Characterization of the *Cannabis sativa* glandular trichome proteome

Lee James Conneely, Ramil Mauleon, Jos Mieog, Bronwyn J. Barkla, Tobias Kretzschmar

Southern Cross Plant Science, Southern Cross University, Lismore, NSW, Australia

* tobias.kretzschmar@scu.edu.au

Abstract

*Cannabis sativa* has been cultivated since antiquity as a source of fibre, food and medicine. The recent resurgence of *C. sativa* as a cash crop is mainly driven by the medicinal and therapeutic properties of its resin, which contains compounds that interact with the human endocannabinoid system. Compared to other medicinal crops of similar value, however, little is known about the biology of *C. sativa*. Glandular trichomes are small hair-like projections made up of stalk and head tissue and are responsible for the production of the resin in *C. sativa*. Trichome productivity, as determined by *C. sativa* resin yield and composition, is only beginning to be understood at the molecular level. In this study the proteomes of glandular trichome stalks and heads, were investigated and compared to the proteome of the whole flower tissue, to help further elucidate *C. sativa* glandular trichome biochemistry. The data suggested that the floral tissue acts as a major source of carbon and energy to the glandular trichome head sink tissue, supplying sugars which drive secondary metabolite biosynthesis. The trichome stalk seems to play only a limited role in secondary metabolism and acts as both source and sink.

Introduction

*C. sativa* is an annual, predominantly dioecious [1], monotypic species of the Cannabaceae in the order Rosales [2]. Its center of diversity is in central Asia, which was also proposed as its center of origin [3]. The use of *C. sativa* can be traced back for millennia through both written and genetic evidence with the earliest plant remains found in burial chambers in Yanghai, China, dated to approximately 2,500 years ago [4].

*C. sativa* has many uses. The seeds are a source of oil and protein rich nutrition [5], the fibres are raw material for the production of clothing and rope [6], and the resin has proven therapeutic properties with medicinal, religious, cultural, and recreational applications [7]. Due to the narcotic effect of some cultivars, the *C. sativa* plant was declared a controlled drug or prohibited substance in the first half of the 20th century in most jurisdictions and, under the United Nations single convention on narcotic drugs [8], remains highly regulated in most countries. Cultivation of *C. sativa* as a medicinal crop, however, is increasing rapidly and is driving a research renaissance in the plant science community [9]. This is in part due to
relaxation of Cannabis legislation in several developed countries, which has fuelled an unprecedented growth of legal Cannabis and derived products, creating multi-billion dollar markets in the US alone [10, 11].

Glandular trichomes are specialised multicellular epidermal protrusions that function as miniature secondary metabolite producing factories [12]. They are found in approximately one third of all angiosperms, where they fulfill a variety of roles in plant-environment interactions [13]. The production of distinct secondary metabolites in glandular trichomes is often species-specific [14, 15]. While terpenoids are arguably the largest class of secondary metabolites produced by glandular trichomes, other common compound classes include phenylpropanoids [16], acyl sugars [17], flavonoids [18] and methylketones [19]. Cannabinoids are a class of terpenophenolic compounds [20] that interact with the human endocannabinoid system, and are exclusively found in C. sativa trichomes [21].

Capitate stalked glandular trichomes are the most abundant trichome type found on C. sativa [22]. Cannabinoids and terpenoids are synthesised by a cluster of secretory cells located at the base of the glandular trichome head, referred to as disc cells. Secondary metabolites accumulate in the sub-cuticular cavities located between the disc cells and the cuticle of the glandular trichome head. The trichome head sits on top of a multicellular stalk connected by stipe cells [23]. The stalk is continuous with the epidermis of the flower tissue, and the walls are notably cutinised. It has been suggested that cutinisation of the stalk cell wall is to prevent leakage of resin produced by the gland head into the apoplastic space [24].

The capitate stalked glandular trichomes are particularly abundant on the female reproductive organs—floral leaves and bracts—of C. sativa [25]. This is likely an adaptation to protect developing seed from herbivory, thus enhancing chances of reproductive success [26]. The exceptionally high productivity of C. sativa glandular trichomes with respect to cannabinoids, however, is likely a consequence of artifical selection and targeted breeding [27].

The two main cannabinoids, cannabidiol (CBD) and tetrahydrocannabinol (THC), are well documented for their therapeutic properties [28, 29] which are largely mediated through interaction with the human endocannabinoid system [30]. CBD has been shown to have anti-inflammatory properties and is capable of increasing the pro-apototic abilities of human immune cells [31], whereas THC is the main psychoactive compound [32]. In addition to CBD and THC, over 100 additional cannabinoids have been detected using chromatography and mass-spectrometry technology [33, 34]. They are typically referred to as minor cannabinoids and include cannabigerolic acid (CBGA), cannabichromenic acid (CBCA), cannabionic acid (CBNA), and cannabicyclolic acid (CBLA), with several showing potential to alleviate different disease states [35, 36].

A key enzyme in the biosynthesis of cannabinoid precursor molecules is polyketide synthase III, a tetraketide synthase that catalyses the condensation of three malonyl Co-A with one molecule of hexanoyl Co-A to produce polyketide intermediates for olivetolic acid cyclase (OAC) to convert to olivetolic acid (OA) [37]. Cannabigerolic acid (CBGA) is synthesised through conjugation of OA and the isoprenoid geranyl pyrophosphate (GPP) via the enzymatic activity of a prenyltransferase, C. sativa prenyltransferase 4 (CsPT4), sometimes referred to as cannabigerolic acid synthase [37]. CBGA is a substrate for tetrahydrocannabinolic acid synthase (THCAS), cannabidiolic acid synthase (CBDAS) and cannabichromenic acid synthase (CBCAS), producing tetrahydrocannabinolic acid (THCA), cannabidiolic acid (CBD), and cannabichromenic acid (CBCA), respectively. Prenyltransferases such as CsPT4 demonstrate flexibility in substrate specificity and are known to accept derivatives of OA with variable alkyl side chain lengths [38].

Another class of cannabinoids known as cannaflavins are produced through the enzymatic activity of C. sativa prenyltransferase 3 (CsPT3). CsPT3 catalyses the covalent linkage of GPP
or dimethylallyl pyrophosphate (DMAPP) with the methylated flavone chrysoeriol to produce cannafaflavins A and B [39]. While the biosynthetic pathways of the major cannabinoids have been mapped to a large degree, little is known about the drivers of trichome productivity that enable these compounds to be accumulated in such high abundance.

Driven by the economic value of trichome-specific secondary metabolites, which have found use as pharmaceuticals, food flavourings and insecticides, extensive research has been conducted on the biology of glandular trichomes. A main focus has been on understanding the proteins, pathways and regulatory mechanisms governing the biosynthesis of secondary metabolites [40, 41]. In the long term, these activities have been proposed to aid in developing strategies to bioengineer systems for increased production of commercially valuable secondary metabolites [13, 42–45]. In this context, the proteomes of trichomes across a range of different species, including Solanum lycopersicum, Artemisia annua, Ocimum basilicum L., Olea europaea and Nicotiana tabacum [46–50], have been analysed. Investigation of the proteins found in Artemisia annua glandular trichomes, by means of a comparative proteomics approach, revealed that proteins involved in the electron transport chain, isoprenoid biosynthetic pathway, translation and proteolysis [47] were more abundant in the trichomes as compared to leaf tissue. A shotgun proteomic analysis of glandular trichomes isolated from Solanum lycopersicum revealed all steps of the methylerythritol 4-phosphate (MEP) pathway, as well as proteins involved in rutin biosynthesis and terpene biosynthesis [51], to be over-represented. Furthermore, glandular trichomes were associated with high energy production and protein turnover rates.

Proteomic analysis of C. sativa was pioneered by Raharjo et al. in 2004 [52]. Using two-dimensional gel electrophoresis and mass spectrometry, 300 proteins were identified from the late-stage flowers of C. sativa. A further 100 proteins were identified from “gland extracts”, likely a mix of epidermal tissues enriched for glandular trichomes. Proteins identified included zinc-finger type proteins, F-box family of proteins and a range of secondary metabolite synthases. Notably lacking were proteins involved in the biosynthesis of cannabinoids. A recent report C. sativa focused on the development of optimal protein extraction methods from C. sativa apical flower buds and isolated glandular trichomes, however shotgun proteomic analysis of the isolated samples only identified 160 proteins in mature apical buds and glandular trichomes [53]. The approach taken also made it difficult to distinguish proteins specific to glandular trichomes from those proteins specific to the apical buds. However, a number of secondary metabolite biosynthetic proteins, including those in the cannabinoid pathway, were identified. The combined proteome of the apical bud and glandular trichome included terpene synthases, members of the plastidal MEP pathway, and three members of the cannabinoid biosynthetic pathway, olivetolic acid cyclase (OAC), tetrahydrocannabinolic acid synthase (THCAS), and cannabidiolic acid synthase (CBDAS).

In this study, 1240 proteins were identified in glandular trichome head protein isolates, 396 proteins in glandular trichome stalk protein isolates and 1682 proteins in C. sativa late-stage flower protein isolates, using quantitative time of flight mass spectrometry (QTOF-MS/MS) and matching assembled peptides to the C. sativa cs10 reference proteome (NCBI). The relative protein abundances of glandular trichome heads, stalks and late-stage flowers were analysed to identify proteins that were shared between tissues, those that were tissue-specific and proteins which showed significantly different levels of relative abundance. Comparison of glandular trichome protein head isolates to the C. sativa late-stage flower protein isolates showed the extent to which glandular trichome heads are governed by secondary metabolite biosynthesis, secondary metabolite transport, and other processes such as carbon refixation.
Methods

Plant growth conditions

Seeds of *C. sativa* cultivar hemp No.8 were germinated on paper towels moistened with 0.01% gibberellic acid in water. After 7 days, seedlings were transferred to 1 L pots containing a mix of 1:1:1 vermiculite, perlite, peat moss, supplemented with 1 g/L dolomite. Seedlings were grown under conditions promoting vegetative growth for five weeks in a Sanyo growth cabinet (18hr light, light level 5). Plants were watered every three days with CANNA veg (CANNA) (40 mL A + 40 mL B/ 10 L water). For reproductive growth, plants were transferred to a growth room and placed under a Vipar spectra grow light (LED model: R900) (900 W)) under a 12:12 h light—dark cycle with both veg and bloom settings switched on. During flowering, plants were watered every three days with water and once per week with CANNA flora (40 mL A + 40 mL B/ 10 L water) and grown for approximately eight weeks or until two thirds of stigmas were brown in colour, indicating floral maturity.

Scanning electron microscopy and fluorescence microscopy

Scanning electron microscopy—Late-stage *C. sativa* flowers were harvested and the calyx was removed and placed on an adhesive carbon strip mounted on a loading stage for imaging. A Hitachi TM4000 plus scanning electron microscope was used with the cold stage set to -30˚C for sample mounting. Voltage was set to 10 kV—mode three and standard (M) vacuum.

Fluorescence microscopy—Calyx from late-stage *C. sativa* flowers were harvested using a blade and mounted on a glass slide. Images were taken using a Nikon E600 fluorescent microscope at an excitation wavelength of 400–440 nm with Dichromatic filter (DM) set at 455 nm and Barrier filter (BA) set at 480 nm. Images were captured using an Olympus DP72 camera controlled through the Nikon Cellsense software.

Isolation of glandular trichomes

Epidermal tissues were harvested from late-stage (week 7 post-floral induction) *C. sativa* flowers through abrasion, after which glandular trichome tissues were enriched through sieving followed by density centrifugation. One gram of late-stage flowers was flash frozen in liquid N₂ and grated against a 425 μm mesh sieve. Grated tissue was collected into a 150 μm mesh sieve. Liquid N₂ was poured over the 150 μm mesh to facilitate passage of particles onto a steel Pyrex™ collecting dish. The sieved material was then collected using precooled waxed paper, and 3 mL was rapidly transferred into a prechilled 15 mL falcon tube, followed by the addition of 3 mL of prechilled mannitol buffer (0.2 M mannitol, 0.05 M Tris-HCl, 0.02 M sucrose, 0.005 M MgCl₂, 0.01 M KCl, 0.0005 M K₂HPO₄, and 0.001 M EGTA).

The epidermal tissue extract was layered onto previously established discontinuous Percoll® density gradients consisting of a cushion of 80% Percoll® followed by 3 mL of 60%, 45%, and 30% Percoll® in mannitol buffer. Fractionation of the epidermal tissue mixture was achieved by centrifugation of the discontinuous Percoll® density gradient at 400 g at 4 °C for 10 min [54]. Glandular trichome heads were recovered at the 0/30% Percoll® interface. Glandular trichome stalks were recovered at the interface of the 30% and 45% Percoll layers. Fractions were collected from the gradient using a P1000 pipette and transferred into 2 mL Eppendorf tubes. Samples were washed free of residual Percoll® using 5 volumes Milli-Q water. Material was pelleted by centrifugation at 1000 g for 5 min in a refrigerated benchtop centrifuge (Heraeus® Megafuge®) and the supernatant was removed. The wash step was repeated three times. Samples were flash-frozen in liquid N₂ and stored at -80 °C.

Purity of the 0–30% fraction and 30–45% fraction was estimated by analysis of images taken through light microscopy. The total number of glandular trichome heads and glandular
trichome stalks were counted, using the multi-point analysis tool in software package ImageJ, for ten images of each of the 0–30% fraction and the 30–45% fraction. Glandular trichome heads and stalks were then expressed as a percentage of the total number of heads and stalks for each image, and the average of these percent totals was calculated.

Protein isolation

Total protein was isolated using approximately 100 mg of glandular trichome heads, 100 mg of glandular trichome stalks, or 1 g of flowers. Frozen samples were ground into a powder using a mortar and pestle. Powdered tissue was collected and placed into prechilled Eppendorf tubes, five volumes of (1:9) trichloroacetic acid (TCA)/acetone were added to each sample and mixed by inverting the tube several times. The suspension was filtered into a falcon tube using a 40 \( \mu \)m nylon mesh sieve. The filtrate was then stored overnight (O/N) @ -20 °C to allow for complete precipitation of protein. Samples were then centrifuged at 2000 g for 10 min in a tabletop refrigerated centrifuge, and the supernatant removed. Pellets were washed and resuspended with 5 mL of ice-cold acetone, and re-centrifuged at 2000 g for 10 min. The acetone wash step was repeated three times and the supernatant removed. Protein pellets were dried in a fume hood.

Trypsin enzymatic digest

Protein pellets were resuspended (1 mg/mL) in a buffer containing 2 M urea and 50 mM ammonium bicarbonate (pH 8) by vortexing and sonicated for 10 min. Tryptic digestion of protein samples was carried out by combining 20 \( \mu \)g of trypsin with 100 \( \mu \)L aliquots of each protein suspension. Tryptic digest reactions were carried out O/N @ 37 °C. Samples were lyophilized using a vacuum centrifuge. Lyophilised samples were dissolved in 100 \( \mu \)L 1% trichloroacetic acid (TCA)/Milli-Q water and transferred to glass sample vials.

nanoHPLC mass spectrometry

Protein samples were analysed on an Eksigent, Ekspert nano LC400 ultra HPLC coupled to a Triple time of flight (TOF) 6600 mass spectrometer (SCIEX) with a picoview nanoflow ion source. Five \( \mu \)L injections were run on a 75 \( \mu \)m x 150mm chromXP C18CL 3 \( \mu \)m column with a flow rate of 400 nL per minute and a column temperature of 45 °C. Solvent A consisted of 0.1% formic acid in water and solvent B consisted of 0.1% formic acid in acetonitrile. IonSpray voltage was set to 2600V, de-clustering potential (DP) 80V, curtain gas flow 25, nebuliser gas 1 (GS1) 30, and interface heater at 150 °C. A linear gradient of 5–30% solvent B over 120 minutes at 400 nL/minute flow rate, followed by a steeper gradient of 30% to 90% solvent B for 3 minutes, then 90% solvent B for 17 min was carried out for peptide elution. The mass spectrometer acquired 100 ms full scan TOF-MS followed by up to 50 ms full scan product ion data in an information dependent acquisition manner. Full scan TOF-MS data was acquired over the range 350–1500 m/z and for product ion MS/MS 100-1500 ions observed in the TOF-MS scan exceeding a threshold of 100 counts and a charge state of +2 to +5 were set to trigger the acquisition of product ion, MS/MS spectra of the resultant 50 most intense ions.

Protein identification

Spectral data acquired from the nanoHPLC MS/MS was matched to peptides derived from protein sequences of the list of all proteins available for hemp on uniport, as well as the list of predicted proteins derived from the C. sativa cs10 project (https://www.ncbi.nlm.nih.gov/genome/gdv/browser/genome/?id=GCF_900626175.1), using the input file from ProteinPilot™ (software version 5.0.1.).
The resulting MZidentifier file was exported to Scaffold4 software (Proteomesoftware) in order to view and quantitate the identified proteins found in our sample dataset.

**Protein data processing: Normalisation and relative protein abundance**

Normalization and relative protein abundance determinations were carried out using Scaffold4 software to allow for semi-quantitative comparisons of protein abundances between samples. Total spectra were normalized per sample using the normalized spectral abundance factor (NSAF) [56] whereby the assigned NSAF score for a protein is the number of spectra for a given protein in a sample divided by the length of that protein, further divided by the sum of all spectra of all proteins for a given sample.

Total spectral counts of the aforementioned samples and their biological replicates were comma-separated variant (CSV) formatted and exported to PANDA view proteomics software [57]. For 0 value spectral counts, 0.001 was used to replace null values and the natural logarithm (Log_e) was determined for all spectral counts for all samples.

To compare the relative protein abundance between two sample types (e.g. glandular trichome heads and stalks) a t-test (p < 0.05) with Benjamini-Hochberg multiple test correction was implemented. The pairwise comparison of relative protein abundance of two sample groups (e.g. glandular trichome heads and stalks) could then be plotted on a volcano plot (i.e. Log_2 Fold change on the x-axis vs –Log_{10} p-value). Allowing estimation of the proteins that are differentially abundant or exclusive to a given sample. Only those proteins present in all three biological replicates, with at least two unique peptides and passing the significance threshold with a fold change of at least 2x (Log2(fold change) ≥ 1; ≤ -1) were analysed further.

**Identification of metabolic pathways and other processes by Gene Ontology (GO) enrichment and MapMan**

GO enrichment analysis of the subset lists of cannabis cs10 proteins against the C. sativa cs10 proteome (NCBI Refseq GCF_900626175.2) was carried out using the fgsea Bioconductor package (https://bioconductor.org/packages/release/bioc/html/fgsea.html). GO annotation of the cs10 proteome was carried out by a stringent BLASTP [58], (E < 1E-15, bit score > 40, percent ID >30% and > = 50% of the shorter amino acid sequence length of the aligned pair) of the cs10 protein set against the GO-annotated Arabidopsis TAIR10 proteome, and associating the TAIR10 GO annotation with the corresponding cs10 homolog.

For enrichment analysis of the subset list of proteins, the protein IDs on the list were matched against the GO-annotated cs10 proteome, and the number of hits are indicated by the size column. The leading edge column lists the protein IDs that have significant hit (P < 0.05) to the GO-annotated cs10 proteome.

Metabolic pathways and various processes represented by the lists of proteins (from the previous step) were identified using MapMan software (v. 3.5.1R2, https://mapman.gabipd.org/home). The C. sativa cs10 proteome was mapped to the MapMan-curated Arabidopsis TAIR10 pathways (downloaded 2020-March 10 from https://mapman.gabipd.org/mapmanstore) via BLASTP, filtering for alignments of cs10 –to–TAIR10 protein with percent ID >30% and > = 50% of the shorter amino acid sequence length of the aligned pair.

**Results**

**Microscopy**

_Cannabis_ capitate stalked glandular trichomes can be divided, morphologically, into two very distinct regions: the glandular trichome head and the stalk. The head appears as a
bulbous sack on top of the stalk, connected by a short constricted neck that is continuous
with the stalk (Fig 1[A]). The base of the head is made up by a cluster of stipe cells that sub-
tend the discoid layer of disc cells (Fig 1[B]), which are connected to the stalk. While the
stipe cells emit red autofluorescence, indicative of photosynthetically active chloroplasts, the
surrounding layer of disc cells are devoid of red autofluorescence, suggesting the absence of
chloroplasts. The glandular trichome stalk is multicellular and is continuous with the epider-
mal layer (Fig 1[C]). The cell boundaries of the glandular trichome stalk with the epidermis
cannot be easily observed and indicate that the outer layer of stalk cells are similar in mor-
phology to epidermal cells and develop perpendicular to the plane of epidermal cells. Fluor-
escence microscopy shows glandular trichome heads emit characteristic blue fluorescence
indictative of the presence of phenolic compounds as previously observed in glandular tri-
chomes from tomato [59], while glandular trichome stalks do not show this fluorescence
(Fig 1[D]). In contrast, chlorophyll autofluorescence (400-440nm excitation) was observed
in glandular trichome stalks, suggesting stalk cells are photosynthetically active ([Fig 1B and
1D]). In order to gain more insight into the unique function of the different trichome tissues
a proteomic approach was undertaken.

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Enrichment of trichome fractions

Glandular trichome heads and stalks were purified by Percoll density gradient centrifugation from a heterogeneous mix of epidermal tissues obtained from excised flowers (Fig 2[A]). Light microscopy confirmed that glandular trichome heads were recovered from the interface between the 0% and 30% Percoll layers (Fig 2[B]). Glandular trichome stalks were recovered from the interface between the 30% and the 45% Percoll layer (Fig 2[C]). Tissues found at the 45%-55% and 55%-80% Percoll interfaces were comprised of mesophyll tissues and dense aggregates of heads and stalks and were excluded from further analysis due to lack of purity. Fragments of stigma and hair-type cystolithic trichomes were identified in the pellet following centrifugation. The glandular trichome head and stalk fractions were not entirely free from contamination, with an average of 11% of stalks present in the glandular trichome head fraction (Fig 2[D]) and an average of 28% heads found in the glandular trichome stalk fraction (Fig 2[E]). Still, there was considerable enrichment in the respective fractions allowing their use for downstream comparative proteome analysis.

Proteomics

Mass spectrometric data was searched against the cs10 C. sativa reference proteome. The results obtained were analysed with Protein Pilot 5.0.2 software (SCIEX), then visualised and validated using Scaffold 4.8.7 (Proteome Science) employing a minimum protein threshold of 99.9% and peptide threshold of 95%. Raw spectral data was submitted to the SCU research portal (https://researchportal.scu.edu.au/) and is available under DOI 10.25918/data.112. A total of 128,242 spectra were mapped to 1820 unique proteins across all tissues and replicates.
There were marked differences in the number of proteins identified between the sample types, with 1682 proteins identified in late-stage *C. sativa* flower, 1240 in glandular trichome heads and 396 proteins identified in glandular trichome stalks. Of the 1820 total proteins, 370 (20%) were found within all three sample types tested, 569 (31.3%) were exclusive to late-stage flowers, 122 (6.7%) were exclusive to glandular trichome heads and one protein (0.1%) was found to be unique to glandular trichome stalks (Fig 3[A]) (S1 Table).

Gene Ontology (GO) enrichment analysis revealed that the biological processes (BP) GO terms translational initiation and mitotic cell cycle, molecular function (MF) GO terms DNA binding and structural constituent of cytoskeleton, and cellular component GO terms (CC) nucleosome and microtubule were all overrepresented among the 370 proteins common to all tissues suggesting common housekeeping functions among all tissues (S2 Table).

Among the 569 proteins unique to late-stage flowers, BP GO terms relating to photosynthesis and chlorophyll biosynthetic process, and CC GO terms relating to chloroplast and chloroplast thylakoid membrane were significantly overrepresented, supporting a dominant role in photo-assimilation for this tissue (S2 Table). Interestingly, the set unique to flowers contained SNF1-related protein kinase regulatory subunit gamma-1-like (XP_030495483.1), implicated in metabolic signalling.

Among the 122 proteins unique to trichome heads, BP GO terms ion transmembrane transport and MF GO terms relating to ATPase activity were overrepresented, underlining the importance of ATP-dependent membrane energization and transport in trichome head function (S2 Table). In addition, several ATP binding Cassette (ABC) transporters were exclusively found in trichome heads.

Apart from common and unique proteins, 15 proteins (0.8%) were identified in both glandular trichome heads and glandular trichome stalks, 10 proteins (0.6%) were identified in glandular trichome stalks and late-stage flowers and 733 proteins (40%) identified in glandular trichome heads and late-stage flowers (S1 Table). Among the latter were several proteins related to raffinose metabolism.

Hierarchical clustering of total spectral counts across three biological replicates for each tissue type was applied to investigate the integrity of the data. Biological replicates for all samples were found to cluster together suggesting the three tissue types are distinct with respect to their proteomes. Furthermore, the glandular trichome head and stalk proteomes are more closely related to one another than either are to the late-stage flower proteome as indicated by the horizontal dendrogram (Fig 3[B]).

Pairwise comparison of relative protein abundance between the three tissue types. ([Fig 4A–4C]) showed significant differences in protein abundance (blue circles) for individual proteins including presence/absence variation (red triangles). Of those proteins found in glandular trichome heads, 4.2% were more abundant (*p* < 0.0263; log2 fold change ≥ 1) when compared to late stage flowers while 2.4% of proteins found in late stage flowers were more abundant compared to heads (*p* < 0.0263; log2 fold change ≥ 1) (Fig 4[A]). Four and a half percent of proteins found in glandular trichome stalks were more abundant (*p* < 0.02014 log2 fold change ≥ 1) compared to late-stage flowers while 19.2% of proteins found in late-stage flowers were more abundant (*p* < 0.2014; log2 fold change ≥ 1) when compared to stalks (Fig 4[B]). A total of 14.3% of proteins found in glandular trichome heads were more abundant in heads compared to glandular trichome stalks (*p* < 0.01009; log2 fold change ≥ 1), while 2.5% of proteins found in glandular trichome stalks were more abundant when compared to glandular trichome heads (*p* < 0.01009; log2 fold change ≥ 1) (Fig 4[C]). The majority of data points did not not pass the significance threshold (grey circles and triangles in ([Fig 4A–4C]).

**Comparison of trichome heads vs flowers.** Fifty-two proteins showed increased abundance in glandular trichome heads ([Fig 4A] and S3 Table [1]). Twelve were found to be
mitochondrial or plastidal in origin. The largest group among these representing 52 proteins was related to secondary metabolism (15%), followed by energy metabolism (12%), amino acid metabolism (10%), lipid metabolism (8%) and detoxification (8%) (Fig 4[D]). More than half of the proteins within the secondary metabolism category related to terpenoid and cannabinoid biosynthesis, including enzymes in the MEP pathway; isopentyl diphosphate delta isomerase (IDI) (ARE72265.1), alloromadendrene synthase (ARE72260.1), prenyltransferase 4–CBGAS (DAC76710.1), olivetolic acid cyclase (AFN42527.1) and prenyltransferase 3-CsPT3 (DAC76713.1). Other secondary metabolite biosynthesis related proteins included chalcone isomerase (AFN42529.1) involved in flavonoid biosynthesis and strictosidine synthase-like 10 (XP_030482574.1) involved in alkaloid biosynthesis [60].

Forty proteins were found with increased abundance (p < 0.00263) in late-stage flowers (Fig 4[A] and S3 Table [2]) and many were chloroplast-derived proteins. Proteins related to photosynthesis were most abundant (23%), followed by protein metabolism (18%), detoxification (5%) and amino acid metabolism (5%) (Fig 4[D]). Proteins related to photosynthesis included photosystem I (XP_030491785.1) and photosystem II (XP_030496367.1, XP_030482568.1) components as well as Calvin cycle proteins, while protein metabolism included translation related proteins, chaperones and disulphide isomerases. Other noteworthy proteins included elongation factors and those related to chromatin assembly, detoxification, carotenoid degradation/pigmentation—carotenoid 9,10(9',10')-cleavage dioxygenase 1-like (XP_030493365.1) and fruit ripening -2-methylene-furan-3-one reductase (XP_030483081.1) (S3 Table [2]).

Mapping of the highly abundant proteins in heads vs. late-stage flowers (Fig 5) indicated that late-stage flowers were comparatively enriched (blue colours) in photosynthesis and starch production, redox activity and nitrogen assimilation while glandular trichome heads were comparatively more abundant in terpene and lipid biosynthetic processes as well as elements of the mitochondrial electron transport chain, citrate metabolism and amino acid metabolism. These results indicate a primary sugar producing role for late-stage flowers while glandular trichome heads are strongly associated with the production and consumption of energy to drive secondary metabolite biosynthesis.

Comparison of trichome stalks vs flowers. Pairwise comparison of relative protein abundance between glandular trichome stalks and late-stage flowers is shown in (Fig 4[B]). A total of 24 proteins were found to be increased in abundance (p <0.02014) in glandular trichome stalks (S3 Table [3]). These proteins were mostly related to energy metabolism (20%) and detoxification (17%) (Fig 4[D]). The remaining proteins included those involved in the MEP pathway for isoprenoid biosynthesis -HDS partial (ARE72266.1), cannabinoid biosynthesis -olivetolic acid cyclase (AFN42527.1) and cannabidiolic acid synthase (AKC34410.1), the citric acid cycle malate dehydrogenase (XP_030508797.1), chromatin assembly and transmembrane secondary active transport V-type proton ATPase subunit F (XP_030502093.1).

Three-hundered and fifty two proteins were found in increased abundance (p<0.02014) in late-stage flowers when compared to glandular trichome stalk proteins (S3 Table [4]). Of those proteins 27% (97) were chloroplast derived proteins, and 9% (33) mitochondria derived. The primary GO categories of these more abundant proteins in late-stage flowers related to protein metabolism (17%), photosynthesis (10%) and amino acid metabolism (10%) (Fig 4[D]).
Mapping of the proteins found more abundant in the pairwise comparison of glandular trichome stalks and late-stage flowers (Fig 6) indicated that many processes are largely underrepresented in the stalks when compared to the late-stage flower proteome. Notable exceptions to this include mitochondrial electron transport related proteins which were found to be more abundant in the stalks, while processes relating to nucleotide metabolism and terpene secondary metabolism were only modestly more abundant in the stalks. Late-stage flowers were comparatively more abundant in processes such as sugar and starch metabolism, cell wall biosynthesis, photosynthesis and respiration, and the biosynthesis of amino acids and lipids. The results indicate that glandular trichome stalks are limited in respect to their biological processes and most likely have a highly specialised biological role.

Comparison of trichome heads vs trichome stalks. Pairwise comparisons of relative protein abundance between glandular trichome heads and glandular trichome stalks is shown in (Fig 4[C]). One hundred and seventy-seven proteins displayed increased abundance in glandular trichome heads and late-stage flowers (Fig 6) indicated that many processes are largely underrepresented in the stalks when compared to the late-stage flower proteome. Notable exceptions to this include mitochondrial electron transport related proteins which were found to be more abundant in the stalks, while processes relating to nucleotide metabolism and terpene secondary metabolism were only modestly more abundant in the stalks. Late-stage flowers were comparatively more abundant in processes such as sugar and starch metabolism, cell wall biosynthesis, photosynthesis and respiration, and the biosynthesis of amino acids and lipids. The results indicate that glandular trichome stalks are limited in respect to their biological processes and most likely have a highly specialised biological role.

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glandular trichome heads ([S3 Table [5]]. These proteins were categorized into protein (16%) and energy metabolism (9%), followed by lipid metabolism (8%), amino acid metabolism (8%) and secondary metabolism (7%) (Fig 4[D]).

Protein metabolism related proteins included: translation – 60S ribosomal protein L10a (XP_030483002.1) and 40S ribosomal protein S19-3 like (XP_030484107.1), protein turnover—(ubiquitin-like protein SMT3 (CAI11094.1) and 26S proteasome non-ATPase regulatory subunit 4 homolog (XP_030485940.1). Energy metabolism included the citric acid cycle enzyme isocitrate dehydrogenase [NAD] regulatory subunit 1 (XP_030485874.1), and the electron transport chain–complex I (XP_030490934.1), complex III (XP_030500833.1), and complex IV proteins (XP_030496363.1), and ATP synthase subunit O (XP_030493792.1). Secondary metabolism included: MEP pathway proteins–DXR (ARE72264.1), MCT (XP_030499547.1), and DXS (XP_030507676.1), terpene biosynthesis pathway proteins -alloaromadendrene synthase (ARE72260.1) and α-pinene synthase (ARE72261.1), cannabinoid biosynthesis proteins–CBGAS prenyltransferase 4 (DAC76710.1), cannabavin biosynthesis—CsPT3 (DAC76713.1), flavonoid biosynthesis protein—chalcone isomerase (AFN42529.1), and alkaloid biosynthesis protein—strictosidine synthase-like 10 (XP_030482857.1). Lipid metabolism-related proteins included many related to fatty acid biosynthesis. Of particular interest was lineolate 13S lipoxygenase 2 (XP_030504574.1), a key enzyme in the biosynthesis of fatty alkyl groups, used in the biosynthesis of alkylresorcinolic acids—precursors to cannabinoid biosynthesis ([S3 Table [5]].

Ten proteins were increased in abundance in glandular trichome stalks (S3 Table [6]) and were categorised into pathways/function (Fig 4[D]). Proteins with increased abundance in glandular trichome stalks (p<0.01) were associated with detoxification—peroxiredoxin-2E-2 (XP_030493857.1) and superoxide dismutase (XP_030504009.1), energy metabolism—ATP
synthase F1 subunit (ALF04039.1) and secondary metabolism—olivetolic acid cyclase (AFN42527.1). Others included chromatin assembly—Histones: H2B.1 (XP_030504034.1), H4 (XP_030481652.1) and H2A.5 (XP_030492994.1), and putative desiccation resistance -BURP domain protein RD22 (XP_030485614.1) and desiccation-related protein PCC13-62-like (XP_030492994.1).

Mapping of the proteins found increased in abundance in the pairwise comparison of both glandular trichome heads and glandular trichome stalks (Fig 7) indicated that glandular trichome heads (proteins shown in red) are hubs of secondary metabolic processes such as terpene, phenylpropanoid, and phenolic biosynthesis. Aside from secondary metabolism, glandular trichome heads were comparatively more abundant in ascorbate and glutathione metabolism, amino acid biosynthesis, sulfur metabolism, energy production and consumption. Glandular trichome stalks were comparatively lacking in notable biosynthetic processes, similar to the comparison between glandular trichome stalks and late-stage flowers. These results provide further support to the view that trichome stalks play a highly specialised role in the trichomes.

Discussion

Considering that major cannabinoids stored in trichome heads can account for over 15% of the *C. sativa* female flower dry weight [61], trichome heads constitute a considerable carbon sink. In fact, in the absence of fertilization and subsequent grain filling, as is the case for medicinal *Cannabis* production, female floral trichomes can be assumed to be the major carbon sink during reproductive growth.
Though it cannot be ruled out that larger fan leaves contribute to source strength of *C. sativa* during reproductive stages, it is more likely that female flowers themselves act as the primary source for carbon. While fan leaves showed clear signs of senescence, female inflorescences remained green and appeared healthy and photosynthetically active, and are thus postulated to function as the main source tissue to fuel the filling of the trichome head subcortical cavities during the late stages of flowering [62].

Pairwise comparisons of trichome heads, trichome stalks and whole late-stage flowers supported this model and provided a more detailed picture of source-sink interactions at the trichome level.

Around one quarter of the proteins displaying increased abundance in late-stage flowers compared to glandular trichome heads were related to photosynthesis and sugar production (Figs 4[D] and 5), indicating a strong role for late-stage flowers in the generation of photo-assimilates that is speculated to serve as a carbon and energy source for trichome head sink tissues. Source-sink-interactions and underlying metabolic signalling at reproductive stages during grain filling have been well studied [63–66]. Sucrose is a key signalling compound, which, through the activity of trehalose-6-phosphate (T6P), regulates primary metabolism. The signalling molecule T6P is thought to act as a sucrose gauge that has been demonstrated to directly inhibit SNF1 (sucrose-non-fermenting 1) related protein kinase (SnRK1) [67]. SnRK1 in turn acts as a central integrator of metabolic signals and governs over a switch from anabolism to catabolism in what is known as a feast or famine response. SnRK1 signalling cascades activate transcription factors that govern energy starvation responses [68].

Interestingly, SNF-1 related protein kinase regulatory subunit gamma-1 like protein (XP_030495483.1) was identified among the proteins in late-stage flowers (S1 Table [3]). Presence of SnRK1 in late-stage flowers suggested that with the development of glandular trichomes a strong sink is established, and that flow of carbon, likely in the form of C₆ sugars, from flower mesophyll to trichome heads would be regulated through established metabolic signalling pathways.

Following a multi-omics approach to study glandular trichome function in tomato, sucrose has been proposed as a carbon and energy source for trichome sinks [69]. Tomato glandular trichomes, however, are photosynthetically active, and for non-photosynthesising glandular trichomes, raffinose has been proposed as the C₆ carbon source. In peppermint glandular trichomes raffinose has been demonstrated to fuel MEP-dependent terpenoid biosynthesis in leucoplasts [70]. In *C. sativa*, chlorophyll-a autofluorescence was present in trichome stalks ([Fig 1B and 1D]) and in the stipe cells that subdend the disc cell layer of glandular trichome heads ([Fig 1B]), but absent from the disc cells ([Fig 1B]). Distinction between chloroplast-containing stipe cells and chloroplast-devoid disc cells seems to suggest a functional separation of disc and stipe cells, which could not be further unravelled with the experimental set up. It is possible that the stipe cells are functionally more related to the cells of glandular trichome stalks, i.e. playing a role in photosynthesis and sugar transport. Stipe cells are typically found with disc cells following separation of the glandular trichome head from the glandular trichome stalk ([Fig 1B]), which is not surprising considering they subdend the disc cell layer. The close relationship between the disc and stipe cells of *Cannabis* glandular trichomes is shown by scanning and transmission electron microscopy studies [71, 72]. Several chloroplast-specific and photosynthesis associated proteins were indeed found in the glandular trichome head fraction, supposedly originating from stipe cells (S1 Table [1]), which is supportive of photosynthesis taking place in these cells.

It has been proposed that light reactions in tomato photosynthetic trichomes supply the reducing power (NADH) demand of the MEP and MVA isoprenoid biosynthesis pathways and that carbon required for secondary metabolism is supplied from source tissues [69], or
refixed respiratory CO$_2$ [73]. The raffinose biosynthetic enzymes, galactinol-sucrose galactosyltransferase 2 (XP_030493448.1) and 5 (XP_030482143.1), were detected in late-stage flowers (S1 Table [3]), while the enzyme alpha-galactosidase (XP_030508592.1), which catalyses the breakdown of raffinose into sucrose and D-galactose, was found in glandular trichome heads (S1 Table [1]), suggesting a potential role for raffinose as a mobile photo-assimilate in C. sativa glandular trichome source-sink metabolism. Moreover, in peppermint, a trichome specific isozyme ferredoxin NADP$^+$ reductase supplies electrons via a ferredoxin for the reductases of the MEP pathway, thus shunting production of monoterpenes via the MEP pathway enzymes (E)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate (HMB-PP) synthase (HDS) and HMB-PP reductase (HDR). In contrast, in photosynthetic glandular trichomes such as those from Solanum habrochaites, this trichome-specific ferredoxin reductase is missing, and terpenoids are derived from both the MEP and the MVA pathway [69, 74, 75]. Ferredoxin NADP reductase (XP_030479992.1) was indeed found in the glandular trichome head proteome (S1 Table [1]), suggesting a high flux of MEP derived terpenoids in this cell type. This peppermint-like model for MEP pathway-derived isoprenoids is further supported by the enrichment of MEP pathway proteins in glandular trichome heads (S3 Table [1]), while MEV related proteins were present in both glandular trichome heads and late-stage flowers, but not significantly more abundant in either. Interestingly, phosphoenolpyruvate (PEP)-carboxylase (PEPC) (XP_030480704.1), associated with CO$_2$ fixation during C$_4$ and CAM metabolism, showed increased abundance in heads when compared to both flowers and stalks (S3 Table [1 and 5]). It has been speculated that PEPC could act in the refixation of respiratory CO$_2$ in trichome heads to maximize carbon efficiency and supply for secondary metabolism [73, 76].

While our approach did not allow us to discriminate between the different cell types in the glandular head fraction, it suggested a model for C. sativa glandular trichome secretory cells akin to the glandular trichomes of the non-photosynthetic secretory system outlined by Shuurink and Tissier [77]. The layer of disc cells notably lacks chlorophyll-a fluorescence (Fig 1[B]) and is subtended by a layer of photosynthetically active stipe cells (Fig 1[B]) [71]. Enrichment of the oxidative pentose phosphate pathway ([Figs 5 and 7] as well as enrichment of the non-photosynthetic isozyme of ferredoxin reductase, which has been proposed to supply the reducing power to the enriched MEP pathway [75], indicates similarity to non-photosynthetic secretory cells such as in peppermint, with specialised leucoplasts [78] as the site of secondary metabolite biosynthesis relying on refixed carbon through PEP-carboxylase [73] and imported carbon in the form of sucrose/raffinose to maintain a high flux of MEP-derived cannabinoids and terpenoids (Fig 8).

When compared to the late-stage flower proteome (S3 Table [3]), stalk tissues showed an enrichment of the light dependant reaction enzyme photosystem II D1 (AJK91402.1), as well as chlorophyll-a autofluorescence throughout glandular trichome stalks (Fig 1[D]). Chlorophyll-a is a light absorbing pigment molecule that works in concert with the photosystem II D1/D2 complex, during the production of ATP and NADH. This suggested glandular trichome stalks play a role in light dependant photosynthesis. The glycolytic enzyme fructose bisphosphate 3-aldolase (XP_030496648.1), a marker for carbohydrate metabolism, including the Calvin cycle, was also highly abundant. We speculate that the resulting sugars from stalk-based photosynthesis could act as an additional source of carbon and reducing power for glandular trichome heads or, equally likely, as local substrates to fuel high rates of mitochondrial respiration. In fact, energy metabolism-related proteins (TCA cycle and respiratory chain components) made up the largest group of proteins with increased abundance in stalks (20%) compared to late flowers (S3 Table [3]), followed by proteins relating to cellular detoxification (17%). High abundance of peroxidases and superoxide dismutase might indicate a harsh cellular environment rich in peroxides and other reactive oxygen species (ROS) in glandular
trichome stalks. Peroxides and ROS abundance could be the result of high photorespiration and mitochondrial respiration rates respectively, supported by the observed enrichment of photosynthesis and mitochondria related proteins in glandular trichome stalks [79, 80].
addition, high irradiation under the controlled conditions of our experimental set up could also contribute to the production of superoxide species [81]. While disc cells are shielded from high irradiation by a large head space full of light absorbing terpenoids and cannabinoids, stalk cells are more exposed. Collectively, increased abundance of proteins involved in energy metabolism and detoxification in the stalks suggested a strong focus of these cells towards generation of ATP and reducing equivalents. ATP is speculated to be needed in stalks cells for membrane energization that drives primary or secondary active membrane transport. ATPases (V-type proton ATPase subunit) that provide the electrochemical gradient to fuel secondary active transport were found to be more abundant in the stalk proteome compared with the late-stage flower proteome (S3 Table [3]). The finding that they were not more abundant in the stalk proteome when compared with the head proteome might be due to the fact that membrane energization to maintain transport is also important in glandular trichome heads, which will be discussed below.

If C₆ sugars are the mobile photo-assimilate in *C. sativa* glandular trichomes, then the presence of secondary active C₆ sugar transporters would be a reasonable assumption and could explain the need for high ATPase activity in the stalk cells. Plant sucrose symporters and antiporters are well described and characterized [82–84], however no sucrose or other related transporters were identified in the stalk proteome. Absence of any form of sugar transporters could indicate that transport mechanisms other than secondary active trans-membrane transport are responsible for carbon shuttling from mesophyll through the stalk to the glandular trichome head. *C. sativa* It must be considered, however, that with a total of 1820 proteins identified (Fig 3[A]), only a fraction of the actual proteome was captured. Moreover, with only 396 proteins identified in (Fig 3[A]) the stalk fraction, it was disproportionally poorly represented in the overall data set and membrane bound transport proteins are generally under-represented in proteome datasets due to their low levels of expression [82]. Thus, absence of any active sugar transporters detected in the stalk proteome could be simply due to lack of resolution of the overall approach.

Surprisingly, two cannabinoid biosynthetic proteins (OAS and CBDAS) displayed increased abundance in stalk vs late-stage flower proteome. While this could indicate contribution of the stalks to biosynthesis of cannabinoids, it is more likely a result of contamination of the stalk fraction with disc cell proteins from the heads ([Fig 2C and 2E]), which were estimated to be around 27%.

In conclusion, while there is a clear indication of *Cannabis* glandular trichome stalks contributing to energy production, photosynthesis and detoxification, details on how they take part in active sugar transport and the biosynthesis of secondary metabolites remains unclear.

Taken together, these results support a model for carbon generation, flow and utilization within glandular trichomes and underlying mesophyll tissue (Fig 8). C₆ sugars, likely raffinose or sucrose, are produced by the mesophyll cells of the late-stage flower and transported via yet unknown mechanisms through trichome stalks to disc cells at the base of the trichome heads. There, they would be broken down into simple sugars and enter sugar catabolic pathways including glycolysis (GLY) and the oxidative pentose phosphate pathway (OPP) [85]. Indeed, a total of 6% of overly abundant proteins in heads compared to stalk proteins and 4% in the comparison between heads and flowers were associated with these pathways (S3 Table [1 and 5]).

C₃ pyruvate can enter energy metabolism, TCA cycle and respiratory chain (RES) for ATP production. Nine percent (9%) of the proteins with increased abundance in heads vs stalks were associated with energy metabolism, suggesting high rates of energy production. In the case of trichome heads compared to late-stage flowers, that figure is 12% (Fig 4[D]). The comparatively higher demand for energy in glandular trichome heads supports an extensive secondary metabolism and possibly membrane energization (S3 Table [5]).
Intermediates and end products of glycolysis (GLY) and pentose phosphate pathway (OPP) can enter the fatty acid metabolism (FAM) pathway to drive lipid biosynthesis needed to support the increase in membranes required for trichome head expansion and possible vesicular trafficking of secondary metabolites, as well as to provide precursors such as olivetolic acid for cannabinoid biosynthesis. Eight percent of the overly abundant proteins in heads vs stalks and 8% in heads vs late flowers were associated with lipid metabolism (Fig 4D), underlining the importance of these pathways in glandular trichome heads. Ultimately the majority of carbon and energy reaching the disc cells of trichome heads is speculated to be invested in secondary metabolite biosynthesis pathways including cannabinoid and terpenoid biosynthesis. While cannabinoid, terpenoid, and MEP pathway biosynthesis associated proteins made up 5% of the proteins increased in abundance in the head vs stalk, they comprised 10% in head vs flower. Proteins contributing to other secondary metabolite pathways made up 3% in head vs stalk and 6% in head vs flowers of the enriched proteins. Notably, 10% of proteins found preferentially more abundant in glandular trichome heads compared to late-stage flowers were related to amino acid (AA) metabolism. The abundance of AA metabolism-related proteins supports the hypothesis of glandular trichome heads as secondary metabolite biosynthetic factories, as AA’s serve as precursors for many of these compounds [86]. Moreover, plasma membrane-type, V-type, and mitochondrial ATPases are found comparatively more abundant in heads compared to late-stage flowers (S3 Table [1]) and were among the most enriched GO categories in the head fraction (S2 Table), presumably to support the filling of subcuticular trichome cavities with secondary metabolites through ion (H+) gradients [87, 88] and to produce ATP to fuel the diverse primary and secondary metabolism of glandular trichome heads [89]. With respect to secondary metabolite transport, an ABC transporter B family member (XP_030500887.1) and pleiotropic drug resistance (PDR) 1 like-protein (XP_030488377.1) were found only in glandular trichome heads (S1 Table [1]). Plant PDR and ABC subfamily B transporters are largely plasma membrane localised transporters that function in the efflux of secondary metabolites from cells via the hydrolysis of ATP [90], contributing to the overall demand for ATP. A PDR type transporter of petunia has previously been implicated in the trichome-specific transport of steroidal compounds or their precursors, contributing to trichome-mediated herbivory defense [91].

Our model supports the paradigm of glandular trichomes as highly specialised secondary metabolite factories and contributes to the understanding of C. sativa glandular trichome biology. By drawing on the differences and similarities in protein composition between the components of glandular trichomes and flowers, we have developed an initial framework for the functions and processes that govern C. sativa glandular trichome productivity. Our findings lay the foundation for future research into metabolic regulation of medicinally important secondary metabolites in C. sativa glandular trichomes.

**Supporting information**

S1 Table. [1–10] List of proteins for each tissue, and all Venn diagram-based comparisons. Total proteins found in the protein isolates of glandular trichome heads [1], glandular trichome stalks [2] and late-stage flowers [3]. Proteins found in Venn diagram-based comparisons of head-stalk-flower consensus [4], stalk only [5], stalk-flower consensus [6], head-stalk consensus [7], head-flower consensus [8], head only [9] and flower only [10]. (XLSX)

S2 Table. [1–4] Gene Ontology (GO) enrichment for unique and common proteins. List of unique and common proteins [1] and GO enrichment for common proteins [2], proteins unique to late flowers [3] and unique to heads [4]. Enriched GO pathways are categories under
molecular function (MF), cellular component (CC) or biological process (BP). P-values (pval),
category size (size) and underlying protein accession IDs (leading edge) are shown.

S3 Table. [1–6] List of proteins found in relative abundance in pairwise comparisons of all
tissues. Overly abundant proteins in head vs late flowers [1], in late flowers vs head [2], in
stalk vs late flowers [3], in late flowers vs stalk [4], in head vs stalk [5] and in stalk vs head [6].
In addition to providing protein accession ID, protein name, p-value and log2 fold change,
proteins were categorized according to main metabolic function (category).

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Author Contributions
Conceptualization: Lee James Conneely, Bronwyn J. Barkla, Tobias Kretzschmar.
Data curation: Ramil Mauleon.
Formal analysis: Lee James Conneely.
Funding acquisition: Bronwyn J. Barkla, Tobias Kretzschmar.
Investigation: Bronwyn J. Barkla, Tobias Kretzschmar.
Methodology: Lee James Conneely.
Project administration: Tobias Kretzschmar.
Resources: Jos Mieog, Tobias Kretzschmar.
Software: Ramil Mauleon.
Supervision: Bronwyn J. Barkla, Tobias Kretzschmar.
Writing – original draft: Lee James Conneely, Bronwyn J. Barkla, Tobias Kretzschmar.
Writing – review & editing: Ramil Mauleon, Jos Mieog.

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