Title:
Metabolic process of raffinose family oligosacharrides during cold stress and recovery in cucumber leaves

Short running title: RFOs metabolism during cold stress and recovery

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Highlight: This study provide several evidences that raffinose family oligosacharrides accumulated during cold stress in cucumber leaves are catabolized in different subcellular compartments by different alpha-galactosidases after temperature recovery.

Abstract

Raffinose family oligosacharrides (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 relative carbohydrates, the expression and activities of main metabolic enzymes and their subcellular compartments were investigated during low temperature treatment and recovery period in cucumber leaves. Cold stress induced the accumulation of stachyose in vacuoles, galactinol in vacuoles and cytosols, and sucrose and raffinose in vacuoles, cytosols and chloroplasts. After cold stress removal, levels of these sugars decreased gradually in respective compartments. Among 4 galactinol synthase genes (CsGS), CsGS1 was not affected by the cold stress, while other three CsGSs were up-regulated by the low temperature. RNA levels of acid-α-galactosidase (GAL) 3, alkaline-α-galactosidase (AGA) 2 and 3, and the activities of GAL and AGA were up-regulated after cold stress removal. The GAL3 protein and GAL activity were exclusively located in the vacuole, whereas the protein of AGA2 and AGA 3 were found in the cytosol and chloroplast respectively. The results indicate that RFOs accumulated during the cold stress in different subcellular compartments in cucumber leaves could be catabolized in situ by different galactosidases after stress removal.

Key Words: cold stress, cucumber, de-acclimation, galactosidase, raffinose family oligosacharrides, subcellular compartment
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 detailedly 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 other several families (Keller and Pharr, 1996; ElSayed et al., 2014; Sengupta et al., 2015).

The important role of RFOs in the stress defence mechanism was also well established. RFOs are characterised 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). In most sucrose-translocating plants, like rice (Oryza sativa) and Arabidopsis (Arabidopsis thaliana) that neither transport nor accumulate large quantities of RFOs in their tissues under normal conditions, the accumulation of RFOs and the induced expression of their biosynthetic enzymes, galactinol synthase (GS) and raffinose synthase (RS), were found in response to diverse abiotic stresses such as temperature extremes, drought and salinity (Nishizawa et al., 2008; Saito and Yoshida, 2011; Gangl and Tenhaken, 2016).

Under stressed conditions, the increase of RFOs was also observed in RFOs-translocating plants. Ajuga reptans, a frost-hardy perennial labiate, accumulates much more RFOs in winter leaves than in summer leaves, and cold treatment would significantly increase the RFOs 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 RFOs-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 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 RFOs 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 synthesis of storage RFOs while *ArGolS2* is for the synthesis of transport RFOs (Sprenger and Keller, 2000). In the cucumber genome, 4 putative GS genes were found (Wang et al., 2016). However, the exact 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 extravacuolar (most probably cytosolic), galactosyltransferase, stachyose and higher RFOs were vacuolar, and sucrose and raffinose were found in cytosol, vacuole and chloroplast (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 stacyose, plays an important role in stabilizing photosystem II in chloroplasts during low temperature stress in Arabidopsis (Iftime et al., 2011; Knaupp et al., 2011). As a crop of subtropical origin which translocate RFOs but not store large quantities of RFOs under the normal condition, does cucumber have different RFOs subcellular localization with the frost-hardy RFOs-translocating plant *Ajuga reptans* and the sucrose-translocating plant Arabidopsis under cold stress remains unknown.

In contrast to cold acclimation, cold de-acclimation is an important regulatory mechanism to ensure plants restoring to their normal growth state when the stress condition was removed. Unfortunately, although the accumulation of RFOs and its physiological significance under stress conditions have been well studied in several plants, how RFOs were catabolized after stress removal has received little attention. Alpha-galactosidases are responsible for the terminal galactose residue removing during RFOs catabolism (Keller and Pharr, 1996). There are 6 putative α-galactosidase genes in the cucumber genome. These genes are divided into 2 groups,
3 α-galactosidase genes (GAL) and 3 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 both RFOs and α-galactosidases reveal multiple subcellular localizations, it is interesting to know if different α-galactosidases catabolize the 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, the expression and activities of metabolic enzymes and their subcellular compartments were investigated during cold treatment and the recovery period in cucumber leaves. We emphasized the observation of the expression pattern, activity and intracellular localization of 6 α-galactosidases to elucidate the catabolic process of RFOs after stress removal.

Materials and methods

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. In the growth chamber, the temperatures were 28°C/22°C (day/night) and the relative humidity 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 4-leaf stage. After 3-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 everyday from the day before treatment to the third day after cold stress.
removal (named C0, C1, C2, C3, R1, R2, R3, respectively). Samples were frozen in liquid nitrogen immediately after harvest and stored at –80 °C.

**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 3,200 × g and 4 °C and the sediment was resuspended again in the heptane:tetrachloroethylene mixture (density 1.3 g ml⁻¹). The suspension was added to an exponential heptane-tetrachlorethylene gradient with a density between 1.27 and 1.50 g ml⁻¹. After centrifugation for 60 min at 5,000 × g and 4 °C, six fractions were collected, aliquots of which were taken for the determination of the marker enzymes, RFOs metabolic enzymes and RFOs related sugars. The calculation was carried out by the software BestFit (Krueger et al., 2014).

**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 enzymes assay, fractions from gradient centrifugation were washed by 3 volumes of C₇H₁₆ and lyophilized. The dried samples were extracted by Hepes buffer (50 mM Hepes-NaOH pH 7.4; 5 mM MgCl₂; 1mM EDTA; 1 mM EGTA; 0.1 % Triton X-100; 10% glycerol; 2mM benzamidine; 2mM aminocaproic acid; 1.5mM PMSF; 1g l⁻¹ PVPP) (Krueger et al., 2014). Activities of GS and α-galactosidases were assayed according to (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 galactinol was replaced by raffinose in the reaction system.

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 bank accession No.: AF206894.1) was used for
normalization in all the analyses performed. The primer sequences for RS and STS
(Gene bank accession No.: EU096498) are listed in Supplementary Table S1. Primers
were confirmed to be approximately 90% to 100% efficient in amplification, and
2−ΔΔCT method (Livak and Schmittgen, 2001) was used for analyses.

Northern blot analysis of the expression of GS and α-galactosidases genes
Total RNA (20μg) was extracted as mentioned above and electrophoresed on a 1.2%
(w/v) agarose gel containing formaldehyde, then transferred onto positively charged
nylon membrane (Amersham-Pharmacia Biotech, Uppsala, Sweden) with 10×SSC
buffer (1×SSC containing 150 mM NaCl and 15 mM sodium citrate, pH 7.0). DNA
fractions of 4 CsGS genes and 6 α-galactosidases genes were cloned into pMD 18-T
vector (Takara, China) as DNA templates for RNA probe synthesis. The primer sequences for cloning and the gene bank accession No. of 10 genes are listed in Supplementary Table S1. The probe synthesis and northern blot was performed using DIG Northern Starter Kit (Roche Applied Science, China) following the manufacturer’s instructions.

**Subcellular localization of 6 α-galactosidases**

To obtain the “cauliflower mosaic virus (CaMV) 35S promoter::GFP::gene ORF” fusion protein expression structure, the CaMV 35S promoter sequence was amplified with primers 5’-ccggaattccatggagtcaaagat-3’ (EcoRI restriction site added) and 5’-ataaggatccagtcccccgtgtt-3’ (BamHI restriction site added) from the vector pCambia1303. The enhanced GFP (EGFP) sequence was amplified with primers 5’-aatgtcgacatggtgagcaagggcgagg-3’ (SalI restriction site added) and 5’-gaatctgcagcttgtacagctcgtccatg-3’ (PstI restriction site added) from the vector pEGFP-N1(U55762). These two fragments were inserted into the vector pCambia1381*c using restriction enzymes mentioned above. All α-galactosidase genes were further cloned into the vector “pCambia1381*c::CaMV 35S::EGFP”.

The primers and restriction enzymes used are list in the Supplementary Table S2. The construction of GAL3 fusion protein expression system is the same as other 5 α-galactosidase genes except the restriction enzymes ApaI and EcoRI were used for CaMV 35S promoter cloning, and EcoRI and BamHI were used to clone EGFP fragments.

Transient transformation of onion epidermis was carried out according to Xu et al. (2004) with modifications. The vectors pCambia1381*c containing “CaMV 35S promoter::EGFP::gene ORF” structures were transformed into *Agrobacterium tumefaciens* strain LBA4404. *Agrobacterium* cultivated overnight at 28°C was harvested at OD600 of 1.5 to 2.0, centrifuged at 5000 rpm for 10 min and resuspended in 50 ml of infiltration liquid (50g l⁻¹ sucrose+100mg l⁻¹ acetosyringone). Adaxial onion epidermis 1 cm² was incubated in the infiltration liquid for 30s and then
cultured on the 1/2 MS solid medium (30g l\(^{-1}\) sucrose, 7g l\(^{-1}\) agar, pH 5.8) for 2 d (25°C, 16h/8h). Plasmolysis cells were obtained by adding 300g l\(^{-1}\) KNO\(_3\) on the tissue for 5min before fluorescence microscope observation (Zeiss LSM710).

Transient transformation of tobacco leaves was mainly according to Sparkes et al. (2006). Basically, the underside of the tobacco leaf was punctured with a small needle. Took up resuspended Agrobacterium infiltration liquid (10 mM MgCl\(_2\), 10 mM MES, 100μM acetosyringone) in a 1 ml syringe (no needle). Placed the tip of the syringe against the underside of the leaf over the needle mark and pressed down gently on the plunger. Placed plants in a growth cabinet under normal conditions for 3d, and then excised 1-2 cm\(^2\) segments of leaf tissue for confocal microscope observation (Zeiss LSM710).

**Results**

*Subcellular distribution of sugars and relative enzymes in cold-stressed cucumber leaves*

Since RFOs will accumulate after cold stress (Sui et al., 2012), cucumber leaves were sampled after a 3-day cold treatment. The distribution of the sugars and relative enzymes among the vacuolar, chloroplast, and cytosolic compartment was measured by non-aqueous fractionation method and the results are summarized in the Table 1. Stachyose and galactose were almost exclusively found in the vacuole, sucrose and raffinose were distributed in all three compartments, while galactinol was located mainly in the vacuole and cytosol. Three RFOs synthesis enzymes, GS, RS and STS, were mostly located in the cytosol. The GAL activity was mainly found in the vacuole whereas the AGA activity was mostly distributed in both chloroplast and cytosol (Table 1).

**Suggested position of Table 1**

*Change of sugar levels during cold treatment and recovery*

According to the subcellular distribution of sugars (Table 1), levels of galactinol, sucrose, raffinose, stachyose and galactose in vacuole, sucrose and raffinose in chloroplast, and galactinol, sucrose and raffinose in cytosol were investigated during
the cold stress and recovery period (Fig. 1). The levels of all measured sugars except galactose increased from 0 d to 3 d of cold treatment and then decreased when the temperature was recovered to the normal level, indicating low temperature promoted the accumulation of these sugars in different subcellular compartments. The galactose level showed an opposite pattern, which decreased during cold stress and increased during the recovery period in the vacuole. The amplitude of fluctuation of sugar levels was more significant in vacuole and chloroplast than in cytosol. No significant fluctuations of sugar levels were found in control plant leaves during the treatment (Supplementary Fig. S1).

The expression and activities of RFOs synthesis enzymes during cold treatment and recovery

Four GSs showed different expression patterns under cold stress. Under normal temperature, the expressions of CsGS1 and CsGS4 were detected and the expression of CsGS4 was stronger than that of CsGS1, while the expressions of CsGS2 and CsGS3 were not detected by the northern blot. Cold treatment had no significant effect on the expression of CsGS1. However, CsGS2, CsGS3 and CsGS4 expressions were enhanced by the low temperature, and RNAs of CsGS2 and CsGS3 could be detected at the 3rd day of cold treatment. After the stress removal, the RNAs levels of these three genes declined gradually. At the 3rd day of temperature recovery, the expression of CsGS3 fell below the detection limit (Fig. 2A). The GS activity was closely correlated to the expression pattern of the CsGS2, CsGS3 and CsGS4, which increased under the cold stress and decreased after the stress removal (Fig. 2B).

The RS showed a similar expression pattern to that of the CsGS2, CsGS3 and CsGS4. The RNA level of RS was significantly up-regulated by the low temperature, and then declined after the stress removal (Fig. 3A). The RS activity also increased, and was higher than that of control plant at the 3rd day of recovery (Fig. 3B). No clear effect of cold stress on the STS expression and enzyme activity was observed in this study (Fig. 3C, D).
The expressions and activities of galactosidases during cold treatment and recovery

As shown in Fig. 4A, among 3 GALs, only GAL1 RNA was detected under normal temperature, and low temperature treatment and recovery have no significant effect on its level. GAL2 mRNA was not detected under both treatments. The expression of GAL3 was undetectable under both normal and low temperature, but was induced remarkably when the cold stress was removed. mRNAs of all 3 AGAs were detected under the normal temperature. No significant effect of temperature change on the AGA1 expression was found. However, the RNA levels of both AGA2 and AGA3 were down-regulated by the cold stress and then restored to normal levels after cold stress removal (Fig. 4 B). Both GAL and AGA activities were measured in different subcellular compartments (Fig. 5). Two substrate, raffinose and stachyose were used in the enzyme assay. The GAL activity was higher with the substrate raffinose, while AGA activity was higher with stachyose. The fluctuation patterns of enzyme activities with two substrates were similar in all treatments. In vacuole, the GAL activity remained unchanged during cold stress and increased significantly after stress removal. In chloroplast and cytosol (Fig. 5A, C), the AGA activities were down-regulated by the low temperature and then up-regulated by the stress removing (Fig. 5 B, D). No significant fluctuation of GAL or AGA activities was found in control plant leaves during the treatment (Supplementary Fig. S2). These results indicated that GAL3, AGA2 and AGA3 were important for RFOs catabolism during the temperature recovery.

Subcellular localization of galactosidase proteins

To obtain further insight into the mechanism of RFOs catabolism after cold stress removal, an EGFP protein was fused to the N-terminus of 6 galactosidases, and placed under the control of the CaMV 35S promoter. These constructs were transiently expressed in the onion epidermal cells. To distinguish the cell wall and the cytosol in these highly vacuolate cells, plasmolysis was carried out before transformation. Fluorescence microscope imaging showed that GAL1 and GAL2 were located near the cell wall, GAL3 was found in the vacuole, while all 3 AGAs were distributed in...
the cytosol (Fig. 6). The subcellular localization of AGA2 and AGA3, which were found to play a role in catabolizing RFOs in cytosol or chloroplast, were further determined by transiently expressing the EGFP fusion construct in the tobacco mesophyll cell. The results revealed that AGA2 was located in the cytosol, while AGA3 was mostly found in the chloroplast (Fig. 7).

Discussion

The subcellular compartment study of RFOs and relative carbohydrates after cold stress in cucumber leaves using non-aqueous fractionation method showed similar results with previous reports, i.e., stachyose and galactose were mostly in the vacuole, galactinol in the vacuole and cytosol, and sucrose and raffinose in the vacuole, cytosol and chloroplast (Schneider and Keller, 2009; Knaupp et al., 2011; Nägele and Heyer, 2013; Findling et al., 2015). In cucumber leaves, besides accumulating after cold stress, RFOs are synthesized in the cytosol of intermediary cells in the minor veins for phloem transport (Turgeon and Wolf, 2009). The effect of this “transport RFOs pool” on the results in this study seemed negligible, since little stachyose are found in the cytosol (Table 1). The levels of galactinol, sucrose and raffinose increased under the low temperature treatment and decreased after the stress removal, similar phenomena were also found in other plant species (Cunningham et al., 2003; Brenac et al., 2013; ElSayed et al., 2014). The results of this study, together with data from Ajuga reptans (Bachmann et al., 1994), suggest that accumulations of RFOs are also important for RFOs translocating species to deal with cold stress. Iftime et al. (2011) have shown that stachyose accumulation in transgenic Arabidopsis plants did not increase the freezing tolerance. However, the up-regulated level of this tetrasccharide in the vacuole in cold acclimated cucumber and Ajuga reptans leave tissues (Findling et al., 2015; this study) suggest that stachyose may exert its protective role in these RFOs-translocating plants. Unlike Ajuga reptans, cucumber does not accumulated higher RFO oligomers in leaves (Meng et al., 2008). The physiological importance of stachyose accumulated in the vacuole during cold stress in cucumber leaves awaits
further research.

GSs are always encoded by multiple genes in plant genomes. Evidences indicated that these isoforms have different cellular locations and physiological functions. In Arabidopsis, AtGolS1 and AtGolS2 were induced by drought and high-salinity stresses, while AtGolS3 was induced by cold stress (Taji et al., 2002). In the stachyose-translocating plant Ajuga reptans, ArGolS1 expressed in the mesophyll for storage RFOs synthesis and ArGolS2 mainly in the intermediary cell for transport RFOs synthesis (Sprenger and Keller, 2000). In the melon (Cucumis melo), Cucumber mosaic virus and heat stress did not affect the expression level of CmGolS1, but caused a significant increase in the relative expression level of CmGolS2 (Gil et al., 2012). In this study, in cucumber leaves CsGS1 expressed constitutively and was not affected by the cold stress, while the expressions of other three CsGSs were up-regulated by the low temperature. Phylogenetic analysis based on the amino acid sequences indicated that cucumber CsGS1 and CmGloS1 are closely related, while cucumber CsGS4 and CmGloS2 cluster into one group (Supplementary Fig. S3). It is not clear if the function of CsGS1 and CmGloS1 is similar to that of the Ajuga reptans ArGolS2 (for transport RFOs synthesis). CsGS2 and CsGS3 showed similar expression pattern during the temperature treatment, i.e., could not be detected under normal temperature and induced by cold stress. In the phylogenetic tree, cucumber CsGS2 is closely related to the SmGloS3, which has a low constitutive expression and could be induced by several abiotic stresses in Salvia miltiorrhiza (Wang et al., 2012). CsGS3, AtGolS2 and AtGolS3 belong to the same group (Supplementary Fig. S3). It seemed that the function of GSs in abiotic stress is mainly determined by which element exists in their promoter area, rather than the amino acid sequences (Taji et al., 2002). The response patterns of 4 CsGSs to other abiotic stresses need further investigation. Other two RFOs biosynthetic enzymes, RS and STS, revealed different expression and activity patterns during temperature treatment, RS was up-regulated by cold stress but STS not. The increased level of substrate raffinose, rather than the biosynthetic enzymes STS, may result in the stachyose accumulation under low temperature. In addition, the cytosol compartment of GS, RS and STS, the chloroplast
and vacuole localization of raffinose and the vacuole localization of stachyose, further
confirm that there are RFOs transporters on the tonoplast and chloroplast envelope in
RFOs-translocating plants (Greutert and Keller, 1993; Schneider and Keller, 2009;
Nägele and Heyer, 2013).

Up to date, little research has been focused on the catabolism process of RFOs
after stress conditions are removed in leaf tissues. Subcellular localizations of 6
cucumber galactosidases, which are considered key enzymes in the pathway, were
studied in this research. Tapernoux-Lüthi et al. (2007) concluded that a C-terminal
oligopeptide extension is a non-sequence-specific vacuolar sorting determinant of
plant galactan:galactan galactosyltransferase and acid galactosidase. Sequence
analysis revealed that among 3 cucumber GALs, only GAL3 has this C-terminal
oligopeptide extension (Supplementary Fig. S4), indicating the vacuolar compartment
of GAL3, and the apoplastic location of GAL1 and GAL2. The results of our EGFP
fusion protein transiently expression experiments confirmed that GAL3 is the only
acid galactosidase located in the vacuole. Combining with the expression and activity
pattern during the temperature treatment, we concluded that GAL3 was responsible for
the RFOs catabolism in vacuoles after stress removal. Alkaline galactosidases were
always considered to be distributed in the cytosol (Keller and Pharr, 1996). Osh69, a
rice alkaline galactosidase, was found to be located in the chloroplast and play a role
during leaf senescence (Lee et al., 2004). In this study, cucumber AGA3 protein was
found in the chloroplast and its expression was down-regulated by the cold stress and
up-regulated by the temperature recovery, strongly suggests an important role for
AGA3 in chloroplast RFOs catabolism after cold stress removal. The alkaline
environment of chloroplast stroma is suitable for the AGA3 to exert its catalytic
function (Findling et al., 2015). The data of this study also indicated that another
alkaline galactosidase, AGA 2, is responsible for RFOs catabolism in the cytosol after
temperature was recovered to normal level.

In conclusion, our results indicate that RFOs accumulated during cold stress in
different subcellular compartments in cucumber leaves could be catabolized in situ by
derent galactosidases after stress removed. The data do not rule out the possibility
that RFOs are translocated to other subcellular compartments for degradation.

Supplementary data

**Fig. S1.** Changes of sugar levels in different subcellular compartments of control plant leaves during treatment.

**Fig. S2.** Changes of α-galactosidase activities in different subcellular compartments of control plant leaves during treatment.

**Fig. S3.** Phylogenetic tree representing the relationship of galactinol synthase genes from different plant species with the full-length protein sequence reported.

**Fig. S4.** Sequence comparison of C-terminal peptides of three cucumber acid alpha-galactosidase genes.

**Table S1.** Primers used for real-time PCR and Northern hybridize probe synthesis.

**Table S2.** Primers used for EGFP fusion protein vector construction.

Acknowledgements

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Table 1. Percentage distribution of carbohydrates and enzymes of RFOs metabolism among the cytosols, chloroplasts and vacuoles of leaf mesophyll cells in cucumber seedlings under cold stress for 3 days. The data represent the mean ±SE of three samples.

<table>
<thead>
<tr>
<th>Carbohydrates/enzymes</th>
<th>Cytosol (%)</th>
<th>Chloroplast (%)</th>
<th>Vacuole (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Galactinol</td>
<td>53.2±6.3</td>
<td>9.4±4.2</td>
<td>38.4±10.2</td>
</tr>
<tr>
<td>Sucrose</td>
<td>37.2±17.2</td>
<td>40.2±13.8</td>
<td>22.6±8.4</td>
</tr>
<tr>
<td>Raffinose</td>
<td>42.1±12.4</td>
<td>21.8±9.6</td>
<td>36.1±15.3</td>
</tr>
<tr>
<td>Stachyose</td>
<td>1.7±1.3</td>
<td>2.1±1.7</td>
<td>96.2±3.6</td>
</tr>
<tr>
<td>Galactose</td>
<td>0.9±1.4</td>
<td>0.8±2.3</td>
<td>98.3±4.3</td>
</tr>
<tr>
<td>Acid-α-Galactosidase</td>
<td>3.2±0.8</td>
<td>1.3±0.5</td>
<td>95.5±7.2</td>
</tr>
<tr>
<td>Alkaline-α-Galactosidase</td>
<td>66.3±8.3</td>
<td>27.4±5.6</td>
<td>6.3±3.2</td>
</tr>
<tr>
<td>Galactinol synthase</td>
<td>100±0.0</td>
<td>0.0±0.0</td>
<td>0.0±0.0</td>
</tr>
<tr>
<td>Raffinose synthase</td>
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<td>0.0±0.0</td>
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Figure legends

**Fig.1.** Changes of sugar levels in different subcellular compartments during cold stress and recovery. C0: cold stress for 0 d; C3: cold stress for 3d; R3: the 3rd day after stress removal. Means±SE (5 samples) followed by different letters are significantly different (P <0.05).

**Fig.2.** Changes of expression and activity of galactinol synthases (GSs) in cucumber leaves during cold stress and recovery. A: expression; B: activity. N: normal temperature (28°C/22°C); C: Cold stress (15°C /8°C); C0, C1, C2, C3: cold stress for 0 d, 1 d, 2 d and 3 d; R1, R2, R3: temperature was restored to normal level for 1 d, 2 d and 3 d. Each point is the average of 5 samples. Error bars represent SEs.

**Fig.3.** Changes of expression and activity of raffinose synthase (RS) and stachyose synthase (STS) in cucumber leaves during cold stress and recovery. A: RS expression; B: RS activity; C: STS expression; D: STS activity. Control: 28°C/22°C; Cold stress: 15°C/8°C; C0, C1, C2, C3: cold stress for 0 d, 1 d, 2 d and 3 d; R1, R2, R3: temperature was restored to normal level for 1 d, 2 d and 3 d. Each point is the average of 5 samples. Error bars represent SEs.

**Fig.4.** Expression of α-galactosidases in cucumber leaves during cold stress and recovery. A: acid-α-galactosidases (GAL); B: alkaline-α-galactosidases (AGA); N: Normal temperature (28°C/22°C); C: Cold stress(15°C /8°C); C0, C1, C2, C3: cold stress for 0 d, 1 d, 2 d and 3 d; R1, R2, R3: temperature was restored to normal level for 1 d, 2 d and 3 d.

**Fig.5.** Changes of α-galactosidase activities in different subcellular compartments of cucumber leaves during cold stress and recovery. A and C: using raffinose as substrate; B and D: using stachyose as substrate; C0: Cold stress for 0 d; C3: cold stress for 3d; R3: the 3rd day after stress removal. Means±SE (5 samples) followed by different letters are significantly different (P <0.05).
Fig. 6. Subcellular localization of acid-α-galactosidases (GALs) and alkaline-α-galactosidases (AGAs). GFP-GAL and GFP-AGA fusion proteins and GFP alone expressed under the control of the CaMV35S promoter in onion epidermal cells were observed under a fluorescent microscope. A1 and A2: GAL1; B1 and B2: GAL2; C1 and C2: GAL3; D1 and D2: AGA1; E1 and E2: AGA2; F1 and F2: AGA3; G1 and G2: GFP alone. A1, B1, C1, D1, E1, F1, G1: Differential interference contrast images; A2, B2, C2, D2, E2, F2, G2: GFP fluorescence signals. To distinguish the cell wall and the cytosol in these highly vacuolate cells, plasmolysis was carried out before transformation. Bars=50 μm.

Fig. 7. Subcellular localization of alkaline-α-galactosidases (AGAs). GFP-AGA2 fusion protein (A1, A2 and A3), GFP-AGA3 fusion protein (B1, B2 and B3) and GFP alone (C1, C2 and C) expressed under the control of CaMV35S promoter in tobacco leaf cells were observed under a confocal microscope. The left column (A1, B1 and C1) shows green channel (GFP signal, 488 nm), the middle column (A2, B2 and C2) shows red channel (chloroplast autofluorescence signal, 633 nm), and the right column (A3, B3 and C3) shows merged images. Bars=10 μm.
Fig. 1

A. In Vacuole

B. In Chloroplast

C. In Cytosol

The graph shows the concentration of carbohydrates in vacuoles, chloroplasts, and cytosol across different samples (C0, C3, K3). Bars with different letters indicate significant differences among treatments.
Fig. 2

A

C0  C1  C2  C3  R1  R2  R3

CsGS1 N
CsGS1 C
CsGS2 N
CsGS2 C
CsGS3 N
CsGS3 C
CsGS4 N
CsGS4 C
RNA

B

Galactin synthase activity (pmol h⁻¹ g⁻¹ FW)

Days of cold stress and recovery

Control
Cold stress
Fig. 3

A: Relative expression level of RsOs

B: POD activity (μmol h⁻¹ g⁻¹ FW)

C: Relative expression level of SYS

D: SYS activity (μmol h⁻¹ g⁻¹ FW)
Fig. 4

A

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Fig. 6
Fig. 7