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Short-term proteomic dynamics reveal metabolic factory for active extrafloral nectar secretion by *Acacia cornigera* ant-plants

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SUMMARY

Despite the ecological and evolutionary importance of nectar, mechanisms controlling its synthesis and secretion remain largely unknown. It is widely believed that nectar is 'secreted phloem sap', but current research reveals a biochemical complexity that is unlikely to stem directly from the phloem. We used the short daily peak in production of extrafloral nectar by *Acacia cornigera* to investigate metabolic and proteomic dynamics before, during and after 2 h of diurnal secretion. Neither hexoses nor dominating nectar proteins (nectarins) were detected in the phloem before or during nectar secretion, excluding the phloem as the direct source of major nectar components. Enzymes involved in the anabolism of sugars, amino acids, proteins, and nectarins, such as invertase, β–1,3–glucanase and thaumatin-like protein, accumulated in the nectary directly before secretion and diminished quantitatively after the daily secretion process. The corresponding genes were expressed almost exclusively in nectaries. By contrast, protein catabolic enzymes were mainly present and active after the secretion peak, and may function in termination of the secretion process. Thus the metabolic machinery for extrafloral nectar production is synthesized and active during secretion and degraded thereafter. Knowing the key enzymes involved and the spatio-temporal patterns in their expression will allow elucidation of mechanisms by which plants control nectar quality and quantity.

Keywords: carbohydrate metabolism, invertase, nectar protein, nectarine, plant secretory process.

INTRODUCTION

Nectar plays multiple roles in plant pollination (floral nectar, FN) and in the indirect defence of plants against herbivores (extrafloral nectar, EFN) (Heil, 2008, 2011; Brandenburg *et al.*, 2009). In addition to its chemical composition, the quantity of nectar secreted also represents an important trait that is positively correlated with pollination success or the resulting indirect defence (Heil *et al.*, 2009; Brandenburg *et al.*, 2012). Plants are therefore capable of adjusting nectar secretion rates to the current needs, and may even re-absorb unconsumed nectar (Pederson *et al.*, 1958; Ziegler and Lüttge, 1959; Búrquez and Corbet, 1991; Nicolson, 1995; Heil *et al.*, 2000; Nepi *et al.*, 2001, 2011b; Escalante-Perez *et al.*, 2012). However, little is known about the mechanisms that underlie the genetic control or phenotypic plasticity of nectar secretion rates, or where in the plant dominant nectar components others than sugars are synthesized.

The classical idea that nectar represents 'secreted phloem sap' (Agthe, 1951; Lüttge, 1961; de la Barrera and Nobel, 2004; Heil, 2011) is challenged by several observations. First, nectaries are commonly characterized by a highly sophisticated ultrastructure consisting of typical secretory parenchyma, and many of them lack a direct connection to the vascular system (Pacini and Nepi, 2007; Escalante-Pérez and Heil, 2012). Second, floral nectaries in many species accumulate starch grains, which are

degraded during anthesis (Horner *et al.*, 2007; Kram *et al.*, 2009). Gene expression analyses confirmed a shift from starch anabolism to catabolism when floral nectaries of Arabidopsis and ornamental tobacco start to secrete FN (Ren *et al.*, 2007a,b; Kram *et al.*, 2009), and an extracellular invertase that is essential for both starch accumulation and nectar secretion in Arabidopsis flowers is exclusively expressed in nectaries (Kram *et al.*, 2009; Ruhlmann *et al.*, 2010; Bender *et al.*, 2012). Similarly, many genes related to exocytosis, hormone metabolism and sugar metabolism were over-expressed in poplar extrafloral nectaries compared to the leaf tissue (Escalante-Perez *et al.*, 2012). Third, proteins found in the phloem sap of leek (*Allium porrum*) showed little overlap with those in the nectar (Peumans *et al.*, 1997). It has been shown that *NECTARIN* genes are expressed in the nectary tissue of ornamental tobacco (Carter and Thornburg, 2003, 2004). Some of these *NECTARIN* genes are under the control of an MYB305 transcription factor, which is required for full secretion activity (Liu *et al.*, 2009; Liu and Thornburg, 2012). All these observations suggest a much stronger metabolic contribution of the nectary itself than was originally considered.

Nectar secretion is a highly dynamic process that depends on the ontogenetic stage of the nectar-secreting structure (Ren *et al.*, 2007a,b; Liu and Thornburg, 2012) and on environmental factors such as consumption rate (Corbet and Delfosse, 1984; Gill, 1988; Pyke, 1991; Heil *et al.*, 2000), and, in the case of EFN, herbivory and current light conditions (Heil *et al.*, 2001; Radhika *et al.*, 2010; Bixenmann *et al.*, 2011). However, most of the studies performed so far were based on comparisons between nectaries and leaf tissue. Therefore, they did not allow identification of the spatio-temporal dynamics of the complete metabolic machinery that is required for nectar production. In order to circumvent the problems that arise from the lack or small size of nectaries in most genetically tractable model species, we used a proteomics approach in combination with biochemical analyses and degenerate primers to study the role of the extrafloral nectaries of the ant-plant, *Acacia cornigera*, in the synthesis of the major nectar components: sugars (Heil *et al.*, 2005b), amino acids (González-Teuber and Heil, 2009) and nectarins (Carter and Thornburg, 2004; González-Teuber *et al.*, 2009, 2010; Nepi *et al.*, 2011a, 2012; Zha *et al.*, 2012). We observed highly dynamic processes in the accumulation and activity of key metabolic enzymes in the nectary tissue, demonstrating that the entire metabolic machinery required for the synthesis of nectar is established and active directly before and during the hours of peak nectar secretion.

RESULTS

The study system

Acacia cornigera is an obligate ant-plant that bears conspicuous extrafloral nectaries on its petioles (Figure 1a). As long as the plants remain inhabited by mutualistic ants, these nectaries secrete large quantities of a biochemically complex EFN with a predictable and sharp diurnal peak, i.e. only between 8:00 and 10:00 am (Figure 1b). Secretion rates decrease dramatically from one day to the next as soon as EFN consumption ceases (Heil *et al.*, 2000, 2004). Although production of EFN in most species represents a jasmonic acid (JA)-dependent induced response to herbivory (Heil, 2011), EFN secretion by *A. cornigera* is not induced by herbivory or JA application (Heil *et al.*, 2004) (Figure 1b). We used this independence of nectar secretion

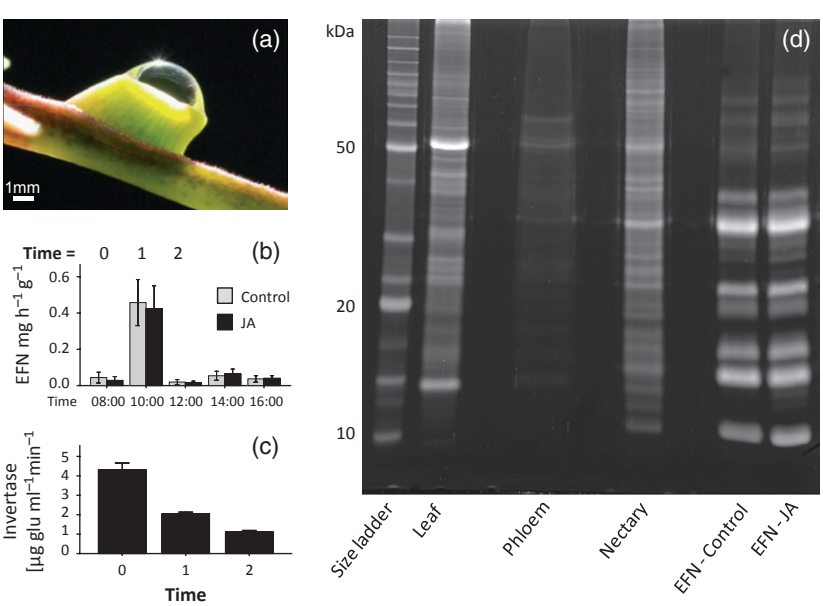

Figure 1. The nectary is the major site of synthesis of important nectar components.
(a) Extrafloral nectary of *Acacia cornigera*.
(b) Mean diurnal secretion activity ± SE (mg soluble solids secreted per hour and g leaf dry mass) of extrafloral nectar (EFN) (n = 12).
(c) Mean invertase activity ± SE (µg glucose released per minute per ml, measured at 340 nm), n = 5 replicates before (t = 0), during (t = 1) and after (t = 2) active EFN secretion.
(d) Proteomes obtained via SDS–PAGE for leaf tissue, phloem exudate, nectary tissue and secreted EFN with and without prior application of JA. The result shown is a representative example for n = 3 replicates per sample type.

on external factors that are related to herbivory and its short diurnal peak to search for enzymes and nectarins that accumulate in the nectary in a temporal pattern directly related to the active secretion.

Nectar is not secreted phloem sap

Invertase (EC 3.2.1.26, β–fructofuranosidase, catalysing the hydrolysis of sucrose to glucose and fructose) was suggested many years ago as an enzyme with a general role in nectar secretion (Lüttge, 1961). The highest level of invertase activity in the *A. cornigera* extrafloral nectary was observed in the hours directly before the secretion process began (at time $t = 0$, Figure 1c). Gas chromatography/electron impact mass spectrometry (GC-EIMS) confirmed the dominance of sucrose in the phloem (2.0 ± 0.8 mg g^{-1} dry mass, $n = 3$), whereas the EFN contained fructose and glucose, but no sucrose (180 ± 4.1 mg g^{-1} fructose and 400 ± 27 mg g^{-1} glucose, $n = 3$). By contrast, the amino acid profiles of phloem exudates and EFN were similar at the qualitative and quantitative level (Table S1).

Next, we compared the proteomes of leaves, phloem exudates, nectary and EFN using one-dimensional SDS–PAGE, tryptic digestion and subsequent analysis of the resulting peptides using nano-electrospray liquid chromatography/tandem mass spectrometry (nanoLC-MS/MS). Although approximately 100 times more phloem sample than EFN sample was loaded onto the gel, absolute protein quantities in the phloem sample were very low (Figure 1d). None of the peptides obtained gave hits to any *A. cornigera* nectarin (González-Teuber *et al.*, 2009), and only two of the phloem proteins were also detected in the nectary tissue (Tables S2 and S3). Thus, whereas little overlap was found among the proteomes of leaves, phloem exudate and nectar, the proteome of the secreted EFN represented essentially a subset of the nectary proteome (Figure 1d), i.e. most (if not all) nectarins were found in the nectary tissue before secretion. Finally, the EFN proteome was highly similar among control plants and JA-treated plants (Figure 1d). Because plant tissues ususally respond to JA application with dramatic changes in their gene expression and thus proteomic patterns, this finding demonstrates the independence of the EFN proteome from general expression patterns in the rest of the plant.

Proteomic dynamics during peak secretion

We followed the development of the nectary proteome by extracting proteins from the nectary tissue and separating them via two-dimensional gel electrophoresis (2DE; Figure 2a–c). Tandem mass spectrometric (MS/MS) analysis of 592 quantitatively dominant protein spots was performed independently for the three time points, and revealed 203 annotated proteins (Figures 2 and 3, and Table S3), the majority of which represented enzymes of carbohydrate metabolism (9% of the spots; e.g. invertase and sucrose synthase) and metabolism of amino acids and proteins (26%, e.g. methionine synthase, glutamine synthetase, proteolytic enzymes). Further quantitatively important functional groups included proteins related to other metabolic processes (9%) and defence and stress (23%, e.g. heat shock protein 70, trypsin inhibitor) (Figure 3). Pathogenesis-related (PR) proteins (van Loon and van Strien, 1999) were particularly common (Figure 2d,e), which explains their dominance in the secreted EFN (González-Teuber *et al.*, 2009, 2010). An invertase with 90% similarity to AtCWINV4 was detected, and temporal patterns in its absorbance/optical density (Figure 2e) resembled those of invertase activity (Figure 1c).

The optical densities of the 48 dominant protein spots were quantified using the program 2D Image Master in Coomassie-stained gels, and were found to be highest before secretion ($t = 0$), diminished during secretion ($t = 1$) and reduced after secretion ($t = 2$) (Figure 2d,e and Table S4). In general, a significant reduction in optical densities during the secretion process was observed for enzymes involved in sucrose hydrolysis (cell-wall invertase) and amino acid metabolism (methionine synthetase) and for secreted nectarins (González-Teuber *et al.*, 2009, 2010), such as thaumatin-like protein and β–1,3–glucanase (Figure 2d,e and Table S4). By contrast, enzymes involved in the degradation of proteins remained quantitatively constant until after the secretion peak. The nectary proteome was mainly composed of enzymes that are directly involved in the synthesis of important nectar components (hexoses, amino acids and nectarins) and the secreted nectarins themselves, many of which accumulated immediately before the active secretion process and quantitatively decreased during and after the active secretion process.

Proteolytic activity

The 2DE and subsequent MS/MS analysis revealed numerous proteolytic enzymes (13%, e.g. proteases, ubiquitin-related proteins and a subunit of the proteasome, see Table S3), many of which remaining at stable or slightly increasing abundances over the secretion peak. Enzymes with proteolytic activity are common in plants and play multiple roles, including defence against herbivores and pathogens, mobilization of storage proteins, liberation of amino acids and the degradation of proteins (Vierstra, 1996; Muntz *et al.*, 2001; Schaller, 2004). We quantified trypsin- and chymotryspin-like proteolytic activity in the nectary tissue (Figure 4), and observed that trypsin-like activity increased significantly over the secretion period [$P < 0.001$ for an effect of time on activity, univariate ANOVA, $n = 5$; all three times differed at $P < 0.05$ according to an Least Significant Difference (LSD) *post hoc* test]. Chymotrypsin-like activity increased from $t = 0$ to $t = 1$, and then remained stable ($P < 0.001$ for an effect of time on activity, univariate ANOVA, $n = 5$; $t = 0$ different at $P < 0.05$ from $t = 1$

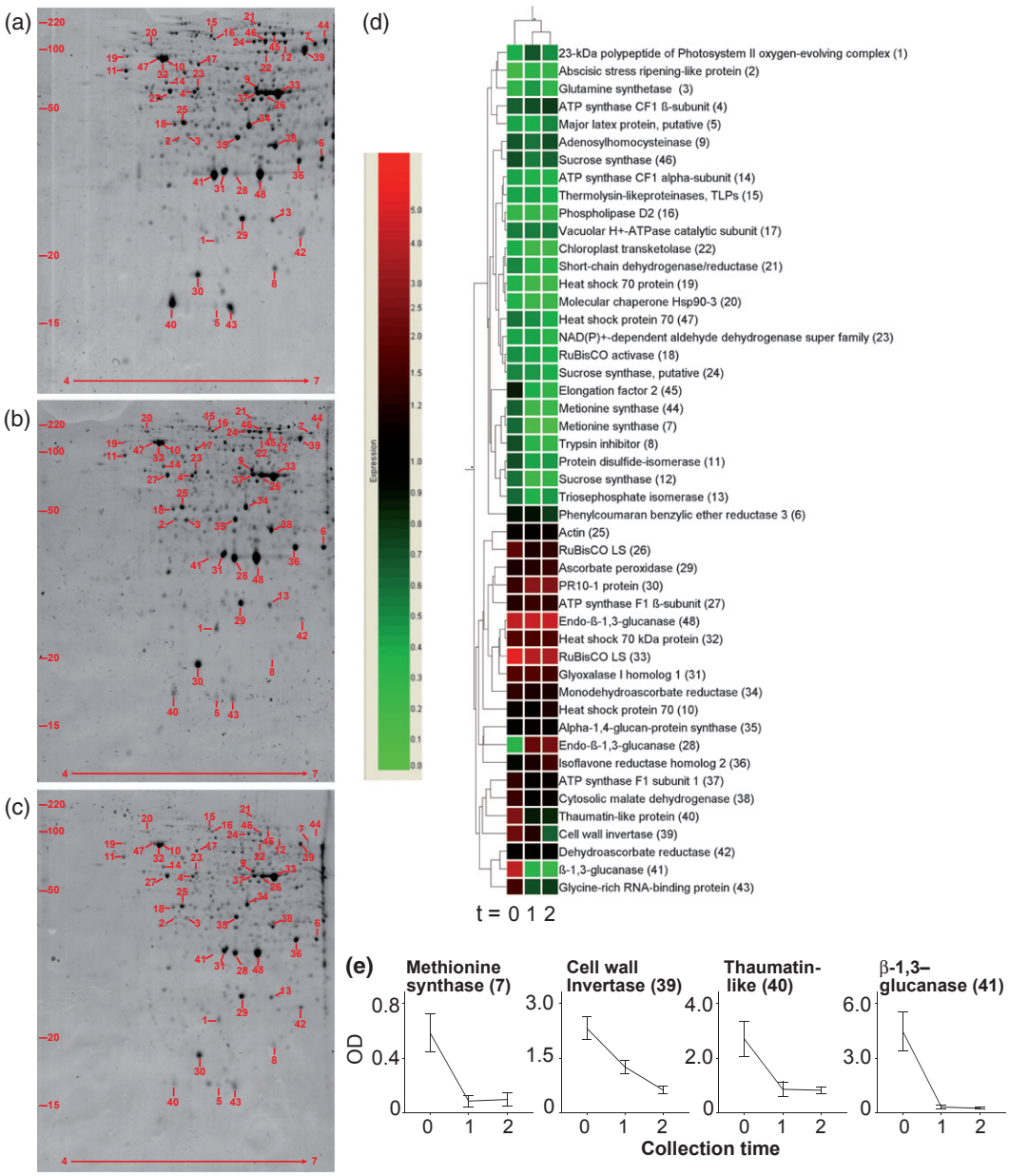

Figure 2. Diurnal proteomic dynamics in the *Acacia cornigera* extrafloral nectary.
(a–c) Nectary proteome (2DE, only pH 4-7 shown) before (t = 0) (a), during (t = 1) (b) and after (t = 2) (c) active EFN secretion.
(d) Heat map of optical densities (means of n = 3 replicates per time point) for the functionally and quantitatively most important proteins.
(e) Optical densities (OD, means ± SE) of four representative proteins involved in amino acid metabolism and sucrose hydrolysis, or representing dominant nectarins. See Figures S1–S3 for full proteomes (pH 3-10).

and t = 2 according to an LSD *post hoc* test). Thus, the proteolytic activity increased during secretion and was highest after the secretion peak.

Tissue-specific gene expression

We aimed to identify the site of synthesis of important sugar metabolic enzymes (sucrose synthetase and invertase) and some nectarins [invertase (Heil *et al.*, 2005b) and β–1,3 –glucanase, PR10 and thaumatin-like protein (González-Teuber *et al.*, 2009)] by demonstrating expression of the corresponding genes in the nectary tissue. The isolation of specific members of gene families in species with no genetic information available typically involves use of degenerate primers for regions that are conserved across many members of the gene family of interest. We selected six functionally important genes (the sugar metabolic genes invertase *CWIN4* and sucrose synthase *SS1*, and the dominant nectarins β–*1,3–glucanase*, *endo*-β–*1,3–glu-*

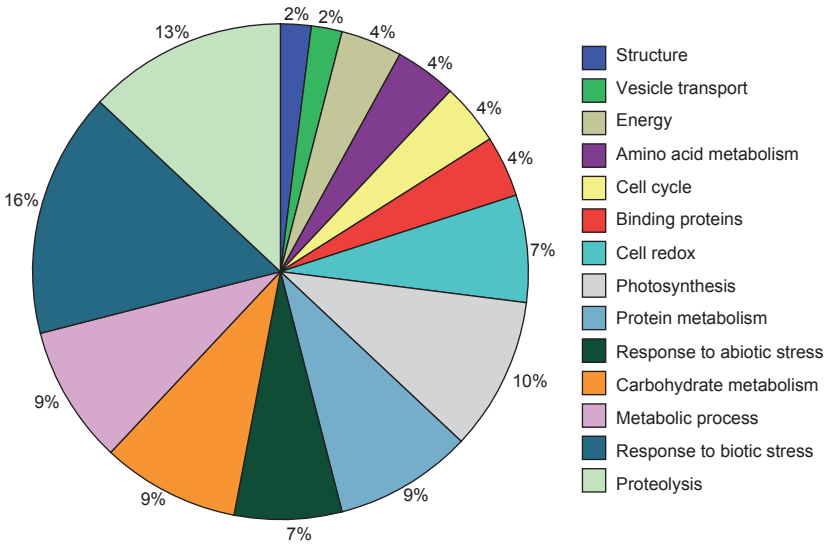

Figure 3. Functional categories of nectary proteins.
Pie chart illustrating the major functional classes of proteins in the *Acacia cornigera* extrafloral nectary (percentages of annotated proteins).

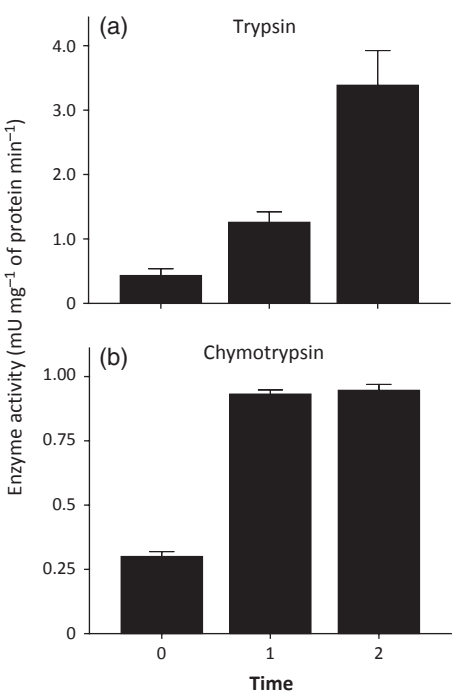

Figure 4. Proteolytic activity in the nectary tissue.
Proteolytic activity [trypsin-like activity (a); chymotrypsin-like activity (b)] was quantified before (t = 0), during (t = 1) and after (t = 2) the diurnal secretion peak, and shown in mUnits per gram protein per min. Values are means and SE (n = 5 replicates). The experiment was repeated five times with biologically independent samples, which gave similar results.

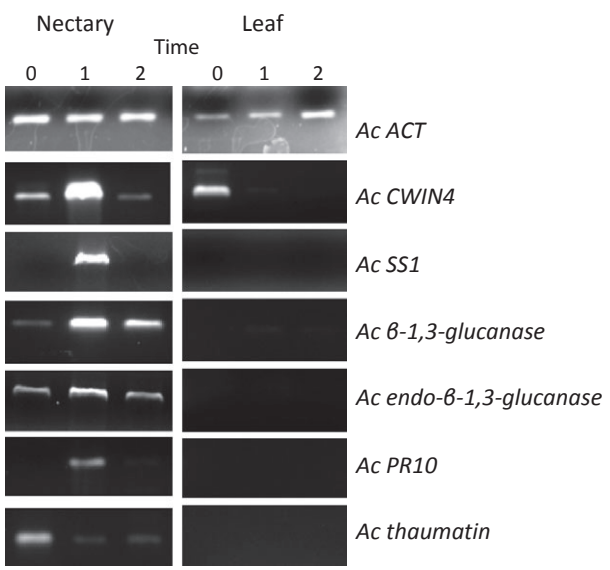

Figure 5. Expression of functionally important genes.
Expression (bands obtained via PCR) is shown for the housekeeping gene actin (*ACT*), genes central to sugar metabolism (invertase, *CWIN4*; sucrose synthase, *SS1*) and genes encoding the PR proteins β–1,3–glucanase, endo-β–1,3–glucanase, PR10 and thaumatin-like protein), in nectary tissue and in leaves before (t = 0), during (t = 1) and after (t = 2) active EFN secretion. All PCR experiments were repeated (n = 3) with similar results.

canase, *PR10* and *thaumatin*; Table S5). Degenerate PCR based on mRNA extracted from nectary and leaf tissues indicated that all of these genes were expressed in the nectary tissue, and most of them exclusively so (Figure 5). Only *CWIN4* was expressed in the leaves as well, and only before EFN secretion (Figure 5). The identity of all PCR products was confirmed by sequencing (Table S6). Genes encoding major nectarins and sugar metabolic genes are expressed in the nectary itself rather than in the leaves, and are at their highest levels during active secretion.

DISCUSSION

The secretion of nectar represents a common plant trait of high ecological, evolutionary and economic importance (Brandenburg *et al.*, 2009; Heil, 2011). However, the mecha-

nisms by which plants adjust nectar secretion rates at the genetic or phenotypic level to environmental factors and current requirements remain to be elucidated. Our understanding of the enzymatic and genetic mechanisms that control these adaptive responses remains rudimentary, most likely because research into the mechanisms that control nectar production is impaired by the lack of conspicuous nectaries in most genetically tractable species. The highly predictable short-term changes in secretion activity (Figure 1) make the extrafloral nectaries of *A. cornigera* an ideal model system to study any changes in the nectary proteome and gene expression patterns that are directly related to the secretion activity at a high temporal resolution.

Nectar and phloem sap significantly differed in their chemical composition, particularly in terms of hexoses and nectarins. These differences between EFN and phloem exudate exclude the phloem as a direct source of the sugars and proteins that are secreted into the EFN. A cell-wall invertase of Arabidopsis (*AtCWINV4*) is the only gene known to encode a metabolically active enzyme with an essential role in FN secretion (Ruhlmann *et al.*, 2010). We found the highest invertase activity directly before active secretion, and its expression in the nectary tissue was highest during the concurrent secretion process. Transcriptomic studies also found invertases to be over-expressed compared to leaf tissue in both floral and extrafloral nectaries (Kram *et al.*, 2009; Escalante-Perez *et al.*, 2012). All these observations underline the importance of invertases in the secretion of nectar, probably because they are required for unloading of sucrose from the phloem and for secretion of hexose-rich nectars (Heil, 2011). Moreover, the stability of the EFN proteome after JA treatment (Figure 1d) was in accordance with the insensitivity of the EFN quantities to external application of JA (Heil *et al.*, 2004). Hundreds or thousands of genes change their expression in response to exogenous JA in most plant tissues (Chini *et al.*, 2007; Thines *et al.*, 2007), and *A. cornigera* leaves exhibit a functioning octadecanoid signalling pathway, elevating their endogenous JA levels in response to damage (Heil *et al.*, 2004). All these observations underline the independence of the regulatory pathways in the nectary from those in the rest of the leaf.

Taken together, our results demonstrate that the nectary represents a metabolically independent organ, and that most synthetic processes that are required for production of important nectar components occur in the nectary itself. Therefore, we focused on the proteomic dynamics in the nectary tissue and found numerous biosynthetic enzymes responsible for the synthesis of amino acids, sugars and proteins, and the secreted nectar proteins themselves. Most anabolic enzymes were present at high optical densities before secretion and decreased during and after the secretion peak (Figures 2 and 3 and Figures S1–S3), but proteolytic enzymes showed the opposite pattern (Figure 4). The presence of numerous proteolytic enzymes (13%, e.g. proteases, ubiquitin-related proteins and a subunit of the proteasome, see Table S3) is consistent with the proteolytic activity in the nectary, which reached highest overall values after the secretion peak (Figure 4). Several proteolytic genes appeared down-regulated in poplar extrafloral nectaries in comparison with leaves, whereas a serine carboxypeptidase (PtpAffx.80486.1.A1_at) was up-regulated (Escalante-Perez *et al.*, 2012). Similarly, multiple serine proteases and ubiquitin-like proteins were over-expressed in floral nectaries of Arabidopsis (Kram *et al.*, 2009). Proteolytic enzymes may play multiple roles in the secretion process, including protection from infection and the liberation of amino acids. However, their high activity in the hours directly after the secretion peak is more consistent with the interpretation that proteolysis is involved in subsequent removal of enzymes from the nectary tissue (Figure 2e). Thus, the activity of proteolytic enzymes in the extrafloral nectaries of *A. cornigera* appears to be involved in the termination of nectar production.

We present here proteomic information from nectary tissue at various stages of the diurnal secretion peak, consider the synthesis of three classes of major nectar components, and report on short-term dynamics in proteomic patterns that are directly related to the active nectar secretion process. How representative are these findings for the mechanisms that underlie nectar secretion in general? Our observations in the extrafloral nectary of *A. cornigera* are in agreement with previous analyses of floral nectaries, which demonstrated the essential role of an extracellular invertase in nectar secretion in Arabidopsis flowers (Kram *et al.*, 2009; Ruhlmann *et al.*, 2010), and indicated that *NECTARIN* genes are expressed in the nectary tissue of ornamental tobacco (Carter and Thornburg, 2003, 2004; Liu *et al.*, 2009; Liu and Thornburg, 2012). However, these studies used floral nectaries with an ontogenetically fixed secretion program, and hence were limited to time scales with an order of magnitude of days. For example, the phase dominated by starch anabolism in ornamental tobacco flowers lasts approximately 8 days (Liu and Thornburg, 2012).

Using extrafloral nectaries with a short diurnal secretion peak, we demonstrate that gene expression, protein accumulation and invertase activity in the nectary are highly dynamic and coincide at the time scale of hours with the concurrent secretion activity. We conclude that accumulation of metabolically important enzymes and nectarins in the nectary immediately before the active secretion process is required for the production of extrafloral nectar. The proteomic approach helped to identify metabolically important enzymes in a non-model plant growing in the wild, and degenerate primers were successfully used to demonstrate tissue-specific expression of the corresponding genes in a species for which no genetic information is available. Similar approaches applied to other species will provide a more complete understanding of the metabolic processes that

control nectar quantity and quality in the multitude of plant species for which this process is essential.

EXPERIMENTAL PROCEDURES

Plant material and study site

Acacia cornigera L. Willdenow (Mimosoidea, Fabaceae) (Janzen, 1974) plants were investigated in the south of Mexico (approximately 15°55′N and 097°09′W). All plants grew in full sun, had not been visibly damaged by pathogens or herbivores, and were inhabited by the mutualist ant *Pseudomyrmex ferrugineus* (Ward, 1993). For an initial screening of the diurnal secretion pattern, the two youngest branches on 12 plants were identified: one of these branches received an aqueous 1 mM solution of JA, the other received the same amount of distilled water. The next day, EFN secretion was quantified every 2 h as the total amount of soluble solids (Heil *et al.*, 2004). This experiment was repeated at two sites on three further days (n = 5 plants each). The resulting EFN secretion curve (Figure 1b) determined the sampling times for all biochemical, enzymatic, proteomic and gene expression studies (n = 3 biologically independent samples for all experiments). Samples of nectary tissue, leaves, EFN and phloem exudates were collected on dry ice before (t = 0: 06:00–08:00 am), during (t = 1: 08:00–10:00 am) and after secretion (t = 2: 10:00–12:00 am), and then stored in liquid nitrogen.

Collection of phloem exudates and EFN

Extrafloral nectar (EFN) was collected as described previously (Heil *et al.*, 2004). For phloem collection, the cut rachis of each leaf was immediately transferred into the pre-incubation chamber of a home-made Plexiglas device (Deeken *et al.*, 2008; Plexiglass, http://www.plexiglas-shop.com) containing 1 mM Na_2EDTA buffer, pH 7.5, and protease inhibitor (Roche complete mix; Roche, http://www.roche.com/), osmotically adjusted to 260 mOsmol with sorbitol. The petioles were re-cut under EDTA buffer to prevent sieve-tube embolism (Deeken *et al.*, 2008). The pre-incubation chamber was washed by sucking EDTA buffer through the chamber and replacing it with fresh buffer. Leaves were illuminated with natural light, and incubated at ambient temperature in CO_2- and H_2O-saturated air (0.1 M $NaHCO_3$). After 1.5 h bleeding, the EDTA buffer containing phloem exudates was removed, frozen and subsequently lyophilized.

Enzymatic activities

The activity of *A. cornigera* cell-wall invertase from nectaries was assayed as described previously (Ruhlmann *et al.*, 2010) with modifications. Briefly, 0.5 g of ground sample was mixed with 500 μl ice-cold 50 mM HEPES/NaOH (pH 8.0, containing 5 mM $MgCl_2$, 2 mM EDTA, 1 mM $MnCl_2$ and 1 mM $CaCl_2$). Samples were incubated on ice for 10 min and then centrifuged at 13 000 ***g*** for 10 min at 4°C. The supernatant was discarded, and pellets containing the cell walls with associated invertases were washed three times with 500 μl extraction buffer (5 sec) by resuspension and centrifugation as above. Finally, pellets were washed with 500 μl of 80 mM sodium acetate (5 sec), pH 4.8. Invertase activity was measured as described previously (Heil *et al.*, 2005a) with modifications. Briefly, 300 μl of 80 mM sodium acetate (pH 4.8) were added to the pellets, and the mixture was incubated at 37°C. Every 5 min, 20 μl of samples were taken and mixed with 200 μl of HK reaction solution (glucose (HK) assay kit; Sigma-Aldrich, http://www.sigmaaldrich.com). After reaching steady state (Heil *et al.*, 2005a), 100 μl of an aqueous 100 mM solution of sucrose was added, and the absorption was measured at 340 nm in a μQuant® microplate reader every 5 min for 30 min (Biotek, http://www.biotek.com).

For quantification of trypsin- and chymotrypsin-like activity, soluble proteins were extracted by grinding 0.1 g tissue in 300 μl ice-cold 50 mM phosphate buffer (pH 6.0) with 0.02% polyvinylpyrrolidone. The mixture was incubated for 15 min at 4°C in a mixer; after this time, samples were centrifuged (10 000 ***g***) for 15 min at 4°C, and supernatants were placed in new tubes and stored at −70°C. Then the activity of serine proteases such as trypsin and chymotrypsin was estimated in photometric assays using the chromogenic substrates *N*–α–benzoyl-D,L–arginine-*p*–nitroanilide (BApNA) for trypsin-like activity and *N*–succinyl-Ala-Ala-Pro-Phe-*p*–nitroanilide (SAAPPpNA) for chymotrypsin-like activity (all materials were purchased from Sigma-Aldrich). All substrates were used at a final concentration of 0.01 M dissolved in DMSO (4.35 mg ml^{-1}). The final volume of the reaction mixture was adjusted to 220 μl by varying the volume of the buffer (0.1 M Tris/HCl, pH 7.4). Aliquots of 20 μg protein were used, and the mixture was pre-incubated for 15 min at 37°C. Then 20 μl of the specific substrate was added, and changes in absorbance were measured at 405 nm using a μQuant® microplate reader every 10 min for 1 h.

Protein extraction for 2DE

For analysis of phloem proteins (Giavalisco *et al.*, 2006), we used 25 mg of lyophilized phloem exudate to which 800 μl of a mixture of acetone/methanol/dithiothreitol (90%, 10%, 10 mM) and 200 μl water were added (final volume 1 ml). Samples were incubated overnight at −20°C, and then centrifuged at 14 000 ***g*** at 4°C for 15 min. Pellets were washed twice in 100% of acetone (5 sec) and centrifuged as above.

Nectary tissue was ground in liquid nitrogen. To extract proteins (Wang *et al.*, 2006), 0.1 g of sample was placed in 1 ml 10% trichloroacetic acid/acetone and centrifuged at 16 000 ***g*** for 3 min (4°C). The pellet was washed with 80% methanol/0.1 M ammonium acetate (5 sec) and centrifuged again. The pellet was then washed three times in a 1:1 mixture of 0.4 ml phenol (Tris-buffered, pH 8.0; Sigma-Aldrich) and 0.4 ml of dense SDS buffer (30% sucrose, 2% SDS, 0.1 M Tris/HCl, pH 8.0, 5% β–mercaptoethanol) and once each with 100% methanol and 80% acetone. After centrifugation, the recovered proteins were dissolved in 2DE rehydration solution (7 M urea, 2 M thiourea, 2% CHAPS, 2% IPG buffer, 0.002% bromophenol blue and 5% complete inhibitor) containing protease inhibitor (Roche complete mix).

Two-dimensional gel electrophoresis

The protein content was quantified using a Bradford protein concentration kit (Bio–Rad, www.bio-rad.com). Protein (300 μg per sample) was re-suspended in 250 μl rehydrating solution (7 M urea, 2 M thiourea, 2% CHAPS, 65 mM dithiothreitol, 2% IPG buffer, pH 3–10), loaded onto 13 cm IPG dry strips, and allowed to rehydrate for 15 h. The proteins were then separated by two-dimensional gel electrophoresis. Protein bands of interest were cut from the gel matrix, and tryptic digestion was performed as described previously (Shevchenko *et al.*, 2006).

LC-MS/MS and data analysis

Protein digests were analysed by nanoelectrospray liquid chromatography/tandem mass spectrometry (nanoLC-MS/MS) on a Synapt HDMS quadrupole time-of-flight mass spectrometer

(Waters, http://www.waters.com) (González-Teuber *et al.*, 2009, 2010). Data were processed using PLGS software version 2.4 (Waters), with baseline subtraction, smoothing and deisotoping of acquired spectra. MS/MS spectra were searched against a sub-database containing common background proteins (human keratins and trypsin) to exclude the proteins that produce these spectra from *de novo* sequencing. The search parameters were: mass tolerances for precursor and fragment ions, 15 ppm and 0.03 Da, respectively; instrument profile, ESI-Trap; fixed modification, carbamidomethyl (cysteine); variable modification, oxidation (methionine); up to one missed cleavage was allowed. Spectra that remained unmatched by database searching were interpreted *de novo* to yield peptide sequences. A 0.002 Da mass deviation for *de novo* sequencing was allowed, and sequences with a ladder score exceeding 40 were selected for homology-based searching using an MS BLAST program (Shevchenko *et al.*, 2001) installed on an in-house server. MS BLAST searches were performed against the comprehensive NCBInr database (http://www.ncbi.nlm.nih.gov/, updated on 10 August 2011) using previously described settings (Shevchenko *et al.*, 2001). In parallel, pkl files of MS/MS spectra were generated and searched against the NCBInr database (updated 11 September 2011, installed on a local server) using MASCOT version 2.3 (http://www.matrixscience.com) and the above-described search parameters. Hits were considered as confident if at least three peptides were matched with ion scores above 30, or proteins were identified by one or two peptides with a score of 55 or better.

cDNA synthesis

RNA was extracted using a buffer consisting of 2% w/v Cetyltrimethyl Ammonium Bromide (CTAB) (Sigma), 2% w/v polyvinylpyrrolidone K25 (Sigma), 100 mM Tris/HCl (pH 8.0), sodium EDTA (pH 8.0), 2.0 M sodium EDTA (pH 8.0), 25 mM NaCl. All solutions used, except Tris/HCl, were prepared using Millipore purified water (Millipore, www.millipore.com), treated with diethylpyrocarbonate (Sambrook *et al.*, 1989) and autoclaved. Extraction buffer (600 μl) was added to 50 mg of frozen ground plant material with β–mercaptoethanol to a final concentration of 2% v/v. This mixture was extracted twice using an equal volume of chloroform: isoamyl alcohol (24:1). The water phase was cleaned using LiCl, sodium acetate and ethanol precipitation steps. The mRNA was subsequently isolated using a Dynabeads mRNA DIRECT kit (Vector Nti software version 11.0. www.invitrogen.com) and used for first-strand cDNA generation using the SuperScript First-Strand Synthesis System for RT–PCR (Invitrogen) according to the manufacturer's instructions.

PCR and sequencing with degenerate primers

Degenerate primers (Table S5) were designed using Vector Nti software (Invitrogen). Degenerate PCR was performed using Platinum PCR Supermix (Invitrogen) with a ramp program as follows: 3 min denaturation starting at 95°C, followed by 40 cycles each consisting of 30 sec denaturation at 94°C and annealing at 48°C with a ramp of 0.2°C sec^{-1} and an increase of 8 sec per cycle, and 1 min elongation at 72°C. The PCR products were analysed by agarose 2% electrophoresis, and sent for sequencing to Laboratorio nacional para la diversidad-Irapuato.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.

Figure S1. Proteome of the *Acacia cornigera* extrafloral nectary directly before the secretion peak.

Figure S2. Proteome of the *Acacia cornigera* extrafloral nectary during the secretion peak.

Figure S3. Proteome of the *Acacia cornigera* extrafloral nectary immediately after the secretion peak.

Table S1. Relative composition of sugars and amino acids in phloem exudate and extrafloral nectar of *Acacia cornigera.*

Table S2. Proteins detected in phloem exudate of *A. cornigera.*

Table S3. Annotation results for proteins from the nectary proteome at $t = 0$, 1 and 2.

Table S4. Development in optical densities of nectary proteins from before secretion to after secretion.

Table S5. Sequences of degenerate primers and actin primers.

Table S6. Sequences and annotation results for degenerate PCR products.

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