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Light-induced decrease of reflectance provides an insight in the photoprotective mechanisms of ripening apple fruit

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ABSTRACT

The magnitude of real-time changes in reflectance of ripening apple (*Malus* × *domestica* Borkh., cv. Antonovka) fruit induced by PAR irradiation (500 μE m^{-2} s^{-1}) was monitored together with pigment composition and non-photochemical dissipation of absorbed light energy. In fruit with high chlorophyll content irradiation induced, within 180 s, a notable decrease of reflectance in the bands centred at 520, 692 and 740 nm. These changes were strongly inter-correlated and exhibited tight relationships with chlorophyll fluorescence intensity, violaxanthin de-epoxidation, NPQ, qN and qP. The decrease of the reflectance in the blue-green is suggested to be related mainly with irradiation-induced conversion of violaxanthin to zeaxanthin whereas the decrease of the reflectance in the red and NIR was obviously due to the quenching of chlorophyll fluorescence. Based on these findings, spectral index AVI (*A*pple *V*iolaxanthin cycle *I*ndex) for non-destructive assessment of violaxanthin cycle operation in apple was developed. According to the results of violaxanthin cycle monitoring with the use of AVI, its activity as well as non-photochemical quenching declined in the course of apple ripening on the background of the increase in the proportion of extrathylakoid carotenoids (mainly fatty acid-esterified xanthophylls) together with the relative amount of light intercepted by these pigments. Comparative importance of violaxanthin cycle and light screening by extrathylakoid carotenoids for photoprotection at different stages of fruit development is discussed.

1. Introduction

Measurements of optical reflectance represent a rich source of valuable information about green plant biochemical composition, physiological condition and development [1–4]. Recent insights in plant reflection properties made it possible to develop a conceptual model for non-destructive assay of chlorophyll (Chl), carotenoid (Car), anthocyanin and flavonol content in leaves and fruit as well as to reliably diagnose damages to and infectious lesions of plants [1,3]. Reflectance spectra could also give a clue about the state and operation of photoprotective mechanisms in photosynthesizing tissues [5–9]. Thus, a technique for estimation of photochemical utilization efficiency of the absorbed PAR was suggested employing the decrease of reflectance in the blue-green region of the spectrum due to up-regulation of violaxanthin cycle (VC) by high light; the magnitude of these changes was shown to correlate with violaxanthin (Vio) de-epoxidation state as well as quenching of chlorophyll fluorescence [6]. This approach has been successfully applied for monitoring of the photochemical efficiency both on the leaf and canopy scales [5,8].

Apart from spectral signature of pigment absorption, the reflected signal from plant surface may contain a small (*ca.* 1–2%) contribution of fluorescence emitted by Chl [10,11]. It should be stressed that the analysis of Chl fluorescence (ChlF) signals *per se* proved to be a powerful tool for investigation of the photosynthetic apparatus [12]. Thus, a decrease of ChlF induced by strong irradiation (>600 μmol quanta m^{-2} s^{-1}), R_{Fd}, is a marker of potential photosynthetic quantum conversion capacity of leaves [10]. Diverse techniques based on modulated ChlF measurements (so called pulse-amplitude modulation, PAM) for probing of photosynthetic apparatus state and functioning have evolved and became widespread over the last two decades [12]. At the same time, only a few attempts have been made to use the fluorescence signal extracted from the total reflected radiation. In this connection, it could be advantageous to employ optical reflectance measurements for simultaneous real-time monitoring of the VC and quenching of Chl fluorescence.

For this study, ripening apple fruit were selected as a model. This selection is warranted, apart from easier handling, by lower in comparison with leaves content of pigments, the bulk of which is situated within several cell layers of skin located above strongly

Abbreviations: Antn, antheraxanthin; Car, carotenoids; Chl, chlorophyll; ChlF, chlorophyll fluorescence; FAXE, fatty acid xanthophyll esters; R, reflectance; PRI, photochemical reflectance index; STD, standard deviation; Vio, violaxanthin; VC, violaxanthin cycle; Zea, zeaxanthin.

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scattering flesh. As a result, reflectance spectra of apple fruit are more resolved than those of leaves [2]. The existence and operation of violaxanthin-dependent ChlF quenching mechanisms has been confirmed for apples [13,14]. Interestingly, the transformation of pigments accompanying apple fruit ripening is characterized by a considerable accumulation of Vio (together with neoxanthin) and progressive esterification of xanthophylls by fatty acids released from thylakoid membranes decomposed during ripening [15,16]. However, to the best of our knowledge, there were no reports in the literature about an increase in VC activity along with fruit ripening [16]. In view of these facts it would be interesting to reveal the physiological significance of the increase in Vio content observed in ripening apples.

In this work we analysed, to the best of our knowledge for the first time, the simultaneous changes in the blue-green and red-to-NIR regions of whole-fruit apple reflectance induced by high irradiance, in real time and in the course of fruit ripening. We tried to show that these changes are correlated with zeaxanthin (Zea) build-up and simultaneous ChlF quenching under strong irradiation. We also attempted to link the light-induced real-time changes in whole-fruit reflectance and patterns of skin pigment transformation characteristic of fruit ripening.

2. Materials and methods

2.1. Plant material and experimental design

Undamaged anthocyanin-free (<0.2 nmol cm^{-2} anthocyanins) apple (*Malus* × *domestica* Borkh.) fruit (cv. Antonovka) grown in Botanical Garden of M.V. Lomonosov Moscow State University (Moscow, Russia) during the seasons 2004 and 2005 were used in this study. Sampling was started in August and continued untill the beginning of October. Seven fruit adapted to low fluxes of diffuse solar radiation were randomly picked *ca.* bi-weekly from the inner part of canopy at the height of 1.5–2 m. Sampling took place at 10 a.m. when no direct sunlight fell on the fruit and were kept in darkness thereafter. The ripening stage of the harvested apple was estimated using optical reflectance-based approach developed in our laboratory [17], the variation of ripeness at any picking date did not exceed 10%. The measurements were performed within 1 h from picking. Skin samples were taken prior to (control) and immediately after irradiation and reflectance measurements, frozen in liquid nitrogen and used for pigment extraction and HPLC analysis.

2.2. Pigment extraction and analysis

Peel pigment extracts were obtained as described in [18] employing extraction with chloroform: methanol (2:1, v/v) mixture, Chl and Car were assayed in chloroform using coefficients reported by Wellburn [19]. Molecular weight of 570 for Car was accepted [20]. The chloroform extracts obtained during the above-mentioned procedure were used for HPLC analysis of pigments in accord with the earlier developed protocol [16]. Fatty-acid xanthophyll esters FAXE were quantified using the calibration curve obtained for Vio, which (together with neoxanthin) was reported to be the main carotenol substrate for fatty acid esterification in ripening apple fruit [15,21]. The extent of Vio de-epoxidation was calculated as $DE = (0.5[Antn] + [Zea])/([Antn] + [Zea] + [Vio])$ [22].

2.3. Reflectance measurements and irradiation conditions

Whole-fruit reflectance spectra, $R_{(\lambda)}$, were recorded in 400–800 nm range using a custom-made spectrophotometer equipped with a 20 W tungsten-halogen lamp (Osram, Germany) as a light source, 50-mm integrated sphere covered with a Munsell reflectance coating (Munsell Color Company; New Windsor, NY, USA), and a OceanOptics USB 2000 (grating #3) spectrometer (OceanOptics; Dunedin, FL, USA) against $BaSO_4$ plate as a standard. The irradiance provided by the measuring beam of the spectrophotometer (500 μmol m^{-2} s^{-1} PAR at the sample surface level) was sufficient to trigger the light-induced changes in reflectance signal and Vio de-epoxidation but did not induce a measurable pigment bleaching which was monitored non-destructively via fruit reflectance [2,23]. According to the results of pilot experiments, no detectable changes of $R_{(\lambda)}$ and ChlF emission occurred after 3 min of irradiation, therefore the irradiation time of 180 s was selected for routine experiments.

2.4. Assessment of light screening by extrathylakoid carotenoids

The attenuation of PAR by extrathylakoid Car was estimated *via* the spectrum reconstruction method [24,25] which is similar to that previously used for spectral reconstruction of pigment absorption in extracts from higher plant leaves [26]. Briefly, measured reflectance spectra, $R_{(\lambda)}$, were transformed into reciprocal reflectance $[R_{(\lambda)}]^{-1}$, which were represented as a linear combination of the contributions, $F(\lambda)$, of individual apple pigment pools and scattering according to the model:

$$M_{(\lambda)} = \alpha_0 + \alpha_1 F_T(\lambda) + \alpha_2 F_X(\lambda) + \alpha_3 F_P(\lambda) + \alpha_4 s(\lambda) \quad (1)$$

where $M_{(\lambda)}$, simulated reciprocal reflectance spectrum; $F_T(\lambda)$, contribution of photosynthetic pigments (Chl and Car) tightly associated with thylakoid membranes, $F_X(\lambda)$, contribution of extrathylakoid Car (mainly xanthophylls and FAXE) accumulating during fruit ripening in the lipid matrix of chloroplast/chromoplast plastoglobuli [21,27]; $F_P(\lambda)$, 'tail' absorption by cuticular and vacuolar phenolics; $s(\lambda)$, contribution of light losses due to scattering [28–30], and α_1–α_4 are constants. The least squares approach was used to find the optimum values of the fitting parameters to minimize the deviation between the measured and the simulated reciprocal reflection spectra of fruit. The data were treated in Microsoft Excel spreadsheet using the Solver tool. The fitting parameters α_1–α_4 were adjusted to minimize ρ, the sum of the residuals in the spectral range 400–750 nm:

$$\rho = \sum_{j}^{n} [\{R(\lambda_j)\}^{-1} - M(\lambda_j)]^2 \quad (2)$$

All spectra were fitted within relative error, $E(\lambda) = [\{R(\lambda)\}^{-1} - M(\lambda)]/\{R(\lambda)\}^{-1}$, range of ±5%. Screening of PAR by extrathylakoid Car at a given wavelength was estimated as a ratio of the amounts of light intercepted by these pigments and photosynthetic Car and Chl:

$$S_{(\lambda)} = \frac{F_X(\lambda)}{F_T(\lambda)} - 1 \quad (3)$$

or, in the whole PAR range, as follows:

$$S^{\mathrm{PAR}} = \int_{\lambda=400}^{\lambda=750} \left(\frac{F_X(\lambda)}{F_T(\lambda)} - 1\right) d\lambda \quad (4)$$

2.5. Chlorophyll fluorescence measurements

The ChlF emission spectra, $F_{(\lambda)}$, were recorded in real time with same setup as used for reflectance measurements equipped with SZS-22 band-pass filter (full width at half-maximum = 90 nm; T_{max} = 80% at 400 nm; $T < 1\%$ at wavelengths beyond 550 nm; Krasnogorsk, Russia) as excitation filter after 30-min dark adaptation. In routine measurements ChlF spectra were calculated as difference of the reflectance spectra of unirradiated fruit, $R^0_{(\lambda)}$, and the same fruit after 180-s irradiation,