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Dehydration induces expression of *GALACTINOL SYNTHASE* and *RAFFINOSE SYNTHASE* in seedlings of pea (*Pisum sativum* L.)

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SUMMARY

The exposition of 7-day-old pea seedlings to dehydration induced sudden changes in the concentration of monosaccharides and sucrose in epicotyl and roots tissues. During 24 h of dehydration, the concentration of glucose and, to a lesser extent, fructose in seedling tissues decreased. The accumulation of sucrose was observed in roots after 4 h and in epicotyls after 8 h of stress. Epicotyls and roots also began to accumulate galactinol and raffinose after 8 h of stress, when small changes in the water content of tissues occurred. The accumulation of galactinol and raffinose progressed parallel to water withdrawal from tissues, but after seedling rehydration both galactosides disappeared. The synthesis of galactinol and raffinose by an early induction (during the first hour of treatment) of galactinol synthase (*PsGolS*) and raffinose synthase (*PsRS*) gene expression as well as a later increase in the activity of both enzymes was noted. Signals possibly triggering the induction of *PsGolS* and *PsRS* gene expression and accumulation of galactinol and raffinose in seedlings are discussed.

Introduction

Soluble carbohydrates, products of primary metabolism, play a variety of functions in plants. Among sugars, sucrose and raffinose family oligosaccharides (RFOs) represented by raffinose, stachyose and verbascose are the focus of much attention, owing to their protective role in tissue tolerance to abiotic stresses. RFOs are ubiquitous storage material in seeds (Obendorf and Górecki, 2012) or in vegetative tissues (Bachmann and Keller, 1995; Nägele and Heyer, 2013), and in some plant species they are used for the transport of carbon skeletons from source to sink tissues (Keller and Pharr, 1996). RFOs are not transported to developing seeds; instead, they are synthesized *de novo* in both embryonic and seed coat tissues (Peterbauer and Richter, 2001). The biosynthesis of RFOs is initiated by the synthesis of galactinol (1-*O*-α-D-galactopyranosyl-L-*myo*-inositol) from UDP-galactose and *myo*-inositol (catalyzing by galactinol synthase, GolS, EC 2.4.1.123), an essential galactosyl residues donor for the synthesis of RFOs. The RFOs biosynthetic pathway includes the transfer of galactosyl residue from galactinol to sucrose by raffinose synthase (RS, EC 2.4.1.82), production of raffinose and transfer of galactose to raffinose and stachyose by multifunctional stachyose synthase (STS, EC 2.4.1.67), producing stachyose and verbascose, respectively (Peterbauer and Richter, 2001). Raffinose, stachyose and verbascose sequentially accumulate late in seed maturation, and their accumulation is accelerated by natural or precocious seed maturation drying (Górecki et al., 2000a). In developing and maturing seeds, the accumulation of RFOs during the drying process coincides with the acquisition of desiccation tolerance (Obendorf, 1997; Górecki et al., 2000b). RFOs in maturing seeds have been proposed to play various roles in desiccation tolerance as compatible solutes (Obendorf, 1997) involved in the osmotic potential of tissues, or agents stabilizing the macromolecules and glassy state in dry seeds (Hoekstra et al., 2001), which can be important for seed longevity (Horbowicz and Obendorf, 1994; Verdier et al., 2013). As storage material, RFOs are quickly degraded during seeds germination (Górecki et al., 2000b). In the embryonic axis, the disappearance of RFOs determinates the seedling's initial growth rate (Blöchl et al., 2007; Lahuta and Goszczyńska, 2009) and coincides with the loss of tissue desiccation tolerance (Obendorf, 1997). However, seedlings seem to retain their ability to synthesize raffinose and stachyose, which is revealed only under water stress conditions (Downie et al., 2003; Lahuta and Górecki, 2011; Brenac et al., 2013).

The accumulation of RFOs in vegetative tissues of different plant species in response to abiotic stresses, like drought, salinity, cold, heat, osmotic and oxidative stress, discovered in the last decade, can be a promising goal for biotechnology of crops (Toldi et al., 2009), and has recently been reviewed in detail (ElSayed et al., 2014).

Abbreviations: RFOs, raffinose family oligosaccharides; GolS, galactinol synthase; RS, raffinose synthase.

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The biosynthesis of galactinol (and raffinose) under stress conditions is triggered by the expression of galactinol synthase genes (Taji et al., 2002; Kant et al., 2008; Evers et al., 2010; Weston et al., 2011; Wang et al., 2012b; Zhou et al., 2013) and expression of genes encoding UDP-glucose-4-epimerase, catalyzing the formation of UDP-galactose, necessary for synthesis of galactinol (Liu et al., 2007; Evers et al., 2010). In effect, tissues accumulate increased amounts of galactinol and raffinose and indicate elevated tolerance to some abiotic stresses. The over-expression of galactinol synthase genes increases tolerance to multiple stresses in transgenic tobacco plants (Zhuo et al., 2013) and tolerance to drought (Taji et al., 2002), high salinity and osmotic stresses (Sun et al., 2013) in *Arabidopsis thaliana*. Moreover, the over-expression of *myo*-inositol phosphate synthase gene (*MIPS*) in tobacco increases MIPS activity and levels of *myo*-inositol, galactinol and raffinose, resulting in enhanced resistance to chilling, drought and salt stresses in transgenic tobacco plants (Tan et al., 2013). Although increasing concentration of *myo*-inositol can stimulate the accumulation of RFOs in both seeds (Karner et al., 2004) and vegetative tissues (Valluru and van den Ende, 2011; Tan et al., 2013), the mechanism of this stimulation remains to be explained. Changes in the expression of raffinose synthase genes in vegetative tissues (mostly in leaves) under abiotic stresses have recently been demonstrated in rice (Wu et al., 2009; Saito and Yoshida, 2011), Arabidopsis (Kant et al., 2008; Egert et al., 2013), poplar (Ko et al., 2011), cucumber (Sui et al., 2012), maize (Zhou et al., 2012a) and pea (Lucau-Danila et al., 2012). The induced or increased accumulation of raffinose can reinforce plants' stress tolerance (ElSayed et al., 2014).

Mature or expanded leaves, shoots and roots are predominant objects in studies concerning the participation of RFOs in abiotic stress tolerance of vegetative tissues (ElSayed et al., 2014). However, during seed germination and seedling establishment, tissues are most susceptible to water stress, and the ability to survive stress reflects on the plant development. Therefore, changes in metabolism during the early response of seedlings to dehydration need deeper explanation.

Several-day-old seedlings are able to accumulate raffinose after 1–2 days of dehydration (Bogdan and Zagdańska, 2006; Zhou et al., 2012a), chilling (Saito and Yoshida, 2011), salinity (Morsy et al., 2007) and osmotic stress (Lahuta and Górecki, 2011). Although the amounts of raffinose accumulated under stress conditions are low and insufficient for instilling stress tolerance in seedlings, seedlings can be a good object for study of mechanisms regulating synthesis of RFOs in response to abiotic stresses. In contrast to fully expanded leaves, shoots and roots, in which raffinose appear after a few days of stress duration (ElSayed et al., 2014), seedlings indicate the ability to an earlier response (Lahuta and Górecki, 2011). We have found that osmotic stress induces the activity of GolS and RS in 7-day-old winter vetch seedlings during the first 6 h of treatment, and tissues transiently accumulated appropriate galactosides (Lahuta and Górecki, 2011). However, the expression levels of the genes involved in the above process during osmotic stress remained unexplained. The results of the present studies demonstrate, for the first time, the dynamic changes in concentrations of soluble carbohydrates, levels of the expression of pea *GALACTINOL SYNTHASE* (*PsGolS*) and *RAFFINOSE SYNTHASE* (*PsRS*) genes, activity of both enzymes and accumulation of galactinol and raffinose at early response of 7-day-old pea seedlings to dehydration.

Materials and methods

Plant material

Seeds of pea (*Pisum sativum* L.) cv Ramzes were surface sterilized in 60% ethanol:water solution for 1 min, rinsed several times in sterilized double distilled water, placed (25 seeds in each of 20 replicates) on wet sheet germination paper (Eurochem BDG, Poland), rolled and transferred into 250 mL glass cylinders. After addition of 50 mL water, the cylinders were incubated in a germination chamber (ILW 115-T STD, Pol-Eko-Aparatura, Poland) at 22 °C in the dark for 7 days. The content of soluble carbohydrates was monitored in the axis and cotyledons to assess the stage at which tissues did not contain any detectable amounts of raffinose family oligosaccharides. Intact 7-day-old seedlings or excised epicotyls and roots (cotyledons were removed) were transferred on open glass Petrie dishes (15 cm diameter, 10 seedlings per dish) and dried at laboratory conditions (22 °C and 28–30% air relative humidity, RH) for 24 h. The intact seedlings after 24 h of dehydration were placed on wet sheet germination paper (Eurochem BGD, Poland), rolled and transferred into 250 mL glass cylinders. After addition of 50 mL of water, the cylinders were incubated in a germination chamber at 22 °C in the dark for 24 h. Epicotyls and roots (10 in each of six replicates) dried separately were collected after 0, 1, 4, 8 and 24 h of dehydration, immersed in liquid nitrogen and stored in an ultra-freezer at −76 °C (PLATINUM 340V, Cheminst, Italy). Samples of epicotyls and roots for carbohydrate analysis and enzyme activity assay were lyophilized (freeze dryer Alpha 1-2LD, Christ, Germany), crushed in a mixed mill (MM200, Retsch, Verder Group, Netherlands) and stored (2 weeks) in a freezer (at −18 °C) until extraction of sugars end enzymes.

The water content in the epicotyl and roots was calculated as a difference between fresh and dry weight (after lyophylisation) of tissues.

Analysis of soluble carbohydrates

Soluble carbohydrates were extracted from 40 to 45 mg of meal with 800 μL of ethanol:water (1:1, v/v, at 90 °C for 30 min), containing 100 μg of xylitol (internal standard). Homogenates were centrifuged and aliquots of clear supernatant were deionized and dried in a speed vacuum rotary evaporator to dryness. The content of soluble carbohydrates was analyzed by high resolution gas chromatography on a ZEBRON ZB-1 capillary column (Phenomenex, USA), according to the method described previously (Lahuta and Górecki, 2011). The carbohydrate content was calculated using the internal standard method. Standards of carbohydrates were purchased from Sigma (USA). Results of analyses (in mg g^{-1} of dry weight, DW) are means of three independent replicates ± SE.

Enzymes activity assay

Extraction of enzymes from excised epicotyl and root tissues was carried out as described earlier (Lahuta and Górecki, 2011). The activity of galactinol synthase (GolS) and raffinose synthase (RS) was determined by the incubation of desalted tissue extract with appropriate substrates, according to the method described by Peterbauer et al. (2001). Products of reactions were determined by the gas chromatography method. All reactions were performed on three independent samples of epicotyl and root tissues. A unit of enzymatic activity corresponds to the amount of product (in picomoles) formed during 1 min of reaction by 1 mg of protein.

PsRS and PsGolS cloning

Genomic DNA was extracted from epicotyls with an innuSPEED Plant DNA Kit (Analitik Jena, Jena, Germany). The PCR reaction consisted of 50 ng of template, 1 μM each primers, 0.2 mM of each dNTPs, PfuPlus buffer and 2.5 U PfuPlus polymerase