THE UNIVERSITY *of* EDINBURGH

Edinburgh Research Explorer

Proteomics and post-secretory content adjustment of *Nicotiana tabacum* nectar

Citation for published version:

Ma, X-L, Milne, R, Zhou, H, Song, Y-Q, Fang, J-Y & Zha, H-G 2019, 'Proteomics and post-secretory content adjustment of *Nicotiana tabacum* nectar', *Planta*, vol. 250, pp. 1703-1715. <https://doi.org/10.1007/s00425-019-03258-4>

Digital Object Identifier (DOI):

[10.1007/s00425-019-03258-4](https://doi.org/10.1007/s00425-019-03258-4)

Link:

[Link to publication record in Edinburgh Research Explorer](#)

Document Version:

Peer reviewed version

Published In:

Planta

Publisher Rights Statement:

This is a post-peer-review, pre-copyedit version of an article published in [insert journal title]. The final authenticated version is available online at: [http://dx.doi.org/\[insert DOI\]](http://dx.doi.org/[insert DOI])

General rights

Copyright for the publications made accessible via the Edinburgh Research Explorer is retained by the author(s) and / or other copyright owners and it is a condition of accessing these publications that users recognise and abide by the legal requirements associated with these rights.

Take down policy

The University of Edinburgh has made every reasonable effort to ensure that Edinburgh Research Explorer content complies with UK legislation. If you believe that the public display of this file breaches copyright please contact openaccess@ed.ac.uk providing details, and we will remove access to the work immediately and investigate your claim.

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19

Proteomics and post-secretory content adjustment of
***Nicotiana tabacum* nectar**

Authors: Xue-Long Ma¹, Richard I Milne², Hong-Xia Zhou¹, Yue-Qin Song¹, Jiang-Yu Fang¹, Hong-Guang Zha^{1*}

1. College of Life and Environment Sciences, Huangshan University, Huangshan, China
2. Institute of Molecular Plant Sciences, University of Edinburgh, Edinburgh, UK

Running title: *Nicotiana tabacum* nectar proteome and post-secretory adjustment

CORRESPONDENCE:

Dr. Hong-Guang Zha

zhahg@hsu.edu.cn

arctos725@outlook.com

Abstract

Main conclusion

The tobacco nectar proteome mainly consists of pathogenesis-related proteins with two glycoproteins. Expression of nectarins was nonsynchronous, and not nectary specific. After secretion, tobacco nectar changed from sucrose-rich to hexose-rich.

Floral nectar proteins (nectarins) play important roles in inhibiting microbial growth in nectar, and probably also tailoring nectar chemistry before or after secretion; however very few plant species have had their nectar proteomes thoroughly investigated. Nectarins from *Nicotiana tabacum* (NT) were separated using two-dimensional gel electrophoresis, and then analyzed using mass spectrometry. Seven nectarins were identified: acidic endochitinase, β -xylosidase, α -galactosidase, α -amylase, G-type lectin S-receptor-like serine/threonine-protein kinase, pathogenesis-related protein 5, and early nodulin-like protein 2. An eighth nectarin, a glycoprotein with unknown function, was identified following isolation from NT nectar using a Qproteome total glycoprotein kit, separation by SDS-PAGE, and identification by mass spectrometry. Expression of all identified nectarins, plus four invertase genes, were analysed by qRT PCR; none of these genes had nectary-specific expression, and none had synchronous expression. The total content of sucrose, hexoses, proteins, phenolics and hydrogen peroxide were determined at different time intervals in secreted nectar, both within the nectar tube (*in vivo*) and following extraction from it during incubation at 30 °C for up to 40 h in plastic tubes (*in vitro*). After secretion, the ratio of hexose to sucrose substantially increased for *in vivo* nectar, but no sugar composition changes were detected *in vitro*. This implies that sucrose hydrolysis *in vivo* might be done by fixed apoplastic invertase. In addition, both protein and hydrogen peroxide levels declined *in vitro* but not *in vivo*, implying that some factors other than nectarins act to maintain their levels in the flower, after secretion.

Keywords: Floral nectar; Gene expression pattern; Glycoprotein; Invertase; Nectarin; Post-secretory adjustment; Tobacco

50 Abbreviations

51

52 2-DE Two-dimensional gel electrophoresis

53 ENOD-2 Early nodulin-like protein 2

54 HPLC High-performance liquid chromatograph

55 MS Mass spectrometry

56 NT *Nicotiana tabacum*

57 PR Pathogenesis-related

58

Introduction

More than 80% of angiosperms are estimated to be pollinated by animals, which are usually rewarded with floral nectar or pollen during visits to flowers (Ollerton et al. 2011). For some animal pollinators, such as honeybirds, moths, and butterflies, nectar is the only reward to promote their visitation. Hence nectar is a key determinant that mediates the interactions of plants with pollinators and defenders, and therefore also enhances plant reproductive success (Heil 2011; Pyke 2016; Roy et al. 2017). Coevolutionary relationships have been assumed to exist between nectar traits and pollinator type (Pyke 2016; Stevenson et al. 2017; Parachnowitsch et al. 2018). Carbohydrates, mainly sucrose, fructose, and glucose, are the major constituents of nectar and serve in the attraction and nutrition of pollinators. Floral nectar also contains other compounds, e.g. amino acids, proteins, several classes of secondary metabolites, minerals, scents and colours (Nicolson and Thornburg 2007; Heil 2011). Nectar chemical composition varies between species, and this is an important aspect of pollination syndromes, because it strongly affects the identity and behaviour of the attracted animals.

It has been known for a long time that floral nectar contains proteins, but only very recently have for researchers began to investigate the nectar proteome, or the activity of nectar proteins (nectarins). This is largely because most of the model plants used over recent decades, such as *Arabidopsis thaliana* (Kram and Carter 2009), *Solanum tuberosum* (Buchmann and Cane 1989), *Glycine max* (Horner et al. 2003), *Oryza sativa*, and *Zea mays* (Bernardello 2007), contain no nectar, or far too little for in-depth proteome analysis. Generally, the concentration of nectarins is less than 100 $\mu\text{g mL}^{-1}$, representing a minute component of the nectar (Nicolson and Thornburg 2007). However, in some species, such as *Mucuna sempervirens* (Fabaceae), nectarin concentration could reach up to 600 $\mu\text{g mL}^{-1}$, making it the second largest component in nectar (Liu et al. 2013).

Nectar proteomes from less than 20 plant species have been thoroughly investigated, and all were shown to be small, containing distinct, consistent arrays of proteins (Heil 2011; Roy et al. 2017). Peumans et al. (1997) identified the first two nectar proteins from *Allium porrum*: lectin and alliinase. From then on, it was realized that, despite the nutritional aspect of nectar, nectarins can be enzymes and hence catalyse reactions in nectar. So far, more than 20

nectarins have been identified from different species, and these identified nectarins can be classified into several classes. The primary class among these is glycoside hydrolase, which includes glucanase, galactosidase, xylosidase, and chitinase (Roy et al. 2017). Notably, other than occasional reports of xylose (Jackson and Nicolson 2002), neither products nor substrates for these enzymes tend to be spontaneously present in floral nectar (e.g. glucan, galactose, chitin), indicating that they might target substrates introduced from outside the nectar, for example by microbes. It is also noticeable that these glycoside hydrolases identified from nectar, combined with other nectarins such as thaumatin, S-RNase, peroxidase, are all classified as pathogenesis-related (PR) proteins. Thus far, 10 of the 17 known groups of PR proteins have been identified in nectar from different species (Jain and Khurana 2018). Certain types of nectarins, such as chitinase and glucanase have been detected in most of the studied nectar samples, and overall the number of different nectarin proteins identified is surprisingly low. These factors both indicate that the nectar proteomes are likely to be conserved, and have evolutionary implications. Comparative nectar proteome studies between phylogenetically related species could test this idea, but have rarely been performed (Seo et al. 2013).

Microorganisms, yeast or bacteria, are commonly reported in nectar that is nutrient-rich (Álvarez-Pérez et al. 2019). Some of the nectar-inhabiting microorganisms have the potential for deleterious infections (Vannette and Fukami 2018). Given that most nectarins uncovered so far are glycoside hydrolase and PR proteins, the function of nectarins is likely to both limit microbial growth in nectar and adjust nectar chemistry for manipulating their animal mutualists' behaviour. It is well established that nectarins play a defensive role (Park and Thornburg 2009). Five nectarins identified in the floral nectar of an ornamental tobacco have been shown to be involved in a redox cycle that generates high concentrations of hydrogen peroxide, which limits microbial growth in nectar (Park and Thornburg 2009). Some isolated nectarins, e.g. a non-specific lipid transfer protein, showed strong and direct antifungal activity (Schmitt et al. 2018).

The presence of nectarins with enzymatic activities, especially the glycoside hydrolase and peroxidase activities identified in various nectar samples, indicates that nectar might not be a static product. The nectar composition could be modified by enzymes before and after

secretion. For example, apoplastic invertase (also known as extracellular or cell-wall invertase) was proved to be an important factor for nectar production in the Brassicaceae (Ruhlmann et al. 2010). Sucrose could be hydrolysed into glucose and fructose by invertase, altering nectar sugar composition (Heil 2011); this was detected in extrafloral nectar of myrmecophytic *Acacia* species (Heil et al. 2005) and in the floral nectar of *Cucurbita pepo* (Nepi et al. 2012). A lipase identified in *Jacaranda mimosifolia* nectar functions in the hydrolysis of nectar lipids (Kram et al. 2008). However, no post-secretory changes in nectar sugar composition could be detected in day- and night- flowering *Nicotiana* species (Tiedge and Lohaus 2018). Hence post-secretion alterations of nectar by nectarins may not be universal, and seem to vary between species, but general trends are as yet unclear.

Other unanswered questions about nectarins include whether the expression of nectarin genes are nectary specific, and if they are synchronously expressed. Furthermore, secreted proteins are usually glycosylated, and this might be an important post-translational modification of secretory proteins in all eukaryotes (Strasser 2016). Even though some nectarins (e.g. NEC 1, 4, and 5 from *Nicotiana langsdorffii* $\times$ *N. sanderae* (Park and Thornburg 2009) are glycoproteins, it is still unknown whether all nectarins have to be glycosylated when secreted.

In this study, we aim to elucidate the nectar proteome of *Nicotiana tabacum*, the well-known model species whose complete genome sequences have been assembled, annotated and published (Sierro et al. 2014). We will compare it with the previous reported nectar proteomes of its relatives, *N. attenuata* (Seo et al. 2013) and *N. langsdorffii* $\times$ *N. sanderae* (Carter et al. 1999) to test the hypothesis that the nectar proteome is evolutionarily conserved. Glycosylated nectarins will be isolated and identified. Nectar sugar, total phenolics, hydrogen peroxide, and nectarin content changes after secretion will be investigated.

Materials and methods

Nicotiana tabacum floral nectar collection and nectarin concentration

N. tabacum (NT) seeds were provided by Dr. Tao Liu (Yunnan Agricultural University, Kunming, China). Twenty NT plants grown under natural light in a greenhouse, located at Huangshan University (29° 41'N, 118° 17'E; Anhui province, China) were used in this study. Daily temperatures in the greenhouse fluctuated between 20 and 32 °C. NT flowers begin to secrete nectar from the stage S3 (Fig. 1) and this stage will last for about 4 h. At this stage, the NT corolla is half-opened and changes color to pink. Three pooled mixes of nectar (1, 2 and 3) were collected as follows.

For NT nectar proteomics analysis and glycoprotein isolation, raw nectar was collected from fully opened flowers (stage 4, Fig. 1) of the 20 NT plants using pipettes, from February to April in 2018, and all nectar was pooled giving a total volume of 10 mL, from around 300 flowers. The pooled sample was filtered through 0.22 µm syringe filters (EMD Millipore) to remove dirt, microorganisms, and pollen granules, and stored at -80 °C prior to use. Fresh NT nectar protein was concentrated by 40 times via ultracentrifuge using Amicon Ultra centrifugal filters (10-kD cutoff; EMD Millipore). Final protein concentration was assessed following Bradford (1976) with bovine serum albumin as a standard. This formed nectar mix 1.

Nectar mix 2 and 3 each comprised a total of 1.2 mL of nectar from around 40 flowers, gathered in the same way as mix 1 except that only young (stage 3) flowers were sampled for mix 2 and 3, and the filtering stage was omitted for mix 3, to check for an effect from microbes. In all these cases, pooling of nectar was used to avoid the confounding variable of variation between individuals.

In vivo nectar collection is described below.

Proteomic analysis of NT nectar

Two-dimensional gel electrophoresis (2-DE) coupled with mass spectrometry was conducted for the identification of NT nectarin. Prior to this analysis, the concentration of protein from nectar mix 1 was increased 40-fold via ultracentrifuge filtering using Amicon

Ultra centrifugal filters (10-kD cutoff; EMD Millipore). NT nectarin separation by 2-DE was accomplished as described by Zhou et al. (2018). Each test used 30 µg total NT nectarins, and samples were run in triplicate. After second-dimension electrophoresis, nectarins were visualized by Coomassie brilliant blue (CBB) G250 staining. Visible protein spots were manually excised from 2-DE gels and subjected to in-gel digestion using trypsin as the protease. The samples were analysed in a MALDI-TOF/TOF mass spectrometer (Model 5800, Applied Biosystems-Sciex). The combined mass spectrometry (MS) and tandem MS (MS/MS) peak lists were analysed using GPS (Global Proteome Server) Explorer Software 3.6 (Applied Biosystems) with a Mascot search engine (MASCOT version 2.3; Matrix Science, London, UK). The following settings were selected for searching: cysteine carbamidomethylation as fixed modifications; methionine oxidation as variable modifications; peptide mass tolerance of 50 ppm at the most and a general fragment mass tolerance of 0.5 Da. Protein identifications were accepted if they contained at least two identified peptides. According to the search engine, a score of 54 represents a significant identification ($P < 0.05$) when the database is restricted to the Viridiplantae taxonomy (NCBI nr 20170707). The mass proteomics data have been deposited to the ProteomeXchange Consortium (<http://www.proteomexchange.org/submission/index.html>) via the PRIDE partner repository (Vizcaino et al. 2016) with the data set identifier PXD011658.

NT nectar glycoprotein isolation and identification

Total glycoprotein was isolated from concentrated nectar mix 1, using a Qproteome total glycoprotein kit (Qiagen) according to the manufacturer's instructions. This kit contains lectins, concanavalin A (ConA) and wheat germ agglutinin (WGA), that can be used for a general enrichment of the total glycoproteins from nectar. At first, a 10 mL pooled NT nectar sample was concentrated to 150 µL using an ultracentrifuge as for 2-DE. Sugars in a sample can impede the binding of glycoprotein onto the lectin column; therefore sugars were removed by repeating the ultracentrifuge filtering step described above, three times with purified water, until the Brix value fell below two degrees (this was monitored using a low-volume hand-held refractometer; Eclipse, Bellingham & Stanley, Tunbridge Wells, UK). After that, concentrated NT nectarin solution was combined with the binding buffer from the

glycoprotein kit, along with protease inhibitor solution, before they were loaded onto the lectin column according to the manufacturer's instructions. Finally, the glycoproteins were eluted three times with 100 μ L of elution buffer ME from the kit, and pooled. The protein concentration was determined following Bradford (1976).

20 μ L of eluted NT nectar glycoproteins was incubated at 65 °C for 10 min in sample buffer under reducing conditions (with 0.1 M dithiothreitol in sample buffer) before being loaded into 12% (w/v) self-poured sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE), and separated using electrophoresis following Laemmli (1970). Concentrated NT nectarin was used as a reference sample. Prestained molecular weight protein markers were used as standards, and proteins were visualized by staining with CBB G250. On a parallel gel, the same set of samples was loaded, but protein detection was by a GelCode Glycoprotein Staining Kit (Thermo Fisher Scientific) according to the manufacturer's instructions.

Analysis of nectarin and invertase gene expression

Eight nectarin genes that were identified by the above proteomic analysis, plus four invertase genes that have been suggested to play a role in the hydrolysis of sucrose (apoplastic, vacuolar and cytosolic invertase, and apoplastic invertase Nin88; Ruhlmann et al. 2010; Tiedge and Lohaus 2018) were selected for relative expression analysis across plant organs and different flower stages. For each gene, total RNA samples were extracted using the RNeasy kit with DNase treatment (Qiagen) from root, stem, leaf, stalk, calyx, upper corolla (i.e. spreading part, beyond the tube, with pink pigment), lower corolla (i.e. corolla tube up to 1 cm from the top of the ovary, with dense trichomes inside), stamen, stigma, ovary, and nectaries at five developmental stages (S1~S5) (Fig. 1). All non-nectary tissues were chosen at the developmental stage S4 (flower fully opened). Tobacco flowers secrete nectar from stage S3 (first opening) until they are pollinated (Zha HG; personal observation). The quality and concentration of extracted RNA was assessed using a Nanodrop spectrophotometer (ND-2000, Thermo Fisher Scientific).

cDNA synthesis was performed according to the manual using the PrimeScript™ RT reagent Kit (Takara, Dalian, china) with oligo(dT) primer. Gene-specific primers (for 8 NT nectarins identified in this study, plus four invertases and a reference gene), were designed

according to the extracted gene sequences from the tobacco genome, as listed in Table S1. Quantitative PCR was performed with the LightCycler 96 system (Roche Applied Science) and a TB Green™ Premix Ex Taq™ II kit (Takara). The PCR conditions were as follows: 94 °C for 5 min and then 45 cycles of PCR (95 °C for 15 s, 55 °C for 15 s and 72 °C for 15 s). The abundance of transcripts was analysed using the relative quantitative ddCt method (Schmittgen and Livak 2008) with normalizing to the housekeeping genes: *EF-1α*. All qPCR experiments were repeated with three independent biological replicates, and amplicon specificity was checked by high-resolution melting curve analysis.

Post-secretory nectar sugar, total protein, hydrogen peroxide, and total phenolics content analysis

For *in vitro* analysis, nectar from nectar mix 2 was divided into five sets, each set comprising 8 samples (30 µL nectar in a plastic microtube). All these processes were done on ice. One set sample was stored at -80 °C immediately after allocation. The other four sets were placed in a shaking incubator, at 30 °C and 100 rpm for different time intervals (6, 16, 24, 40 h). The temperature of 30 °C was chosen because it was around the typical temperature for the glasshouses where our plants were grown. After incubation, the samples were stored at -80 °C prior to use. These experiments were repeated for nectar mix 3.

For *in vivo* analysis, individual nectar samples of at least 30 µL were generated, with each sample representing a specific time period after the onset of stage 3. A total of 100 NT flowers at stage 3 were identified and labelled at 16:00, on Apr. 2, 2018. Where 30 µL or more was available from a single flower, this formed a single sample; where flowers had <30 µL nectar, two or more flowers' nectar were combined to form a sample containing at least 30 µL in total. Otherwise, these samples were not pooled, filtered or concentrated. Samples were collected 15 at a time, at each of five time points (0, 6, 16, 24, 40 h after onset of stage 3), and in all cases stored immediately after collection at -80 °C, until they were used.

For each sample replicate from each analysis, each of total sugar concentration, sugar ratios, total phenolics, total protein, and hydrogen peroxide content were determined. For each sample, 5 µL nectar was used to determine the total sugar concentration (Brix value), obtained with a low-volume hand-held refractometer (Eclipse). The sugar ratio is defined as

the ratio by weight of sucrose to that of glucose and fructose combined, S/(G+F). Nectar was defined as hexose-rich if the sugar ratio was between 0.1 and 0.5, or sucrose-rich if it is 0.5-1.0 (Nicolson and Thornburg 2007). The determination of sugar ratios of NT nectar was performed with an EClassical 3100 high-performance liquid chromatograph (HPLC) (Elite, Dalian, China) equipped with a refractive index detector (RI-201H, Shodex, Japan) and a carbohydrate column (SC1011, Shodex). 2 μ L nectar was injected for each test, and distilled water was used as an eluent at a flow rate of 0.8 mL min⁻¹ at 85 °C as described in Zhou et al. (2018). 5 μ L nectar was used for each measurement for the total phenolics, protein, and hydrogen peroxide contents in the samples. The concentration of hydrogen peroxide in nectar was measured using a commercially available colorimetric assay kit (Sangon Biotech, Shanghai, China) according to the user's manual. The total phenolics content was measured spectrophotometrically using the Folin–Ciocalteu reagent with gallic acid as standard (Liu et al. 2013). The protein content in nectar samples was spectrophotometrically assayed at 595 nm according to Bradford's (1976) method. Differences in each measurement between groups were tested by one-way analysis of variance (ANOVA) followed by a Tukey post-hoc test. Statistical significance was set at $P < 0.05$. Differences between nectar sample collection and treatment methods for *in vivo* versus *in vitro* tests might cause differences between initial samples, especially for some chemically active ingredients such as phenolics and hydrogen peroxide. Therefore, we did not make direct comparisons between the *in vitro* and *in vivo* samples at specific time points, but focused instead on the dynamic changes over time within each set.

NT nectarin invertase activity assay

To investigate the invertase activity in nectar, concentrated NT nectarin (0.5 mg mL⁻¹) from nectar mix 1 was used. The residual sugars from the nectarin sample were removed using an ultracentrifuge and distilled water until no sugar could be detected by HPLC as described previously (Ma et al. 2017). NT nectarin invertase activity was measured according to Tiedge and Lohaus (2018), but the products were assayed by HPLC.

Results

Proteomic analysis: *Nicotiana tabacum* (NT) nectar proteins are mainly glycoside hydrolase (GH)

The floral nectarin concentration of NT ($18.2 \pm 5.0 \mu\text{g mL}^{-1}$; mean $\pm$ SE, $n = 15$) was ten times lower than in *N. rustica*, *N. bonariensis*, or *N. langsdorffii* (Silva et al. 2018). An individual NT flower secretes up to 40 μL nectar, which is similar to the amount produced by an ornamental tobacco, *N. langsdorffii* $\times$ *N. sanderae* (Carter et al. 1999) and higher than that of *N. attenuata* which has much smaller flowers than NT (Seo et al. 2013). Two-dimensional gel electrophoresis revealed a constant number of 21 spots for the NT nectar, 19 of which were successfully identified by mass spectrometry, and from which seven distinct proteins could be distinguished (Fig. 2; Supplementary Table S2). These were acidic endochitinase, β -xylosidase, α -galactosidase, α -amylase, G-type lectin S-receptor-like serine/threonine-protein kinase (LECRK4), pathogenesis-related protein 5, and early nodulin-like protein 2 (ENOD-2). The first four of these nectarins were classified as glycoside hydrolases (GH), belonging to GH families 19, 3, 27, and 13, respectively. LECRK4 and Enod-2 were identified from floral nectar for the first time. According to the size of the spots on the 2-DE gel (Fig. 2), β -xylosidase and acidic endochitinases were the major nectarins in NT nectar. Some weak spots with apparently small molecular weight, such as spot numbers 15 and 17, were also identified to be acidic endochitinases. They were probably truncated acidic endochitinase.

Two NT nectarins are glycosylated

In this study, we performed the first glycoproteomic analysis for nectarins. Surprisingly, after affinity chromatography and glycoprotein staining, only two glycoproteins were identified from NT nectar (Fig. 3a). The first was β -xylosidase (band 1; accession number: XP_016497975.1) identical to the protein identified by 2-DE coupled with mass spectrometry, which is consistent with plant β -xylosidases, usually being glycosylated (Hrubá et al. 2005). The second was an uncharacterized protein (band 2; accession number: XP_016437493.1), which was not detected by 2-DE and mass spectrometry, indicating that it was present at very

low level in NT nectar but was enriched during glycoprotein affinity chromatography. Apoplastic invertase was proposed to play important roles in the sucrose hydrolysis in the nectar production and is a well-known secretory glycoprotein (Ruhlmann et al. 2010). However, we didn't detect any invertases by this approach. An extra band (band 3; Fig. 3b) with a MW of 27kDa was visualized by SDS-PAGE and CBB staining, this band was subsequently identified as concanavalin-A eluted from the column.

***N. tabacum* nectarin genes were neither nectary-specific, nor synchronously expressed**

Expression analysis was conducted on the 8 nectarin genes detected (including the uncharacterized glycoprotein) plus four invertase genes (Fig. 4). It is interesting that none of the tested genes were expressed exclusively in the nectary. Even within the nectary, expression of these nectarins was non-synchronous with the flower development. α -Galactosidase was mainly expressed in the nectary (Fig. 4d) and its function in nectar has been investigated before (Zha et al. 2012). β -Xylosidase is a major protein in NT nectar, and showed much greater expression in the corolla and stamens than in the nectary (Fig. 4h). Whether β -xylosidase that is synthesised in the corolla could be transported into the nectary or nectar via trichomes inside of the corolla is worth further investigation. Another major nectarin, acidic endochitinase, was also expressed in non-reproductive organs, and its transcripts continuously accumulated in the nectary until the flower began withering (Fig. 4a). In this study, both pathogenesis-related protein 5 and apoplastic invertase could be deemed as being flower-specifically expressed (Fig. 4f and i). Both transcripts began to accumulate in the nectary at stage S2 and decline after stage S4. As well as in the nectary, pathogenesis-related protein 5 was also expressed in the stigma, whereas apoplastic invertase was expressed in stamens. Hence these two nectarins differed in activity and physiological function. The remaining three nectarins, LECRK4, ENOD-2, and α -amylase, were identified in floral nectar for the first time, although in each case their gene expression was detected in other organs as well as the nectary (Fig. 4b, c, e). They are very likely to be involved in some physiological activities other than in nectar production. In this study, the apoplastic invertase gene (accession number: KF308284; the counterpart of cell-wall invertase 4 in *Arabidopsis thaliana*) displayed very highly enriched expression in the NT nectary from stages S2 to S4,

but the expression declined in the later developmental stage of flowering (Fig. 4i). Invertase nin88 is an analogue of the apoplastic invertase, but is defective with no invertase activity (Le Roy et al. 2013). In this study, it was generally expressed and showed the lowest expression in nectary among the tested organs (Fig. 4j). Two other kinds of invertase, vacuolar and cytosolic invertase, also showed no nectary exclusivity in their expression, and can't be linked with any function with nectary (Fig. 4k, l).

NT nectar composition post-secretory modification

For *in vitro* analysis, there was no detectable difference in results between filtered and unfiltered nectar, for all variables assayed (the total content of sucrose, hexoses, protein, phenolics and hydrogen peroxide in nectar). Therefore only results from filtered nectar (mix 2) are discussed here. Nectar sugar content (Brix value), the ratio of sucrose-to-hexose (glucose + fructose), protein, and hydrogen peroxide concentration were measured at different time intervals *in vivo* and *in vitro* to monitor the post-secretory changes in NT nectar (Fig. 5). Nectar total sugar concentration can increase because of evaporation after secretion (Nicolson and Thornburg 2007), but in this study, no significant different changes in nectar sugar concentration were detected *in vivo* or in *in vitro* after 40 h (Fig. 5a). Perhaps evaporation had a negligible effect due to NT nectar being stored deep within the flower tube.

However, no invertase activity was detected in NT nectar (data not shown) and the protein of invertase was not detected by the proteomic approach in this study. The sucrose-to-hexose ratio in the nectar in plastic tubes with pollen and microorganisms removed was unchanged for 40 h in spite of incubating at 30 °C with gentle shaking. In addition, no glucose or fructose was generated after NT nectarins incubated with sucrose for up to 12 h, and therefore no NT nectarin could be deemed to have any invertase activity. The relatively low sucrose-to-hexose ratio for *in vitro* nectar might be caused by the much longer time for the collection, pooling, and filtering process.

A significant decrease of the sucrose-to-hexose ratio over time was detected within nectar of the *in vivo* group (Fig. 5b; Fig. S1). Because overall sugar content did not change during this time, and it is highly unlikely that sucrose was absorbed while the monosaccharides were secreted, this result appears to show hydrolysis of sucrose.

The concentration of both hydrogen peroxide and total protein decreased significantly in *in vitro* nectar, but neither did so in *in vivo* (Fig. 5c, d). Hydrogen peroxide is an active ingredient in nectar and can break down naturally especially in a complex solution like nectar (Carter and Thornburg 2004). Proteins can be degraded by abiotic factors, among which incubation at 30 °C and/or shaking, for long periods, might have caused degradation during this study. Both protein and hydrogen peroxide concentrations remained stable in *in vivo* nectar but the controlling mechanism, which might involve inhibition of breakdown, is yet to be discovered. In this study, the concentration of total phenolics in *in vivo* nectar increased from 7.3 ± 3.5 to 15.1 ± 2.4 ($\mu\text{g mL}^{-1}$; mean $\pm$ SD, $n = 15$) but remained constant in *in vitro* nectar (Fig. 5e).

Discussion

Nectar proteome may vary between closely related species.

The nectar proteome is usually small with fewer than 20 proteins detected in any one species' floral nectar (Heil 2011; Roy et al. 2017). PR proteins, e.g. chitinase, and glucanase, are deemed as basic proteinaceous components in nectar in different studied species (Zha et al. 2016). In this study, eight nectarins were identified in NT nectar, and acidic endochitinase was the major one. Another NT nectarin, xylosidase, was also detected in the nectar from *Nicotiana attenuata* (Seo et al. 2013), *Cucurbita pepo* (Nepi et al. 2011), and *Liriodendron tulipifera* (Zhou et al. 2016). This is consistent with β -xylosidase playing important roles in flower and pollen development (Hrubá et al. 2005). To our knowledge, besides NT in this study, at least three other Solanaceae taxa's nectar proteomes have been investigated: *N. attenuata* (Seo et al. 2013), *N. langsdorffii* $\times$ *N. sanderae* (Carter et al. 1999), and *Petunia hybrida* (Hillwig et al. 2011). A total of five proteins have been identified in *N. langsdorffii* $\times$ *N. sanderae* nectar and have been proven to play important roles in so called "Nectar Redox Cycle" for hydrogen peroxide generation and anti-microbial function in the nectar (Park and Thornburg 2009). However, none of those nectarins were detected in NT nectar during this study. Therefore, if the main function of nectarins is antimicrobial, then NT might employ a different mechanism from *N. langsdorffii* $\times$ *N. sanderae*'s, even though they are closely related. S-RNase is a predominant protein detected in *P. hybrida* nectar, but it was only detected as a very minor proteinaceous component in *N. attenuata* nectar (Seo et al. 2013) and was not detected in *N. langsdorffii* $\times$ *N. sanderae* (Park and Thornburg 2009) nor in NT nectar in this study. In addition, three nectarins here detected in NT nectar, LECRK4, ENOD-2, and α -amylase, have never been detected in any other species' nectar. All this indicates that the nectar proteome profile can be dramatically different even between very closely related species; however the controlling mechanisms for this are largely unknown.

No free invertase detected in NT floral nectar

Floral nectar is currently viewed as a pollinator manipulant as well as an attractant, and sugars play central roles therein (Pyke 2016). It is well known that the main sugar

components of floral nectar are sucrose, fructose, and glucose, and in most of the cases the mole ratio between fructose and glucose is 1:1 regardless of sucrose concentration in nectar (Nicolson and Thornburg 2007). From this, it is almost certain that fructose and glucose in nectar are the products of sucrose hydrolysis by invertase, either inside or outside the nectary, and apoplastic invertases are proposed to play important roles in both nectar production and post-secretory sugar composition adjustment (Heil et al. 2005; Nepi et al. 2011). Invertase activity has only ever been detected from floral nectar in one species, *Cucurbita pepo*, but even there it was too low to change the sugar profile significantly during the short period of anthesis (Nepi et al. 2012). Within *Nicotiana*, strong invertase activity has been detected in several species' nectaries, but never in their floral nectar (Tiedge and Lohaus 2018). So far, to our knowledge, despite the use of cutting-edge proteomic techniques (gel-based or gel-free), no invertase protein has been identified in any well studied floral nectar, despite examinations of *Cucurbita pepo* (Nepi et al. 2009), *Nicotiana langsdorffii* $\times$ *N. sanderae* (Carter et al. 1999), *Nicotiana attenuata* (Seo et al. 2013), *Liriodendron tulipifera* (Zhou et al. 2016), *Canavalia gladiata* (Ma et al. 2017), *Ricinus communis* (Nogueira et al. 2018), and *Mucuna sempervirens* (Zhou et al. 2018).

In this study, no invertase proteins or activity were detected in the NT nectar, even using liquid chromatography mass spectrometry-based proteomics techniques (LC MS/MS) which provide higher resolution than regular gel-based proteomic approaches (data not shown). Moreover, we found that NT floral nectar changes from sucrose-rich to hexose-rich type *in vivo*, but that this does not happen *in vitro* (Fig. 5b; Fig. S1). Hence sucrose hydrolysis occurs in NT nectar despite the apparent absence of free invertase, but only when it remains within the flower. Therefore, free nectarins are not responsible. Though it cannot be ruled out entirely, microbial activity is also unlikely to be the cause, for two reasons. First, unfiltered *in vitro* nectar (from which microbes had not been removed) showed no hydrolysis, suggesting that hydrolysis *in vivo* has a different cause. Secondly, the glasshouses excluded most or all insect visitors to the flowers, giving little opportunity for microbes to arrive.

A likely candidate for sucrose hydrolysis *in vivo* is cell-wall-bound apoplastic invertase on the inner side surface of the flower tube. Among three classes of invertase, only apoplastic invertase (CELL WALL INVERTASE 4, AtCWINV4) has been proved to function in nectar

production in the nectary of *Arabidopsis thaliana* (Ruhlmann et al. 2010). In this study, apoplastic invertase (AtCWINV4 analogue) was the only gene whose expression showed a positive correlation with nectary development, suggesting an important role for this compound in NT nectar formation. Its transcripts were also detected in stamens, which is consistent with its analogue having been detected in the stamen of *Arabidopsis thaliana* and *Oryza sativa*, where it functions in sucrose accumulation or anther and pollen sterility (Ruhlmann et al. 2010). Apoplastic invertase contains a signal peptide and can be secreted into extracellular space, whereas other kinds of invertases are non-secretory proteins. However, it also reported that plant apoplastic invertase is glycosylated, and exists as non-soluble proteins that are ionically bound to the cell wall (Sturm 1999), indicating that it is almost impossible for them to be released into nectar. Hence we infer that apoplastic invertases do not enter the nectar as a free nectarin, but are nonetheless likely to hydrolyse sugars in NT nectar after secretion.

High levels of invertase activity and invertase protein have been detected in the extrafloral nectar of myrmecophytic *Acacia* species, where they play roles in adjusting its sugar composition to exclude non-mutualistic ants (Heil et al. 2005). In addition, invertases have also been detected in gymnosperm pollination drops, where they hydrolyse sucrose in the ovular secretion into its monomeric constituents and influence pollen selection and development (Poulis et al. 2005). However, both pollination drops and extrafloral nectar have different purposes and different evolutionary and developmental origins from floral nectar (Nepi et al. 2009). It is still unknown whether the invertases detected in extrafloral nectar and pollination drops are the same type of invertase as the apoplastic invertase functioning in the NT floral nectary in this study.

Floral nectar composition post-secretory adjustment is a complex process.

Nectar has never been considered to be a static product, even after secretion (Pedersen et al. 1958). Nectar properties (total sugar concentration, volume, and viscosity) can change dynamically in time mainly because of the microclimate within the flower or outside it (Nicolson and Thornburg 2007; Nepi and Stpicyńska 2008). For example, in the post-secretory stage, *Leucosceptrum canum* nectar changes in colour from light to dark purple,

as the concentration of 5-hydroxyflavylium, a purple anthocyanidin, increases (Zhang et al. 2012). Ongoing reactions following secretion are also implied in those nectars that continuously release odours; these odours would directly impact plant reproductive fitness, by attracting pollinators or deterring nectar robbers and florivores (Raguso 2004). The variation of secondary metabolites across time, space, phenotype, climate, and even within a plant can also be attributed to post-secretory changes of nectar components (Stevenson et al. 2017). The detection of enzymes and microorganisms from nectar of various plant species has inspired scientists to hypothesize that further, biologically controlled changes to the biochemistry of nectar can occur after secretion (Raguso 2004; Roy et al. 2017).

Because of the significance of sugars in nectar ecological function, overall sugar content has often been monitored for post-secretion changes, and has generally been found to be stable in extracted floral nectar, and hence deemed to be phylogenetically constrained (Nepi et al. 2012; Abrahamczyk et al. 2017; Tiedge and Lohaus 2018). In this study, we proved that, following secretion, NT floral nectar changes from sucrose-rich to hexose-rich type *in vivo*, but not *in vitro*, and suggest cell-wall-bound apoplastic invertase as a possible cause (see above). Nectar sugar composition has often been related to the pollination syndrome of the plant species, with sucrose-rich nectar being found in flowers visited by moths, long-tongued bees, and the normal pollinators of NT, e.g. hummingbirds and butterflies (Nicolson and Thornburg 2007; Tiedge and Lohaus 2018). Based on this, we hypothesized that the pollinators of NT might prefer the sucrose-rich nectar of young flowers, encouraging them to visit more often, and for longer, during the young stage when the pollen is abundant and the stigma receptive. If so, the progressive conversion of sucrose to hexoses might be an adaptive mechanism to manipulate pollinators into favouring younger flowers, coupled with floral cues (e.g. flower color, scent) to indicate the age of the flower to the pollinator. Those pollinators that tend to be associated with hexose-rich nectar generally do not visit NT, and in most cases would not be able to access its nectar due to its long corolla tubes; these include flies, short-tongued bees, passerine birds, and Neotropical bats (Nicolson and Thornburg 2007). Hence it might not be possible for nectar conversion from sucrose-rich to hexose rich to be associated with a switch to attracting different pollinator types. A large-scale ecological

experiment involving artificial flowers would be a way to test whether varying sugar ratios within the nectar has a direct effect on pollinator behaviour.

Post-secretory adjustment of floral nectar composition is a complex but little-studied process, yet might be an important way to manipulate pollinator behaviour and so maximise pollen transfer, seed set, and biological fitness. As well as sugars, changes may involve nectarins, scents, hydrogen peroxide, alkaloids, amino acids, and others. It was proved that phenolics in nectar are active and have complex effects on the behaviours of pollinators or non-pollinating visitors (Afik et al. 2006). In this study, the concentration of phenolics in *in vivo* nectar doubled towards the end of anthesis, although neither the mechanisms of synthesis and secretion nor the effects of increasing phenolics concentration in nectar are known. The mechanisms for post-secretion content changes might include enzymatic activity from nectarins, and fixed surface enzymes as noted above, but also abiotic environmental factors such as sunlight, oxygen, and temperature. Equally, nectar dwelling microorganisms can consume nutrients and/or otherwise modify the nectar composition; whether this happens will depend on how easily they can reach the nectar of a newly opened flower, and whether it has chemical defences against them. Microbial density in nectar can vary dramatically between individual flowers of the same species (Vannette and Fukami 2018). Therefore, plant nectar post-secretory changes should be a process that is relatively independent to the effects of invasive microbes. In this study, we cannot completely exclude the effect of microorganismal activity in altering the content of NT nectar *in vivo*, but our preliminary test showed that the levels of fungi in NT nectar remained low and static during the post-secretory phase (less than 1000 cells μL^{-1} in nectar 40 h after secretion; HG Zha, unpublished data). The absence of animal pollinators in the greenhouse might be the reason for such low microbial density in NT nectar in this study.

Many components of nectar are dynamically regulated by both secretion and resorption (Nepi and Stpiczyńska 2008), and post-secretory adjustment may for some compounds serve to maintain their levels, rather than alter them. This might apply especially to unstable compounds that tend to naturally degrade, such as hydrogen peroxide, which is an active and commonly detected component of nectar (Carter and Thornburg 2004). Consistent with this, hydrogen peroxide concentration in NT nectar remained stable *in vivo*, but declined *in vitro*,

544 suggesting that some biological process in flowers acts to either prevent hydrogen peroxide
545 degradation, or continuously generate more. This might involve nectarins and/or surface
546 enzymes, but the mechanism is unknown.
547

Conclusion

The *Nicotiana tabacum* nectar proteome is small and mainly consists of pathogenesis-related proteins which are conserved across different species. Only two nectarins from the NT nectar proteome, xylosidase and an uncharacterized protein, were proved to be glycosylated. This indicates that glycosylation might not be a necessary post-translational modification for nectarin secretion. None of the NT nectarin genes so far identified were exclusively expressed in the nectary, and nor were their expressions synchronous. In flowers, NT nectar changed from sucrose-rich to hexose-rich after secretion, even though no invertase activity was detected in NT nectar. From this, post-secretory sugar composition adjustment in NT nectar involves factors other than nectarins, with a likely role for fixed apoplastic invertases. Further studies on functional interactions among nectarins, and nectar-inhabiting microorganisms' influence on nectar chemistry, especially under natural conditions, will help us understand the whole mechanism of nectar post-secretory content adjustment.

563

564 **Conflict of Interest:** The authors declare that they have no conflict of interest.

565

566 **Author Contributions**

567 HGZ and XLM conceived and designed research. XLM, YQS, and HXZ conducted
568 experiments. JYF and RIM analysed data. RIM and HXZ wrote the manuscript. All authors
569 read and approved the manuscript.

570

571 **Acknowledgements**

572 This work was supported by Natural Science Foundation of Anhui Province (1608085MC65
573 to HG Zha) and Science Foundation of Huangshan University (2014XKJQ004 to HG Zha,
574 and 2015 XKJQ 005 to HX Zhou).

575

576

Figure legends

Fig. 1 *Nicotiana tabacum* flower. **a** Different developmental stages of *N. tabacum* flower. S1 stage, very young flower (corolla less than 1.5 cm long). S2 stage, young flower (corolla ca 3 cm long, almost half the length of the adult flower). S3 stage, near adult flower with weak pink but not fully opened corolla and nectar production. This stage tends to last around 4 hours for NT. S4 stage, mature flower with nectar and anther dehiscence. This stage can last for more than 36 hours, depending on insect visitation and growing conditions, such as temperature, watering, etc. Therefore, in our experiments, enough flowers still contained nectar at 40 hours from onset of S3 to allow nectar sampling at that time point. S5 stage, post-fertilization flower without nectar and corolla beginning to wilt. **b** The structure of *N. tabacum* flower, with nectary indicated by an arrow

Fig. 2 Coomassie blue stained 2D gel of *N. tabacum* nectarins. Proteins were separated by isoelectrofocusing (pH 3–10) in the first dimension and using 12% SDS–PAGE gel in the second dimension. The molecular masses of standards are shown on the left. Identified nectarins on the gel are indicated by arrows

Fig. 3 SDS-PAGE (12% gel) analysis of glycoproteins from *N. tabacum* nectar. **a** Glycoprotein staining (Glycan staining). Two identified glycoproteins (β -xylosidase and an uncharacterized protein) on the gel are indicated by arrows. **b** Coomassie blue G250 staining (protein staining). G: isolated glycoproteins from NT floral nectar; FN: floral nectar; M: prestained molecular weight markers

Fig. 4 Tissue-specific transcript expressions of nectarins and invertases. RNA was isolated from different organs: root (R), stem (STE), leaf (L), stalk (STA), calyx (CA), upper corolla (CO, spreading part, beyond the tube, with pink pigment), lower corolla (CM, corolla tube up to 1 cm from the top of the ovary, with dense trichomes inside), stamen (STM), stigma (STI), Ovary (O), and nectaries at five developmental stages (S1~S5). All data were normalized to *EF-1 α* transcript levels and relative to the target gene expression in the leaf. Values represent

mean $\pm$ SE from three biological repeats. Different lower-case letters indicate statistically significant differences ($P < 0.05$ according to one way ANOVA). **a** acidic endochitinase; **b** α -amylase; **c** early nodulin-like protein 2; **d** α -galactosidase; **e** G-type lectin S-receptor-like serine/threonine-protein kinase (LECRK4); **f** pathogenesis-related protein 5; **g** uncharacterized protein; **h** β -xylosidase; **i** apoplastic invertase; **j** apoplastic invertase NIN88; **k** vacuolar invertase; **l** cytosolic invertase

Fig. 5 Post-secretory adjustment of NT nectar, *in vivo* (black bars) and *in vitro* (gray bars). **a** brix; **b** sucrose:(fructose + glucose); **c** protein; **d** hydrogen peroxide; **e** phenolics. Bars represent means $\pm$ SE ($n = 15$ for *in vivo* nectar and $n = 8$ for *in vitro* nectar). Statistical analysis was only performed within each data set, *in vivo* or *in vitro* test. Significant differences ($P < 0.05$ according to one way ANOVA) are indicated by different letters. Letters are in bold for *in vivo* tests and regular font for *in vitro* tests

Fig. S1 HPLC chromatogram of *in vivo* NT floral nectar at different time intervals after secretion

References

- Abrahamczyk S, Kessler M, Hanley D, Karger DN, Müller MPJ, Knauer AC, Keller F, Schwerdtfeger M, Humphreys AM (2017) Pollinator adaptation and the evolution of floral nectar sugar composition. *J Evol Biol* 30 (1):112-127. doi:10.1111/jeb.12991
- Afik O, Dag A, Shafir S (2006) The effect of avocado (*Persea americana*) nectar composition on its attractiveness to honey bees (*Apis mellifera*). *Apidologie* 37 (3):317-325. doi:10.1051/apido:2005064
- Álvarez-Pérez S, Lievens B, Fukami T (2019) Yeast–bacterium interactions: The next frontier in nectar research. *Trends Plant Sci* 24 (5):393-401. doi:10.1016/j.tplants.2019.01.012
- Bernardello G (2007) A systematic survey of floral nectaries. In: Nicolson SW, Nepi M, Pacini E (eds) *Nectaries and nectar*. Springer Netherlands, Dordrecht, pp 19-128. doi:10.1007/978-1-4020-5937-7_2
- Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 72 (1-2):248-254. doi:10.1016/0003-2697(76)90527-3
- Buchmann SL, Cane JH (1989) Bees assess pollen returns while sonicating *Solanum* flowers. *Oecologia* 81 (3):289-294. doi:10.1007/bf00377073
- Carter C, Graham RA, Thornburg RW (1999) Nectarin I is a novel, soluble germin-like protein expressed in the nectar of *Nicotiana* sp. *Plant Mol Biol* 41 (2):207-216. doi:10.1023/A:1006363508648
- Carter C, Thornburg RW (2004) Is the nectar redox cycle a floral defense against microbial attack? *Trends Plant Sci* 9 (7):320-324. doi:10.1016/j.tplants.2004.05.08
- Heil M (2011) Nectar: generation, regulation and ecological functions. *Trends Plant Sci* 16 (4):191-200. doi:10.1016/j.tplants.2011.01.003
- Heil M, Rattke J, Boland W (2005) Postsecretory hydrolysis of nectar sucrose and specialization in ant/plant mutualism. *Science* 308 (5721):560-563. doi:10.1126/science.1107536
- Hillwig MS, Kanobe C, Thornburg RW, MacIntosh GC (2011) Identification of S-RNase and peroxidase in petunia nectar. *J Plant Physiol* 168 (7):734-738. doi:10.1016/j.jplph.2010.10.002
- Horner HT, Healy RA, Cervantes-Martinez T, Palmer RG (2003) Floral nectary fine structure and development in *Glycine max* L. (Fabaceae). *Int J Plant Sci* 164 (5):675-690. doi:10.1086/377060
- Hrubá P, Honys D, Twell D, Čapková V, Tupý J (2005) Expression of β -galactosidase and β -xylosidase genes during microspore and pollen development. *Planta* 220 (6):931-940. doi:10.1007/s00425-004-1409-0
- Jackson S, Nicolson SW (2002) Xylose as a nectar sugar: from biochemistry to ecology. *Comp Biochem Physiol B Biochem Mol Biol* 131 (4):613-620. doi:10.1016/S1096-4959(02)00028-3
- Jain D, Khurana JP (2018) Role of pathogenesis-related (PR) proteins in plant defense mechanism. In: Singh A, Singh IK (eds) *Molecular aspects of plant-pathogen interaction*. Springer Singapore, Singapore, pp 265-281. doi:10.1007/978-981-10-7371-7_12
- Kram BW, Bainbridge EA, Perera MADN, Carter C (2008) Identification, cloning and characterization of a GDSL lipase secreted into the nectar of *Jacaranda mimosifolia*. *Plant Mol Biol* 68:173-183. doi:10.1007/s11103-008-9361-1
- Kram BW, Carter CJ (2009) *Arabidopsis thaliana* as a model for functional nectary analysis. *Sex Plant Reprod* 22 (4):235-246. doi:10.1007/s00497-009-0112-5

666 Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage
 667 T4. *Nature* 227 (5259):680-685. doi:10.1038/227680a0
 668 Le Roy K, Vergauwen R, Struyf T, Yuan SG, Lammens W, Matrai J, De Maeyer M, Van den Ende W
 669 (2013) Understanding the role of defective invertases in plants: tobacco Nin88 fails to
 670 degrade sucrose. *Plant Physiol* 161 (4):1670-1681. doi:10.1104/pp.112.209460
 671 Liu T, Shah A, Zha H-G, Mohsin M, Ishtiaq M (2013) Floral nectar composition of an outcrossing bean
 672 species *Mucuna sempervirens* Hemsl (Fabaceae). *Pak J Bot* 45 (6):2079-2084
 673 Ma XL, Milne RI, Zhou HX, Fang JY, Zha HG (2017) Floral nectar of the obligate outcrossing *Canavalia*
 674 *gladiata* (Jacq.) DC. (Fabaceae) contains only one predominant protein, a class III acidic
 675 chitinase. *Plant Biol* 19 (5):749-759. doi:10.1111/plb.12583
 676 Nepi M, Bini L, Bianchi L, Puglia M, Abate M, Cai G (2011) Xylan-degrading enzymes in male and
 677 female flower nectar of *Cucurbita pepo*. *Ann Bot* 108 (3):521-527. doi:10.1093/aob/mcr165
 678 Nepi M, Soligo C, Nocentini D, Abate M, Guarnieri M, Cai G, Bini L, Puglia M, Bianchi L, Pacini E (2012)
 679 Amino acids and protein profile in floral nectar: Much more than a simple reward. *Flora* 207
 680 (7):475-481. doi:10.1016/j.flora.2012.06.002
 681 Nepi M, Stpiczyńska M (2008) The complexity of nectar: secretion and resorption dynamically regulate
 682 nectar features. *Naturwissenschaften* 95 (3):177-184. doi:10.1007/s00114-007-0307-2
 683 Nepi M, von Aderkas P, Wagner R, Mugnaini S, Coulter A, Pacini E (2009) Nectar and pollination drops:
 684 how different are they? *Ann Bot* 104 (2):205-219. doi:10.1093/aob/mcp124
 685 Nicolson S, Thornburg R (2007) Nectar chemistry. In: Nicolson SW, Nepi M, Pacin E (eds) *Nectaries and*
 686 *nectar*. Springer Netherlands, Dordrecht, pp 215-264. doi:10.1007/978-1-4020-5937-7_5
 687 Nogueira FCS, Farias ARB, Teixeira FM, Domont GB, Campos FAP (2018) Common features between
 688 the proteomes of floral and extrafloral nectar from the castor plant (*Ricinus communis*) and
 689 the proteomes of exudates from carnivorous plants. *Front Plant Sci* 9 (549).
 690 doi:10.3389/fpls.2018.00549
 691 Ollerton J, Winfree R, Tarrant S (2011) How many flowering plants are pollinated by animals? *Oikos*
 692 120 (3):321-326. doi:10.1111/j.1600-0706.2010.18644.x
 693 Parachnowitsch AL, Manson JS, Sletvold N (2018) Evolutionary ecology of nectar. *Ann Bot* 123
 694 (2):247-261. doi:10.1093/aob/mcy132
 695 Park S, Thornburg R (2009) Biochemistry of nectar proteins. *J Plant Biol* 52 (1):27-34.
 696 doi:10.1007/s12374-008-9007-5
 697 Pedersen MW, Lefevre CW, Wiebe HH (1958) Absorption of C14-Labeled sucrose by alfalfa nectaries.
 698 *Science* 127 (3301):758-759. doi:10.1126/science.127.3301.758-a
 699 Peumans WJ, Smeets K, Nerum KV, Leuven FV, Damme EJM (1997) Lectin and alliinase are the
 700 predominant proteins in nectar from leek (*Allium porrum* L.) flowers. *Planta* 201 (3):298-302.
 701 doi:10.1007/s004250050070
 702 Poulis BAD, O'Leary SJB, Haddow JD, von Aderkas P (2005) Identification of proteins present in the
 703 Douglas fir ovular secretion: An insight into conifer pollen selection and development. *Int J*
 704 *Plant Sci* 166 (5):733-739. doi:10.1086/431808
 705 Pyke GH (2016) Floral nectar: pollinator attraction or manipulation? *Trends Ecol Evol* 31 (5):339-341.
 706 doi:10.1016/j.tree.2016.02.013
 707 Raguso RA (2004) Why are some floral nectars scented? *Ecology* 85 (6):1486-1494.
 708 doi:10.1890/03-0410

709 Roy R, Schmitt AJ, Thomas JB, Carter CJ (2017) Review: Nectar biology: from molecules to ecosystems.
 710 Plant Sci 262:148-164. doi:10.1016/j.plantsci.2017.04.012
 711 Ruhlmann JM, Kram BW, Carter CJ (2010) CELL WALL INVERTASE 4 is required for nectar production in
 712 *Arabidopsis*. J Exp Bot 61 (2):395-404. doi:10.1093/jxb/erp309
 713 Schmitt AJ, Sathoff AE, Holl C, Bauer B, Samac DA, Carter CJ (2018) The major nectar protein of
 714 *Brassica rapa* is a non-specific lipid transfer protein, BrLTP2.1, with strong antifungal activity.
 715 J Exp Bot 69 (22):5587–5597. doi:10.1093/jxb/ery319
 716 Schmittgen TD, Livak KJ (2008) Analyzing real-time PCR data by the comparative CT method. Nat
 717 Protoc 3:1101. doi:10.1038/nprot.2008.73
 718 Seo PJ, Wielsch N, Kessler D, Svatos A, Park CM, Baldwin IT, Kim SG (2013) Natural variation in floral
 719 nectar proteins of two *Nicotiana attenuata* accessions. BMC Plant Biol 13:101-101.
 720 doi:10.1186/1471-2229-13-101
 721 Sierro N, Battey JN, Ouadi S, Bakaher N, Bovet L, Willig A, Goepfert S, Peitsch MC, Ivanov NV (2014)
 722 The tobacco genome sequence and its comparison with those of tomato and potato. Nat
 723 Commun 5:3833. doi:10.1038/ncomms4833
 724 Silva FA, Guirgis A, Thornburg R (2018) Nectar analysis throughout the genus *Nicotiana* suggests
 725 conserved mechanisms of nectar production and biochemical action. Front Plant Sci 9 (1100).
 726 doi:10.3389/fpls.2018.01100
 727 Stevenson PC, Nicolson SW, Wright GA (2017) Plant secondary metabolites in nectar: impacts on
 728 pollinators and ecological functions. Funct Ecol 31 (1):65-75. doi:10.1111/1365-2435.12761
 729 Strasser R (2016) Plant protein glycosylation. Glycobiology 26 (9):926-939.
 730 doi:10.1093/glycob/cww023
 731 Sturm A (1999) Invertases. Primary structures, functions, and roles in plant development and sucrose
 732 partitioning. Plant Physiol 121 (1):1-7. doi:10.1104/pp.121.1.1
 733 Tiedge K, Lohaus G (2018) Nectar sugar modulation and cell wall invertases in the nectaries of day-
 734 and night- flowering *Nicotiana*. Front Plant Sci 9 (622). doi:10.3389/fpls.2018.00622
 735 Vannette RL, Fukami T (2018) Contrasting effects of yeasts and bacteria on floral nectar traits. Ann Bot
 736 121 (7):1343-1349. doi:10.1093/aob/mcy032
 737 Vizcaino JA, Csordas A, del-Toro N, Dianas JA, Griss J, Lavidas I, Mayer G, Perez-Riverol Y, Reisinger F,
 738 Ternent T, Xu QW, Wang R, Hermjakob H (2016) 2016 update of the PRIDE database and its
 739 related tools. Nucleic Acids Res 44 (D1):D447-456. doi:10.1093/nar/gkv1145
 740 Zha HG, Milne RI, Zhou HX, Chen XY, Sun H (2016) Identification and cloning of class II and III chitinases
 741 from alkaline floral nectar of *Rhododendron irroratum*, Ericaceae. Planta 244 (4):805-818.
 742 doi:10.1007/s00425-016-2546-y
 743 Zha HG, Flowers VL, Yang M, Chen LY, Sun H (2012) Acidic alpha-galactosidase is the most abundant
 744 nectarin in floral nectar of common tobacco (*Nicotiana tabacum*). Ann Bot 109 (4):735-745.
 745 doi:10.1093/aob/mcr321
 746 Zhang FP, Cai XH, Wang H, Ren ZX, Larson-Rabin Z, Li D-Z (2012) Dark purple nectar as a foraging signal
 747 in a bird-pollinated Himalayan plant. New Phytol 193 (1):188-195.
 748 doi:10.1111/j.1469-8137.2011.03894.x
 749 Zhou HX, Milne RI, Ma XL, Song YQ, Fang JY, Sun H, Zha HG (2018) Characterization of a
 750 L-Gulonono-1,4-Lactone oxidase like protein in the floral nectar of *Mucuna sempervirens*,
 751 Fabaceae. Front Plant Sci 9 (1109). doi:10.3389/fpls.2018.01109

752 Zhou Y, Li M, Zhao F, Zha H, Yang L, Lu Y, Wang G, Shi J, Chen J (2016) Floral nectary morphology and
753 proteomic analysis of nectar of *Liriodendron tulipifera* Linn. Front Plant Sci 7 (826).
754 doi:10.3389/fpls.2016.00826