In *Arabidopsis thaliana*, the invertase inhibitors AtC/VIF1 and 2 exhibit distinct target enzyme specificities and expression profiles

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Abstract Plant cell wall (CWI) and vacuolar invertases (VI) play important roles in carbohydrate metabolism, stress responses and sugar signaling. Addressing the regulation of invertase activities by inhibitor proteins (C/VIF, cell wall/vacuolar inhibitor of fructosidase), we have identified two C/VIFs from *Arabidopsis thaliana*. AtC/VIF1 showed specific inhibition of VI activity, whereas AtC/VIF2 inhibited both, CWI and VI. Expression analysis revealed that expression of AtC/VIF1 was restricted to specific organs, AtC/VIF2, however, was weakly expressed throughout plant development. Promoter::GUS transformants confirmed pronounced differences of tissue/cell type-specific expression between both isoforms. Growth of an AtC/VIF1 T-DNA KO mutant was unaffected, but VI activity and hexose content were slightly increased.

Keywords: Invertase inhibitor; Posttranslational regulation; Functional genomics; *Arabidopsis thaliana*

1. Introduction

Plants translocate carbohydrates from assimilating organs to sites of consumption such as storage organs and rapidly growing tissues. Most higher plants use sucrose as transport form. Within certain plant tissues like transport phloem or storage cells sucrose can be accumulated up to 20%, which is more than 500 mM. Plants possess two different classes of enzymes to break down sucrose for entry into metabolism: sucrose synthase (SuSy) and invertase. The first enzyme yields UDP-glucose and fructose in a reversible reaction in the cytosol, whereas invertases in the cytosol, the vacuole or the cell wall release glucose and fructose in an irreversible reaction. Both types of enzymes have been shown to be essential for plants, especially during growth, storage compound accumulation, and stress responses [1,2].

According to their pH optima, plant invertases can be divided into two categories: neutral and acid invertases. Acid invertases are of exceptional importance as they are the only enzymes able to cleave sucrose in extracellular compartments such as the vacuole (vacuolar invertase; VI) or the apoplastic space (cell wall invertase; CWI). Acid invertases are responsible for sucrose unloading from the conducting tissues and for the adjustment of the hexose/sucrose ratio, which affects plant development including programmed cell death [3]. Thus, the hexose/sucrose ratio acts as an important metabolic signal, which dramatically affects gene expression profiles [4–8]. Consequently, the expression patterns of CWI and VI have to be tightly controlled, both temporally and spatially. CWI and VI exist in small gene families, *Arabidopsis thaliana* having 6 putative CWI isoforms and 2 VI isoforms [9,10]. Induction of CWI or VI activities is mediated via increased transcription of the corresponding genes in response to a wide range of stress-related and developmental cues [11–13]. In contrast, rapid downregulation of invertase activity is not yet fully understood but appears to require mechanisms additional to transcriptional repression.

Given that CWI and VI show glycan decoration [14] and are therefore intrinsically very stable enzymes, silencing of CWI and VI activities depends on post-translational mechanisms. In particular, a well orchestrated downregulation of CWI and/or VI activities appears to be important during developmental transitions from high meristematic activity to differentiation and accumulation of storage compounds as observed during seed development and in the formation of vegetative storage organs. In these developmental processes, the hexose/sucrose ratio declines rapidly which correlates with an efficient silencing of CWI and/or VI activities [15,16]. The molecular mechanisms of this downregulation of CWI and VI activities are not yet fully understood but one specific mechanism operates via complex formation of CWI and VI with proteinaceous invertase inhibitors [17–19]. A recent study proposed a crucial role to an apoplastic invertase inhibitor in the control of leaf senescence [3].

Invertase inhibitors have been known for a long time [20,21], but have only recently been cloned [17] and characterized [22,23]. Functional genomics approaches revealed that invertase inhibitors and inhibitors of pectin methylesterase (PMEI) [24] belong to the same diverse protein family. Within this protein family, PMEIs are more abundant than invertase inhibitors. Moreover, invertase inhibitors and PMEIs show a significant homology with the pro-domains of type I pectin methylesterase. Therefore, the entire protein family is referred to as PMEI-related proteins (PMEI-RP). Here, we report that two PMEI-RP genes from *Arabidopsis thaliana* indeed encode invertase inhibitors with different target enzyme specificities. Furthermore, a detailed expression analysis is presented and implications for invertase regulation are discussed.

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2. Materials and methods

2.1. Plant material

Arabidopsis thaliana cv Wassilewskija plants were grown in a growth chamber in standard potting soil (9 cm pots) under short-day conditions (8 h light at 24 °C, 16 h dark at 18 °C, and approx. 50% humidity).

2.2. Construction of expression plasmids

The coding regions of AtC/VIF1 (at1g47960) and AtC/VIF2 (at5g64620) without the signal peptide (predicted by psort: http://psort.nibb.ac.jp/form.html) were amplified by polymerase chain reaction (PCR) from flower cDNA using the following primers (RE sites underlined): AtC/VIF1, sense 5′-GATAGCCATGGAAGGAAGTATAATAGAGCCAA-3′, antisense 5′-TATAAGCGGCCGCTAAAGCAACATTCTCACAAT-3′; AtC/VIF2, sense 5′-ATCGTAACCATGGGAGCATCAACCCTAATCT-3′, antisense 5′-TATATGCGGCCGCTATTCAACAAGGCGATCAA-3′. The amplification products were digested with *Nco*I/*Not*I and after gel purification (Kit from Qiagen, Hilden, Germany) ligated into the *Nco*I/*Not*I restricted pETM-20 vector (http://www.emblheidelberg.de/ExternalInfo/geerlof/draft_frames/flowchart/clo_vector/pETM/pETM-20.pdf). Expression from this vector produces 6 × His-tagged thioredoxinA-AtC/VIF fusion proteins with a TEV protease cleavage site to separate the fusion partners after purification.

2.3. Expression and purification of recombinant AtC/VIF1 and 2 proteins

The expression and purification of recombinant AtC/VIF1 and 2 proteins was performed following the protocol earlier reported for the invertase inhibitor NtCIF [22].

2.4. Acid invertase and PME enzyme assays

Partially purified acid invertase preparations were isolated from *A. thaliana* source leaves. The tissue was ground in liquid nitrogen and homogenized in 2 ml/g extraction buffer (30 mM MOPS, 250 mM sorbitol, 10 mM $MgCl_2$, 10 mM KCl, and 1 mM PMSF, pH 6). After centrifugation (10 min, 3500 × *g*), the pellet was washed once (10 min) with extraction buffer containing 1% Triton X-100, twice with extraction buffer, finally resuspended in 1 ml/g assay buffer (20 mM triethanol amine, 7 mM citric acid, and 1 mM PMSF, pH 4.6), and used for the determination of cell wall invertase activity. The supernatant was mixed with 1/9 volume ConA buffer (500 mM sodium acetate, 10 mM $CaCl_2$, 10 mM $MgCl_2$, 10 mM $MnCl_2$, and 1 mM PMSF, pH 6.3), 2 ml/100 g fresh weight concanavalin A (ConA)-sepharose (Sigma, Steinheim, Germany), and incubated for 1 h on ice under constant agitation. After centrifugation (5 min, 3000 × *g*) and washing with 10-fold diluted ConA buffer, the bound protein fraction was eluted with 5 volumes (of initially used ConA-sepharose) of elution buffer (10-fold diluted ConA buffer containing 10% (w/v) methyl α-D-glucopyranoside), and used for the determination of vacuolar invertase activity.

The acid invertase assay was performed by mixing 50 μl invertase preparation, 100 μl substrate (100 mM sucrose, in assay buffer), and assay buffer up to a volume of 300 μl. After a 1 h incubation at 37 °C, acid invertase activity was measured by enzymatic determination of released glucose in a coupled assay with hexokinase and glucose-6-phosphate dehydrogenase according to [25]. The PME assay was performed with commercially available PME from orange peel (Sigma) as previously described [24,26].

For inhibition studies, enzyme preparations were mixed with recombinant AtC/VIF proteins and coincubated in assay buffer without substrate for 30 min at 37 °C (invertase assay) or 25 °C (PME assay). Thereafter, substrate was added and enzyme activity determined. As a control, invertase or PME preparations were pre-incubated without inhibitory proteins for the same period of time before activity measurement.

2.5. Transcript estimation by real-time PCR

Total RNA was extracted from various tissues of *A. thaliana* WT plants using the RNeasy Plant Mini Kit (Qiagen) following the manufacturer's instructions. To eliminate residual genomic DNA present in the preparations, the samples were treated with RNase-free DNaseI (Promega, Mannheim, Germany) and subsequently the RNA was bound to RNeasy Spin columns (Qiagen) for purification. After elution with RNase-free water, 2 μg of RNA was transcribed into first strand cDNA using the Omniscript RT Kit (Qiagen) with an oligo dT primer.

Real-time PCR was performed using the Platinum Taq-DNA polymerase (Invitrogen, Karlsruhe, Germany) and SYBR-Green as fluorescent reporter in Biorad iCycler. Primers were designed against the coding region of AtC/VIF1 (sense 5′-TGGCCTCGCTCTCATCCTCATTG-3′; antisense 5′-GCTTCTATGGCTTCGGGAACATC-3′) and AtC/VIF2 (sense 5′-GTTGGTATGACAAACGCCACCTC-3′; antisense 5′-ATGGAGGCATAGTCATAAGCTTCAT-3′). Primers against actin were described previously [27]. A serial dilution of source leaf cDNA was used as standard curve to optimize amplification efficiency for AtC/VIF and actin primers. Each reaction was performed in triplicates, and specificity of amplification products was confirmed by melting curve and gel electrophoresis analysis. Relative expression levels of AtC/VIF1 and AtC/VIF2 were calculated and normalized with respect to Act2/8 mRNA according to the method in [28].

2.6. Generation of promoter::GUS plants

The promoter regions of AtC/VIF1 and AtC/VIF2 were amplified from genomic DNA with the following primers (RE sites underlined): AtC/VIF1, sense 5′-GGCGGCAAGCTTATTGAAAGTTTACTCGAA-3′, antisense 5′-AGTTCTCCCGGGCTTCTTTGATGATTATCT-3′; AtC/VIF2, sense 5′-AGCCTAAAGCTTTCTTCGAAGCATCCGATT-3′, antisense 5′-ATTATTCCCGGGTTCAGGAAGAAGGTTTTG-3′. The amplification products were digested with *Hin*dIII/*Sma*I and after gel purification ligated into the *Hin*dIII/*Sma*I restricted pGPTV-bar vector [29]. The resulting constructs consisted of the promoter in front of a beta-glucuronidase (uidA) gene. After mobilizing the constructs in *Agrobacterium tumefaciens*, *A. thaliana* cv Wassilewskija plants were transformed using the floral dip method [30]. Transformants were screened for resistance to the herbicide Basta™.

For analysis of GUS activity, tissue samples of T2 transformants were treated with GUS staining buffer (100 mM Na_2HPO_4/NaH_2PO_4), pH 7.0, 10 mM Na_2EDTA, 0.5 mM $K_3[Fe(CN)6]$, 0.5 mM $K_4[Fe(CN)_6]$, and 0.08% X-GlcA (Duchefa, Haarlem, The Netherlands) for 20 h at 37 °C. Green tissues were bleached with ethanol before examination.

2.7. Isolation of a T-DNA-tagged AtC/VIF1 KO mutant

Access to T-DNA-tagged *Arabidopsis* knockout lines was available through the Arabidopsis Knockout Facility at the University of Wisconsin [31]. The screening for mutants was performed as described at http://www.biotech.wisc.edu/Arabidopsis/.

3. Results and discussion

In a functional genomics approach to PMEI-RPs, we currently analyse the entire protein family in *Arabidopsis*. Recently, we have identified two of these genes as coding for inhibitors of PME [24]. Here, we identify two other genes from the same family as invertase inhibitors.

3.1. Heterologous expression of recombinant AtC/VIF1 and 2 proteins and in vitro proof of function

To express AtC/VIF1 and 2 as recombinant proteins, the open reading frames without the predicted N-terminal signal sequences were cloned into the pETM-20 vector. Expression in the *E. coli* strain Origami(DE3) yielded recombinant AtC/VIF1 and 2 as N-terminal thioredoxinA-AtC/VIF fusion proteins. From the fusion proteins, AtC/VIF1 and 2 were released by cleavage with TEV protease [32]. ThioredoxinA and TEV protease are both provided with his-tags, therefore pure AtC/VIF1 and 2 proteins were recovered in the flow-through of a Ni-affinity chromatography column (data not shown). The

E. coli strain Origami(DE3) was chosen because of its deficiencies in thioredoxin reductase and glutathione reductase activities, thus providing an oxidizing environment to facilitate disulfide bridge formation.

Purified AtC/VIF1 and 2 proteins were analyzed by SDS–PAGE using sample buffer with and without reductant. Under non-reducing conditions their mobility increased, indicating the presence of intramolecular disulfide bridges in the recombinant proteins (data not shown). The presence of disulfide bridges was previously observed for the recombinant tobacco invertase inhibitor NtCIF and for the pectin methylesterase inhibitors AtPMEI1 and 2 [24]. The recent crystallographic analysis of NtCIF has highlighted the importance of these disulfide bridges for structural stabilization of both the four-helix-bundle core of the inhibitor as well as an N-terminal α-hairpin module [23]. Thus, it was assumed that the AtC/VIF1 and 2 proteins were correctly folded, and their in vitro activities were determined with CWI and VI enzyme preparations (Figs. 1 and 2).

The analysis of in vitro activities of recombinant AtC/VIF1 and 2 clearly defined both proteins as inhibitors of invertases. Conversely, both proteins showed no activities against PME preparations which were completely inhibited by AtPME1 and 2 proteins, respectively (data not shown). Interestingly, AtC/VIF1 and 2 were clearly distinct in their target specificities. AtC/VIF1 appeared to be rather selective for VI with little activity against CWI (Fig. 1). However, AtC/VIF2 inhibited both enzymes (Fig. 2), but the affinity for VI was about 10-fold higher than for CWI. Based on these results, AtC/VIF1 is likely to operate as an inhibitor of VI also in vivo, whereas the situation is less clear for AtC/VIF2. Previous studies suggested that individual members of the PMEI-RP family show either PMEI activity or C/VIF activity, but never both (S. Wolf, S. Grsic-Rausch, S. Greiner and T. Rausch, unpublished results; [33]). This notion has now been extended to two C/VIF proteins from *Arabidopsis thaliana*. However, within the subgroup of C/VIF proteins, different inhibitor isoforms may in vitro exhibit either narrow or broad specificities. Thus, NtCIF, an experimentally confirmed cell wall isoform, inhibits in vitro

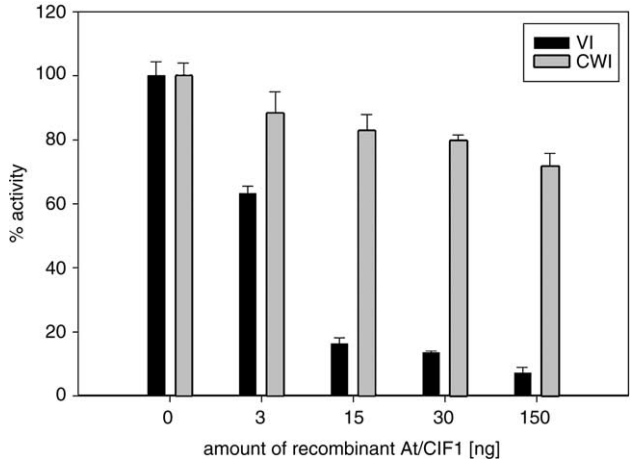

Fig. 1. Inhibitory effect of recombinant AtC/VIF1 protein on different invertase preparations. Dose-dependent effects of AtC/VIF1 protein on VI and CWI activities isolated from *Arabidopsis* leaves are shown. Target enzyme preparations were preincubated with inhibitor proteins for 30 min prior to enzyme assay.

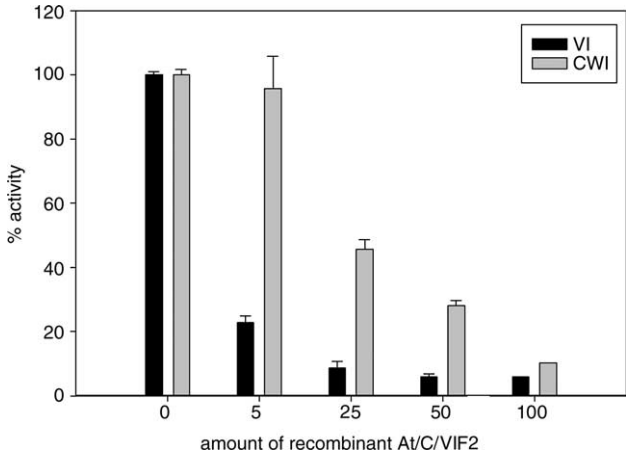

Fig. 2. Inhibitory effect of recombinant AtC/VIF2 protein on different invertase preparations. Dose-dependent effects of AtC/VIF2 protein on VI and CWI activities isolated from *Arabidopsis* leaves are shown. Target enzyme preparations were preincubated with inhibitor proteins for 30 min prior to enzyme assay.

CWI and VI, whereas recombinant NtVIF appears to be selective for VI. Conversely, an invertase inhibitor from sugar beet, for which the vacuolar targeting has been demonstrated, inhibited in vitro VI and CWI (Jan Eufinger, unpublished data). The structural basis for the target enzyme specificities of PMEI-RPs, i.e., PMEI versus C/VIF activity, is currently being investigated.

3.2. Expression analysis by real time PCR and promoter::GUS fusions

To compare the expression of *AtC/VIF1* and *2* mRNAs in different tissues, transcripts were quantitatively estimated by real time PCR, using *Actin2/8* for normalization (Fig. 3). The results revealed a low but consistent expression of *AtC/VIF2* in different plant organs, whereas *AtC/VIF1* showed an overall higher expression, with highest transcript levels in roots, senescent leaves and flowers. To explore the expression of *AtC/*

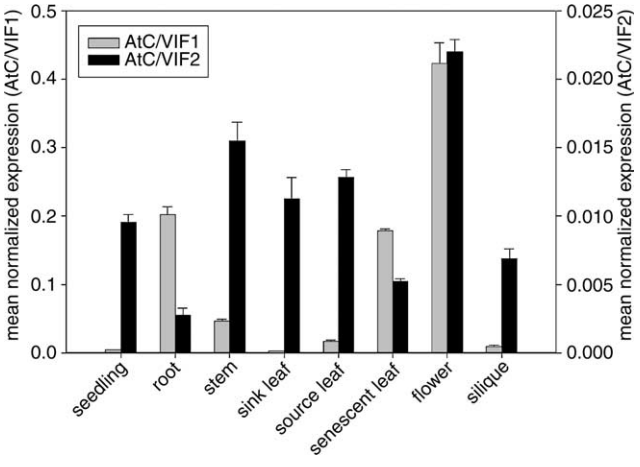

Fig. 3. Quantitative determination of AtC/VIF1 and 2 transcripts in different organs of *Arabidopsis thaliana* by real time PCR. Data for AtC/VIF1 and AtC/VIF2 are presented as relative expression normalized with respect to Actin2/8 mRNA (= 1). Tissue samples were collected from 8-week-old flowering plants.

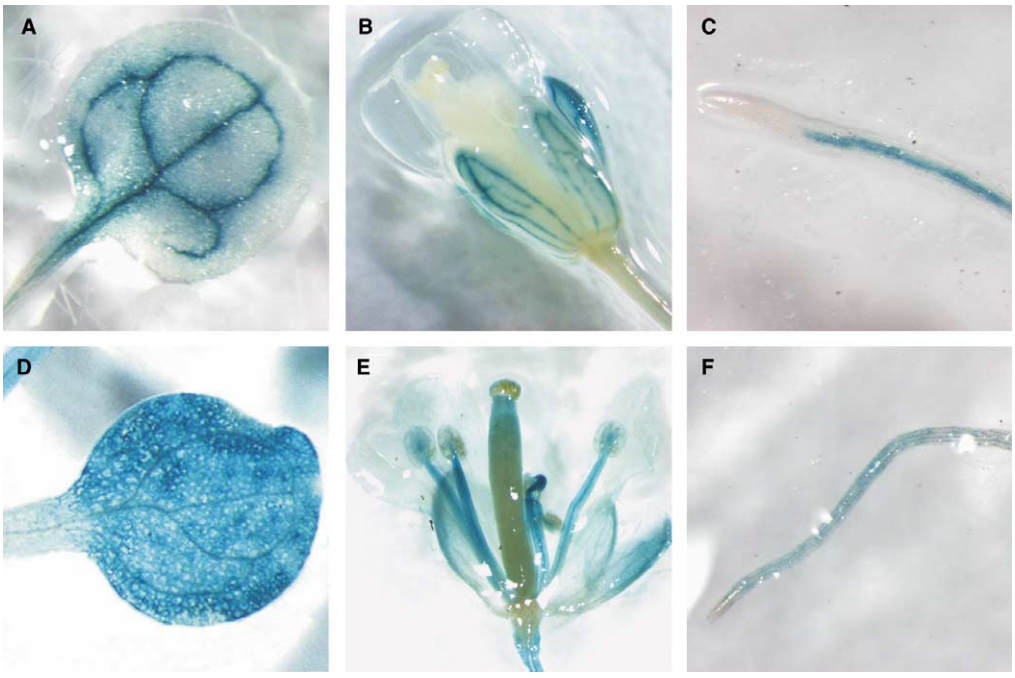

Fig. 4. Expression analysis of AtC/VIF1 and 2, using promoter::GUS fusions. Panels A, B, and C represent AtC/VIF1 promoter::GUS fusions, panels D, E, and F AtC/VIF2 promoter::GUS fusions. Cotyledons and roots (A/D, and C/F) were taken from 3 week old *Arabidopsis* plants, whereas flowers (B/E) were taken from 8 week old plants. The strong expression of *AtC/VIF1* was localized to the vascular tissue. Conversely, *AtC/VIF 2* expression was more evenly distributed.

VIF1 and *2* at higher spatial resolution, we generated *promoter::GUS* lines for both isoforms, containing 1486 and 1525 bp of 5′-upstream sequence for *AtC/VIF1* and *2*, respectively. The analysis of *promoter::GUS* transformants revealed pronounced differences between the inhibitor isoforms (Fig. 4). In cotyledons and roots (Fig. 4 A/D, and C/F), the strong expression of *AtC/VIF1* was localized to the vascular tissue, and the same was true for all leaf stages (data not shown). Cross sections revealed that the GUS-staining was confined to the phloem (data not shown). Conversely, *AtC/VIF 2* expression was more evenly distributed. Likewise, conspicuous qualitative differences of GUS-staining patterns were observed in flowers (Fig. 4B/E). While strong *GUS* expression in *AtC/VIF1* lines was concentrated in the vascular tissue of the sepals only, all floral organs were stained in *AtC/VIF2* plants.

3.3. Acid invertase activities and sugar levels in a T-DNA-tagged AtC/VIF KO mutant

To address the in vivo functions of the strongly expressed AtC/VIF1 isoform, we have analyzed a T-DNA tagged AtC/VIF1 KO mutant obtained from the Arabidopsis Knockout Facility at the University of Wisconsin [31]. The T-DNA insert was localized in the only intron, 96 bases downstream of the exon-intron border (base 295 relative to the translational start site). Real time PCR analysis confirmed the absence of *AtC/VIF1* transcripts (data not shown). A comparison of extractable total VI activities in leaf extracts from wild type and KO plants revealed a 43% increase in the latter (Table 1), whereas CWI activities were not affected. This effect is most likely underestimated, since expression of *AtC/VIF1* is confined to the vascular (phloem) tissues (see above), whereas at least one of the two VI isoforms in *Arabidopsis thaliana* appears to be expressed in the entire leaf blade (Christina Hofmann, unpublished results). Therefore, the effect within the vascular tissue may be more pronounced. When comparing the tissue concentrations of sucrose, fructose and glucose, only minor changes were observed in the KO mutant (Table 1), the hexose/sucrose ratio being 4.42 in the mutant and 3.72 in the WT plants. Again, this effect could be underestimated for the reasons given above. When plants were cultivated under optimum growth conditions (green house, growth chamber), a preliminary analysis of KO mutant plants showed no visible changes in phenotype compared to wild type plants during vegetative growth, flowering and seed production. However, as the expression of the target enzymes, VI (and possibly CWI), is strongly regulated in response to various biotic and abiotic stress factors, a comprehensive search for possible mutant phenotypes under stress exposure has been initiated.

Table 1
VI activities, hexose and sucrose contents in source leaves of 6 week old WT and AtC/VIF1 T-DNA KO mutant plants grown in the green house under non-stressed conditions

		WT	KO
Invertase activities (nkat)	VI	54.26 ± 13.31	77.77 ± 14.48
	CWI	3.36 ± 1.24	3.40 ± 0.89
Sugar contents (μmol/g)	glucose	4.00 ± 1.51	5.02 ± 1.61
	fructose	1.44 ± 0.58	2.02 ± 0.71
	sucrose	1.46 ± 0.50	1.59 ± 0.62

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