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CELL WALL INVERTASE 4 is required for nectar production in *Arabidopsis*

Jeffrey M. Ruhlmann, Brian W. Kram and Clay J. Carter*

Department of Biology, 1035 Kirby Dr, University of Minnesota Duluth, Duluth, MN 55812, USA

* To whom correspondence should be addressed: E-mail: cjcarter@d.umn.edu

Abstract

To date, no genes have been reported to directly affect the de novo production of floral nectar. In an effort to identify genes involved in nectar production, the Affymetrix® ATH1 GeneChip was previously used to examine global gene expression profiles in *Arabidopsis thaliana* nectaries. One of the genes displaying highly enriched expression in nectaries was *CELL WALL INVERTASE 4* (*AtCWINV4*, At2g36190), which encodes an enzyme that putatively catalyses the hydrolysis of sucrose into glucose and fructose. RT-PCR was used to confirm the nectary-enriched expression of *AtCWINV4*, as well as an orthologue from *Brassica rapa*. To probe biological function, two independent *Arabidopsis cwinv4* T-DNA mutants were isolated. Unlike wild-type plants, *cwinv4* lines did not produce nectar. While overall nectary morphology appeared to be normal, *cwinv4* flowers accumulated higher than normal levels of starch in the receptacle, but not within the nectaries themselves. Conversely, wild-type, but not *cwinv4*, nectarial stomata stained intensely for starch. Cell wall extracts prepared from mutant flowers displayed greatly reduced invertase activity when compared with wild-type plants, and *cwinv4* flowers also accumulated significantly lower levels of total soluble sugar. Cumulatively, these results implicate CWINV4 as an absolutely required factor for nectar production in the Brassicaceae, specifically by maintaining constant sink status within nectaries, thus allowing them to accumulate the sugars necessary for nectar production. In addition, CWINV4 is probably responsible for the hexose-rich composition observed for many Brassicaceae nectars.

Key words: *Arabidopsis thaliana*, *Brassica rapa*, cell wall invertase, invertase, nectar, nectaries, nectary.

Introduction

Nectar is secreted by many angiosperms as a reward to promote pollinator visitation (Simpson and Neff, 1983). This mutually beneficial process increases pollination efficiency and, ultimately, the propagation of plant species. Nectar is a complex solution that consists not only of sugars, which can range from 8% to 80% (w/w) depending on the species (Baker and Baker, 1983), but also a multitude of additional components, including: amino acids (Baker and Baker, 1973), organic acids (Baker and Baker, 1975), terpenes (Ecroyd *et al.*, 1995), alkaloids (Deinzer *et al.*, 1977), flavonoids (Ferreres *et al.*, 1996), glycosides (Roshchina and Roshchina, 1993), vitamins (Griebel and Hess, 1990), phenolics (Ferreres *et al.*, 1996), metal ions (Heinrich, 1989), oils (Vogel, 1969), free fatty acids (Kram

et al., 2008), and proteins (Carter and Thornburg, 2004). These non-sugar compounds have been shown to perform a wide variety of tasks, from acting as a deterrent to certain visitors (Baker, 1978), to promoting pollination by attracting cognate pollinators (Carter *et al.*, 2006; Raguso and Pichersky, 1999).

Of the sugars found in nectar, the most prevalent are sucrose, glucose, and fructose (Baker and Baker, 1983). The predominant forms of these three carbohydrates in *Arabidopsis thaliana* nectar are the simple hexoses glucose and fructose. For example, it was previously reported that *Arabidopsis* nectar has a hexose (glucose+fructose)-to-sucrose ratio close to 33.33 (Davis *et al.*, 1998). Various nectars also contain multiple forms of other carbohydrates

in minor concentrations (Baker and Baker, 1983), which are thought to play important roles as supplementary nutrition for potential pollinators.

The floral organ responsible for nectar synthesis and secretion is the nectary. *Arabidopsis* flowers contain two types of nectaries, lateral and median, with lateral nectaries being more pronounced and responsible for nearly all nectar production (Davis *et al.*, 1998). Furthermore, the lateral nectary is much more heavily innervated by sieve tubes than the median, which could also account for its higher level of nectar secretion (Davis *et al.*, 1986). Although it is known that the nectary is responsible for nectar production and secretion, the specific genes responsible for the underlying processes are not. With this in mind, there is a clear understanding of some of the cellular processes that take place in nectariferous cells (nectar-secreting cells) during secretion. At the initiation of secretion, vacuoles found inside these cells increase in volume, dictyosome numbers are reduced, the endoplasmic reticulum becomes more active, and mitochondrial numbers increase (Zhu and Hu, 2002). Along with increased organelle activity, starch grains found in the chloroplasts become reduced in size, or disappear altogether, indicating that they probably contribute to the carbohydrate component of the nectar itself (Ren *et al.*, 2007; Zhu and Hu, 2002).

With an understanding of the basic processes taking place at an ultrastructural level, a general pathway for nectar secretion out of *Arabidopsis*, and other merocrine-type, nectaries has been proposed. Starting from the phloem, pre-nectar metabolites are transported via plasmodesmata to pre-secretory nectariferous tissue (Fahn, 1988; Pacini and Nepi, 2007). Here the pre-nectar is stored as starch grains in chloroplasts until nectar production begins; although it is also possible that some photosynthate is produced *in situ*, as has been reported for *Brassica napus* nectaries (Davis *et al.*, 1986). When needed, starch grains are degraded and the endoplasmic reticulum and dictyosomes modify the sugars derived from degraded starch grains and package them into vesicles that fuse with the plasma membrane for secretion. It is also likely that some nectar carbohydrate, in the form of sucrose, is directly transported to secretory cells from underlying sieve tubes via plasmodesmata, and that plasma membrane transporters may play a direct role in the secretion of some nectar solutes (Fahn, 1988; Pacini and Nepi, 2007). The specific point of secretion from the nectary is thought to be modified stomata, which remain permanently open, providing a direct path out of the nectary (Davis AR, in Bowman, 1994; Zhu *et al.*, 1995; Zhu and Hu, 2002). Similar mechanisms for nectar production have been proposed for a number of other species (Ge *et al.*, 2000; Wist and Davis, 2006; Horner *et al.*, 2007; Pacini and Nepi, 2007; Ren *et al.*, 2007).

As described above, a number of ultrastructural and physiological aspects of nectary function are known; however, the impacts of individual genes on *de novo* nectar production are largely unknown. To link individual genes to nectar synthesis and secretion, the Affymetrix® ATH1 GeneChip was previously used to assess global gene expression profiles within nectaries (Kram *et al.*, 2009). This analysis identified a large number of genes with relatively nectary-specific expression profiles. Amongst these genes, *CELL WALL INVERTASE 4* (*AtCWINV4*, At2g36190) was particularly up-regulated in nectaries. The characterization of two independent *Arabidopsis cwinv4* mutant lines is described here, the results of which implicate cell wall invertase activity as an absolutely required factor for nectar production in the Brassicaceae. Since nectar volume and composition can significantly affect the frequency of flower visitation by pollinators (Silva and Dean, 2000), knowledge of the genes required for nectar production may have broad implications, ranging from a better understanding of the co-evolution of plant–pollinator interactions, to increasing pollination efficiency in multiple crop species.

Materials and methods

Plant material and growth conditions

Arabidopsis thaliana ecotype Columbia-0 was the primary genotypic background used for this study. A previously described transgenic *Arabidopsis* line expressing plasma membrane localized GFP (35S::GFP:LTI6b; Cutler *et al.*, 2000) was used for confocal microscopy studies. T-DNA mutant lines, SALK_130163 and SALK_017466C, were obtained from the *Arabidopsis* Biological Resource Centre (Alonso *et al.*, 2003), and homozygous mutants were renamed *cwinv4-1* and *cwinv4-2*, respectively. Individual plants were genotyped by PCR from genomic DNA, with oligonucleotide primers designed in locations flanking both ends of the T-DNA insertion, along with a third primer located on the left border of the T-DNA insertion. Genotyping primers were designed with the *iSct Primers* tool of the Salk Institute Genomics Analysis Laboratory (http://signal.salk.edu/tdnaprimers.2.html); the nucleotide sequences of all primers used in this study are provided in Table 1. Genotyping PCR reactions were performed with GoTaq Green Master Mix according to the manufacturer's instructions (Promega Corp., Madison, WI, USA).

Rapid-cycling *Brassica rapa* (CrGC 1-33) was used for *BrCWINV4* studies. Plants were grown in individual pots on a peat-based medium with vermiculite and perlite (Pro-Mix BX; Premier Horticulture, Rivière-du-Loup, Quebec, Canada). All plant growth was performed in Percival AR66LX environmental chambers with settings of: 16/8 h day/night cycle, photosynthetic photon flux of 150 μmol m^{-2} s^{-1} and temperature of 23 °C.

Microarray data mining

The mean probe set signal intensities for all invertase genes expressed in *Arabidopsis* nectaries, as identified by Affymetrix ATH1 GeneChip® microarray, were compared to those in 13 different tissues at multiple developmental stages, and are presented in Supplementary Table S1 at *JXB* online. The raw normalized microarray data used for the analyses presented here were originally described by Kram *et al.* (2009).

Chemicals and reagents

Unless noted otherwise, all chemicals were obtained through Sigma Aldrich Chemical Co. (St Louis, MO, USA) or Thermo Fisher Scientific (Waltham, MA USA).

RT-PCR analyses

The RNAqueous-Micro® micro scale RNA isolation kit (Ambion, Austin, TX) was used, in conjunction with the Plant RNA Isolation Aid (Ambion, Austin, TX), to extract RNA from plant

tissues. For floral tissues, RNA was extracted from Stage 11–12 and 14–15 flowers from *Arabidopsis thaliana* (stages defined by Smyth *et al.*, 1990), or the equivalent of Stage 14–15 for *Brassica rapa*. The nectaries of Stage 11–12 flowers are pre-secretory (pre-anthesis), whereas those from Stage 14–15 are secretory (post-anthesis). Standard agarose gel electrophoresis and UV spectrophotometry were used to evaluate RNA quality for all samples. Reverse transcription-PCR (RT-PCR) was used to examine the presence of CWINV4 transcripts with the Promega Reverse Transcription System (A3500) according to the manufacturer's instructions with 0.1 µg of input RNA. Partial cDNA sequences for *BrCWINV4* and *BrUBQ* were identified through a *B. rapa* nectary EST project (M Hampton *et al.*, unpublished data), and deposited into GenBank as accession numbers GQ146458 and GR719937, respectively. All primers used for RT-PCR analyses are listed in Table 1.

AtCWINV4::AtCWINV4:GFP analyses

A 4837 bp *AtCWINV4* genomic DNA fragment was amplified with the AtCWINV4-GFP-F and AtCWINV4-GFP-R primers (Table 1) using Phusion polymerase (New England BioLabs, Ipswich, MA), and directly cloned into the pCR® Blunt II-TOPO® vector (Invitrogen, Carlsbad, CA). This fragment contained 1895 bp of sequence upstream of the *AtCWINV4* transcriptional start site, along with the full AtCWINV4 coding region, minus the stop codon. The sequence of the *AtCWINV4* insert was confirmed via dideoxy sequencing at the University of Minnesota DNA Sequencing and Analysis Facility (St Paul, MN, USA), and the fragment was then subcloned into the *EcoR*I and *Spe*I sites of the binary vector pORE-R4. The coding region of this fragment was cloned in-frame with the GFP coding region found in pORE-R4. The resultant construct was transformed into *Agrobacterium tumefaciens* (GV3101), which was then used to transform *Arabidopsis* by the floral dip method (Clough and Bent, 1998). Transformed seedlings were selected on solid Murashige and Skoog (MS) media with kanamycin (50 µg ml^{-1}). Plants confirmed to carry the above construct were either observed with a Nikon SMZ1500 stereomicroscope configured for the detection of GFP fluorescence, or a Nikon C1 Spectral Imaging Confocal Microscope at University of Minnesota Imaging Center. Sample preparation simply consisted of sepal removal from flowers to expose the nectaries prior to imaging.

Enzyme assays

Cell wall invertase activity was assayed as previously described (Wright *et al.*, 1998). In summary, individual samples consisted of 25 mg of Stage 14–15 flowers, which were freshly collected and flash frozen in liquid nitrogen. Flowers were then transferred to 1.5 ml microcentrifuge tubes containing 400 µl of extraction buffer (50 mM HEPES-NaOH, pH 8.0; 5 mM MgCl$_2$; 2 mM EDTA; 1 mM MnCl$_2$; 1 mM CaCl$_2$; 1 mM dithiothreitol; and 0.1 mM phenyl-methyl-sulphonyl fluoride), where they were homogenized with a pestle and incubated on ice for 10 min. Samples were then centrifuged at 13 000 *g* for 10 min at 4 °C. The supernatant, containing vacuolar and cytoplasmic invertases, was transferred to a new microcentrifuge tube. The remaining pellet was washed three times with 400 µl of extraction buffer by resuspension and centrifugation at 13 000 *g* for 10 min at 4 °C. The pellet was washed a final time with 400 µl of 80 mM sodium acetate, pH 4.8. Following the wash, the pellet was resuspended in 400 µl of 80 mM sodium acetate, pH 4.8. The total volume was divided into four 100 µl aliquots. One of the aliquots was boiled for 15 min to serve as a negative control. To each aliquot, 800 µl of staining solution (0.024% nitroblue tetrazolium; 0.014% phenazine methosulphate; 25 units glucose oxidase; 1% sucrose; in 80 mM sodium acetate, pH 4.8) was added as previously described (Wittich and Willemse, 1999). Supernatant fractions, containing the vacuolar and cytoplasmic invertases, were also examined for activity and used as positive controls. Absorbance at 550 nm was then used a measure of total invertase activity.

Histochemical staining

Flowers were examined for starch accumulation by iodine–potassium iodide (IKI) staining (Jensen, 1962). Briefly, freshly collected flowers were placed in IKI solution (Thermo Fisher S93409) and subjected to a 1 min vacuum infiltration, followed by a 5 min incubation at 20 °C. Flowers were then rinsed twice in deionized water and immediately imaged. Sepals were removed from flowers prior to staining when examining nectary starch accumulation.

Soluble sugar analysis

Total soluble sugar (sucrose and glucose) within flowers was analysed as previously described (Bethke and Busse, 2008). Briefly, Stage 14–15 flowers were collected, weighed, and flash frozen in liquid nitrogen. The flowers were then transferred to a 1.5 ml microcentrifuge tube containing 500 µl of 80% ethanol, homogenized with a pestle, and incubated in a rotisserie incubator at 60 °C for 24 h. After incubation, the samples were centrifuged for 10 min at 4000 rpm, with the supernatant being retained. The remaining pellet was resuspended with 500 µl of 80% ethanol and again

Table 1. Oligonucleotide primers used in this study

Oligo name	Sequence	Purpose
cwinv4-1 F	ACATCCACAAAACCAGCAAAC	*cwinv4-1* genotyping
cwinv4-1 R	ACAATGAACGGTTCAGCATTC	*cwinv4-1* genotyping
cwinv4-2 F	AAATGTGAACGTAAACTGCGG	*cwinv4-2* genotyping
cwinv4-2 R	TTTGTTAAATGTTTTTGGCCG	*cwinv4-2* genotyping
LBb1.3	ATTTTGCCGATTTCGGAAC	T-DNA left border, genotyping
AtCWINV4 RT-F	CAACGGTGTCAGATTCATTAG	*AtCWINV4* RT PCR
AtCWINV4 RT-R	CGGTACGAGTATTACACGC	*AtCWINV4* RT PCR
BrCWINV4 RT-F	TCAGGCCGATGTGGAAGTGACATT	*BrCWINV4* RT PCR
BrCWINV4 RT-R	ATCCACAAAGCCAGCAAACGATGG	*BrCWINV4* RT PCR
AtGAPDH-F	AGGGTGGTGCCAAGAAGGTTG	*AtGAPDH* RT PCR
AtGAPDH-R	GTAGCCCCACTCGTTGTCGTA	*AtGAPDH* RT PCR
BrUBQ-F	TTGAGGTGGAAAGCTCTGACACGA	*BrUBQ* RT PCR
BrUBQ-R	AATCGGCCAATGTACGACCATCCT	*BrUBQ* RT PCR
AtCWINV4-GFP-F	gaattcGAAATCAGAGTCACTGTGCC	Cloning of *AtCWINV4* into pORE-R4, *EcoR*I site
AtCWINV4-GFP-R	actagtTTCCTAATTTCACAGTATCTCTC	Cloning of *AtCWINV4* into pORE-R4, *Spe*I site

incubated in a rotisserie incubator at 60 °C for 24 h. Following incubation, samples were again centrifuged and the supernatant collected and combined with previous supernatant. The supernatant was diluted 1:40 with 10 mM sodium acetate, pH 5.0. From the dilute supernatant, 25 µl was added to a new microcentrifuge tube and mixed with 25 µl of 38 U ml^{-1} invertase in 10 mM sodium acetate, pH 5.0 and incubated at room temperature for 15 min. After incubation, 25 µl of 150 mM sodium phosphate, pH 7.4 and 25 µl of enzyme mix (100 µl of 10 mM Ampliflu Red in DMSO; 1.725 ml dH$_2$O; 0.575 ml 150 mM sodium phosphate, pH 7.4; 100 µl of 10 U ml^{-1} HPR in 50 mM sodium phosphate, pH 7.4; 100 µl of 100 U ml^{-1} glucose oxidase in 50 mM sodium phosphate, pH 7.4) were added to the sample and incubated for 30 min in the dark at 20 °C. Samples were then analysed via spectrophotometry at 560 nm—the wavelength at which absorbance is proportional to glucose concentration. To obtain soluble sugar concentrations, the assay was repeated with and without invertase. The assay performed in the absence of invertase provided information on baseline glucose concentrations. To then obtain sucrose concentrations, the glucose concentration obtained without invertase was subtracted from the glucose concentration observed with invertase.

Scanning electron microscopy

Flowers were manually removed of sepals and flash frozen in liquid nitrogen. Imaging was performed under low vacuum conditions (~65 kPa) and an accelerating volt of 15 kV, with a JEOL Ltd. (Tokyo, Japan) JSM-6490 scanning electron microscope at the University of Minnesota Duluth Scanning Electron Microscope Laboratory.

Results

AtCWINV4 *is highly expressed in nectaries*

By microarray analysis, *AtCWINV4* was previously identified as having a largely nectary-specific expression profile in wild-type plants (Kram *et al.*, 2009). To determine if other invertase genes shared similar expression patterns to *AtCWINV4*, raw normalized probe set signal intensity data (Kram *et al.*, 2009) were examined. A total of nine invertase genes, including *AtCWINV4*, were identified as being confidently expressed in *Arabidopsis* nectaries (see Supplementary Table S1 at *JXB* online; Kram *et al.*, 2009); however, *AtCWINV4* was the only invertase gene that displayed extreme up regulation in nectaries via microarray (see Supplementary Table S1 at *JXB* online). Moreover, AtCWINV4 was the sole nectary-expressed invertase predicted to be localized to the cell wall (based on TAIR annotation; prediction due to shared sequence similarity with known cell wall invertases). *AtCWINV4* encodes a 591 amino acid protein with a theoretical molecular mass of 67 412.2 Da. PSORT analysis (Nakai and Kanehisa, 1991) predicted AtCWINV4 to be secreted from the cell via a 22 amino acid signal peptide, which would be consistent with cell wall localization.

To confirm the nectary-enriched expression of *AtCWINV4*, reverse transcription polymerase chain reaction (RT-PCR) was performed on RNA isolated from 11 major tissues, including nectaries. Results shown in Fig. 1A demonstrate that *AtCWINV4* was highly expressed in both the lateral and median nectaries of flowers at pre- and post-anthesis (lanes 9–11); it was also expressed in pistils and roots at much lower levels. Similar expression patterns were also observed for a *CWINV4* orthologue from *Brassica rapa* (*BrCWINV4*, Fig. 1B).

To examine *AtCWINV4* expression further, a genomic fragment encoding AtCWINV4, including 1.8 kb of the promoter region, was cloned in-frame with GFP within the binary vector pORE-R4 (to generate *AtCWINV4*::AtCWINV4:GFP). Wild-type *Arabidopsis* transformed with this construct displayed nectary-specific GFP fluorescence (Fig. 1C, D). Examinations of these plants via laser scanning confocal microscopy further supported AtCWINV4 tissue-specific expression (see Supplementary Video S1 at *JXB* online), with GFP appearing to be expressed throughout the entire nectary. Although not definitive, confocal results (see
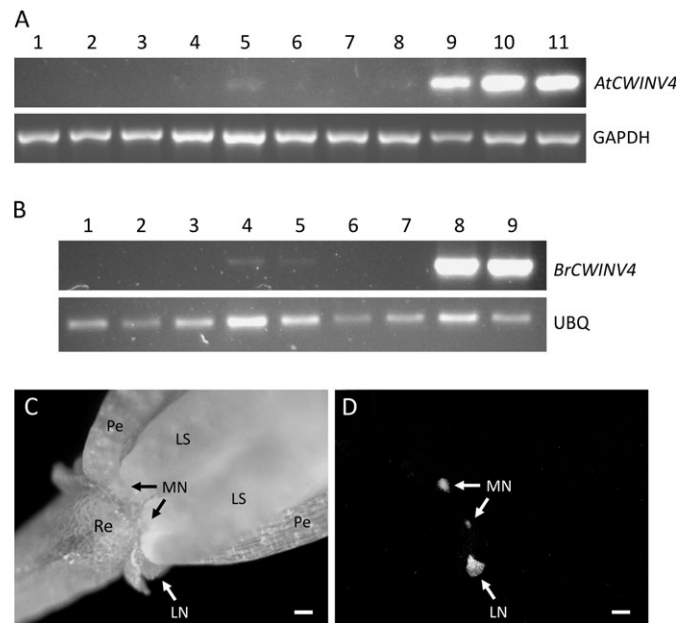
Fig. 1. *CWINV4* is highly expressed in nectaries. Reverse transcription-polymerase chain reaction (RT-PCR) was used to examine the expression profiles of *AtCWINV4* (A) and an orthologue from *Brassica rapa* (B; *BrCWINV4*, accession number GQ146458). The tissues examined in (A) included: (1) petal; (2) sepal; (3) rosette leaf; (4) stamen; (5) pistil; (6) root; (7) internode shoot; (8) silique; (9) mature median nectaries (Stage 14–15); (10) immature lateral nectaries (Stage 11–12); and, (11) mature lateral nectaries (Stage 14–15). (B) Tissues included: (1) petal; (2) sepal; (3) leaf; (4) stamen; (5) pistil; (6) root; (7) stem; (8) mature median nectaries; and (9) mature lateral nectaries. GAPDH (At3g04120) and a *B. rapa* ubiquitin gene (*BrUBQ*, accession number GR719937) were used as constitutively expressed controls. All *B. rapa* floral tissues examined were from the equivalent of Stage 14–15 *Arabidopsis* flowers. The images shown are the results obtained after 27 cycles of RT-PCR. (C) Bright field image of Stage 15 *Arabidopsis* flower expressing an AtCWINV4:GFP fusion, under control of the native *AtCWINV4* promoter, and (D) GFP fluorescence derived from same flower. Sepals were removed prior to imaging. Re, receptacle; Pe, petal; LS, long stamen; MN, median nectary; LN, lateral nectary; scale bars, 100 µm.
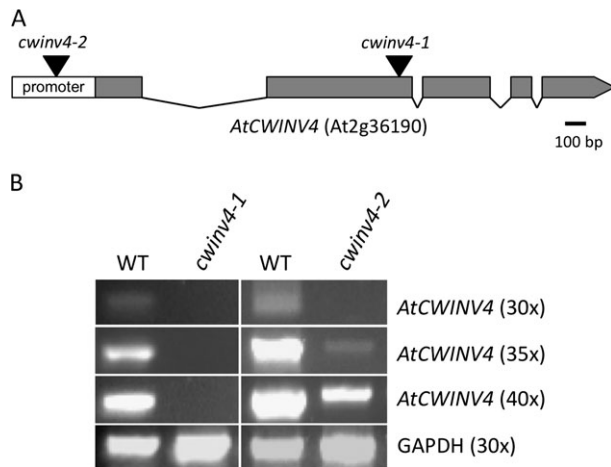
Supplementary Video S1 at *JXB* online) also suggested that most fluorescence was localized to cell walls due to diffuse patterns of GFP accumulation, which appeared to outline nectary cells. This observation was in contrast to the well-defined localization of a plasma-membrane targeted GFP fusion protein in nectaries (see Supplementary Video S2 at *JXB* online; 35S::GFP:LTI6b fusion described in Cutler *et al.*, 2000).

cwinv4 *mutant lines*

To probe the biological function of *AtCWINV4*, two independent T-DNA mutant lines were identified, *cwinv4-1* and *cwinv4-2*. *cwinv4-1* had a T-DNA inserted in the second exon, whereas *cwinv4-2* had an insertion ~200 nucleotides upstream of the transcriptional start site (Fig. 2A). Significantly, *cwinv4-1* was identified as a null mutant, whereas *AtCWINV4* expression was greatly reduced in *cwinv4-2* (Fig. 2B). Indeed, while *AtCWINV4* transcripts were readily detected from whole flowers of wild-type plants following 30 cycles of RT-PCR, faint bands derived from *cwinv4-2* cDNA were only visible at 35 cycles or greater.

cwinv4 *flowers have decreased cell wall invertase activity*

Once confirmed as mutants, both *cwinv4-1* and *cwinv4-2* flowers were subsequently examined for extracellular (cell wall) invertase activity. Invertases catalyse the hydrolysis of the disaccharide sucrose into glucose and fructose (Berger *et al.*, 2007; Winter and Huber, 2000*b*). Cell wall extracts were prepared as previously described (Wright *et al.*, 1998) from whole flowers of *cwinv4-1*, *cwinv4-2*, and wild-type

plants. Both *cwinv4* lines displayed large decreases in total cell wall invertase activity (Fig. 3), whereas intracellular fractions displayed similar activities to wild-type plants (data not shown). The higher cell wall invertase activity observed in *cwinv4-2* than *cwinv4-1* flowers was probably due to the leaky expression of AtCWINV4 in *cwinv4-2* (i.e. *cwinv4-1* is a null mutant, whereas *cwinv4-2* is a knockdown mutant). It was also noted that cell wall invertase activity was not reduced to zero in cell wall extracts derived from flowers of either *cwinv4* mutant. Whether this result is indicative of activity derived from other cell wall invertases, or represents minor contamination from intracellular invertases is unknown. However, it should be noted that whole flowers were used for these assays, not isolated nectaries (i.e. other cell wall invertases may be expressed in petals, stamen, etc).

cwinv4 *mutants do not secrete nectar*

To determine if mutations occurring within *AtCWINV4* have an impact on nectar production, individual flowers were examined for the presence of nectar droplets, which accumulate inside the cups formed by sepals surrounding the lateral nectaries. Figure 4A illustrates a typical analysis, and shows a nectar droplet clinging to the inside of a sepal of a wild-type flower; nectar droplets were never observed within either *cwinv4-1* or *cwinv4-2* flowers (Table 2; Fig. 4B). Interestingly, extremely limited indications of
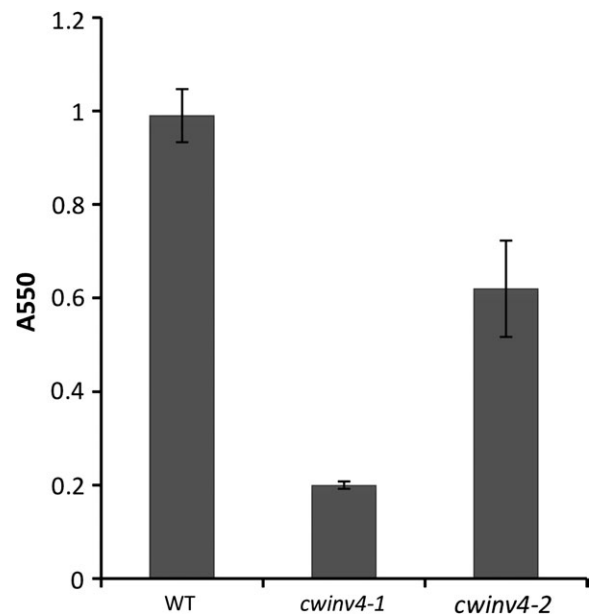

Fig. 2. Identification of *cwinv4* mutants. Two independent *Arabidopsis* T-DNA mutant lines (Alonso *et al.*, 2003), *cwinv4-1* (SALK_130163) and *cwinv4-2* (SALK_017466C), were obtained from the *Arabidopsis* Biological Resource Centre and genotyped to obtain homozygous mutant plants. The relative locations of each T-DNA insertion are shown in (A). RT-PCR, performed on RNA isolated from whole flowers, demonstrated that *cwinv4-1* is a null mutant, and that *AtCWINV4* is expressed significantly lower in *cwinv4-2* flowers than in wild-type (B).

Fig. 3. Cell wall invertase activity is greatly reduced in *cwinv4* flowers. Cell wall extracts were prepared from whole Stage 14–15 flowers (25 mg fresh weight) of wild-type and *cwinv4* plants, which were subsequently tested for invertase activity. The results from one experiment with *cwinv4-1* and *cwinv4-2* plants are shown ($n=3$ independent preparations). Independent and repeated experiments with both *cwinv4-1* and *cwinv4-2* plants displayed consistent results, with varying amplitudes of total activity for both wild-type and mutant plants.
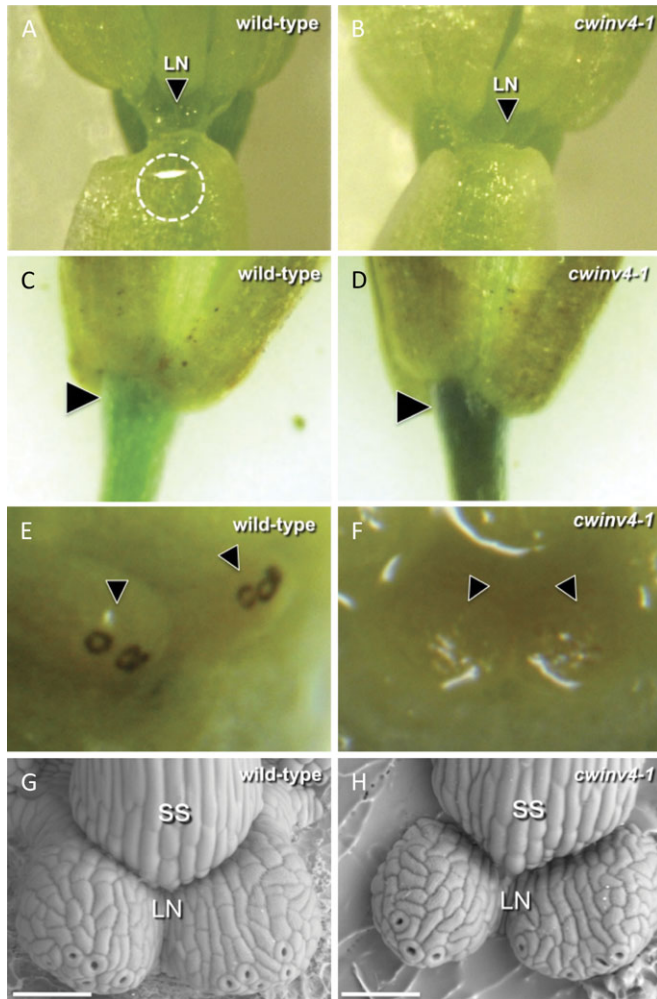
Fig. 4. *cwinv4* flowers do not secrete nectar. Nectar production in *Arabidopsis* flowers can be examined by gently peeling back the sepal. Nectar droplets accumulating within the sepal cups surrounding lateral nectaries (LN) were consistently present in wild-type plants (circled in A), whereas neither *cwinv4* mutant secreted nectar (e.g. B; Table 2). Both *cwinv4* mutants also accumulated higher than normal levels of starch within the receptacle (arrowheads), which often extended into the pedicel (C, D). Conversely, the stomata of wild-type nectaries stained heavily for starch (E, arrowheads), whereas those of *cwinv4* flowers did not (F). The morphology of *cwinv4* nectaries was examined to determine if aberrant nectary structure might account for the lack of nectar secretion. In all flowers examined, *cwinv4* nectaries (H) appeared to have similar morphology to wild-type plants (G). Scale bars are 50 μm; LN, lateral nectary; SS, short stamen.

secretion very rarely appeared inside the sepals of *cwinv4-2* flowers; however, nectar droplets were never present (only a thin glistening film), and due to the extremely rare occurrence (*c.* 5% of flowers) and limited amount of fluid produced, these putative secretions could neither be collected nor analysed. If this rarely observed fluid truly represented nectar secretion, it would be consistent with the leaky expression of CWINV4 found in *cwinv4-2*.

Table 2. Examination of nectar production in wild-type and *cwinv4* flowers

	Wild-type	***cwinv4-1***	***cwinv4-2***
No. of flowers with nectar[a]	250/250	0/250	0[b]/250

[a] Flowers were examined for nectar droplets accumulating inside sepals surrounding lateral nectaries. A minimum of ten individual plants were examined for each line.
[b] Extremely limited indications of fluid very rarely appeared inside the sepals of *cwinv4-2* flowers; however, no nectar droplets ever accumulated inside the flowers of these plants. These findings are in line with the leaky expression of AtCWINV4 found within *cwinv4-2*.

cwinv4 *flowers display altered patterns of starch accumulation*

Invertases play key roles in sugar source–sink relationships (Roitsch, 1999; Roitsch and Ehness, 2000). Thus, whole flowers were examined for altered patterns of starch accumulation via iodine–potassium iodide (IKI) staining. *cwinv4* lines displayed an obvious increase in starch accumulation within the floral receptacle, which often extended into the pedicel (Fig. 4C, D). Conversely, wild-type flowers accumulated high levels of starch within nectarial stomata, whereas *cwinv4* plants did not (Fig 4E, F).

### Nectary ultrastructure is normal in cwinv4 *flowers*

Scanning electron microscopy was used to examine if any defects in nectary development or morphology could account for the lack of nectar production observed in the *cwinv4* mutant lines. Both mutant lines displayed all structural hallmarks of wild-type nectaries, including having multiple modified stomata (in an open conformation) and being covered by a highly reticulated cuticle (Fig. 4G, H). Confocal microscopy utilizing lipophilic fluorescent dyes (i.e. FM4-64) also demonstrated normal nectary morphology within *cwinv4* flowers (data not shown).

cwinv4 *flowers accumulate less soluble sugar*

Related to the starch analyses described above, it seemed logical to examine whether *cwinv4* mutants displayed changes in the accumulation of soluble sugars. Simple sugars were extracted from whole flowers and assayed as previously described (Bethke and Busse, 2008). Results shown in Fig. 5 demonstrate that both *cwinv4-1* and *cwinv4-2* flowers accumulated significantly lower levels of soluble sugars, specifically sucrose and glucose, than wild-type plants. Notably, the overall ratio of glucose-to-sucrose was similar in wild-type and *cwinv4* flowers (~90% glucose and 10% sucrose in all lines examined, data not shown).

Discussion

Arabidopsis flowers are highly autogamous, which poses the question as to why functional and energetically costly nectaries are maintained in this species. The answer to this
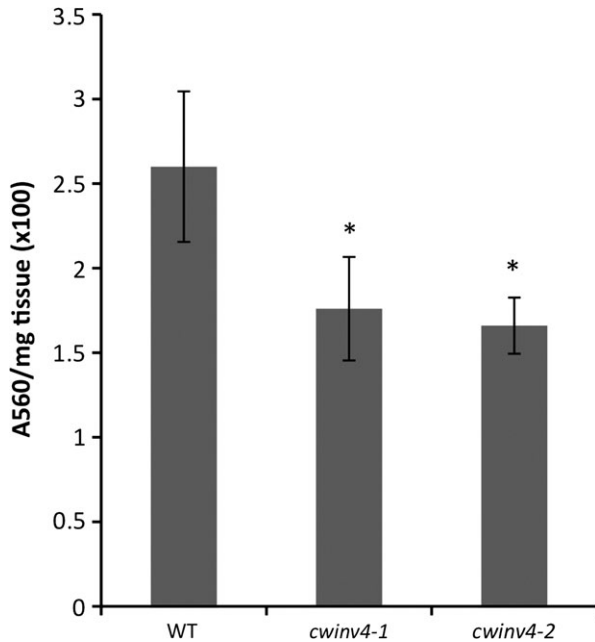
Fig. 5. *cwinv4* flowers accumulate decreased total soluble sugar. Whole flowers (Stage 14–15) from both *cwinv4-1* and *cwinv4-2* displayed significantly lower levels of total soluble sugar (sucrose and glucose) than wild-type plants (n=3, *P=0.027 and 0.013, respectively). Results are displayed as total absorbance per mg of floral tissue ×100. The relative proportion of sucrose-to-glucose was similar between *cwinv4* and wild-type flowers (data not shown).

question may lie in the revelation that insects do visit *Arabidopsis* flowers in their native setting, and a small but significant amount of outcrossing does occur (Hoffmann *et al.*, 2003). The fact that many species share similar nectary structure with *Arabidopsis* (e.g. *Brassica* sp.), and produce relatively large amounts of nectar (Davis *et al.*, 1996, 1998), suggests that similar mechanisms for nectar production may exist amongst the Brassicaceae. Many of these species are not self-fertile, and are dependent on pollinators to achieve full fecundity (Pearson, 1933; Rahman, 1940; Vesely, 1962; Nieuwhof, 1963). Notably, *Arabidopsis* nectaries also seem to share common developmental mechanisms with much of the eudicot clade (Lee *et al.*, 2005). As such, *Arabidopsis*, with its fully sequenced genome and genetic resources, can serve as a valuable model for the molecular dissection of nectary form and function. This report describes initial efforts towards characterizing *CELL WALL INVERTASE 4*, a gene required for nectar production in *Arabidopsis*. To the best of our knowledge, this is the first report of an individual gene being required for *de novo* nectar production in flowering plants.

Plant invertases have been classified into several general types based upon subcellular localization and pH optima. Cell wall invertases, in particular, are adventitiously bound to cell walls and function optimally in an acidic pH (Sturm, 1999; Sturm and Tang, 1999; Tymowska-Lalanne and Kreis, 1998*a, b*). The model plant *Arabidopsis thaliana* encodes over 20 known or putative invertases (The *Arabidopsis* Information Resource (TAIR), www.arabidopsis. org). Of these gene products, at least four are known or predicted to be localized to the cell wall. Significantly, cell wall invertases have been shown to exhibit both temporal and spatial specificity in their expression (Sherson *et al.*, 2003). Of interest in this study was *AtCWINV4*, which displayed exceptionally enhanced expression in *Arabidopsis* nectaries of both pre- and post-anthesis flowers (Fig. 1; Kram *et al.*, 2009). These results are consistent with previous reports, which demonstrated that *AtCWINV4* is highly expressed in *Arabidopsis* flowers by RT-PCR (Sherson *et al.*, 2003).

The observed expression patterns for *AtCWINV4*, combined with the relationship between invertases and sucrose, and the composition of *Arabidopsis* nectar (hexose-rich), imply a central role for this gene in mediating nectary function. Sucrose is one of the most important products of photosynthesis, and is the form in which most carbohydrates are transported between tissues (Roitsch, 1999; Roitsch and Ehness, 2000; Winter and Huber, 2000*a, b*). This disaccharide has also been shown to play important roles in plant signalling, growth, and developmental processes (Weber *et al.*, 1998; Sturm, 1999; Sturm and Tang, 1999; Winter and Huber, 2000*a, b*; Sherson *et al.*, 2003; Berger *et al.*, 2007). Because of the catabolic nature of invertases, it is thought that these enzymes could be prime mediators of source–sink relationships, driving the flow of sucrose from source to sink tissues and, in turn, regulating plant growth and development.

Perhaps most significantly in this study, nectar was found to be absent in *cwinv4* flowers (Fig. 4; Table 2), which could be explained by the suggested role of invertases in establishing sucrose gradients between source and sink tissues. If this interpretation is accurate, the loss of invertase function would be paired with a reduction in the extracellular hydrolysis of sucrose into glucose and fructose. A role for CWINV4 acting in this manner was supported by the finding that *cwinv4* flowers displayed markedly decreased CWINV activities when compared to wild-type plants (Fig. 3). Such disruption of the sucrose gradient from source to sink cells would result in the cessation of sugar flow into nectaries, thus depriving nectariferous tissue of the sugars required for nectar production.

The mechanistic impact of CWINV4 activity on nectar production is perhaps 2-fold. CWINV4 may drive the movement of sucrose into nectaries from source tissues, which, in turn, is deposited as starch (supported by Fig. 4C–F). Significantly, starch accumulation within *Arabidopsis* nectaries has been previously reported (Bowman, 1994; Zhu *et al.*, 1995; Zhu and Hu, 2002; Ren *et al.*, 2007); at anthesis, starch grains are reportedly degraded and are a presumed source of nectar carbohydrate (Ren *et al.*, 2007). CWINV4 involvement in starch accumulation in nectaries is supported by two pieces of evidence in this study: (i) increased starch accumulation within the receptacle and pedicel of *cwinv4* flowers (Fig. 4C, D), and (ii) intense IKI staining in the stomata of wild-type

nectaries, as opposed to *cwinv4* flowers (Fig. 4E, F). Taken together, these findings suggest that CWINV4-deficient nectaries lack the sink status necessary to accumulate starch, with excess photosynthate apparently being stored within the receptacle and pedicel instead. Significantly, pedicels have been suggested to serve as sources of nectar carbohydrate via the direct production of photosynthate (Pacini and Nepi, 2007), which would also be consistent with the observed starch staining patterns observed in Fig. 4D. The fact that *cwinv4* flowers accumulated less soluble sugar than wild-type flowers (Fig. 5) is a final piece of evidence suggesting a role for CWINV4 in supplying nectariferous tissue with the sugars necessary for nectar production.

A second, but non-mutually exclusive, hypothesis for CWINV4 involvement in nectar production can be posited for flowers at anthesis. Without hydrolysis of sucrose to glucose and fructose within the apoplast of nectary cells, an extracellular hexose-rich environment (high solute) would not be established and, in turn, osmotic pressure might be insufficient to draw out water—a suggested requirement for the secretion of hexose-dominant nectars (Fahn, 1988; Nicolson, 1998). Moreover, the spontaneity of sucrose transport across the plasma membrane of nectariferous cells, probably via sucrose transporters, would be greatly diminished in *cwinv4* nectaries. Interestingly, genes representing the full canonical sucrose biosynthesis pathway are highly up-regulated in mature *Arabidopsis* nectaries, along with several putative sucrose transporters (Kram *et al.*, 2009). These overall findings are consistent with a mechanism for generating the high hexose-to-sucrose ratio previously reported for the nectars of multiple Brassicaceae species (Davis *et al.*, 1998). Indeed, it is likely that CWINV4 activity is at least partially responsible for the observed hexose-rich composition of *Arabidopsis* nectar. Invertase activity has even been reported to occur within the nectars of multiple species, and implicated as a factor required for the generation of hexose-dominant nectar (Baker and Baker, 1983; Nicolson, 1998; Heil *et al.*, 2005). Reports of this activity within nectar itself are also consistent with cell wall localization or, at a minimum, the secretion of invertases from nectary cells.

In sum, all current evidence indicates that cell wall invertase activity, specifically derived from AtCWINV4, plays an essential role in the production of *Arabidopsis* nectar. The fact that an orthologue from *B. rapa*, *BrCWINV4*, displayed nearly identical expression patterns to *AtCWINV4* (Fig. 1B) suggests that the requirement of cell wall invertases in nectar production is conserved amongst the Brassicaceae, and possibly many other nectar-producing plants. Indeed, investigations into petunia pollination syndromes have identified a single QTL, which may encode an invertase, responsible for the control of nectar volume (Stuurman *et al.*, 2004; Galliot *et al.*, 2006*a*, *b*). Teasing apart the proposed mechanisms of CWINV4 involvement in nectar production will require further experimentation; however, its nectary-specific expression profile, coupled with its apparent involvement in sugar metabolism, suggests it is a principal player in *Arabidopsis* nectary function.

Supplementary data

Supplementary data can be found at *JXB* online.

Supplementary Table S1. Normalized Affymetrix ATH1 GeneChip® probe set signal intensity for *Arabidopsis* invertase genes expressed in nectaries (.xls format).

Supplementary Video S1. Projection video of *Arabidopsis* flower expressing *AtCWINV4*::AtCWINV4:GFP fusion (.mov format).

Supplementary Video S2. Projection video of *Arabidopsis* nectary expressing 35S::GFP:LTI6b, a plasma membrane-localized marker (.mov format).

Acknowledgements

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