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Differential localization of flavonoid glucosides in an aquatic plant implicates different functions under abiotic stress

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Abstract

Flavonoids may mediate UV protection in plants either by screening of harmful radiation or by minimizing the resulting oxidative stress. To help distinguish between these alternatives, more precise knowledge of flavonoid distribution is needed. We used confocal laser scanning microscopy (cLSM) with the "emission fingerprinting" feature to study the cellular and subcellular distribution of flavonoid glucosides in the giant duckweed (*Spirodela polyrhiza*), and investigated the fitness effects of these compounds under natural UV radiation and copper sulphate addition (oxidative stress) using common garden experiments indoors and outdoors. cLSM "emission fingerprinting" allowed us to individually visualize the major dihydroxylated B-ring-substituted flavonoids, luteolin 7-O-glucoside and luteolin 8-C-glucoside, in cross-sections of the photosynthetic organs. While luteolin 8-C-glucoside accumulated mostly in the vacuoles and chloroplasts of mesophyll cells, luteolin 7-O-glucoside was predominantly found in the vacuoles of epidermal cells. In congruence with its cellular distribution, the mesophyll-associated luteolin 8-C-glucoside increased plant fitness under copper sulphate addition but not under natural UV light treatment, whereas the epidermis-associated luteolin 7-O-glucoside tended to increase fitness under both stresses across chemically diverse genotypes. Taken together, we demonstrate that individual flavonoid glucosides have distinct cellular and subcellular locations and promote duckweed fitness under different abiotic stresses.

KEYWORDS
2-aminoethyl diphenylborinate, apigenin, confocal laser scanning microscopy (cLSM), copper sulphate, flavonoids, glucosides, heavy metal, luteolin, oxidative stress, UV light

1 | INTRODUCTION

Plants produce an exceptionally diverse arsenal of secondary metabolites, with over 200,000 individual compounds, many of which facilitate interaction with the environment (Dixon & Strack, 2003). Biochemically closely related compounds are often unevenly distributed within organs and tissues, and show distinct cellular and subcellular distributions, suggesting different functions. For instance, among flavonoids, the concentration of flavonoid glucosides is often higher in surface appendages and the adaxial epidermis (or the abaxial epidermis in case of steeply angled leaves) than in the mesophyll, which were interpreted as optimal locations to screen harmful solar

UV radiation (Bidel, Meyer, Goulas, Cadot, & Cerovic, 2007; Caldwell, Robberecht, & Flint, 1983; Hutzler et al., 1998; Schnitzler et al., 1996; Strack, Heilemann, Mömken, & Wray, 1988). Likewise, the high concentrations of anthocyanins in the abaxial (water-exposed) epidermis of the hydrophyte *Spirodela polyrhiza* and the induction of this compound under chromium stress suggested that these compounds alleviate oxidative stress induced by water-derived sources (Oláh, Combi, Szollosi, Kanalas, & Mészáros, 2009; Oláh, Tóth, Szöllosi, & Kiss, 2008). However, a frequent limitation in relating a compounds' localization to its function remains the ability to localize individual metabolites and differentiate them from closely related compounds.

Flavonoids are one of the most diverse groups of plant secondary metabolites structurally, phylogenetically and functionally, and have a long history as subjects of localization studies. Flavonoids can be visualized in vitro and *in planta* by confocal laser scanning microscopy (cLSM) due to their fluorescence properties when stained with 2-aminoethyl diphenylborinate (2-APB, (also known as DPBA, Naturstoffreagenz A, Naturstoff Agent or Neu's reagent) (e.g., Sheahan & Rechnitz, 1993; Peer et al., 2001; Tattini et al., 2004; Agati, Stefano, Biricolti, & Tattini, 2009; Agati et al., 2013; Matteini, Agati, Pinelli, & Goti, 2011). 2-APB staining produces fluorescence emission spectra under UV and blue light excitation with significant differences in relative quantum yields that may distinguish one flavonoid from another (Agati, Azzarello, Pollastri, & Tattini, 2012; Buer, Muday, & Djordjevic, 2007; Filippi et al., 2015). These spectra have been exploited, for instance, to differentiate 2-APB-stained hydroxycinnamic derivatives from a mixture of mono- and dihydroxylated flavonoid glucosides in *Phillyrea latifolia* (Agati, Galardi, Gravano, Romani, & Tattini, 2002). Similarly, 2-APB-staining allowed visualization of the movement and conversion of synthetic flavonoid aglycones in flavonoid deficient *Arabidopsis transparent testa4* (*tt4*) mutants (Buer et al., 2007). However, the visualization of individual native flavonoid glucosides within a plant species has not been achieved to date.

Flavonoids are known to serve diverse functions in plants including protection against insect predators, attraction of pollinators, defence against microbes, pollen germination and modification of development by involvement in auxin transport (Mouradov & Spangenberg, 2014; Panche, Diwan, & Chandra, 2016). It is generally agreed that flavonoids also function to protect plants against UV and other radiation, but whether these compounds function as screening pigments or antioxidants has been controversial (Agati et al., 2009, 2013; Burchard, Bilger, & Weissenböck, 2000; Di Ferdinando, Brunetti, Agati, & Tattini, 2014; Harborne & Williams, 2000; Hernández, Alegre, Van Breusegem, & Munné-Bosch, 2009; Mouradov & Spangenberg, 2014; Robson, Klem, Urban, & Jansen, 2015). At one time, the role of flavonoids was considered to be primarily as filters of UV radiation, consistent with their frequent location in cell walls and vacuoles of epidermis cells or trichomes (Barnes et al., 2016; Hutzler et al., 1998; Schnitzler et al., 1996; Strack et al., 1988). However, more recent studies challenged this view, as flavonoids accumulate also in vacuoles of mesophyll cells (Agati et al., 2002; Liu, Gitz, & Mcclure, 1995), chloroplasts (Agati, Matteini, Goti, & Tattini, 2007; Saunders & McClure, 1976) and in the nucleus (Feucht, Schmid, & Treutter, 2014). Furthermore, flavonoids are relatively poor UV-B absorbers compared to other phenylpropanoid classes such as hydroxycinnamic acids (Agati et al., 2013). In addition, the ratio of flavonoids to hydroxycinnamates strongly increases upon exposure to UV-B or strong sunlight: for instance, the flavonol quercetin replaced hydroxycinnamic acid derivatives during shade-to-sun transition (Agati et al., 2009, 2013). These observations suggest that UV-B screening is not the sole function for flavonoids. In particular, flavonoids may attenuate oxidative stress that is caused by UV radiation (Ravanat, Douki, & Cadet, 2001). The role of flavonoids in alleviating oxidative stress is further supported by the observation that flavonoid biosynthesis is up-regulated by a plethora of abiotic and biotic stresses that all lead to the generation of reactive oxygen species (ROS) (Agati et al., 2011; Babu et al., 2003; Vogt, Gülz, & Reznik, 1991). For example, copper, a metal that has dramatically increased in the environment due to the present industrial and agricultural practices (Fernandes & Henriques, 1991), generates ROS by auto-oxidation and metal-dependent Fenton reactions (Michalak, 2006; Sytar et al., 2013). Flavonoids, particularly the *ortho*-dihydroxylated B-ring-substituted flavonoids, are assumed to benefit plant performance under copper addition by scavenging ROS and suppressing ROS-formation as chelating agents (Babu et al., 2003; Brown, Khodr, Hider, & Rice-evans, 1998; Rice-Evans, Miller, & Paganga, 1996). The structural diversity and the cellular and subcellular distribution of individual flavonoids may strongly affect their ability to function as radiation filters or antioxidants.

The giant duckweed, *Spirodela polyrhiza* (L.) Schleid. (Text S1), is a free-floating, cosmopolitan hydrophyte (Bog et al., 2015) with a relatively simple flavonoid chemistry. The four major flavonoids, luteolin 7-O-glucoside (lut 7-O-glc, cynaroside), luteolin 8-C-glucoside (lut 8-C-glc, orientin), apigenin 7-O-glucoside (ap 7-O-glc, apigetrin) and apigenin 8-C-glucoside (ap 8-C-glc, vitexin), are structurally closely related differing only in the position of the glucose residue and by the presence of one or two hydroxyl groups in the B-ring (Casas, Duarte, Doseff, & Grotewold, 2014; Gitz, Liu-Gitz, McClure, & Huerta, 2004; Li, Bonawitz, Weng, & Chapple, 2010; Nugroho, Choi, & Park, 2016; Qiao et al., 2011; Wallace, Mabry, & Alston, 1969) (Figure 1a). While luteolin and apigenin glucosides have similar UV-absorption spectra (Agati et al., 2013), the *ortho*-dihydroxylated B-rings of luteolin and its derivatives have higher chelating and anti-oxidative potential than those of their monohydroxylated apigenin counterparts (Rice-Evans et al., 1996). Furthermore, based on 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2-azinobis (3-ethyl-benzothiazoline-6-sulphonic acid (ABTS) and ferric reducing antioxidant power (FRAP) assays, luteolin-8-C glucoside has higher radical scavenging and antioxidant potential than its 7-O-counterpart (Zhang, Xinyu, Cheng, Jin, & Zhang, 2014). The differences in anti-oxidative capacity may relate to the distribution of the individual flavonoids within the cell and tissue layers. The four flavonoids accumulate to high concentrations in the thallus-like, green vegetative plant tissue, the so-called "frond" (Landolt, 1986). *Spirodela polyrhiza* fronds reproduce almost exclusively asexually by budding with duplication rates of 2–3 days under optimal growth conditions (Landolt, 1957; Ziegler, Adelmann, Zimmer, Schmidt, & Appenroth, 2015). As *S. polyrhiza* proliferates best in nutrient-rich water, this hydrophyte often grows in proximity to agriculture fields and thus is often subjected to full sunlight as well as copper from anthropogenic sources.

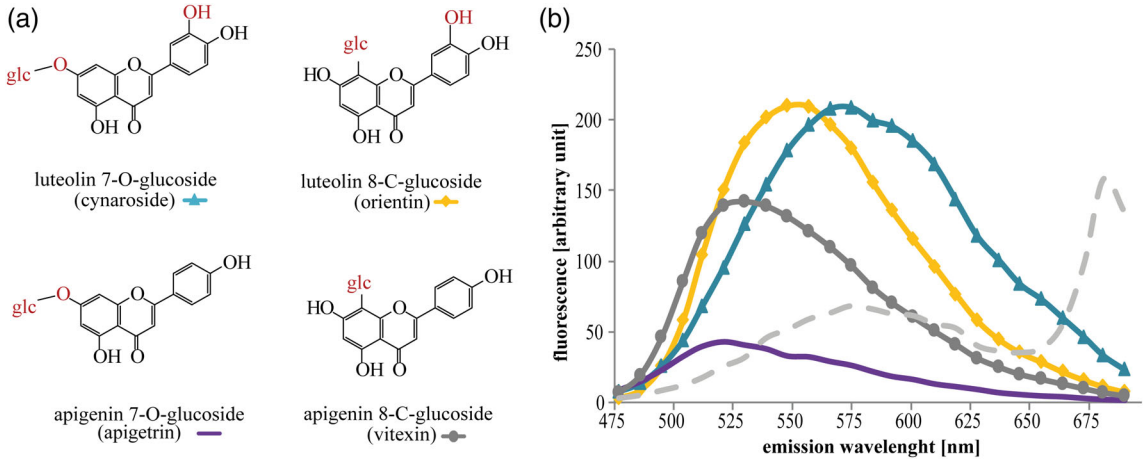

FIGURE 1 (a) Chemical formulae and (b) fluorescence emission spectra of the major *Spirodela polyrhiza* flavonoid glucosides and chlorophyll. Each synthetic standard (5 mM in DMSO) was dissolved 1:1 with 0.25% 2-aminoethyl diphenylborinate (2-APB) resulting in a final concentration of 2.5 mM and was excited at 405 nm. The chlorophyll spectrum was extracted from an unstained cross-section and excited at 405 nm. Images were acquired in the λ-mode and emission spectra were extracted via manual landmarks in the cLSM software and stored in the software database to subsequently enable "emission fingerprinting" in 2-APB-stained cross-sections of *S. polyrhiza*

Here, we aimed to explore the individual localization of the four major flavonoid glucosides in *S. polyrhiza* under copper sulphate and natural sunlight treatment to learn about their tissue-specific and subcellular distribution. We hypothesized that variation in the cellular or subcellular localization of specific flavonoids with different anti-oxidative capacity but similar UV absorption would indicate that the flavonoids act at least partially as antioxidants. We first developed a cLSM method to determine the cellular and subcellular distribution of individual flavonoids without interference from one another. We then determined the effect of copper sulphate addition and UV light exposure on flavonoid accumulation in different locations and correlated metabolite accumulation with plant fitness under stress treatment in diverse *S. polyrhiza* genotypes to help understand the role and ecological relevance of individual flavonoid glucosides under changing environmental conditions.

2 | MATERIALS AND METHODS

2.1 | Plant growth conditions

Spirodela polyrhiza (L.) Schleid. plants were cultivated in liquid full nutrient medium (N-medium: KH_2PO_4 150 μM, $Ca(NO_3)_2$ 1 mM, KNO_3 8 mM, H_3BO_3 5 μM, $MnCl_2$ 13 μM, Na_2MoO_4 0.4 μM, $MgSO_4$ 1 mM, FeNaEDTA 25 μM) (Appenroth, Teller, & Horn, 1996). The *Spirodela polyrhiza* genotype 7,498 originating from North Carolina (United States) was used for cLSM analysis. A full list of the registered accessions involved in the fitness assays is provided in supplemental Table S1 (Zhao, Appenroth, Landesman, Salmeán, & Lam, 2012). For indoor experiments, plants were pre-cultured in the same growth chamber under identical conditions as the subsequent experiments for at least 1 week unless indicated otherwise. Indoor experiments were performed in a climate chamber at 26°C under long-day conditions (16:8 hours light: dark) with a light intensity of 130–160 μmol m^{-2} s^{-1} (Osram Lumilux L36 W/ 865 cool daylight; Osram GmbH, Munich, Germany). Plants were cultivated in transparent 250 ml plastic bowls covered with transparent and perforated plastic lids (diameter 11 cm). The plastic bowls were set on green plastic trays to avoid water condensation on the lid.

2.2 | Statistical analyses

All statistical analyses were performed in R version 3.3.1 (R Core Team, 2016). Pairwise comparisons were performed with the multcomp package (Hothorn, Bretz, & Westfall, 2008). Linear mixed model analyses were performed using the lme4 package (Bates, Mächler, Bolker, & Walker, 2015). More details are given in the experimental section below with further references to detailed information in the supplemental materials.

2.3 | Establishment of a database of fluorescence emission spectra for major *S. polyrhiza* flavonoids

To establish a database of the fluorescence emission spectra of the major *S. polyrhiza* flavonoid glucosides, we recorded the emission spectra of synthetic standards using a cLSM. Luteolin 7-O-glucoside (cyranoside, Extrasynthese, Genay Cedex, France), luteolin 8-C-glucoside (orientin, Extrasynthese, Genay Cedex, France), apigenin 7-O-glucoside (apigetrin, Extrasynthese, Genay Cedex, France) and apigenin 8-C-glucoside (vitexin, Roth GmbH and Co. KG, Karlsruhe, Germany) were individually dissolved in pure DMSO at 5 mM. Samples were further diluted 1:1 with 0.25% (w/v) of the staining agent 2-aminoethyl diphenylborinate (2-APB, Sigma Aldrich, Steinheim, Germany) reaching a final flavonoid glucoside

concentration of 2.5 mM each. Using a confocal laser scanning microscope (ZEISS 880, Carl Zeiss, Oberkochen, Germany) 40 µl of 2-APB mixed standards were placed on a microscopy slide, covered with a 22×22 mm coverslip, excited at 405 nm and emission was detected between 410 and 695 nm in 9 mm bins (λ-mode). Firstly, an image in the λ-mode was acquired to record an emission spectrum of each compound. The acquired spectra were stored in the spectra database (which included the autofluorescence spectrum of chlorophyll from a cross-section excited with 405 nm) and subsequently used for the online emission fingerprinting mode. In this mode, the fluorescence emission from the cross-sections, containing all four flavonoids, excited by 405 nm is compared to the previously acquired and stored spectra of the standards and sorted/unmixed into the respective channels based on the similarity of the spectra pixel-by-pixel. The emission fingerprinting tool is based on spectral differences among compounds such as curve shape/skewness and the positions of peaks, but not intensity differences. The microscope was equipped with an EC-Plan-Neofluar 10×/0.3 dry type objective and a C-Apochromat 40×/1.20 W Korr M27 water immersion type objective and ZEN 2.1 [black, 64-bit] software. Identical settings were used for all images and channels used for fluorescence quantification: excitation wavelength was 405 nm using a laser diode, 10× objective, 21% transmission, 625 gain, pinhole set to 1 Airy Unit (~39 µm). The focus for the image recordings was chosen (a) by finding the maximum when using a solution drop or (b) by focusing on the chloroplasts for in vivo images of *S. polyrhiza* cross-sections (details in Text S2). In order to test the robustness of our recorded emission spectra, we performed additional tests extracting spectra under varying pH conditions, 2-APB concentrations and DMSO levels. Furthermore, we tested for the association of compound concentration and fluorescence intensities (details in Text S2).

2.4 | Visualization and quantification of secondary fluorescence across tissue layers of the S. polyrhiza frond

To qualitatively and quantitatively investigate the distribution of the flavonoid glucosides across the *S. polyrhiza* frond, we visualized the compounds in cross-sections using cLSM with the established fluorescence database. Mature fronds were freehand sectioned along a distinct peripheral axis (Figure S1) with a section thickness of about 0.5 mm. Cross-sections were stained with 0.25% 2-APB for 1 min and immediately analysed without washing the sections under the same cLSM settings as described above. Chloroplasts seen by autofluorescence of chlorophyll were chosen to define the focal plane for all recorded in vivo images. The "online emission fingerprinting" feature of cLSM was used to separate the mixed signals of stained cross-sections pixel-by-pixel using the entire emission spectrum of each of the previously stored reference spectra.

Fluorescence intensities in the adaxial epidermis, mesophyll and abaxial epidermis were estimated by randomly arranging five distinct circular regions of interest (ROI) within each of these tissue layers using ImageJ. The ROI size (adjusted to the size of each layer) was 365, 5,795 and 365 square microns for the adaxial epidermis, mesophyll and abaxial epidermis, respectively. In this work, mesophyll refers to the area between the upper epidermis and beginning of aerenchyma in cross-sections. ROIs were placed randomly across each tissue layer except that aerenchyma was avoided. The ROI calculation output is the mean grey value per pixel within a ROI ranging from 0 to 255. Averaging the means of five ROIs within one layer resulted in the final fluorescence intensity per replicate. For statistical analysis of this experiment, see Text S3.

2.5 | Verification of cLSM-based flavonoid visualization in vivo

To verify our visualization method *in planta*, we first investigated the effect of the phenylalanine ammonia lyse (PAL)-inhibitor, 2-aminoidane-2-phosphonic acid (AIP), an inhibitor of the first step on the phenylpropanoid pathway (Gitz et al., 2004), in *S. polyrhiza* on both fluorescence signals and HPLC-based flavonoid content analysis. For details on the plant handling, HPLC and statistical analysis, see Text S4.

2.6 | Effect of frond age on flavonoid glucoside accumulation

To investigate the effect of frond age on flavonoid glucoside accumulation, 12 mother fronds with a small attached daughter frond each were divided up among three bowls and grown for 14 days under control conditions. On days 0, 1, 2, 3, 5, 7 and 14, one daughter frond from each bowl was harvested for HPLC analysis (remaining fronds were grown as backups). The medium was replaced once a week and additional generations of fronds were discarded to ensure space and nutrients for fronds to be analysed. To test for differences in flavonoid content over the lifespan of fronds, we compared mean flavonoid levels between different days using ANOVA and Tukey's post hoc test.

2.7 | Investigation of subcellular localization of flavonoids

To investigate the subcellular localization of the flavonoids, we additionally stained cross-sections with the vacuole specific staining agent, neutral red. Cross-sections of mature plants were incubated for 3 min in 0.01% neutral red (3-amino-7-dimethylamino-2-methylphenazine hydrochloride, Sigma Aldrich, Steinheim, Germany) in purified water (w/v). Sections were then washed three times in tap water for 2 min prior to cLSM microscopy. Samples were excited with a 543 nm HeNe laser (40× objective, 0.6 zoom).

2.8 | Tissue-specific induction of flavonoids under copper sulphate treatment

To investigate the magnitude and tissue layer specificity of flavonoid glucoside induction under copper sulphate addition, we performed

cLSM and HPLC analyses on plants growing in the presence and absence of $CuSO_4$. Three single fronds were placed into 250 ml plastic bowls filled with 180 ml full nutrient medium with and without 10 μM $CuSO_4$ (n = 12). After 5 days, one mature granddaughter fronds (Figure S1) from half of the bowls of each treatment was used for cLSM analysis as described above. At the same time, one mature granddaughter of each of the remaining bowls was harvested and analysed by HPLC as described above. In addition, the fresh mass of all emerged fronds was recorded. For statistical analysis procedure, see Text S5.

2.9 | Induction of flavonoids under natural UV light

To investigate the effect of natural midsummer UV radiation on flavonoid content and accumulation pattern, we performed outdoor experiments under natural UV light exposure with UV-shielded conditions as control. Plants were transferred from the growth chamber to a sun-exposed field site in Jena, Germany, in July 2017. Plants were grown in full nutrient medium-filled 200 ml plastic cups (diameter 5/6.5 cm (bottom/top, height 8.5 cm, Pöppelmann, Lohne, Germany) that were fitted into the cut holes of white polyvinyl chloride inserts (3 mm thickness) floating inside water-filled 10 L buckets (weather data see Table S2). To prevent excessive heating of the medium by the sun, buckets were buried to the rim in soil. UV radiation was manipulated by covering the buckets with either UV-blocking (UV Gallery100, Sandrock Kunststoffe, Germany) or UV-transmitting poly(methyl methacrylate) sheets (GS 2458, Sandrock Kunststoffe, Germany) leaving a 2–3 cm gap to allow for airflow. Sheets differed mostly in UV-A but also in UV-B transmission (Figure S1 in [Xu et al., 2019]). To allow for acclimation of the plants to outdoor conditions prior to the start of the experiments, plants were cultivated under UV-blocking sheets that were covered with two layers of green plastic foil, which were successively removed after 1 and 2 days, respectively. Fronds were pre-cultured outdoors for another 5 days under UV-blocking sheets. Due to enhanced evaporation outdoors, medium was replaced twice a week during pre-cultivation and experiment. Subsequently, three single fronds were placed into full nutrient medium-filled plastic cups floating inside UV-transmitting and UV-blocking buckets (n = 10). After 7 days, one mature granddaughter frond from half of the replicates was used for cLSM as described above and one granddaughter of the remaining replicates were used for HPLC analysis as described above, except that frozen fronds were ground for 1.5 min in 5 ml tubes since fronds grown outdoors were firmer. Biomass production of all emerged fronds was recorded. For statistical analysis procedure, see Text S6.

2.10 | Fitness assay of genotypes exposed to copper sulphate

To test whether individual flavonoid content correlates to plant fitness under copper sulphate treatment, 53 worldwide distributed *S. polyrhiza* genotypes were grown in the presence and absence of copper sulphate. Genotypes, which were selected based on their geographically dispersed origin (Table S1), were pre-cultivated for 7 days in 250 ml Erlenmeyer flasks containing 100 ml full nutrient medium in the above-mentioned climate chamber. For each genotype, five mature plants with a small daughter frond each were placed into 250 ml plastic bowls that were filled with 150 ml full nutrient medium with and without 10 μM $CuSO_4$ (n = 3) in a climate chamber operating under the following conditions: 26°C constant, 16:8 hr light:dark, illumination at 160 μmol m^{-2} s^{-1} supplied by metal halide lamps (mt400DL, EYE Iwasaki). The bowls were covered with transparent and perforated plastic lids and a white fleece (Agrarvlies, 17 g/m^2) to avoid water condensation on the lid. After 7 days, plants were harvested, weighed and approximately 50 mg fresh mass frozen in liquid nitrogen. Samples were stored at −20°C until further analysis. Methanol extractions and HPLC analysis to measure flavonoid concentrations were performed as described above. Detailed information on statistical analysis can be found in Text S7.

2.11 | Fitness assay of genotypes exposed to ambient UV light

To test whether individual flavonoids benefit plant fitness under natural UV light, 38 worldwide distributed *S. polyrhiza* genotypes, including 33 genotypes used in the copper sulphate experiment (Table S1), were cultivated under UV-exposed and -shielded conditions outdoors in late August and early September 2016. Plants were cultivated inside the cavities (Ø 5 cm) of white seedling trays floating inside 52 L transparent plastic boxes (79 × 58 × 17.5 cm, Bauhaus, Germany) filled with 30 L full nutrient medium at the field site in Jena, Germany as described above. Boxes were covered with either UV-transmitting or UV-blocking Plexiglas as described above, leaving 2–3 cm distance between the box edges and Plexiglas to allow air circulation. For acclimatization, approximately 50 marked plants per genotype were transferred on August 25, 2016 from the indoor incubator into an outdoor box under UV-blocking Plexiglas. The Plexiglas was additionally covered with a total of two layers of blue and green plastic sheets that were subsequently removed after 3 and 4 days to allow for plant acclimation. Seven days after removal of the protective sheets, three single daughter plants per genotype were placed into the cavities of floating seedlings trays inside three boxes covered with either UV-blocking or UV-transmitting Plexiglas, respectively (weather data see Table S2). Genotype position was randomized in each box. The medium was exchanged after 1 week. After 2 weeks, fronds were collected in pre-weighed Eppendorf tubes, frozen in liquid nitrogen and stored at −20°C until further processing. All tubes were weighed to determine fresh mass. To measure flavonoid concentrations, one replicate per treatment was ground under liquid nitrogen to fine powder with mortar and pistil. A 50 mg ground sample was extracted with 1 ml methanol by vortexing for 15 s. HPLC analysis of the supernatant was performed as described above. For details on statistical analysis, see Text S8.

3 | RESULTS

3.1 | Confocal laser scanning microscopy allows differentiation and relative quantification of individual flavonoid glucosides in duckweed fronds

In order to distinguish the four major *S. polyrhiza* flavonoid glucosides under cLSM, we investigated their fluorescence emission spectra using synthetic standards incubated with 2-aminoethyl diphenylborinate (2-APB) at 405 nm (Figure 1b). While apigenin 7-O-glucoside (ap 7-O-glc) and apigenin 8-C-glucoside (8-C-glc) showed highly congruent emission spectra between 480 and 690 nm, luteolin 7-O-glucoside (lut 7-O-glc) and luteolin 8-C-glucoside (lut 8-C-glc) had distinct emission spectra in terms of their shape and maxima separated by ~30 nm (Figure 1b). Preliminary investigations of 2-APB-stained cross-sections of the aerial parts of *S. polyrhiza* using the traditional cLSM technique of sequential imaging recording showed crosstalk between fluorescence signals and did not allow distinct visualization of the individual flavonoids. Instead, the emission spectra in vivo were analysed using the "online emission fingerprinting" feature of the cLSM software. The lut 7-O-glc, lut 8-C-glc and ap 7-O-glc channels showed distinct signals (Figure 2). However, the ap 8-C-glc channel had a weak signal-to-noise ratio and considerable crosstalk with the ap 7-O-glc channel in stained cross-sections, and so was discarded, with the ap 7-O-glc channel designated as a mixture of both ap 7-O-glc and ap 8-C-glc. Thus, we were able to differentiate three out of the four major *S. polyrhiza* flavonoid glucosides with largely overlapping emission spectra.

We next investigated the selectivity, linearity and robustness of this visualization method. Non-stained cross-sections showed the autofluorescence signal of chlorophyll only, indicating that conjugation of 2-APB to the flavonoid glucosides is required for fluorescence. Cross-sections of plants that had been grown with the PAL-inhibitor 2-aminoidane-2-phosphonic acid (AIP) (Figure S2), which reduces total flavonoid concentrations by over 70% (Figure S3b, $p < 1.2e^{-06}$, Student's *t* test), displayed clearly reduced emission levels (Figure S3a, Figure S3d) and slightly altered distribution patterns of the individual flavonoids (Figure 2 and Figure S3c). Standard curves with synthetic standards showed positive correlations between fluorescence intensity and metabolite concentrations (Figure S4). However, individual compounds emitted at different intensities with lut 7-O-glc emitting up to a fivefold higher signal at a concentration of 2.5 mM than ap 7-O-glc (Figure S4). pH value and DMSO concentration also affected absolute fluorescence intensities of compounds, but in similar ways across the four compounds in vitro (Figure S5, S6). The emission spectra, however, were distinguishable under all tested pH values, DMSO and 2-APB concentrations (Figure S5, S6, S7). In *S. polyrhiza* fronds, individual flavonoid concentrations measured by HPLC were dynamic during the early stages of frond development but became stable once the plants matured (Figure S8). Taken together, these data show that "online emission fingerprinting" of 2-APB-stained *S. polyrhiza* frond cross-sections in cLSM is selective for the individual flavonoid glucosides of mature plants and can be used to identify compounds present and quantify their relative amounts within a single compound but not between them.

3.2 | Individual flavonoids have distinct tissue-specific and subcellular distributions

To quantitatively investigate the distribution of individual flavonoid glucosides in *S. polyrhiza* fronds, we measured fluorescence intensities in frond cross-sections from plants growing under controlled conditions using cLSM with "online emission fingerprinting." Lut 7-O-glc accumulated predominantly in the upper epidermis, whereas lut 8-C-glc as well as ap 7-O- and 8-C-glc were mostly present in the mesophyll (Figure 2a, b). To investigate the subcellular occurrence of individual compounds, we visually compared the fluorescence pattern of the flavonoid glucosides with that of chlorophyll (natural fluorescence in chloroplasts) and of neutral red, which selectively stains vacuoles when applied to cross-sections (Figure 3, Figure S9). The fluorescence of both luteolin glucosides showed a distribution pattern similar to that of the neutral red stained vacuoles (Figure 3, Figure S9). In addition, the fluorescence of lut 8-C-glc overlapped with that of the chloroplasts (Figure 3, Figure S9). Apigenin glucosides on the contrary were associated with chloroplasts only (Figure 3, Figure S6). Views of the adaxial epidermis of neutral red and 2-APB-stained sections revealed a lack of fluorescence signals in cell wall regions (Figure S10).

3.3 | Copper sulphate addition but not UV radiation induces flavonoid glucoside concentrations in a tissue-specific manner

To investigate whether the copper sulphate addition affects flavonoid glucoside accumulation in *S. polyrhiza*, we analysed flavonoid glucoside concentrations using HPLC and determined the individual distribution patterns of compounds using cLSM in fronds grown after copper sulphate treatment indoors. Under the experimental conditions used, copper sulphate addition reduced plant biomass production by 54% (Figure S11, $p = 2.2e^{-07}$, Student's *t* test). The dihydroxylated B-ring-substituted lut 7-O-glc and lut 8-C-glc were induced by 125 and 94% by copper sulphate addition, respectively (lut 7-O-glc: $p = 3e^{-06}$, lut 8-C-glc $p = 7e^{-06}$), whereas the monohydroxylated B-ring-substituted apigenin glucosides increased by only 54% (ap 7-O-glc: $p = 0.001$, ap 8-C-glc $p = 4e^{-04}$, Student's *t* test) (Figure 4a); consequently, the induction level significantly differed between compounds with mono- and dihydroxylated B-rings (Tukey's post hoc test). cLSM analysis on cross-sections of mature granddaughter fronds growing in the presence and absence of copper sulphate revealed that flavonoid accumulation was disproportionally affected across the different tissue layers. Under copper sulphate addition, intensities of lut 7-O-glc increased mostly in the mesophyll ($p = .005$) and abaxial epidermis ($p = .045$), but not the adaxial epidermis ($p = .9$, Figure 5a). Similarly, intensities of lut 8-C-glc tended to increase in mesophyll cells ($p = .07$), decrease in the abaxial epidermis ($p = .09$)

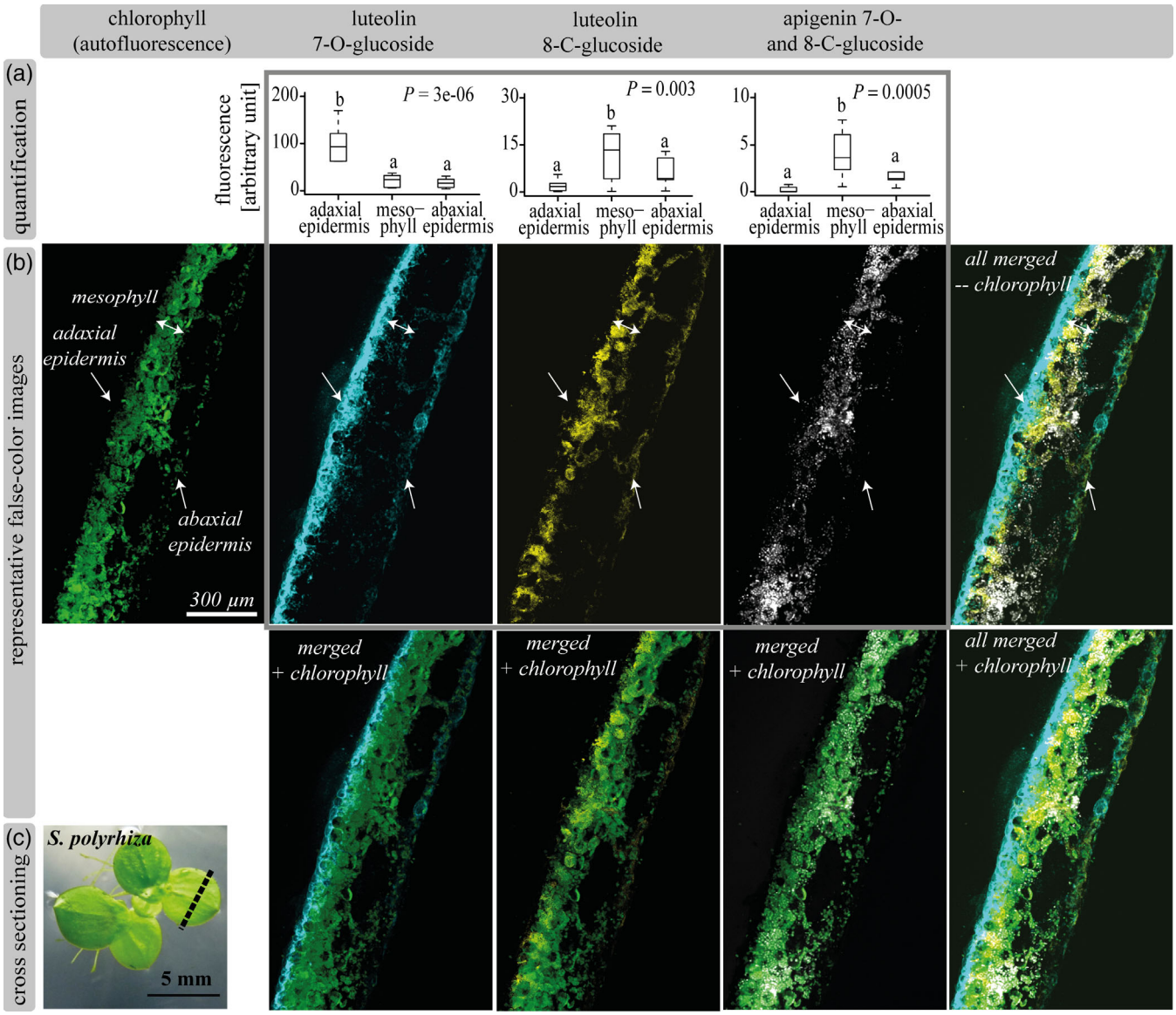

FIGURE 2 Tissue-specific distribution of individual flavonoid glucosides in *Spirodela polyrhiza* based on fluorescence quantification. (a) Quantification of fluorescence of each compound in different tissues. Statistics give the likelihood ratio tests comparing two linear mixed effect models that differ in the factor tissue layer as a fixed effect. Different lower-case letters indicate significant differences in fluorescence intensity according to Tukey's HSD test. n = 6, 2-APB = 2-aminoethyl diphenylborinate. (b) cLSM false-colour-images of 2-APB-stained *S. polyrhiza* cross-sections of the green plant body of fully mature plants acquired using the emission spectra recorded from the respective standard shown in Figure 1b. *In planta* fluorescence signals of the apigenin 7-O-glucoside and 8-C-glucoside showed crosstalk and could not be separated reliably, so these compounds were quantified together. Brightness and contrast of images were adjusted individually in ImageJ for illustrative purpose. (c) Mature *S. polyrhiza* fronds were sectioned along a distinct peripheral axis (dashed line)

and did not change in the adaxial epidermis (p = .1) under copper sulphate addition. The intensity of the apigenin glucosides was not affected by copper sulphate addition except in the abaxial epidermis, in which copper sulphate reduced apigenin fluorescence (p = .03). Linear mixed model analysis revealed a significant effect of copper sulphate on the accumulation of lut 8-C-glc across the tissue layers (significant interaction term of treatment × tissue layer, p = .01), whereas the models did not show any effect of copper sulphate on the tissue-specific distribution of the other flavonoid glucosides (lut 7-O-glc p = .3, apigenins p = .1). There were also no apparent differences in the subcellular distribution of flavonoid glucosides.

To investigate whether natural UV light alters the amounts and distribution patterns of flavonoid glucoside accumulation, we cultivated plants under UV-blocking and UV-transmitting Plexiglas covers for 7 days outdoors. Ultraviolet light did not affect plant biomass production (Figure S12) nor concentrations of individual flavonoids (Figure 4b). Linear mixed effects model did not reveal differences in treatment effects on flavonoid accumulation pattern (Figure 5b).

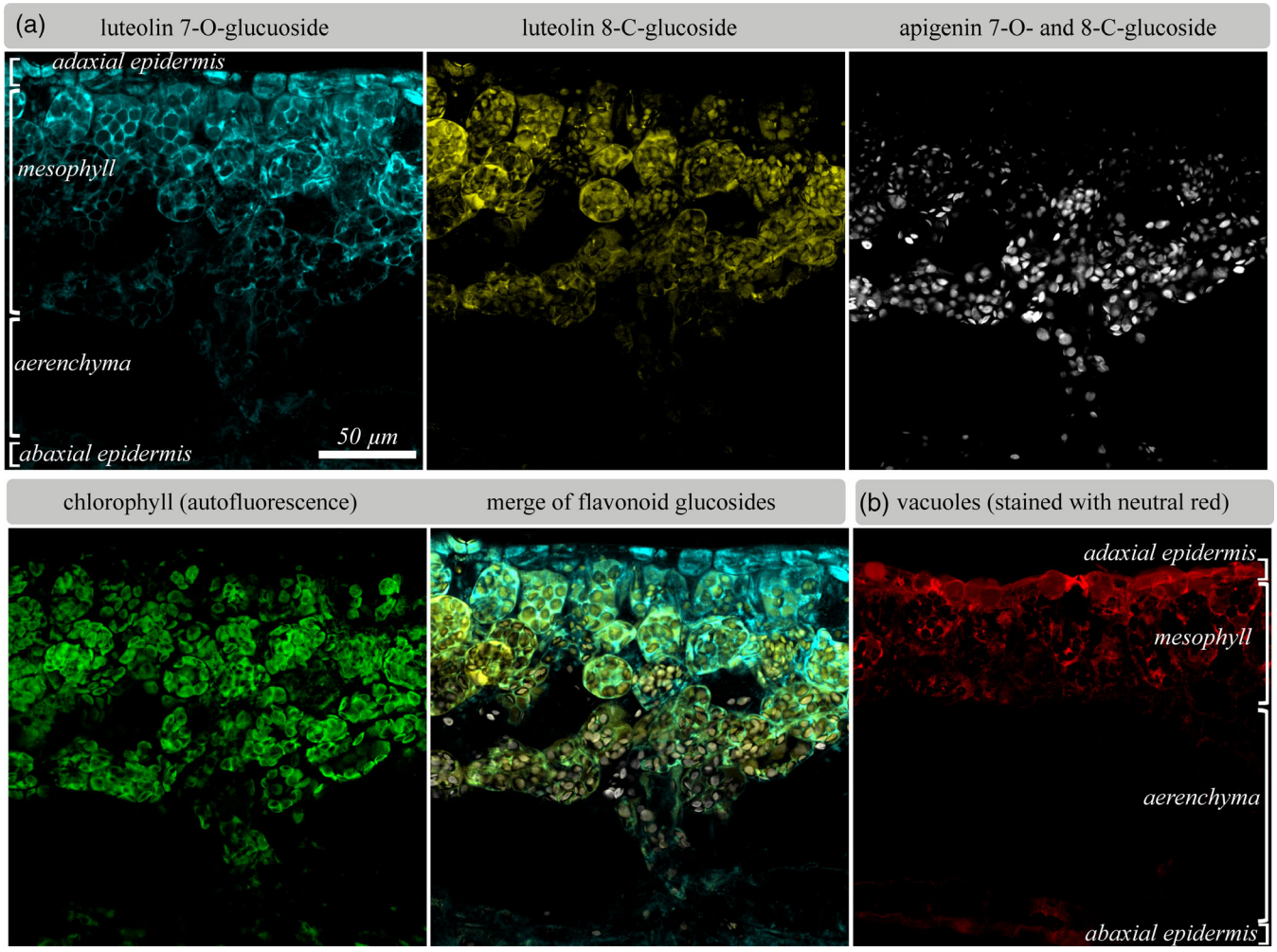

FIGURE 3 Subcellular distribution of individual flavonoid glucosides based on cLSM false-colour-images of a (a) 2-APB and a (b) neutral red stained *Spirodela polyrhiza* cross-section. Both luteolin glucosides accumulated mostly in vacuoles, and luteolin 8-C-glucoside was associated with chloroplasts. Apigenin glucosides were almost exclusively associated with chloroplasts. cLSM was performed with the "online emission fingerprinting" feature again based on the stored emission spectra of the respective standards shown in Figure 1b. 2-APB-stained cross-sections were excited with 405 nm; in another cross-section, vacuoles were stained with neutral red and excited with 543 nm. Brightness and contrast of all images were adapted in ImageJ for illustrative purpose. 2-APB = 2-aminoethyl diphenylborinate. Additional images of merged channels are shown in Figure S9

Taken together, the data show that copper sulphate addition increased the amount of dihydroxylated B-ring-substituted luteolin glucosides, especially lut 8-C-glc, in a tissue-specific manner, whereas natural UV light did not affect the amount or distribution of flavonoid glucosides.

3.4 | Certain flavonoid glucosides correlate with resistance to copper sulphate addition and UV stress

The distinct cellular and subcellular distribution of the four major flavonoids of *S. polyrhiza*, as well as their differential and partially tissue-specific induction under copper sulphate addition suggest that the compounds may have differential fitness effects under natural UV light exposure and copper sulphate addition. We tested this hypothesis by growing chemically diverse *S. polyrhiza* genotypes under these conditions. We standardized growth across genotypes by comparing the mean biomass production of exposed plants (with copper sulphate or with UV light) of each genotype to the mean biomass production of non-exposed plants (without copper sulphate addition; without UV light) of each genotype ("relative plant fitness"; values below one indicate growth suppression, whereas values above one imply growth promoting effects under copper and UV light, respectively).

After 7 days of growth indoors, copper sulphate addition reduced biomass production by average of 30% across 53 *S. polyrhiza* genotypes ($p = 2.2e^{-16}$, paired *t* test, Figure S13). The constitutive concentrations of both luteolin glucosides positively correlated to relative plant fitness under copper sulphate addition across the genotypes (lut 7-O-glc, $p = .046$; lut 8-C-glc $p = .04$, linear models, Figure 6a),

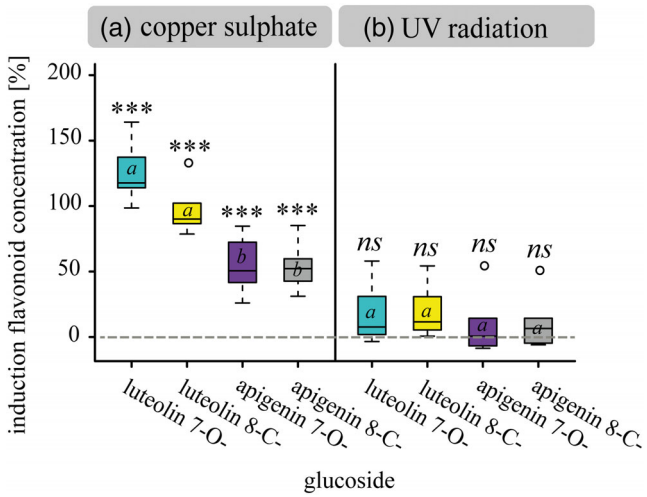

FIGURE 4 Percentage induction of flavonoid glucosides in whole *Spirodela polyrhiza* plants after treatment with copper sulphate (a) or natural UV radiation (b). Flavonoid levels of stressed plants were compared to mean flavonoid levels of control plants (HPLC-based quantification). Different lower-case letters indicate significant differences in the percentage induction according to Tukey's post hoc tests. The second generation (granddaughters) of plants that had been subjected to the original treatment was analysed. $n = 6$ (a), $n = 5$ (b)

whereas no correlations were observed for the apigenin glucosides ($p > .4$, linear models, Figure 6a), despite the constitutive concentrations of all flavonoid glucosides being highly correlated to each other (Figure S14, $p < .001$, Pearson's moment correlations). Total constitutive flavonoid glucoside concentration was weakly correlated to relative fitness ($p = .06$, linear model, Figure S15). Similar patterns for relative plant fitness and individual and total flavonoid glucoside concentrations were found when the induced metabolite concentrations were analysed (Figure S16a). In absolute terms, both luteolin glucosides were negatively correlated to biomass production under control conditions (lut 7-O-glc: $p = 5.72e^{-07}$, lut 8-C-glc: $p = 1.89e^{-05}$, linear models) and only weakly negatively (lut-7-O-glc, $p = .099$) or not correlated to biomass production (lut 8-C-glc, $p = .39$) under copper sulphate addition (interaction metabolite concentration * copper $p < .0014$ for both luteolin glucosides, linear models, Figure S17). Ap 7-O-glc was negatively correlated to plant biomass production regardless of treatment (linear models, $p = .017$ with copper sulphate addition, $p = .002$ without copper sulphate addition, interaction ap 7-O-glc * treatment = 0.13, Figure S17). Ap 8-C-glc was not correlated to biomass production in the presence or absence of copper sulphate addition ($p > .76$ with and without copper sulphate addition, interaction ap 8-C-glc * treatment $p = 0.77$, Figure S17).

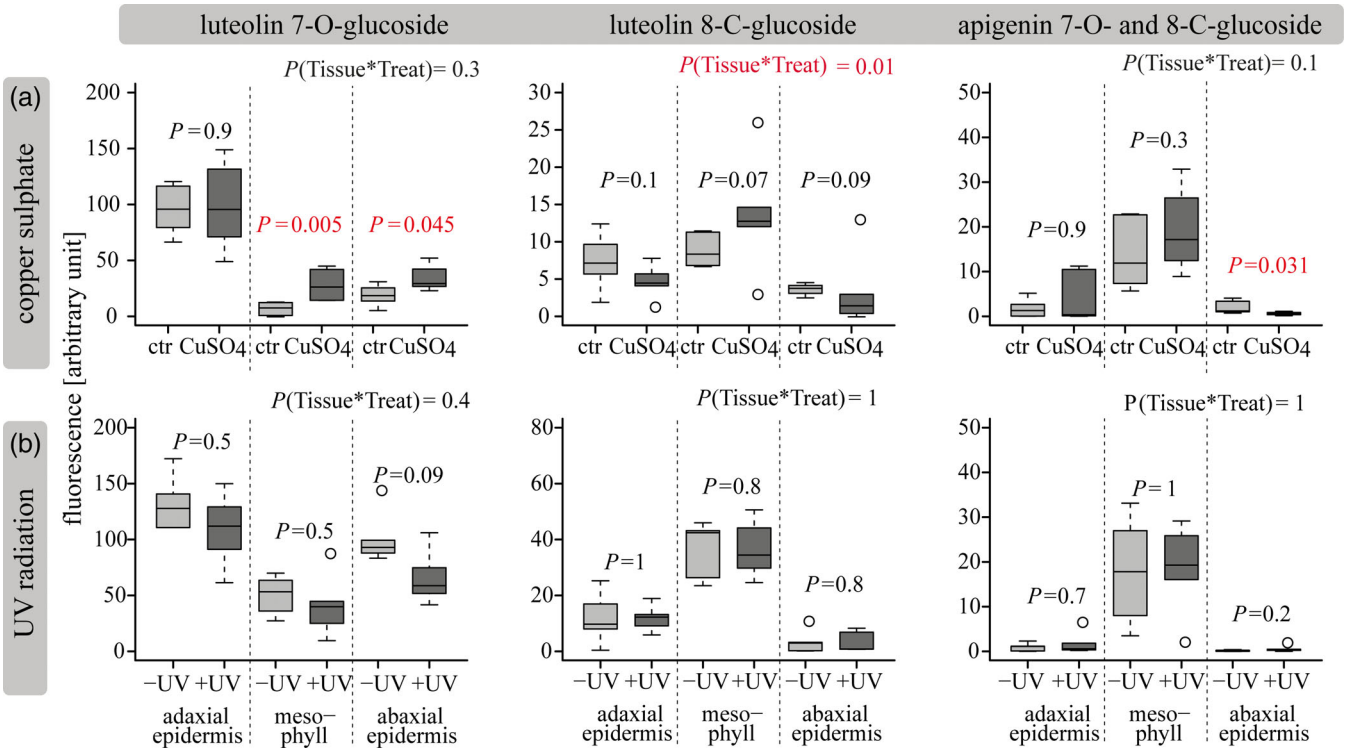

FIGURE 5 Induction of flavonoid glucosides in different tissues of *Spirodela polyrhiza* after treatment with copper sulphate (a) natural UV light (b). Copper but not UV treatment had tissue-specific effects on flavonoid accumulation, particularly on luteolin 8-C-glucoside levels. Plants were grown in the absence and presence of copper sulphate or natural UV light until the granddaughter generation had matured, which was subsequently free hand-sectioned and 2-APB-stained for cLSM analysis. *p* values of likelihood ratio tests comparing two linear mixed effect models that differed in the interaction term of treatment and tissue layer are displayed above each figure. Wilcoxon–Mann–Whitney tests were then used to test for differences of individual flavonoid fluorescence intensities between treatments within a tissue layer. $n = 6$ (a), $n = 5$ (b), ctr = control

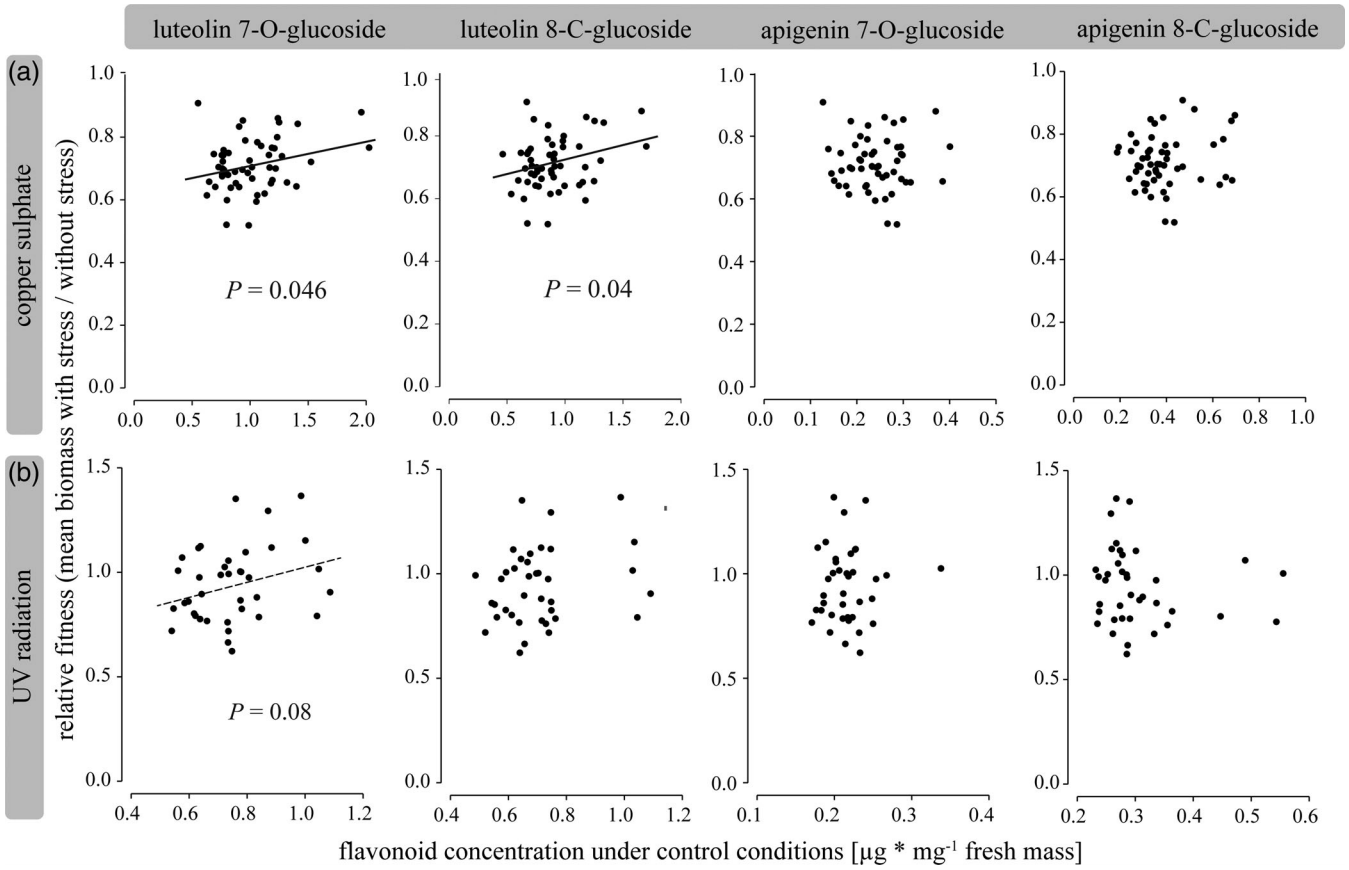

FIGURE 6 Correlation of individual flavonoid glucosides to relative plant fitness across *Spirodela polyrhiza* genotypes in the presence and absence of copper sulphate (a) and natural UV light (b). Relative fitness is the mean biomass of exposed plants (A: with copper sulphate; B: with natural UV light) of each genotype compared to the mean biomass production of control plants (A: without copper sulphate; B: without natural UV light) of each genotype. Data points below the horizontal dashed line indicate reduction in biomass under copper excess and UV light, respectively. Fifty-three genotypes were grown indoors for 7 days in the presence and absence of 10 µM copper sulphate, whereas 38 genotypes were grown outdoors for 14 days in the presence and absence of natural UV light. Each data point represents the mean of one genotype. *p* values linear models based on mean values per genotype are shown

We next assessed the correlation of the individual flavonoids to plant fitness in plants growing under UV-exposed and -shielded conditions outdoors. After 14 days of growth outdoors, natural UV light reduced biomass production an average of 8% across 38 *S. polyrhiza* genotypes (Figure S18, $p = .013$, paired *t* test). Ultraviolet light reduced the biomass production of most genotypes, but positive effects of UV light on plant growth were also observed in about 30% of the genotypes (Figure S19). The concentration of the adaxial epidermis-enriched lut 7-O-glc (measured under UV-shielded conditions) tended to positively correlate to relative plant fitness under UV light ($p = .08$, linear model, Figure 6b), whereas no correlations were observed for the other flavonoid glucosides ($p > .2$, linear models, Figure 6b). Total flavonoid concentration was not correlated to relative plant fitness ($p = .16$, linear model, Figure S20). Similar but non-significant associations between relative plant fitness and individual or total flavonoid concentrations were found when metabolite concentrations under UV-exposed were analysed (Figure S16b). In absolute terms, both luteolin glucosides were negatively correlated to plant biomass production in the presence and absence of UV light (lut 7-O-gluc: $p < .0005$, lut 8-C-glc: $p < .06$, linear models; $p > .82$ for interaction of metabolite concentration * treatment, linear models; Figure S21). Ap 7-O-glc concentration was not correlated to biomass production regardless of UV treatment ($p > .18$ linear models, $p = .64$ for interaction metabolite concentration * treatment, linear model, Figure S21). Ap 8-C-glc was positively associated with plant fitness in the presence and absence of UV light ($p < .002$, linear models; $p = .57$ for interaction metabolite concentration * treatment, linear model, Figure S21).

Taken together, these data provide evidence that lut 8-C-glc and possibly lut 7-O-glc benefit plant fitness under copper sulphate addition, while lut 7-O-glc may alleviate the negative effects of UV radiation.

4 | DISCUSSION

The ways in which flavonoids protect plants under UV radiation have long been controversial with opinion divided between UV screening and anti-oxidative mechanisms. Here, we show that both possibilities

may be correct for different flavonoid glucosides in the duckweed *S. polyrhiza*. The two major flavone glucosides with *ortho*-dihydroxy B-ring substitution, luteolin-7-O-glucoside and luteolin-8-C-glucoside, have distinct cellular and subcellular localization patterns. In addition, these compounds are correlated with resistance to different stressors (natural UV radiation vs. copper sulphate addition) when comparing many genotypes in common garden experiments suggesting different mechanisms for mediating UV protection.

4.1 | Duckweed flavonoid glucosides have distinct tissue and subcellular localization patterns

Using cLSM, we found that the major *S. polyrhiza* flavonoid glucosides have distinct tissue and subcellular distributions. Lut 7-O-glc accumulates mostly in the vacuoles of the adaxial epidermis, lut 8-C-glc predominantly in the vacuoles and chloroplasts of mesophyll cells and 7-O- and 8-C-apigenin glucosides almost exclusively in the chloroplasts of mesophyll cells. The association of the apigenin glucosides with chloroplasts is consistent with a previous study identifying ap 7-O-glc in isolated chloroplasts of *S. polyrhiza* (Saunders & McClure, 1976). In contrast to our work, lut 7-O-glc was previously identified in isolated chloroplast preparations of *S. polyrhiza* (Saunders & McClure, 1976). However, a microscopic survey of fresh, unwashed sections, such as performed here, may be more accurate for localization of low molecular weight metabolites than subcellular fractionation due to the tendency of many metabolites to adhere to membrane fractions. Flavonoid glucosides in *S. polyrhiza* may be additionally associated with other cell compartments in concentration levels not detectable with our methods, especially as our protocols are less sensitive to the apigenin glucosides and other non-*ortho*-dihydroxylated B-ring flavonoids that do not form adducts with 2-APB at typical cellular concentrations (Agati et al., 2009).

Uneven distribution of flavonoid glucosides across plant tissue layers was reported before, for example, for *Phillyrea latifolia* (Agati et al., 2002, 2012) and *Ligustrum vulgare* (Tattini et al., 2004), and several studies suggest that individual flavonoids occur in different subcellular compartments using HPLC, cLSM and organelle isolation (Agati et al., 2012; Hernández et al., 2009). Here, we showed that closely related flavone glucosides had partially contrasting tissue and subcellular locations, which suggest different functions in the plant.

4.2 | The cLSM software enables visualization of individual flavonoids in planta

In demonstrating the differential localization of individual flavonoid glucosides, we showed for the first time, to our knowledge, that individual flavonoid glucosides can be selectively visualized *in planta* using the flavonoid complexing agent 2-APB, which forms a fluorescent adduct. We employed the linear unmixing feature of the cLSM software to separate a spectral signal recorded from a single or a group of pixels containing various fluorophores into a separate intensity signal for each compound (Talamond, Verdeil, & Conéjéro, 2015). In *Coffea* species, this unmixing feature was used to visualize distinct fluorescence signals of the phenolic compounds chlorogenic acid and mangiferin (Conéjéro, Noirot, Talamond, & Verdeil, 2014). In our study, lut 8-C-glc and lut 7-O-glc were successfully distinguished in cross-sections of *S. polyrhiza* fronds despite largely overlapping emission curves that would have hindered selective visualization using traditional cLSM techniques. Furthermore, we were able to locate monohydroxylated B-ring flavonoid glucosides, which are also known to fluoresce when stained with 2-APB albeit upon excitation with 365 nm instead of 405 nm as used in our experiments (Agati et al., 2002). Experiments to determine whether the linear unmixing algorithms are sufficient to individually trace other flavonoids will help to assess the suitability of the described methods for other organisms.

4.3 | Individual duckweed flavonoid glucosides may have separate roles in resistance to copper and UV stress

Luteolin 7-O- and 8-C-glucoside share many chemical features that may be important for copper and UV stress resistance. Firstly, these compounds have the same aglycone moieties, and thus identical conjugated double bond systems and arrangements of functional groups except glucose, resulting in nearly identical UV-absorption spectra (Figure S22). Secondly, both compounds share the same *ortho*-dihydroxylated B-ring patterns, which are important for their radical scavenging capacity (Burda & Oleszek, 2001; Sekher, Chan, O'Brien, & Rice-Evans, 2001), Cu^{+}-chelating activity (Brown et al., 1998) and the suppression of the Fenton reaction (Cheng & Breen, 2000). Although the *ortho*-dihydroxylated B-ring (catechol group) is very important for the antioxidant properties of these compounds, the glycosylation site still may affect antioxidant capacity. Indeed, based on 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2-azinobis (3-ethyl-benzothiazoline-6-sulfonic acid (ABTS) and FRAP assays, luteolin-8-C glucoside has higher radical scavenging and antioxidant potential than its 7-O-counterpart (Zhang et al., 2014).

Indeed, the two luteolin glucosides appear to have partially different functions in *S. polyrhiza* fronds (Table 1). In a survey of chemically diverse *S. polyrhiza* genotypes, both compounds were correlated with high fitness under copper sulphate addition and both were induced by this treatment. This is in line with previous studies showing that *ortho*-dihydroxylated B-ring-substituted flavonoids have high chelating and antioxidant potential that may reduce heavy metal stress (Brown et al., 1998; Mira et al. 2002), and that flavonoid deficient *A. thaliana* plants are more susceptible to cadmium and zinc stress than controls (Keilig and Ludwig-Müller, 2009). However, lut 8-C-glc accumulated mainly in the mesophyll, while lut 7-O-glc accumulated mostly in the adaxial epidermis. Although both compounds increased in the mesophyll under copper sulphate treatment, the fact that lut 8-C-glc is the dominant compound points to its importance for copper stress resistance. At the subcellular level, lut 8-C-gluc was stored mainly in vacuoles and was also associated with chloroplasts, in which

TABLE 1 Summary of tissue-specific distribution, subcellular occurrence, induction and fitness benefits of the major flavonoid glucosides in *Spirodela polyrhiza*

	Distribution within plant body cLSM		Subcellular occurrence cLSM	Flavonoid induction under stress		Fitness benefit under stress	
				Copper sulphate addition	UV	Copper sulphate addition	UV
Luteolin 7-O-glucoside	Adaxial epidermis	++	Vacuole	125% $p = 3e^{-06}$	*ns*	Positive $p = .046$	Positive ($p = .08$)
	Mesophyll	+					
	Abaxial epidermis	+					
	$p = 3e^{-06}$						
Luteolin 8-C-glucoside	Adaxial epidermis	+	Vacuole, chloroplast	94% $p = 7e^{-06}$	*ns*	Positive $p = .04$	*ns*
	Mesophyll	++					
	Abaxial epidermis	+					
	$p = 0.003$						
Apigenin 7-O-glucoside Apigenin 8-C-glucoside	Adaxial epidermis	+	Chloroplast	54% $p = .001$	*ns*	*ns*	*ns*
	Mesophyll	++					
	Abaxial epidermis	+		54% $p = .004$	*ns*	*ns*	*ns*
	$p = .0005$						

Note: Classification of the tissue-specific distribution flavonoids in fronds was based on Tukey's HSD tests (genotype 7498, from Figure 2). Flavonoid induction under stress is based on Student's *t* test comparing induced and non-induced flavonoid levels determined by HPLC analysis (genotype 7,498, from Figure 4). *p* values of fitness benefits under stress relate to linear models correlating relative fitness to individual metabolite concentration across worldwide distributed *S. polyrhiza* genotypes (from Figure 6). ++, high concentrations; +, low concentrations; ns, not significant.

flavonoids have been suggested to reduce hydrogen peroxide (H_2O_2) and to maintain ROS concentrations at sub-lethal levels in combination with vacuolar class III peroxidases (Ferreres et al., 2011). The sites of lut 8-C-glc in vacuoles and chloroplasts are also locations where copper from the environment can be accumulated (Printz, Lutts, Hausman, & Sergeant, 2016). However, in both compartments, the pH is compatible with the formation of flavonoid–copper complexes (Berkowitz & Wu, 1993; Malešev & Kuntić, 2007), allowing the chelation of copper leading to copper stress resistance. The localization and induction patterns of lut 8-C-glc, its high radical scavenging potential relative to lut 7-O glc (Zhang et al., 2014) and its correlation with fitness under copper sulphate addition suggest that this compound alleviates oxidative stress that may be induced by copper treatment.

In contrast to lut 8-C-gluc, lut 7-O-glc not only correlated to plant fitness under copper sulphate but also showed a tendency to be correlated to fitness under natural UV light exposure in a survey of chemically diverse *S. polyrhiza* genotypes. In addition, lut 7-O-glc accumulated not in the mesophyll, but mostly in the adaxial epidermis, the cell layer that receives most UV radiation through its exposure to sunlight. This O-glucoside was mainly stored in vacuoles, and so may shield the underlying cell layers from UV radiation as vacuoles occupy almost the entire surface of the epidermis (Figure 3, Figure S10). On the other hand, lut-7-O-glc may also act by scavenging light-induced ROS. Thus, this compound may protect the adaxial epidermal cells from oxidative damage both through reducing the formation of ROS (UV screening) and through quenching the radicals (antioxidant capacity). Protection of the epidermal cells may be particularly important in *S. polyrhiza* due to the proximity of the underlying reproductive cells.

In recent years, some researchers suggested that the UV-protecting role of flavonoids is due more to their antioxidant than UV-screening properties since though the maximum absorption of flavonoids lies in the UV-A region, they are induced by light in UV-B region and visible ranges also (Agati et al., 2012; Brunetti, Fini, Sebastiani, Gori, & Tattini, 2018). However, the fact that lut 7-O-glc, primarily localized in the adaxial epidermis, was associated with greater fitness under both UV radiation and copper sulphate treatment, but that lut 8-C-glc, localized in the mesophyll, was associated with greater fitness only under copper sulphate addition, indicates that individual flavonoids may have antioxidant and/or UV-screening functions, depending on their location. The localization of apigenin glucosides, which are considered to have low anti-oxidative potential (Rice-Evans et al., 1996), in the chloroplast, a cellular compartment that generates oxidative stress appears contradictive and raises questions about the role of apigenin derivatives. Targeted manipulation of apigenin derivatives through genetic engineering may provide insights into the compounds' function in vivo.

Whatever the mechanism of protection, flavonoids are known to alleviate the effects of UV radiation indoors and under natural settings outdoors (Guidi et al., 2011; Jansen & Bornman, 2012; Kliebenstein, 2004; Robson et al., 2015). Experiments that specifically manipulate the major *S. polyrhiza* flavonoids through genetic engineering or chemical modification may provide further insights into the ecological relevance of these compounds under different stresses and their functional specificity.

As one of the most common and phylogenetically widespread groups of secondary metabolites, flavonoids have received much attention in recent years due to their potentially positive effects on

human health (Panche et al., 2016). Yet, the ecological roles of flavonoids remain controversial, partially due to their structural diversity, different accumulation patterns and varying abundance, all of which affect their biological activity (Hernández et al., 2009). By using a combination of cLSM and common garden experiments with chemically diverse *S. polyrhiza* genotypes, we showed that chemically similar flavonoids have distinct tissue-specific and intra-cellular distributions that may be associated with distinct functions in abiotic stress resistance. Knowledge of the localization of plant metabolites can be very valuable in understanding their ecological relevance.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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Additional supporting information may be found online in the Supporting Information section at the end of this article.

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