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Biochemistry

Enhanced expression of the proline synthesis gene *P5CSA* in relation to seed osmopriming improvement of *Brassica napus* germination under salinity stress

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ABSTRACT

Osmopriming is a pre-sowing treatment that enhances germination performance and stress tolerance of germinating seeds. *Brassica napus* seeds showed osmopriming-improved germination and seedling growth under salinity stress. To understand the molecular and biochemical mechanisms of osmopriming-induced salinity tolerance, the accumulation of proline, gene expression and activity of enzymes involved in proline metabolism and the level of endogenous hydrogen peroxide were investigated in rape seeds during osmopriming and post-priming germination under control (H_2O) and stress conditions (100 mM NaCl). The relationship between gene expression and enzymatic activity of pyrroline-5-carboxylate synthetase (P5CS), ornithine-δ-aminotransferase (OAT) and proline dehydrogenase (PDH) was determined. The improved germination performance of osmoprimed seeds was accompanied by a significant increase in proline content. The accumulation of proline during priming and post-priming germination was associated with strong up-regulation of the *P5CSA* gene, down-regulation of the *PDH* gene and accumulation of hydrogen peroxide. The up-regulated transcript level of *P5CSA* was consistent with the increase in P5CS activity. This study shows, for the first time, the role of priming-induced modulation of activities of particular genes and enzymes of proline turnover, and its relationship with higher content of hydrogen peroxide, in improving seed germination under salinity stress. Following initial stress-exposure, the primed seeds acquired stronger salinity stress tolerance during post-priming germination, a feature likely linked to a 'priming memory'.

Introduction

Seed germination is an important stage of plant development. Several physiological and biochemical changes take place during seed germination, such as resumption of respiratory activity (Bewley et al., 2013), activation of repair mechanisms, protein synthesis from stored and newly synthesized mRNA, and reserve mobilization (Bewley, 1997). The proper course of germination determines the establishment of the mature plant. Germination can be influenced by many abiotic factors that restrict or inhibit it. Among these, salinity is one of the major abiotic stresses affecting seed germination, plant growth and reproduction (Zhu, 2002).

Seed priming is widely used in the cultivation of plants to improve germination efficiency and field emergence under adverse environmental conditions (Carvalho et al., 2011; Jisha et al., 2013). Seed osmopriming is a pre-sowing treatment that exposes seeds to a low external water potential that allows partial hydration but prevents radicle protrusion through the seed coat (Bradford, 1986). Osmopriming with polyethylene glycol (PEG) has been described as a good technique for improving seed germination of different

Abbreviations: P5CS, pyrroline-5-carboxylate synthetase; P5CR, pyrroline-5-carboxylate reductase; PDH, proline dehydrogenase; OAT, orn-δ-aminotransferase; PEG, polyethylene glycol; Pro, proline; Orn, ornithine; GSA, glutamate semialdehyde; P5C, pyrroline-5-carboxylate; UP, unprimed; P, primed; UP_d, dry unprimed seeds; P_{nd}, primed non dried seeds; P_d, primed dried seeds; UP_{7H_2O}, unprimed seeds germinating 7 h on water; P_{7H_2O}, primed seeds germinating 7 h on water; $UP_{7\ NaCl}$, unprimed seeds germinating 7 h on 100 mM NaCl; $P_{7\ NaCl}$, primed seeds germinating 7 h on 100 mM NaCl; UP_{11H_2O}, unprimed seeds imbibed 11 h on water; $UP_{16\ NaCl}$, unprimed seeds imbibed 16 h on 100 mM NaCl.

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species under salt and drought stress (Jisha et al., 2013). Several reports have demonstrated priming-improved germination performance, but the underlying mechanisms of priming-mediated stress tolerance are still poorly understood. It has been suggested that stress tolerance acquired by osmopriming treatment may be associated with the accumulation of dehydrins (DHNs) and a more robust antioxidant system in relation to activation of pre-germinative metabolism might imprint in seeds a sort of 'stress memory' or 'priming memory' (Chen and Arora, 2011, 2013; Chen et al., 2012). Gallardo et al. (2001) reported accumulation of stress-related proteins, such as low molecular heat shock proteins (HSPs) during osmopriming in Arabidopsis seeds. Similarly, Kubala et al. (2015) reported that proteins and genes involved in the stress response, such as HSPs and members of the enzymatic antioxidative defense system, were up-regulated during osmopriming of *Brassica napus* seeds. Moreover, the up-regulation of P5CSA encoding pyrroline-5-carboxylate (P5C) synthase A, a key enzyme in proline (Pro) synthesis, was observed in osmoprimed rape seeds (Kubala et al., 2015).

Proline accumulation is a common physiological response to salinity and osmotic stress in many plants species (Ashraf and Foolad, 2007). Proline is mainly synthesized from L-glutamic acid (Glu), which is reduced to glutamate semialdehyde (GSA) by pyrroline-5-carboxylate synthetase (P5CS); next, GSA spontaneously cyclizes to form P5C. P5C reductase (P5CR) further reduces the P5C intermediate to Pro. This pathway is found in the cytosol and in plastids (Hu et al., 1992; Verbruggen and Hermans, 2008). Formation of GSA/P5C from ornithine (Orn) via orn-δ-aminotransferase (OAT) was postulated to constitute an alternative pathway of Pro synthesis and accumulation (Roosens et al., 1998; Verbruggen and Hermans, 2008). There is also evidence for a pathway in which OAT does not seem to contribute to Pro biosynthesis, but generates P5C, which is used for the production of glutamate (Funck et al., 2008).

Pro is catabolized to Glu in mitochondria by Pro dehydrogenase (PDH) and P5C dehydrogenase (P5CDH) (Hu et al., 1992; Verbruggen and Hermans, 2008). Pro acts as a compatible osmolyte and is a way to store carbon and nitrogen (Delauney and Verma, 1993; Hare and Cress, 1997; Verbruggen and Hermans, 2008). Moreover, Pro is thought to be a component of the antioxidative defense system, a regulator of cellular redox potential, a stabilizer of subcellular structures and macromolecules or a component of signal transduction pathways that regulate stress-responsive genes (Szabados and Savouré, 2010; Székely et al., 2008). Pro accumulation during osmotic stress is due to increased synthesis and reduced degradation (Rentsch et al., 1996; Verbruggen and Hermans, 2008). Proline synthesis can also play an important role in promoting germination. An increase in free Pro was observed prior to radicle emergence in Arabidopsis seeds and feedback inhibition of Pro synthesis by exogenous Pro decreased germination rate (Hare et al., 2003). Moreover, radicle emergence of transgenic Arabidopsis seeds that expressed an antisense copy of the gene encoding the Pro biosynthetic enzyme (P5CS), was delayed in relation to reduction of Pro accumulation during germination (Hare et al., 2003).

In addition to Pro accumulation, various abiotic and biotic stresses also activate endogenous production of reactive oxygen species (ROS). Although most of them induce cellular damage, it has been demonstrated that hydrogen peroxide can play an important role as a signaling molecule that triggers the acclimation to adverse environmental conditions (Rejeb et al., 2014). The accumulation of Pro in the course of osmotic stress is partially due to ABA and H_2O_2 signaling (Savouré et al., 1997; Strizhow et al., 1997; Rejeb et al., 2014). Yang et al. (2009) reported that the H_2O_2 molecule induced Pro accumulation, rapid increase of activity of Δ^1-pyrroline-5-carboxylate synthetase and up regulation of Δ^1-pyrroline 5 carboxylate synthetase gene expression in coleoptiles and radicles of maize seedlings. Many studies have indicated that hydrogen peroxide promotes seed germination of several plants, such as Arabidopsis, barley, wheat, rice, sunflower and soybean (Ishibashi et al., 2013). H_2O_2 can act as a signaling molecule in the beginning of seed germination, involving specific changes at proteomic, transcriptomic and hormonal levels (Barba-Espin et al., 2012).

Although Pro accumulation has sometimes been reported in primed seeds (Sivritepe et al., 2003; Farhoudi et al., 2011), no data are available concerning putative gene activation or H_2O_2 involvement in this process. In this study, we used *B. napus* seeds osmoprimed in PEG solution and subsequently germinating under salinity conditions to elucidate the biochemical and molecular mechanism of osmopriming-mediated Pro titer modifications. Pro accumulation, expression of genes encoding the key enzymes of Pro metabolic turnover and accumulation of endogenous hydrogen peroxide are considered at the pivotal phases of osmopriming treatment and during post-priming germination under both stress and control conditions.

Materials and methods

Seeds osmopriming treatment, germination tests and morphological analysis of seedlings

The seeds of rape (*Brassica napus* L. cv Libomir) were kindly provided by OBROL Company. Priming was carried out in polyethylene glycol (PEG) 6000 solution (osmotic potential −1.2 MPa) during 7 d at 25 °C in the darkness on Petri dishes lined with 3 layers of filter paper wetted with PEG as described previously by Kubala et al. (2015). After incubation, seeds were washed 3 times with sterile deionized water to remove the osmotic agent and dried at room temperature until they reach initial moisture content (water content 5%, primed dried seeds, P_d) or were frozen in liquid nitrogen and stored at −80 °C (seeds at the end of soaking, primed non dried seeds, P_{nd}).

Germination tests were carried out on Petri dishes lined with three layers of filter paper Whatman no. 2 moistened with 10 mL of water or 100 mM NaCl. One hundred primed (P) and unprimed (UP) seeds in 10 replicates for each analyzed condition were used for germination tests. A seed was considered germinated when the radicle protruded the seed coat. The parameters, such as germination curve, maximum percentage of germination (G_{max}), time to reach 50% of germination (T_{50}) and area under the curve (ACU), were used for interpretation of germination performance and salinity tolerance using "Germinator_curve-fitting1.27.xls" Microsoft Excel script (Joosen et al., 2010) and the mathematical approach described by El-Kassaby et al. (2008). Morphological analyses were performed on 2-, 3-, 7- and 14-day-old seedlings. After measuring shoot and root length, seedling vigor index was determined by following formula:

Seedling length vigor index (SLVI) = (mean shoot length + mean root length) × G_{max}.

Measurements of chlorophyll a fluorescence

Chlorophyll *a* fluorescence measurements were performed in cotyledons of 7- and 14-day-old seedlings grown under control and stress conditions using the Fluorescence monitoring system FMS1 (Hansatech Instruments LTD, Kings Lynn, England). The experimental protocol of Lichtenthaler et al. (2005) was used. The following chlorophyll fluorescence parameters were measured: minimum Chl fluorescence in the dark-adapted state (F_o), maximum Chl fluorescence in the dark-adapted state (F_m), maximum Chl fluorescence in the light-adapted state (F'_m), steady state Chl fluorescence in the light-adapted state (F_s), Chl fluorescence level induced by non-saturating irradiation (F). F_m was measured after

30 min of dark adaptation. The maximum quantum efficiency of PSII (F_v/F_m) and effective quantum yield of photochemical energy conversion in PSII (Φ_{PSII}), were calculated using following formulas $F_v/F_m = (F_m - F_0)/F_m$ and $\Phi_{PSII} = (F_m' - F_s)/F_m'$, respectively. Fluorescence decrease ratio also known as vitality index (R_{Fd}) was calculated according to formula $R_{Fd} = F_d/F_s = (F_m - F_s)/F_s$.

Proline (Pro), hydrogen peroxide, gene expression and enzyme activity analyses were conducted on seeds collected at crucial points of osmopriming treatment according to Kubala et al. (2015) as shown in Fig. 1, i.e. at the end of soaking (P_{nd}), after drying of osmoprimed seeds to initial moisture content (P_d), and also during germination *sensu stricto*, prior to radicle emergence. Analyses were also performed on dry unprimed seeds (UP_d). Primed and UP seeds germinating on water and 100 mM NaCl were collected after 7 h of imbibition corresponding to achievement of 1% of germination of P seed on both water and 100 mM NaCl. Moreover, UP seeds germinating on water and 100 mM NaCl were collected also after 11 h and 16 h of imbibition, corresponding to achievement of 1% of germination of UP seeds on water and 100 mM NaCl, respectively.

Proline content

Proline content was measured as described by Bates et al. (1973) and modified by Khedr et al. (2003). Frozen seeds (0.5 g) were homogenized in 10 mL of 3% sulphosalicylic acid and then centrifuged at 10,000 × *g*. The supernatant (0.5 mL) was mixed with 1 mL of glacial acetic acid and 1 mL of 2.5% acid ninhydrin (2.5 g of ninhydrin dissolved in a mixture of 60 mL glacial acetic acid and 40 mL 6 M phosphoric acid). The mixture was incubated for 1 h at 100 °C and then the reaction was terminated by cooling in an ice bath. The reaction mixture was extracted with 2 mL of toluene, mixed vigorously with the test tubes stirrer for 15 s. The chromophore-containing toluene was warmed to room temperature and absorbance was read at 520 nm using toluene as a blank. The Pro concentration was determined from standard curve and calculated per g of dry weight.

Hydrogen peroxide determination

Spectrophotometric determination of hydrogen peroxide was performed based on the titanium (Ti^{4+}) method as described by Arasimowicz et al. (2009). Rapeseeds (0.5 g) were homogenized in 6 mL of 100 mM phosphate buffer pH 7.8. The homogenate was centrifuged at 15,000 × *g* for 30 min at 4 °C. The reactive mixture for spectrophotometric measurement contained 100 mM phosphate buffer pH 7.8, plant extract and the titanium reagent consisting of 0.3 mM 4-(2-pyridylazo)resorcinol and 0.3 mM titanium potassium tartrate mixed at the ratio 1:1. Absorbance was measured at 508 nm wavelength against a calibration curve prepared for the content of H_2O_2 from 0 to 100 nM. Hydrogen peroxide levels were calculated per g of dry weight.

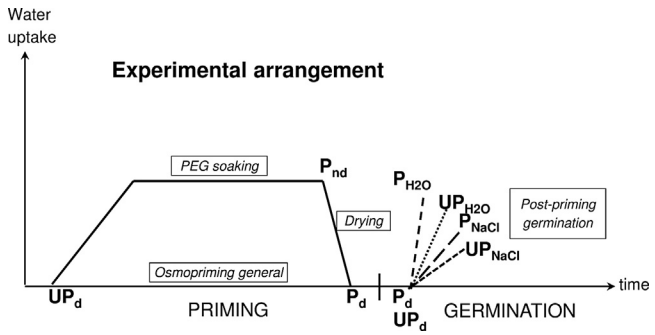

Fig. 1. Experimental design used in this study. Analyses were conducted at crucial points of osmopriming treatment and during the germination of control (unprimed) and treated (osmoprimed) seeds. (UP_d) dry unprimed seeds; (P_d) dry primed seeds; (P_{nd}) seeds at the end of priming treatment; ($7UP_{7H_2O}$) unprimed seeds imbibed 7 h on water; P_{7H_2O} primed seeds imbibed 7 h on water; ($UP_{7\ NaCl}$) unprimed seeds imbibed 7 h on 100 mM NaCl; ($P_{7\ NaCl}$) primed seeds imbibed 7 h on 100 mM NaCl; (UP_{11H_2O}) unprimed seeds imbibed 11 h on water; ($UP_{16\ NaCl}$) unprimed seeds imbibed 16 h on 100 mM NaCl.

RNA isolation

RNA was isolated as described by Asif et al. (2000) and DNase treatment was realized using RQ1 RNAse-free DNase (Promega, Leiden, The Netherlands) according to the manufacturer's instructions. RNA quality was verified by absorbance ratios (A260/A280) of 1.8–2.0 measured on the NanoDrop ND-1000 (Isogen Life Science, De Meern, The Netherlands), agarose gel electrophoresis and RNA capillary electrophoresis using the Bioanalyzer Agilent 2100 (Agilent Technologies, Santa Clara, CA, USA) with Agilent 6000 RNA Nano kit (Agilent Technologies, Santa Clara, CA, USA). The concentration of RNA was measured using the NanoDrop ND-1000.

cDNA synthesis and PCR conditions

Reverse transcription was performed with 2 μg of total RNA using the "Revert Aid H minus first strand cDNA synthesis kit" (Fermentas, St Leon-Rot, Germany), following the manufacturer's instructions. RT-PCR was performed using 10 times diluted cDNA (200 ng). For normalization purposes, *ACTIN2.1* (NCB GenBank Accession: FJ529167.1; GI: 241740071) was chosen as a reference gene. Since *B. napus P5CSA* and *P5CSB* genes show high level of homology in coding sequences (85%), specific primers were designed in less conserved regions. The following primers were thus used: *P5CSA* (NCBI GenBank accession: AF314811.1; GI:12667248) forward: 5′-CCAGGAGATCAAATGCTATCTTACA-3′ and reverse: 5′-GAACGACCGTGCTTCTGGTA-3′; *P5CSB* (NCBI GenBank accession: AF314812.1; GI:12667250) forward: 5′-CTGAACATTCCGGAAGTAAAATCAT-3′ and reverse: 5′-AGCGACTCCATTGTCTCCAT-3′; *OAT* (NCBI GenBank accession: EU375566.1, GI:169665582) forward:5′-GAAACCGCTTTGAAAGTTGC-3′ and reverse: 5′-CATGTCGGGACGAATCTCTT-3′; *PDH* (NCBI GenBank accession: EU375567.1, GI: 169665584) forward: 5′-GATAGGTCCCATGGTGGATG-3′ and reverse: 5′-ATCGAAGCAAATCGCTCACT-3′; *ACTIN2.1* forward:5′-TGCAGACCGTATGAGCAAAG-3′ and reverse: 5′-AATGCTTGGAGTCCTGCTTG-3′. Amplifications were carried out using Dream Taq Green DNA Polymerase (Fermentas, St Leon-Rot, Germany). An initial denaturation step was conducted at 95 °C for 2 min, each cycle consisted of 45 s at 95 °C, 30 s at an annealing temperature depending on the primer combination, and 25 s extension at 72 °C, followed by a final extension of 5 min at 72 °C. The following annealing temperature were used 60 °C for *P5CSA, P5CSB and OAT*, 55 °C for *PDH* and 53 °C for *ACTIN2.1*. The range of linearity between cycle numbers and relative amounts of RT-PCR products were checked in preliminary experiments to ensure that PCR products were recovered from the linear phase (Fig. S1A). Thirty cycles during PCR were used for all genes. For accurate quantification, measurements must be taken in the linear phase of PCR, where the cDNA concentration is directly proportional to signal intensity. We used five different cDNA concentrations to determine whether this linear phase had been covered (Fig. S1B). The products were separated on 1.8% agarose gels and stained with ethidium bromide. Expression differences were analyzed by gel densitometry using Gelix One software and expressed as relative values compared to actin expression (peak size of target gene/peak size of actin) and addition ratio to the UP_d value. At

least six independent PCR amplifications were conducted for each gene and produced similar results.

PCR products sequencing

PCR products for all genes were confirmed by sequencing to ensure that designed primers flank the target cDNA. PCR products were purified with thermosensitive Exonuclease I and FastAP Alkaline Phosphatase (Thermo Scientific) and sequenced with BigDye Terminator v3.1 on an ABI Prism 3130XL Analyzer (Applied Biosystems) according to manufacturer's instructions. Sequence chromatograms were checked for accuracy using FinchTV 1.3.1 (Geospiza Inc.). The similarity searches of coding sequences between *Brassica* and *Arabidopsis* genes and between *Brassica* isoforms were performed on the NCBI server (http://www.ncbi.nlm.nih.gov) using NCBI Standard Nucleotide BLAST (Altschul et al., 1990).

Enzyme extraction

Seeds were collected, frozen and ground in liquid nitrogen using a mortar and pestle to obtain a fine powder, and were then homogenized in an appropriate extraction buffer. Ratios for buffer volume: g fresh weight were 2:1. Extraction of all enzymes was carried out in cold room at 4 °C.

P5CS extraction was carried out according to Ruiz et al. (2002), the PDH and OAT extraction was done as described by Lutts et al. (1999) and Ruiz et al. (2002). For P5CS and PDH extraction 50 mM Tris–HCl buffer (pH 7.4) containing: 0.6 M KCl, 7 mM $MgCl_2$, 3 mM EDTA, 1 mM DTT, 5% (w/v) insoluble PVP was used. Homogenate was filtrated through 2 layers of Miracloth and centrifuged at 39, 000 × *g*, 4 °C for 20 min. After centrifugation, the supernatant was collected and desalted on Sephadex G-25 column (GE Healthcare PD-10 column) equilibrated with 50 mM Tris–HCl (pH 7.4) supplied with 10% glycerol (Ruiz et al., 2002; Lutts et al., 1999). The medium used for OAT extraction consisted of 100 mM K-Pi buffer (pH 7.9) supplied with 1 mM EDTA, 15% glycerol, 10 mM β-mercaptoethanol. The homogenate was centrifuged at 15,000 × *g*, 4 °C for 15 min. After centrifugation, the supernatant was treated with $(NH_4)_2SO_4$ at 60% saturation for 45 min (Lutts et al., 1999). After ammonium sulfate treatment sample was centrifuged at 15,000 × *g* for 15 min and supernatant was desalted on Sephadex G-25 column (GE Healthcare PD-10 column) equilibrated with extraction buffer.

Enzyme assays

The PDH and OAT activity assays were conducted according to methods described by Lutts et al. (1999). The P5CS activity was estimated as described by Silva-Ortega et al. (2008). PDH activity was examined by monitoring the $NADP^+$ reduction at 340 nm in 0.15 M Na_2CO_3 buffer (pH 10.3) containing 15 mM L-proline and 1.5 mM $NADP^+$ (Lutts et al., 1999; Ruiz et al., 2002). The OAT activity was measured by monitoring the decrease in absorbance of NADH at 340 nm in 0.2 M Tris–KOH buffer (pH 8.0) containing 5 mM ornithine (Orn), 10 mM α-ketoglutarate and 0.25 mM NADH. The P5CS activity was determined as γ-glutamyl kinase in the enzyme extract by monitoring the formation of γ-glutamyl hydroxamate. Reaction mixture contained 50 mM Tris–HCl buffer (pH 7.0) with: 50 mM L-glutamate, 20 mM $MgCl_2$, 100 mM hydroxylamine-HCl, 10 mM ATP in 0.5 mL final volume. Reaction was started by addition of enzyme extract and the probe was incubated at 37 °C for 15 min. The reaction was stopped by addition of 1 mL of stop buffer (2.5% $FeCl_3$ and 6% TCA in 2.5 N HCl). Precipitated proteins were removed by centrifugation and absorbance of clear supernatant was read at 535 nm against a blank identical to the above but without ATP. The amount of γ-glutamyl hydroxamate complex produced during the reaction was estimated from the molar extinction coefficient 250 mol^{-1} cm^{-1} reported for Fe^{3+} hydroxamate complex of the compound. The activity was expressed in U mg^{-1} protein which represents the amount of enzyme required to produce 1 mmol of γ-glutamyl hydroxamate min^{-1}. Protein content was determined according to Bradford, 1976. For each treatment, samples were extracted from three independent biological replicates. For each extraction, enzyme activities were estimated in triplicate.

Statistical analysis

Differences in measured parameters were analyzed for statistical significance by using one-way analysis of variance (ANOVA) and the Tukey–Kramer Multiple Comparison Test. Means were considered significantly different at $P < 0.01$.

Results and discussion

Osmopriming improves seed germination and seedling performance under salinity stress

Osmopriming improved the germination performance of *B. napus* seeds and salinity tolerance at this developmental stage (Fig. 2A). The seed coat rupture of P seeds germinating under both salinity and control conditions occurred after 7 h of imbibition, while for UP seeds, early visible radicle protrusion occurred after 11 h and 16 h of imbibition on water and in the presence of NaCl, respectively (Fig. 2B). The osmopriming had a beneficial effect on germination speed under both stress and control conditions (Fig. 2B). The time required for germination of 50% of seeds was ~0.5 and 0.3 times shorter in P seeds germinating under control and salinity conditions, respectively, as compared to UP seeds germinating under the same conditions (Fig. 2C). The value of area under curve showed a strong difference in germination performance and salinity tolerance between P and UP seeds. For P seeds this value was ~100% higher as compared to UP seeds germinating under control and stress conditions, respectively (Fig. 2D). Moreover, the maximum percentage germination was ~10% and 30% higher for P seeds than for UP ones under control and salinity conditions, respectively. Salinity stress caused a decrease of maximum percentage germination by 20% in UP seeds, while in P seeds, it was reduced by only 2% (Fig. 2B). Seedlings grown from P seeds on both water and NaCl for 2 and 3 d had higher seedling vigor indices as compared to those grown from UP ones, and this parameter was even higher in seedlings from P seeds subjected to salinity as compared to seedlings from UP seeds grown on water (Fig. 2E). The 7- and 14-day-old seedlings from P seeds were characterized by higher SLVI in response to salinity (Fig. 2E) in comparison to seedlings from UP seeds. However, after 14 d, these differences were not statistically significant. The photosynthetic performance of rape seedling cotyledons was estimated as chlorophyll *a* fluorescence parameters i.e. maximum quantum efficiency of PSII (F_v/F_m), effective quantum yield of photochemical energy conversion in PSII (Φ_{PSII}), and fluorescence decrease ratio (R_{Fd}) (Fig. 3). There were no significant changes in F_v/F_m and Φ_{PSII} in younger seedlings grown from UP and P seeds on either water or NaCl, but both parameters were higher in 14-day-old seedlings grown from P seeds in the presence of NaCl as compared to seedlings from UP seeds exposed to salinity (Fig. 3A and B). The decrease of R_{Fd} was observed in 7-day-old seedlings upon salinity stress, but after an additional seven days of salinity treatment, R_{Fd} values were not statistically different regardless of whether seedlings from UP and P seeds were grown on water or NaCl, respectively. However, this parameter was still higher in seedlings grown from P seeds and exposed to NaCl in

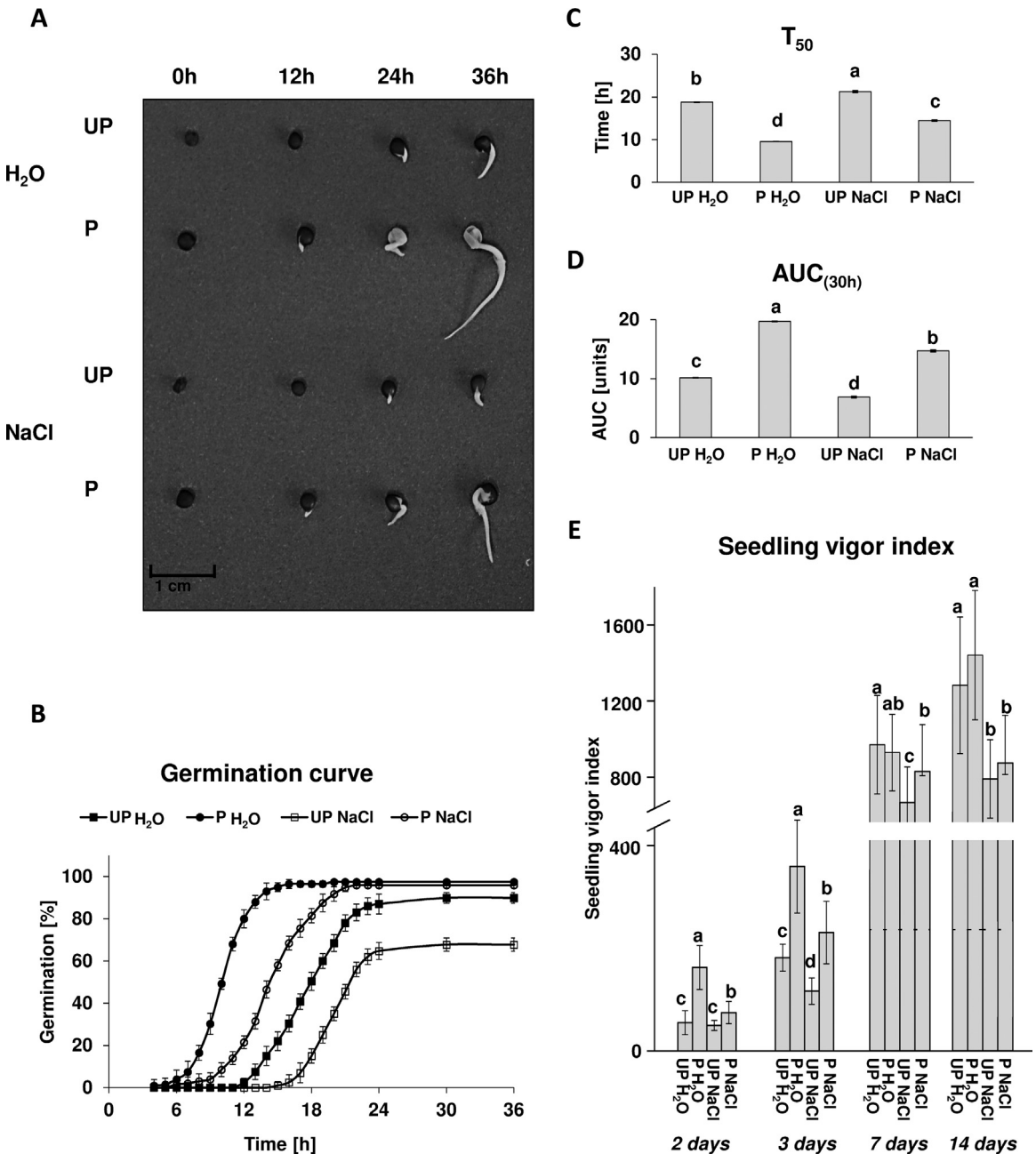

Fig. 2. Germination and seedlings vigor index of control (UP—unprimed) and osmoprimed (P) *Brassica napus* seeds. The pictures of UP and P seeds at 0, 12, 24 and 36 h of germination under control conditions (H_2O) and salinity stress (100 mM NaCl) (A). Germination curve (B). Time needed to reach 50% germination, (T_{50}) (C). Area under the curve until 30 h ($AUC_{(30h)}$) (D). The vigor index of seedlings (E) grown from P and UP seeds for 2, 3, 7 and 14 d on water and 100 mM NaCl. The same letters on bars describe not significant differences between means (on Fig. 2E calculated separately for seedlings grown 2, 3, 7 and 14 d; $n = 30$).

comparison to seedlings grown from UP seeds, indicating more efficient photosynthesis of the former. It has been reported that salinity stress can predispose plants to photoinhibition and photodamage (Qiu et al., 2003). In rape seedlings of the salt stress-tolerant cultivar the efficiency of PSII photochemistry and photochemical quenching transiently decreased after 4 d of salinity treatment followed by progressive recovery after 6 d of stress (Benincasa et al., 2013). The observed increase in F_v/F_m under salinity in 14-day-old seedlings grown from P seeds implies an increase in photochemical conversion efficiency of PSII. The increased efficiency of PSII photochemistry under salt stress has been detected previously in other species (Stepien and Johnson, 2009; Li et al., 2010). The higher salt tolerance of primed seeds and seedlings could also result from the higher activity of antioxidant enzymes. Mittova et al. (2002) found that salt tolerance was higher in the wild tomato than in the cultivated tomato due to the increased activities of superoxide dismutase (SOD), ascorbate peroxidase (APX) and guaiacol peroxidase (POD). The enhanced activities of APX, CAT and SOD as well as increased expression rates of *APX*, *CAT* and *SOD* genes have also been shown in rape seeds germinating under salinity conditions (Kubala et al., 2013). These results show that osmopriming had a profitable effect on *B. napus* seed germination and salinity tolerance. Priming overcame the negative effect of salt stress in relation to seed germination and seedling establishment. Our results are in agreement with the data published by other authors showing that priming improves not only germination, but also stress tolerance of germinating seeds and seedlings (Ashraf and Foolad, 2005; Farhoudi et al., 2011; Chen and Arora, 2013).

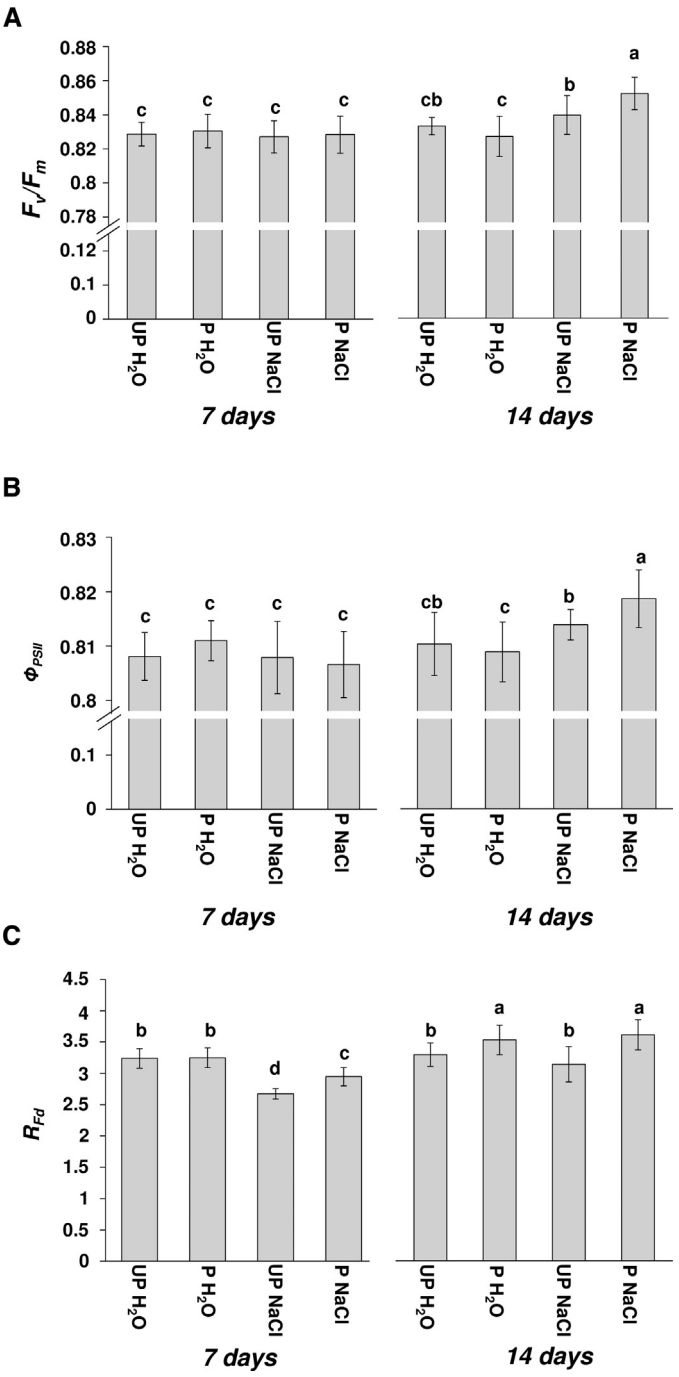

Fig. 3. Chlorophyll fluorescence parameters of seedlings cotyledons grown from primed (P) and unprimed (UP) seeds 7 and 14 d on water and 100 mM NaCl. Maximum quantum efficiency of PSII (F_v/F_m) (A), effective quantum yield of photochemical energy conversion in PSII (Φ_{PSII}) (B) and fluorescence decrease ratio (R_{Fd}) (C). The differences were statistically significant, as determined by one-way analysis of variance (ANOVA) and Tukey–Kramer Multiple Comparison Test ($n = 6$, $P < 0.05$).The same letters on bars describe not significant differences between means (calculated separately for seedlings grown 7 and 14 d for $n = 6$).

Accumulation of proline during osmopriming may improve seed germination capacity

Osmoprimed seeds accumulated significantly more Pro than untreated ones (Fig. 4A) and the most significant increase of Pro level was noted mainly during the first step of priming treatment (soaking), reaching in P_{nd} seeds ~270% higher level as compared to UP ones. Moreover, a slight increase of Pro content occurred during drying of osmoprimed seeds. The accumulation of Pro in response to priming appears to be the result of moderate osmotic stress generated during soaking in PEG solution and partial hydration of seed tissues. A high level of Pro was maintained during the germination of P seeds under both water and NaCl conditions and was 150% and 60% higher than in UP ones at the same developmental stage, i.e. after 11 h and 16 h of imbibition on water and under salinity, respectively. Slight depletion of Pro content in response to salinity was observed after 7 h of imbibition of UP seeds compared to germination on water. However, it increased after 16 h of salinity treatment. Similar results, showing a transient decrease of Pro content after 6 h and 12 h of salt treatment followed by an increase at 24 h post-salinity treatment were noted in seedlings of a canola (*B. napus*) salt-tolerant cultivar (Saadia et al., 2012). The higher level of Pro in P seeds compared to UP ones could partly explain the higher resistance of P seeds to salt stress during germination. Exogenous application of Pro during germination has indeed been reported to improve seed germination and seedling growth under salt stress conditions in several species amongst others canola (*B. napus*) (Posmyk and Janas, 2007; Athar et al., 2009). Pro accumulation is often observed in response to stress. Pro accumulation occurred during osmotic and salt stress in *B. napus* (Xue et al., 2009) and osmotic stress in *Arabidopsis* (Yoshiba et al., 1995). Moreover, the increased Pro content was observed in response to drought and salt stress in *Petunia hybrida* (Yamada et al., 2005). It should be noted that, in P seeds, the Pro concentration was lower in seeds germinating in the presence of NaCl than in the presence of water. In addition to its involvement in stress resistance, Pro also assumes crucial functions in relation to energy demand of young dividing cells at the root tip. Indeed, cycling of Pro substrates may be coupled to maintaining the $NADP^+$/NADPH ratio via the oxidative pentose phosphate pathway (Hare et al., 2003). Thus, the Pro concentration in P seeds may be sufficient to ensure stress resistance and a greater consumption of Pro in the presence of NaCl may reflect a greater need for NADPH to support root growth.

Proline accumulation during osmopriming and post-priming germination is mainly associated with higher expression level of P5CSA *gene and increased P5CS activity*

Pro biosynthesis via the glutamate pathway is controlled by the activity of P5CS encoded by two distinct genes*: P5CS1* and *P5CS2*. Analysis of coding sequences using NCBI's BLAST showed that *B. napus P5CSA* and *P5CSB* genes share up to 89% and 91% sequence identity with *Arabidopsis thaliana P5CS1* and *P5CS2*, respectively (Figs. S2 and S3). *B. napus P5CSA* and *P5CSB* genes showed an 85% homology level in coding regions (Fig. S4). PCR products from amplification of specific regions of DNA strands of both genes were verified by sequencing. The expression of the *P5CSA (Arabidopsis P5CS1* homolog*)* gene was strongly activated due to osmopriming and this gene seems to be the most activated gene of Pro metabolism during priming and post-priming germination under both control and salt-stress conditions (Fig. 5A). Indeed, the expression of *P5CSA* increased by ~2700% due to osmopriming, mainly during the soaking phase (P_{nd}) followed by a slight decrease during drying (P_d) (Fig. 5A). For P seeds, *P5CSA* expression decreased during germination on water, while in seeds germinating on NaCl, it was maintained at the same level as in primed non-dried seeds. Moreover, during germination under salinity, the expression level of *P5CSA* was ~270% higher in P than in UP seeds regardless of whether the seeds were compared at the same time point or at the same developmental stage. It is noteworthy that activation of *P5CSA* also occurred during the germination of UP seeds on both water and NaCl, reaching almost the same level after 7 and 16 h of imbibition under stress and after 11 h in control sample. Even if less obvious, our results showed also an accumulation

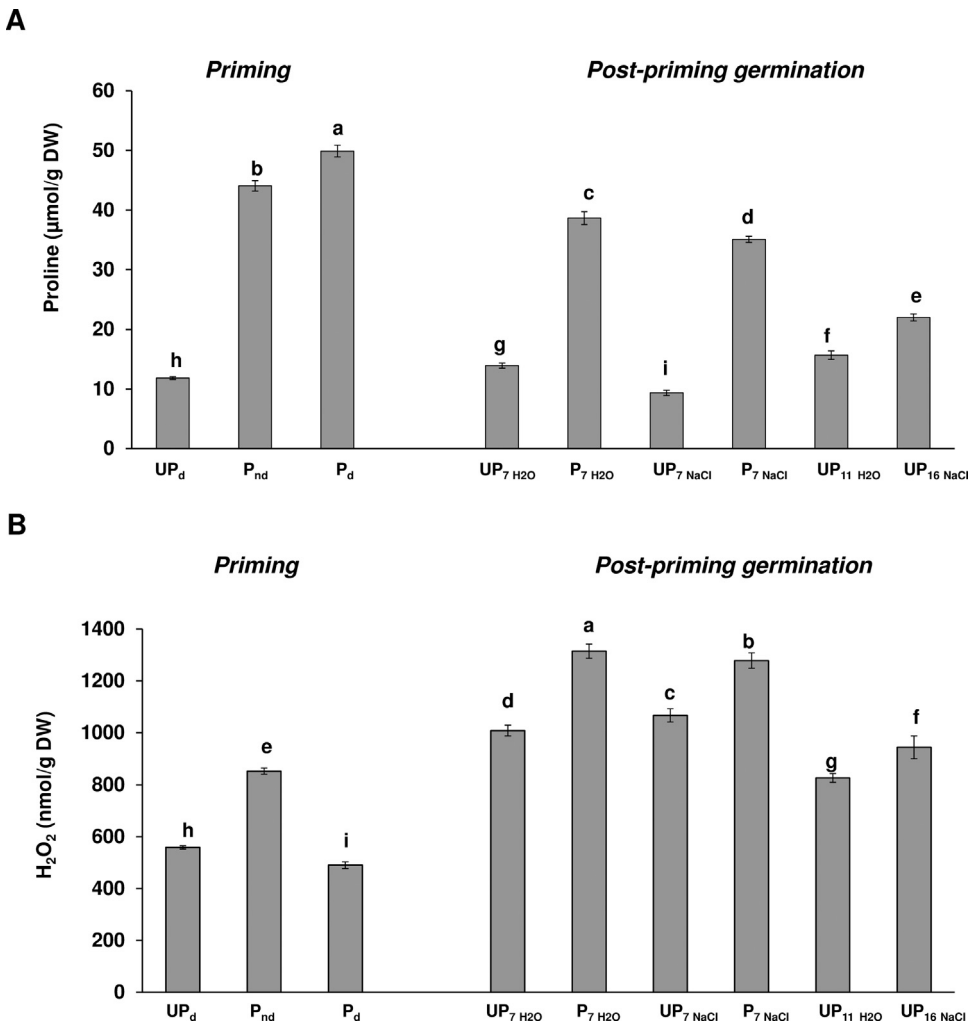

Fig. 4. Proline (A) and hydrogen peroxide (B) content in seeds of *Brassica napus* during priming treatment (UP_d; P_{nd} and P_d) and postpriming germination (UP_{7H_2O}; P_{7H_2O}; $UP_{7\ NaCl}$; $P_{7\ NaCl}$, $UP_{11\ H_2O}$; $UP_{16\ NaCl}$). (UP_d—dry unprimed seeds), (P_{nd}—primed non dried seeds) (P_d—primed dried seeds), ($UP_{7\ H_2O}$, P_{7H_2O} —unprimed and primed seeds germinating 7 h on water, respectively), ($UP_{7\ NaCl}$, $P_{7\ NaCl}$—unprimed and primed seeds germinating 7 h on 100 mM NaCl, respectively), ($UP_{11\ H_2O}$, $UP_{16\ NaCl}$—unprimed seeds germinating 11 h and 16 h on water and 100 mM NaCl, respectively). The differences were statistically significant, as determined by one-way analysis of variance (ANOVA) and Tukey–Kramer Multiple Comparison Test ($n = 9$, $P < 0.01$). The same letters on bars indicate not significant differences between means.

of *P5CSB* (*Arabidopsis P5CS2* homolog) transcript level during PEG soaking (Fig. 5B). *P5CSB* showed more stable expression level than *P5CSA* during post-priming germination under control conditions but not under stress. With respect to the priming process, the *P5CSB* expression level increased by 190% at the end of the PEG soaking phase (P_{nd}) as compared to dry unprimed seeds (UP_d), followed by decrease during drying (P_d), (Fig. 5B). The decreased expression during the drying process was also observed for *P5CSA* but this decline was proportionally higher for *P5CSB*. During germination, the *P5CSB* gene was specifically activated in P seeds germinated under salinity conditions, and to a lesser extent, in UP ones germinated 11 h on water. The orthologs of *P5CS1* were often reported as the main genes activated in response to osmotic and salt stress in different plant species. The induction of P5CS activity occurred during osmopriming, mainly at the drying phase, and in dry P seeds enzyme activity increased by 60% as compared to dry unprimed ones (Fig. 6A). During germination of P seeds on water and NaCl, P5CS activity decreased and represented 80% and 65% of that in dry primed seeds, respectively. However, in the presence of NaCl, enzyme activity was 170% and 40% higher in P seeds compared to UP seeds at the same time point and developmental stage, respectively (Fig. 6A). The *P5CS1* gene was activated in *Arabidopsis* in response to osmotic (Yoshiba et al., 1995) and salt stress (Strizhow et al., 1997). Tobacco plants overexpressing the *P5CS1* gene showed higher activity of the P5CS enzyme and gene expression level and synthesized 10–18 fold more Pro than control plants resulting in higher tolerance to salt stress (Kishor et al., 1995). Furthermore, *Ailanthus altissima* plants exposed to drought stress and 300 mM NaCl showed increased activity of the P5CS enzyme and accumulation of free Pro (Filippou et al., 2014). During stress, in *Arabidopsis*, the *P5CS1* but not the *P5CS2* gene was required for Pro accumulation (Fabro et al., 2004; Székely et al., 2008). Furthermore, in *Arabidopsis*, activation of *P5CS1* occurred by an ABA-dependent and ABA insensitive regulatory pathway (Strizhow et al., 1997) and H_2O_2-derived signals (Versules et al., 2007), and was recently reported to be regulated by epigenetic control and alternative splicing (Kesari et al., 2012). The *Arabidopsis P5CS2* gene is a housekeeping gene that is active in dividing meristematic tissues, such as the shoot and root tips (Székely et al., 2008). Moreover, this gene can be also activated by a virulent bacteria, salicylic acid, and ROS signals, which trigger a hypersensitive response (Fabro et al., 2004; Szabados and Savouré, 2010).

We observed that the expression level of the *OAT* gene slightly increased during osmopriming of rape seeds (Fig. 5C) as a result of its activation, mostly during PEG soaking. A further increase of *OAT* expression was observed during post-priming germination on

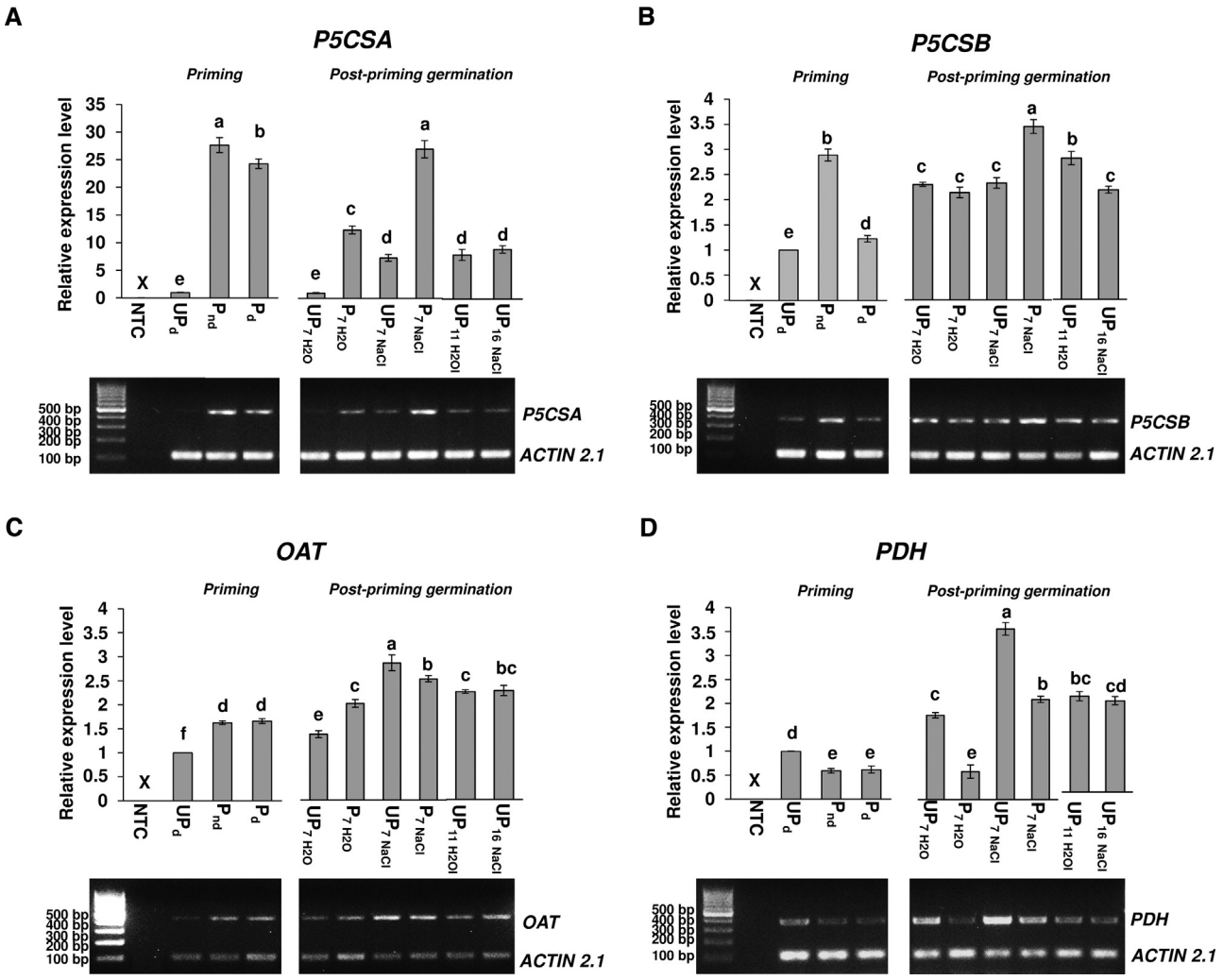

Fig. 5. Semi quantitative RT-PCR analysis of: *P5CSA* (A), *P5CSB* (B), *OAT* (C) and *PDH* (D) genes expression level in seeds of *Brassica napus* during priming treatment (UP_d; P_{nd} and P_d) and postpriming germination ($UP_{7\ H_2O}$; P_{7H_2O}; $UP_{7\ NaCl}$; $P_{7\ NaCl}$; $UP_{11\ H_2O}$; $UP_{16\ NaCl}$). (UP_d—dry unprimed seeds), (P_{nd}—primed non dried seeds), ($UP_{7\ H_2O}$, P_{7H_2O}—unprimed and primed seeds germinating 7 h on water, respectively), ($UP_{7\ NaCl}$, $P_{7\ NaCl}$—unprimed and primed seeds germinating 7 h on 100 mM NaCl, respectively), ($UP_{11\ H_2O}$, $UP_{16\ NaCl}$—unprimed seeds germinating 11 h and 16 h on water and 100 mM NaCl, respectively), NTC—none template control. The differences were statistically significant, as determined by one-way analysis of variance (ANOVA) and Tukey–Kramer Multiple Comparison Test ($n = 6$, $P < 0.01$). The same letters on bars indicate not significant differences between means. Note that the *Y*-axis scales are the same for B, C and D graphs and different for A.

both water and NaCl. However, the *OAT* gene was more activated in seeds germinated on NaCl as compared to those germinated on water. Moreover, the accumulation of *OAT* transcripts also occurred in the course of germination of UP seeds. After a transient increase at 7 h from start of imbibition in the presence of NaCl, the activity of *OAT* was maintained at the same level under both control and salinity conditions. Furthermore, the *OAT* gene was up-regulated during post-priming germination on water (Fig. 5C). With respect to the activity of the OAT enzyme, opposite results to that of gene expression were observed. The activity of OAT slightly decreased during osmopriming (Fig. 6B) and a further decrease was observed during germination under both water and salinity conditions (Fig. 6B). However, there was no significant difference between primed seeds germinating on water versus primed non dried and primed dry seeds. Both P and UP seeds showed the decreased OAT activity in response to salinity stress, but in P seeds, enzyme activity was maintained at higher level. The differences in the *OAT* expression levels between P and UP seeds were less than those observed for *P5CSA*. The Orn pathway contributed to Pro accumulation in young *A. thaliana* plantlets growing up under osmotic stress (Roosens et al., 1998) and in rice cultivars sensitive to NaCl exposed to salt stress (Lutts et al., 1999), but may also be involved in glutamate synthesis and thus in nitrogen recycling (Kavi Kishor and Sreenivasulu, 2014).

Proline degradation is a process to opposite to that of Pro biosynthesis and also regulates the Pro level. Pro degradation is controlled by activity of the *PDH* gene. The transcription of the *PDH* gene was inhibited during osmopriming of rape seeds (Fig. 5D). The *PDH* expression level decreased by 40% during PEG soaking as compared to UP dry seeds (Fig. 5D) and both primed non dried and primed dry seeds showed the same level of *PDH* expression. Moreover, the down-regulation of *PDH* was observed during post-priming germination under both control and stress conditions (Fig. 5D), resulting in accumulation of Pro. The expression level of this gene was about 70% and 40% lower in P seeds germinating on water and NaCl, respectively, as compared to UP ones germinated 7 h on water and NaCl. On the other hand, the expression level of *PDH* remained at the same level in UP seeds germinating 11 and 16 h on water and NaCl, respectively (i.e. in seeds representing the same developmental stage under control and stress). Furthermore, salinity enhanced the expression of *PDH* in both P and UP seeds, but in UP it was higher than in P ones. The discrepancy between gene expression (Fig. 5D) and PDH activity (Fig. 6C) was observed during osmopriming. Generally, the activity of PDH was lower in response to post-priming

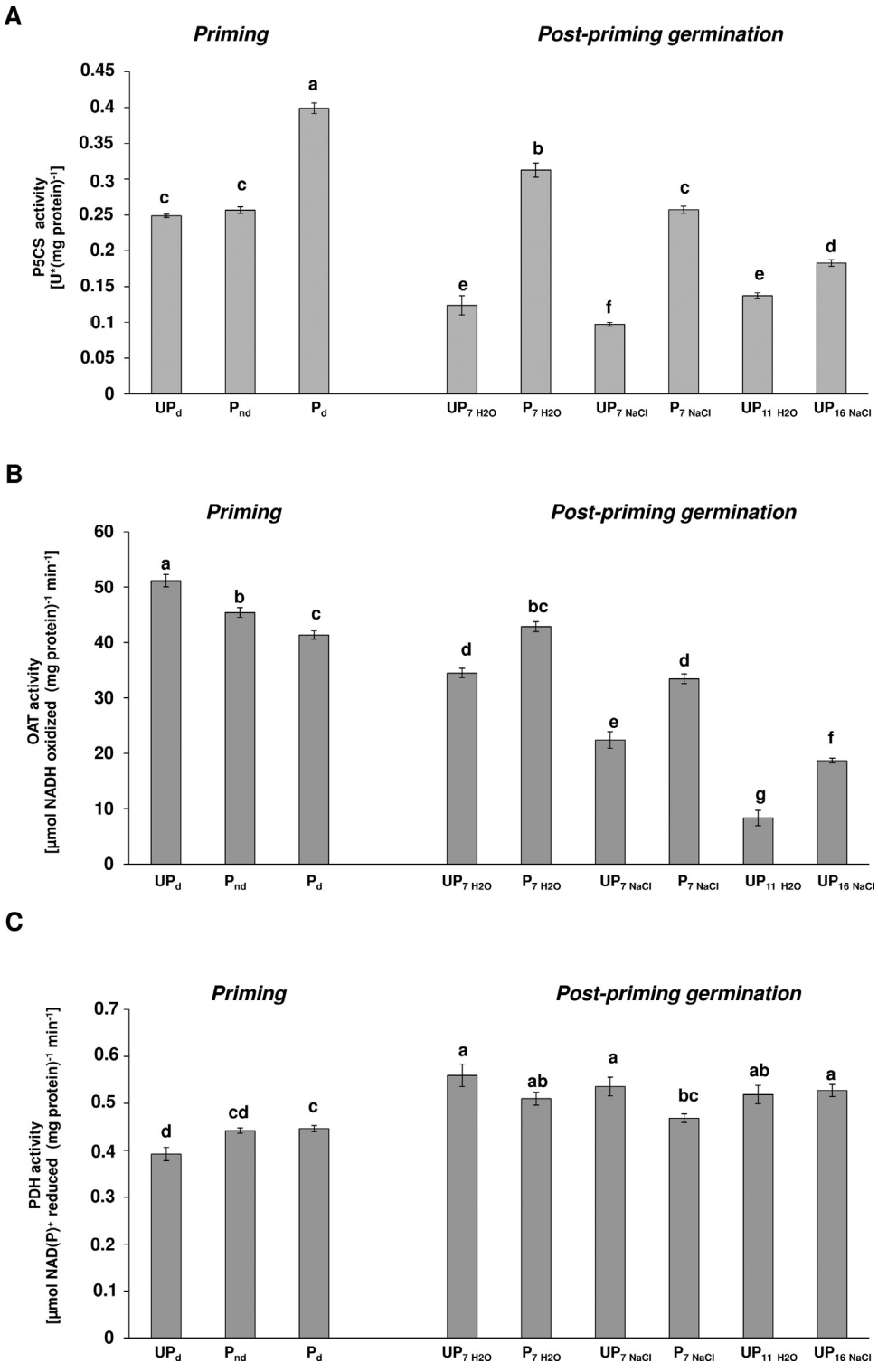

Fig. 6. Activity of P5CS (A), OAT (B) and PDH (C) in seeds of *Brassica napus* during priming treatment (UP_d; P_{nd} and P_d) and postpriming germination ($UP_{7\ H_2O}$; P_{7H_2O}; $UP_{7\ NaCl}$; $P_{7\ NaCl}$; $UP_{11\ H_2O}$; $UP_{16\ NaCl}$), (UP_d—dry unprimed seeds), (P_{nd}—primed non dried seeds), (P_d—primed dried seeds), ($UP_{7\ H_2O}$, P_{7H_2O} —unprimed and primed seeds germinating 7 h on water, respectively), ($UP_{7\ NaCl}$, $P_{7\ NaCl}$—unprimed and primed seeds germinating 7 h on 100 mM NaCl, respectively), ($UP_{11\ H_2O}$, $UP_{16\ NaCl}$—unprimed seeds germinating 11 h and 16 h on water and 100 mM NaCl, respectively). The differences were statistically significant, as determined by one-way analysis of variance (ANOVA) and Tukey–Kramer Multiple Comparison Test ($n = 9$, $P < 0.01$). The same letters on bars indicate not significant differences between means.

germination under both stress and control conditions. Usually, *PDH* transcription is activated by rehydration and Pro but repressed by dehydration, thus preventing Pro degradation during abiotic stress (Kiyosue et al., 1996). The *Arabidopsis PDH* gene was shown to be inhibited by salt stress. This inhibition was correlated with increased Pro accumulation (Peng et al., 1996). Moreover, Roy et al. (1992) indicated that a salt-resistant rice cultivar accumulated Pro under NaCl salinity, which was attributed to the decrease in PDH activity. The observed down-regulation of *PDH* during PEG soaking and up-regulation in P and UP rape seeds germinating under salt as

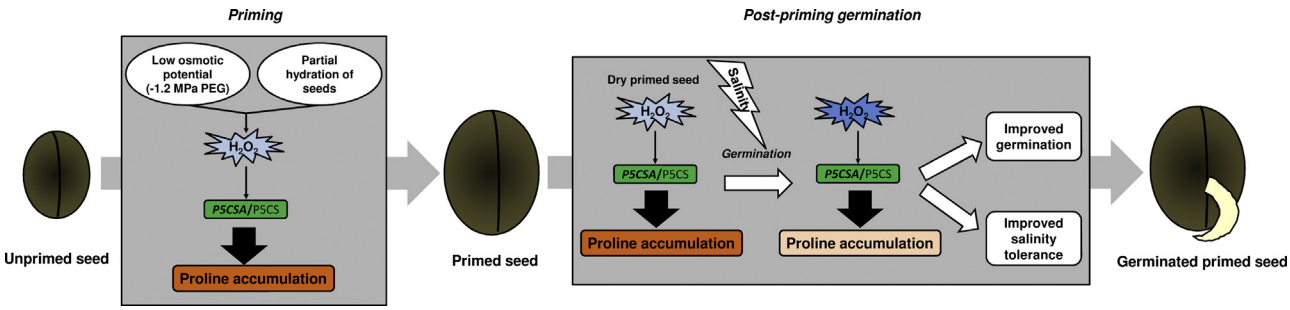

Fig. 7. Schematic presentation of osmopriming-dependent mechanism improving salinity tolerance of germinating *Brassica napus* seeds. Osmotic stress generated during soaking seeds in PEG solution and partial hydration of seed tissues stimulate stress response i.e. proline (Pro) accumulation during osmopriming and post-priming germination, as a result of hydrogen peroxide-induced expression of Pro synthesis gene *P5CSA* and P5CS enzyme activity. Green: gene up-regulation (bold, italic)/induction of enzyme activity (regular). The intensity of the colors is proportional to gene/enzyme activity and metabolite levels. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

compared to seeds germinating on water suggests that other mechanisms than rehydration–dehydration regulate *PDH* activation and repression during osmopriming and post-priming germination.

Our results showed that there is no evident correlation between transcript level and enzyme activity during priming: gene expression was significantly increased at the end of seed soaking for P5CS, while activity in this phase was maintained at the same level as in dry unprimed seeds (Fig. 6). Similarly, gene expression was increased for OAT, while activity was reduced. On the contrary, *PDH* gene expression was reduced during osmopriming, while activity slightly increased. The more obvious correlation between mRNA abundance and enzyme activity was observed for post-priming germination. The best correspondence between changes in mRNA level and enzyme activity was observed for P5CS and PDH in (primed seeds germinating 7 h on 100 mM NaCl ($P_{7\ NaCl}$)) as compared to UP ones (unprimed seeds germinating 7 h on 100 mM NaCl ($UP_{7\ NaCl}$)), in which transcript accumulation and enzyme activities were similar in profile and scale, which was not a case for OAT. Transcriptomic and proteomic analyses conducted on rape seeds collected at crucial points of osmopriming treatment and also during germination *sensu stricto* demonstrated differences between transcriptome and proteome data sets (the match between genes and proteins was limited to only 12 gene–protein pairs), emphasizing the importance of the regulation of mRNA translation and post-translational processes during priming and post-priming germination (Kubala et al., 2015). Posttranscriptional processing such as transcript de/stabilization, translation, posttranslational modifications and protein degradation influence the quality and quantity of expressed proteins and thus affect the correspondence between transcript and enzyme activity.

Endogenous hydrogen peroxide induces Pro accumulation during osmopriming and post-priming germination

The increased *P5CSA* transcript level and enzymatic activity of P5CS accompanied by decreased gene expression and enzymatic activity of PDH and Pro accumulation in P seeds germinated on 100 mM NaCl was associated with a higher level of endogenous hydrogen peroxide. During the first step of osmopriming (PEG soaking), the level of hydrogen peroxide increased by 50% as compared to the unprimed dry seeds and subsequently decreased during drying (Fig. 4B). The higher content of hydrogen peroxide was observed during post-priming germination under both control and stress conditions. The role of ROS in signaling networks during germination was reviewed by Bailly (2004), Bailly et al. (2008). Hydrogen peroxide can be a positive regulator of germination and may promote this process. Also, Pro was shown to play an important role in promoting germination. An increase in the free Pro level was observed prior to radicle emergence in *Arabidopsis* seeds, while feedback inhibition of Pro synthesis by exogenous Pro decreased germination rates (Hare et al., 2003). Moreover, radicle emergence of transgenic *Arabidopsis* seeds, which expressed an antisense copy of the gene encoding the Pro biosynthetic enzyme (P5CS), was delayed. It was correlated with a reduction of the Pro level during germination (Hare et al., 2003). The accumulation of Pro occurring during osmotic stress is partially regulated by ABA signaling (Savouré et al., 1997; Strizhow et al., 1997; Rejeb et al., 2014) and H_2O_2 which is part of ABA dependent pathway (Versules et al., 2007; Rejeb et al., 2014). In maize seedlings treated with exogenous hydrogen peroxide accumulation of Pro, up-regulation of *P5CS* and activation of Δ^1-pyrroline-5-carboxyl synthetase was observed (Yang et al., 2009). Moreover, Filippou et al. (2014) showed free Pro accumulation as a consequence of higher activity of P5CS enzyme in *Ailanthus altissima* seedlings exposed to drought and salinity stress, accompanied by H_2O_2 accumulation. Hydrogen peroxide was reported to specifically activate *P5CS1* (Neil et al., 2008). Greater accumulation of H_2O_2 in P seeds associated with higher Pro content as well as gene expression and enzymatic activity of P5CS support this view and suggest that hydrogen peroxide and Pro play a crucial role in relation to osmopriming improvement salinity tolerance (Fig. 7). As shown for maize seedlings, exogenous hydrogen peroxide caused a decrease of Pro dehydrogenase (PDH) activity (Yang et al., 2009). Taken together, our results reveal that the promotion of germination as well as salinity stress tolerance of osmoprimed seeds may be due to Pro accumulation through the glutamate pathway. In addition to its role as an osmoregulator (which indeed requires a much higher concentration), Pro has been noted as a protector of cellular structures (which may be of high importance during the rehydration phase of germination, when all these structures could be modified by an abrupt inflow of water). Proline itself has been suggested to act as an antioxidant.

Osmopriming-improved germination of Brassica napus *seeds and priming-dependent salinity stress tolerance as a consequences of 'priming memory'*

Many papers have reported that seed priming enhances the stress tolerance of germinating seeds (Ashraf and Foolad, 2005; Iqbal and Ashraf, 2007; Farooq et al., 2008; Chen et al., 2010; Chen et al., 2012). Bruce et al. (2007) considered priming as a pre-germination stress exposure that can leave seeds with a 'stress-memory'. Chen and Arora (2013) proposed a hypothetical model that illustrates the physiology of priming-induced stress tolerance, achieved via two strategies. The first strategy includes the osmopriming related events that facilitate the transition of quiescent dry seed into germinating state and lead to improved seed germination.

The second strategy is correlated to the imposition of abiotic stress on seeds during priming that represses radical protrusion but stimulates stress response, potentially inducing cross-tolerance. The authors suggest that these two strategies together constitute a 'priming memory' in seeds, which can be recruited upon a subsequent stress-exposure and mediates greater stress-tolerance of germinating P seeds. Our work supports this hypothesis and shows that, in rape, osmopriming stimulates the stress response by hydrogen peroxide-mediated accumulation of Pro and this accumulation subsequently improves germination under salinity stress.

Conclusions

To the best of our knowledge, this is the first study investigating Pro accumulation and the modulation of gene expression and activity of Pro turnover enzymes in response to osmopriming. Our results show that osmopriming improves *B. napus* seed germination and salinity tolerance during post-priming germination and seedling establishment and this germination performance is linked with Pro accumulation as a result of hydrogen peroxide-induced *P5CSA* expression and P5CS activity. The initial exposure to osmotic stress created during priming results in greater salinity stress tolerance during post-priming germination, a feature likely linked to a 'priming memory'.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jplph.2015.04.009

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