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Research paper

Patterns of low temperature induced accumulation of dehydrins in *Rosaceae* crops—Evidence for post-translational modification in apple

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ABSTRACT

Important crop plants of *Rosaceae* family are often damaged during winter due to the lack of acclimation and cold hardiness. One of the cellular responses of plants to cold stress is the accumulation of dehydrin proteins. We studied the expression of dehydrins in several *Rosaceae* species during low temperature treatment *in vitro*. Microshoots of *Pyrus communis*, *Malus* × *domestica*, *Fragaria vesca*, *Fragaria* × *ananassa*, *Prunus cerasus* and *Prunus avium* cultivars were grown in low temperature conditions. Genotype −specific accumulation of dehydrins was detected by immunoblot analysis of the extracted proteins. Untargeted difference gel electrophoresis of *Malus x domestica* microshoots revealed an extensive accumulation of three dehydrins. In a protein phosphatase assay, MdDHN2 and MdDHN4, but not MdDHN6 proteins were found to be extensively phosphorylated. In terms of the amount of protein synthesized, dehydrins are a major protein-level adaptation mechanism to low temperature in *M. x domestica.* In addition to dehydrins, the induction of proteins involved in the response for oxidative stress were observed. Additionally, a Xero2 −like dehydrin of *F. vesca* was detected by difference gel electrophoresis and identified by nano LC–MS/MS.

1. Introduction

Dehydrins, or group 2 LEA proteins, are an ubiquitous group of highly hydrophilic, intrinsically disordered proteins, with a wide range of molecular masses, found throughout the plant kingdom. They accumulate in seeds and vegetative tissues in response to water stress, such as drought, high salinity and freezing (Battaglia et al., 2008; Graether and Boddington, 2014). Dehydrins are divided in five subclasses depending on the organization of their conserved motifs, such as the K-segment, tyrosine-rich Y-segment and the serine-track containing S-segment. These subclasses are K_n, SK_n, K_nS, Y_xK_n, and Y_xSK_n, where x and n represent the number of repeats (Close, 1996). Several studies have provided evidence for the involvement of dehydrins in cold stress tolerance. For example, the accumulation of barley dehydrin 5 was found to correlate with frost tolerance in 21 barley cultivars (Kosova et al., 2008). The wheat dehydrin WCOR410 was found to be associated with frost tolerance (Danyluk et al., 1998) and to confer tolerance in transgenic strawberry (Houde et al., 2004). The protective effects of dehydrins are thought to arise from their interactions with membranes and proteins, stabilizing these structures and preventing aggregation (Hanin et al., 2011). Recent results indicate that the disordered structure of dehydrins is important for effective cryoprotection (Hughes et al., 2013). Particular dehydrins have also been reported to bind negatively charged lipid vesicles (Koag et al., 2003), multivalent cations such as Fe^{3+}, Ca^{2+}, Zn^{2+}, Co^{2+}, Ni^{2+} and Cu^{2+} (Kruger et al., 2002), as well as nucleic acids (Hara et al., 2009).

Some dehydrins are post-translationally modified, mainly phosphorylated, under stress conditions. This phosphorylation occurs at the S-segment, and may be associated with their translocation to the nucleus (Goday et al., 1994). The phosphorylation of the S segment may be significant in stress tolerance. For example, the wheat stress-inducible dehydrin DHN-5 was found to be differentially phosphorylated in contrasting wheat cultivars in response to drought stress (Brini et al., 2007). Phosphorylation also seems to affect the cation binding properties of some dehydrins (Alsheikh et al., 2005).

Plants of *Rosaceae* family, such as strawberry, apple, sweet and sour cherry, are often damaged during winter at temperate regions due to the lack of acclimation and cold hardiness. Several studies have shown the expression of dehydrin genes during cold stress in *Rosaceae*. For example, dehydrin genes were found to be differentially expressed

Abbreviations: ACN, acetonitrile; DIGE, difference gel electrophoresis; FA, Formic acid; LC–MS/MS, liquid chromatography-tandem mass spectrometry; LEA protein, late embryogenesis abundant protein; ROS, reactive oxygen species

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during dormancy in bark and bud tissues of *Malus x domestica* (Garcia-Bañuelos et al., 2009; Falavigna et al., 2014). In diploid *F. vesca* genotypes, dehydrin content was found to correlate with freezing tolerance (Davik et al., 2013). *Rosaceae* species often express several different dehydrins, which posses functional and tissue-specific variation. The existence of multiple dehydrin proteins has been shown by immunoblotting (Baniulis et al., 2012; Koehler et al., 2012), as well as *in silico* from published genome databases, and their expression in various tissues confirmed for grapevine (Yang et al., 2012), apple (Liang et al., 2012; Falavigna et al., 2015; Porto et al., 2015) and Asian pear (Hussain et al., 2015). Using the hmmsearch program and dehydrin domain sequences, Liang et al. (2012) identified nine dehydrins from the apple genome, of which the *MdDHN2*, *MdDHN4* and *MdDHN6* expression was found to be up-regulated by chilling. The *MdDHN2* sequence was found to have high similarity with a previously characterized dehydrin gene associated with cold hardiness and dormancy in apple (Garcia-Bañuelos et al., 2009).

We studied the accumulation of dehydrin proteins in *Rosaceae* during cold acclimation *in vitro*. Microshoots of *Pyrus communis*, *Malus* × *domestica*, *Fragaria vesca*, *Fragaria* × *ananassa*, *Prunus cerasus* and *Prunus avium* cultivars were acclimated, and the accumulation of dehydrins was detected by immunoblot analysis of the extracted proteins using polyclonal antibodies against the K-segment (Close et al., 1993). The protein identity was further determined from samples of *M. domestica*, and *F. vesca*, two species of *Rosaceae* family with available genome information (Velasco et al., 2010; Shulaev et al., 2010). Proteins were separated and detected using 2D difference gel electrophoresis (DIGE) and differentially abundant proteins were identified using high-resolution nano liquid chromatography-tandem mass spectrometry (LC–MS/MS).

2. Materials and methods

2.1. Plant material and acclimation conditions

Microshoots of European pear (*Pyrus communis*) cv. Senryo and Karaliene Jadvyga, domestic apple (*Malus* × *domestica*) cv. Golden Delicious and Gala, wild strawberry (*Fragaria vesca*) and garden strawberry (*Fragaria* × *ananassa*) cv. Holiday, sour cherry (*Prunus cerasus*) cv. Vietine rugscioji, Molodeznaya and sweet cherry (*Prunus avium*) cherry cv. Merchant and Jurgita were propagated and grown in tissue culture jars on Murashige and Skoog medium, supplemented with 3.2 µM 6-Benzylaminopurine, 3% sucrose and 0.8% plant agar at 22 ± 3 °C, under fluorescent lamp illumination of 50–150 µmol m^{-2} s^{-1} intensity and 16/8 h photoperiod. The cold-treated microshoots were grown for 28 days at 4 ± 1 °C under fluorescent lamp illumination of 25–50 µmol m^{-1} s^{-1} intensity and 8/16 h photoperiod (8 h light and 16 h dark).

2.2. Protein extraction

Total cell protein was prepared using phenol extraction, coupled with ammonium acetate precipitation, as described previously (Isaacson et al., 2006). Two hundred milligrams of acclimated and control microshoots from a single jar were pooled, flash frozen with liquid nitrogen and ground using mortar and pestle. Ground plant tissue was transferred to a clean 15 mL test tube and 5 mL of ice cold extraction buffer (0.7 M Sucrose, 0.1 M KCl, 0.5 M Tris-HCl, 50 mM EDTA, 2% (v/v) b-mercaptoethanol and 1 mM PMSF, pH 7.5) and 5 mL of tris buffered phenol was added. The tube was incubated at +4 °C in a rotary laboratory mixer for 30 min, centrifugated at 5000 *g* for 30 min at +4 °C, and the upper phenolic layer was collected to a clean tube. The upper phase was back-extracted twice with the extraction buffer, and the proteins were precipitated by adding 5 vols of cold methanol, containing 0.1 M ammonium acetate. After overnight precipitation at −20 °C, the protein pellet was collected by centrifugation at 5000 *g* at +4 °C and washed twice with cold methanol and once with acetone. The pellet was dried for 5 min in a vacuum centrifuge and stored at −70 °C until further analysis.

2.3. Immunoblotting

Protein samples were dissolved in SDS sample buffer and separated on a 5–15% gradient polyacrylamide gel. After transfer onto polyvinylidene difluoride membrane and blocking with 5% defatted milk protein in TBS, dehydrins were identified by immunoblotting, using 1:7500 dilution of a polyclonal rabbit antibody (Enzo Life Sciences, Inc, NY) against the conservative lysine-rich sequence, characteristic of dehydrins (Close et al., 1993). Immunoreactive proteins were detected by HRP-conjugated anti-rabbit antibody at 1:5000 dilution and Clarity Western ECL kit (Bio-Rad), according to the manufacturer's instructions.

2.4. Difference gel electrophoresis

Phenol extracted proteins from acclimated and control samples were dissolved in DIGE lysis buffer (7 M Urea, 2 M Thiourea, 4% CHAPS, 30 mM Tris, pH 8.5). The protein concentration was measured from diluted samples by Bradford assay. Fifty micrograms of protein from control and acclimated samples was labeled using 400 pmol of Cy3 and Cy5 dyes (Lumiprobe, Hannover), using manufacturer's instructions. Fifty micrograms of each of the protein samples was used to create the pooled internal standard, which was labeled with the Cy2 dye (Lumiprobe) for 2DE difference electrophoresis (Unlu et al., 1997). The preparative gels were loaded with 200 µg of protein, consisting of 100 µg of control and 100 µg of treated samples, also labeled with Cy3 and Cy5. Samples were isoelectrically focused on a 24 cm pH 3-11NL DryStrip using Ettan IPGphor 3 (GE Healthcare, USA). Second dimension separation was carried out on 12% polyacrylamide gels using Ettan DALTSix electrophoresis system (GE Healthcare). Fluorescent proteins were visualized using Typhoon FLA 9000 scanner (GE Healthcare). The gels were aligned using the internal standard with the RAIN software (Dowsey et al., 2008). Spots were quantified using the Pinnacle software (Morris et al., 2008). Statistical analyses were performed using the Limma package (Ritchie et al., 2015). The spot volumes were log-transformed and normalized using the loess and quantile methods, for normalizing within-gel and between-gel variation, respectively.

2.5. Alkaline phosphatase treatment

Hundred micrograms of phenol extracted protein from the cold-treated *Malus x domestica* cv. Golden Delicious, dissolved in DIGE lysis buffer, was diluted to 180 µL with milli-Q water, and 20 µL of 10 x reaction buffer (100 mM Tris–HCl, 1 M KCl, 50 mM $MgCl_2$, 0.2% Triton X-100, pH 8.0 at 37 °C) was added. Fifty units of FastAP thermolabile alkaline phosphatase (Thermo Fisher, USA) was added to the samples, and the same volume of water was added to the controls. The reaction was allowed to proceed for 1 h at 37 °C. After treatment, the proteins were cleaned by chloroform precipitation (Wessel and Flugge, 1984) for 2D electrophoresis. Briefly, 800 ul of methanol and 200 ul of chloroform were added to the sample. After mixing, 600 µL of milli-Q water was added to initiate phase separation. The samples were mixed again, centrifugated for 1 min at 14000 *g*, and the aqueous top layer was discarded, taking care not to disturb the interphase which contains the proteins. Six hundred microliters of methanol was added and proteins were pelleted by centrifugation for 1 min at 14000 *g*. After discarding the supernatant, proteins were dried in vacuum centrifuge, and dissolved in 20 µL of DIGE lysis buffer. The treated and control samples were labeled with Cy3 and Cy5 dyes, respectively, and run on a 2DE gel as described above.