Journal of Plant Physiology 169 (2012) 965–970

Contents lists available at SciVerse ScienceDirect

Journal of Plant Physiology

journal homepage: www.elsevier.de/jplph

Effect of CMV infection and high temperatures on the enzymes involved in raffinose family oligosaccharide biosynthesis in melon plants

Lidor Gil[a], Julius Ben-Ari[b], Robert Turgeon[c], Shmuel Wolf[a,*]

[a] The Robert H. Smith Institute of Plant Sciences and Genetics in Agriculture and the Otto Warburg Minerva Center for Agricultural Biotechnology, The Hebrew University of Jerusalem, The Robert H. Smith Faculty of Agriculture, Food and Environment, Rehovot 76100, Israel
[b] Interdepartmental Analytical Unit, The Hebrew University of Jerusalem, The Robert H. Smith Faculty of Agriculture, Food and Environment, Rehovot 76100, Israel
[c] Department of Plant Biology, Cornell University, Ithaca, NY 14853, USA

ARTICLE INFO

Article history:
Received 14 December 2011
Received in revised form 30 January 2012
Accepted 21 February 2012

Keywords:
Cucumber mosaic virus
Cucumis melo
Galactinol synthase
Phloem loading
Raffinose synthase
Stachyose synthase

ABSTRACT

Ultrastructural and molecular studies have provided experimental evidence for the classification of cucurbits as symplastic loaders, mainly translocating the raffinose family oligosaccharides (RFOs) raffinose and stachyose. Earlier studies established that cucumber mosaic virus (CMV) infection causes a significant increase in the sucrose-to-RFO ratio in the phloem sap of melon plants. The alteration in phloem sap sugar composition was associated with upregulation of *CmSUT1* transcript within the vascular bundles.

The current research aimed to explore the effect of CMV infection on the enzymes involved in symplastic phloem loading and RFO biosynthesis. Viral infection did not affect the activity of either raffinose or stachyose synthases in source leaves, but caused upregulation of the respective transcripts. Interestingly, activity of galactinol synthase was higher in CMV-infected leaves, associated with upregulation of *CmGAS2*. A significant increase in *CmGAS2* expression in source leaves of melon plants exposed to high temperatures indicated that this response is common for both biotic and abiotic stresses. However, the effect of CMV or heat stress on phloem sap sugar composition is not due to alteration in RFO biosynthesis.

Introduction

The raffinose family oligosaccharides (RFOs) raffinose and stachyose are synthesized from sucrose by the sequential addition of galactose moieties donated by galactinol. Galactinol is formed from UDP-galactose and myo-inositol via the activity of galactinol synthase (GAS), which is considered to be a key regulator in the pathway. GAS has been found exclusively in plants and serves as the sole galactosyl donor for RFO biosynthesis (Keller and Pharr, 1996). Galactinol and sucrose generate raffinose and myo-inositol; galactinol and raffinose produce stachyose and myo-inositol. The production of the trisaccharide raffinose and tetrasaccharide stachyose is catalyzed by raffinose synthase (RafS) and stachyose synthase (StS), respectively (Bachmann et al., 1994).

Raffinose and stachyose are the major translocated sugars in various plants species (Turgeon, 1996). They serve as carbon storage in seeds and play a role in dessication tolerance (Obendorf, 1997). As non-reducing sugars, they constitute suitable storage material and are accumulated to relatively high levels during seed maturation in *Arabidopsis* (Taji et al., 2002; Sicher, 2011), soybean (Blackman et al., 1992), pea (Blöchl et al., 2007) and maize (Brenac et al., 1997).

Accumulation of RFOs has been demonstrated under various abiotic stresses, for example, in *Arabidopsis* plants subjected to low temperatures or drought stress (Taji et al., 2002), *Xerophyta viscosa* subjected to water deficit (Peters et al., 2007), and *Ajuga reptans* subjected to cold stress (Bachmann et al., 1994). Nevertheless, the molecular mechanism governing the action of these metabolites remains unclear. It has been suggested that they act as signals or as reactive oxygen species (ROS) scavengers when they accumulate to high concentrations in particular tissues (Valluru and den Ende, 2011). Nishizawa et al. (2008) demonstrated that galactinol and raffinose scavenge hydroxyl radicals in *Arabidopsis*, suggesting their role in protecting plant cells from oxidative damage. Further support for the role of galactinol and RFOs in the stress response was provided by the increased expression of the gene coding for GAS (*GolS1*) in *Ajuga reptans* leaves in response to chilling stress (Sprenger and Keller, 2000) and upregulation of *AtGolS1* in *Arabidopsis* in response to heat stress (Panikulangara et al., 2004).

The finding that *AtGolS1* is a target gene for heat-shock factors supported the notion that the encoded enzyme is indeed essential for heat-stress-dependent raffinose synthesis (Panikulangara et al., 2004). Interestingly, expression of a reporter gene coding for GUS under the *AtGolS1* promoter revealed that, under control

* Corresponding author. Fax: +972 8 9462385.
E-mail address: swolf@agri.huji.ac.il (S. Wolf).

0176-1617/$ – see front matter © 2012 Elsevier GmbH. All rights reserved.
http://dx.doi.org/10.1016/j.jplph.2012.02.019

conditions, the expressed gene is localized to minor veins. However, marked GUS expression was observed throughout the leaf tissues upon exposure of the plants to high temperatures (Panikulangara et al., 2004).

The Cucurbitaceae translocate significant quantities of RFOs in the phloem (Haritatos et al., 1996). These plants are characterized by numerous plasmodesmata interconnecting the specialized companion cells (intermediary cells) and the surrounding bundle sheath. According to the "polymer trap" model of symplastic loading (Turgeon, 1991), sucrose diffuses from the mesophyll into intermediary cells, where it is then converted to RFOs. These sugars are too large to leak back through the narrow plasmodesmata to the surrounding cells and therefore accumulate in the phloem (Haritatos et al., 1996).

We have previously reported that infection of melon plants with cucumber mosaic virus (CMV) dramatically increases the sucrose-to-RFO ratio in phloem sap collected from cut petioles or stems (Shalitin and Wolf, 2000). Based on this finding, we proposed that CMV infection initiates an apoplastic mode of sugar loading into the sieve tube of melon plants such that both symplastic and apoplastic mechanisms are operating in concert. Support for this idea was provided by analyses of *CmSUT1* expression in healthy and CMV-infected plants, revealing significant upregulation of *CmSUT1* in the vascular bundles of CMV-infected source leaves (Gil et al., 2011). The accumulated data suggest that melon plants are capable of loading sugars into the sieve tube via either the symplastic or apoplastic route. CMV infection induces expression of *CmSUT1* to load sucrose into the sieve tube from the apoplast, resulting in a higher sucrose-to-RFO ratio in the phloem sap of the infected plants.

In the present research, we examined the effect of CMV infection on the expression of transcripts and activity of enzymes involved in RFO biosynthesis. The goal of this study was to determine whether the increased sucrose-to-RFO ratio in the phloem sap of CMV-infected melon plants is due to reduced RFO biosynthesis, in addition to the increased sucrose loading into the sieve tube. The results indicated that CMV infection did not reduce the expression of the respective genes, nor did it cause a reduction in their activity. On the contrary, CMV infection resulted in increased GAS activity in source leaves and upregulation of *CmGAS2*. This phenomenon was also evident under abiotic stress, i.e. high temperature.

Materials and methods

Plant materials

Melon (*Cucumis melo* L. cv. Hale's Best Jumbo) plants were grown in a coconut mixture in 15-cm diameter plastic pots. Two-week-old seedlings were transferred to an insect-free temperature-controlled growth chamber with a temperature of 25–28 °C/18–20 °C (day/night, respectively), and infected with cucumber mosaic virus (CMV) (Fny strain) as described previously (Shalitin and Wolf, 2000).

RNA isolation and RT-PCR analysis

Total RNA was extracted from 50 mg leaves of same-aged CMV-infected and healthy plants, using Tri-reagent (Sigma, http://www.sigmaaldrich.com/) according to the manufacturer's protocol. RNA was quantified by RNA/DNA analyzer (GeneQuant II, Pharmacia Biotech, http://www.americanlaboratorytrading.com/). cDNA was prepared from the same amount of RNA (2 μg) per sample pretreated with 2 unit μg^{-1} of DNAse (Promega, http://www.promega.com/), using Verso™ cDNA synthesis kit (Thermo Scientific AB-1453, http://www.thermoscientific.com/).

Real-time RT-PCR was carried out using 0.5 μl of 2.5 pmol of each primer, 4 μl cDNA and 5 μl ABsolute™ Blue QPCR SYBR® Green ROX Mix. PCR conditions were 95 °C for 15 min (enzyme activation), and then the following cycle, repeated 45 times: 95 °C for 10 s, 59 °C for 15 s and 70 °C for 25 s. The obtained cycle temperature (CT) values were analyzed with Rotor-Gene 6000 Series software by averaging the two independently calculated normalized expression values of the duplicates. The calculated numerical values were divided by the values obtained for the housekeeping gene *tubulin* in each respective sample. The primers used in this study were (5′ → 3′):

CmGAS1_F610 TGCAAGGCCACTACCCAACTTT
CmGAS1_R940 ACAGAACAGAGATCAAAGGTGTGAG
CmGAS2_F597 TCTACGTGTAACGGCACCAA
CmGAS2_R812 ATGTTTGCTTCTTGCCCTGT
CmRafS_F22 CATTTTGGCTCCAAGGATGT
CmRafS_R200 ATGCTTCCCCACAGAATCAC
CmStS_F202 ATTATCCACACGACCCCAAA
CmStS_R449 CATTTCTTCGGTGACCCACT
Tub_F262 CCGCAGACAAGCGTTCCAAAA
Tub_R381 GACCGAGTGCGAAAATTAGCC

Enzyme extraction and galactinol synthase (GAS), raffinose synthase (RafS) and stachyose synthase (StS) activity assays

Enzyme extraction was performed according to Peters et al. (2007) with some modifications: 250 mg fresh leaf material was ground in 500 μl chilled extraction buffer [50 mM HEPES/KOH pH 7.5, 2 mM $MnCl_2$, 2 mM $MgCl_2$, 1 mM Na–EDTA, 2% (w/v) polyvinylpyrrolidone (PVP K30), 2% (w/v) polyethyleneglycol (PEG 20,000), 0.1% (w/v) Triton X-100]. Prior to use, 40 mM DTT and one complete mini EDTA-free protease inhibitor tablet (Roche, http://www.roche.com/) were added to 10 ml extraction buffer. Samples were centrifuged at 12,000 × *g* (10 min, 4 °C). A 250-μl aliquot of supernatant was desalted by gel filtration at 1400 × *g* (2 min, 4 °C) through 5 ml Sephadex G-25 columns (fine, final bed volume of 3 ml). Columns were pre-equilibrated twice with 2 ml assay buffer (50 mM HEPES/KOH pH 7.5, 2 mM $MnCl_2$, 10 mM DTT).

Aliquots (20 μl) of desalted extract were assayed for GAS activity in a final volume of 40 μl assay buffer containing final concentrations of 100 mM myo-inositol and 10 mM UDP-gal, at 30 °C for 25 min. Desalted extract (20 μl) was assayed for RafS activity in a final volume of 40 μl assay buffer containing final concentrations of 20 mM galactinol and 150 mM sucrose, at 30 °C for 3 h. Similarly, 20 μl desalted extract was assayed for StS activity in a final volume of 40 μl assay buffer containing final concentrations of 20 mM galactinol and 150 mM raffinose, at 30 °C for 3 h. The reactions were stopped by boiling in a water bath for 10 min. After addition of 100 μl DDW, the samples were stored at −80 °C. For LC–MS analysis, samples were thawed and centrifuged at 12,000 × *g* for 5 min. They were then diluted (1:15) in DDW and derivatized as described below. Protein concentration was determined by the method of Bradford with bovine serum albumin as the standard.

Sample preparation and derivatization for LC–MS analysis

Calibration solutions of carbohydrates were prepared in water at the following concentrations: 4000, 2000, 1000, 500, 200, 100, 25, 10, 5 ng ml^{-1}. Sorbitol was added as an internal standard to the calibration and biological samples at a final concentration of 100 ng ml^{-1}. The samples (1 ml) were evaporated under dry nitrogen stream at 40 °C. Peracetylated carbohydrates were prepared with acetic anhydride using iodine as the catalyst (Kartha and Field, 1997). Briefly, 50 μl of iodine solution in acetic anhydride (2 mg ml^{-1}) was added to a dried sample followed by sonication