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Stress-induced flavonoid biosynthesis and the antioxidant machinery of plants

Alessio Fini,[1] Cecilia Brunetti,[1] Martina Di Ferdinando,[1] Francesco Ferrini[1] and Massimiliano Tattini[1,2,*]

[1]Dipartimento di Scienze delle Produzioni Vegetali; del Suolo e dell'Ambiente Agroforestale; Università di Firenze; [2]Consiglio Nazionale delle Ricerche; Istituto per la Protezione delle Piante; Sesto Fiorentino, Firenze Italy

There is a growing body of evidence that flavonoids do not primarily function as UV-B screening pigments in photoprotection. Recent findings support the idea that excess light stress, irrespective of the relative proportions of the solar wavebands reaching the leaf surface, upregulates the biosynthesis of dihydroxy B-ring-substituted flavonoid glycosides, as a consequence of and aimed at countering the generation of ROS. Intriguingly, the very conditions that lead to the inactivation of antioxidant enzymes can also upregulate the biosynthesis of antioxidant flavonoids, which suggests flavonoids constituting a secondary ROS-scavenging system in plants exposed to severe/prolonged stress conditions. H_2O_2 may diffuse out of the chloroplast at considerable rates and be transported to the vacuole, the storing site for flavonoids, by tonoplast intrinsic proteins, under severe excess light conditions. We suggest that the unanticipated key role of the vacuole in the ROS homeostasis might be mediated by flavonoids.

Key words: antioxidant enzymes, excess light stress, flavonol metabolism, hydrogen peroxide (H_2O_2), mesophyll flavonoids, quercetin glycosides, oxidative stress

Abbreviations: APX, ascorbate peroxidase; CAT, catalase; CHS, chalcone synthase; F3'H, flavonoid 3'-hydroxylase; FLS, flavonol synthase; MAA, mycosporin-like aminoacid; PAL, phenylalanine ammonia lyase; ROS, reactive oxygen species; SOD, superoxide dismutase

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*Correspondence to: Massimiliano Tattini; Email: tattini@ipp.cnr.it

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The ancient and widespread flavonol metabolism has been widely reported to be mostly involved in the response mechanisms of plants to a wide range of stressful conditions.[1] The loss of mycosporin-like aminoacid (MAA) in favor of flavonol metabolism is a strong evidence that flavonoids did not likely serve a primary UV-B screening function during the evolution of early land plants.[2,3] In fact (1) MAA are excellent UV-B absorbers and flavonols are less effective UV-B attenuators with respect to most flavonoid structures;[4] (2) antioxidant flavonols accumulate to a great extent as a consequence of sunlight irradiance in the absence of UV-radiation.[5,6] These findings lead to the hypothesis that excess light stress, irrespective of the relative proportions of the solar wavebands reaching the leaf surface, upregulates the biosynthesis of flavonoids, as a consequence of and aimed at countering the generation of reactive oxygen species (ROS).[3] In other words, flavonoids behave mostly as antioxidants in photoprotection.[3,7]

We have recently reported that mild root-zone salinity stress and UV-irradiance increased to a very similar degree the biosynthesis of dihydroxy B-ring-substituted flavonoid glycosides (i.e., the antioxidant flavonoid structures usually encountered in leaf tissues)[8] in *Ligustrum vulgare* leaves.[9] Our findings are consistent with the expression of genes of the biosynthesis of antioxidant flavonols (e.g., quercetin 3-O-glycosides), i.e., *FLS* (flavonol synthase) and *F3'H* (flavonoid 3'-hydroxylase) being strongly induced by a plethora of abiotic stresses,[10-12] including UV-B radiation.[13] Since different stresses have been reported to generate ROS, it has been speculated that stress-induced changes in ROS/REDOX homeostasis activate the biosynthesis of antioxidant flavonols,[3,14,15] this idea conforming to R2R3MYB transcriptor factors, which regulate the biosynthesis of flavonols, being themselves REDOX-controlled.[16]

There is a large consensus for flavonoids to function as ROS scavengers, as they may inhibit the generation and reducing ROS once formed,[17] but the actual

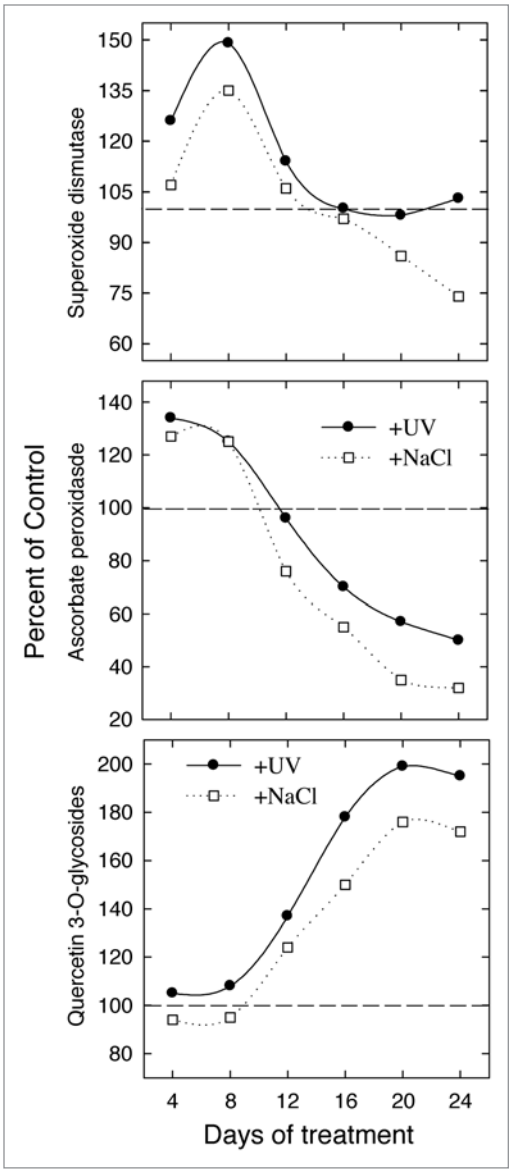

Figure 1. Time-course of SOD and CAT activities, and of quercetin 3-O-glycosides concentration in leaves of *Ligustrum vulgare* as affected by UV-radiation (UV-A: 803 and UV-B: 38.8 kJm^{-2}, respectively) or root-zone salinity (125 mM NaCl). Control refers as to plants growing at full sunlight irradiance in the absence of UV-radiation.

significance of their antioxidant function in a planta situation has been strongly criticized. These criticisms originate from the observations that (1) flavonoids occur almost exclusively in the vacuoles of epidermal cells, and hence, physically separated from the main sources of ROS;[18] (2) plants posses a highly efficient antioxidant machinery to keep the level of ROS under a tight control.[19]

However, we have recently given compelling evidence that antioxidant flavonoids accumulate to a large extent in the vacuole of mesophyll cells in leaves experiencing severe excess light stress,[6,9] and mesophyll anthocyanins have been reported to reduce hydrogen peroxide (H_2O_2) generated upon mechanical wounding.[20] Furthermore, the view that the "constitutive" system of antioxidant defenses is activated as a consequence of different stresses is not true in many instances. The activities of different antioxidant enzymes have long been reported to decline greatly under severe excess-light stress, a condition to which plants are faced with, when suffering from the concomitant action of two or more stresses.[21-23] Here (**Fig. 1**) we show that (1) the activity of ascorbate peroxidase (APX) declined steeply in *L. vulgare* leaves growing at 100% photosynthetic active radiation (PAR) when additionally exposed to UV-radiation, and particularly to root-zone salinity stress (in salt-treated plants the SOD activity also declined as compared with control plants), for more than two weeks; (2) the accumulation of quercetin 3-O-glycosides reached a maximum after three weeks of treatment.

It might be no a mere coincidence that the very conditions that lead to the inactivation of antioxidant enzymes can also induce the biosynthesis of antioxidant flavonoids (including anthocyanins).[23] Excess PAR-irradiance led to a substantial decrease of SOD activity on a long-term basis,[24] the reverse being observed for the accumulation of quercetin derivatives in epidermal cells.[25] The biosynthesis of quercetin glycosides has been shown to be inversely correlated with the increase in SOD and CAT activities as a consequence of high light stress in two Oleaceae,[26] and, consistently, the activity of antioxidant enzymes increased more in a soybean line with a lower flavonoid content in response to UV-B-induced oxidative damage.[27]

Antioxidant enzymes have long been proposed as representing the first line of defense against ROS generation, but their action needs to be complemented by that of other ROS scavenging systems during severe stress conditions.[28] We suggest flavonoids as constituting a secondary ROS-scavenging system in plants suffering from severe excess excitation energy to the photosynthetic apparatus. Actually, excess excitation energy has been recently shown to specifically increase the biosynthesis of the antioxidant dihydroxy B-ring flavone derivatives.[14] Fiorani et al. have reported *PAL* (phenylalanine ammonia lyase, the entry point in the phenylpropanoid metabolism) and *CHS* (the first committed step in the flavonoid biosynthetic branch-pathway) being strongly induced in plants overexpressing alternative oxidase (AOX, which is involved in stress-induced variations of the cellular REDOX state).[30]

Excess light may allow H_2O_2 to diffuse out of the chloroplast at considerable rates (as a consequence of APX depletion),[21,31,32] and tonoplast intrinsic proteins may allow

H_2O_2 to enter the vacuole,[33] the storing site for flavonoids. Flavonoids are superb substrates for class III peroxidases to reducing H_2O_2, whereas ascorbic acid functions primarily to the recycling of flavonoid radicals to their reduced forms.[34,35] There is intriguing evidence of a large redistribution of the ascorbate pool to the vacuolar compartment under excess light stress.[36] It may be hypothesized that mesophyll flavonoids may effectively reduce H_2O_2 escaping from the chloroplast, when the pool of chloroplast antioxidants is depleted as a consequence of severe excess light. The unanticipated key role of the vacuole in the ROS homeostasis[37] might be, therefore, mediated by flavonoids. There is a very narrow range of H_2O_2 concentration as a threat to the cell, including the programmed cell death, or as a signaling molecule responsible for increasing tolerance,[37,38] and flavonoids may serve a key functional role to keeping the concentration of H_2O_2 at a sub-lethal level.

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