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Research paper

Metabolomics reveals the within-plant spatial effects of shading on tea plants

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It is well known that green tea made from fully developed leaves located at the base of young shoots is of lower quality than that made from the still developing leaves located on the top of the shoot. It has additionally been shown that plant shading can significantly improve green tea quality. Here, we aimed to get more insight into the effects of shading on the overall metabolome in different parts of the tea shoots. To do this, field-grown tea plants were shaded by coverage with either a straw layer or a black net, both blocking the daylight intensity for more than 90%. Both the first (i.e. still developing) leaf and the fourth (i.e. fully developed) leaf, as well as the stem of young shoots were harvested and subjected to complementary untargeted metabolomics approaches, using accurate mass LC-Orbitrap-Fourier transform mass spectrometry (FTMS) for profiling both semi-polar and lipid-soluble compounds and GC-TOF-MS for profiling polar compounds. In total, 1419 metabolites were detected. Shading resulted in a decreased ratio of polyphenols to amino acids (which improves the quality of green tea) and lower levels of galloylated catechins in the shoots. The positive effect of shading on the amino acid/catechin ratio was more pronounced in the fully developed (fourth) than in the developing (first) leaves. Furthermore, many metabolites, especially organic acids, carbohydrates and amino acids, showed differential or opposite responses to the shading treatments between the three shoot tissues investigated, suggesting a within-plant spatial regulation or transport/redistribution of carbon and nitrogen resources between the tissues of the growing young shoots. This work provides new insight into the spatial effects of shading on tea plants, which could further help to increase tea quality by improving cultivation measures for plant shading.

Highlights:

- **Using complementary untargeted metabolomics approaches, 1419 metabolites has been detected in three young tea shoot tissues (stem, first and fourth leaves) and their responses to two plant shading treatments determined.**
- **In contrast to its marked increase in both first and fourth leaves, the key tea quality-related amino acid theanine significantly decreased in the stems under shading.**
- **The positive effect of shading on the amino acid/catechin ratio was more pronounced in the fully developed (fourth) than in the young still-developing (first) leaves.**
- **Differences in shading-induced changes in metabolites (e.g. organic acids, carbohydrates and amino acids) between the three young shoot tissues (suggest a within-plant spatial redistribution of carbon and nitrogen resources.**

Keywords: flavonoid and amino acid metabolism, leaf position, shading, tea quality, untargeted metabolomics.

Introduction

The quality of a cup of tea, including its aroma, taste and color, is determined by the content and proportion of its chemicalconstituents, which are directly related to the metabolite composition of the harvested plant shoots and subsequent conversions during the manufacturing process of tea leaves and stems (Zhang and Ruan 2016). Although it is the key to tea quality, the exact metabolite composition in young tea shoots depends on multiple factors: specific genetic strain, climatic conditions (temperature, humidity, light), soil nutrient status, horticultural practices, harvest season and leaf age (position on stem), among others (Wang et al. 2011).

The biosynthesis of flavonoids (polyphenols), the most important group of secondary metabolites and quality components of tea (Zhang and Ruan 2016) may be influenced to a large extent by environmental factors (Liu et al. 2016). Temperature, for example, has critical effects on the biosynthesis and accumulation of flavonoids and other phenolic compounds (Lin et al. 2011; Cheynier et al. 2013), frequently even more than light has (Yao et al. 2005; Bernal et al. 2013). Studies have shown that high light levels strongly induce gene expression and activation of key enzymes involved in flavonoid biosynthesis, which coincide with flavonoid accumulation, in many plant species (Lo and Nicholson 1998; Cheynier et al. 2013). Other key quality compounds in tea, such as amino acids and caffeine, are also at least partially regulated by local environmental factors (Lee et al. 2010; Wang et al. 2011). In general, plants may suffer from high-light stress when the available radiation energy exceeds its consumption or dissipation through their photosynthetic reaction centers, as betrayed by a reduced plant photosynthetic capacity that leads to photoinhibition (Long et al. 1994). Several studies, however, suggest that moderate shading can promote the accumulation of amino acids, chlorophyll and caffeine in young tea shoots, since under normal light conditions the excess in photosynthetic activity may exacerbate nitrogen deficiency and accelerate the decomposition of quality-related amino acids (Ku et al. 2009; Wang et al. 2012; Lee et al. 2013; Zhang et al. 2014). Yet, little is known about the influence of plant shading on the metabolite composition in the various parts of the harvested young tea shoots and how these are affected by changes in light conditions (Lee et al. 2010; Wang et al. 2011).

Previous work has shown that shading of tea plants can influence the composition of quality-related components in their young shoots, which improves the taste quality of the resulting green tea brew. Shading of tea plants to decrease the light intensity reduces their content of polyphenols and improves the taste quality of green tea (Ku et al. 2009; Wang et al. 2012; Lee et al. 2013). Wang et al. (2012) suggested that this reduction arises from both the polymerization of catechins and the glycosylation of flavonols, resulting in the accumulation of larger, more complex flavonoid structures with a concomitant reduction in their smaller precursor forms. However, while most of previous studies have focused on the metabolites in the youngest, still developing leaves and buds, relative little is known about the effects of shading on the fully developed tea leaves, which are a major part in most green teas (Wang et al. 2012; Lee et al. 2013; Zhang et al. 2014).

By adding pure compounds to the brew, free amino acids (e.g. theanine and glutamate) have been recognized as the principal contributors to the taste of mellowness, while phenolic compounds (e.g. catechins and flavonols) and their oxidized derivatives are the principal contributors to astringent and bitter tastes of tea beverages, especially of green teas (Zhang and Ruan 2016). A high-quality green tea is characterized by high contents of free amino acids with appropriate concentrations of catechins and caffeine (Wang et al. 1988). In brief, a low ratio of total-phenolics/amino-acids is beneficial for green tea quality. The contents and ratios of tea quality-related compounds, such as amino acids and polyphenols, in different tissues/parts of the harvested young tea shoots (buds, leaves and stems) may also show tissue specificity; e.g. gallic acid and theanine are found at relatively high levels in stems (Lee et al. 2011). Because of their lower ratio of total phenols to amino acids, the younger (still developing) leaves provide green tea of higher quality than older (fully developed/expanded) leaves do (Lee et al. 2011). Therefore, examining this amino acid: polyphenol ratio in young versus older leaves in the harvested shoots is critical for predicting and confirming the quality of the final tea products (Lee et al. 2011).

In recent years, metabolite profiling has been applied in various studies related to tea plants and their products (Zeeb et al. 2000; Ku et al. 2009; Lee et al. 2010; Fujimura et al. 2011; Lee et al. 2011; Vrhovsek et al. 2012; Liu et al. 2017). However, the metabolic networks of tea plants remain far from being clearly elucidated, mainly due to the fact that plants' biosynthetic pathways of amino acids, sugars, organic acids, flavonoids, alkaloids and lipids are highly interrelated. Compared to a single analytical platform, multiplatform comprehensive metabolomics will provide more insight, which makes it a powerful approach for rebuilding metabolic networks and getting deepest insight into the effects of, for instance, changes in growth conditions on the global plant metabolome.

We investigated whether the accumulation and metabolism of quality-related compounds in the various parts of the young tea shoots might be differentially affected by shading. In this study, we determined the within-plant spatial effects of two frequently applied shading treatments on the metabolome of both the first (still developing) leaves, the fourth (fully expanded) leaves and the stem of young shoots from field-grown tea plants. We applied a multiplatform-metabolomics approach enabling

the analysis of hundreds of compounds from a wide range of polarity classes. Additionally, we performed quantitative gene expression analysis to determine the effects of shading at the level of specific transcripts. Our main aim was to get a better insight into the spatial effects of shading on the global metabolism in young tea shoots, which information could then be used to increase tea quality by improving cultivation measures for plant shading.

Materials and methods

Tea plant cultivation and experimental design

The shading experiments were conducted in a 14-year-old tea field at a commercial plantation in Shaoxing, Zhejiang Province, China (Shaoxing Royal Tea Village Co., Ltd). The plants (*Camellia sinensis* (L.) cv. Longjing 43) were planted with a row spacing of 1.8 m. When new shoots had grown to one terminal bud and two young leaves, in March 2016, tea plants were covered by either a layer of STRAW (5-cm thickness) or a NET (black high-density polyethylene netting), both installed 1 m above the surface of the plant canopy (Figure S1 available as Supplementary Data at *Tree Physiology* Online). Plants without any covering served as the non-shaded controls. Tea plants in four consecutive rows (15 m) were defined as one plot (covering an area of ca. 90 m^2), and each treatment was replicated three times. Five spots in the canopy of each plot were randomly selected to measure leaf temperature and incoming light intensity. Temperature and humidity were recorded on the surface of tea leaves during the entire experiment. After 20 days, when the newly formed young shoots consisted of 1 bud and 5–6 leaves, the first and fourth leaves as well as the stems were separately harvested (each in six biological replicates, each biological replicate was a pool of 30 leaves/stems from six plants), quickly frozen in liquid nitrogen, and stored at −80°C in an ultra-freezer until further analysis. Harvested samples were freeze-dried and then ground into a fine powder using a ball mill (M301, Retsch GmbH, Germany) prior to further analysis. All 54 samples were firstly analyzed for chlorophylls, (epi)catechins, alkaloids, polar compounds and semi-polar compounds. Due to the limited amount of sample, especially of the young leaves, subsequent gene expression analysis could only be performed on the stems and the fourth leaves, while for both lipidomics, flavonols and lignin analyses only sufficient sample of the fourth leaves was left.

Quantitative determination of chlorophylls, catechins, alkaloids and lignin

For analyzing chlorophylls, 50 mg of the powdered samples (were extracted with Tris-buffer/MeOH/chloroform (with 0.1% w/v butylated hydroxytoluene as antioxidant and Sudan 1 as internal standard), as recently described by Mokochinski et al. (2018). The extracted powders were washed twice with chloroform. After combining and drying the chloroform phases, the lipid-soluble compounds were dissolved in 0.5-mL ethyl acetate (+0.1% butyl hydroxyl toluene, w/v); then, 10 µL of this was injected into an HPLC (Waters Alliance e2695, Milford, MA, USA) coupled to a photodiode array detector (Waters 996 PDA, Milford, MA, USA), which enabled the recording of absorbance spectra of eluting compounds at 240–700 nm. Chlorophyll a and b were identified based on comparisons of retention time and absorption spectra with authentic standards. External calibration curves were constructed to enable quantification of compounds after correcting for the variation in the internal standard (i.e. Sudan 1). Waters Empower 3 software (Waters, Milford, MA, USA) was used for raw data processing. Catechins were extracted and quantified by HPLC-DAD as described by Zhang et al. (2017); their separation was performed using a C18 reverse-phase column (250 × 4.6 mm i.d., Phenomenex, Torrance, CA, USA). Alkaloids were extracted with 75% MeOH in MQ water (v/v) and detected using HPLC-DAD, as described in Zhang et al. (2017). Lignin determination was performed as described by Van Soest (1963).

Gas chromatography–mass spectrometry-based metabolomics

The extraction of polar primary metabolites (mainly amino acids, organic acids, sugars) was adapted from Mokochinski et al. (2018). Specifically, 20 mg of each tea leaf powder was extracted with 1.4 mL of 80% (v/v) methanol in MQ water containing ribitol as an internal standard. After vortexing (10 s) and centrifugation (12,000 rpm, 10 min), 500 µL of the supernatant was mixed with 375 µL of chloroform and 750 µL of MQ water. Then, 50 µL of the upper (polar) phase was dried by vacuum centrifugation. Automated derivatization of the metabolites, with methoxyamine and *N*-methyl-*N*-(trimethylsilyl) trifluoroacetamide (MSTFA), was performed by a CombiPAL pipetting-autosampler robot (CTC Analytics AG, Zwingen, Switzerland) mounted on top of the GC unit. After completing this derivatization, a series of alkanes were automatically added to each sample as well. Extracts were then analyzed using gas chromatography coupled to time-of-flight mass spectrometry (GC-TOF-MS), comprising of an Agilent 6890 gas chromatograph (Agilent Technologies, Santa Clara, CA, USA) and a Pegasus III TOF MS (Leco Instruments, Saint Joseph, MI, USA). Chromatographic separation was conducted using a capillary column (Agilent DB-5, 30 m × 0.25 mm i.d., 0.25 µm, Santa Clara, CA, USA). Hard ionization was performed at 70 eV.

Liquid chromatography–mass spectrometry-based metabolomics

Semi-polar extracts To compare samples for their composition of semi-polar metabolites (mainly phenolic acids, polyphenols,

alkaloids), all samples were extracted by essentially following the method proposed by De Vos et al. (2007). Specifically, 25 mg of dry powder was extracted with 1 mL of 75% MeOH in MQ water (v/v) acidified with 0.1% (v/v) of formic acid (FA). After vortexing (1 min), sonification (15 min) and centrifugation (10 min), 2 µL of the resulting supernatant was injected in a high-mass-resolution liquid chromatography–mass spectrometry (LC–MS) system consisting of a Waters ACQUITY UHPLC coupled to a Waters PDA detector (DAD) and a Thermo Linear Ion Trap (LTQ)-Orbitrap FTMS XL hybrid system. The compounds in each extract were separated with a Luna C18 column (Phenomenex, 150 × 2 mm i.d., 3 mm—Torrance, CA, USA) using a 45-min gradient of 5–75% acetonitrile (eluent B) in MQ water (eluent A), both acidified with 0.1% (v/v) FA, as described by Mokochinski et al. (2018). The column was kept at 40°C, and the flow rate was 0.19 mL/min. The PDA detector was set at a wavelength range of 210–600 nm. The Orbitrap FTMS was set in negative electrospray ionization mode, with a mass resolution of 60,000 (FWHM) and an m/z range of 90–1350 D. For a selected set of samples, additional mass fragmentation experiments were performed using data-dependent acquisition in the discovery mode, by setting the ion trap to select the three most intense ions per full scan for fragmentation up to MS3 with a normalized collision energy of 30 eV; the exclusion time for the same mass was set at 20 s, and the detection of the accurate masses of the fragments by the Orbitrap FTMS was at a resolution of 7500.

Nonpolar extracts (lipidomics) Untargeted analysis of lipid-soluble compounds was essentially performed as described in Remmers et al. (2018), using four biological replicates of the fourth leaves. Specifically, 25 mg of the dry tea powder were extracted with 4.6 mL of chloroform/methanol (1:1, v/v) containing 10 µmol/L of internal standard (1,2-didecanoyl-sn-glycero-3-phosphocholine, Sigma P7081) and 0.1% (w/v) of the antioxidant butyl hydroxyl toluene (2,6-di-tert-butyl-4-methylphenol, Sigma B1378). After incubation on ice for 15 min, the samples were sonicated for 20 min and centrifuged. Then, 400 µL of the supernatant was dried in a Speedvac and re-dissolved in 1 mL of ethanol. After centrifugation, 5 µL was injected into the same accurate mass LC–MS system as described above with the same MS settings, but with different chromatographic conditions: these lipid-soluble compounds were separated on an HSS T3 column (Waters, 1.8 µm, 1.0 × 100 mm) using 20% acetonitrile +10 mmol/L NH4Ac (eluent A) and 10% acetonitrile +90% isopropanol +10 mmol/L NH4Ac (eluent B) as the mobile phases. The gradient started at 35% eluent B (+65% eluent A), increased linearly to 70% eluent B (+30% eluent A) in 3 min, then to 85% eluent B (+15% eluent A) in 7 min, and finally ended with a washing step at 90% eluent B (+10% eluent A), from 15 to 17 min. The column temperature was maintained at 55°C, and the flow was 0.2 mL/min.

Untargeted data processing

For each metabolomics platform, the raw data files were processed in an unbiased manner using the dedicated Metalign-MSClust workflow. First, MetAlign software (Lommen and Kools 2012) was used for baseline correction, peak picking and alignment of all mass signals across all the samples. The resulting lists of peak intensities (peak heights) of mass signals were filtered for mass features present in at least four samples. Absent peaks—i.e. with a signal to noise ratio < 3—were replaced by random values between 40 and 60% of the local noise inputted by MetAlign. After this filtering step, 24,916 reproducible mass features were retained for the GC-TOF-MS analysis of derivatized polar extracts (GC-TOF-MS polar), 14,398 for the LC–MS analysis of the semi-polar extracts (LC–MS semipolar) and 19,010 for the LC–MS analysis of nonpolar extracts (LC–MS lipidomics). These peak lists were then brought into MSClust software (Tikunov et al. 2012), in order to cluster the mass signals originating from the same metabolite (including e.g. isotopes, adducts and in-source fragments) based on their corresponding retention time and intensity pattern across all samples. This clustering yielded 180, 695 and 544 mass clusters (i.e. reconstructed, putative metabolites with their mass spectra) for the GC-TOF-MS polar, LC–MS semipolar and LC–MS lipidomics platforms, respectively; this represented a total of 1419 metabolites for further study (Tables 1, S1, S2, S3).

Multivariate analysis and statistics

The preprocessed metabolite intensity data were introduced to SIMCA-P v13.0 (demo, Umetrics, Sweden) for principal component analysis (PCA) after their log-transformation and Pareto-scaling. Univariate statistical analyses were performed in R (http://www.r-project.org/). 'Adonis' was applied for the permutational multivariate analyses of variance. Statistically significant differences among mean values were tested using one-way ANOVA, with a multiple correlation test using false discovery rate (FDR) estimation. Tukey's *post hoc* test was applied for the pairwise comparison of multiple groups' mean values. Differences were considered significant when $P < 0.01$.

Identification of selected metabolites

To annotate the differentially accumulating metabolites—top 20 of each comparison between treatments in the three tissue types—the extracted data from the chromatograms were matched with those in available metabolite libraries. For the LC–MS data, observed retention time, accurate mass, isotopic composition, UV spectra and MS/MS information were manually matched with publicly accessible (KNApSAcK, METLIN, LipidMAps and MassBank) and in-house metabolite databases obtained from previous studies on tea and other plant species

Table 1. Number of primary metabolites (GC, derivatized polar extracts detected by the GC-TOF-MS platform), secondary metabolites (LC, detected by the LC–MS semipolar platform) and lipid-soluble metabolites (lip, nonpolar extracts detected by the LC–MS lipidomics platform) of which the abundance in tea shoots grown under shading by either NET or STRAW was significantly different ($P < 0.01$) from the unshaded (control; CK) shoots (six biological replicates per treatment).

		Net/CK Down-regulated in Net	UP-regulated in Net	Straw/CK Down-regulated in Straw	UP-regulated in Straw	Straw/Net Down-regulated in Straw	UP-regulated in Straw
LC (695 metabolites)	First leaf	227(33%)	109(16%)	267(38%)	106(15%)	46(7%)	9(1%)
	Fourth leaf	254(37%)	136(20%)	284(41%)	143(21%)	75(11%)	26(4%)
	stem	153(22%)	85(12%)	183(26%)	96(14%)	54(8%)	31(4%)
GC(180 metabolites)	First leaf	26(24%)	52(48%)	30(28%)	42(39%)	22(20%)	9(8%)
	Fourth leaf	21(19%)	61(56%)	28(26%)	75(69%)	22(20%)	50(46%)
	stem	82(76%)	20(19%)	37(34%)	42(39%)	14(13%)	89(82%)
Lip(544 metabolites)	Fourth leaf	117(22%)	99(18%)	126(23%)	106(19%)	39(7%)	43(8%)

(Moco et al. 2007; Van der Hooft et al. 2012; Ridder et al. 2013). For the GC-TOF-MS data, annotations were based on comparing both the spectra and the retention index (RI) to standard compounds previously analyzed on the same system, and by checking the mass spectral databases of NIST and the Max Planck Institute Gölm, Germany (Fiehn et al. 2000).

Quantification of flavonol levels

The levels of total (mainly glycosylated) quercetin, kaempferol and myricetin were determined from 22.5 mg of tea leaf powder (that was hydrolyzed in 75% MeOH containing 1.2 mol/L of HCl (+ 0.1% TBHQ as antioxidant) at 90–95°C for 1 h (Muir et al. 2001). Released flavonol aglycons were then analyzed by HPLC (Waters Alliance e2695, Milford, MA, USA) coupled to a photodiode array detector (Waters 996 PDA, Milford, MA, USA), using the same C18reversed-phase column and gradient as described above for the LC–MS analysis of (non-hydrolyzed) semi-polar extracts. External calibration curves with pure compounds (kaempferol, quercetin and myricetin) were built to quantity the detected flavonols. Waters Empower v3 software (Waters, Milford, MA, USA) was used for peak integration.

Quantitative real-time PCR analysis

From the fourth leaves and stems, total RNA was isolated with an RNAplant_plus kit (Tiangen, China). The cDNA was synthesized with a PrimeScriptTM RT reagent kit (TaKaRa) and quantitative real-time PCR (qRT-PCR) performed on an Applied Biosystems 7300 machine (Carlsbad, CA, USA). Primer pairs used for the qRT-PCR can be found in Table S4 available as Supplementary Data at *Tree Physiology* Online. *GAPDH* was used as the reference gene. For each target gene, triplicate reactions were performed; mean values of these three technical replicates were calculated for each biological replicate. Relative transcript levels were then calculated against those of the internal control (household gene *GAPDH*) by using the formula $2^{-\Delta\Delta Ct}$. All data shown are expressed as the mean ± SD ($n = 3$ biological replicates).

Results

Shading treatment and phenotypic characterization

During the experimental period, the daytime light radiation levels at plant canopy level under NET shading, STRAW shading, and in the non-shaded control were 100–300, 15–60 and 800–2500 μmol m^{-2} s^{-1}, respectively; hence, ca. 90 and 96% of the solar light was blocked by the NET and STRAW treatments, respectively (Figure 1A and B). The maximum daytime temperatures of the tea plant canopy were 27, 23 and 32°C under the NET, STRAW and control conditions, respectively; while the average evening/night temperature was 15°C for all (Figure 1D). Thus, during daylight hours, the shaded tea plants had significantly lower canopy temperatures than the non-shaded controls ($P < 0.01$), with the cooling effect of STRAW exceeding that of NET. Air humidity was significantly higher ($P < 0.01$) under both NET and STRAW than in the uncovered canopy, especially during the light period, with STRAW maintaining the highest air humidity (Figure 1C). Thus, the shading treatments not only affected the plants' exposure to light, but also to temperature and humidity: the lower the light intensity on the canopy, the lower was the local temperature and the higher the relative humidity during daytime.

Shaded leaves (all leaves from young tea shoots) were heavier (51.7 ± 2.7 and 50.2 ± 1.9 g fresh weight per 0.1 m^2 of tea plant canopy under NET and STRAW shading, respectively) than non-shaded leaves (47.0 ± 3.8 g, $P < 0.01$). The levels of chlorophyll *a* and b were ca. 50% higher in both NET-and STRAW-shaded plants than in the non-shaded controls (Figure 2D), corroborating their darker green color (Figure 2B).

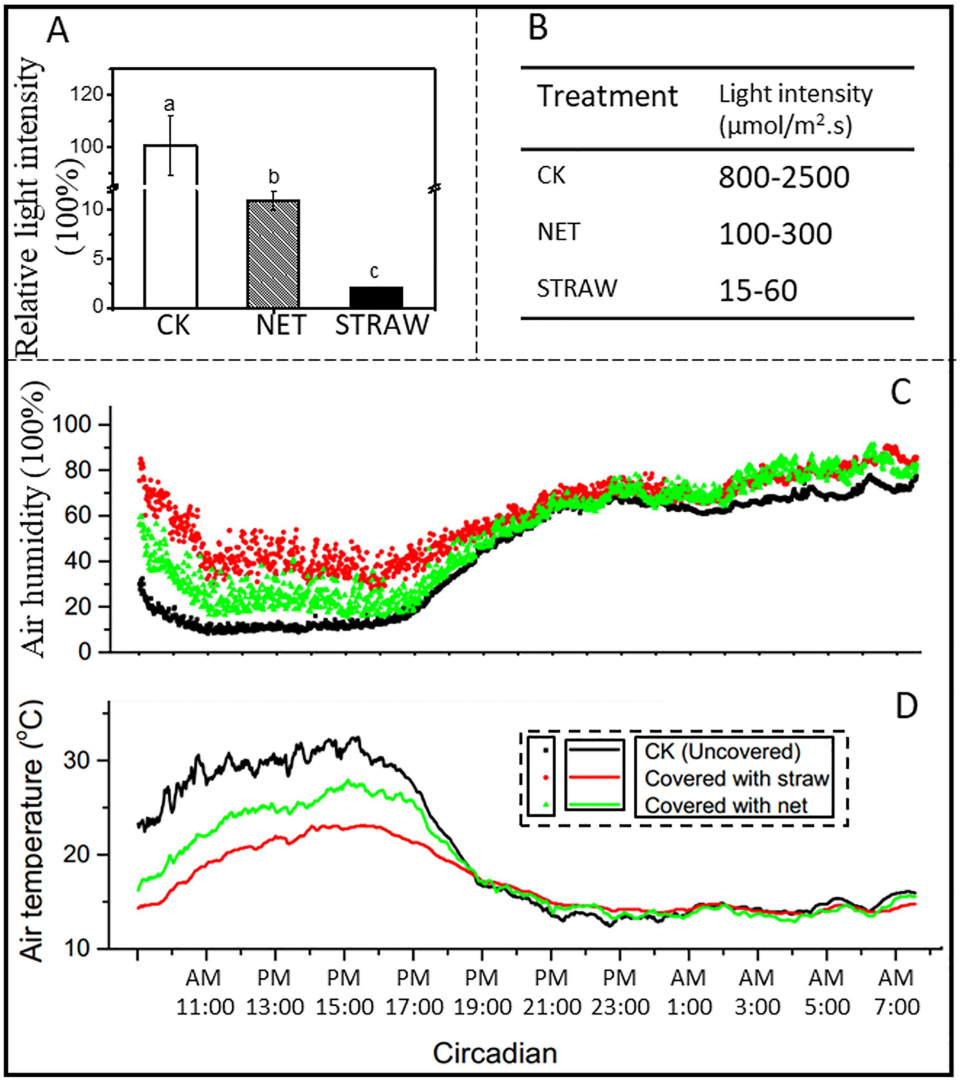

Figure 1. Light radiation (A, B), air temperature (C) and air humidity (D) measured in the canopy of tea plants (means ± SD, five biological replicates per treatment groups) under NET and STRAW shading and in the canopy of uncovered control plants (CK). 'NS' and different letters above the bar indicate significant ($P < 0.01$) differences, respectively, among the shading treatments.

Moreover, in the fourth leaves, there was less ($P < 0.01$) lignin in shaded than in non-shaded (control) leaves, corroborating the softer texture of these shaded leaves (Figure S2 available as Supplementary Data at *Tree Physiology* Online).

Impact of shading on the overall metabolome of tea shoots

As exemplified by the highlighted chromatographic peaks in the LC–MS profiles of semi-polar compounds (Figure S3 available as Supplementary Data at *Tree Physiology* Online), the metabolite compositions of the shaded (NET and STRAW) tea leaves differed greatly from the non-shaded (control) leaves. We used complementary mass spectrometry-based platforms for deep-phytochemical profiling coupled to unsupervised principal component analysis (PCA) (Figure 3), to reveal the global metabolome differences between three parts of the young shoots of tea plants, i.e. the first (developing) leaf, the fourth (full-grown) leaf, and the stem, and their response to shading treatments. The PCA score plot based on secondary metabolites detected by the LC–MS semipolar platform (Figure 3A) clearly distinguished the leaf and stem samples on PC1 (explaining 43.9% of the total variation) with separation of the first and fourth leaves on PC2. Moreover, PC2 corresponded to the shading effect in all three tissues types (Figures 3A and S4), with the STRAW-shaded tissues further from their non-shaded controls than those shaded with NET. These results indicate that both shading treatments generally have similar effects on the secondary metabolites in each shoot tissue type, with STRAW being more effective than NET. In contrast, the overall effect of shading on polar primary metabolites, as detected by GC-TOF-MS, was partially opposite between leaves and stems, with the fourth leaves being more strongly affected than the first leaves (Figure 3B). According to the PCA results of the

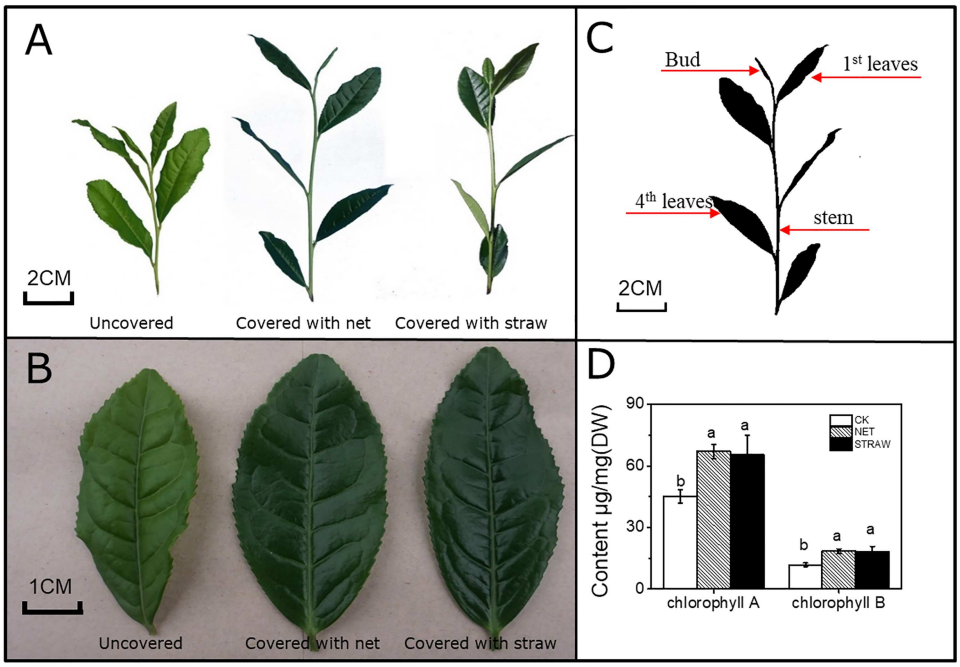

Figure 2 Phenotypic characterization (A, B), leaf position (C), and chlorophyll content (D) of tea plant shoots grown under NET and STRAW shading and of the non-shaded (control) plants (means ± SD, six biological replicates per treatment groups). 'NS' and different letters above the bar indicate significant ($P < 0.01$) differences, respectively, among the shading treatments.

LC–MS lipidomics platform (fourth leaves only), both shading treatments greatly affected the lipid-soluble metabolites, in which PC1 corresponded to the shading effect (Figure 3C). In order to obtain a more general picture of the effect of shading on the spatial metabolome of the shoot, we merging the metabolomics data sets that were obtained for all tissues, i.e. from the LCMS-semipolar and the GC-TOF-MS-polar platforms. A PCA score plot based on these merged data sets (Figure 3D) shows that the fourth leaves under NET shading (sample code N-4) are close to the nonshaded first leaves (sample code CK-1), while under STRAW shading the fourth leaves (code S-4) are close to the shaded first leaves of both NET and STRAW treatments (codes S-1 and N-1; the samples of the nonshaded fourth leaves (code CK-4) are clearly different from all others. These PCA results suggest that the shading of tea plants can shape the metabolome, at least the polar and semi-polar compounds, of especially the fourth (fully developed) leaves into a composition more similar to that of the first (still developing) shaded leaves, as compared to these tissues under non-shading conditions.

Table 1 gives the numbers of metabolites that significantly differed ($P < 0.01$) in their relative abundance among the three treatments. Clearly, both shading treatments have substantial, and in leaves also differential effects on both polar primary, semi-polar secondary and lipid-soluble compounds (the latter determined for the fourth leaves only) in the young shoots.

Changes in primary metabolism in tea shoots by shading

Regarding the primary metabolites detected by the GC-TOF-MS polar platform a higher number of compounds was up-regulated than down-regulated by shading, in both the first and fourth leaves of the tea plants (Table 1). Those polar primary metabolites that were significantly changed in their abundance by either or both shading treatments are listed in Table S5 available as Supplementary Data at *Tree Physiology* Online. In the first leaves, the relative level of glycine, glutamine and serine was all significantly lower ($P < 0.01$) in both NET and STRAW treatments compared with the unshaded controls, whereas the level of glutamic acid, aspartic acid and especially theanine were significantly ($P < 0.01$) increased (Figure 4). In the fourth leaves, shading treatments, and especially STRAW, significantly increased ($P < 0.01$) the relative levels of most amino acids, of which the key tea flavor compound theanine was again the most responsive (average ratio between STRAW and control treatment = 108.38). In contrast to its marked increase in both first and fourth leaves, the level of theanine was significantly decreased in the stems by shading ($P < 0.01$, ratio between STRAW and control was 0.0035).

The contents of many carbohydrates in leaves and stems were also significantly changed ($P < 0.01$) by the shading treatments (Figure 4). For example, the contents of D-xylopyranose, fructose, maltose and glucose were reduced under the shading treatments, while those of sucrose, 2-furanacetic acid, D-xylose, fructofuranosyl-fructofuranose and D-glucose were increased;

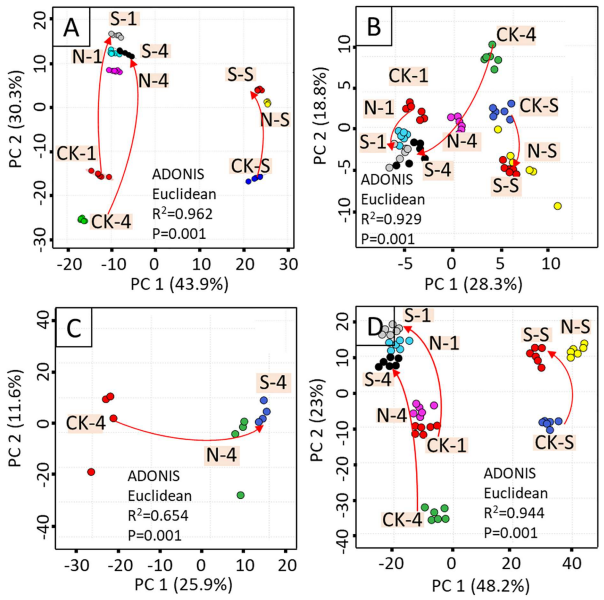

Figure 3 PCA score plot of tea samples based on the relative variation of all 695 compounds detected by the LC–MS semipolar platform (A), all 180 compounds detected by the GC-TOF-MS polar platform (B) and all 544 compounds detected by the LC–MS lipidomics platform (C), merged data of two subsets (GC-TOF-MS polar and LCMS-semipolar metabolites) of the tea foliar metabolome (D), using the leaves and stems harvested from tea shoots grown under either NET or STRAW shading and those from uncovered control shoots. 'N-', 'S-' and 'CK-' denote the tea shoots shaded by NET, STRAW and uncovered, respectively, while the additional codes '1', '4' and 'S' denote the first, fourth leaves and stem, respectively (six biological replicates per treatment group). *P* and R^2 indicate the confidence level and explanatory power of grouping (to total variation), respectively, from the permutational multivariate analyses of variance.

for D-glucose in the fourth leaves, the ratio of shaded vs. control plants was 44.94 and 101.12 under the NET and STRAW treatments, respectively.

Like the carbohydrates, the organic acids showed variable responses to shading, depending on the shoot part analyzed. For instance, shading treatments induced decreases in the relative contents of succinic acid and quinic acid, and increases in the content of both D-glucuronic acid, malic acid, gallic acid, citric acid and α-ketoglutaric acid in the first leaves; the alkaloid caffeine was increased in the first leaves as well. In the fourth leaves, citric acid, malic acid, acetic acid and α-ketoglutaric acid were increased by shading, while in stems most significantly changed ($P < 0.01$) organic acids decreased, except quinic acid, which compound was slightly increased; remarkably, citric acid decreased under NET, while it increased under STRAW, in both cases about a factor 2.

Changes in secondary metabolism in tea shoots under shading

As shown in Table S6 available as Supplementary Data at *Tree Physiology* Online and Figure 4, the shading treatments caused marked changes in secondary metabolite composition as detected by the LC–MS semi-polar platform. In all three shoot tissues, the number of down-regulated secondary metabolites in shaded vs. control leaves was much higher than the number of up-regulated ones (Table 1). Furthermore, the number of metabolites altered by shading in the fourth leaves exceeded those in both the first leaves and stems (Table 1), suggesting the fourth leaves being the most responsive tissue regarding its secondary metabolism.

Shading, and especially the STRAW treatment, resulted in a significant increase in the caffeine content of both the fourth leaves and the stem, while in the first leaves, this compound was unaffected as compared to the unshaded control shoots (Table 2).

To determine differential effects between shading treatments, in each tissue type we compared STRAW and NET applications for their relative impact on metabolite levels as compared to the non-shaded (control) plants. In the first leaves, the largest relative differences between STRAW versus NET levels were found for quercetin 3,7,4'-O-triglucoside (ratio 0.01; i.e. 100-fold less in STRAW than NET) and theaflavin (ratio 1.65); in the fourth leaves, the largest differences between these shading treatments were detected for the quercetin-based acylated triglycoside (ratio 0.12) and catechin 7-O-apiofuranoside (ratio 6.59), while quercetin-3-O-(glucoside-6-O-rhamnoside) (ratio 0.05) and 3-O-methylgallate (ratio 2.81) were found most differentiated in the stem.

Quantitative determination of the various tea catechins and epicatechins, based on HPLC-DAD, showed that in the first leaves the contents of the catechin-related structures—including gallocatechin, catechin, epigallocatechin gallate, gallocatechin gallate and catechin gallate—all significantly decreased ($P < 0.01$) under shading treatments, while the epicatechin structures, including epigallocatechin, epicatechin and epicatechin gallate, did not at all or hardly change in their contents (Table 2). Expression analysis of known structural genes related to the biosynthesis of these catechins and other flavonoids (including CHI, FLS, *F3 '5' H* and *DFR*) indicated that these genes were highly down-regulated, compared to control plants, by both shading treatments and in both the first leaves and, to a lesser extent, the stem of the young shoots (Figure 5). Quantification of flavonols, after acid-hydrolysis of samples, indicated that quercetin, myricetin and kaempferol were all decreased by the STRAW shading, to levels that were 25, 20 and 70% of those in control plants, respectively (Figure 6), a result which is in line with the down-regulation of the *FLS* gene and all other genes of the flavonoid pathway tested (Figure 5).

Changes in lipids in tea shoots by shading

The fourth leaf samples were additionally subjected to profiling of their lipid-soluble compounds (lipidomics), using accurate mass LC–MS. As shown in Table S7 available as

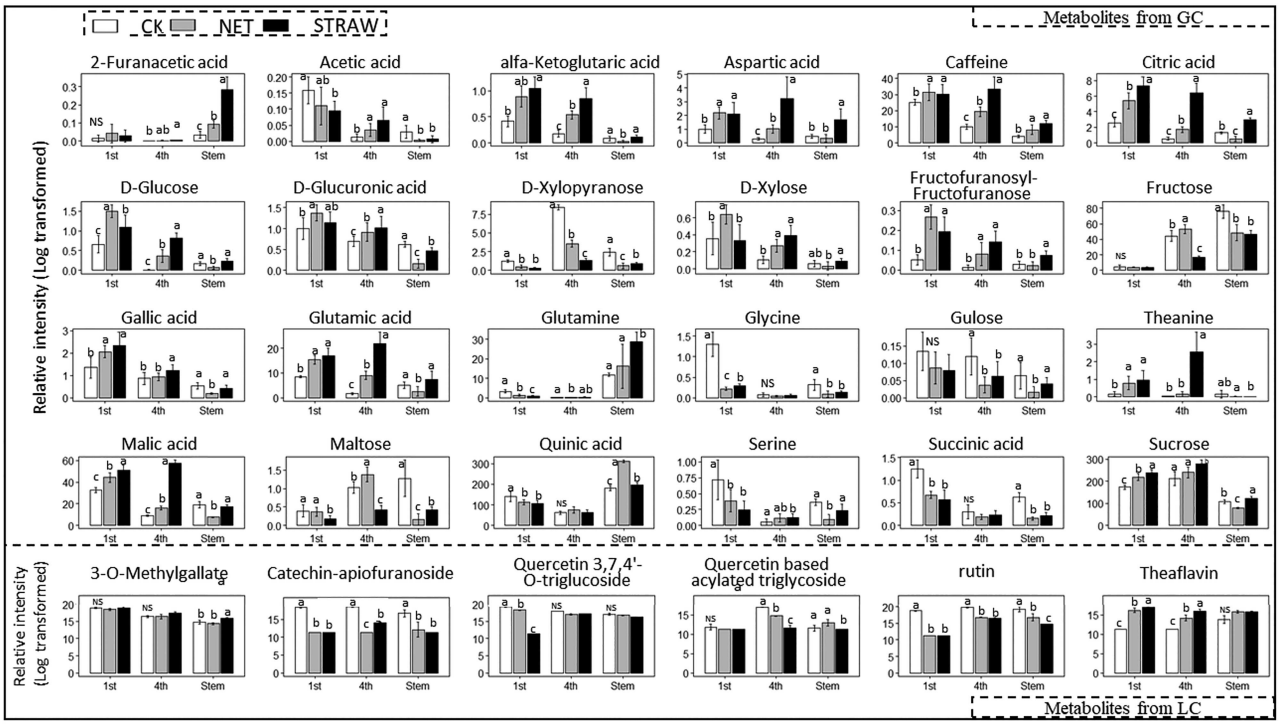

Figure 4 Putatively identified metabolites showing significant changes in their relative abundance (means ± SD; $n = 6$) in young shoots of tea plants grown for 20 days under shaded conditions using either NET (grey bars) or STRAW (black bars), compared with the non-shaded (control) plants (CK; white bars). 'NS' and different letters above the bar indicate insignificant and significant ($P < 0.01$) differences, respectively, between shading treatments.

Table 2. Concentrations (mg/g dry weight, means ± SD) of the principal catechins and alkaloids in young tea shoots grown under shading by either NET or STRAW, compared with the unshaded (control) shoots (six biological replicates per treatment). Different letters indicate significant ($P < 0.01$) differences, between the three shading treatments or tissues

	First leaf			Fourth leaf			Stem		
	control	Net	Straw	control	Net	Straw	control	Net	Straw
Gallocatechin	1.17 ± 0.09a	0.37 ± 0.02b	0.29 ± 0.01c	0.25 ± 0.01d	0.04 ± 0 h	0.07 ± 0 g	0.16 ± 0.02e	0.13 ± 0f	0.13 ± 0f
Catechin	7.41 ± 0.53a	1.57 ± 0.02 g	1.08 ± 0.02 h	3.41 ± 0.27b	2.05 ± 0.06d	1.16 ± 0.06 h	2.79 ± 0.52c	1.77 ± 0.03e	1.61 ± 0.01f
Epigallocatechin gallate	73.78 ± 3.49a	45.79 ± 11.44d	58.13 ± 1.57c	64.29 ± 0.99b	55.66 ± 1.45c	42.18 ± 0.29d	13.3 ± 2.16e	12.1 ± 0.29e	14.73 ± 0.39e
Gallocatechin gallate	1.64 ± 0.16b	0.92 ± 0.03d	0.92 ± 0.1d	0.95 ± 0.13d	0.44 ± 0.04e	0.44 ± 0.02e	2.36 ± 0.16a	0.95 ± 0.02d	1.36 ± 0.03c
Epigallocatechin	12.54 ± 0.87a	10.48 ± 0.49c	13.73 ± 0.56b	6.44 ± 0.25e	5.99 ± 1.48e	7.5 ± 0.09d	3.13 ± 0.29f	1.72 ± 0.13 h	2.26 ± 0.05 g
Epicatechin	6.42 ± 0.92b	6.23 ± 0.25b	6.24 ± 1.01b	3.91 ± 0.14d	1.47 ± 0.01e	4.69 ± 0.33c	13.55 ± 1.07a	4.21 ± 0d	4.1 ± 0.31d
Epicatechin gallate	15.71 ± 0.74a	14.29 ± 0.35b	13.37 ± 0.55c	10.18 ± 0.45e	13.66 ± 0.49c	11.7 ± 0.12d	2.15 ± 0.32 g	2.05 ± 0.03 g	2.73 ± 0.02f
Catechin gallate	1.32 ± 0.05a	0.71 ± 0.02c	0.7 ± 0.03c	0.92 ± 0.04b	0.63 ± 0.02d	0.57 ± 0.01e	0.14 ± 0.02f	0.05 ± 0.01 h	0.07 ± 0 g
Gallate	2.87 ± 0.15c	4.06 ± 0.03b	5.63 ± 0.36a	0.33 ± 0.02i	0.52 ± 0.01 h	0.94 ± 0.03e	0.58 ± 0.06 g	0.83 ± 0.01f	1.36 ± 0d
Theophylline	2.05 ± 0.27a	1.79 ± 0.32b	2.03 ± 0.01a	0.85 ± 0.04d	1.42 ± 0.01c	1.73 ± 0.03b	0.47 ± 0.05e	0.2 ± 0.02 g	0.35 ± 0.02f
Caffeine	20.5 ± 1.05a	19.44 ± 0.46a	20.13 ± 0.67a	14.39 ± 0.23c	17.57 ± 0.49b	19.1 ± 0.45a	4.7 ± 0.6f	6.26 ± 0.01e	9.6 ± 0.07d

Supplementary Data at *Tree Physiology* Online, some lipid structure (e.g. putatively identified as L-olivosyl-oleandolide, DG(18:0/18:1(9Z)/0:0), TG(18:0/18:2(9Z,12Z)/18:3(6Z,9Z,12Z))) levels were decreased by shading as compared to the control treatment, whereas others (e.g. PI(18:4(6Z,9Z,12Z,15Z)/0:0), anandamide (18:2, n-6)) increased. It worth noting that glycerophosphoserines levels significantly decreased while other glycerophospholipids increased by the shading treatment. The gene expression analysis using qRT-PCR revealed higher expression of the *LOX* gene (encoding lipoxygenase enzyme) in the leaves from NET shading than in either the control or STRAW shading treatment (Figure 5). By contrast, the expression of the gene encoding chlorophyll decomposition enzyme (CHL) was significantly ($P < 0.01$) inhibited under both STRAW and NET conditions (Figure 5).

Discussion

The youngest, still developing leaves and buds represent the growth centers of new shoots of tea plants, while the older, fully developed leaves function as the photosynthetic center. Consequently, these contrasting leaf tissues form a sink-source relationship that transfer resources via the stem tissue (Pfautsch et al. 2015). For example, circulation and reuse of stored

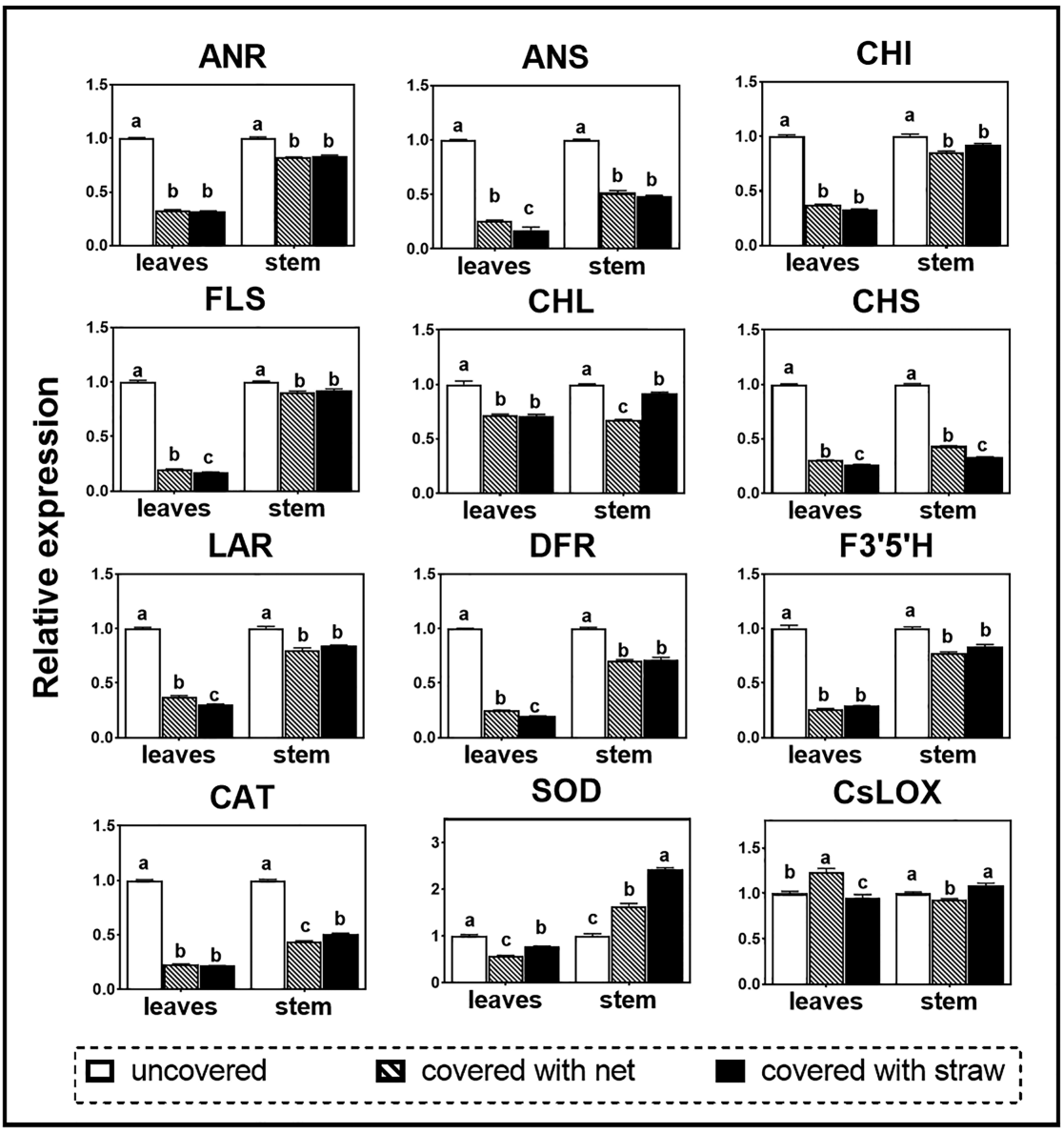

Figure 5 Expression of a selected set of genes in leaves (fourth) and stems from young tea plant shoots grown under NET or STRAW shading and non-shaded (control; uncovered) conditions. For each gene, its expression was calculated relative to GAPDH gene; bars indicate means with SD of three biological replicates. ANR, anthocyanidin reductase; ANS, anthocyanidin synthase; CAT, catalase; CHI, chalcone isomerase; CHL, chlorophyll decomposition enzyme; CHS, chalcone synthase; CsLOX, lipoxygenase; DFR, dihydroflavonol-4-reductase; F3'5'H, flavonoid 3′,5′-hydroxylase; FLS, flavonol synthase; LAR, leucoanthocyanidin reductase; SOD, superoxide dismutase. 'NS' and different letters above the bar indicate insignificant and significant ($P < 0.01$; $n = 3$ biological replicates) differences, respectively, between shading treatments.

nitrogen is an important strategy for plants to utilize limited nitrogen effectively (Staswick 1994; Masclaux-Daubresse et al. 2010). By investigating distinct parts of the young tea shoots—i.e. the first (still developing) and fourth (fully developed) leaf and the stem—our results suggested that the effects of shading were differential among those shoot tissues, indicating spatial effects in the shoot parts normally harvested together for tea preparations. For example, the shading-induced changes in both catechins and amino acids were far more pronounced in the old (fourth) leaves than in the young (first) leaves and indicated that the usually positive effect of shading on the amino acid/catechin ratio, in which parameter is key to green tea quality, was more pronounced in the fully developed (fourth) than in the developing (first) leaves. Additionally, our results show that the shading-induced changes in carbohydrates (e.g. D-glucose) in the first leaves differed from those of the fourth leaves (Figure 4). This difference in leaf positions may be linked to the priority of providing carbon and nitrogen resources to tea plants' growth center, which includes the first leaf (Royer et al. 2013; Pfautsch et al. 2015). It is worth noting that in

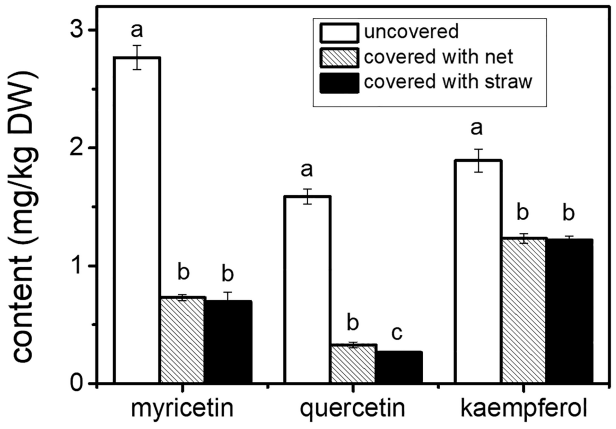

Figure 6 Total content of flavonols (means ± SD; $n = 6$; determined as aglycons after acid hydrolysis) in the fourth leaves of shoots from tea plants grown under NET and STRAW shading and non-shaded (control) conditions. Different letters above the bar indicate significant ($P < 0.01$) differences with control plants and between shading treatments.

contrast to its marked increase upon shading in both first and fourth leaves, theanine contents significantly decreased in the stems. A previous study suggested that about 70% of nitrogen in the new sprouts of spring tea derives from the redistribution from the old leaves (Okano et al. 1994). Ruan and Gerendas (2015) applied ^{15}N urea on the old leaves and found that this urea was mainly transported to the young tea shoots (developing leaves). Considering that theanine is the main form of nitrogen storage in tea plants, our results suggest a within-plant spatial regulation or transport/redistribution of both carbon and nitrogen resources between the different tissues of the growing young shoots.

Previous studies suggested that shading of tea plants can markedly elevate the levels of amino acids and decrease the accumulation of catechins in young shoots (Saijo 1980; Wang et al. 2012), which is beneficial to the quality of green tea. Subsequently, many more metabolites responding to shading in young tea shoots have been identified (Lee et al. 2013; Zhang et al. 2014; Zhang and Ruan 2016). However, most of these compounds are the end-products rather than the intermediates from biosynthetic pathways, and the effect of shading on the regulation of the accumulation of quality-related compounds in tea shoots such as amino acids and flavonoids, including all biosynthetic pathway steps from precursors to the end-products through intermediates, are far from clear. In this comprehensive metabolomics study, both semi-polar, lipid-soluble and polar compounds were considered and taken into account to determine which compounds in which tea shoot tissues are mostly influenced by two different practical shading treatments to improve green tea quality, i.e. NET and STRAW resulting in a filtering of 90 and 96%, respectively, of the incoming sunlight. Most importantly, many responding compounds, including malic acid, citric acid, α-ketoglutaric acid and PI (18:4(6Z,9Z,12Z,15Z)/0:0) have been newly identified, which will help us in understanding the effects of shading on the metabolism of amino acids, sugars, organic acids, flavonoids, alkaloids and lipids.

Our study also provides novel insights into the metabolism and spatial distribution of product quality-related compounds in tea shoots in response to shading. For example, rather than an overall increase in amino acids, our study shows that glycine, glutamine and serine were significantly ($P < 0.01$) decreased in the first leaves under plant shading. While free amino acids infused in a green tea brew have been recognized as the principal contributors to the taste of mellowness, each amino acid species has its own taste that may be sweet, salty, sour, bitter or umami (Zhang and Ruan 2016). Our results suggest that the taste of tea may be altered by changing the composition of certain amino acids instead of the total amount. Meanwhile, the abundance of catechins, including their galloylated forms, decreased while that of free gallic acid increased under the shaded cultivation of tea plants, indicating that light can affect the galloylation of catechins. Since galloylated catechin esters have a lower taste threshold for astringency than their nongalloylated forms (Zhang and Ruan 2016), we should reevaluate the effect of shading on quality improvement of green tea.

Light energy beyond the acceptable range of the photoreaction center in plant causes light stress, leading to photoinhibition (Alboresi et al. 2011). Apart from influencing tea flavor, flavonol glycosides are effective attenuators of sunlight and are therefore considered to play a key role in the photoprotection of plants (Fujimura et al. 2011; Landi et al. 2014; Landi et al. 2015; Jay Allemand et al. 2015; Zhang et al. 2017). In the present work, quercetin glycosides, myricetin glycosides and kaempferol glycosides were all down-regulated by both shading treatments and especially by the more effective STRAW shading, consistent with results reported before (Zhang et al. 2014), and suggest the involvement of flavonol glycosides in light adaptation of tea shoots (Agati and Tattini 2010; Agati et al. 2011; Prochazkova et al. 2011). In general, flavonoids with an ortho di- or trihydroxy structure in their B-ring (catechol unit), like quercetin and myricetin, are more effective antioxidants than flavonoids having only one hydroxy substitution, like kaempferol (Tattini et al. 2004; Brunetti et al. 2013). In this study, the contents of total kaempferol, quercetin and myricetin all decreased upon shading, but the relative effect was larger for both the two catechol-containing flavonol species than for kaempferol. Since quercetin and myricetin have higher antioxidant potential than kaempferol (Rice Evans et al. 1996; Firuzi et al. 2005), these shading-mediated differential decreases between flavonol species suggest that the light-dependent metabolism of flavonols in tea plants is related to their differential antioxidant bioactivity.

Tea is a shade plant, originally growing in the tropical forests under the light protection from trees (Carr and Stephens 1992).

Upon cultivation in a plantation, such as in our study, the plants will receive much more solar light than in the forest, which may result in light-induced stress and photoinhibiton. There is indeed evidence that the photosynthesis of tea is decreased by strong solar radiation, although the mechanism is unclear yet (Carr and Stephens 1992). Moreover, previous studies have shown that the photosynthetic capacity of tea is greater for leaves grown in the shade than in full sun (Rahman 1988). Mohotti and Lawlor (2002) also suggested that photosynthesis of tea is decreased by strong solar radiation and that shading may decrease loss of energy due to photoinhibition. In our experiments, the relative levels of glucose and sucrose in tea leaves, most specifically the fourth leaves, were markedly increased by shading (Table S6 available as Supplementary Data at *Tree Physiology* Online, Figure 4). Moreover, as compared to the unshaded controls, shaded leaves had an increased biomass, an increased chlorophyll content (resulting in a darker green phenotype), a lower expression of the gene encoding for chlorophylase (the enzyme involved in chlorophyll breakdown) and less markers of photoinhibition/photorespiration due to light stress. All these results fit well with an increased photosynthesis and thus an increase rate of carbon assimilation (e.g. higher sugar levels) by shading the tea plants cultivated in open fields.

Conclusion

By using complementary targeted and untargeted metabolomics approaches, combined with an expression analysis of selected genes, we have showed that the metabolome, including green tea quality-related compounds like amino acid and catechins, in the older fully developed fourth leaves are differentially affected by shading than those in the younger still-developing first leaves and the stem of young tea shoots. We hypothesize that the stem crucially functions in the source-sink and carbon-nitrogen balance of different-aged leaves of young tea shoots, thereby playing a potential role in determining the actual metabolite composition of the leaves and thus in the quality of derived teas. Our work suggests that tea quality, including its flavor, color and potentially health-beneficial properties, can be targeted by making a distinction between the various shoot parts, like fully developed leaves, still-developing leaves and stems, of tea plants at harvest and by optimizing plant growth conditions through dedicated shading treatments.

Supplementary data

Supplementary data for this article are available at *Tree Physiology* online.

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Conflict of interest

None declared.

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