Research article

Developmental patterns of enzyme activity, gene expression, and sugar content in sucrose metabolism of two broomrape species

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A R T I C L E   I N F O

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Reducing sugars
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A B S T R A C T

A better understanding of broomrape physiological features opens up new perspectives for developing specific management strategies. For this purpose, activities of key enzymes involved in osmoregulation (SAI1, CWI, M6PR, and SUS1) were considered at developmental stages of two important broomrape species (Egyptian and branched broomrape) on tomato. While Egyptian broomrape tubercles had high activities of invertases, branched broomrape shoots revealed high activities of M6PR and SUS1 during both pre- and post-emergence stages except for M6PR at post-emergence stages of P. aegyptiaca. Interestingly, the main accumulation of total reducing sugars was detected in tubercle during pre- and in shoot during post-emergence. Unlike low levels of genes expression (except for CWI) before parasite emergence, significantly higher expression levels of SAI1, SUS1 and M6PR were detected after parasite emergence. Matching the expression levels of SAI1 and SUS1 genes with their corresponding enzymes activities makes them as the suitable candidates for gene silencing strategies.

1. Introduction

Broomrapes (Phelipanche spp. Syn. Orobanche spp.) are achlorophyllous root-parasitic plants damaging many economically important crops in the Mediterranean, central and eastern Europe, and Asia (Parker, 2009). The crops attacked by Phelipanche are mainly from the Compositae, Solanaceae, Fabaceae, Umbelliferae, Cruciferae, Cucurbitaceae, Labiatae, Rosaceae, ASTRACEAE, Chenopodiaceae plant families (Abang et al., 2007). The Egyptian (P. aegyptiaca) and branched (P. ramosa) broomrape, which are two important broomrape species, distributed similarly in Europe and the Middle East. P. aegyptiaca has lower dispersal to other continents than P. ramosa. The host range of P. aegyptiaca and P. ramosa are very similar to each other, but the occurrence of P. aegyptiaca may be more frequent than P. ramosa on cucurbit crops (https://www.cabi.org/isc/datasheet/37742, 1 April 2018). Tomato (Solanum lycopersicum), which is an important crop with high economic value, is damaged by broomrape and faces 50%-100% crop loss every year (Parker and Riches, 1993), especially with two above-mentioned broomrape species. Selective control of this weed is extremely difficult since the weed strictly attaches to the host root, concealed for most of life cycle with an underground growth, has achlorophyllous nature, and produces a significant number of seeds that may remain viable in the soil for more than 15 years (Fernández-Aparicio et al., 2016). These issues make this parasitic plant a challenge in the weed management (Eizenberg et al., 2012) and further studies are necessary to understand the physiological features of this root-parasitic plant for developing new specific control strategies such as gene silencing (Aly et al., 2009).

Since both transpiration rates and water flux in xylem elements of broomrape are low (Hibberd et al., 1999), osmotic adjustment through sucrose degradation is an essential process in water movement in the host plant and in the growth of parasite (Abbes et al., 2009a). Several studies (Delavault et al., 2002; Draie et al., 2011; Péron et al., 2012) suggested that osmotic strength of the parasite might depend on the activity of sucrose-splitting enzymes. Catalysis of sucrose cleavage is done by invertases (EC 3.2.1.26), yielding glucose and fructose, and by sucrose synthases (SUSs), yielding fructose and UDP-glucose (EC 2.4.1.13) (Draie et al., 2011). The study of Draie et al. (2011) on P. ramosa revealed that soluble acid invertase (SAI1) activity and PrSal1 transcripts, among the five invertases, are dominant in growing organs and affect cell turgor and growth during the parasite’s life cycle. Also, they showed that cell wall invertase (CWI) activity played an important role in sink strength of the infected roots during the host root penetration (Draie et al., 2011). According to the study of Péron et al. (2012) on the role of a P. ramosa sucrose synthase encoding gene (PrSus1), once the parasite connects to the host (tomato) vascular system, its transcripts accumulate at their highest level. They emphasized on the role of PrSus1 in utilizing the host-derived sucrose in meristematic areas.

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and in cellulose biosynthesis in discriminating vascular elements (Péron et al., 2012). Also, high level of mannitol is the other factor increasing the osmotic pressure in broomrape (Aly et al., 2009). Studying the changes in mannose 6-phosphate reductase (M6PR), the key enzyme of mannitol production, activity and M6PR gene expression in *P. ramosa* growing on tomato roots showed that the elevation of mannitol accumulation is high due to M6PR activity in the parasite attached to root (Delavault et al., 2002). However, there is no study on these above-mentioned enzymes in *P. aegyptiaca*.

Various studies reported different osmotic pressure for various broomrape species (*P. sulphurea*, *P. aegyptiaca*) on the same host (tomato or tobacco) (Kokina, 1946), the given broomrape species (*P. aegyptiaca*) on different hosts (*Nicotiana tabacum*, *Petunia hybrida*, and *Solanum melongena*) (Singh et al., 1968), and different host genotypes (*P. foetida*) on susceptible and tolerant faba bean lines (Abbes et al., 2011b). There is no simultaneous comparison of the gene expression described by Labrousse et al. (2001) and Draie et al. (2011), *P. aegyptiaca* and *P. ramosa* and compared them at different organs during developmental stages as described by Labrousse et al. (2001) and Draie et al. (2011), respectively. Conditioned seeds and plants’ organs were immediately frozen in liquid nitrogen and stored at −80°C prior to RNA and enzyme extraction.

2.2. Enzymes extraction

Invertases (SAI and CWI) extractions were conducted as described by Draie et al. (2011), M6PR extraction was done according to Delavault et al. (2002) and the SUS extraction was established as described by Yang et al. (2013). The enzymes extracts were desalted on a PD-10 Sephadex column prior to assay (GE Healthcare, Sigma). An aliquot of every desalted crude extract was taken for protein determination (Bradford, 1976). All assays were carried out in triplicate. Substrates were omitted from control reactions.

2.3. Enzymes assay

Invertases and M6PR assays were done as Draie et al. (2011) and Robert et al. (1999), respectively. Briefly, the produced NADPH in glucose conversion, as a result of invertases activity, and the produced NADP due to NADPH-dependent M6PR activity were determined with a plate reader (340 nm) in UV-transmissive flat bottom 96-well plates (UV-Star; Greiner Bio One, Kremsmünster, Austria). Standard curves

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### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>SAI</td>
<td>Soluble acid invertase</td>
</tr>
<tr>
<td>CWI</td>
<td>Cell wall invertase</td>
</tr>
<tr>
<td>SUS1</td>
<td>Sucrose synthase</td>
</tr>
<tr>
<td>M6PR</td>
<td>Mannose 6-phosphate reductase</td>
</tr>
<tr>
<td>TubIII</td>
<td>Growing tubercle</td>
</tr>
<tr>
<td>Tub-SIV</td>
<td>Tubercle bearing the growing subterranean shoot</td>
</tr>
<tr>
<td>SIV</td>
<td>Subterranean shoot</td>
</tr>
<tr>
<td>Tub-FS</td>
<td>Tubercle bearing the emerged flowering shoots</td>
</tr>
<tr>
<td>AP</td>
<td>Apical part of the flowering shoots</td>
</tr>
<tr>
<td>BP</td>
<td>Basal part of the flowering shoots</td>
</tr>
<tr>
<td>FCDS</td>
<td>Flowering shoot bears fruits containing developing seeds</td>
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</tbody>
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Fig. 1. Different organs during developmental stages in *P. aegyptiaca* parasitizing tomato plants: (a) growing tubercle (TubIII); (b) tubercle (Tub-SIV) bearing the growing subterranean shoot (SIV); (c) tubercle (Tub-FS) bearing the emerged flowering shoot. Apical part (AP) and basal part (BP) of the flowering shoot is growing; flowering shoot bears fruits containing developing seeds (FCDS). Bar, 1 cm.
were created by first preparing a set of standard solutions with known concentrations of the NADPH and NADP solutions, respectively. Invertases and M6PR activities were calculated from the amounts of NADPH and NADP (nmol) produced per second, respectively, and expressed in terms of nkat g\(^{-1}\) FW. SUS1 assay was conducted as Yang et al. (2013) and the produced reducing sugars were measured using the 3,5-dinitrosalicylic acid-based (DNS) method according to Miller (1959) and were detected at 540 nm by the plate reader. Glucose solutions with the concentration ranging from 0.1 μg ml\(^{-1}\) to 20 mg ml\(^{-1}\) were used to create standard curve.

2.4. Gene expression levels of key enzymes in sucrose metabolism of broomrape

Frozen tissues (0.1 g) were grounded into fine powder in liquid nitrogen and total RNA was extracted using a total RNA isolation kit (DENAZist Asia, Mashhad, Iran). Extracts were treated with DNase I to eliminate residual genomic DNA. The integrity of total RNA was determined by electrophoresis on 1.5% (w/v) agarose gel. Using oligo dT20 as a primer, the cDNAs were synthesized by PrimeScriptTM first-strand cDNA synthesis kit (Takara) based on manufacture’s instruction for 1 μg of total RNA. The qPCR was performed on a Rotor-Gene Q system (QIA-Gene). The cDNA was added to the reaction mixture (Eva probe qPCR Mix Plus (no ROX)) together with specific primers (final concentration of 0.2 pmol μL\(^{-1}\)) in 20 μL total volume. The gene-specific primers were designed using Primer Blast online software based on nucleotide sequences from *P. ramosa* at NCBI (http://www.ncbi.nlm.nih.gov/) with accession numbers GU997130, JN048797, GU997132, and AF055910 for SAI1, SUS1, CWI, and M6PR, respectively (Table 1) and confirmed with the PPGP website (http://ppgp.huck.psu.edu). There were three technical replicates for each cDNA and the qPCR reaction conditions were as follows: a 10-min activation at 95 °C, followed by 40 cycles of denaturation at 95 °C for 15 s, annealing at 58 °C for 20 s, and extension at 72 °C for 20 s. The cycle threshold (Ct) was set at 0.01. To ensure that only single products were generated, a melting curve was acquired for each primer at the end of each run. The relative gene expression levels in different tissues were calculated by comparative Ct method (Pfafl (2001)). The significance of differences in the gene expression was determined by t-test using the Rest 2009 Software (http://rest.gene-quantification.info). The reported values represent the average of three biological replicates. *P. ramosa* elongation factor 1-α gene (PrEF1α1) (Accession number of HM219554) was used as internal control (Draie et al., 2011; Péron et al., 2012) (Table 1) and at the different developmental stages test, the relative expression levels of target genes were compared with the TubIII as control sample.

2.5. Total reducing sugars determination

Total reducing sugars were measured using the DNS method according to Miller (1959) as previously detailed in the subsection of Enzyme assay.

2.6. Statistical analyses

Data were processed with Excel 2003 and given as means ± SE.

<table>
<thead>
<tr>
<th>Product (bp)</th>
<th>Reverse primer (5’-3’)</th>
<th>Forward primer (5’-3’)</th>
<th>Gene</th>
</tr>
</thead>
<tbody>
<tr>
<td>177</td>
<td>CITGGCAGGTTGCTCTTTTAG</td>
<td>TTAGTACCTTTGAGGGGAC</td>
<td>EF1</td>
</tr>
<tr>
<td>153</td>
<td>CGTCAATTTTGACTCCATATGAG</td>
<td>GACGTGCGCACTCAGGGCC</td>
<td>SUS1</td>
</tr>
<tr>
<td>170</td>
<td>TGCAATGAGGCGCCTGTAAG</td>
<td>TGCCCTACATAAAGCTTGAAG</td>
<td>CWI</td>
</tr>
<tr>
<td>155</td>
<td>GCACTACCTTCTTCTCTC</td>
<td>TGGTGAAGACTATGGGAGG</td>
<td>M6PR</td>
</tr>
<tr>
<td>180</td>
<td>AGTCATGCACTCCACATACCGT</td>
<td>GGACCGCGAGCGCGCTGG</td>
<td>SAI1</td>
</tr>
</tbody>
</table>
3.2. Development-related changes in enzymes activity

The activities of SAI1, CWI, SUS1, and M6PR enzymes at different organs during developmental stages of the broomrape species including *P. ramosa* and *P. aegyptiaca* have been shown and compared in Fig. 3. According to the figure, low activities of SAI1, CWI, SUS1, and M6PR were observed in the conditioned seeds of both species. For *P. aegyptiaca*, the highest activities of SAI1, CWI, M6PR, and SUS1 were observed in Tub-SIV, Tub-SIV, Tub-FS, and BP (and AP), respectively. In case of *P. ramosa*, the maximum activities of SAI1 and CWI were observed in Tub-FS (and FCDS) and Tub-SIV (and TubIII), respectively, and the peak values of M6PR and SUS1 activities were recorded in AP. While both broomrape species showed no significant variations in CWI activity at all organs during developmental stages except for Tub-SIV, they were significantly different in the other three enzymes activities. As shown in Fig. 3B, in both species, CWI activity was high in tubercles during pre-emergence growth stages (TubIII, Tub-SIV), significantly decreased in the emerged flowering spike, and then remained unchanged during the late stage of the parasite development. Tubercles (Tub-SIV and Tub-FS) showed significantly higher activities of invertases (SAI1 and CWI), except for CWI after emergence, than their corresponding shoots (SIV and AP, respectively) in both species. In contrast, the activities of M6PR (Fig. 3C) and SUS1 (Fig. 3D) were higher in shoots (SIV and AP) than those in tubercles (Tub-SIV and Tub-FS, respectively) in both broomrape species, except for M6PR activity during post-emergence of *P. aegyptiaca* from the soil.

The activities of the four above-mentioned enzymes were also affected by parasite species. While higher activities of M6PR and SUS1 (*p* < 0.001) were displayed in *P. ramosa*, *P. aegyptiaca* showed higher activities of CWI and SAI1 (*p* < 0.05) (statistical analyses not presented here). High activities of SAI1 and CWI during pre-emergence (Tub-SIV and SIV), and SUS1 and M6PR during post-emergence (Tub-FS and AP) were observed in Egyptian broomrape (except for M6PR activity in shoots). In branched broomrape, while activity of CWI was high during pre-emergence stages of parasite, high activities of the three other enzymes were detected during post-emergence stages (Fig. 3).

3.3. Development-related changes in gene expression levels

Changes in the relative expression levels of target genes at each organ during developmental stages compared to the stage TubIII, as the control sample, have been represented in Fig. 4. Like the enzymes activities, the expression levels of genes were low in conditioned seeds and then increased following parasite attachment to tomato roots for both broomrape species. Higher SAI1 expression was observed in shoots (SIV and AP) than that in tubercles (Tub-SIV and Tub-FS), except for branched broomrape (Fig. 4A). Similar to CWI activity, expression of CWI was higher in tubercles than that in shoots. Tub-FS and FCDS in Egyptian broomrape and FCDS in branched broomrape showed the highest CWI expression (Fig. 4B). Despite constant expression levels of M6PR and SUS1 before emergence of parasite from the soil, their expression levels significantly increased after emergence of parasite especially in shoots (Fig. 4C and D).

The expression levels of genes were not affected by parasite species, except for SUS1 in which gene expression was higher in branched broomrape compared to that in Egyptian broomrape (*p* < 0.001) (statistical analyses not presented here).

3.4. Relationships among total reducing sugars, enzyme activity, and gene expression

The correlation analyses among total reducing sugars, activities of SAI1, CWI, M6PR, and SUS1, and expression levels of SAI1, CWI, M6PR, and SUS1 for each broomrape species have been presented in Table 2. According to Table 2, significantly positive linear correlations between total reducing sugars and activities of M6PR (*r* = 0.43, *p* < 0.05), SAI1 (*r* = 0.43, *p* < 0.05), and SUS1 (*r* = 0.61, *p* < 0.01) were observed across different organs during developmental stages of branched broomrape. While there was no significant correlation between CWI expression (like CWI activity) and reducing sugars (*r* = −0.05), significantly positive correlation were detected between reducing sugars and expression levels of M6PR (*r* = 0.48, *p* < 0.05), SAI1 (*r* = 0.51, *p* < 0.05), and SUS1 (*r* = 0.52, *p* < 0.05) in this species. In Egyptian broomrape, reducing sugars had significant correlations with activity of SAI1 (*r* = 0.49, *p* < 0.05) and expression of SAI1 (*r* = 0.42, *p* < 0.05). We also considered the correlations between the enzymes and expression (like CWI activity) and reducing sugars (*r* = 0.49, *p* < 0.05), and expression levels of SAI1 (r = 0.42, p < 0.05). We also considered the correlations between the enzymes and expression (like CWI activity) and reducing sugars (*r* = 0.49, *p* < 0.05), and expression levels of SAI1 (r = 0.42, p < 0.05).
activities and the genes expression levels in both species. Significantly higher correlations were found between SAI1 expression and SAI1 activity \( (r = 0.79, p < 0.001) \) and between SUS1 expression and SUS1 activity \( (r = 0.48, p < 0.05) \) in branched broomrape and between SAI1 expression and SAI1 activity \( (r = 0.74, p < 0.01) \) in Egyptian broomrape. However, CWI and M6PR activities had no significant correlations with the corresponding genes expression levels (i.e. CWI and M6PR, respectively) in both broomrape species.

4. Discussion

Broomrape functions as a foreign supernumerary and dominant sink organ for the host plant by lowering its osmotic potential to a value much more negative than the host (Harloff and Wegmann, 1987) and increasing sink strength through strong accumulation of auxin, potassium, amino-acids, mannitol, hexoses, and starch (Abbes et al., 2009a; Delavault et al., 2002). It is demonstrated that SAI1 (Draie et al., 2011), CWI (Draie et al., 2011), SUS1 (Péron et al., 2012), and M6PR (Delavault et al., 2002) enzymes play important role in osmoregulation of branched broomrape, but no study has yet been done on Egyptian broomrape. We investigated whether and how different broomrape species and different organs during developmental stages affect activity of enzymes involved in broomrape sink strength. Total reducing sugar contents and transcription levels of genes, in addition to enzyme activity, were examined in this study. All enzyme activity, gene expression, and reducing sugar contents increased after attachment of both broomrape species to tomato roots (Figs. 2–4). This is an efficient developmental strategy after attachment which allows the rapid conversion of the host-derived sucrose into reducing sugars through several enzymes involved in this process (Delavault et al., 2002). The precise contribution of these enzymes may be depending on the way by which the sucrose enters the cell (symplastic transport through the plasmodesmata or apoplastic pathway) (Bowsher et al., 2008), or it may be due to energetic reason (Geigenberger, 2003).

Enzyme activity was affected by species and organs during developmental stages of parasite. While Egyptian broomrape tubercles showed high invertases activities (SAI1 and CWI), branched broomrape shoots had high activities of M6PR and SUS1 during both pre- and post-emergence stages except for M6PR at post-emergence stages of P. aegyptiaca (Fig. 3A–D). Sucrose accumulation in tubercles as the main sink tissues of the parasite probably induces high activities of invertases. The gene expression level was not affected by parasite species, except for SUS1. Unlike the enzyme activity, the gene expression level was not considerable before parasite emergence from the soil, except for CWI and SAI1. Consistent with CWI activity, significantly higher gene expression of CWI was observed in tubercles than shoots during both pre- and post-emergence stages of two broomrape species (except for CWI during post-emergence of P. ramosa). This result was inverse for the other three genes so that significantly higher expression levels of SAI1 (during both pre- and post-emergence of parasite from the soil), SUS1 and M6PR (during post-emergence) were detected in shoots than those in tubercles. The study of Harloff and Wegmann (1987) also showed that although synthesis of hexoses and mannitol occurred in tubercle from the host-derived sucrose, the main accumulation was in shoots, supporting the occurrence of a long-distance transport of these sugars in the parasite (Delavault et al., 2002). In this study, the main accumulation of reducing sugars in both species was detected in tubercle (Tub-SIV) during pre- and in shoot (AP) during post-emergence of both broomrape species. It seems that the accumulation of reducing sugars in
Tub-SIV is more related to higher SAI1 and CWI activities during subterranean growth, and its accumulation in AP is more related to higher activities of SUS1 and M6PR during aerial growth. Tub-FS in branched broomrape also showed high amounts of reducing sugars content. Similar results were observed on Egyptian broomrape at this stage, as reported by Nativ et al. (2017). They explained that the accumulation of reducing sugars in tubercles acts as a reservoir of carbohydrates for the upper organs after removing the upper shoot, and/or after reducing the sullies from the host (Nativ et al., 2017).

There were significant positive correlations between reducing sugars and the activities of SAI1, SUS1, and M6PR enzymes as well as the expression levels of SAI1, M6PR, and SUS1 genes across different organs during developmental stages of branched broomrape. It suggests that high amounts of reducing sugars accumulation in this species may depend mainly on the SAI1, SUS1, and M6PR activities than the CWI enzyme activity. Since more reducing sugar contents were detected in branched broomrape than Egyptian broomrape, and due to higher biomass and number of branched broomrape shoots per pot (data not shown) observed in this study, it seems that reducing sugar contents in a parasite species is an important factor for recognition of dominance among different parasite species on a given host. In Egyptian broomrape, in contrast, total reducing sugar contents showed significant correlations with activity of SAI1 (r = 0.49, p < 0.05) and expression level of SAI1 (r = 0.42, p < 0.05). This species also showed higher invertases (SAI1 and CWI) activities and earlier appearance in pots (data not shown) than those in branched broomrape. Considering that Abbès et al. (2009b) reported low broomrape growth after attachment to resistant host's lines in concordance with low invertase capacity, it seems that there is a possible positive relationship between invertases activities (especially SAI1) and earlier parasite appearance on the soil. However, this assumption requires further testing. Comparing two studied invertases, SAI1 activity was always higher than CWI activity in both broomrape species (Fig. 3A–B). This is probably because of lower Km value of the SAI compared to CWI for sucrose (Pan et al., 2005) that have also been reported in literature, e.g. Wakabayashi et al. (2015) on clover broomrape, Draie et al. (2011) on branched broomrape, Zhang et al. (2006) on grape bryony, and Nie et al. (2010) on Chinese jujube.

Higher invertases (SAI1 and CWI) activities during pre-emergence stages especially in Egyptian broomrape suggests that earlier targeting of these enzymes may result in parasite destruction before irretrievable damage to host plants. Draie et al. (2011) have already reported the early involvement of CWI enzyme during the branched broomrape penetration into the tomato roots. In addition, no significant difference in SAI1 expression in Egyptian broomrape between Tub-SIV and Tub-FS and between SIV and AP justifies early management of the parasite in this study (Fig. 4). The expression of this gene matched well with its activity in both broomrape species (Table 2). This result suggests that this gene regulation occurs at the transcriptional level, making it as the suitable candidate for gene silencing strategy. Besides, SUS1 expression matched well with its activity in branched broomrape. Yang et al. (2013) also highlighted the role of SUS and SUS in sucrose metabolism and their possible regulation at the transcriptional levels in the aril of Litchi chinensis. In contrast, there was no matching between CWI and M6PR activities with CWI and M6PR transcriptional patterns in both studied species. It may be due to either high stability/long half–lives of enzymes or regulation of their activity at the post-transcriptional level. Delavault et al. (2002) has also stated that M6PR expression is actually constitutive in P. ramosa and so, it is difficult to estimate the M6PR activity from mRNA expression levels. High activity of M6PR and its corresponding gene expression in both broomrape species especially in branched broomrape which were detected in this study are promising and interesting for controlling this destructive species (Figs. 3C–4C). Our results call for additional studies to evaluate the efficiency of new transgenic resistance in host plants based on either the production of proteinaceous inhibitors or the gene silencing technology against these genes in the parasite. The efficiency of gene silencing strategy in the other parasites based on the genetic modification of the host plant is also revealed (Aly et al., 2009; Alakonya et al., 2012; Bandaranayake et al., 2010; Tomilov et al., 2008). On the whole, this study provided evidence in sugar metabolic difference in two species that can be considered for management. To achieve a complete picture of the sink strategy in broomrape species, more studies are necessary to investigate the mechanisms of sucrose unloading and sucrose allocation among mannitol, hexoses, and starch in sink cells, and the involvement of other classes of sucrose-degrading enzymes.

5. Author contribution statement

HA and HA conceived and designed research. ZF conducted experiments and analyzed the data. ZF wrote the first version of the manuscript. All authors read and approved the manuscript.

Conflicts of interest

The authors declare that they have no competing interests.

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