*, the transcripts of *PP2C40*, *PP2C59*, and *PP2C6*, on the whole, increased first and then decrease (Fig. 3). These results indicated that *PP2Cs* expression could be induced by ABA or be inhibited by ABA in different RSM potted.

Effect of water loss on transcripts of TaPP2Cs downstream signals under different relative soil moisture: To further confirm the response of *PP2C* with water loss, the mRNA expression levels of *ABF*, *SnRK2.4* and *SnRK2.5* of *PP2C* downstream components were carried out by real-time PCR analysis under different RSM. The results showed that in addition to an insignificant variation at $p < 0.01$ in *SnRK2.4* expression under different RSM, the same trends were observed in both *SnRK2.5* and *ABF* expression. With soil water loss, both transcripts declined rapidly from 85% to 65% RSM, then increased rapidly from 65% to 45% RSM (Fig. 4). These results suggest that in slight loss of soil water condition, *SnRK2.5* and *ABF* expression could be inhibited by water loss, supporting the notion that *PP2Cs* expression could be induced by slight loss of water.

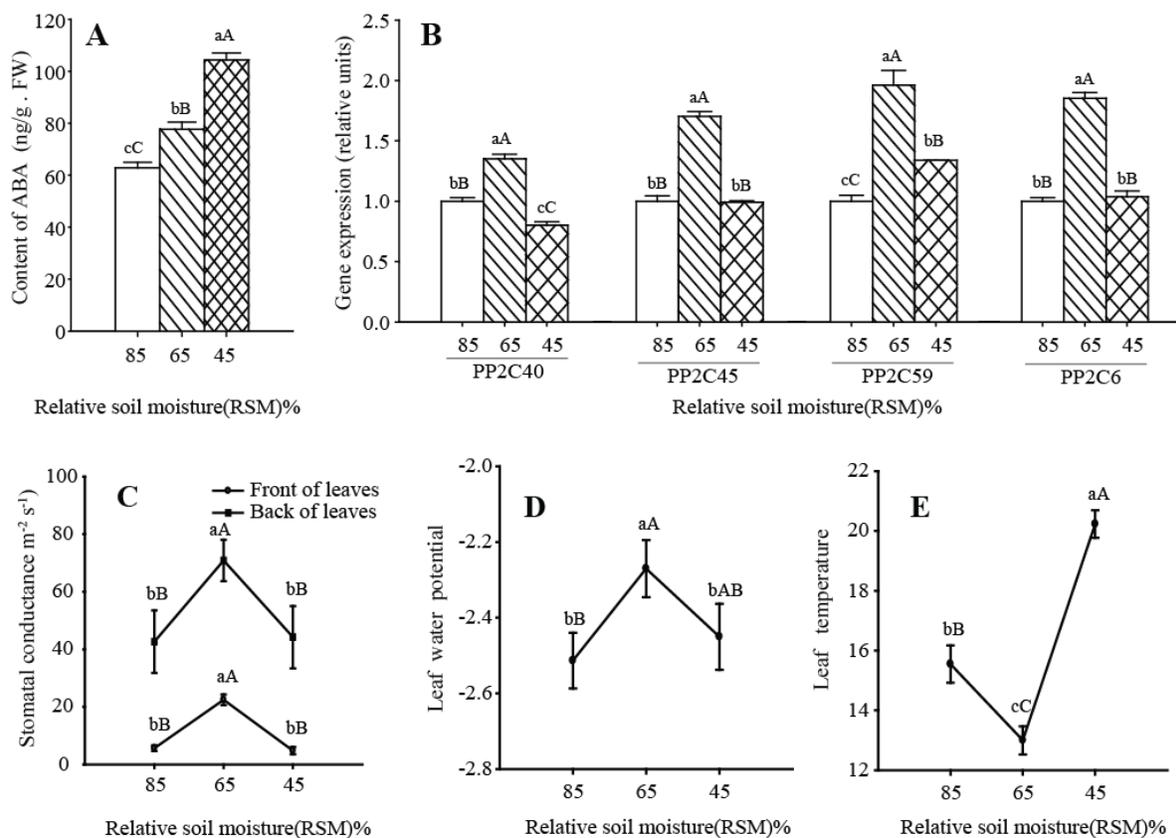


Fig. 1. ABA content and *PP2Cs* transcripts under different RSM in potted wheat.

A: ABA contents; B: The transcripts of *PP2Cs*. C: Stomatal conductance; D: Leaf water potential; E: Leaf temperature. Different small and capital letters within the same column indicate significant differences at $p < 0.05$ and $p < 0.01$, respectively. Error bars represent SE ($n=3$).

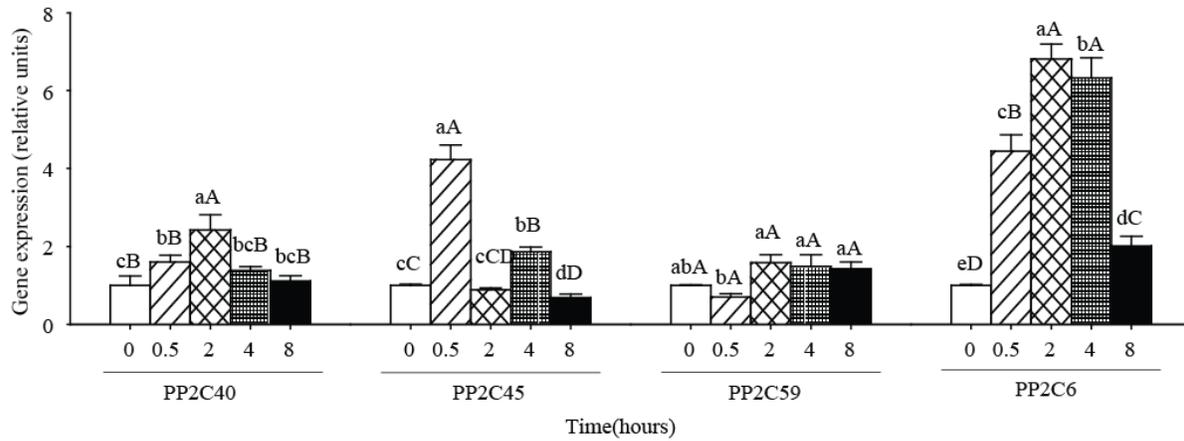


Fig. 2. Transcripts of *PP2Cs* in detached wheat leaves with water loss. Different small and capital letters within the same column indicate significant differences at $p < 0.05$ and $p < 0.01$, respectively. Error bars represent SE (n=3).

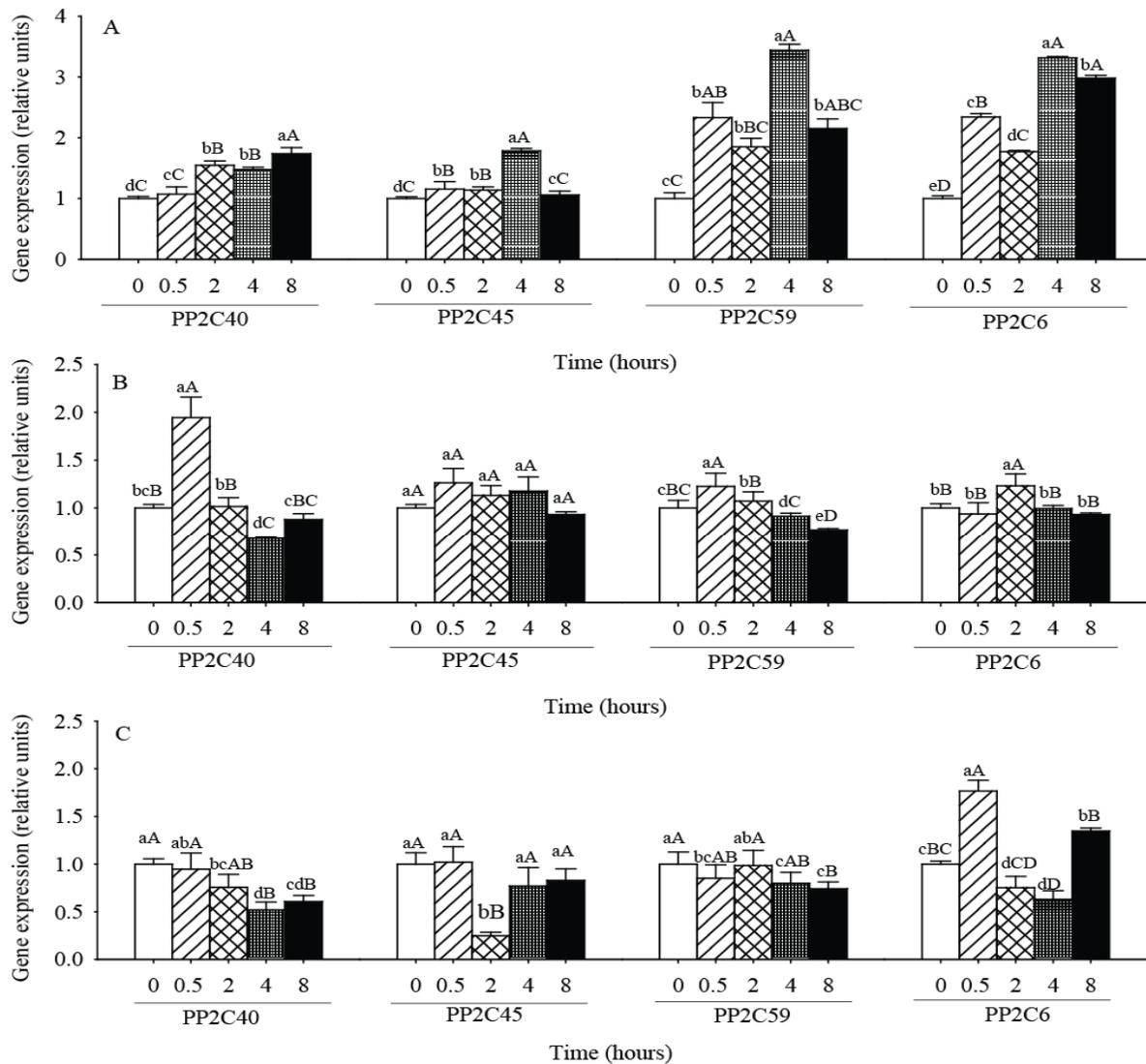
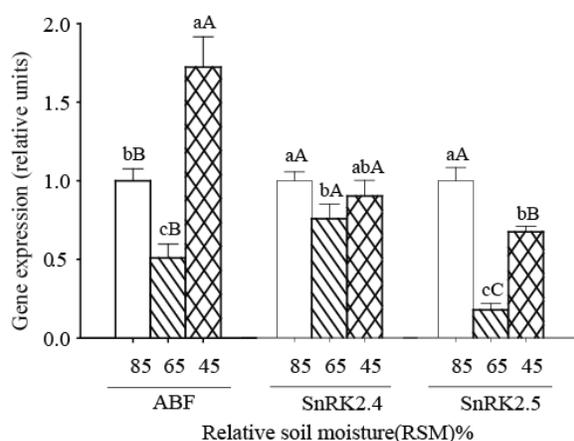


Fig. 3. Transcripts of *PP2Cs* in 100µM ABA treated, different RSM potted wheat. A: in RSM 85% RSM; B: in RSM 65% RSM; C: in RSM 45% RSM. Different small and capital letters within the same column indicate significant differences at $p < 0.05$ and $p < 0.01$, respectively. Error bars represent SE (n=3).

Table 1. Primers used for real-time PCR.

NCBI ID No.	Gene names	Primer seq (5'-3')
(HQ391079.1)	<i>TaPP2C40</i>	5'-TTGGCAGTGATGGCTTAT-3' 5'-GGATACCTGCTTCTTGG-3'
(HQ287800.1)	<i>TaPP2C45</i>	5'-GGGTCAACAGCATCAACT-3' 5'-CCCTGCCACATAACAAA-3'
(EF672269.1)	<i>TaPP2C59</i>	5'-CGTGTCAAGGGACCATAAGC-3' 5'-CACCAAATGCCCGAGAAA-3'
(AB238930.1)	<i>TaPP2C6</i>	5'-GAATGAAGAGGTATGCGATGT-3' 5'-CAATAACCGTGATGTTGTCC-3'
(GQ384359.1)	<i>TaSnRK2.4</i>	5'-CACGATGAGGGAGATAAAAG-3' 5'-TCCGAGAAAAGAAGGCAC-3'
(AF519804.1)	<i>TaABF</i>	5'-TCAGTCTTGCGAGAGAAGCA-3' 5'-CTTTCAGACGAGCGTTCTCC-3'
(AB181991.1)	<i>Actin</i>	5'-TACTCCCTCACAACAACCG-3' 5'-AGAACCTCCACTGAGAACA-3'
	<i>TaSnRK2.5</i>	5'-GCGACCTCAAGCTGGAG-3' 5'-CCGAGCAGAAGAAGAATC-3'

Fig. 4. Transcripts of *ABF*, *SnRK2.4* and *SnRK2.5* under different RSM potted wheat.

Different small and capital letters within the same column indicate significant differences at $p < 0.05$ and $p < 0.01$, respectively. The error bars represent the SE ($n=3$)

Discussion

It is known that the initial physiological response to drought stress is a loss of water, as a result which leads to stomatal closure, in turn, repression of cell growth and photosynthesis, disturbance of metabolism, and even plant death (Smirnov, 1993). Owing to a key role of ABA in stomatal movement (Zhang & Outlaw, 2001; Wikinson and Davies, 2002; Davies *et al.*, 2005), over the past years, much progress has been made toward understanding the mechanisms of ABA action (Hirayama and Shinozaki, 2007; Raghavendra *et al.*, 2010; Guo *et al.*, 2011; Jia *et al.*, 2013b).

Earlier studies by the identification of the strong ABA-insensitive loci *ABI1* and *ABI2* establish the negatively-regulated role of PP2Cs in ABA response in Arabidopsis (Finkelstein *et al.*, 2002). In recent years, this notion was definitely clarified in the ABA-PYR1-PP2C core signaling pathways: as no or less ABA, PP2C can inhibit target gene expression of ABA response; in contrast, as more ABA, PP2C can promote target gene

expression of ABA response. In the current study, slightly loss water both in potted and in detached wheat leaf tests, could activated *PP2Cs* expression, finally inhibited ABA responses (Figs. 1 and 2). This situation (slightly loss water) might be the same as 'as no or less ABA'. This notion is further confirmed by the effect of exogenous ABA on *PP2C* expression in different RSM potted test, namely, *PP2C* expression, on the whole, was induced by ABA in 85% RSM; was first induced then inhibited in 65% RSM; was inhibited in 45% RSM (Fig. 3).

To further confirm the response of PP2C with water loss, the mRNA expression levels of the downstream PP2C signals were also carried out by real-time PCR analysis under different RSM. The results showed that with soil water loss, the transcripts of both *SnRK2.5* and *ABF* declined rapidly from 85% to 65% RSM coupled with an opposite trend in both ABA levels and *PP2C* expression. These results suggest that *SnRK2.5* and *ABF* expression could be inhibit by water loss, supporting that *PP2Cs* expression could be induced by slight water loss.

Conclusion

ABA accumulation is controlled by plant water condition. The hub gene *PP2C* expression is dependent on plant water condition, namely, in early loss of water, *PP2Cs* expression could be induced by ABA; in severe loss of water, the *PP2Cs* expression could be inhibited by ABA, suggesting that PP2Cs might be served as an early signal molecular for water stress in wheat, and this gene may be useful in agricultural production in drought area.

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