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Esculetin and esculin (esculetin 6-O-glucoside) occur as inclusions and are differentially distributed in the vacuole of palisade cells in *Fraxinus ornus* leaves: A fluorescence microscopy analysis

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ABSTRACT

The location of individual coumarins in leaves of *Fraxinus ornus* acclimated at full solar irradiance was estimated using their specific UV- and fluorescence spectral features. Using a combination of UV-induced fluorescence and blue light-induced fluorescence of tissues stained with diphenylborinic acid 2-aminoethylester, in wide field or confocal laser scanning microscopy, we were able to visualize the distribution of esculetin and esculetin 6-O-glucoside (esculin) in palisade cells. Coumarins are not uniformly distributed in the cell vacuole, but accumulate mostly in the adaxial portion of palisade cells. Our study indeed shows, for the first time, that coumarins in palisade cells accumulate as vacuolar inclusions, as previously reported in the pertinent literature only for anthocyanins. Furthermore, esculetin and esculin have a different vacuolar distribution: esculetin largely predominates in the first 15 μm from the adaxial epidermis. This leads to hypothesize for esculetin and esculin different transport mechanisms from the endoplasmic reticulum to the vacuole as well as potentially different roles in photoprotection. Our study open to new experiments aimed at exploring the mechanisms that deliver coumarins to the vacuole using different fluorescence signatures of coumarin aglycones and coumarin glycosides.

1. Introduction

Polyphenols, as most secondary metabolites, serve multiple functions in plant–environment interactions. Relatively recent evidence indeed severely constrains early view that phenylpropanoid metabolism evolved merely to protect plants against their natural enemies [1,2]. The biosynthesis of secondary metabolites, including polyphenols, is costly for plants, since it diverts freshly assimilated (as well as stored) carbon and energy from growth [3]. Therefore, these (usually very stable) metabolites have long been suggested as serving multiple functions in plants challenged against a wide array of stress agents, of abiotic and biotic origin [4–6]. The relative significance of different functions potentially served by polyphenols in plant–environment interactions depends on leaf developmental stage and the stress severity [7–9], both factors being capable of tightly impacting on the network of other defenses *sensu lato* [10,11]. An increasing body of evidence suggests that stress agents of abiotic origin activate the biosynthesis of very few polyphenol structures, mostly phenylpropanoids with a catechol group in the benzene ring [7,12,13]. Therefore, it has been hypothesized that phenylpropanoids may play a key role as antioxidants during intense stress events [14,15], e.g., when solar energy largely exceeds that usable in photosynthesis (excess light stress *sensu stricto*), a condition at which plants are faced with on seasonal and daily basis [16]. Early view that vacuolar location of polyphenols is not suitable for performing antioxidant functions [17] has been recently questioned, since H_2O_2 may freely diffuse through both the chloroplast and the peroxisome under severe excess excitation energy [7,18,19].

Coumarins, as most polyphenols, have the potential to serve more than one role in photoprotection [20,21]. Coumarins may

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indeed absorb photons over the UV-waveband (λ_{max} in the 330–355 nm spectral range) and may effectively quench reactive oxygen species (ROS) [22] at very low concentrations (i.e., to behave as effective antioxidants *sensu* Asensi-Fabado and Munné-Bosch [23]). Nonetheless, this vast class of phenylpropanoids has been poorly explored for its involvement in plant photoprotection [20,24]. It was shown that 3,6,7-trihydroxycoumarin was effective in protecting the green alga *Dasycladus vermicularis* from an excess of UV-radiation [24]. More recently, Fini et al. [12] have shown that in response to severe excess light stress, driven by the concomitant action of intense water deficit and high sunlight, the biosynthesis of esculetin (6,7 di-hydroxycoumarin, effective antioxidant) was favored over the biosynthesis of esculetin 6-O-glucoside (esculin, poor antioxidant) [22,25] in *Fraxinus ornus*. The increase in esculetin concentration was paralleled by an enhanced activity of vacuolar peroxidases and steep depressions in the activities of catalase and ascorbate peroxidase. It was therefore argued that esculetin in conjunction with peroxidases may constitute a secondary antioxidant system aimed at reducing H_2O_2 entering the vacuole under severe excess light stress, when primary antioxidant defenses are depleted [12].

The knowledge of the inter-cellular and intra-cellular distribution together with the chemical features of individual phenylpropanoids is of outstanding significance for understanding their putative functions in response to different environmental stimuli, including high solar irradiance [7,26]. Most polyphenols detected in leaf cells are glycosylated (e.g., at the 3-position in flavonols or at the 7-position in flavones) [7], thus increasing their solubility in the aqueous cellular milieu. Glycosylation also facilitates phenylpropanoid transport from the endoplasmic reticulum, the putative site of their biosynthesis, to various cell compartments as well as their secretion out of the cell [26–29]. In the case of *F. ornus* leaves, both coumarin aglycones and glycosides have been detected in high concentrations [12,30]. Esculetin is very poor soluble in water due to the absence of glycosylation as compared with its glycosides, i.e., esculetin 6-O-glucoside (esculin) and esculetin 7-O-glucoside (chicorin). This raises the question of how these coumarins accumulate in leaf cells: are they uniformly distributed in the vacuole (as is apparently the case for other glycosylated phenylpropanoids [7] or do they occur as vacuolar inclusions, as previously reported for anthocyanins [31–33] and still unidentified yellow fluorescent bodies [34]?

Our study investigated the location of major individual coumarins in *F. ornus* leaves, i.e. esculetin and its glycosides, with the aim of exploring their functional roles in photoprotection. We took advantage of the presence of the 6,7 dihydroxy-substitution in esculetin or the 6(7)-glucosylation in esculin (chicorin) (see chemical structures in Fig. 2) to perform an in-depth fluorescence

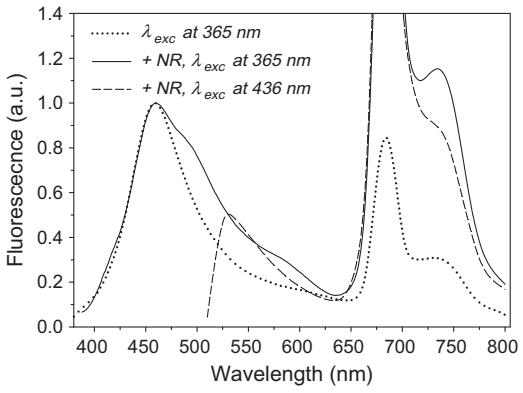

Fig. 1. Fluorescence spectra of the palisade tissue in a *Fraxinus ornus* leaf cross section under 365 nm excitation, with and without Naturstoff treatment, and under 436 nm with Naturstoff treatment.

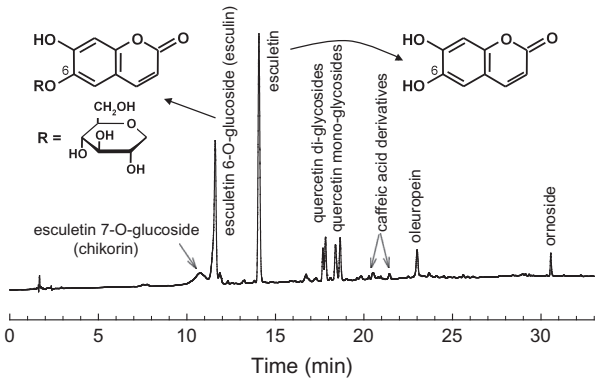

Fig. 2. A representative HPLC–DAD chromatographic profile (recorded at 280 nm) of a *Fraxinus ornus* leaf extract. Quercetin di-glycosides include quercetin 3-O-rutinoside (rutin), quercetin 3-O-robinobioside (tentatively, but see [30]), and mono-glycosides include quercetin 3-O-glucoside and quercetin 3-O-galactoside.

microscopy analysis. It comprised multispectral fluorescence micro-spectroscopy and micro-imaging (performed at wide-field and confocal laser scanning level), using UV-excited fluorescence and blue-excited Naturstoff (diphenylborinic acid 2-amino-ethylester) reagent-induced fluorescence of individual compounds. This is the first report showing the sub-cellular location of individual coumarins, an issue of outstanding significance in photobiology.

2. Materials and methods

2.1. Plant material and growth conditions

Two-month-old leaves were sampled from one-year-old *F. ornus* plants that had been grown outdoor from May to July in Florence (43°46′17″N, 11°15′15″E). UV irradiance (280–400 nm) and PAR (photosynthetic active radiation over the 400–700 nm waveband) were measured by a SR9910-PC double-monochromator spectroradiometer (Macam Photometric Ltd., Livingstone, UK), and a calibrated Li-190 quantum sensor (Li-Cor Inc., Lincoln, NE, USA), respectively. Irradiance rates were the following: UV-A 961.7 ± 56.3 kJ m^{-2}, UV-B 47.2 ± 2.9 kJ m^{-2}, PAR 1246 ± 77 μmol quanta m^{-2} s^{-1} on a daily basis, over the whole-experimental period.

2.2. Analysis of coumarins and flavonoids

Internal phenylpropanoids, mostly coumarins and flavonols [30], were extracted, identified and quantified following the protocol of Tattini et al. [35], with some modifications. Freeze-dried leaf tissue (30–40 mg) was extracted with 3 × 5 mL 75% EtOH (adjusted to pH 3.0 with formic acid), and the supernatant partitioned with 3 × 8 mL of *n*-hexane. The ethanol fraction was reduced to dryness under vacuum at room *T* with a Büchi P12 Multivapor unit equipped with a Büchi V-855 vacuum controller (Büchi, Flawil, Switzerland). The residue was dissolved in 1 mL of MeOH/H_2O (9:1, v:v), and 10 μL aliquots injected in Perkin–Elmer Flexar chromatograph equipped with a quaternary 200Q/410 pump and LC 200 diode array detector (DAD, all from Perkin–Elmer, Bradford, CT, USA). Analysis of phenylpropanoids secreted by glandular trichomes (other members of the *Oleaceae* family, except *Olea europaea*, do have secretory trichomes mostly distributed on the abaxial surface in fully-developed leaves) [7,26] was performed as follows. Leaves were dipped in CH_3Cl (3 × 45 s) and the CH_3Cl solution reduced to dryness, rinsed in 70% EOH (pH 3.0 using formic acid) and injected in the HPLC equipment described above. Phenylpropanoids were separated using a 250 mm × 4.6 mm Hypersil BDS