Flavonoids as Antioxidants in Plants Under Abiotic Stresses

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Abstract

Flavonoids make a relevant contribution to the response mechanisms of higher plants to a plethora of abiotic stresses. In addition to the long-reported functions as screeners of damaging short-wave solar radiation, flavonoids have been suggested as playing key functions as antioxidants in stressed plants, by inhibiting the generation and reducing reactive oxygen species (ROS) once formed. The ROS-scavenging properties of flavonoids are restricted to few structures, namely, the dihydroxy B-ring-substituted flavonoid glycosides. This structure–activity relationship conforms to the well-known stress-induced preferential biosynthesis of dihydroxy B-ring-substituted both flavones and flavonols. These flavonoids, especially the derivatives of quercetin, have been shown to greatly affect the movement of auxin at intra- and intercellular levels, and hence to tightly regulate the development of individual organs and the whole plant. The effectiveness of flavonoids to inhibit the activity of the auxin efflux facilitator proteins tightly depends on the chemical features that confer the antioxidant potential. In this review article, we discuss about (1) the effect of different abiotic stresses on the accumulation of individual flavonoids, (2) the potential role served by antioxidant flavonoids in the antioxidant machinery of plants exposed to severe stress conditions, and (3) the function of flavonoids as developmental regulators.

Keywords

Abiotic stresses • Antioxidant enzymes • Auxin movement • Dihydroxy B-ring-substituted flavonoids • Reactive oxygen species • UV-radiation

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1 Introduction

Flavonoids, the vast class of secondary metabolites encompassing more than 10,000 structures (Harborne and Williams 2000), have long been reported to display wide range of uses in plant–environment interactions (Winkel-Shirley 2002; D'Auria and Gershenzon 2005; Agati and Tattini 2010). In the recent past, the idea behind functioning of flavonoids primarily as attenuators of short solar wavelengths in plants exposed to UV-B or full solar irradiance has been questioned (Gerhardt et al. 2008; Agati and Tattini 2010; Agati et al. 2011; Akhtar et al. 2010), as flavonoids most responsive to UV-radiation are far from being the most effective UV-B absorbers among the thousands of polyphenol structures (Cockell and Knowland 1999; Harborne and Williams 2000). These suggestions are consistent with the early authoritative views of Swain (1986) and Stafford (1991) of flavonols, the most ancient and widespread of the flavonoids (Rausher 2006; Winkel 2006), having served key antioxidant or "internal regulatory" functions during the evolution of early terrestrial plants.

The biosynthesis of flavonoids is upregulated not only as a consequence of UV-radiation but also in response to a wide range of other abiotic (and biotic) stresses, ranging from nitrogen/phosphorus depletion to cold and salinity/drought stress (Tattini et al. 2004, 2005; Lillo et al. 2008; Olsen et al. 2009; Agati et al. 2011). Since different stresses have in common the generation of reactive oxygen species (Mittler et al. 2004; Mittler 2006), it has been postulated that flavonoids are synthesized to effectively counter the stress-induced oxidative damage. Flavonoids may accomplish their antioxidant functions by both preventing the generation of ROS (through their ability to chelate transition metal ions such as Fe and Cu, Brown et al. 1998; Melidou et al. 2005; Hernández et al. 2009; Agati and Tattini 2010) and scavenging ROS once formed (Ryan et al. 2002; Babu et al. 2003; Tattini et al. 2004; Agati et al. 2007; Jaakola and Hohtola 2010).

How flavonoids may perform their scavenging activity in an in vivo condition is still a matter of conflict, and major criticisms regarding the localization functional relationships (Halliwell 2009; Hernández et al. 2009) have been only partially addressed (Yamasaki et al. 1997; Agati et al. 2007; Agati and Tattini 2010; Akhtar et al. 2010). However, we note that flavonoids do not exclusively occur in the vacuoles of epidermal cells (as long been reported, Saunders and Mc Clure 1976), and hence far enough from the sites of ROS production. Relatively recent experiments have reported a large accumulation of flavonoids in mesophyll cells both in the vacuole (Agati et al. 2002, 2009, 2011; Gould et al. 2002; Tattini et al. 2005, 2006; Kytridis and Manetas 2006) and in the chloroplasts (Agati et al. 2007). This subcellular distribution of flavonoids is, therefore, consistent with a their putative role as ROS-quenchers. Chloroplast flavonoids (chloroplasts have been reported to be both capable of flavonoid biosynthesis and may represent an important site of flavonoid accumulation), (Oettmeier and Heupel 1972; Saunders and Mc Clure 1976; Takahama 1982; Takahama and Oniki 1997; Zaprometov and Nikolaeva 2003) have been shown to effectively quench singlet oxygen generated upon excess blue-light irradiance (Agati et al. 2007). A vacuolar distribution of mesophyll flavonoids may be of key significance in reducing hydrogen peroxide (H_2O_2) that may freely escape from the chloroplast under severe stress conditions, as also hypothesized to occur for flavonoids located in the vacuoles of epidermal cells (Yamasaki et al. 1997; Sakihama et al. 2000). Nevertheless, the matter is far being conclusively elucidated and poses serious concerns from an analytical and technical point of view, mostly concerning the simultaneous visualization of reactive oxygen species and flavonoid distribution at inter and intracellular levels (Hernández et al. 2009; Agati and Tattini 2010).

We highlight that even though polyphenols, particularly flavonoids have long been shown to scavenge various forms of reactive oxygen "in vitro", the flavonoids usually encountered in plants, e.g., in leaf tissues, are the glycosylated structures, as glycosylation both increases the solubility of carbon-based metabolites in an aqueous cellular milieu and preserve the most reactive

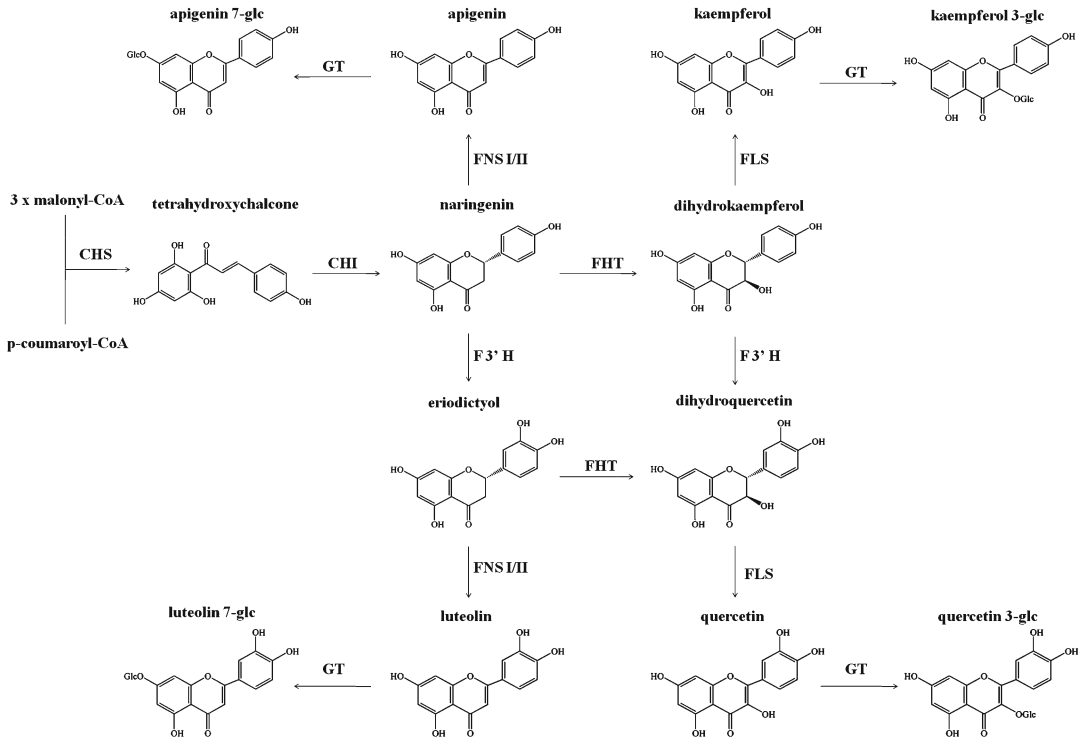

Fig. 9.1 Scheme of the main flavonoid pathway leading to the most common flavanones, flavones, dihydroflavonols, flavonols, and flavonol glycosides. Enzymes – *CHS* chalcone synthase, *CHI* chalcone isomerase, *FNS I/II* flavone synthase I and II, *F3′H* flavonoid 3′-hydroxylase, *FHT* flavanone 3β-hydroxylase, *FLS* flavonol synthase, *GT* glycosyl transferases

functional groups from autooxidation (Pearse et al. 2005). Actually, few glycosylated flavonoids are effective antioxidants, whereas most flavonoid aglycones (i.e., lacking the sugar moiety) are actually capable of quenching ROS (Rice-Evans et al. 1996). Quercetin 3-O- and luteolin 7-O-glycosides that posses a catechol group (ortho-dihydroxy B-ring substitution, Fig. 9.1) in the B-ring of the flavonoid skeleton, but not kaempferol 3-O- or apigenin 7-O-glycosides, display an appreciable antioxidant activity, in the molar concentration-range likely encountered in plant cells (Tattini et al. 2004).

Noticeably, stress-responsive flavonoids have the greatest antioxidant potential, and the ratio of "effective antioxidant" to "poor antioxidant" flavonoids has been conclusively shown to increase steeply in response to a plethora of abiotic stresses (Kolb et al. 2001; Schmitz-Hoerner and Weissenböck 2003; Tattini et al. 2004; Lillo et al. 2008; Kotilainen et al. 2008; Jaakola and Hohtola 2010; Agati et al. 2009, 2011). The issue of "how significant are flavonoids as antioxidants in plants" has been recently explored by Hernández et al. (2009): these authors have suggested of minor significance the contribution of flavonoids to the highly integrated and constitutive antioxidant defense system (which includes antioxidant enzymes, ascorbic acid, and glutathione) operating in plants suffering from various abiotic stresses (as early authoritatively suggested by Halliwell 2009).

Nevertheless, the first line of defense against stress-induced enhancement in ROS concentration – the antioxidant enzymes – have been reported to be ineffective to protect cells from oxidative damage during severe stress conditions (Polle 2001; Apel and Hirt 2004; Hatier and Gould 2008). Hatier and Gould (2008) have recently suggested that the very conditions that lead to the accumulation of flavonoids are those that may inactivate key antioxidant enzymes

(Casano et al. 1997; Streb et al. 1997). In other words, the actual significance of stress-responsive "antioxidant" flavonoids in the context of the well-coordinated antioxidant defenses operating in stressed plants has to be explored on the basis of the stress severity at which plants are faced with.

Flavonoids with the greatest antioxidant potential have the additional capacity to inhibit the polar auxin transport (PAT, Jacobs and Rubery 1988; Brown et al. 2001; Besseau et al. 2007; Peer and Murphy 2007), and, hence, capable of regulating the development of individual organs and the whole plant (Taylor and Grotewold 2005; Lazar and Goodman 2006; Agati and Tattini 2010). Flavonoids have long been shown to inhibit the activity of PIN and MDR P-glycoproteins, that regulate the cellular auxin homeostasis and the cell-to-cell auxin transport (Geissler et al. 2005; Peer and Murphy 2007; Friml and Jones 2010). In this regard, flavonoids do not perform any reducing activity (i.e., antioxidant activity *sensu stricto*), but the chemical features that confer the antioxidant potential are required to effectively interact with the auxin transport proteins (Peer and Murphy 2006; Agati and Tattini 2010). These "internal regulatory" or "physiological" functions of flavonoids, early hypothesized by Stafford (1991) as the most prominent *in planta*, have to be regarded with special attention. In fact, an increasing body of evidence suggests that the health-promoting effect of flavonoids in mammals depends not only upon their ability to scavenge a wide array of reactive oxygen species – free radicals and H_2O_2 – but on their affinity with several proteins (including the mitogen activated protein kinases, MAPK) that supersede key steps of cell growth and differentiation (Williams et al. 2004; Taylor and Grotewold 2005; Peer and Murphy 2006; Lamoral-Theys et al. 2010). Flavonoids might behave, therefore, as "signaling molecules" (Peer and Murphy 2006) or "developmental regulators" (Taylor and Grotewold 2005) in plants, as well as in animals, and their functional roles going well beyond their ability to merely scavenge reactive oxygen.

In this article, we (1) review the pertinent literature on the effect of most common abiotic stresses on the biosynthesis of "UV-absorbing" flavonoids (2) discuss on the potential contribution of flavonoids in the antioxidant machinery of plants under severe stress conditions and (3) their functional roles in the control of plant growth.

2 Stress-Induced Biosynthesis of Flavonoids

A brief summary of stress-induced changes in secondary metabolism, particularly in the biosynthesis of flavonoids has been reported in Table 9.1.

2.1 UV-Radiation

Hundreds of experiments conducted over the last three decades have conclusively shown that flavonoid biosynthesis is mostly upregulated as a consequence of high UV-B and/or full solar irradiance (it is noted that UV-B radiation does not account for more than 5% of the total UV-radiation at mid-latitudes, Ballaré 2003). It is interesting to note that the biosynthesis of other phenolics compounds, both hydroxycinnamic acid derivatives and poly-galloyl derivatives (i.e., hydrolyzable tannins) that are in constitutively greater concentrations than flavonoids under low-light conditions, is very poorly affected by an increase in UV-radiation (Ollson et al. 1999; Burchard et al. 2000; Hofmann et al. 2003; Tattini et al. 2004, 2005, 2006). Since hydroxycinnamic acid derivatives and some hydrolyzable tannins have been reported to display a superior molar extinction coefficient than flavonoids over the 280–320 nm region of the solar spectrum (Harborne and Williams 2000; Tattini et al. 2004), it may be questioned that flavonoids play the primary role as UV-B attenuators in response to UV-B or full solar irradiance (Cockell and Knowland 1999; Agati and Tattini 2010). Similarly, the loss of mycosporin-like aminoacid (MAA) in favor of flavonoid (actually flavonol) metabolism during the colonization of land by plants was likely for fulfilling several uses, as MAA are effective UV-B absorbers (Cockell and Knowland 1999).

Table 9.1 Changes in phenylpropanoid and flavonoid metabolism in response to a variety of abiotic stresses

Species	Treatment	Flavonoids or gene expression affected by treatment	References
Arabidopsis thaliana (hybrid lines differing in freezing tolerance)	Freezing	Upregulation of *CHS*, *CHI*, *DFR*, *FLS1*, and *F3'H* during cold acclimation in the cold tolerant lines	Hannah et al. (2006)
A. thaliana (hybrid lines differing in freezing tolerance)	Freezing	Flavonoid accumulated during cold acclimation and leaf flavonoid content strongly correlated to freezing tolerance	Korn et al. (2008)
A. thaliana	Various treatments	Quercetin biosynthesis enhanced by N deprivation when coupled with another stressor, e.g., cold or high light	Lillo et al. (2008)
A. thaliana	Cold + darkness	Upregulation of flavonoid 3-O-transferase, PAL, CHS and other genes of the phenylpropanoid pathway induced by cold + light treatment	Soitamo et al. (2008)
A. thaliana ecotype Colombia	Nitrogen deprivation + cold	Greater quercetin than kaempferol accumulation	Olsen et al. (2009)
Arnica montana	Cold + UV-exclusion	Ortho-dihydroxy flavonoid biosynthesis was induced by low temperature, but not UV	Albert et al. (2009)
Betula pendula	O_3-fumigation + CO_2 enrichment	O_3 increased the concentration of quercetin derivates at ambient CO_2	Peltonen et al. (2005)
Brassica napus	UV-B enhancement and exclusion	Greater biosynthesis of quercetin glycosides than acylated kaempferol glycoside	Gerhardt et al. (2008)
Brassica oleracea (different cultivars)	Cold	Cold induces higher quercetin to kaempferol ratio in all cultivars, and increases the tissue antioxidant activity	Schmidt et al. (2010) and Zietz et al. (2010)
Fagus sylvatica	O_3-fumigation	Upregulation of all shikimate pathway genes and increased concentration of kaempferol 3-O-glycosides	Betz et al. (2009)
Ginkgo biloba	O_3-fumigation	Ozone steeply increased the quercetin to kaempferol ratio	He et al. (2009)
Lemna gibba	Cu^{2+} + different light environment	Copper and simulated solar radiation induced the biosynthesis of the very same flavonoids, as a consequence of ROS accumulation	Babu et al. (2003)
L. gibba	DMCU/DBMIB + cold + different light environments	Flavonoids accumulate in response to a photosynthetic electron transport chain redox signal, which can operate independently from the ROS signal	Akhtar et al. (2010)
Ligustrum vulgare	High light + drought	Upregulation of quercetin 3-O-rutinoside, luteolin 7-O-glucoside and echinoside in response to high light	Tattini et al. (2004)
L. vulgare	High light + UV-exclusion	Greater accumulation of quercetin and luteolin in response to high light, even in the absence of UV-radiation	Agati et al. (2009)
L. vulgare	High light + NaCl + UV-exclusion	Biosynthesis of quercetin 3-O-rutinoside, luteolin 7-O-glucoside similarly induced by UV-radiation and root zone salinity. Accumulation of di-hydroxylated flavonoids upregulated by excess light even in absence of UV-radiation	Agati et al. (2011)

(continued)

Table 9.1 (continued)

Species	Treatment	Flavonoids or gene expression affected by treatment	References
L. vulgare and *Phyllirea latifolia* (shade tolerant and sun-demanding respectively)	High light	High light induced accumulation of di-hydroxylated flavonoids in the mesophyll to a greater extent in the shade-tolerant than in the sun-demanding species	Tattini et al. (2005)
Mahonia repens	High light	High light induced greater accumulation of anthocyanins and chlorogenic acid	Grace et al. (1998)
Marchantia polymorpha	UV-B enhancement and/or exclusion	Enhancement of UV-B increased luteolin to apigenin ratio	Markham et al. (1998)
Myrtus communis (salt-sensitive) and *Pistacia lentiscus* (salt-tolerant)	NaCl and high light	Greater carbon allocation to myricetin and quercetin glycosides in the salt-sensitive than in the salt-tolerant species	Tattini et al. (2006)
Olea europaea	NaCl + high light	Flavonoid accumulation was increased by high light and salinity, but the effect of high light was greater	Remorini et al. (2009)
Oryza sativa (two genotypes differing in salt tolerance)	NaCl	Upregulation of genes involved in the flavonoid pathway in the salt-sensitive genotype	Walia et al. (2005)
O. sativa (flavonoid deficient mutants vs. parent line)	UV-B enhancement	Accumulation of saponarin and lutonarin reduced DNA damage in the parent line if compared to the flavonoid-knock out mutants	Schmitz-Hoerner and Wissenbock (2003)
Panax ginseng	Cu^{2+}	Upregulation of PAL and increased flavonoid content	Ali et al. (2006)
Petunia (wild type vs. mutants with enhanced anthocyanin production vs. mutants with antisense FLS)	UV-B enhancement	UV-B increased quercetin to kaempferol ratio, and the effect was greater in the mutants with antisense FLS	Ryan et al. (1998)
Petunia (wild type vs. F3H deficient mutants)	UV-B enhancement	Increased quercetin to kaempferol ratio in wild type	Ryan et al. (2002)
Phaseolus vulgaris	O_3-fumigation	Increased *PAL, CHS,* and *CHI*	Paolacci et al. (2001)
P. vulgaris	O_3-fumigation	Increased accumulation of kaempferol 3-O-glucuronide	Kanoun et al. (2003)
Pinguicola vulgaris	UV-B enhancement	Supplemental UV-B increased anthocyanis, and this resulted in enhanced high light and cold tolerance	Mendez et al. (1999)
Pinus sylvestris	Cd^{2+}	Root exposition to Cadmium caused an accumulation of soluble phenolics in the cytosol of root cells	Schutzendubel et al. (2001)
Salix (*S. myrsinifolia* and hybrids)	UV-B enhancement + drought	UV-B increased quercetin derivates in all plants, while the effect of drought was clone-specific	Turtola et al. (2005)

Schefflera arboricola	H_2O_2	Evidences for the dramatic superior antioxidant activity of quercetin than kaempferol derivatives	Yamasaki et al. (1997)
Solanum lycopersicon	Nitrogen deprivation + cold + different light intensities	Maximum increases in quercetin biosynthesis were found in the low nitrogen + cold + high light treatment. Cold, nitrogen deprivation and light had a synergetic effect on *PAL*, *CHS*, *F3H*, *FLS* upregulation	Løvdal et al. (2010)
Trifolium pratense	Mild O_3-exposure	Mild ozone stress increased total leaf phenolics	Saviranta et al. (2010)
Vitis vinifera	Different light environments	High light induced the biosynthesis of antioxidant flavonoids in the presence or in the absence of UV	Kolb et al. (2001)
V. vinifera	UV-exclusion + ABA or water control	Quercetin and kaempferol were decreased by UV-exclusion and increased by ABA	Berli et al. (2010)
Various species: *Cistus creticus*, *Photinia x fraseri* (which accumulate anthocyanins in the mesophyll) and *Rosa* spp. and *Ricinus vulgaris* (which accumulate in the epidermis)	Methyl viologen	Evidences for the antioxidant activity of anthocyanins	Kytridis and Manetas (2006)
Various species	Cold + UV-exclusion	Flavonoids accumulation was upregulated by cold in the absence of UV-radiation	Bilger et al. (2007)

The old statement that flavonoids are UV-screening pigments that allow the penetration of photosynthetic active radiation in photosynthetic cells is of limited significance (Caldwell et al. 1983), as all phenolics display the same physical–chemical properties (Harborne and Williams 2000). Instead, the UV-B-induced biosynthesis of anthocyanins (actually flavonoids sensu stricto) is hard to be explained on the basis of their UV-screening features (Mendez et al. 1999; Manetas 2006; Kytridis and Manetas 2006), as most anthocyanins (with the exception of few acylated forms) do not appreciably absorb in the 280–390 (UV-waveband) region of the solar spectrum (Harborne and Williams 2000).

Furthermore, UV-induced biosynthesis of flavonoids is actually restricted to very few structures, i.e., the dihydroxy B-ring-substituted flavonoid glycosides, such as quercetin 3-O or luteolin 7-O-glycosides. UV-induced increase in the ratio of dihydroxy to monohydroxy B-ring substituted flavonoid glycosides, e.g., the luteolin to apigenin or quercetin to kaempferol ratios, has long been reported in different plant species exposed to various proportions of UV-radiation (Markham et al. 1998; Ryan et al. 1998, 2002; Schmitz-Hoerner and Weissenböck 2003; Kotilainen et al. 2008). Gerhardt et al. (2008) have shown that the ratio of quercetin glycosides to acylated kaempferol glycosides, which are capable to effectively absorb in both the UV-B and UV-A region of the solar spectrum, increased greatly as a consequence of UV-B irradiance. This differential accumulation of flavonoid glycosides has been explained by different authors in terms of the large variations in the free radical scavenger properties of mono with respect to dihydroxy B-ring substituted flavonoids (Rice-Evans et al. 1996; Tattini et al. 2004; Gerhardt et al. 2008; Akhtar et al. 2010).

It has been reported that glycosylation, firstly in 3-postion in the case of flavonols or in 7-position in the case of flavones (Fig. 9.1), is necessary to make these compounds soluble in the aqueous cellular milieu, in addition to preserving the most reactive groups from autooxidation (Pearse et al. 2005). The catechol group in the B-ring of the flavonoid skeleton is mostly responsible to conferring "antioxidant" capacity to glycosylated flavonoids in the concentration range that may be actually encountered in cell compartments (Rice-Evans et al. 1996; Yamasaki et al. 1997; Tattini et al. 2004; Agati et al. 2009; Agati and Tattini 2010). Luteolin and quercetin glycosides may effectively chelate Fe and Cu-ions, thus preventing the generation of ROS (Brown et al. 1998; Melidou et al. 2005), in addition to reducing a wide range of ROS, from singlet oxygen (Agati et al. 2007) to superoxide anion (Tattini et al. 2004), and the stable hydrogen peroxide (Takahama and Oniki 1997; Yamasaki et al. 1997). Tattini et al. (2004) reported that upon high solar irradiance the ratio of the antioxidant luteolin 7-O-glucoside to the poor antioxidant luteolin 4′-O-glucoside was steeply enhanced, suggesting a fine tuning exerted by high light in the flavonoid metabolism to specifically synthesize antioxidant metabolites, as also reported for the light-induced increase in chlorogenic to other mono-hydroxy hydroxycinnamates (Grace et al. 1998). Recently, Jaakola and Hohtola (2010) have reported the latitude-induced enhancement in the quercetin to kaempferol ratio in different species was for protecting plants from cold-induced oxidative damage rather than for merely absorbing UV-B-radiation. Flavonoids with the greatest antioxidant potential have been reported to accumulate in response to high solar irradiance, either in the presence or in the absence of UV-radiation (Kolb et al. 2001; Agati et al. 2009, 2011). Berli et al. (2010) have shown that in grape leaves exposed to UV-B radiation some of the key components of the antioxidant machinery, e.g., CAT, APX, and carotenoids were unaltered, while the biosynthesis of quercetin derivatives mostly upregulated. It is noted that hydroxycinnamic acid derivatives, including caffeic acid, were unaffected by UV-B irradiance. These findings once again confirm that UV-B irradiance did not enhance the biosynthesis of effective UV-B attenuators (i.e., hydroxycinnamates) but that of ROS-scavenging compounds, i.e., the flavonoids. On the whole, these findings strongly support the hypothesis of a key antioxidant function served by flavonoids in photoprotection (Agati and Tattini 2010).

It may be hypothesized that the flavonoid biosynthesis is upregulated under high-sunlight irradiance, irrespective of the proportion of various solar wavelengths reaching the leaf surface, as a consequence of changes in ROS and/or REDOX homeostasis. This hypothesis is further corroborated by the observation that R2R3MYB transcription factors that regulate the flavonol biosynthesis are themselves REDOX-controlled (Taylor and Grotewold 2005; Dubos et al. 2010).

2.2 Nitrogen-Depletion and Cold

Low nitrogen availability and cold have been reported to have a strong impact on flavonoid, particularly flavonol metabolism. Bilger et al. (2007) have shown that UV-absorbing compounds, particularly flavonoids, accumulated to a great extent as a consequence of a decrease in temperature – from 19 to 11°C – in the epidermal cells of various species. Since this increase was detected in plants exposed to light irradiance in the absence of UV-radiation, the authors suggested for flavonoids an antioxidant function in response to cold-induced photooxidative damage. Similar conclusions were also drawn by Korn et al. (2008), who observed a positive correlation between the concentration of flavonoids and the cold-tolerance of *Arabidopsis thaliana* accessions, even though these authors did not discharge a protective function of flavonoids in membrane stability (Erlejman et al. 2004). These findings are consistent with those coming from the experiments of Hannah et al. (2006), as the expression of *CHS*, *CHI*, *DFR*, *FLS1*, and *F3'H*, i.e., the whole set of genes involved in the biosynthesis of di-hydroxylated B-ring flavonols (Fig. 9.1) was induced to a substantially greater degree in cold-tolerant than in cold-sensitive accessions, and positively correlated with the biosynthesis of quercetin derivatives and anthocyanins.

Several experiments conducted by the Cathrine Lillo's group have shed new light on the impact of nitrogen depletion and low T on the biosynthesis of flavonoids, particularly flavonols. Kaempferol glycosides were less responsive to nitrogen depletion than corresponding quercetin derivatives, and the interactive effects of nitrogen and low T were significantly greater on the accumulation of quercetin as compared with the kaempferol accumulation (Olsen et al. 2009). It is interesting to note that in *Arabidopsis*, the mere depletion of nitrogen in plants growing under low light irradiance was unable to substantially affect the biosynthesis of quercetin, which was at trace level in leaves growing under normal greenhouse conditions (Lillo et al. 2008). Lillo et al. (2008) have suggested that a second factor, such as low temperature or high light, seems to be actually required to trigger the biosynthesis of the antioxidant quercetin derivatives. This suggestion have to be considered, however, with some cautions, as most experiments have been conducted under growth conditions typical of the understorey or mild-to-deep shaded environments, since light irradiance did not exceed 200 μmol quanta m^{-2} s^{-1}. However, results are in partial agreement with the recent suggestion reported in Jaakola and Hohtola (2010), for the effect of latitude on the flavonol biosynthesis. In detail, Albert et al. (2009) and Schmidt et al. (2010) observed that the increase in quercetin to kaempferol ratio was strongly correlated with a decrease in external temperature in *Arnica montana* and *Brassica oleracea*, respectively, under natural conditions. It has been suggested that the enhancement in the quercetin to kaempferol ratio depended more on the decrease of T than on the increase of UV-B radiation because of an increase in latitude (Jaakola and Hohtola 2010).

On the whole, cold and nitrogen deprivation affect the biosynthesis of flavonoids and lead to a preferential biosynthesis of the antioxidant quercetin derivatives with respect to that of the corresponding monohydroxy B-ring-counterparts, i.e., kaempferol glycosides. These effects are very similar to those exerted by high light irradiance, irrespective of the portions of various solar wavebands reaching the leaf surface (Lillo et al. 2008; Jaakola and Hohtola 2010). Even more, the synergic effect of cold and high light in inducing the expression of both the flavonoid and the flavonol-branch biosynthesis is similar to that found in response to nitrogen and cold treatments on the

biosynthesis of quercetin derivatives (Soitamo et al. 2008; Løvdal et al. 2010).

The issue, however, deserves further investigation, as an in-depth analysis of the stress-induced effects on the regulation of flavonoid biosynthesis has not been supported with a detailed analysis on the sites of flavonoid accumulation, a prerequisite to conclusively address their "antioxidant" functions in vivo.

2.3 Drought and Salinity Stress

There are few, but interesting experiments that have detailed the impact of "osmotic stress", as drought and salinity, on the flavonoid metabolism in higher plants. Walia et al. (2005) have reported an upregulation of the flavonoid biosynthetic genes, as a consequence of NaCl stress. The increase in the expression of *F3′H*, which leads to the biosynthesis of ortho-dihydroxylated B-ring "antioxidant flavonoids", was superior in the salt-sensitive genotype than in the salt-tolerant rice genotype. The enhancement in flavonoid biosynthesis was paralleled with the increase in glutathione-S-transferase, which is involved in the transport of flavonoids to the vacuole (Zhao and Dixon 2009). The increase in carbon allocated to myricetin and quercetin glycosides, two well-known antioxidant flavonols, was significantly greater in the salt-sensitive *Myrtus communis* than in the salt-tolerant *Pistacia lentiscus* (Tattini et al. 2006), and supposed to be involved in the peroxidase-catalyzed reduction of H_2O_2, based on their large accumulation in the palisade parenchyma cells. Recently, Agati et al. (2011) have shown that root-zone salinity stress had a very similar effect on flavonoid metabolism of that exerted by UV-radiation. In both cases the biosynthesis of quercetin 3-O-glycosides was mostly upregulated, while the biosynthesis of apigenin 7-O-glycosides (mono-hydroxy B-ring flavones) remaining unaffected by mild NaCl stress. It was additionally observed that salinity stress and UV-radiation greatly enhanced the biosynthesis of luteolin 7-O-glycosides. These findings support the idea that different osmotic stressors lead to a similar plant response: the upregulation of the biosynthesis of those flavonoids, as the dihydroxy B-ring-substituted flavones, like luteolin 7-O-glycosides, which are effective antioxidants (Tattini et al. 2004). A fine tuning exerted by root-zone salinity on the flavonoid metabolic pathway was observed, as the ratio of luteolin 7-O-glucoside to the monohydroxy B-ring-substituted luteolin 4′-O-glucoside steeply increased passing from control to NaCl-treated plants. We note that luteolin 4′-O-glucoside lacking the catechol group in the B-ring of the flavonoid skeleton does not display effective free radical scavenger activity in the molar concentration range that may reasonably occur in leaf cells. Vasquez-Robinet et al. (2008) have found that the maximum flavonoid gene expression (*CHS* and *GST*) was observed during the period of more intense drought stress, which suggests for flavonoids a key protective role against water stress. The decrease of maximum carbon assimilation (A_{max}) induced by osmotic stresses exacerbates the deleterious effects of high solar irradiance, as it has been found in some Mediterranean species (Guidi et al. 2008, 2011; Melgar et al. 2009; Agati et al. 2011). In grape berries, Castellarin et al. (2007a, b) found a steep induction of the whole set of genes involved in the biosynthesis and transport of flavonoids because of water stress. These data are consistent with those of Tattini et al. (2004) in *Ligustrum vulgare* leaves, as both water stress and sunlight irradiance led to a steep enhancement in the biosynthesis of flavonoids with an orthodihydroxy B-ring substitution. Although drought stress and sunlight irradiance have been shown to synergistically enhance the expression of flavonoid biosynthetic genes, drought-stressed plants growing at 35 or 100% sunlight irradiance in the field had a significantly smaller concentration of both quercetin 3-O- and luteolin 7-O-glycosides than the well-watered counterparts. It was shown that drought stress depressed steeply the amount of "newly assimilated" or "fresh" carbon available to flavonoid biosynthesis. The matter needs to be explored in depth as, transcript or expression abundance may not directly translate in protein abundance, i.e., enzyme activity (Sweetlove and Fernie 2005), and the tissue flavonoid accumulation may additionally largely depend upon the extent to which different

stresses may affect carbon gain and tissue specific concentration in nonstructural carbohydrates. Since it is the molar concentration of flavonoids in different plant tissues coupled with their intracellular distribution that supersede the potential ROS-scavenging functions in vivo, the issue merits further investigation (Turtola et al. 2005; Melgar et al. 2009; Remorini et al. 2009).

2.4 Ozone

Ozone, a secondary pollutant formed in the troposphere by the interaction of hydrocarbons, nitrogen oxides and sunlight, is a powerful oxidizing agent capable of reacting with any biomacromolecule, although it is neither a free radical species nor a reactive oxygen species, such as H_2O_2 (Mustafa 1990). It is not clear whether oxidative burst occurs and whether visible lesions are caused by ozone through programmed cell death (Sandermann 1996), but the upregulation of aromatic secondary metabolism, including the shikimate and the flavonoid pathways, has been commonly reported during O_3-stress (Janzik et al. 2005; Betz et al. 2009; Iriti and Faoro 2009).

In *Arabidopsis*, ozone induced mRNA levels of PAL within 3 h from exposure, whereas increases in the mRNA levels of antioxidant enzymes (e.g., a neutral peroxidase and a cytosolic CuZn-superoxide dismutase) were found after 12 h (Sharma and Davis 1994). Other works report an induction of PAL and glutathione S-transferase (GST, which conjugates most flavonoids and allowed transport to different cellular compartments, Agati and Tattini 2010; Zhao and Dixon 2009) transcription and activity within 2–3 h from treatment (Eckey-Kaltenbach et al. 1994; Sgarbi et al. 2003; Guidi et al. 2009), followed by a twofold increase of flavone glycoside concentration (Eckey-Kaltenbach et al. 1994). An increase of chalcone synthase (CHS) and chalcone isomerase (CHI), the enzymes involved in the first committed steps of flavonoid biosynthesis (Fig. 9.1), has been reported after ozone fumigation (Kangasjarvi et al. 1994; Paolacci et al. 2001) in a variety of plant species. Kanoun et al. (2003) detected a linear relationship between O_3-levels and the accumulation of kaempferol 3-O-glucuronide, and Betz et al. (2009) found an increase of kaempferol 3-O-glycoside in ozone-treated beeches. Higher concentration of quercetin derivates were found in O_3-treated birch growing at ambient CO_2 concentration (Peltonen et al. 2005). Interestingly, ozone both decreased total phenolics and did not display any significant effects on kaempferol biosynthesis, while steeply enhancing the biosynthesis of quercetin derivatives in leaves of *Ginkgo biloba* (He et al. 2009). Saviranta et al. (2010) have recently reported a specific induction of flavonoid biosynthesis in response to mild ozone stress, and supposed to be involved in countering O_3-induced oxidative damage. As previously reported, ozone enters the leaf through stomata, which are located, in most dicotyledonous species, on the lower side of the leaf lamina, where the density of glandular trichomes producing epicuticular flavonoids is also high (Valkama et al. 2003). Moreover, flavone aglycones have antioxidant activity (Rice-Evans et al. 1997) and can react directly with O_3 deposited on leaf surface.

2.5 Heavy Metals

Other potential abiotic stresses that may expose plants to oxidative damage have been investigated with respect to flavonoid metabolism. The functional roles of flavonoids in the response mechanisms to heavy-metal stress have received some attention. Kováčik et al. (2009) have reported an increase in both flavonoids and caffeic acid in *Matricharia camomilla* leaves as a consequence of high Ni^{2+}-supply, whereas coumaric acid derivatives and phenolic acids did not vary. These findings led authors to hypothesize an antioxidant function of phenolics under heavy-metal stress. Ali et al. (2006) have shown an increase in flavonoid content and free radical scavenging activity in root suspension culture because of excess Cu^{2+}. Excess-Cu^{2+} ions have been reported to stimulate the biosynthesis of flavonoids, mostly luteolin-glycosides, in the absence of UV-irradiance, closely resembling the effect to exposing plants to simulated solar

irradiance in leaves of *Lemna gibba* (Babu et al. 2003). These authors showed a positive correlation between the stress-induced increase in ROS concentration and the accumulation of flavonoids with the greatest antioxidant potential. It may be, therefore, postulated that the flavonol metabolism responds to alterations in ROS homeostasis and flavonoids contributed to its restoration, to maintain ROS concentration at a sublethal level. Similar conclusions were drawn by Schützendübel et al. (2001) for the role of root polyphenols in plants suffering from a severe Cd^{2+}-stress. These authors found that the accumulation of polyphenols was inversely related with the activities of key antioxidant enzymes, that declined steeply as Cd^{2+}-stress progressed, and hypothesized for polyphenols a ROS-scavenging functions to compensate for the decrease in the activity of primary antioxidant defenses. More recently, Potters et al. (2007) have suggested that flavonoids may exert their beneficial effects on Cd^{2+}-stress by affecting the movement of auxin and hence exert a tight control on the root architecture.

3 Antioxidant Flavonoids and the Antioxidant Machinery of Plants

3.1 Stress-Induced Alterations in the Antioxidant Enzymes System

There is a large consensus that a well-coordinated system of constitutive antioxidant defenses is activated in plants upon a plethora of abiotic stresses (for a recent review, see Gill and Tuteja 2010). Superoxide dismutase (SOD), the well-known first-line of defense against ROS generation (aimed at removing the highly reactive superoxide anion) ($O_2^{\cdot-}$), and both ascorbate peroxidase (APX) and catalase (CAT), the enzymes that are devoted at detoxifying the "relatively stable" H_2O_2, have long been reported to play a key role in protecting plants from stress-induced oxidative injuries (Schwanz and Polle 2001a, b; Polle 2001). The extent to which the activity of antioxidant enzymes increases upon stress imposition has been widely reported to correlate positively with tolerance to various abiotic stresses (Hernández et al. 1999, 2000, 2003; Tattini et al. 2005; Sekmen et al. 2007), although it has been also reported that the constitutive activity of antioxidant enzymes is correlated with stress tolerance (Pasqualini et al. 2001; Schwanz and Polle 2001a, b; Guidi et al. 2010). However, under prolonged stress, the activities of key components of the antioxidant machinery of stress-sensitive species have been reported to decline (Hatier and Gould 2008). For example, high doses of UV-B radiation have been reported to decrease the activity of both SOD and APX in *Ulva fasciata* (Shiu and Lee 2005). Several papers of the Andrea Polle's group have conclusively reported of a depression in the activity of antioxidant enzymes in plants exposed to the concomitant action of two or more stresses (Peltzer and Polle 2001; Polle 2001; Peltzer et al. 2002). These findings lead to the hypothesis that the stress severity, which depends on both the intensity and duration of the stress imposed, in addition to the species-specific ability to counter the stress-induced impairments of the photosynthetic machinery, may detrimentally affect the first line of defense against the generation of ROS (Schwanz and Polle 2001a, b; Schützendübel et al. 2001; Wang et al. 2007, 2008).

On the whole, these findings do not support the general view that the whole set of constitutive antioxidant defenses is activated as a consequence of stressful conditions of different origin, and poses the question of the extent to which key components of the antioxidant machinery may actually integrate to counter stress-induced ROS generation. The action of antioxidant enzymes have long been suggested to need of being be complemented by the action of other antioxidant defenses on a long-term basis, when the severity of stress increases (Apel and Hirt 2004).

3.2 Antioxidant Enzymes and Antioxidant Flavonoids: Is There a Relation?

Hatier and Gould (2008) has recently suggested that flavonoids may serve an important antioxidant function when the activity of other components of the antioxidant machinery is steeply depressed

under severe conditions of excess light. Their hypothesis is based upon the observation that the very conditions that lead to enzyme inactivation are those responsible for the maximum biosynthesis of anthocyanins.

Indeed, the biosynthesis of antioxidant flavonoids is mostly upregulated under excess light stress, both in the absence and in the presence of UV-irradiance. Kolb et al. (2001) have reported a great increase in the biosynthesis of quercetin derivatives with respect to kaempferol derivatives in response to visible sunlight irradiance. Agati et al. (2009) in *L. vulgare*, a sun-sensitive species (Tattini et al. 2005), detected a sevenfold increase in the concentration of quercetin 3-O-rutinoside passing from 20 to 100% sunlight irradiance in the absence of UV-radiation. Even more, the ratio of phenylpropanoids with catechol group in the B-ring of the flavonoid skeleton (quercetin 3-O- plus luteolin 7-O-glycosides) or in the benzene ring (echinacoside, a caffeic acid derivative) to monohydroxy substituted counterparts (*p*-coumaric, apigenin 7-O-glycosides) increased as much as 360% because of an increase in PAR irradiance. Babu et al. (2003) have reported of a preferential biosynthesis of luteolin as compared with apigenin derivatives in *L. gibba* as a consequence of excess Cu^{2+} in the absence of UV-radiation. In addition to the known ROS-related signaling pathway leading to the induction of flavonoids, a recent work showed the existence of a second retrograde signaling pathway that operates during various stress situation and influences flavonoid biosynthesis (Akhtar et al. 2010). This second signaling pathway has been related to photosynthetic electron transport chain (PETC) redox state. The PETC redox signal can operate independently of ROS and override the effects of ROS on flavonoid biosynthesis (Akhtar et al. 2010).

Few experiments have been conducted to specifically address the issue of flavonoid biosynthesis as a consequence of stress-induced alteration in antioxidant enzyme activity. Xu et al. (2008) have reported a greater accumulation of antioxidant enzyme proteins in a soybean line with reduced flavonoid content. Aguilera et al. (2002) and Shiu and Lee (2005) have shown that in *U. fasciata* exposed to high UV-B doses, a condition that leads to the maximal flavonoid accumulation, the activity of antioxidant enzymes, particularly CAT and APX, declined greatly. Under severe excessive light stress, the expression of genes involved in the biosynthesis and conjugation of flavonoids were mostly upregulated, whereas the activity of SOD was unaltered (Soitamo et al. 2008). In two *Oleaceae* species differing in their ability to withstand excessive sunlight radiation (estimated in terms of chlorophyll loss and the leaf lipid peroxidation), which exhibited a constitutively different antioxidant enzyme activity, the activity of phenylalanine ammonia-lyase and the biosynthesis of antioxidant quercetin glycosides was steeply greater in the sun-sensitive than in the sun-tolerant species. Agati et al. (2011) have recently reported that the accumulation of epidermal flavonoids was completed after 2 weeks of treatment, when the activities of key antioxidant enzymes declined, as a consequence of UV-irradiance (Guidi et al. 2011).

On the whole, these findings may lead to the hypothesis that the biosynthesis of flavonoids is mostly upregulated under severe stress conditions, when the activities of antioxidant enzymes decline, and, hence, flavonoids may complement the action of other ROS-scavenging systems. Flavonoids have, therefore, to be regarded as a "secondary" antioxidant system, activated upon a severe ROS/REDOX unbalance because of the depletion of primary antioxidant defense systems. This hypothesis is supported by the observation that the greatest antioxidant flavonoid biosynthesis is correlated with the greatest oxidative damage, which conforms to the depletion of antioxidant defenses primarily, also in terms of time, aimed at detoxifying ROS. Under high solar radiation, a greater increase of di-hydroxylated flavonoids was in plants of *L. vulgare*, a shade-tolerant species, when compared to *Phillyrea latifolia*, a sun-requiring species (Tattini et al. 2005). The greater shift toward the flavonoid biosynthetic pathway detected in *L. vulgare* than in *P. latifolia* was related to the greater need of the former species to counter oxidative damage. Similarly, when *M. communis* and *P. lentiscus* were exposed to high solar radiation and root-zone salinity, the allocation of carbon to flavonoid metabolism increased more in the former than in

the latter species, and appeared to be related to leaf oxidative damage (Tattini et al. 2006).

It may not be a mere coincidence that flavonols, the most ancient and widespread of flavonoids, are effective antioxidants. Excess light stress is experienced by plants not only on a seasonal, but also on a daily basis, under natural conditions (Li et al. 2009), and the flavonol metabolism has been highly conserved from the colonization of land by plants, even though the evolution of flavonoid metabolism have produced more than 10,000 structures.

The issue of how flavonoids may perform their reducing activity if confined in cellular compartments, the vacuole, far from the chloroplast, the main source of ROS still generates conflict (Hernández et al. 2009). The old view that flavonoids accumulate almost exclusively in the vacuole of epidermal cells has been recently confuted by a series of experiments in which flavonoids have been shown to largely accumulate in the vacuole of mesophyll cells, and, hence, in the proximity of ROS generation centers. Gould et al. (2002) have shown that vacuolar anthocyanins in the mesophyll may quench H_2O_2 generated upon mechanical injury. Antioxidant quercetin and luteolin glycosides have been reported to accumulate in the vacuole of mesophyll cells exposed to excess PAR irradiance, and it has been speculated that they may help reducing hydrogen peroxide. It is noted that ascorbic acid is a very poor substrate for vacuolar peroxidases that may act to reduce H_2O_2 using flavonoids as preferential substrates (Yamasaki et al. 1997). Ascorbic acid has long been reported to serve as a secondary reducing agent to recycle the flavonoid radicals to their original forms (Sakihama et al. 2000). In a recent experiment Zechmann et al. (2011) have interestingly noted that the pool of vacuolar ascorbate increased dramatically as a consequence of excess light stress, and it may be speculated to be involved in the peroxidase-catalyzed reduction of H_2O_2 using flavonoids as substrates.

Recently, Agati et al. (2007) have reported of a chloroplastic distribution of antioxidant flavonoids using three-dimensional deconvolution microscopy. These findings conform to early views of chloroplast localization of flavonoids (Oettmeier and Heupel 1972; Saunders and Mc Clure 1976; Takahama 1982), and of chloroplast being capable of flavonoid biosynthesis (Zaprometov and Nikolaeva 2003). Chloroplast quercetin and luteolin 7-O-glycosides were effective in quenching singlet oxygen generated upon excess visible light in vivo. Feucht et al. (2004) and Polster et al. (2006) have reported of the occurrence of flavonoids in the nucleus of emerging leaflets of various species, which conforms to a nuclear distribution of both chalcone synthase (CHS) and chalcone isomerase (CHI) (Saslowsky et al. 2005). Hernández et al. (2009) have suggested nuclear flavonoids being capable to chelate transition metal ions and, hence, to inhibit the generation of H_2O_2. This function is to be considered as an antioxidant function, even though there is not a reducing activity here, as it prevents the oxidative damage (Halliwell 2009).

Nevertheless, the issue of the subcellular distribution of flavonoids is far from being conclusively addressed. Flavonoids do not display fluorescence when dissolved in the aqueous cellular milieu and, hence, they need to become pseudofluorescent upon the addition of "fluorescent" probes. The largely used Naturstoff reagent (NR), 2-amino ethyl diphenyl borinic acid, has long been reported to have difficulty to enter cellular compartments, because of its acidic nature (Shehan et al. 1998), and, it is highly specific for dihydroxy B-ring-substituted flavonoid glycosides (Agati et al. 2009). By contrast, the alkalinization of flavonoid solutions with NH_3 under UV-excitation is not specific for flavonoid fluorescence (Kolb et al. 2001; Agati et al. 2002). We recall here that the fluorescence signature of NR-stained tissue under blue light excitation at 488 nm, as commonly used in confocal laser scanning microscopy (CLSM), has to be considered with some cautions.

4 "Antioxidant" Flavonoids as Developmental Regulators

The term "developmental regulators" for flavonoids has been proposed recently by Taylor and Grotewold (2005), and resemble closely that of

"internal regulators" early coined by Stafford (1991). Stafford speculated that in early terrestrial plants the concentration of flavonoids had to be relatively low, and, hence, their UV-B screening functions of relatively scarce significance, as flavonoid concentrations capable to effectively attenuating UV-radiation need to be in the mM range (Edwards et al. 2008; Agati and Tattini 2010). By contrast, flavonoid concentration in the high nM to low μM range may be effective in regulating the auxin movement (Besseau et al. 2007) and quenching reactive oxygen species (Tattini et al. 2004). Recent findings of the localization of the auxin efflux facilitator protein, PIN5, to the endoplasmic reticulum (Friml and Jones 2010), the site of flavonoid biosynthesis, are of particular interest. The localization of the moss PIN proteins to the endoplasmic reticulum leads to the hypothesis that the ancestral functions of these "short" PIN proteins was likely to mediate the cellular auxin homeostasis, which further reinforces the Stafford's hypothesis.

Flavonoids are, in fact, well-known endogenous regulators of auxin movement (Jacobs and Rubery 1988; Brown et al. 2001; Peer and Murphy 2007), and, interestingly, antioxidant flavonoids display the greatest ability to regulate the transport of auxin in vivo (Taylor and Grotewold 2005; Besseau et al. 2007). Quercetin aglycone (which lacks the glycosyl moiety in the 3-postion of the flavonoid skeleton, and hence, display the greatest antioxidant potential, Rice-Evans et al. 1996) is more effective than both kaempferol aglycone and quercetin 3-O-rutinoside to inhibiting the basipetal transport of auxin (PAT, Jacobs and Rubery 1988; Besseau et al. 2007). Flavonoids may profoundly alter the tissue- and cell-specific auxin concentrations by tightly affecting the IAA-oxidation (Mathesius 2001), not just by modulating its intra- and intercellular movements. Monohydroxy B-ring flavonoids have long been shown to behave as cofactors and dihydroxy B-ring flavonoids as inhibitors of the peroxidase-mediated oxidation of auxin (Galston 1969). Jansen (2002) has provided strong evidence that the increase in quercetin to kaempferol ratio may confer UV-tolerance because of the strikingly different affinities of the two flavonols on class III peroxidases (Yamasaki et al. 1997).

Plants suffering from different abiotic stresses display a marked redistribution of growth (Baena González 2010), the so-called stress-induced morphogenic responses (SIMR) (Potters et al. 2007). This "unspecific" response of plants suffering from different stressful conditions is a part of their acclimation strategy to "flight" away from unfavorable environments (Potters et al. 2007). It has become clear that ROS-production and altered phytohormone transport and/or metabolism, that are traits common to different stresses, are involved in SIMR (for review articles, Pritzschke and Hirt 2006; Peer and Murphy 2006; Beveridge et al. 2007), thus making flavonoids as ideal candidates to greatly impact on stress-induced redistribution of growth (Thibaud-Nissen et al. 2003; Lazar and Goodman 2006). The involvement of phenolics in the tolerance mechanisms to Cd^{2+}-stress (Schützendübel et al. 2001; Potters et al. 2007) depends on their ability to both scavenging ROS (as a consequence of Cd^{2+}-induced depression in the activities of antioxidant enzymes, Schützendübel et al. 2001) and inhibiting the basipetal transport of auxin and hence the redirection of root growth (Potters et al. 2007).

It may not be a coincidence, therefore, that stress-responsive flavonoids are effective antioxidants. The chemical features that confer reducing ability against a wide array of free radicals and H_2O_2 allow flavonoids to also display the greatest affinity for proteins involved in key processes of growth and development (Peer and Murphy 2006). Flavonoids have long been reported to behave as transcript regulators in eukaryotic cells mostly through the inhibition of phosphorylation signaling cascades or specific kinases (Peer and Murphy 2006; Lamoral-Theys et al. 2010). This effect is believed to be responsible for the flavonoid-induced inhibition in the activity of PIN/MDR-PGP auxin transport proteins (Muday and DeLong 2001; Taylor and Grotewold 2005), and the catechol group in the B-ring of the flavonoid skeleton is the key feature responsible for the high affinity of flavonoids for different protein kinases (Williams et al. 2004; Lamoral-Theys et al. 2010). As a consequence, stress-responsive dihydroxy B-ring-substituted flavonoids may have a dual role on SIMR, behaving

as regulators of both ROS (H_2O_2) concentration and ROS-induced MAPK signaling cascades.

5 Conclusion and Future Perspective

Flavonoids have long been shown to be involved in the response of plants to a plethora of stressful agents of both abiotic and biotic origin. This general finding is consistent with flavonoids being capable of displaying a wide range of functional roles in stressed plants. However, few flavonoid structures are capable of multiple functions, ranging from UV-screening, ROS scavenging, and inhibition of the activity of auxin efflux facilitator proteins. These flavonoids, which are the most responsive to various abiotic stresses, display the greatest potential to behave as antioxidants. The flavonol metabolism, which was at work in early terrestrial plants, has remained intact for millions of years despite the evolution of flavonoid metabolism and has produced more than 12,000 structures.

To serve such a variety of functional roles, antioxidant flavonols have to be distributed in different tissues and cellular compartments. Flavonols have been detected in the nucleus and suggested to protect DNA from damage, in the vacuole of mesophyll cells as well as in the chloroplasts, and suggested to scavenge highly reactive free radicals and the relatively stable H_2O_2. Flavonols have also been detected at the plasma membrane and hence optimally located to interfere with PIN/PGP-glycoproteins that mediate the cell-to-cell movement of auxin. Even more, flavonoids, which are synthesized in the endoplasmic reticulum, may exert a tight control on the cellular auxin homeostasis, through their interaction with ER-located "short" PIN proteins.

Nevertheless, there are still relevant issues that need to be deeply explored to address the actual relevance of stress-responsive flavonoids in an *in planta* situation. In our opinion, the relative contribution of flavonoids into the well-coordinated antioxidant machinery "activated" as a consequence of different abiotic stress is to be primarily assessed. In this regard, time-course experiments aimed not only at determining the transcript abundance or the gene expression of different antioxidant components but also at quantifying their activities or concentrations are actually required. At the same time, the stress-induced alterations on the inter- and intracellular distribution of key components of the antioxidant machinery, with special emphasis to flavonoids and ascorbic acid, should be routinely estimated as the severity of stress increases.

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