Journal of Plant Physiology 224–225 (2018) 112–120

Contents lists available at ScienceDirect

Journal of Plant Physiology

journal homepage: www.elsevier.com/locate/jplph

Metabolic process of raffinose family oligosaccharides during cold stress and recovery in cucumber leaves

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ARTICLE INFO

Keywords:
Cold stress
Cucumber
De-acclimation
Galactosidase
Raffinose family oligosaccharides
Subcellular compartment

ABSTRACT

Raffinose family oligosaccharides (RFOs) accumulate under stress conditions in many plants and have been suggested to act as stress protectants. To elucidate the metabolic process of RFOs under cold stress, levels of RFOs, and related carbohydrates, the expression and activities of main metabolic enzymes and their subcellular compartments were investigated during low-temperature treatment and during the recovery period in cucumber leaves. Cold stress induced the accumulation of stachyose in vacuoles, galactinol in vacuoles and cytosol, and sucrose and raffinose in vacuoles, cytosol, and chloroplasts. After cold stress removal, levels of these sugars decreased gradually in the respective compartments. Among four galactinol synthase genes (*CsGS*), *CsGS1* was not affected by cold stress, while the other three *CsGSs* were up-regulated by low temperature. RNA levels of *acid-α-galactosidase* (*GAL*) *3* and *alkaline-α-galactosidase* (*AGA) 2* and *3*, and the activities of GAL and AGA, were up-regulated after cold stress removal. GAL3 protein and GAL activity were exclusively located in vacuoles, whereas AGA2 and AGA 3 proteins were found in cytosol and chloroplasts, respectively. The results indicate that RFOs, which accumulated during cold stress in different subcellular compartments in cucumber leaves, could be catabolized *in situ* by different galactosidases after stress removal.

1. Introduction

Raffinose family oligosaccharides (RFOs) are galactosyl extensions of sucrose that exist widely in the plant kingdom. The physiological functions of RFOs in higher plants have been studied in detail in the past decades. RFOs are important storage carbohydrates in various plant tissues including leaves, stems, tubers, fruits, and seeds, temporarily or terminally (Keller and Pharr, 1996; ElSayed et al., 2014; Sengupta et al., 2015; Ivamoto et al., 2017). In addition, RFOs are used for phloem transport in some plants in Cucurbitaceae, Lamiaceae, Oleaceae, Scrophulariaceae, and several other families (Keller and Pharr, 1996; ElSayed et al., 2014; Sengupta et al., 2015).

The important role of RFOs in the stress defense mechanism has also been well established. RFOs are characterized as osmoprotectants or antioxidants, and may serve as signals in response to several abiotic or biotic stresses (Zuther et al., 2004; ElSayed et al., 2014; Sengupta et al., 2015). Most sucrose-translocating plants, like rice (*Oryza sativa*) and Arabidopsis (*Arabidopsis thaliana*), do not accumulate large quantities of RFOs in their tissues under optimum conditions. However, under stressed conditions such as temperature extremes, drought, and salinity, the accumulation of RFOs and the induced expression of the biosynthetic enzymes galactinol synthase (GS) and raffinose synthase (RS) were always found in these plants (Nishizawa et al., 2008; Saito and Yoshida, 2011; Gangl and Tenhaken, 2016).

The increase of RFOs was also observed in RFO-translocating plants in response to abiotic stresses. *Ajuga reptans*, a frost-hardy perennial labiate, accumulates many more RFOs in winter leaves than in summer leaves, and cold treatment significantly increases RFO concentration in leaves (Bachmann et al., 1994). In cucumber (*Cucumis sativus* L.), *RS* expression, RS activity, and the content of raffinose and stachyose increased gradually in the leaves, fruits, stems, and roots under low-temperature stress (Meng et al., 2008; Sui et al., 2012). In these RFO-translocating species, it seems that there are two pools of RFOs: a storage pool in the mesophyll (long-term in *Ajuga reptans* or short-term in cucumber), which is involved in the stress response, and a transport pool in the phloem (Bachmann and Keller, 1995; Sui et al., 2012).

GS is a key enzyme catalyzing the first step in the RFO biosynthetic pathway (Keller and Pharr, 1996). Most plants have more than one isoform of GS coded by different genes. In *Ajuga reptans*, there are two GS genes, *ArGolS1* and *ArGolS2*. *ArGolS1* is mainly involved in the

Abbreviations: AGA, alkaline-α-galactosidase; CaMV, cauliflower mosaic virus; EGFP, enhanced green fluorescent protein; GAL, acid-α-galactosidase; GS, galactinol synthase; RFOs, Raffinose family oligosaccharides; RS, raffinose synthase; STS, stachyose synthase

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https://doi.org/10.1016/j.jplph.2018.03.012
Received 27 November 2017; Received in revised form 22 March 2018; Accepted 22 March 2018
Available online 29 March 2018

synthesis of storage RFOs while *ArGolS2* is for the synthesis of transport RFOs (Sprenger and Keller, 2000). In the cucumber genome, four putative GS genes were found (Wang et al., 2016). However, the exact roles of these roles of these genes in the stress response and phloem transport are not well investigated.

The subcellular localization of RFOs and their biosynthetic enzymes under low-temperature stress were further studied in *Ajuga reptans* and Arabidopsis. The results showed that GS, RS, and stachyose synthase (STS) were extravascular (most likely cytosolic); galactosyltransferase, stachyose, and higher RFOs were vacuolar; and sucrose and raffinose were found in cytosol, vacuoles, and chloroplasts (Bachmann and Keller, 1995; Tapernoux-Lüthi et al., 2007; Schneider and Keller, 2009; Knaupp et al., 2011; Findling et al., 2015). It is suggested that raffinose rather than stachyose plays an important role in stabilizing photosystem II in chloroplasts during low-temperature stress in Arabidopsis (Iftimea et al., 2011; Knaupp et al., 2011). As a crop of subtropical origin that translocates RFOs but does not store large quantities of RFOs under optimum conditions, it remains unknown whether cucumber exhibits different RFO subcellular localization than the frost-hardy RFO-translocating plant Ajuga reptans and the sucrose-translocating plant Arabidopsis under cold stress.

In contrast to cold acclimation, cold de-acclimation is an important regulatory mechanism to ensure that plants are restored to their normal growth state when the stress condition is removed. Unfortunately, although the accumulation of RFOs and its physiological significance under stress conditions have been well studied in several plants, the question of how RFOs are catabolized after stress removal has received little attention. Alpha-galactosidases are responsible for the removal of the terminal galactose residue during RFO catabolism (Keller and Pharr, 1996). There are six putative α-galactosidase genes in the cucumber genome. These genes are divided into two groups, three acid α-galactosidase genes (*GAL*) and three alkaline α-galactosidase genes (*AGA*), according to their activity in response to pH (Wang et al., 2016). GALs are considered to be localized in the apoplast space or vacuole, while AGAs are supposed to be localized in the cytosol (Keller and Pharr, 1996; Tapernoux-Lüthi et al., 2007). Considering that both RFOs and α-galactosidases can be found in multiple subcellular localizations, it would be interesting to investigate whether different α-galactosidases catabolize RFOs in different subcellular compartments when stress conditions are relieved.

In this study, in order to reveal the metabolic process of RFOs during cold stress, levels of RFOs and relative carbohydrates, as well as the expression and activities of metabolic enzymes and their subcellular compartments, were investigated during cold treatment and during the recovery period in cucumber leaves. We focused on assessing the expression pattern, activity, and intracellular localization of six α-galactosidases to elucidate the catabolic process of RFOs after stress removal.

2. Material and methods

2.1. Plant material and temperature treatment

Cucumber (*Cucumis sativus* L.) cultivar Jinchun 5 (from Tianjin Cucumber Institute, China) was used in this study. Seedlings were grown in 10 × 10 cm plastic pots containing a peat–vermiculite mixture (2:1, v/v) in a growth chamber. The seedlings were thinned to one per pot 10 d after germination. Plants were watered once daily and fertilized weekly with the Hoagland nutrient solution (200 ml work solution per pot). In the growth chamber, the temperatures were 28 °C/22 °C (day/night), with a relative humidity of 70%. Light was provided by high-pressure mercury lamps (Philip HPLN 400 W) at about 700 μmol $m^{-2} s^{-1}$ for 12 h per day (7:00–19:00). Plants for cold treatment were transferred to another chamber in which the temperature was lowered to 15 °C/8 °C at the four-leaf stage. After three-day chilling treatment, the temperature in the chamber was restored to 28 °C/22 °C. Control plants remained in the original chamber throughout the experiment. The second leaves from the apical meristem of each plant were collected at 16:00 every day from the day before treatment to the third day after cold stress removal (denoted by C0, C1, C2, C3, R1, R2, and R3, respectively). Samples were frozen in liquid nitrogen immediately after harvest and stored at −80 °C.

2.2. Non-aqueous fractionation of leaves

The procedure was conducted according to Nadwodnik and Lohaus (2008) and Krueger et al. (2014) with a few modifications. After removing the middle rib and larger veins, the samples were ground to a fine powder in liquid nitrogen in a precooled mortar and then lyophilized at −25 °C. The dry leaf powder was suspended in 20 ml of heptane:tetrachloroethylene mixture (density 1.3 g ml^{-1}). Sonication was performed for 2 min, with 6 × 10 cycles at 65% power. The sonicated suspension was filtered through a nylon sieve (40 μm). The sample was centrifuged for 10 min at 3200 × *g* and 4 °C, and then the sediment was resuspended again in the heptane:tetrachloroethylene mixture (density 1.3*g* ml^{-1}). The suspension was added to an exponential heptane-tetrachloroethylene gradient with a density between 1.27 and 1.50 g ml^{-1}. After centrifugation for 60 min at 5000 × *g* and 4 °C, six fractions were collected, aliquots of which were taken for the determination of the marker enzymes, RFO metabolic enzymes, and RFO-related sugars. Glyceraldehyde-3-phosphate dehydrogenase (EC1.2.1.9) was used as a chloroplast marker, UDP-glucose-pyrophosphorylase (EC 2.7.7.9) as a cytosolic marker, and acidic phosphatase (EC 3.1.3.2) as a vacuolar marker. The calculation was carried out by the software BestFit (Krueger et al., 2014).

2.3. Carbohydrate assay and enzyme activity determination

Chloroform methanol extracts were prepared from the aliquots mentioned above for the determination of the carbohydrate concentrations (Nadwodnik and Lohaus, 2008). Galactinol, stachyose, raffinose, galactose, and sucrose were analyzed by HPLC methods as described previously (Miao et al., 2007). For the enzyme activity assay, fractions from gradient centrifugation were washed by three volumes of C_7H_{16} and lyophilized. The dried samples were extracted using Hepes buffer (50 mM Hepes-NaOH pH 7.4; 5 mM $MgCl_2$; 1 mM EDTA; 1 mM EGTA; 0.1% Triton X-100; 10% glycerol; 2 mM benzamidine; 2 mM aminocaproic acid; 1.5 mM PMSF; 1 gl^{-1} PVPP) (Krueger et al., 2014). Activities of GS and α-galactosidases were assayed according to Wang and colleagues (Wang et al., 2016). For the assay of RS, the reaction buffer contained 50 mM HEPES–NaOH (pH 7.0), 1 mM DTT, 10 mM galactinol, and 40 mM sucrose. Mixtures were incubated at 30 °C for 3 h, and the reactions were stopped by boiling for 5 min. The mixture was centrifuged at 28,000 × *g* for 5 min, and the supernatant was passed through a 0.45-μm filter. The content of raffinose was determined by HPLC. Enzyme activity is given as μmol of raffinose formation per hour (Sui et al., 2012). The assay of STS was the same as that of RS, except for galactinol that was replaced by raffinose in the reaction system.

2.4. Total RNA isolation and expression analysis of RS and STS

Total RNA was extracted from approximately 100 mg of the leaf tissues (without middle ribs and larger veins) using TRIzol reagent (Invitrogen, Shanghai, China). Reverse transcription was performed using a Prime Script TM RT Reagent Kit with gDNA eraser (Perfect Real Time, TaKaRa, Dalian, China). Quantitative real-time PCR was performed using the One Step SYBR PrimeScript RT-PCR Kit (TaKaRa, Dalian, China) on an ABI PRISM 7700 Sequence Detection System (Applied Biosystems, Shanghai, China), following the manufacturer's instructions. The real-time PCR was carried out according to the following protocol: 2 min at 94 °C, followed by 39 cycles of 94 °C for 15 s, 60 °C for 15 s and 72 °C for 30 s. The cucumber 18S rRNA gene (Gene