

Letter

Antioxidants by nature: an ancient feature at the heart of flavonoids' multifunctionality

Introduction

Early land plants' ability to adapt to novel environmental pressures associated with an ever-changing terrestrial habitat was the result of a vast set of evolutionary innovations, including metabolic ones (Wagner, 2011; Bowman *et al.*, 2017). Land plants, as sessile organisms, were driven to evolve integrated and modular metabolic pathways. Several of them were true metabolic network innovations, responsible for synthesizing several novel compounds (Cannell *et al.*, 2020; Dadras *et al.*, 2023b). The new specialized metabolites (SMs) contributed to thrive in these new and frequently hostile environments (Rensing, 2018; Cheng *et al.*, 2019; Han *et al.*, 2019; Buschmann, 2020; Fürst-Jansen *et al.*, 2020). There is evidence that metabolic plasticity is a key component of a highly complex network in the plant–environment interaction, which also includes morphoanatomical traits. This network largely and ultimately determines the ability of terrestrial plants to escape from the most severe environmental threats, the so-called 'flight strategy' of sessile organisms (Potters *et al.*, 2007; Lauder *et al.*, 2019). While an elaborate metabolic system was already placed in the closest algal ancestors of land plants (Rieseberg *et al.*, 2021; Dadras *et al.*, 2023a), primary and particularly secondary metabolic networks have grown far more sophisticated throughout plant evolution (Keeling *et al.*, 2010; Wang *et al.*, 2015; Maeda, 2019; Bowles *et al.*, 2020; Li *et al.*, 2024). They contributed to land plant distribution toward more challenging habitats (Stemans *et al.*, 2009). For instance, the R2R3MYB family of transcription factors (TFs), which regulates a wide array of biological processes, including the expression of genes involved in the biosynthesis of phenylpropanoids, has been extraordinarily expanded and diversified in the lineage of angiosperms (Feller *et al.*, 2011; Bowman *et al.*, 2017; Albert *et al.*, 2018; Jiang & Rao, 2020; Davies *et al.*, 2021). Enzymes involved in both the 'decoration' of basic phenylpropanoid skeletons (e.g. the C6-C3-C6 core skeleton of flavonoids) and their transport to different subcellular compartments have also expanded much throughout plant evolution (Kitamura, 2006; Tohge *et al.*, 2018; Alseikh *et al.*, 2020; Davies *et al.*, 2020; Li *et al.*, 2020; Wen *et al.*, 2020). The extraordinary chemical diversity originated from the rise and evolution of multiple SM pathways, coupled with their location in different tissues and cellular compartments, well explains the outstanding plant adaptability to harsh stressful conditions

(*sensu stricto*, that is, distance from pre-existing homeostasis) associated with the terrestrial habitat (Fürst-Jansen *et al.*, 2020; Rensing, 2020).

The pivotal role of SMs in the adaptability of land plants depends not only on their extraordinarily high number and diversified skeletons, synthesized by different taxa (Weng *et al.*, 2021), but also on their inherent ability to play multiple functions (Milo & Last, 2012; Ehlers *et al.*, 2020; Mutwil, 2020; Durán-Medina *et al.*, 2021; Hu *et al.*, 2021; de Vries *et al.*, 2021; Weng *et al.*, 2021). Although SM biosynthesis might have served as a sink for the excess of carbon available to plants during their initial exploration of a highly enriched CO₂ atmosphere (Dadras *et al.*, 2023a,b), SMs multifunctionality efficiently compensates for the energetic cost required for their biosynthesis (Kliebenstein, 2013; Erb & Kliebenstein, 2020). The multifunctional nature of SMs and their high responsiveness to abiotic and biotic stressors provide plants with an unlimited defense arsenal, in which each SM may play different roles depending on the severity of the stress events and the degree of plant body complexity. These factors determine the metabolite distribution at the organ, tissue, cellular, and subcellular levels (Schneider *et al.*, 2019; Wang *et al.*, 2019; Shitan & Yazaki, 2020; Weng *et al.*, 2021). In simpler terms, the evolution of multifunctional SM biosynthesis follows the natural tendency to catch as many flies with one clamp as possible (Wink, 1999; Izhaki, 2002).

Here, we focus on the ancient and ubiquitous class of flavonoids (Fig. 1), which are highly responsive to abiotic and biotic environmental stressors and are capable of regulating key steps in plant growth and development (Pollastri & Tattini, 2011; Schneider *et al.*, 2019; Chapman & Muday, 2021; Garagounis *et al.*, 2021; Venegas-Molina *et al.*, 2021; Daryanavard *et al.*, 2023). However, their multifunctionality makes it difficult to determine the foremost environmental drivers for the emergence and diversification of the flavonoid metabolic network, despite decades of extensive research (Rozema *et al.*, 1997, 2002; Buer *et al.*, 2010; Tripp *et al.*, 2018; Yonekura-Sakakibara *et al.*, 2019; Davies *et al.*, 2020). We provide a detailed analysis of the complex relationship between the multifunctional nature of flavonoids and the environmental stimuli primarily responsible for the rise of the flavonoid metabolic network, offering conclusive evidence for the structural–functional relationship that is at the root of their functional versatility.

Did flavonoid metabolism first emerge in response to biotic pressures?

The emergence of flavonoids represented an outstanding major metabolic innovation during the plants' water-to-land transition (de Vries *et al.*, 2017; Davies *et al.*, 2020; Dos Santos Nascimento & Tattini, 2022). This rise has been initially hypothesized to have

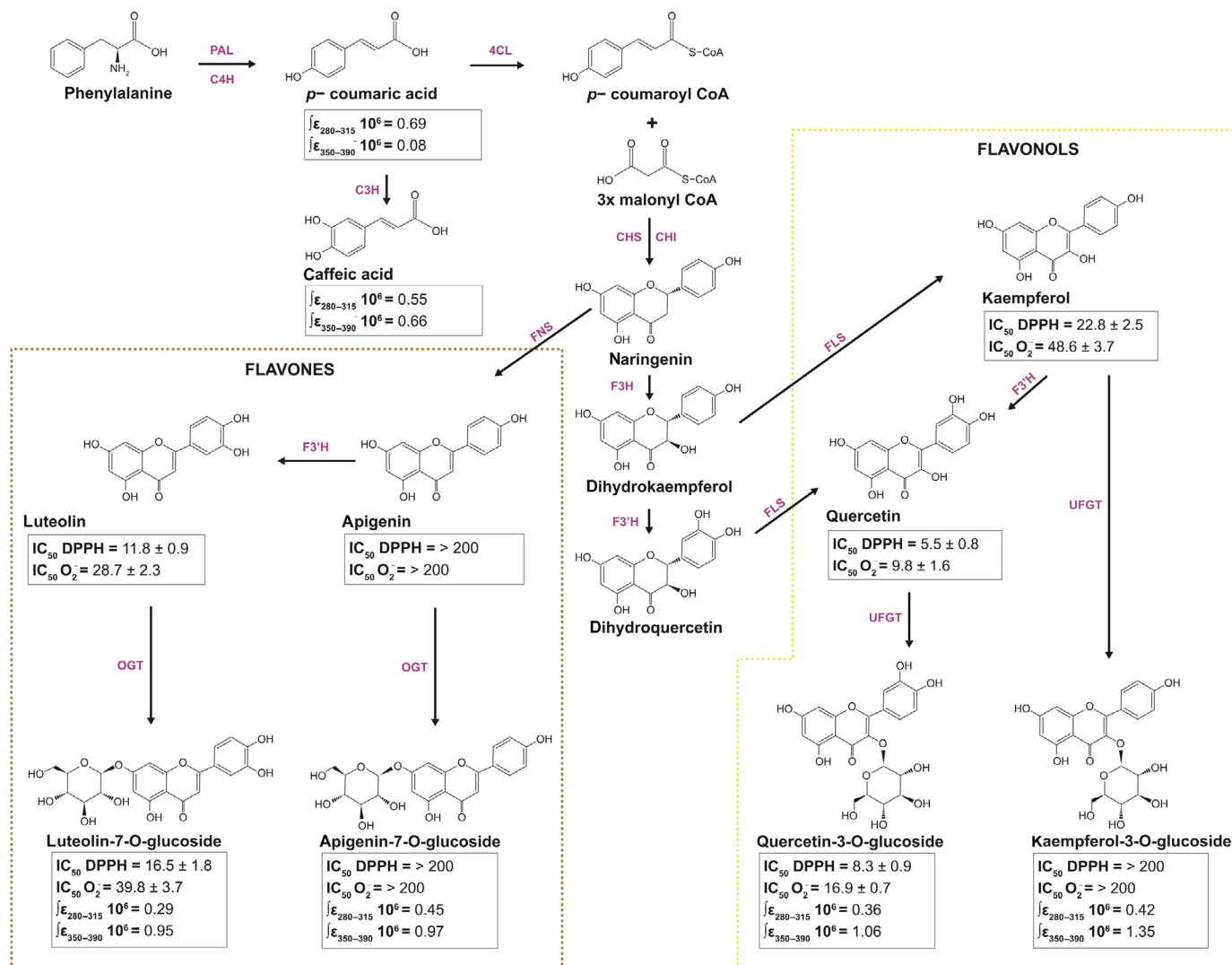


Fig. 1 Simplified scheme of the phenylpropanoid pathway leading to the biosynthesis of hydroxycinnamic acid derivatives (HCAs, here reported are *p*-coumaric and caffeic acids), mono- and dihydroxy B-ring-substituted flavones and flavonols (FLAV). The UV-absorbing capacity of HCAs and FLAV has been measured by integrating individual molar extinction coefficients (ϵ) over the 280–315 (UV-B) and 315–390 nm (UV-A) waveband. The antioxidant capacity of FLAV, both aglycones and glycoside derivatives, has been estimated by calculating the concentration (μM) of individual metabolites capable of reducing by 50% (IC_{50}) that of the synthetic free radical DPPH (2,2-diphenyl-1-picrylhydrazyl) and the superoxide anion (O_2^-), following the spectrophotometric protocols of Baratto *et al.* (2003). Data of IC_{50} are means \pm SD of three replicate measurements. 4CL, 4-coumaroyl-CoA ligase; C3H, *p*-coumarate 3-hydroxylase; C4H, cinnamate 4-hydroxylase; CHI, chalcone isomerase; CHS, chalcone synthase; F3'H, flavanone 3'-hydroxylase; F3H, flavanone 3-hydroxylase; FLS, flavonol synthase; FNS, flavone synthase; OGT; 7-O-glucosyl transferase; PAL, phenylalanine ammonia-lyase; UFGT; UDP glucose-flavonoid 3-O-glucosyl transferase.

occurred in response to herbivore pressure (Swain, 1977; Cooper Driver, 1980), the long-known 'biochemical coevolutionary arms-race theory' (Ehrlich & Raven, 1964). In brief, the rise and the diversification of flavonoids, in terms of number and structural complexity, paralleled with major changes in plant morphology, would have been a direct consequence of the selective pressure caused by predation and diseases (Levin, 1971; Swain, 1975, 1977). This coevolution hypothesis has been proven for several classes of SMs, but questioned in other instances, such as the case of flavonoids and other phenolics (Jones & Firn, 1991; Close & McArthur, 2002; Davies *et al.*, 2020; Erb & Kliebenstein, 2020).

For instance, Rausher (2001) argued that plant enemies are too rare to generate a frequent evolution of defensive features, such as the biosynthesis of many SMs, particularly flavonoids. Close & McArthur (2002) pointed out the relatively minor role of many phenolics, including flavonoids, as anti-herbivore agents, while providing evidence for their main functions as photo-protectants. Although tannins have historically been viewed as defense compounds against herbivore insects, relatively new evidence supports their antioxidant role (Salminen & Karonen, 2011; Constabel *et al.*, 2014; Gourlay & Constabel, 2019). Finally, the vast literature concerning the phenylpropanoid biosynthesis in

response to herbivores and their role in plant resistance has not provided proof of the predominant role of flavonoids as deterrents for herbivores (Serrano *et al.*, 2012; Garcia-Molina & Pastor, 2024). For instance, UV-B radiation, which is known to trigger flavonoid biosynthesis, has been reported to either increase or decrease the resistance to herbivores in a range of species (Izaguirre *et al.*, 2003; Rousseaux *et al.*, 2004; Schneider *et al.*, 2019). The biosynthesis of flavonoids is strongly suppressed by the bacterial *flg22*, which indeed stimulates other phenylpropanoid biosynthetic branch pathways (Serrano *et al.*, 2012), in agreement with the observation that sinapic and caffeic acid derivatives offer higher herbivory resistance than flavonoids (for a review, see Ballaré, 2014). There is also convincing evidence that most angiosperms prioritize immune responses over stress-induced flavonoid accumulation under microbial attack, and this might represent an ancient evolutionary regulatory crosstalk mechanism (Lozoya *et al.*, 1991; Lo & Nicholson, 1998; Logemann & Hahlbrock, 2002; Serrano *et al.*, 2012).

It is conceivable that, despite flavonoids' excellent antibacterial properties, resistance to natural enemies driven by greater production of these compounds may merely be a side consequence of chemicals that evolved to perform other ecological purposes (Rausher, 2001; Erb & Kliebenstein, 2020). This hypothesis is reasonable based on both the multifunctional nature of SMs and the vast range of environmental stresses, other than predators, that plants face on land (Rensing, 2018; Donoghue *et al.*, 2021).

The intriguing relationship between flavonoids and oxidative stress

It is worth noting that once plants moved onto land, they were confronted with a novel set of abiotic environmental stresses, such as the scarcity of water and nutrients, high solar irradiance and changing spectral quality of light, and huge fluctuation in air temperature (Fürst-Jansen *et al.*, 2020; Markham & Greenham, 2021; Xu *et al.*, 2021; Kim *et al.*, 2022). The evolution of a molecular network conferring water stress resistance is indeed the typical feature of all land plants (Rensing, 2020; Schreiber *et al.*, 2022). This supports the view that the simultaneous action of abiotic stressors, predominantly but not exclusively a combination of water scarcity and high sun irradiation, was the fundamental driver for the rise of SM biosynthesis pathways, including for flavonoids (Rensing, 2018; Brunetti *et al.*, 2019; Dixon & Dickinson, 2024). Flavonoid biosynthesis is greatly activated in response to drought stress and high solar irradiation (Tattini *et al.*, 2004, 2015; Nakabayashi *et al.*, 2015; Siipola *et al.*, 2016; Wang *et al.*, 2020), but it is also triggered by nutrient deficiency, salinity and cold (Lillo *et al.*, 2008; Agati *et al.*, 2011; Albert *et al.*, 2018; Bian *et al.*, 2019; Sachdev *et al.*, 2021). This leads to the hypothesis that changes in reactive oxygen species (ROS)/redox homeostasis, as commonly occur in plants exposed to a wide range of environmental stressors (Devireddy *et al.*, 2021; Peláez-Vico *et al.*, 2022; Dietz & Vogelsang, 2024), may have regulated the flavonoid biosynthesis in land plants (Babu *et al.*, 2003, 2005; Taylor & Grotewold, 2005; Xu *et al.*, 2015). This hypothesis fits well with the notion that the activities of most TFs that regulate the

flavonoid biosynthetic genes, including the R2R3MYBs, are under tight ROS/redox control (Heine *et al.*, 2004; He *et al.*, 2018; Imran *et al.*, 2018; Martin *et al.*, 2022; Pratyusha & Sarada, 2022). There is compelling evidence that stress-induced increase in the excitation pressure on PSII and the subsequent change in the redox status of the photosynthetic electron transport chain (PETC) serve as retrograde signals (chloroplast-to-nucleus signaling) to regulate flavonoid biosynthesis (Gerhardt *et al.*, 2008; Akhtar *et al.*, 2010; Richter *et al.*, 2020, 2023).

While ROS/redox regulation of flavonoid production does not necessarily point to a primary function of these molecules as quenchers/scavengers of stress-induced ROS accumulation (i.e. as antioxidants *sensu stricto*), flavonoids are components of the integrated antioxidant network, aimed at keeping the ROS level within a sub-lethal concentration range, under the most severe stressful conditions (to be described later, for details, Agati *et al.*, 2007, 2012, 2020; Nakabayashi *et al.*, 2015; Tattini *et al.*, 2015; Muhlemann *et al.*, 2018; Chapman & Muday, 2021; Martin *et al.*, 2022). We note that the addition of far-red light (FR), which is known to induce a more oxidized PETC, inhibits the biosynthesis of flavonoids and greatly decreases the ratio of quercetin (Que) to kaempferol (Kae) derivatives, which is the inverse of what happens when *Brassica napus* is supplemented with UV-B radiation (Gerhardt *et al.*, 2008). On the contrary, several studies have observed a marked increase in Que to Kae ratio in several angiosperms, such as pea, soybean, and *Arabidopsis thaliana*, supplemented with red light (R) (Furuya *et al.*, 1962; Falcone Ferreyra *et al.*, 2021; Lim *et al.*, 2023). An increase in Que to Kae derivatives, or in dihydroxy B-ring (dihydroxy thereafter) to monohydroxy B-ring-substituted (monohydroxy) flavonoids, is commonly observed in plant lineages of different complexity (such as bryophytes and angiosperms) in response to a wide range of abiotic stressors, including to high PAR and UV-B radiation (for review articles see, Pollastri & Tattini, 2011; Neugart & Schreiner, 2018; Agati *et al.*, 2020; Davies *et al.*, 2020; Dos Santos Nascimento & Tattini, 2022; Singh *et al.*, 2023). While Que and Kae aglycones, the last to a considerably lesser extent, have an effective ability to scavenge free radicals and ROS, this is not the case for Kae derivatives, in which the highly reactive 3-OH (flavonol) group is usually glycosylated (Rice-Evans *et al.*, 1996; Fig. 1). Glycosylation makes flavonoids soluble in the aqueous cellular milieu, prevents their auto-oxidation, facilitates their transport from the endoplasmic reticulum (ER) to different cellular compartments, but depresses to some extent their antioxidant capacity (Fig. 1). The ROS-scavenging activity of flavonoids mostly depends on the presence of the catechol group in the B-ring, followed by the presence of both C2-C3 unsaturation and a 4-oxo function in the C-ring, just like in Que (Rice-Evans *et al.*, 1996; Williams *et al.*, 2004). Consistently, Que 3-*O*-glucoside has a lower ROS-scavenging ability than Que, but considerably higher antioxidant capacity than Kae, whereas Kae 3-*O*-glucoside displays negligible antioxidant capacity (Fig. 1). While we cannot rule out the possibility that glycosylated flavonoids are de-glycosylated, releasing the most active aglycone forms (e.g. plants contain a plethora of β -glucosidase that may perform this function, Roepke & Bozzo, 2015; Le Roy *et al.*, 2016; Baba *et al.*, 2017), there is no

consistent body of evidence showing the presence of flavonoid aglycones in plant cells prone to oxidative stress, such as in epidermal and sub-epidermal tissues (Wollenweber *et al.*, 2011; Ketudat Cairns *et al.*, 2015; Baba *et al.*, 2017; Uehara *et al.*, 2018).

The functional significance of flavonoids as antioxidants in an *in planta* condition has long been debated (for critical review articles, see Hernández *et al.*, 2009; Agati *et al.*, 2012, 2020), owing to early observations of their almost exclusive location in the vacuoles of epidermal cells (Hrazdina *et al.*, 1982; Caldwell *et al.*, 1983; Hutzler *et al.*, 1998). Instead, flavonoids occur in the vacuoles, the cytoplasm, including the chloroplasts, and the nuclei of parenchymatic cells (Fig. 2; Polster *et al.*, 2006; Agati *et al.*, 2007, 2009, 2012; Böttner *et al.*, 2021) in significantly larger amounts than in the epidermal tissues (Gori *et al.*, 2021; Fig. 3). This makes flavonoids ideal for fine-tuning the ROS concentration in different subcellular compartments, as widely reported in several species (Ferrerres *et al.*, 2011; Muhlemann *et al.*, 2018; Chapman *et al.*, 2019; Agati *et al.*, 2020; Singh *et al.*, 2021; Cerqueira *et al.*, 2023). Agati *et al.* (2007) provided conclusive evidence that chloroplast-located dihydroxy flavonoids (Fig. 2) efficiently quenched singlet oxygen generated by a large excess of photosynthetically active radiation. Flavonols distributed in the cytoplasm and the nuclei of stomata guard cells effectively scavenge

H₂O₂ (Watkins *et al.*, 2014, 2017, see the next section for details). Flavonoids' ability to scavenge ROS may be especially advantageous in plants dealing with multiple environmental stresses, such as when solar irradiance causes severe light stress (Fini *et al.*, 2011; Tattini *et al.*, 2015). It is known that plants experience severe photooxidative stress, on a daily and seasonal basis, when light irradiance vastly exceeds that usable for photosynthesis, as occurs during the central hours of the day. Light excess is often accompanied by high temperature and vapor pressure deficit, consequently driving stomata closure. The resulting midday depression of photosynthesis, which results in huge ROS production, is further enhanced due to excess light- and heat-induced reduction in the activity of photosynthetic enzymes (Bagley *et al.*, 2015; Moore *et al.*, 2021). There is evidence that the activity of antioxidant enzymes may fall significantly during the central hours of the day, mostly due to the negative effect of high air temperature (Peltzer & Polle, 2001; Lu *et al.*, 2008; Tattini *et al.*, 2015; Soengas *et al.*, 2018), further enhancing photooxidative stress. The large diurnal variations in flavonoid content recently reported in a range of species, with higher concentrations detected in the midday hours (Barnes *et al.*, 2008, 2016; Gori *et al.*, 2021), equip plants with not only an effective shield against the penetration of higher levels of UV-B but also with a more

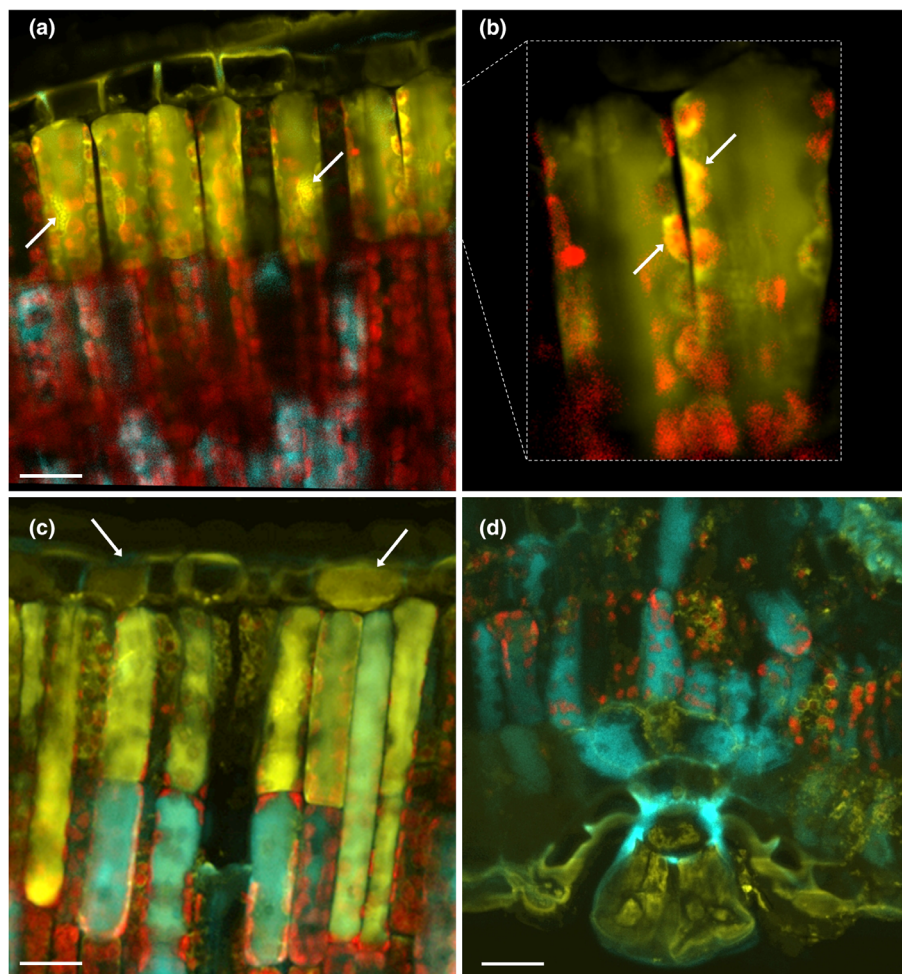


Fig. 2 Inter- and intra-cellular distribution of flavonoids (FLAV) and hydroxycinnamic acid derivatives (HCAs) in 6-month-old *Phillyrea latifolia* leaves newly developed in full sunlight. Cross sections were stained with Naturstoff reagent (NR, phosphate-buffered (pH 6.8) saline (1%, w/v, NaCl) solution of 0.1% (w/v) 2-amino ethyl diphenyl boric acid) and merged fluorescence images (a–d) result from confocal laser scanning microscopy (CLSM) analysis under the following, sequential, excitation (exc)/ emission (em) setups. $\lambda_{exc} = 365/\lambda_{em} = 415\text{--}485$ nm for HCA-derived blue fluorescence; $\lambda_{exc} = 488/\lambda_{em} = 565\text{--}535$ nm for FLAV-derived yellow fluorescence; $\lambda_{exc} = 638/\lambda_{em} = 690\text{--}785$ nm for chlorophyll-derived red fluorescence. FLAV accumulate in the vacuoles and the nuclei of adaxial parenchyma (arrows in a), in the outer envelope membranes of the chloroplasts (arrows in b), and in the vacuoles of adaxial epidermal cells (arrows in c). HCAs occur in abaxial mesophyll cells, which have a palisade-like morpho-anatomy (as typically occurs in sun-adapted leaves), together with yellow fluorescent FLAV (d). The multicellular glandular trichome exclusively accumulates FLAV in the vacuole and likely in the cytoplasm, whereas HCAs are merely distributed in the wall of the trichome stalk cell (d). Bars, 20 μm .

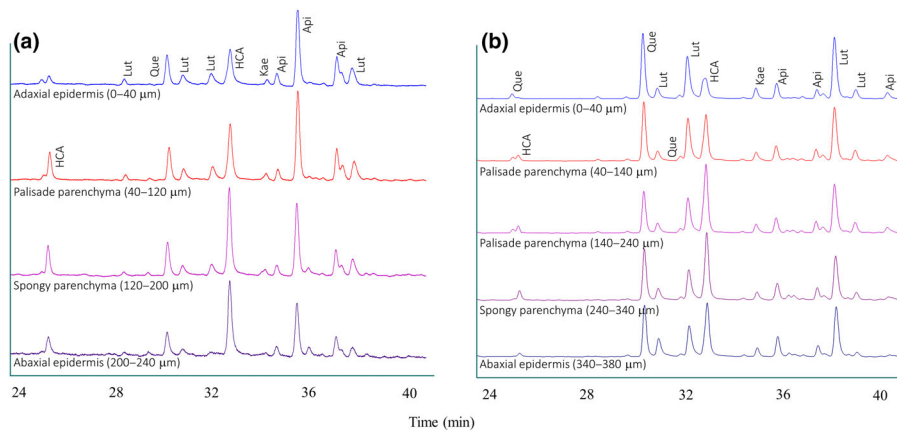


Fig. 3 Representative high performance liquid chromatography (HPLC)-DAD chromatograms of different tissue layers of 3-month-old *Phyllirea latifolia* leaves newly developed in partial shading (25% full sunlight, a) or in full sunlight (b), showing large light-induced changes in phenylpropanoid composition. While hydroxycinnamic acid (HCA) and apigenin (Api) derivatives mostly contribute to the phenylpropanoid pool in shaded leaves, quercetin (Que) and luteolin (Lut) derivatives largely represent the phenylpropanoids synthesized by full-sun exposed leaves. Of note, HCAs accumulate poorly in the mostly light-exposed adaxial epidermis in leaves exposed to the greatest UV-B irradiance (b), despite their greatest ability to absorb solar UV-B wavelengths. Longitudinal sections were cut with a cryo-microtome following the protocols of Ålenius *et al.* (1995) and Tattini *et al.* (2015) from leaves sampled at midday. The quali- and quantitative analysis of phenylpropanoids were performed using the protocol of Tattini *et al.* (2015) and chromatograms (recorded at 350 nm) were normalized based on the fresh weight of different tissue layers.

efficient ROS-scavenging system. We have recently provided evidence that the morning-to-midday increase in flavonoid content observed at the whole-leaf level, almost exclusively involves sub-epidermal tissues and dihydroxy flavonoids (Gori *et al.*, 2021). This is consistent with the common observation that flavonoids with modest ROS-scavenger capacities respond poorly to light stress and to a variety of other abiotic stimuli (Agati *et al.*, 2012; Fig. 3).

While flavonoids have been reported to effectively counter oxidative stress of different origins in a wide range of angiosperms (Agati *et al.*, 2020), there is no conclusive evidence for this role in the bryophyte lineages. Stafford (1991) speculated that a fledgling flavonoid metabolism was unlikely to provide flavonoid concentrations suitable for efficient ROS scavenging. However, it is worth noting that flavonoid concentrations in the low μM range are sufficient to effectively counter the oxidative stress, and the extant bryophyte lineage accumulate appreciable concentrations of flavonoids (high nmol to low $\mu\text{mol g}^{-1}$ DW, Albert *et al.*, 2018; Liu *et al.*, 2022). A recent study has shown that the DELLA TF promotes the exclusive biosynthesis of luteolin 7-*O*-glucuronide in *Marchantia polymorpha* and enhances its tolerance to oxidative stress induced by methyl viologen (which mostly generates superoxide anion and hydroxyl radical, Hernández-García *et al.*, 2021). The increase in luteolin 7-*O* to apigenin 7-*O*-glucuronide in UV-B-treated *M. polymorpha* also poses an antioxidant role of flavonoids in UV photoprotection (Markham *et al.*, 1998, see the next section). This supports the hypothesis of the effective antioxidant role of flavonoids during the evolution of land plant lineages challenged by a wide range of environmental injuries.

Flavonoids in UV photoprotection: a primary antioxidant function?

There is vast, relatively old, literature supporting the idea that an increase in UV, particularly UV-B irradiance, was the primary

driver for the rise of flavonoid metabolism when plants moved from freshwater to colonize land, which is consistent with the notion that UV-B radiation greatly enhances flavonoid biosynthesis (Wellmann, 1976; Robberecht & Caldwell, 1978; Caldwell, 1979). It has been inferred that the accumulation of flavonoids in land plants is to primarily equip these plants with an efficient shield against the penetration of the shortest wavelengths of solar radiation. Nonetheless, a very recent UV-omics investigation indicates that UV radiation likely plays a secondary role compared with water availability during plant terrestrialization (for a review, see Martínez-Abaiagar & Núñez-Olivera, 2022). In other words, while the biosynthesis of protective sunscreens is an ancestral molecular adaptation of land plants (Rensing, 2018), this does not necessarily favor a primary UV-B absorbing function of flavonoids in the photoprotection systems of different land plant lineages, including the bryophytes (Agati & Tattini, 2010). Even though early lineages of land plants did experience an increase in UV-B irradiance when moving from freshwater, it is worth noting that all flavonoids have a relative minimum absorbance at the UV-B portion (280–315 nm), while maximally absorbing at the UV-A region of the solar spectrum (usually in the range 330–365 nm; Fig. 1; Agati *et al.*, 2009, 2013). This leads to the hypothesis that flavonoids are unlikely to fulfill a primary UV-B screening function in land plants of varying complexity (Cockell & Knowland, 1999). It is a prerequisite for a metabolite to serve a primary screening function in the overlap between its absorbance spectrum and the light spectrum responsible for its biosynthesis. The biosynthesis of acyl flavonoids, which absorb effectively over the entire range of solar UV wavelengths (Fischbach *et al.*, 1999) is a derived trait of land plants, and it is limited to a few species (Tohge *et al.*, 2016; Alseikh *et al.*, 2020; Wen *et al.*, 2020). For instance, we have reported unusual mono- and di-coumaroyl derivatives of Kae 3-*O*-glucoside, with outstanding capacity to absorb effectively over the entire solar UV spectrum, in the cell walls of stellate trichomes in leaves of

Cistus salvifolius (a shrub inhabiting most unfavorable areas of Mediterranean basin, Saracini *et al.*, 2005; Tattini *et al.*, 2007).

Furthermore, we observe that hydroxycinnamic acid derivatives (HCA), whose concentrations are comparable to those of flavonoids under low UV-B radiation, are almost unresponsive to increasing UV-B fluence (Burchard *et al.*, 2000; Tattini *et al.*, 2000, 2004; Fig. 3). This finding, which conforms to the general observation of UV-B-induced increase in flavonoids to HCAs ratio (Agati & Tattini, 2010; Fig. 3), offers conclusive support to the idea of a relatively minor role of flavonoids as UV-B absorbers in UV-B photoprotection. HCAs display the greatest absorption capacity over the UV-B portion of the solar spectrum among the phenylpropanoid pool synthesized by most taxa (Agati *et al.*, 2013; Neugart *et al.*, 2014; Fig. 1). We note that HCAs distributed on the cuticle matrix, in both the walls and the vacuole of epidermal cells, may effectively limit the entry of UV-B photons in the leaf, when present in constitutively (i.e. in tissue exposed to low fluence of UV-B irradiance) high concentrations (Schnitzler *et al.*, 1996; Clarke & Robinson, 2008; González Moreno *et al.*, 2022). The presence of cuticular HCAs may represent an ancestral mechanism for efficient energy dissipation (Renault *et al.*, 2017), based on the observation that the level of cuticular HCA (mainly *p*-coumaric and ferulic acids) of most bryophytes largely exceed that of the majority of angiosperms (González Moreno *et al.*, 2022). The functional significance of HCAs, especially those associated with the cuticle and the epidermal cell walls, in UV-B photoprotection, has been often underestimated (Mazza *et al.*, 2000; Kolb *et al.*, 2001; Fabón *et al.*, 2010; Monforte *et al.*, 2018). However, the matter is of interest, especially when examining the photoprotection mechanisms of land plants at a low degree of body complexity (Renault *et al.*, 2017).

Recent evidence of highly conserved mechanisms for sensing and signaling UV-B radiation in the liverwort *M. polymorpha*, the moss *Physcomitrella patens*, and the flowering plant *A. thaliana* is of interest and conforms to the notion that the UVR8-signaling pathway has already originated during the movement of plants from the deeper sea to shallow water (Han *et al.*, 2019). All the species use the UVR8 photoreceptor and the b-ZIP TF HY5, a master regulator of light signaling and photomorphogenesis, to acclimate to changes in UV-B wavelengths (Albert *et al.*, 2018; Soriano *et al.*, 2018; Podolec *et al.*, 2021). Moreover, UV-B radiation similarly changes the flavonoid pool in both bryophytes and angiosperms, since only the biosynthesis of dihydroxy flavones and flavonols is stimulated by UV-B radiation (Markham *et al.*, 1998; Agati & Tattini, 2010; Wolf *et al.*, 2010; Agati *et al.*, 2012; Fig. 3). It has been therefore inferred that flavonoids are more involved in countering the photooxidative stress generated by UV-B radiation, through their ROS-scavenging capacity, than in avoiding photooxidative stress by acting as sunscreens (Ryan *et al.*, 2001; Agati *et al.*, 2012; Emiliani *et al.*, 2013; Dadras *et al.*, 2023b). As a corollary, this offers additional support to early views that high UV-B irradiance is sensed as an oxidative stress (Landry *et al.*, 1995; Jenkins, 2009), just as occurs when plants experience a wide array of abiotic and biotic stressors. Consistently, the very same effective antioxidant flavonoids accumulate to a similar extent in response to high visible or UV-B radiation in

a range of species (Agati *et al.*, 2009, 2011; Siipola *et al.*, 2016; Albert *et al.*, 2018; Taulavuori *et al.*, 2018; Zhang *et al.*, 2018; Falcone Ferreyra *et al.*, 2021). The antioxidant role of flavonoids due to high light intensity may well explain why surface organs such as glandular trichomes, which are autonomous in phenylpropanoid biosynthesis, preferentially accumulate dihydroxy flavonoids at the expense of HCAs in sun-adapted *Phillyrea latifolia* leaves (Tattini *et al.*, 2000; Agati *et al.*, 2002; Fig. 2). It is additionally consistent with the primary ROS-scavenging functions recently attributed to Que 3-*O*-rutinoside in glandular trichomes of tomato (Sugimoto *et al.*, 2022).

We suggest that following the diversification and efficiency of flavonoid metabolism, which led to the sequential production of flavones, flavonols, and anthocyanins (Li *et al.*, 2020) coupled with a versatile transport system, plants had a vast arsenal of metabolites available, capable of limiting the generation (avoidance through light-screening) and allowing the scavenging of ROS once they are formed. This enabled plants to reverse efficiently photooxidative stress of increasing severity, allowing their successful adaptation in more challenging habitats (Pollastri & Tattini, 2011; Dos Santos Nascimento & Tattini, 2022).

Flavonoids as signaling molecules: a robust 'antioxidant-dependent' function

The notion that flavonoids act as signaling metabolites has been widely reported in animal cells, and this ability is primarily responsible for the health benefits usually attributed to flavonoids (Williams *et al.*, 2004). The capacity of flavonoids to modulate the activity of a range of proteins that may act as downstream components in diverse signaling pathways (mostly of oxidative nature) has been explored to a lesser extent in plants, especially in aboveground organs (Taylor & Grotewold, 2005; Peer & Murphy, 2006; Brunetti *et al.*, 2018, 2019; Daryanavard *et al.*, 2023). Nonetheless, Helen Stafford proposed, three decades ago, that flavonoids had key functions as internal physiological regulators and chemical messengers, rather than acting as UV-screening pigments during the colonization of land by plants (Stafford, 1991). She speculated indeed that: (1) a still-evolving flavonoid metabolism combined with an undeveloped transport system is unlikely to furnish the vacuolar compartment with flavonoid concentrations sufficient to allow optimal UV-B screening in early land plants; and (2) a primary UV-screening role does not fit with the extraordinary degree of glycosylation of the flavonoid backbone observed in most plant species. On the contrary, low amounts of flavonoids, which Stafford hypothesized as having been likely synthesized by the first land plants, should have been sufficient to modulate auxin signaling, by acting on both its transport and degradation. Flavonoids had been identified as endogenous regulators of phytochrome-induced asymmetrical auxin (IAA) distribution, through their ability to modulate the activity of IAA oxidase, in early, seminal experiments conducted at Galston's Lab at Yale University and by Stafford at Reed College in Portland (Furuya *et al.*, 1962; Furuya & Thomas, 1964; Bottomley *et al.*, 1965; Stafford, 1965). Notably, both low red light and white light supplementation promoted

asymmetrical IAA distribution, without affecting Kae glycosides biosynthesis, while strongly inducing Que derivatives biosynthesis in *Pisum sativum* (Bottomley *et al.*, 1965). In the same species, Kae derivatives were observed to act as cofactors of IAA oxidase, while Que derivatives successfully hindered the enzyme activity (Furuya *et al.*, 1962; Galston, 1969). At the time of Stafford's hypothesis, there was additional evidence of antioxidant flavonoids being also most effective in modulating IAA efflux, based on their ability to inhibit the binding of the synthetic auxin transport inhibitor *N*-1-naphthylphthalamic acid (NPA) to a plasma membrane protein (Jacobs & Rubery, 1988). As flavonoids modulate IAA movement and local auxin concentrations at extremely low concentration ranges (from nM to low μ M), Stafford speculated this was the ancestral role of flavonoids during plant terrestrialization (Stafford, 1991). Furthermore, she argued that flavonoids might serve these functions in the cytoplasm, near the site of their biosynthesis, that is, the cytoplasmic face of the ER. This argumentation received support later when ancestral IAA auxin efflux PIN proteins, such as the short-chain PIN5 and PIN8, were discovered to be localized at the ER (Mravec *et al.*, 2009; Viaene *et al.*, 2014; Ung *et al.*, 2022). Incidentally, ER is also the site of IAA biosynthesis (Kriechbaumer *et al.*, 2017; Brunetti *et al.*, 2018).

There is evidence of plasma membrane-associated PIN trafficking and polarization mechanisms in *M. polymorpha* and *P. patens* (Skokan *et al.*, 2019; Tang *et al.*, 2024), and auxin has been reported to influence cell growth and differentiation in both bryophytes (Flores-Sandoval *et al.*, 2024). These findings support Stafford's opinion of an ancestral role of flavonoids as modulators of intra- and intercellular IAA movement. We have also hypothesized that flavonoids served a major function as chemical messengers during plant terrestrialization (Brunetti *et al.*, 2018), but this matter is far from being fully elucidated, as we discuss below.

The role of flavonoids as chemical messengers has been widely reported for the growth of belowground organs in angiosperms (Hassan & Mathesius, 2012; Ng *et al.*, 2020; Ghitti *et al.*, 2022), such as in the arbuscular mycorrhizal (AM) association. The effects of flavonoids on AM result from their ability to modulate both local IAA gradients and the level of downstream components of the auxin signaling pathway, as occurs during nodulation (Zhang *et al.*, 2009; Abdel-Lateif *et al.*, 2013). The finding that flavonoid aglycones, which are usually exuded by roots, are more effective in promoting AM compared with corresponding glycosylated forms (Zhang *et al.*, 2009; Tian *et al.*, 2021; Kumar *et al.*, 2024), adds further support to the idea that the multifunctionality of flavonoids relates with their antioxidant character. AM association was an event of crucial significance for the adaptability of rootless bryophytes in water- and nutrient-depleted terrestrial habitats (for recent reviews, see Dos Santos Nascimento & Tattini, 2022; Gille *et al.*, 2024; Martin & van der Heijden, 2024). Although the putative role of flavonoids in AM association in bryophytes is an attractive suggestion, the strong relationship between flavonoids and auxin observed in angiosperms needs conclusive support in bryophytes. Nonetheless, flavonoids have been recently reported to block auxin transport and inhibit auxin response, thus contributing to 2D-3D transition in *P. patens* (Moody *et al.*, 2021). There is also evidence that SHORT-LEAF, a member of the Tandem direct repeat-

containing (TDR) proteins regulates gametophore development in *P. patens* by mediating the auxin distribution pattern through its strong influence on flavonoid biosynthesis (Palit *et al.*, 2024). These findings are remarkable and open the possibility of a putative role of flavonoids as modulators of auxin response and signaling in bryophytes.

The physicochemical features, especially the presence of the catechol group in the B-ring, confer flavonoids (and other polyphenols) the potential to scavenge ROS and interact with a range of macromolecules as well (Pollastri & Tattini, 2011). For instance, flavonoids may inhibit the activities of a wide array of proteins, including protein kinases by strongly competing with their ATP-binding sites (structural similarity), as well as acting at the ATP noncompetitive binding site through the formation of both hydrogen bonds and van der Waals interactions (Barron *et al.*, 2002; Bode & Dong, 2013). There is compelling evidence that the 3'-OH group as seen in dihydroxy flavones and flavonols is pivotal for hydrogen bonds with protein kinase backbone amide groups (for a review, see Hou & Kumamoto, 2010). This conforms to the observation that Que and luteolin are more active than Kae and apigenin, respectively, in inhibiting the activities of a range of tyrosine kinases (Chin *et al.*, 2013; Alizadeh & Ebrahimzadeh, 2022). There is consensus that these features are significantly more important than the conventional hydrogen-donating capacity (antioxidant role *sensu stricto*) to explain the effects of flavonoids in the modulation of human cell growth and metabolism (Hou & Kumamoto, 2010; Gu *et al.*, 2019). Flavonoids can regulate and modulate the activities of a wide range of proteins in plant cells, including but not limited to protein kinases. For instance, flavonoids inhibit the activity of PIDs, which are serine/threonine kinases that phosphorylate the PIN, IAA efflux carriers (Henrichs *et al.*, 2012; Adamowski & Friml, 2015), thus determining their asymmetrical distribution at the plasma membrane, and hence the intercellular IAA fluxes, the well-known polar IAA transport (PAT). However, flavonoids may also modulate the activities of several ATP-binding cassette B subfamily (ABCB)-type IAA transporters (multidrug resistance (MDR) P-glycoproteins, Blakeslee *et al.*, 2005) through bifunctional interactions at both the vicinal ATP-binding site and the steroid-interacting region within the protein cytosolic domain (Conseil *et al.*, 1998; Ferreira *et al.*, 2015). In turn, flavonoids could synergistically inhibit both PIN- and ABCB-based major IAA streams (Mellor *et al.*, 2022), through direct association with PINs (Teale *et al.*, 2020; Kurepa *et al.*, 2023). Indeed, the synthetic inhibitor of IAA transport NPA was shown to lead to conformational perturbation in PIN and hence to decreases in PIN activity (Abas *et al.*, 2021). It is not surprising that the antioxidant dihydroxy flavonoids, particularly the flavonol Que, display the greatest inhibitory effect on the activities of PIN and MDR P-glycoproteins proteins (Mohana *et al.*, 2016), and hence in determining IAA gradients in different tissues and cells (Peer & Murphy, 2006, 2007; Michniewicz *et al.*, 2007; Bailly *et al.*, 2008; Adamowski & Friml, 2015). This may well explain the term 'developmental regulators', coined for flavonols by Taylor & Grotewold (2005), a robust function of these molecules in both plants and animals.

We observe that flavonoids may influence IAA gradients in shoots and roots not only by modifying hormone transport at the organ, tissue, cellular, and subcellular levels, but also by influencing IAA catabolism. Early research established that some flavonoids block IAA oxidase (Furuya *et al.*, 1962; Bottomley *et al.*, 1966), a peroxidase for which flavonoids display strong affinity, as is also the case for vacuolar peroxidases that use flavonoids as preferential substrates to detoxify hydrogen peroxide (H_2O_2 , Yamasaki *et al.*, 1997). This has strong similarities with the mechanisms through which flavonoids inhibit IAA oxidase activity, that is, by serving as preferential substrates compared to IAA for IAA oxidase, and by scavenging H_2O_2 generated during early steps of auxin oxidation (Galston *et al.*, 1950; Mathesius, 2001). It is not surprising, therefore, that Que and its derivatives are much more potent inhibitors of IAA oxidase than the corresponding Kaederived compounds, these last behaving indeed as cofactors of IAA oxidase at certain concentrations (Furuya *et al.*, 1962; Bottomley *et al.*, 1966). The largely different action of Que and Kae derivatives on IAA oxidase activity may be in part explained by the capacity of Que, but not of Kae derivatives, to chelate Mn (II), a well-known cofactor of IAA oxidase (Morgan *et al.*, 1966). The ability of dihydroxy flavonoids to chelate transition metal ions (De Souza & De Giovanni, 2004) has also been used to explain their ability to prevent irreversible oxidative damage in plant nuclei. Dihydroxy flavonoids may efficiently chelate Fe(II)-ions involved in the Fenton reaction ($\text{Fe(II)} + \text{H}_2\text{O}_2 \rightarrow \text{Fe(III)} + \text{OH}^*$), thus limiting the formation of hydroxyl radical (OH^*) (Agati *et al.*, 2012). Recent findings suggest that the major route through which IAA is oxidized in early and modern land plants is by the action of DIOXYGENASE for AUXIN OXIDATION1 protein (DAO1, Zhang *et al.*, 2016), a member of the 2-oxoglutarate and Fe(II)-dependent (2OG Fe(II)) oxygenase superfamily. Interestingly, an *Arabidopsis* mutant overaccumulating the antioxidant flavonol Que displayed the lowest level of ox-IAA (Peer *et al.*, 2013), likely due to the effective inhibition of DAO activity and scavenging of ROS (Zhang & Peer, 2017). The strong inhibitory effect of antioxidant flavonoids on the activity of proteins regulating IAA-oxidation is suggested as being of greater significance than their modulation of inter- and intra-cellular auxin movement in determining auxin gradients at cellular and subcellular levels and, hence, in regulating plant growth (Zhang & Peer, 2017).

Overall, this evidence implies that flavonoids play a critical role in modulating the auxin-signaling network beyond influencing the distribution of IAA at both inter- and intra-cellular levels. Furthermore, relatively recent findings support the notion that flavonoids act as components of a regulatory circuit of the auxin-signaling pathway. Grunewald *et al.* (2012) have shown that IAA enhances the synthesis of Que derivatives, by acting on the WRKY23 TF and, in turn, Que may fine-tune IAA distribution, in a PIN-independent manner. The auxin–flavonol relationship is strong (Blilou *et al.*, 2005; Lewis *et al.*, 2011) and very recent findings provide conclusive evidence that the IAA repressor IAA17.1, a repressor of early IAA response genes, together with the heat shock protein HSF5a, promote flavonol biosynthesis and decrease ROS accumulation in salt-treated roots of *Populus tomentosa* (Song *et al.*, 2024).

There is also recent evidence of a robust relationship between flavonols and the abscisic acid (ABA)-signaling pathway (Gao *et al.*, 2021; Segarra-Medina *et al.*, 2023), which may have contributed greatly to the adaptation of plants to the harsh terrestrial habitat (Brunetti *et al.*, 2019). The high integration of ABA- and light signaling, which occurs at the level of primary signaling components, such as the bZIP TFs ABA Insensitive 5 (ABI5) and HY5 (Chen *et al.*, 2008), may well explain the ABA-induced activation of flavonol biosynthesis, especially of quercetin, in a vast range of species (Berli *et al.*, 2010; Alonso *et al.*, 2016; Song *et al.*, 2022; Castro-Cecri *et al.*, 2023). It is noted that the crosstalk between ABA and light signaling is an ancient and robust trait of terrestrial plants as the structure and function of HY5 and ABI5 are conserved among early and current-day land plants (Komatsu *et al.*, 2013; Gangappa & Botto, 2016). Flavonols, in turn, regulate the ABA signaling, acting at the level of downstream network components, such as H_2O_2 and MAPKs (Brunetti *et al.*, 2019). Studies conducted at Gloria Muday's Lab have conclusively shown that flavonols, accumulated (and likely synthesized) in the cytoplasm and nucleus of stomata guard cells, antagonize the closure of stomata by greatly decreasing the levels of H_2O_2 , a well-known downstream messenger of the ABA signaling network (Watkins *et al.*, 2014, 2017). However, it cannot be excluded that flavonols additionally inhibit the activity of MAPKs that operate downstream of H_2O_2 to induce stomata closure (Jammes *et al.*, 2009; De Zelicourt *et al.*, 2016; Brunetti *et al.*, 2019).

Conclusions: not all flavonoids are equally multifunctional

The functional significance of the diversity and complexity of specialized metabolism has been focused mostly on plant–herbivore interactions and based upon the notions that: (1) most SMs synthesized within specific pathways have low biological activity; and (2) the deployment of a mixture of SMs provides functional synergisms and evolutionary stability (Firn & Jones, 2000; Steppuhun & Baldwin, 2008; Heiling *et al.*, 2022; Blanchard & Holeski, 2024).

Consequently, the extraordinary chemical diversity within the flavonoid class, caused by the vast range of glycosylation and substitution patterns of the C6-C3-C6 skeleton, complicates a deterministic estimation of their multifunctionality. As previously stated, flavonoids differ significantly in antioxidant capacity, especially when considering the forms found in plant cells. Monohydroxy flavonoid derivatives, for example glycosides of apigenin and Kae, are poor antioxidants (Fig. 1), and their putative effects in an *in planta* condition have been erroneously inferred from studies conducted *in vitro* or *ex-vivo* using flavonoid aglycones in too many instances (Williamson, 2002). While studies involving flavonoid aglycones may reveal the functions of distinct flavonoid classes in belowground processes (e.g. lateral root emergence; symbiotic nodulation and/or mycorrhizal association Zhang *et al.*, 2009; Chapman & Muday, 2021), this is not the case for aboveground organs, which often accumulate flavonoid glycosides in their tissues. Once again, we emphasize that Que 3-*O*-glucoside has a lower antioxidant capacity than Que, but has a higher ROS-

scavenging ability than Kae. The antioxidant capacity of Kae 3-*O*-glucoside is indeed negligible in a concentration range consistent with its solubility in the aqueous cellular milieu (Fig. 1).

Accordingly, monohydroxy flavones and flavonols have significantly lesser multifunctional potential than their dihydroxy counterparts. It may not be a mere coincidence that in plants exposed to a variety of environmental stresses, including the increase in UV-B or visible light irradiance, the biosynthesis of antioxidant flavonoids is activated, while the monohydroxy flavonoid pool remains unchanged (for extensive reviews see Agati & Tattini, 2010; Agati *et al.*, 2012, 2020; Fig. 3). Data here reported support flavonoids' key activities in both preventing irreversible stress-induced oxidative damage and modulating different oxidative stress-induced signaling pathways. Flavonoids tune both ROS levels and the activity of downstream components of oxidative signaling pathways, such as a wide range of protein kinases, in plants and animals. The antioxidant function of flavonoids is, therefore, robust and strongly tied to the plant's ability to evolve (i.e. evolvability, *sensu* Lesne, 2008; Wagner, 2011) in an ever-changing terrestrial habitat.

In fact, antioxidant flavonoids play a role in stress-induced morphogenic responses (SIMR), a typical feature of plants exposed to a wide range of stresses (Jansen, 2002; Potters *et al.*, 2007), which are, indeed, strongly dependent on ROS (and IAA) signals (Gayomba & Muday, 2020; Martin *et al.*, 2022). Flavonoids regulate the auxin-signaling pathway by severely reducing the activity of proteins that regulate IAA-oxidation while determining IAA gradients by acting on proteins that escort IAA at intra- and intercellular levels. Consistently, flavonoids have been recognized as modulating plant development (reviewed recently in Daryanavard *et al.*, 2023), particularly root growth and architecture (Mathesius, 2018; Gayomba & Muday, 2020). Studies examining the involvement of flavonoids in the development of aboveground organs, such as shoot architecture, have yielded conflicting results (Beveridge *et al.*, 2007; Buer & Djordjevic, 2009; Buer *et al.*, 2013; Fraser *et al.*, 2017). This is because most research has been conducted under growth conditions different enough from those often experienced by plants concomitantly facing multiple stressors in their natural solar irradiation when SIMR truly makes sense (Robson *et al.*, 2015). For example, high levels of sunlight and UV-B stimulate or inhibit IAA biosynthesis and signaling, respectively (Hersch *et al.*, 2012; Hayes *et al.*, 2014; Huq, 2018), whereas both light regimes stimulate the biosynthesis of antioxidant flavonoids (Agati *et al.*, 2020). In *Arabidopsis*, a high light-induced increase in IAA biosynthesis also triggers the biosynthesis of flavonols, particularly of Que (Lewis *et al.*, 2011; Grunewald *et al.*, 2012). In turn, Que may attenuate local auxin signaling, thus inhibiting apical dominance, as typically occurs in UV-B-treated plants under natural conditions (Hayes *et al.*, 2014; Robson *et al.*, 2015). The mutual regulation of auxin biosynthesis/signaling and flavonoids usually observed in angiosperms is still lacking to be properly described in bryophytes, but very recent studies open new perspectives on this intriguing matter (Moody *et al.*, 2021; Palit *et al.*, 2024).

The functional significance of the regulatory roles of flavonols on the ABA signaling network has not yet received enough attention,

despite the fact they have the potential to significantly regulate the gas exchange performance of plants facing multiple environmental pressures associated with rapid climate change, such as a combination of transient heat waves and rainfall scarcity in high light-stressed habitats. However, the matter is of primary significance for the ecology of plants with highly diverse complexity.

Overall, we have shown that while flavonoids with varying physicochemical properties have similar abilities to absorb UV radiation and repel herbivores, they differ greatly in their ability to scavenge ROS and hence to modulate both hormone and oxidative signaling pathways. We have provided conclusive evidence that these antioxidant-related properties, coupled with the distribution in different tissues and cellular compartments, confer only to antioxidant flavonoids the ability to efficiently serve several functions in plants undergoing changes in cellular homeostasis because of a variety of external stimuli. The observation that the biosynthesis of antioxidant flavonoids is a common response of different land plants lineages when confronted with a range of environmental pressures is remarkable, implying that this might represent an ancient feature of land plants.

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Competing interests

None declared.

Author contributions

LBSN and MT conceived the structure and wrote the MS. CB and AG performed HPLC analysis of phenylpropanoids and estimated the scavenger ability of individual flavonoids for DPPH radical and superoxide anion. GA and ELP determined the UV-absorbing capacities of individual phenylpropanoids and performed CLSM analyses. All the authors revised and edited the MS.

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