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BRIEF COMMUNICATION

Aquaporin expression during seed osmopriming and post-priming germination in spinach

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Abstract

Aquaporins (AQPs) are proteinaceous channels known to regulate transmembrane water transport, and therefore may be important component of imbibition during osmopriming and germination. To explore the association between AQPs and osmopriming-led enhanced germination performance, we studied the expression patterns of four spinach (*Spinacia oleracea*) AQP coding genes (*SoPIP1;1*, *SoPIP1;2*, *SoPIP2;1*, and *SoδTIP*) during osmopriming and subsequent germination under optimal conditions, chilling, and drought. All these genes were up-regulated within 2 - 4 d of priming (phase II-imbibition). We hypothesize such up-regulation to facilitate the pressure potential-driven cell expansion and increase germination potential of primed seeds. Our data during post-priming germination suggest that *SoPIP1;1* and *SoδTIP* were more closely associated with enhanced germination performance. In general, all *AQP*s were down-regulated under chilling and drought. However, under chilling, *SoPIP2;1* was expressed at relatively higher level in primed seeds that also exhibited greater chilling tolerance, whereas *SoPIP1;2* and *SoδTIP* exhibited opposite pattern. Similarly, *SoPIP1;1*, *SoPIP2;1*, and *SoδTIP* exhibited higher expression in primed seeds that also had greater drought tolerance.

Additional key words: chilling, drought, plasma membrane intrinsic protein, polyethylene glycol, tonoplast intrinsic protein.

Seed priming is a pre-sowing treatment that improves germination performance manifested by their greater germination percentage, rate, and uniformity (Bradford 1986, Chen *et al*. 2010). Osmopriming is one type of priming that partially hydrates seeds through exposure to low external water potentials imposed by polyethylene glycol (PEG), or inorganic salts, *etc*. Due to this advanced imbibition ('head-start'), primed seeds have an improved performance during subsequent germination. In recent years, evidence is accumulating to suggest that osmopriming also improves germination performance under stress conditions (Korkmaz and Korkmaz 2009, Chen *et al*. 2010).

It has been proposed that osmopriming improves stress tolerance during germination through two strategies (Chen and Arora 2012). First, it enables a 'head-start' for germination-related activities (Benamar *et al.* 2003, Sung *et al*. 2008) to increase the germination potential. Secondly, it imposes moderate osmotic stress, which prevents radicle protrusion but activates protective systems that confer tolerance to subsequent stresses, *i.e*. 'cross tolerance' (Gallardo *et al*. 2001, Ligterink *et al*. 2007, Chen *et al*. 2012a).

We have previously established an optimal osmopriming protocol for spinach (*Spinacia oleracea* cv. Bloomsdale) that improves seed germination performance as well as tolerance to chilling and drought stresses (Chen *et al*. 2010). We further found a few potential biochemical/molecular markers for greater stress-tolerance of primed seeds (Chen and Arora 2011, Chen *et al*. 2012a). However, a biomarker for increased germination potential during priming is still elusive.

Seed germination, a multiphasic process, is initiated

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Abbreviations: AQP - aquaporin; PEG - polyethylene glycol; PIP - plasma membrane intrinsic protein; qPCR - quantitative real-time polymerase chain reaction; TIP - tonoplast intrinsic protein.
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by water uptake and culminates at radicle protrusion driven by embryo expansion (Bewley and Black 1994, Nonogaki *et al*. 2007). Seed hydration is potentially mediated by aquaporins (AQPs), the proteinaceous channels for transmembrane water movement (Baiges *et al*. 2002, Maurel 2007). Cell-to-cell water transport is mainly regulated by two AQP subgroups: tonoplast intrinsic proteins (TIP) and plasma membrane intrinsic proteins (PIP) (Maurel *et al*. 2002, Maurel 2007, Hussain *et al*. 2011). AQPs have also been implicated for regulating pressure potential-driven cell expansion (Maurel *et al*. 2008 and references therein, Peng *et al*. 2008). Taken together, these observations suggest that AQPs may be markers for seed germination potential.

The association between *AQP* expression and seed germination has been documented in a few species. *OsPIP1;3*-antisense transgenic *Oryza sativa* seeds exhibited reduced germination under optimal conditions and desiccation stress whereas those over-expressing *OsPIP1;3* had an improved tolerance to water deficit (Liu *et al*. 2007). These observations are consistent with the findings in *Nicotiana tabacum* seeds transformed with sense and antisense of *Brassica napus BnPIP1* (Yu *et al*. 2005). Moreover, it appears that NO, a signal molecule, promotes seed germination likely through activating *PIP*s (Liu *et al*. 2007).

Currently, sequences coding for three *PIP*s and one *TIP* have been reported in spinach (*SoPIP1;1*, *SoPIP1;2*, *SoPIP2;1*, and *SoδTIP*) (Johansson *et al*. 1996, Karlsson *et al*. 2000, Fraysse *et al*. 2005). While *SoPIP2;1* was implicated in regulating volume changes of guard cells and mesophyll protoplasts, *SoPIP1*s were believed to participate in phloem loading, transport, and unloading, and stomatal movements (Fraysse *et al*. 2005). However, no specific role was ascribed to *SoδTIP* water-channel (Karlsson *et al*. 2000). Though the role of *SoPIP*s and *SoδTIP* in seed germination has not yet been explored, given the significance of *AQPs* in water absorption, they likely undergo altered expression during osmopriming and germination. This study was conducted to determine and compare the expression dynamics of *SoAQPs* during osmopriming and post-priming germination under both optimal and stress conditions.

Spinach (*Spinacia oleracea* L. cv. Bloomsdale) seeds for osmopriming time-course and germination under optimal and stress treatments were sampled as described in Chen *et al*. (2010) and Chen and Arora (2011). Quantitative real-time polymerase chain reaction (qPCR) was conducted, as described in Chen *et al*. (2012a,b), to study the expression patterns of *SoAQPs* during osmopriming as well as post-priming germination under optimal and stress conditions. *18S rRNA*, with primers adopted from a published sequence (Shou 2003), was used as the internal control (Chen *et al*. 2012a,b). The forward (F) and reverse (R) primers for target genes (as follows) were designed with *Primer 3* software (Whitehead Institute for Biomedical Research, Cambridge, MA, USA) according to the published mRNA sequences (Johansson *et al*. 1996, Karlsson *et al*. 2000, Fraysse *et al*. 2005): *SoPIP1;1* (Genbank acc. No. AJ249384): F-'GGACCA TGCTTGGGATCATC' and R-'CAGCTAAAGCAGC TCCAATGAA'; *SoPIP1;2* (acc. No. AY372191): F-'GGGCAATCCCTTTCAAATCC' and R-'ACATCC ACCCATGAATGAAACC'; *SoPIP2;1* (acc. No. L77969): F-'CCGTCGCCACTGTCATTG' and R-'GGC CAACAGAACCACAAACA'; *SoδTIP* (acc. No. AJ245953): F-'GCCCAGTGTGCTGGTTCTGT' and R-'GCAACACTGTGGATTGGAGTTG'. The relative expression level of target genes in all samples was calculated by normalizing the threshold cycle (Cq) for *AQPs* with that of *18S rRNA* through the ΔΔCq method (*ABI* Manual). The experiments were independently repeated at least twice with similar results. The most representative data (with mean of triplicates ± standard errors) are presented.

In general, seed water uptake during germination includes three phases (Bewley and Black 1994). Phase I is an initial rapid water absorption and mainly a physical

Table 1. *Aquaporin* expression and moisture content in spinach (*Spinacia oleracea* cv. Bloomsdale) seeds during osmopriming. Seeds were primed with -0.6 MPa PEG 8000 at 15 °C, and collected after 1, 2, 4, and 8 d of priming. A subsample of 8-d primed seeds was dried back at 25 °C for 2 d to original moisture (8 d + 2DD). Fresh and dry masses of all samples (including unprimed) were measured to determine seed moisture change during priming. *SoAQPs*' expression was measured by qPCR and calibrated against *18S rRNA* as an internal control according to Chen *et al*. (2012 a,b). The expression of individual *SoAQPs* in 1-d primed seeds was assigned a value of '1' (*) and used as the calibrator for day 2, 4, and 8. The experiment was independently repeated at least twice. Most representative data set is presented as means ± SE (n = 3). Within each column, means followed by the same letter are not significantly different at $P < 0.05$ according to Fisher's least significant difference test.

Priming duration [d]	Relative expression level [fold] *SoPIP1;1*	*SoPIP1;2*	*SoPIP2;1*	*SoδTIP*	Seed moisture content [%]
0	-	-	-	-	8.6 ± 0.08 a
1	1.02 ± 0.11*a	1.00 ± 0.08*a	1.00 ± 0.06*a	0.98 ± 0.07*a	40.3 ± 0.48 b
2	1.01 ± 0.13 a	2.87 ± 0.11 b	2.17 ± 0.07 b	1.00 ± 0.08 a	40.8 ± 0.21 b
4	3.38 ± 0.52 b	1.33 ± 0.02 c	0.79 ± 0.03 c	2.36 ± 0.19 b	41.7 ± 0.17 c
8	1.12 ± 0.07 a	0.49 ± 0.02 d	0.67 ± 0.01 d	1.03 ± 0.13 a	42.9 ± 0.24 d
8 d +2DD	-	-	-	-	8.6 ± 0.07 a

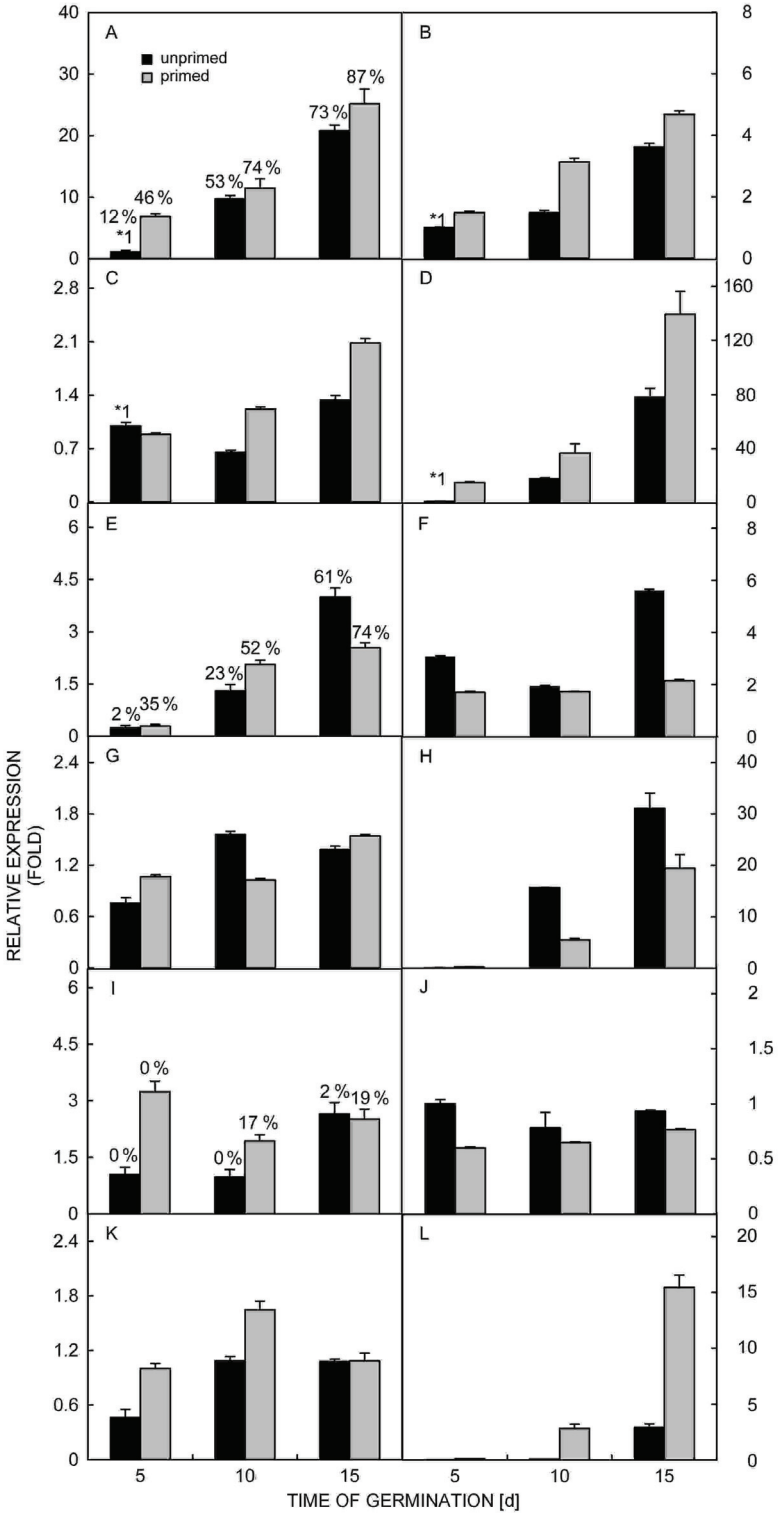

Fig. 1. *SoAQP* expression in spinach seeds during germination under optimal, chilling, and drought conditions. Seeds were primed with PEG 8000 (-0.6 MPa) at 15 °C for 8 d, followed by 2-d slow drying at room temperature. Unprimed and primed-dry seeds were germinated at 10 °C (optimal; *A*, *B*, *C*, and *D*), at 5 °C (chilling; *E*, *F*, *G*, and *H*), and under drought (PEG 8000, -0.8 MPa; *I*, *J*, *K*, and *L*). Seeds were collected when germinated for 5, 10, and 15 d. Germination percentage is indicated in *A*, *E*, and *I*. The expression of four *SoAQP*s was investigated: *SoPIP1;1* (*A*, *E*, and *I*), *SoPIP1;2* (*B*, *F*, and *J*), *SoPIP2;1* (*C*, *G*, and *K*), and *SoδTIP* (*D*, *H*, and *L*). The expression of each *SoAQP per se* in 5-d unprimed seeds germinated under optimal conditions was assigned a value of '1' (*1), and used to calibrate the expression in other treatments. The experiment was independently repeated at least twice. Most representative data set is presented as means ± SE (n = 3)

process; phase II is marked by little net water uptake but accompanies a high metabolic activity that prepares seeds for radicle protrusion; and phase III is a second burst of water uptake coupled with radicle emergence; the latter is precluded during priming where seeds are only brought up to phase II. Our data indicate an up-regulation of all *SoAQPs* within 2 - 4 d of osmopriming, *i.e.* phase II imbibition (Table 1), an expected occurrence since priming involves transmembrane water influx. Possibly, such up-regulation facilitates cell expansion and enhances germination potential as supported by ensuing discussion.

Manz *et al*. (2005) observed that water was mainly distributed in embryos of tobacco seeds during phase II imbibition, a stage where embryo growth initiates and continues by cell expansion (see references in Nonogaki *et al*. 2007). Indeed, the pressure potential-driven embryo expansion during phase II has been considered as essential for radicle protrusion (Nonogaki *et al*. 2007, Sliwinska *et al*. 2009). We propose that such cell expansion could be mediated by *AQPs*. Following studies together support this notion: *1*) over expression of *Panax ginseng PgTIP1* or *Brassica oleracea BobTIP26;1* resulted in increased cell size in *Arabidopsis* and tobacco, respectively (Reisen *et al*. 2003, Lin *et al*. 2007); *2*) *PIP*s are preferentially expressed in elongating tissues (see references in Maurel *et al*. 2008); and *3*) exogenous treatment of mercury (a general AQP inhibitor) delayed radicle protrusion in *Arabidopsis* (De Willigen *et al*. 2006). Accordingly, up-regulation of *SoPIP*s and *SoδTIP* in osmoprimed seeds, in the present study, may be functionally associated with the higher germination potential of primed seeds (Chen *et al*. 2010). Notably, *SoAQP* expression dropped after 4-d priming despite little change in seed moisture (Table 1). This may be due to an arrest of further germination caused by limited water supply during priming, thus likely making it unnecessary to maintain *AQPs* in up-regulated state.

Our results during post-priming germination indicate that the four *AQPs* may be associated with seed germination potential to varying degrees. While *SoAQP*s were generally up-regulated in primed seeds, *SoPIP1;1* and *SoδTIP* exhibit greater up-regulation than the other two under optimal conditions: at 5-d, the expression of *SoPIP1;1* and *SoδTIP* in primed seeds was ~ 7 and 15-folds, respectively, of their corresponding unprimed controls compared to ~1.5 and 0.9-folds for the other two genes (Fig. 1*A-D*). Though only by association, our data suggest new roles for these two genes during seed germination and more in-depth study is warranted to test this notion.

Our data indicate that *PIP1*s, which typically are not as active water channels as *PIP2*s (Kaldenhoff and Fischer 2006), exhibit greater up-regulation in primed seeds *versus* unprimed controls during germination. At day 5, the up-regulation of *SoPIP1;1* and *SoPIP1;2* was ~7 and 1.5-fold, respectively (Fig. 1*A,B*) whereas *SoPIP2;1* expression remained essentially unaltered (Fig. 1*C*) indicating a greater association of *PIP1*s than *PIP2* with germination. In support of this observation, *pip1* tobacco knock-out mutants exhibited retarded testa rupture (hence reduced germination potential) compared to the wild-type whereas no such response was observed for *pip2* mutants (Ernst 2007).

The relationship between *SoAQP* expression and germination under chilling stress seems less straightforward in this study. All four *SoAQPs* were down-regulated in unprimed and primed seeds compared to optimal conditions, with *SoPIP1;1* and *SoδTIP* being relatively more impacted (compare Fig. 1*A-D* with *E-H*). This observation agrees with the finding that cold treatment represses *AQP* expression and/or activity and hence the hydraulic conductance of roots (Aroca *et al*. 2005, Maurel *et al*. 2008, Ionenko *et al*. 2010). Chilling-tolerant tissues, on the other hand, often exhibit greater *AQP* expression as well as enhanced water-channel activity compared to the susceptible ones leading to a rapid recovery from cellular dehydration (Aroca *et al*. 2005, Li *et al*. 2009, Matsumoto *et al*. 2009).

Comparison of primed *versus* unprimed germinating seeds under chilling reveals that *SoPIP2;1* was expressed at higher levels, generally (*i.e*., at two of the three time-points), in primed seeds than unprimed ones (Fig. 1*G*), and the former also had greater chilling tolerance (Chen *et al*. 2010, Chen and Arora 2011, Chen *et al*. 2012a) (compare germination percentages of primed and unprimed seeds in Fig. 1*E*). It is plausible, therefore, that *SoPIP2;1* is more related with regulating chilling tolerance than the other three *SoAQPs*, a notion deserving further investigation. Interestingly, *SoPIP1;2* and *SoδTIP* expression was significantly lower in primed seeds than unprimed ones (Fig. 1*F*,*H*). Taken together, these results suggest a varying response of *AQP* family members to abiotic stresses reflecting their functional diversity, an observation noted also in other studies (Peng *et al*. 2008, Matsumoto *et al*. 2009).

All four *SoAQP*s were down-regulated under drought stress compared to optimal germination (compare Fig. 1*A-D* with *I-L*) which is consistent with earlier report on maize (*Zea mays*) seedlings (Ionenko *et al*. 2006). Here too, a varied response by *SoAQPs* was noted when comparing primed *versus* unprimed seeds. Except for *SoPIP1;2*, other three *AQPs* had higher expression in primed seeds than the unprimed ones (Fig. 1*I-L*). Two opposite views exist for the physiological role of AQPs *vis-à-vis* drought tolerance (Hachez *et al*. 2006, Hussain *et al*. 2011): *1*) *AQPs* up-regulation may help avoid drought stress by providing additional capability of water-uptake for stressed plants (Lian *et al*. 2004, Ermawati *et al*. 2009); and *2*) over-expression of some *AQP*s may exacerbate drought stress by facilitating water loss (Aharon *et al*. 2003). Our data (Fig. 1*I*), in conjunction with greater drought tolerance of primed seeds (Chen *et al*. 2010, Chen and Arora 2011, Chen *et al*. 2012a), support the first view, as do the findings by Gao *et al*. (1999) with PEG- and ABA-primed *Brassica napus* seeds germinated under drought or salt stress.

In conclusion, osmopriming might improve germination performance and the stress tolerance of

germinating seeds *via* altering *AQP* expression. Our data indicate *SoAQP*s' up-regulation during priming (especially *SoPIP1;1* and *SoδTIP*) is associated with enhanced seed germination performance of primed seeds. However, the connection between *AQP* expression and priming-led enhanced chilling tolerance is less straightforward: only *SoPIP2;1* exhibited higher expression in primed seeds than unprimed ones and thus may be more involved in regulating chilling tolerance than other *SoAQP*s. On the contrary, under drought stress, all *SoAQP*s, but *SoPIP1;2,* had higher expression in primed seeds which were also more drought-tolerant than unprimed ones; such up-regulation may increase the water availability for germination of primed seeds.

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