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Review

Flavonoids as antioxidants in plants: Location and functional significance

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ABSTRACT

Stress-responsive dihydroxy B-ring-substituted flavonoids have great potential to inhibit the generation of reactive oxygen species (ROS) and reduce the levels of ROS once they are formed, i.e., to perform antioxidant functions. These flavonoids are located within or in the proximity of centers of ROS generation in severely stressed plants. Efficient mechanisms have been recently identified for the transport of flavonoids from the endoplasmic reticulum, the site of their biosynthesis, to different cellular compartments. The mechanism underlying flavonoid-mediated ROS reduction in plants is still unclear. 'Antioxidant' flavonoids are found in the chloroplast, which suggests a role as scavengers of singlet oxygen and stabilizers of the chloroplast outer envelope membrane. Dihydroxy B-ring substituted flavonoids are present in the nucleus of mesophyll cells and may inhibit ROS-generation making complexes with Fe and Cu ions. The genes that govern the biosynthesis of antioxidant flavonoids are present in liverworts and mosses and are mostly up-regulated as a consequence of severe stress. This suggests that the antioxidant flavonoid metabolism is a robust trait of terrestrial plants. Vacuolar dihydroxy B-ring flavonoids have been reported to serve as co-substrates for vacuolar peroxidases to reduce H_2O_2 escape from the chloroplast, following the depletion of ascorbate peroxidase activity. Antioxidant flavonoids may effectively control key steps of cell growth and differentiation, thus acting regulating the development of the whole plant and individual organs.

Contents

1. The biosynthesis of antioxidant flavonoids is enhanced by severe stress conditions

Flavonoids are a large class of secondary metabolites encompassing more than 10,000 structures. Several lines of evidence corroborate the hypothesis that they have antioxidant functions in higher plants that are challenged with a range of environmental stresses [1–3]. First, enzymes devoted to polyphenol oxidation, i.e., polyphenol-oxidases and peroxidases, occur ubiquitously in higher

Abbreviations: CHS, chalcone synthase; CHI, chalcone isomerase; F3H, flavanone 3-hydroxylase; FLS, flavonol synthase; F3′H, flavonoid 3′-hydroxylase; UDP-Glu, uridin diphosphate-glucose.

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plants [4]. Second, plants undergoing severe stress conditions preferentially accumulate dihydroxy B-ring-substituted flavonoids, which are effective scavengers of reactive oxygen species (ROS) [5–11]. This evidence is consistent with ROS generation as a common trait among plants that are exposed to vastly different stressors [12,13]. Third, the biosynthesis of 'antioxidant' flavonoids increases more in stress-sensitive species than in stress-tolerant species; stress-sensitive species display a less effective first line of defense against ROS under stressful conditions and are subsequently exposed to a more severe 'oxidative stress' [8,14–16]. It has been hypothesized that changes in the cellular redox homeostasis activate the biosynthesis of flavonoids, particularly flavonol metabolism [17] because MYB (myeloblastosis) transcription factors that regulate the biosynthesis of flavonols, and subsequently anthocyanins, are also regulated by changes in the cellular redox potential [18–20].

Severe stress conditions might inactivate antioxidant enzymes, while up regulating the biosynthesis of flavonols [21,22]. Conversely, the increase in the antioxidant enzyme activity upon UV-B radiation is negatively correlated with 'constitutive' flavonol production [5,23]. The biosynthesis of effective antioxidant flavonoids is enhanced when plants growing under strong light are concomitantly faced with other stress agents [9,11,15,21]. These environmental conditions expose plants to an excess of light or an excess of excitation energy. Excess light is stressful to plants on a daily basis and could reduce the activity of chloroplast antioxidants while up-regulating the biosynthesis of flavonoids, even in the absence of UV irradiance [13,21,24–26]. Thus, the activity of flavonoids may constitute a 'secondary' antioxidant system that is activated as a consequence of the depletion of antioxidant enzyme activity [3,22,27].

Flavonols play a more important role than xanthophylls in protecting *Arabidopsis* leaves from long-term visible light-induced oxidative damage [28]. This photo-protective role of flavonoids cannot be attributed to the visible-light screening functions of quercetin-3-O-glucoside 7-O-rhamnoside and kaempferol 3-O-glucoside 7-O-rhamnoside, as these compounds have negligible absorbance beyond 420 nm [10]. Instead, we speculate that quercetin derivatives may protect chloroplasts from the visible light-induced generation of singlet oxygen ($^1O_2$), as previously reported for the dihydroxy B-ring-substituted flavonoids located in the chloroplast envelope of *Phillyrea latifolia* leaves [29]. This hypothesis is consistent with the preferential increase of quercetin derivatives, a dihydroxy B-ring-substituted flavonol, with respect to kaempferol, a monohydroxy B-ring flavonol upon white light irradiance that was reported more than four decades ago [30]. Indeed, glycosylated flavonoids are commonly detected in healthy leaf cells, and the ability to donate electrons or hydrogen atoms, i.e., to perform a reducing activity [31,32], is primarily conferred through ortho-dihydroxy B-ring substitution (e.g., luteolin and quercetin derivatives, Fig. 1).

The ratios of the 'effective antioxidant' quercetin and luteolin glycosides to the 'poor antioxidant' kaempferol and apigenin glycosides significantly increase upon high light irradiance, irrespective of the relative proportions of different solar wavelengths reaching the leaf surface [2,3,6,7,10,11,30,33,34]. The biosynthesis of kaempferol and quercetin glycosides increases under low and under high light irradiance in response to nitrogen depletion in *Arabidopsis* [9]. Quercetin 3-O- and luteolin 7-O-glycosides accumulate similarly in response to UV-B irradiance or root zone salinity in *Ligustrum vulgare* [11]. These findings should be interpreted in terms of the relative abilities of different flavonoids to reduce ROS, and not in terms of their abilities to prevent ROS generation through the absorption of highly energetic solar wavelengths, as mono- and dihydroxy B-ring flavonoids have similar UV-spectral features (Fig. 1) [7].

Quercetin derivatives are more effective than monohydroxy B-ring flavonoids in performing multiple functions in plants, which include the capacity to complex with Cu and Fe ions, thus inhibiting the generation of ROS by the Fenton reaction [35] as well as reducing ROS once formed (Fig. 1). In addition, the catechol group confers a much greater ability to quercetin than kaempferol derivatives to modulate the activity of several proteins that supersede cell growth and differentiation [36,37]. This may exert a fine control on plant architecture which is a key feature for acclimation/adaptation of most species to excess sunlight irradiance [38,39]. As a consequence, quercetin derivatives fully accomplish "nature's tendency to catch as many flies with one clap as possible" [40] and equip plants with versatile compounds capable of countering unpredictable environmental injuries. It is speculated that the primary functions of quercetin derivatives and other dihydroxy B-ring-substituted flavonoids in plants faced with severe excess light depend more on chemical features conferring particular antioxidant and 'antioxidant-related' (see below Section 5) and not UV-screening capacities [2,3,41–43].

However, there is still uncertainty concerning the functions of 'antioxidant' flavonoids *in vivo* [4,32]. We examine this conflicting issue based upon (i) an in-depth analysis of the sub-cellular flavonoid distribution, (ii) the functional robustness of flavonol, particularly quercetin metabolism [4], and (iii) the potential significance of vacuolar flavonoids as H_2O_2-reducing agents in high light-stressed plants, with the aim of exploring the significance of flavonoids as antioxidants in plants.

2. Flavonoid distribution is suitable for antioxidant functions

The multiplicity of the functional roles of flavonoids in plant-environment interactions is consistent with their presence in a wide array of cells and sub-cellular compartments (Figs. 2 and 3). The massive accumulation of flavonoids in external appendices, such as trichomes, is consistent with UV-screening functions [44]. Surface flavonoids are effective UV-B absorbers (as well as anti herbivory agents) [27] but the substitution of hydroxycinnamic acid derivatives with effective antioxidant flavonoids in glandular/secretory trichomes and adaxial epidermal cells in response to UV-B irradiance [7,8,45–47] is difficult to explain in terms of the relative UV-B screening capacities (Fig. 1). Hydroxycinnamic acid derivatives have an ε_{max} (ε, molar extinction coefficient) in the 290–320 nm spectral region, and hence, are much more effective than dihydroxy B-ring-substituted flavonoids, which have an ε_{max} beyond 350 nm, in absorbing the shortest solar wavelengths [8,33,47]. It has been hypothesized that flavonoids do not serve primary UV-B screening functions in UV-B-irradiated cells [2,3,5,43]. Hydroxycinnamates have been found present in the secretory products of the glandular trichomes in shade-adapted *P. latifolia* leaves, but absent in leaves exposed to full sunlight [46,47]. These flavonoids are present in the vacuoles of the glandular trichome cells (Fig. 2), which raises the question of how flavonoids perform reducing activities if confined to compartments that are physically separated from the centers of ROS generation [4,32]. Conversely, the effective ability of vacuolar flavonoids in constituting effective UV-shields has been early questioned, as UV-radiation might freely pass through the anticlinal cell-walls [42,48].

The UV-B screening functions of flavonoids during the plant colonization of land have likely originated from other, ancestral, primary roles. Genes for the biosynthesis of dihydroxy B-ring-substituted flavonoids have been detected in liverworts and mosses [41]. The concentrations of these flavonoids in early terrestrial plants (μM range) [41,42] unlikely constitute an effective shield against the shortest solar wavelengths, for which flavonoid

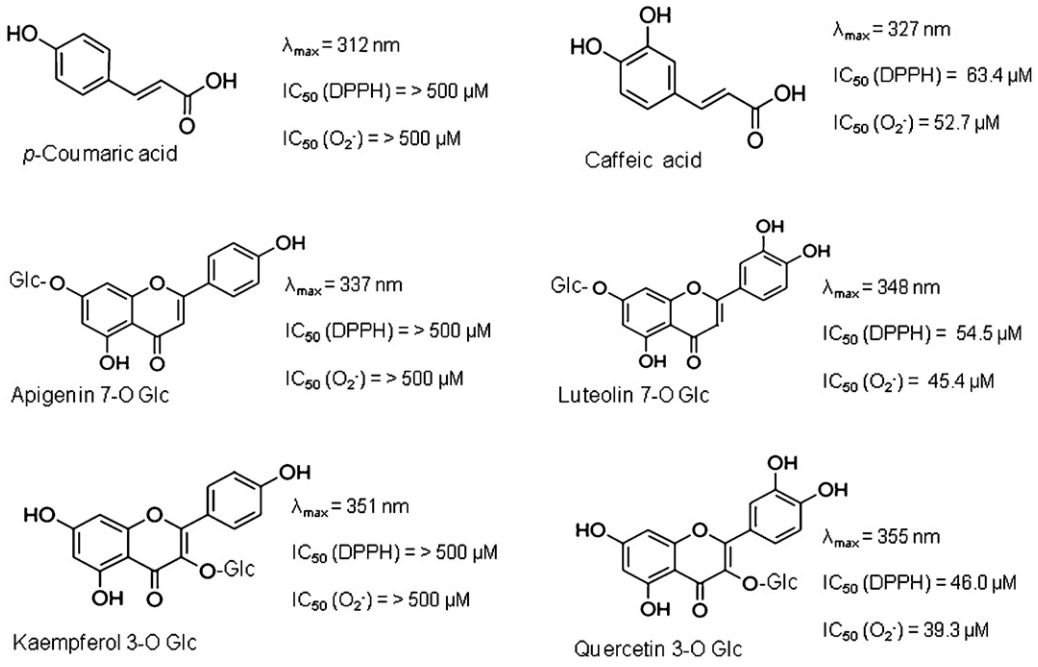

Fig. 1. Chemical structures of mono- and di-hydroxy cinnamic acid and flavonoid derivatives detected in leaves of higher plants. Maximum absorbance wavelength (λ_{max}), scavenger activities (IC_{50}) against the synthetic free radical 2,2-diphenyl-1-picrylhydrazyl radical (DPPH) and the superoxide anion (O_2^-) have been reported for each compound. Absorbance spectra were recorded in phosphate buffer at a metabolite concentration of 50 μM. IC_{50} denotes the metabolite concentration required to reduce by 50% the concentration of free radicals, following the protocols in [7] and [11].

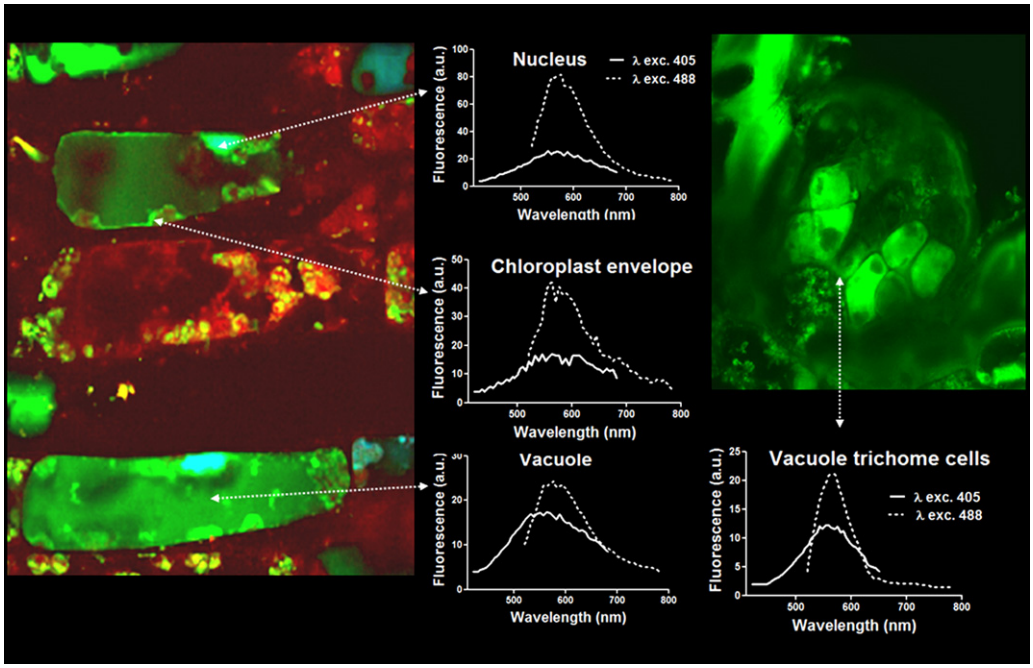

Fig. 2. Fluorescence microimaging of *P. latifolia* leaves showing the sub-cellular distribution of dihydroxy-substituted phenylpropanoids. Cross section, 100-μm-thick, were stained with Naturstoff Reagent [0.1%, w/v, 2-amino ethyl diphenyl boric acid in phosphate buffer, pH 6.8, with addition of 1% NaCl, w/v] and fluorescence recorded with Leica TCS SP5 confocal microscope (Leica Microsystems CMS, Wetzlar, Germany) equipped with an acusto-optical beam splitter (AOBS) and an upright microscope stand (DMI6000), following previous protocols [13,15]. Views refer to the second layer of palisade parenchyma (at 100-μm depth from the adaxial epidermis) and the glandular trichome cells. The nucleus, the chloroplast envelope and the vacuole of glandular trichome cells are compartments of exclusive accumulation of dihydroxy B-ring-substituted flavonoid glycosides. Indeed, the peak of maximal emission, at approx. 575 nm, did not differ depending on the excitation wavelength. By contrast, in the vacuole of palisade cells – which emits at 545 or 575 under 405 or 488 nm excitation, respectively – both caffeic acid derivatives (λ_{em} = 525 nm) and dihydroxy B-ring-substituted flavonoid glycosides are present. Please, note that the light-blue color associated with the nucleus originates from the dark-blue fluorescence of 4′-6-Diamidino-2-phenylindole (DAPI, used for nucleus staining) and the green-fluorescence attributed to flavonoids.

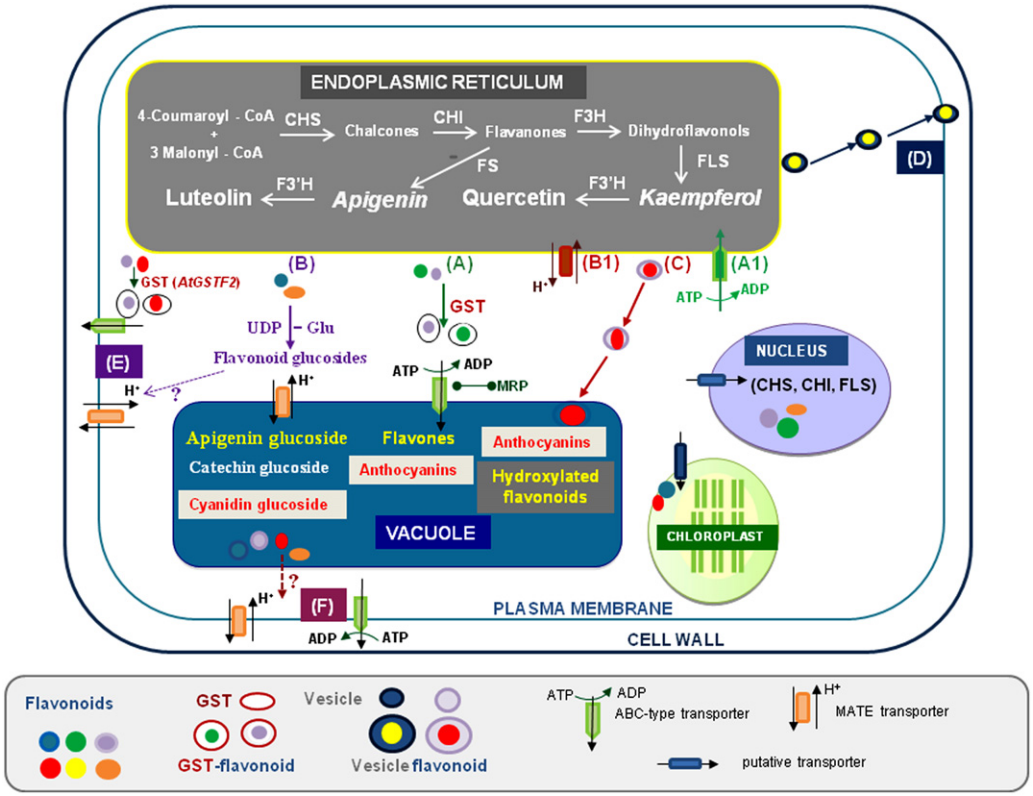

Fig. 3. Transport mechanisms of endoplasmic reticulum (ER)-derived flavonoids to the vacuole and the cell wall, redrawn from [67–71]. Flavonoids may cross the tonoplast membrane through ATP binding cassette (ABC)-type and multidrug and toxic ions extrusion (MATE) transporters. Flavonoids conjugated to Glutathione S-transferase (GST) accumulated in the vacuole by ABC-type transporters (A), mostly through multidrug resistance associated proteins (MRP). Flavonoid glucosides may cross the tonoplast membrane using H^+-energized mechanism (B) through the action of secondary transporter like proteins (MATE). Vesicle mediated transport of flavonoids, particularly of anthocyanins, has been widely observed (C). ABC-type (A1) and MATE (B1) might be involved in the uptake of flavonoids by ER (i.e., escort of flavonoids from the ER cytoplasmic face into the ER lumen) and participate in the vesicle mediated accumulation of vacuolar flavonoids. Flavonoids are extruded from the cell and accumulated into the cell wall through both vesicle mediated transport (D) and membrane transporter mediated transport mechanisms (e.g., using GST-flavonoid complexes as in E). Release of flavonoids from the vacuole has been also reported and flavonoids might cross the plasma membrane using both ABC- and MATE-type proteins (F). Whether flavonoids are transported to or synthesized in the nucleus and the chloroplast is unknown.

concentrations in the mM range are required [2,43]. The UV-B screening functions of flavonoids during the colonization of land by plants are supposed to have followed the evolution of other branch pathways of phenylpropanoid metabolism [43]. *Cistus salvifolius* leaves contain a wide range of kaempferol, quercetin and myricetin derivatives, but only two coumaroyl derivatives of kaempferol 3-O-glucoside (astragalin) have been detected in non-secretory stellate and dendritic trichomes [49]. These acyl kaempferol glycosides were associated with the cell wall of trichome arms, which is an optimal location for effective UV-screeners [48,49]. Instead, epidermal vacuolar dihydroxy B-ring-flavonoids have long been suggested to serve antioxidant functions, as the epidermal cells themselves must be protected, not only aimed preserve the underlying sensitive organs from photo-oxidative damage [42,50]. However, the intercellular movement of ROS, i.e., H_2O_2, in high light stressed leaves has not conclusively been proven.

There is a large body of evidence showing that dihydroxy B-ring substituted flavonoids occur in the vacuole of mesophyll cells (Fig. 2) [7,8,10,46,47,51,52]. It is speculated that tonoplast-located aquaporins transport H_2O_2 to vacuoles containing flavonol glycosides [53] for being then reduced by guaiacol peroxidases (see Section 4.2 for details). Moreover, there is compelling evidence of H_2O_2 scavenging by vacuolar anthocyanins upon mechanical injury *in vivo* [54].

Chloroplasts have long been reported to contain flavonoids and appear to be capable of flavonoid biosynthesis [3,32]. These findings are consistent with the recent evidence obtained from multispectral fluorescence micro-imaging, both wide-field (three-dimensional deconvolution microscopy) [29] and confocal laser scanning microscopy (Fig. 2). Dihydroxy-B-ring substituted flavonoids have been localized to chloroplasts in *P. latifolia* leaves [29]. These antioxidant flavonoids are 'associated' with the chloroplast envelope (Fig. 2) and effectively scavenge singlet oxygen ($^1O_2$) generated from exposure to excess blue light [29]. The chloroplast contains an extraordinary arsenal of defense agents against ROS, particularly $^1O_2$ [55], and the flavonoids might complement the action of other $^1O_2$ scavengers, such as carotenoids, under severe excess light conditions. Singlet oxygen, although highly reactive, and hence, short-lived, has been detected outside the chloroplasts under high light conditions [56]. Chloroplast envelope-located flavonoids may limit the exit of $^1O_2$ from the chloroplast and the $^1O_2$-retrograde signaling to the nucleus which may lead to programmed cell death [55,57].

Flavonoids within the chloroplast also have the potential of preserving the integrity of the envelope membrane through lipid remodeling during cellular dehydration, and hence prevent oxidative damage [58,59]. Cold tolerance in *Arabidopsis* [60,61] has been attributed to the capacity of flavonoids to physically and chemically interact with biological membranes, which greatly depends on the hydrophilic capacity and the number of hydroxyl groups in a molecule. This is consistent with the effective capacity of flavonols to interact with the polar head of phospholipids at water–lipid interface of membranes, which results in lipid ordering and prevention of oxidative damage [62].

Dihydroxy-B-ring flavonoids also occur in nuclei [63,64] (Fig. 2). This localization is consistent with a potential antioxidant function in severely stressed plants, as dihydroxy B-ring-substituted flavonoids, such as quercetin derivatives, may effectively inhibiting the generation of the extraordinary reactive hydroxyl radical in the presence of relatively high H_2O_2 concentrations, thus preserving DNA from oxidative damage [35]. The catechol group in the B-ring of glycosylated flavonoids is primarily involved in the formation of metal ions-flavonoid complexes [2,35]. The nuclear localization of enzymes involved in key steps of flavonol biosynthesis, i.e., chalcone synthase, chalcone isomerase and flavonol synthase, is intriguing [64,65] and suggests a key function for nuclear flavonoids in the control of the transcription of genes required for growth and development, such as the auxin transport facilitator proteins [64,65]. The affinity of flavonoid glycosides for different protein kinases, including mitogen activated protein kinases, depends on the presence of the double C2–C3 bond in the central ring and a 3′–4′ OH-substitution [36,37,66]; notably, these structural requirements are fully satisfied by quercetin derivatives (Fig. 1).

3. Transport of flavonoids

The location of flavonoids within different cells and cellular compartments is potentially related to their multiple functions in plant environment interactions. Since flavonoids are synthesized via a well-characterized multi-enzyme complex localized in the cytoplasmic surface of the endoplasmic reticulum (ER, Fig. 3) efficient flavonoid transport systems deliver these metabolites across different membrane-limited compartments [67]. We describe here two main routes that transport endoplasmic reticulum-derived flavonoids: the intracellular transport to the vacuole and the extracellular transport to the cell wall [68]. The functional significance of vacuolar and cell wall flavonoids is discussed below (Sections 3.2 and 4).

3.1. Transport of flavonoids to the vacuole

Flavonoids are delivered into the vacuole through membrane transporter mediated transport as well as vesicle mediated transport (Fig. 3) [67–70]. Two types of transporters are the primary candidates for catalyzing the vacuolar transport of flavonoids: multidrug and toxic compound extrusion (MATE) transporters and ATP-binding cassette (ABC) proteins. Flavonoids conjugated to glutathione transferases (GST) accumulate in the vacuole mostly by multidrug resistance associated protein (MRP)-type ABC transporters (A in Fig. 3) [68,70], whereas flavonoid glucosides are thought to cross the tonoplast membrane through the action of secondary transporter like proteins belonging to the MATE family (B in Fig. 3) [71].

MRP-mediated vacuolar transport of GS-flavonoid complexes (sometimes referred as to GS-X) does not depend on GST activity, but on the GST protein itself. This suggests that GST behaves as a “ligandin” or as a “carrier” for the transport of flavonoids to the tonoplast [69,70]. GS-X pumps are known that deliver anthocyanins and flavones to the vacuolar compartment, but may also be involved in the uptake of flavonoids by the ER, and participate in vesicle-mediated transport of flavonoids (A1 in Fig. 3) [68].

The delivery of flavonoids into the vacuole may occur via H^+-antiport mechanism via MATE proteins (B in Fig. 3) [71]. The H^+-antiport mechanism at the tonoplast has been recently questioned, and MATE transporters might function as uptake transporters because the cytosolic pH (7.2–7.5) is higher than that of the vacuolar lumen (5.2–5.5) [72]. MATE transporters seem to be mostly involved in the vacuolar transport of (endogenous) glucosides, as in the case of anthocyanins, apigenin and catechins [71]. Uptake experiments conducted in isolated vacuoles show that an endogenous apigenin glucoside (saponarin) in barley is transported to the vacuole by an H^+-antiporter, whereas in *Arabidopsis* that does not synthesize saponarin the transport occurs via ABC-type transporters [73]. ABC-type transporters are much more effective than MATE transporters in accumulating flavonoids in the vacuole [73], possibly because of the pH gradient-dependent MATE protein activity. Therefore, the directly energized transport mechanism of ABC-type transporters might be of crucial significance in the detoxification of large amount of flavonoids produced in response to abiotic or biotic stresses.

The localization of MATE proteins to the tonoplast has been questioned in some cases [74]. These transporters, as also hypothesized for the GS-X pumps, may have multiple localizations. MATE transporters are involved in ER-derived vesicle-mediated transport (B1 in Fig. 3) of quercetin and kaempferol glycosides in the tapetosomes in *Brassica* pollen [75].

Transport mechanisms for flavonol glycosides to the vacuole have not yet been reported, although their massive vacuolar accumulation necessarily requires efficient delivery systems from the ER. The *Arabidopsis* genome has more than 120 putative ABC transporters, the majority of which still needs to be fully characterized [76]. A MRP-type ABC transporter delivers the dihydroxy B-ring substituted luteolin 7-O-diglucuronide to the vacuole in barley [73].

The issue of flavonoid transport into the vacuole is of key significance for flavonoid metabolism, as vacuolar accumulation of flavonoids is a pre-requisite for their biosynthesis [70]. This closely resembles the carbohydrate-mediated feedback regulation of net CO_2 assimilation rate following the decrease in sink strength under severe stressful conditions.

Vesicle mediated transport of flavonoids to the cell vacuole has been detected in different plant species. A model has been proposed in which GS-X pumps associated with the ER reticulum may transport the flavonoids from the ER cytoplasmic surface to the ER lumen, and then flavonoids might move through vesicles that fuse with the vacuolar membrane (C in Fig. 3) [68]. Vesicle-mediated transport of different flavonoids does not seem to involve the Golgi apparatus, as has also been observed for the transport of storage proteins. Vesicle-mediated transport has been mostly investigated for the vacuolar accumulation of anthocyanins. During early stages of anthocyanin accumulation, these flavonoids accumulate in numerous small vesicles (classically termed anthocyanoplasts) in the cytoplasm (C in Fig. 3). Later these vesicles fuse (coalesce) into a single large body; at the same time of vacuole becomes colored. These data are consistent with the presence of vacuolar globules, i.e., anthocyan vacuolar inclusions [69]. In maize cells yellow autofluorescent bodies have been reported to accumulate in the central vacuole likely through a vesicle-mediated transport mechanism [77]. The chemical structures of these yellow fluorescent bodies have not yet been assessed. However, the maximal fluorescence emission of these metabolites at 568 nm under 440–460 nm excitation leads to hypothesize that they contain highly hydroxylated flavonoid structures (Fig. 3).

3.2. Secretion of flavonoids to the cell wall

Some phenylpropanoids and flavonoids are secreted out of the cell. Acylated kaempferol derivatives have been detected in the cell wall of leaf epidermal cells in Scots pine [48]. The increase in cell wall flavonoids as leaf ages is paralleled by a decrease in soluble flavonoids [48]. This suggests that vacuolar efflux of these metabolites and deposition in the cell wall has occurred [70]. Quercetin and kaempferol derivatives have been also observed in the cell wall of epidermal cells in lisianthus flowers petals [78]. How these

flavonoid derivatives are escorted to the cell wall has not yet been addressed.

Phenylpropanoids, particularly hydroxycinnamic acid derivatives contribute to the cell wall formation through esterification with complex carbohydrates [79]. These ER-synthesized compounds are released in small vesicles that fuse in larger bodies and migrate to cell wall, after fusion with the plasma membrane (D in Fig. 3) [80].

GSTs and MATE transporters have been involved in the escort of flavonoids to the plasma membrane and the cell wall in *Arabidopsis* and *Nicotiana tabacum* (E in Fig. 3) [72,81]. Flavonoids are released from the vacuole under elicitor treatment and might cross the plasma membrane using ABC-type transporters (F in Fig. 3) and vesicle-mediated transport [70].

Cell wall phenylpropanoids increase in different plant organs in response to pathogens. They confer tolerance to pathogens in different species [82–84]. This may occur through a multiplicity of functional roles of cell wall located flavonoids. In addition to toxicity for the pathogens flavonoids may produce a physical barrier to fungal penetration through peroxidative cross-linking (lignification) of the cell wall [79,85]. Additionally, flavonoids exert a tight control on auxin movement and peroxidase-mediated auxin oxidation [86–88]. The regulation of flavonols on auxin transport and catabolism may have a crucial role during fungal penetration that is distinct from that played by flavones (i.e., isoflavonoids) [87,88]. Cell wall flavonoids may also exert multiple roles in UV-B tolerance. Acylated kaempferol glycosides in the cell wall of epidermal cells may absorb efficiently UV-B wavelengths, contribute in lignification and tightly regulate the peroxidase-induced oxidation of auxin [88].

Much effort will be required in the near future to fully understand the transport mechanisms and the transport rates of individual metabolite to its final destinations. The issue is complex as the transport of an individual flavonoid may occur via a wide array of different mechanisms. The subcellular localization/functional relationship is further complicated by having individual flavonoids that are capable of playing more than one role, not only in the same cell, but even in the same subcellular compartment.

Flavonoids might serve multiple functional roles in the nucleus and the chloroplast. The mode of transport of flavonoids in these subcellular compartments is unknown (Fig. 3), although such transport has essential roles in plant development, growth and defence [2,3,29,64].

4. The significance of flavonoids as antioxidants

4.1. The stress-induced metabolism of dihydroxy B-ring-substituted flavonoids is a robust trait of terrestrial plants

MYB transcription factors, which regulate the biosynthesis of flavonols, are activated through changes in the redox potential [18–20], and have been present in land plants for more than 500 million years [89]. *Myb* genes regulate anthocyanin biosynthesis, but anthocyanins appeared in land plants much later, approximately 250 million years ago, during land-plant adaptive radiation [41]. Over-expression of the anthocyanin biosynthesis gene *PAP1* (*production of anthocyanin pigment 1*) enhances the biosynthesis of the antioxidant quercetin 3-O-glycosides while repressing the synthesis of the 'poor antioxidant' kaempferol 3-O-glycosides in *Arabidopsis* [90].

MYB transcription factors have long reported to regulate differentiation in *Arabidopsis* (epidermal cell fate and seed coat development) [91]. This regulatory network shows a close relationship with flavonoid biosynthetic pathway, and might have been derived from gene duplication and subsequent divergence events of anthocyanin or flavonol biosynthesis regulators, implying neofunctionalization and perhaps multiple origins in the control of trichome formation [91–93]. Marine algae use N-containing compounds, such as mycosporin-like amino acids as UV-B screening pigments, whereas early terrestrial plants use nitrogen-free organic compounds, i.e., the flavonoids [43]. This difference likely results from the evolution of angiosperms in high-O_2 and high-UV-B environments through enzymatic reactions derived from mechanisms for dealing with ROS [94].

Five enzymes have been identified in the synthesis of various flavonoid structures in liverworts and mosses: chalcone synthase, chalcone isomerase, flavanone 3-hydroxylase, for the synthesis of monohydroxy flavones together with flavonol synthase and flavonoid 3′-hydroxylase for the subsequent synthesis of dihydroxy B-ring substituted quercetin derivatives [41]. These genes are induced early by high light in *Arabidopsis* [95], and are the most responsive genes in current-day plants suffering from a wide range of environmentally induced oxidative damage [3]. This is because the potential of effectively fulfilling several roles in response to a wide range of severe environmental injuries, including the control of developmental processes (see Section 5) [42,96], is restricted to the dihydroxy B-ring flavonoids, particularly quercetin. We hypothesize that quercetin might have improved the adaptability of plant species to an ever-changing environment [97], conferring long-term functional robustness for the 'dynamic selection' of species [98]. Therefore the antioxidant properties of flavonoids represent a robust biochemical trait of organisms exposed to oxidative stress of different origin, and should be considered to be majorly significant for plant-environment interactions. It is worth noting that these functions are fully accomplished at low nM to μM concentrations, as likely occurred in early terrestrial plants [41,42].

4.2. The potential mechanism of antioxidant flavonoids in plants

It has been recently questioned whether flavonoids effectively reduce various forms of reactive oxygen in plants [4,32]. The critiques are reasonable, as the ability of flavonoids to reduce the ROS generated in stressed plants cannot be extrapolated from their *in vitro* scavenger activities or their similar functions in human cell metabolism [99]. An increasing body of evidence suggests that the beneficial effects of plant-derived phytochemicals in humans might marginally depend on their antioxidant functions *sensu stricto* (i.e., reducing activities) [99]. Indeed, the concentrations of flavonoids in the plasma (low μM range) are much lower than those of other low molecular weight antioxidants, e.g., ascorbic acid (high μM range). Thus, the ROS-detoxifying capacity of ascorbic acid would exceed that of flavonoids [66].

The concentration of antioxidant flavonoids in severely stressed leaves may be 'extremely high', up to 25-35 $\mu mol\, g^{-1}$ DW [7,11,46,47]. This concentration exceeds the concentration of the stable ROS, i.e., H_2O_2 [100]. For example, the vacuolar concentration of kaempferol and quercetin glycosides reached approximately 200 μM in *Catharanthus roseus* exposed to high light [101]. The K_m for vacuolar peroxidases of quercetin glycosides, e.g., rutin, is 2 orders of magnitude less than that of quercetin aglycone [50,101]. However, the vacuolar H_2O_2 concentration is much smaller than the flavonoid concentration [100], and may be effectively reduced by dihydroxy B-ring-substituted flavonoid glycosides. The antioxidant potential of flavonoids for peroxidases has been mostly assessed using aglycones *in vitro* [101]. Future experiments have to be performed on the ability of flavonoid glycosides to serve as substrates for guaiacol peroxidases to address the controversial matter on their actual antioxidant functions in plants [2,32,99].

A fundamental issue regarding the significance of flavonoids as antioxidants in higher plants depends upon how an antioxidant is defined. Definitions resulting from experiments conducted in humans consider the ability to "diminish" oxidative stress as an antioxidant function, which includes the capacity of flavonoids to chelate Fe and Cu ions [102,103]. As a consequence quercetin glycosides are better antioxidants than kaempferol glycosides, independent on the relative capacities to donate electrons or hydrogen atoms [104].

The spatio-temporal correlation between oxidative stress events, ROS generation and the ROS-scavenging activity of flavonoids, e.g., the detection of flavonoid oxidation products *in planta*, is still a matter of conflict [32]. The biosynthesis of antioxidant flavonoids is up-regulated as a consequence of severe high-light stress, when the activity of chloroplastic antioxidant enzymes is depleted [11,21,22,24–26]. The idea that the co-localization of antioxidant enzymes, their substrates (i.e., flavonoids) and H_2O_2 occurs only upon the disruption of tonoplast membrane [4,32] is questionable. Peroxidases, flavonoids and ascorbate are present in the vacuolar compartment, and ascorbate recycles flavonoid radicals to their reduced forms [50]. Ascorbate has an extremely low affinity for vacuolar peroxidases [105]. It is conceivable that the concentration of ascorbate exceeds the flavonol concentration in the vacuole [100,101,106]; thus, it effectively sustains the reduction of flavonoid radicals. The peroxidase/flavonoid/ascorbate system has the potential to effectively reduce vacuolar H_2O_2 using the dihydroxy B-ring-substituted structures as preferential substrates [10,22,105]. As a consequence, we have previously suggested that trace amounts of flavonoid radicals are present in plant tissues and hence, undetectable with routinely used analytical techniques [2].

In the final analysis, the mechanism that underlies the reducing activity of flavonoids in the vacuolar compartment involves the intracellular movement of H_2O_2. H_2O_2 diffusion from generation centers to the vacuoles increases greatly in response to high light stress following the depletion of chloroplast ascorbate peroxidase [25,26,53]. H_2O_2 freely diffuses across cellular membranes and aquaporins facilitate the intracellular movement of H_2O_2 [107,108]. Actually, H_2O_2 moves freely within cells and acts as signaling molecule, particularly in cells that are exposed to excess excitation energy [107,109]. Tonoplast intrinsic proteins conduct H_2O_2 in yeast [53] and, in combination with anthocyanins, detoxify the ROS generated from high light [108,110]. The idea of plant cells having compartments that are impermeable to ROS or ROS-derived compounds is likely to be re-considered. H_2O and H_2O_2 may move within the cells through similar transport mechanisms because they have similar physiochemical characteristics [107].

We hypothesize that flavonoids mediate the "unanticipated key role of the vacuole in the control of cellular ROS homeostasis" [13] in conjunction with peroxidases. Class III peroxidases are largely distributed within the vacuole in the inner surface of the tonoplast [101]. Peroxidase activity and vacuolar flavonoids increase in parallel as a consequence of high light [22,101], and a steep enhancement of vacuolar ascorbate upon high light irradiance occurs in the leaves of *Arabidopsis* and *Nicotiana tabacum* [106]. It has been argued that the vacuolar flavonoid-mediated control of whole-cell H_2O_2 homeostasis has limited significance, as the concentration of H_2O_2 in the vacuole is likely much lower than in other cellular compartments [32]. Nonetheless H_2O_2 may be a threat for the cell, leading to the programmed cell death, or function as a signalling molecule activating a network of defences conferring stress tolerance, in a very narrow concentration rage [13,100,111].

Our discussion does not address the issue of the functional roles of monohydroxy B-ring-substituted flavonoids as well as hydroxycinnamates, the concentration of which changes little in response to different stress agents. Thus, the concentration of monohydroxy flavonoids might exceed that of the antioxidant counterparts, even in stressed plants, as monohydroxy flavonoid concentrations as high as 10 $\mu mol\, g^{-1}$ DW have been detected [7,47]. The knowledge of stress-induced changes in inter- and intracellular distribution of different flavonoid classes might help elucidating this controversial matter. Unfortunately, no fluorescent probes are available for selectively forming adducts with individual flavonoids in different matrices. Monohydroxy flavonoid glycosides are autofluorescent when associated with the cell wall matrix [49], but do not form adducts with Naturstoff reagent in solution at concentration ranges that are compatible with the cellular milieu [10]. Therefore, vacuolar kaempferol or apigenin glycosides are difficult to visualize. Likely this objective will benefit from the extraordinarily rapid improvement of the Matrix-Assisted Laser Desorption/Ionization (MALDI) Time-of-Flight Mass Spectrometry (TOF) imaging techniques that allow localizing individual metabolites in cross sections. However, the resolution actually available in such equipments rarely exceeds 10 μm.

The light-induced enhancement in the ratio of dihydroxy (e.g., caffeic acid derivatives) to mono-hydroxycinnamates (e.g., p-coumaric acid derivatives) has been previously interpreted in terms of the relative abilities to scavenge ROS [7,112] (Fig. 1). The location of antioxidant flavonoids in adaxial epidermal and mesophyll cells, and of antioxidant hydroxycinnamates (such as chlorogenic acid and various caffeic glycosyl esters) in cells located deep in the leaf (presumably experiencing less severe UV-irradiance and oxidative stress) remains to be conclusively explained [7,46,47]. We hypothesize that caffeic acid derivatives are mostly destined to enhance lignin biosynthesis more than increasing the concentration of soluble hydroxycinnamic intermediates in highly irradiated cells [10].

5. Flavonoids as developmental regulators: antioxidant-related functions?

Antioxidant flavonoids inhibit a wide array of kinases that supersede key steps of growth and differentiation in eukaryotic cells [36,66,113]. Quercetin effectively inhibits the auxin efflux facilitators Pin-formed and multidrug resistant proteins [114]. These proteins may control developmental processes at the organismal level [2,3,17,38,39] and are potentially involved in the so-called "stress-induced morphogenic responses" and "flight" strategy of sessile organisms [3,115,116]. The stress-induced increase in the activities of class III peroxidases [116] might contribute to stress-induced morphogenic responses by increasing the ROS scavenging system [117] and regulate the tissue-specific levels of auxin through "antioxidant" flavonoids, such as quercetin, as substrates [88,118].

The 'short Pin-formed' protein PIN5 is the only pin-formed protein detected in mosses and is associated with the endoplasmic reticulum, the site of flavonoid biosynthesis [96,119]. The detection of PIN5 in mosses suggests that flavonoids primarily served as physiological/internal regulators during the evolution of early terrestrial plants [42]. We cannot exclude that flavonoids still serve these primary regulatory functions in modern terrestrial plants faced with a wide array of stress agents. Such functions do not conflict with ROS-scavenging activities, as these activities solely depend upon the reducing capabilities of flavonoids. Flavonoids have the potential to regulate auxin gradients (by inhibiting polar auxin transport), local auxin concentrations (inhibiting peroxidase-mediated IAA-oxidation) [39,88,118], and scavenge ROS in the nM to low μM concentration range, which is a concentration much smaller than that detected in leaves growing in full sunlight.

6. Concluding remarks and future prospects

We have proposed that vacuolar flavonoids might constitute a secondary antioxidant system, even on a temporal basis. This is because (1) they are primarily activated upon severe stress conditions, when the activity of antioxidant enzymes is depleted; (2) they specifically counter the stress-induced increase of the oxidant load in the vacuole, where antioxidant enzymes are not normally found and also where ROS are not generated; (3) they are capable of maintaining the H_2O_2 concentration within a sub-lethal concentration range; and (4) they activate a network of events, including stress-induced morphogenesis, which protects plants from further and unexpected injuries of different origins. This view is consistent with the extremely low concentrations of antioxidant flavonoids in a wide array of species under either optimal or slightly stressful conditions. We speculate that signals activating the biosynthesis of flavonoids include drastic changes in ROS or REDOX homeostasis, which address the temporal correlation between flavonoid biosynthesis and oxidative stress events.

The relationship between antioxidant enzymes and flavonoids in the response mechanisms of higher plants to abiotic and biotic stress agents deserves further research. Antioxidant enzyme activity may be severely depressed during the midday hours, as strong light could result in severe excess-light stress, particularly when plants are concomitantly faced with other stresses, such as high temperature and drought. The increase in zeaxanthin as a consequence of high light and drought stress [120,121] counters ROS generation through the thermal dissipation of excess energy via nonphotochemical quenching and antioxidant activity in thylakoid membranes [55,122]. The relative significance of key components of the antioxidant machinery, such as antioxidant enzymes, ascorbic acid, carotenoids and flavonoids, may strongly depend upon their subcellular and temporal distribution.

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