NECTAR COLLECTION AND ANALYSES

COLLECTION PROCEDURES

Important Considerations

One significant consideration when performing nectar collection is timing, both developmental and circadian. This is because nectar production in *Brassica* sp. undergoes circadian oscillations. For example, nectar production was found to be maximal between 4-8 hours after dawn (h.a.d.) in *Brassica napus* (Búrquez and Corbet, 1991). We have observed a nearly identical pattern of nectar production in Arabidopsis (Bender and Carter, unpublished). Thus, when making comparisons, e.g., wild-type vs. mutant, we only collect nectar from flowers 4-8 h.a.d., and alternate between collection from mutant and wild-type plants if more than one biological replicate is being collected. Similarly, samples are only collected from Stage 14-15 flowers on plants of the same age that have been grown side-by-side under the exact same light, water and fertilizer regime.

Arabidopsis thaliana

The vast majority (>99%) of *Arabidopsis thaliana* Col-0 nectar is produced by the lateral nectaries (Davis et al., 1998). Thus, our Arabidopsis nectar samples are prepared by carefully pulling back sepals covering the lateral nectaries and touching the nectar droplet with small, uniform triangular wicks cut from Whatman No. 1 filter paper under a dissecting microscope. For standardization purposes, nectar from a set number of flowers (e.g., usually 20 flowers) is collected on one wick prior to being placed in 100 µl of nuclease free water for solute elution, making one replicate. For metabolite profiling via

GCGC-MS (described below), a total of six replicates are made for Col-0 and each mutant line used. For routine sugar analyses (also described below), Arabidopsis nectar samples are prepared using the same nectar collection procedure as for GCGC-MS analysis, but nectar is collected from ten flowers per wick and then placed in 500 µl of sterile water to elute the solutes. A similar procedure was previously described for nectar collection from Arabidopsis flowers with cut pedicels cultured in sugar solutions (Davis et al., 1998); however, we have found culturing is not necessary to obtain enough sample for metabolite analyses for flowers grown in situ. Nonetheless, culturing Arabidopsis flowers in sugar solutions containing specific treatments (e.g., hormones) is useful for examining regulatory mechanisms of nectar production (Fekete, 2011).

Brassica sp.

Brassica sp. nectar samples are readily collected with 2 µl micropipettes (Drummond Scientific cat. no. 1-000-0020), with the fluid then expelled into microcentrifuge tubes. Significantly, one can collect 100 µL of nectar from wild-type flowers within a day. In our work, nectar samples are clarified by centrifugation and always stored at -80 ^{O}C until analysis to avoid sample degradation.

NECTAR ANALYSES

Sugar Assays as a Measure of Nectar Production

Arabidopsis nectar was previously reported to be hexose dominant. Indeed little or no sucrose was observed, and glucose and fructose was found in close to a 1:1 ratio (Davis et al., 1998). As such, measurement of glucose alone can be used as a proxy for total nectar production. In our studies, glucose concentration of Arabidopsis nectar samples is analyzed according to previously described methods utilizing glucose oxidase and Ampliflu™ Red (Bethke and Busse, 2008; Ruhlmann et al., 2010). Briefly, 75 µl of eluted nectar sample (nectar from 10 flowers collected on a paper wick placed in 500 ul diH_2O) is combined with 25 µl of Amp-Red enzyme mix and incubated for 30 minutes in the dark at room temperature. Sample absorbance is measured at 560 nm, which is directly proportional to glucose concentration. The Amp-Red enzyme mix is prepared by mixing 862.5µl 150 mM $NaPO_4$ (pH 7.4), 1 unit of horseradish peroxidase (Sigma cat. no. P8250), 10 units of glucose oxidase (Sigma cat. no. 49180), and 100µl of 10 mM Ampliflu™ Red in DMSO (Sigma cat. no. 90101) to a volume of 2.6 mL. If desired, standard curves can be generated to provide an estimate of total glucose (Bethke and Buss, 2008); however, relative changes in nectar production can be observed by utilizing the absorbance of wild-type and mutant samples collected side-by-side as described under 'COLLECTION PROCEDURES'. The same approach for nectar sugar analysis is also applicable for the hexose-predominant *Brassica* nectars.

Metabolomic Analyses

While sugars are the predominant solutes found in nectars, other minor constituents play important roles in plant-animal interactions. The W.M. Keck Metabolomics Research Laboratory at Iowa State University has developed methods for in-depth metabolomic analyses of nectars using a novel GCGC-MS technique. This new instrumentation has the ability to separate metabolites by conducting gas chromatography (GC) in tandem. Namely, the instrument separates the metabolites by conducting sequential separation of the metabolites through two tandem GC columns; thus separation though the first GC column is immediately followed by separation in a second GC column; ultimately the analytes that are separated by the two tandem GC columns are detected by a mass-spectrometer (MS), which also provides a means of identifying the individual metabolites. Because analytes are separated by a tandem arrangement of GC columns, this technology, analogous to 2-dimensional PAGE for proteomics analysis, has the power of separating highly complex mixtures of metabolites. The extraction method that we applied separates the metabolites according to the nature of its polarity. As a result, this extraction protocol generates both polar and nonpolar fractions. Both of these fractions are evaluated through this combined GCGC-MS method to provide information on nectar metabolites. In addition to metabolite identification, the relative amount and total concentration of each metabolite can be determined. Pooled nectar samples from wild-type and mutant Arabidopsis and *Brassica* plants as described above have been used for nectar compositional analyses. For example, nectar collected from all six *Brassica* sp. (*B. rapa*, *oleraceae*, *juncea*, *napus*, *nigra* and *carinata*) has been analyzed, and nearly 40 metabolites have been identified. Some of these metabolites include: sugars, amino acids, organic acids, mineral acids, fatty acids, hydrocarbons, and secondary compounds.

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