1 **Title:**

2 **Metabolic process of raffinose family oligosacharrides during cold stress and**
3 **recovery in cucumber leaves**

4

5 **Short running title:** RFOs metabolism during cold stress and recovery

6

7 **Authors and affiliations:**

8 Man Lu(1406061710@qq.com), Zhiping Zhang (zhangzp@yzu.edu.cn), Jinjin Xu

9 (1140315372@qq.com), Wenhua Cao (853942204@qq.com), Minmin Miao

10 (mmmiao@yzu.edu.cn)

11 School of Horticulture and Plant Protection, Key Laboratory of Plant Functional

12 Genomics of Ministry of Education, Yangzhou University, 12 Wenhui East Road,

13 Yangzhou 225009, China

14

15 **Corresponding author:** Minmin Miao; **Telephone number:** 86-514-87979395

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30 **Metabolic process of raffinose family oligosacharrides during cold stress and** 31 **recovery in cucumber leaves**

32

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

37 **Abstract**

38 Raffinose family oligosacharrides (RFOs) accumulate under stress conditions in many
39 plants and have been suggested to act as stress protectants. To elucidate the metabolic
40 process of RFOs under cold stress, levels of RFOs and relative carbohydrates, the
41 expression and activities of main metabolic enzymes and their subcellular
42 compartments were investigated during low temperature treatment and recovery
43 period in cucumber leaves. Cold stress induced the accumulation of stachyose in
44 vacuoles, galactinol in vacuoles and cytosols, and sucrose and raffinose in vacuoles,
45 cytosols and chloroplasts. After cold stress removal, levels of these sugars decreased
46 gradually in respective compartments. Among 4 galactinol synthase genes (*CsGS*),
47 *CsGS1* was not affected by the cold stress, while other three *CsGSs* were up-regulated
48 by the low temperature. RNA levels of *acid- α -galactosidase* (*GAL*) 3,
49 *alkaline- α -galactosidase* (*AGA*) 2 and 3, and the activities of GAL and AGA were
50 up-regulated after cold stress removal. The GAL3 protein and GAL activity were
51 exclusively located in the vacuole, whereas the protein of AGA2 and AGA 3 were
52 found in the cytosol and chloroplast respectively. The results indicate that RFOs
53 accumulated during the cold stress in different subcellular compartments in cucumber
54 leaves could be catabolized *in situ* by different galactosidases after stress removal.

55

56 **Key Words:** cold stress, cucumber, de-acclimation, galactosidase, raffinose family
57 oligosacharrides, subcellular compartment

58

59 **Introduction**

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

68 The important role of RFOs in the stress defence mechanism was also well
 69 established. RFOs are characterised as osmoprotectants or antioxidants, and may
 70 serve as signals in response to several abiotic or biotic stresses (Zuther *et al.*, 2004;
 71 ElSayed *et al.*, 2014; Sengupta *et al.*, 2015). In most sucrose-translocating plants, like
 72 rice (*Oryza sativa*) and Arabidopsis (*Arabidopsis thaliana*) that neither transport nor
 73 accumulate large quantities of RFOs in their tissues under normal conditions, the
 74 accumulation of RFOs and the induced expression of their biosynthetic enzymes,
 75 galactinol synthase (GS) and raffinose synthase (RS), were found in response to
 76 diverse abiotic stresses such as temperature extremes, drought and salinity (Nishizawa
 77 *et al.*, 2008; Saito and Yoshida, 2011; Gangl and Tenhaken, 2016).

78 Under stressed conditions, the increase of RFOs was also observed in
 79 RFOs-translocating plants. *Ajuga reptans*, a frost-hardy perennial labiate, accumulates
 80 much more RFOs in winter leaves than in summer leaves, and cold treatment would
 81 significantly increase the RFOs concentration in leaves (Bachmann *et al.*, 1994). In
 82 cucumber (*Cucumis sativus* L.), RS expression, RS activity and the content of
 83 raffinose and stachyose increased gradually in the leaves, fruits, stems and roots under
 84 low temperature stress (Meng *et al.*, 2008; Sui *et al.*, 2012). In these
 85 RFOs-translocating species, it seems that there are two pools of RFOs: a storage pool
 86 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,

117 3 acid α -galactosidase genes (*GAL*) and 3 alkaline α -galactosidase genes (*AGA*),
 118 according to their activity in response to pH (Wang *et al.*, 2016). *GAL*s are considered
 119 to be localized in the apoplast space or vacuole, while *AGAs* are supposed to be
 120 localized in the cytosol (Keller and Pharr, 1996; Tapernoux-Lüthi *et al.*, 2007).
 121 Considering both RFOs and α -galactosidases reveal multiple subcellular localizations,
 122 it is interesting to know if different α -galactosidases catabolize the RFOs in different
 123 subcellular compartments when stress conditions are relieved.

124 In this study, in order to reveal the metabolic process of RFOs during cold stress,
 125 levels of RFOs and relative carbohydrates, the expression and activities of metabolic
 126 enzymes and their subcellular compartments were investigated during cold treatment
 127 and the recovery period in cucumber leaves. We emphasized the observation of the
 128 expression pattern, activity and intracellular localization of 6 α -galactosidases to
 129 elucidate the catabolic process of RFOs after stress removal.

130

131 **Materials and methods**

132 *Plant material and temperature treatment*

133 Cucumber (*Cucumis sativus* L.) cultivar Jinchun 5 (from Tianjin Cucumber Institute,
 134 China) was used in this study. Seedlings were grown in 10×10 cm plastic pots
 135 containing a peat-vermiculite mixture (2:1, v/v) in a growth chamber. The seedlings
 136 were thinned to one per pot 10 d after germination. Plants were watered once daily
 137 and fertilized weekly with the Hoagland nutrient solution. In the growth chamber, the
 138 temperatures were 28°C/22°C (day/night) and the relative humidity 70%. Light was
 139 provided by high-pressure mercury lamps (Philip HPLN 400 W) at about 700 μmol
 140 $\text{m}^{-2} \text{s}^{-1}$ for 12 h perday (7:00-19:00). Plants for cold treatment were transferred to
 141 another chamber in which the temperature was lowered to 15°C/8°C at the 4-leaf
 142 stage. After 3-day chilling treatment, the temperature in the chamber was restored to
 143 28°C/22°C. Control plants remained in the original chamber throughout the
 144 experiment. The second leaves from the apical meristem of each plant were collected
 145 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 .

148

149 *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 ($40\text{ }\mu\text{m}$). The sample was centrifuged for 10 min at $3,200 \times g$ 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 $5,000 \times g$ 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).

164

165 *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 C_7H_{16} and lyophilized. The dried samples were extracted by Hepes buffer (50 mM Hepes-NaOH pH 7.4; 5 mM MgCl_2 ; 1mM EDTA; 1 mM EGTA; 0.1 % Triton X-100; 10% glycerol; 2mM benzamidine; 2mM aminocaproic acid; 1.5mM PMSF; 1 g 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

176 7.0), 1 mM DTT, 10 mM galactinol and 40 mM sucrose. Mixtures were incubated at
177 30 °C for 3 h and the reactions were stopped by boiling for 5 min. The mixture was
178 centrifuged at 28,000×g for 5 min and the supernatant was passed through a 0.45 μm
179 filter. The content of raffinose was determined by HPLC. Enzyme activity is given as
180 μmol of raffinose formation per hour (Sui *et al.*, 2012). The assay of STS was the
181 same as that of RS, except galactinol was replaced by raffinose in the reaction system.

182

183 *Total RNA isolation and expression analysis of RS and STS*

184 Total RNA was extracted from approximately 100 mg of the leaf tissues (without
185 middle ribs and larger veins) using TRIzol reagent (Invitrogen, Shanghai, China).
186 Reverse transcription was performed using a Prime Script™ RT reagent Kit with
187 gDNA eraser (Perfect Real Time, TaKaRa, Dalian, China). Quantitative real-time
188 PCR was performed using the One Step SYBR PrimeScript RT-PCR Kit (TaKaRa,
189 Dalian, China) on an ABI PRISM 7700 Sequence Detection System (Applied
190 Biosystems, Shanghai, China), following the manufacturer's instructions. The
191 real-time PCR was carried out according to the following protocol: 2 min at 94°C,
192 followed by 39 cycles of 94°C for 15 s, 60°C for 15 s and 72°C for 30 s. The
193 cucumber 18S rRNA gene (Gene bank accession No.: AF206894.1) was used for
194 normalization in all the analyses performed. The primer sequences for RS and STS
195 (Gene bank accession No.: EU096498) are listed in Supplementary Table S1. Primers
196 were confirmed to be approximately 90% to 100% efficient in amplification, and
197 $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001) was used for analyses.

198

199 *Northern blot analysis of the expression of GS and α-galactosidases genes*

200 Total RNA (20μg) was extracted as mentioned above and electrophoresed on a 1.2%
201 (w/v) agarose gel containing formaldehyde, then transferred onto positively charged
202 nylon membrane (Amersham-Pharmacia Biotech, Uppsala, Sweden) with 10×SSC
203 buffer (1×SSC containing 150 mM NaCl and 15 mM sodium citrate, pH 7.0). DNA
204 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.

210

211 *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'-ccggaattccatggagtcacaaagat-3' (*EcoR* I restriction site added) and 5'-ataaggatccagtcaccccggtt-3' (*BamH* I restriction site added) from the vector pCambia1303. The enhanced GFP (EGFP) sequence was amplified with primers 5'-aatgtcgacatggtgagcaagggcgagg-3' (*Sal* I restriction site added) and 5'-gaatctgcagctgtacagctcgtccatg-3' (*Pst* I 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 *Apa* I and *EcoR* I were used for CaMV 35S promoter cloning, and *EcoR* I and *BamH* I 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

234 cultured on the 1/2 MS solid medium (30g l⁻¹ sucrose, 7g l⁻¹ agar, pH 5.8) for 2 d
 235 (25°C, 16h/8h). Plasmolysis cells were obtained by adding 300g l⁻¹ KNO₃ on the
 236 tissue for 5min before fluorescence microscope observation (Zeiss LSM710).
 237 Transient transformation of tobacco leaves was mainly according to Sparkes *et al.*
 238 (2006). Basically, the underside of the tobacco leaf was punctured with a small needle.
 239 Took up resuspended *Agrobacterium* infiltration liquid (10 mM MgCl₂, 10 mM MES,
 240 100μM acetosyringone) in a 1 ml syringe (no needle). Placed the tip of the syringe
 241 against the underside of the leaf over the needle mark and pressed down gently on the
 242 plunger. Placed plants in a growth cabinet under normal conditions for 3d, and then
 243 excised 1-2 cm² segments of leaf tissue for confocal microscope observation (Zeiss
 244 LSM710).

245 **Results**

246 *Subcellular distribution of sugars and relative enzymes in cold-stressed cucumber* 247 *leaves*

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

258 *Suggested position of Table1*

259 *Change of sugar levels during cold treatment and recovery*

260 According to the subcellular distribution of sugars (Table 1), levels of galactinol,
 261 sucrose, raffinose, stachyose and galactose in vacuole, sucrose and raffinose in
 262 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).

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

314

315 *Subcellular localization of galactosidase proteins*

316 To obtain further insight into the mechanism of RFOs catabolism after cold stress
 317 removal, an EGFP protein was fused to the N-terminus of 6 galactosidases, and placed
 318 under the control of the CaMV 35S promoter. These constructs were transiently
 319 expressed in the onion epidermal cells. To distinguish the cell wall and the cytosol in
 320 these highly vacuolate cells, plasmolysis was carried out before transformation.
 321 Fluorescence microscope imaging showed that *GAL1* and *GAL2* were located near
 322 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 tetrasaccharide 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

352 further research.

353 GSs are always encoded by multiple genes in plant genomes. Evidences indicated that

354 these isoforms have different cellular locations and physiological functions. In

355 *Arabidopsis*, *AtGolS1* and *AtGolS2* were induced by drought and high-salinity

356 stresses, while *AtGolS3* was induced by cold stress (Taji *et al.*, 2002). In the

357 stachyose-translocating plant *Ajuga reptans*, *ArGolS1* expressed in the mesophyll for

358 storage RFOs synthesis and *ArGolS2* mainly in the intermediary cell for transport

359 RFOs synthesis (Sprenger and Keller, 2000). In the melon (*Cucumis melo*), Cucumber

360 mosaic virus and heat stress did not affect the expression level of *CmGolS1*, but

361 caused a significant increase in the relative expression level of *CmGolS2* (Gil *et al.*,

362 2012). In this study, in cucumber leaves *CsGS1* expressed constitutively and was not

363 affected by the cold stress, while the expressions of other three *CsGSs* were

364 up-regulated by the low temperature. Phylogenetic analysis based on the amino acid

365 sequences indicated that cucumber *CsGS1* and *CmGloS1* are closely related, while

366 cucumber *CsGS4* and *CmGloS2* cluster into one group (Supplementary Fig. S3). It is

367 not clear if the function of *CsGS1* and *CmGloS1* is similar to that of the *Ajuga reptans*

368 *ArGolS2* (for transport RFOs synthesis). *CsGS2* and *CsGS3* showed similar

369 expression pattern during the temperature treatment, *i.e.*, could not be detected under

370 normal temperature and induced by cold stress. In the phylogenetic tree, cucumber

371 *CsGS2* is closely related to the *SmGloS3*, which has a low constitutive expression and

372 could be induced by several abiotic stresses in *Salvia miltiorrhiza* (Wang *et al.*, 2012).

373 *CsGS3*, *AtGolS2* and *AtGolS3* belong to the same group (Supplementary Fig. S3). It

374 seemed that the function of GSs in abiotic stress is mainly determined by which

375 element exists in their promoter area, rather than the amino acid sequences (Taji *et al.*,

376 2002). The response patterns of 4 *CsGSs* to other abiotic stresses need further

377 investigation. Other two RFOs biosynthetic enzymes, RS and STS, revealed different

378 expression and activity patterns during temperature treatment, RS was up-regulated

379 by cold stress but STS not. The increased level of substrate raffinose, rather than the

380 biosynthetic enzymes STS, may result in the stachyose accumulation under low

381 temperature. In addition, the cytosol compartment of GS, RS and STS, the chloroplast

382 and vacuole localization of raffinose and the vacuole localization of stachyose, further
383 confirm that there are RFOs transporters on the tonoplast and chloroplast envelope in
384 RFOs-translocating plants (Greutert and Keller, 1993; Schneider and Keller, 2009;
385 Nägele and Heyer, 2013).

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

409 In conclusion, our results indicate that RFOs accumulated during cold stress in
410 different subcellular compartments in cucumber leaves could be catabolized *in situ* by
411 different galactosidases after stress removed. The data do not rule out the possibility

412 that RFOs are translocated to other subcellular compartments for degradation.

413

414 **Supplementary data**

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

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

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

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

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

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

425

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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 $\pm$ SE of three samples.

Carbohydrates/enzymes	Cytosol (%)	Chloroplast (%)	Vacuole (%)
Galactinol	53.2 $\pm$ 6.3	9.4 $\pm$ 4.2	38.4 $\pm$ 10.2
Sucrose	37.2 $\pm$ 17.2	40.2 $\pm$ 13.8	22.6 $\pm$ 8.4
Raffinose	42.1 $\pm$ 12.4	21.8 $\pm$ 9.6	36.1 $\pm$ 15.3
Stachyose	1.7 $\pm$ 1.3	2.1 $\pm$ 1.7	96.2 $\pm$ 3.6
Galactose	0.9 $\pm$ 1.4	0.8 $\pm$ 2.3	98.3 $\pm$ 4.3
Acid- α -Galactosidase	3.2 $\pm$ 0.8	1.3 $\pm$ 0.5	95.5 $\pm$ 7.2
Alkaline- α -Galactosidase	66.3 $\pm$ 8.3	27.4 $\pm$ 5.6	6.3 $\pm$ 3.2
Galactinol synthase	100 $\pm$ 0.0	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0
Raffinose synthase	100 $\pm$ 0.0	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0
Stachyose synthase	99.1 $\pm$ 2.6	0.0 $\pm$ 0.0	0.9 $\pm$ 1.2

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 $\pm$ 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 $\pm$ 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 μ m.

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, 633nm), and the right column (A3, B3 and C3) shows merged images. Bars=10 μ m.

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