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Sucrose phloem unloading follows an apoplastic pathway with high sucrose synthase in *Actinidia* fruit

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ABSTRACT

Phloem unloading plays a pivotal role in photoassimilate partitioning and the accumulation of sugars in sink organs, e.g. fruit. Here, we investigated the pathway of sucrose unloading in kiwifruit (*Actinidia deliciasa* cv. Qinmei) using a combination of electron microscopy, transport of the phloem-mobile symplastic tracer carboxyfluorescein and enzyme activity and gene expression assays. Our structural investigation revealed that the sieve element-companion cell complex of bundles feeding the fruit flesh was symplastically isolated from its surrounding parenchyma cells throughout fruit development, whereas numerous plasmodesmata were present between the phloem parenchyma cells. Consistent with this, carboxyfluorescein unloading showed that the dye remained confined in the phloem strands during fruit development. The activities and expression of cell wall acid invertase in fruit flesh were lower than those of other enzymes that catalyze sucrose dissociation. However, sucrose synthase showed higher enzyme activities and mRNA expression in fruit flesh compared with other detected enzymes. These results imply that, in kiwifruit flesh, phloem unloading of sucrose is predominantly an apoplastic pathway during fruit development, and that sucrose synthase is a key enzyme for sucrose post-unloading pathways.

1. Introduction

In the majority of plants, sucrose (Suc) is produced in, and translocated from, photosynthetically active leaves (source, loading) to support non-photosynthetic tissues (sinks, unloading), such as developing seeds, fruits, and tubers. Phloem unloading includes transfer across the sieve element-companion cell (SE/CC) complex boundary (SE unloading) and subsequent transport through a diverse range of sink parenchyma cells (PCs) (post-phloem transport) [1,2]. It is now well accepted that phloem unloading plays an important role in the partitioning of photoassimilates, thereby determining to a large extent crop output and quality [2]. Moreover, carbohydrate accumulation in the fruit relies upon the phloem network to transport sugars from the source to sink organs. Phloem unloading is generally considered to play a key role in the partitioning of photoassimilate [2–4]. For providing neoteric methods to improve fruit quality and increase food security, it is critical to understand the pathway of Suc transfer from the phloem to the fruit. Suc in the phloem moving into the fruit is regarded as the transfer of Suc from SE-CC complexes to sites of utilization/storage in the recipient sink cells [1,2]. There are two routes of Suc unloading: the symplastic pathway and the apoplastic pathway. Sucrose unloading can utilize the symplastic pathway, the apoplastic pathway, or both. If Suc unloads via the symplastic pathway, it moves from the SE-CC complex directly to the surrounding PCs via plasmodesmata, driven by a concentration gradient. However, if Suc unloads via the apoplastic pathway, it is transported directly from the SE-CC complexes into the apoplastic space between cells independently of plasmodesmata. In the apoplastic space, Suc can be transported by sucrose transporters (SUCs or SUTs) into the PCs, meanwhile Suc also can be converted into fructose (Fru) and glucose (Glc) by cell wall invertases (CWINVs), and then be assimilated by hexose transporters (HTs) into the PCs. Different plants can utilize distinct mechanisms to transport Suc from the phloem sieve tubes to PCs in fruit [6]. For example, apple and pear fruit use an apoplastic phloem unloading pathway over the course of fruit development [7,8]. In contrast, for grape berries and tomatoes, Suc unloading shifts from a symplastic to an apoplastic pathway [9,10].

Irrespective of the pathway, Suc in the sink PCs is transferred into vacuoles for storage or enzymatically degraded into Fru and Glc/UDP-Glc to support the growth of the sinks, which affects the ability of Suc post-unloading. In the PCs, Suc imported into the cytoplasm must rapidly be cleaved to decrease the chemical

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gradients in the sink cells, thereby promoting unloading. Hence, sucrose-cleaving enzymes are important factors for determining sink strength of fruit. Two different types of enzymes are responsible for such cleavage: sucrose synthase (EC 2.4.1.13) (SUSY) and invertases (EC 3.2.1.26). SUSY catalyzes the reversible conversion of Suc to Fru and UDP-Glc. The activity of neutral invertase (NINV) is much lower than that of SUSY in apple [11] and kiwifruit (*Actinidia deliciosa* var. Hayward) [12], suggesting that SUSY is the key enzyme catalyzing the progress of Suc degradation in fruit. It has been reported that the activity of SUSY is much more important than that of other enzymes (e.g. invertase) in many biological process, e.g. sugar unloading [13], cellulose and callose synthesis [14,15] and sink strength [16]. Therefore, it is important to understand the activity and expression pattern of SUSY during fruit development.

Kiwifruit is an emerging commodity in international markets due to its unique flavor and nutritional value (e.g., high content of vitamin C, amino acids and minerals). As the health benefits of kiwifruit become better appreciated by consumers, demand is on the rise for cultivars with good size, texture, flavor and nutritional value. All of these characteristics are related to the ability of carbohydrate unloading in fruit. Although Suc, *myo*-inositol and planteose are the major soluble carbohydrates in the leaves of kiwi [17], Suc is the main accumulated photosynthate produced by the crowns in kiwifruit [18]. During kiwifruit development, there are three stages characterized by the dominating metabolism: (1) cell division, (2) starch accumulation and (3) fruit maturation [19]. These stages have been studied in terms of non-structural carbohydrate content [20,21]. Carbohydrate metabolism during development, ripening and the post-harvest period has been described in detail, as have the dynamics of the related enzyme activities [12,22,23], but the unloading pathway of imported carbon and sink strength have received limited attention.

In this work, we conducted a study of the unloading of carbohydrate that is imported from the source leaves into kiwifruit. Our objective was to determine the unloading pathway during kiwifruit development and to identify the activities, and expression pattern of the enzymes responsible for metabolizing the imported Suc.

2. Material and methods

2.1. Plant materials

Nine-year-old *Actinidia deliciosa* var. 'Qinmei' kiwi vines were used in this study. The vines were grown under natural conditions in an experimental orchard at the Horticultural Experimental Station of Northwest A&F University, Yangling, Shaanxi, China. At 44 (early developmental stage), 75 (middle developmental stage) and 135 (late developmental stage) days after blooming (DAB), five independent fruit samples were obtained from the south side of the tree canopy between 4:00 PM and 6:00 PM under full sun exposure; each replicate contained eight fruit harvested from four trees. The samples were weighed immediately, cut into small pieces, and frozen in liquid nitrogen. To compare the expression patterns of relevant genes in the source and sink tissues, mature leaves and shoot tips were obtained at 44 DAB from the vines. All of the frozen samples noted above were stored at −80 °C until use.

2.2. Tissue preparation for ultrastructural observation

The method of tissue preparation for ultrastructural observation was adapted from Zhang et al. [7] with some minor changes. Fruit were harvested from the south side of the trees. The fruit was cut transversely and the vascular bundle zone was cut into small columns (approximately $1 \times 2 \times 3\ mm^3$), which were fixed immediately with 4% (v/v) glutaraldehyde in 100 mM precooled phosphate buffer (pH 7.0). The samples were subjected to a vacuum for 0.5 h and then incubated at 4 °C for at least 6 h. After an extensive rinse with precooled phosphate buffer (pH 7.0), the samples were postfixed in 1% (w/v) OsO_4 for 2–4 h at room temperature. Following another extensive rinse with the precooled phosphate buffer (pH 7.0), the samples were dehydrated using a graded ethanol series (30–100%) at room temperature. The details of the process were as follows: 30% ethanol for 10 min, 50% ethanol for 10 min, 70% ethanol for 10 min, 80% ethanol for 10 min, 90% ethanol for 10 min and 100% ethanol for 20 min. Next, the samples were infiltrated with a graded LR White resin at room temperature. The details of the process was as follows: 3:1 ethanol and LR White resin for 3 h, 1:1 ethanol and LR White resin for 6 h, 1:3 ethanol and LR White resin for 12 h, and 100% LR White resin for 24 h. Then, the tubes containing the samples were placed in capsules and incubated at 55 °C for 2–3 days. The capsules were placed in a dessicator until use. Ultrathin sections (approximately 60–80 nm in thickness) were obtained using an Ultracut microtome (RMC, USA) and mounted on 100-mesh copper grids for ultrastructural observation. At least five observations were made for each ultrathin section. Plasmodesmata were counted at all cell interfaces, including the interfaces between SE and CC, SE and PP, CC and PP, and PP and PP in each selected field. The results of the plasmodesmal counting were given as the number of plasmodesmata per micron of specific cell–cell interface length on transversal section, which is referred to as plasmodesmal density (no. of plasmodesmata μm^{-1}).

2.3. Carboxyfluorescein diacetate labeling

The method of membrane-permeable 5(6)carboxyfluorescein diacetate (CFDA) loading into the fruit through the pedicel was adapted from Zhang et al. [7] with some modifications. First, the CFDA was dissolved in DMSO (50 mg mL^{-1}) as the master stock and stored at −20 °C in a light-resistant container until use. For a working solution, it was diluted to 1 mg mL^{-1} with 0.1 M phosphate buffer (pH 7.0). Approximately 100 μl of CFDA solution was injected into the cotton lines around the outer phloem region of the pedicel, which was gently scratched with a saw without wounding the xylem, and then silver paper was used to surround the cotton. After allowing the plant to transport the dye for 72 h, the fruit was removed and immediately taken into the lab at 4 °C for sectioning and microscopy. Free hand sections, including transverse and longitudinal sections, were obtained; the microscopic examinations were conducted using a fluorescence microscope (Olympus Corporation, Tokyo, Japan) under a blue light (488 nm). Eight fruit were examined on each experimental date.

2.4. Measurements of soluble sugars

Soluble sugars were obtained and derivatized as described by Wang et al. [24]. Briefly, the samples (0.1 g) were extracted in 1.4 ml of 75% methanol, with ribitol added as the internal standard. After the non-polar metabolites were fractionated into chloroform, 5 μl of the polar phase was transferred into 2.0 ml Eppendorf vials to measure the metabolites (Suc, Fru, Glc, and *myo*-inositol) in each sample. These were dried under vacuum without heating and then derivatized sequentially with methoxamine hydrochloride and N-methyl-N-trimethylsilyl-trifluoroacetamide [25]. The metabolites were analyzed using a Shimadzu GC/MS-2010SE (Shimadzu Corporation, Tokyo, Japan). These metabolites were identified by comparing their fragmentation patterns with those from a mass spectral library generated on our GC/MS system, and from an annotated quadrupole GC/MS spectral library downloaded from the Golm Metabolome Database (http://csbdb.mpimp-golm. mpg.de/csbdb/gmd/msri/gmd_msri.html). Quantifi-