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Emissions of carotenoid cleavage products upon heat shock and mechanical wounding from a foliose lichen

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ABSTRACT

Carotenoids constitute a major target of chloroplastic photooxidative reactions, leading to the formation of several oxidized derivatives and cleavage products, some of which are volatile (VCCPs). Among them, β-cyclocitral (β-CC), at least, is a retrograde signaling molecule that modulates the activity of many key physiological processes. In the present work, we aimed to study whether β-CC and other VCCPs are released into the atmosphere from photosynthetic tissues. To overcome stomatal limitations, the foliose chlorolichen *Lobaria pulmonaria* was used as the model system, and the emissions of biogenic volatiles, induced by heat and wounding stresses, were monitored by proton-transfer reaction time-of-flight mass-spectrometry (PTR-TOF-MS) and gas-chromatography (GC–MS). Prior to stress treatments, VCCPs were emitted constitutively, accounting for 1.3% of the total volatile release, with β-CC being the most abundant VCCP. Heat and wounding stresses induced a burst of volatile release, including VCCPs, and a loss of carotenoids. Under heat stress, the production of β-CC correlated positively with temperature. However the enhancement of production of VCCPs was the lowest among all the groups of volatiles analyzed. Given that the rates of carotenoid loss were three orders of magnitude higher than the release rates of VCCPs and that these compounds only represent a minor fraction in the blend of volatiles, it seems unlikely that VCCPs might represent a global stress signal capable of diffusing through the atmosphere to different neighboring individuals.

1. Introduction

Chlorophyll (Chl) is a double-edged sword for plants. This molecule is capable of harvesting sunlight, initiating the process of photosynthesis, but it also involves an unavoidable risk of photooxidation. Thus, a certain proportion of the photons absorbed by Chl cause the generation of reactive oxygen species (ROS), which bring about oxidative damage in lipids, pigments and proteins (Mittler, 2002). One of the most harmful ROS is the singlet oxygen ($^1O_2$), which is generated by the transfer of excitation energy from triplet excited chlorophyll ($^3Chl^*$) to ground triplet oxygen ($^3O_2$) (Krieger-Liszkay, 2005). This process can be exacerbated by environmental factors that reduce photosynthetic efficiency and hence affect the balance between energy absorption and use, leading to the over-reduction of the electron transport chain and the accumulation of $^3Chl^*$ (Munné-Bosch et al., 2013).

To counteract ROS formation, plants possess a plethora of defense mechanisms. Among them, carotenoids play a pivotal role, being able to prevent $^1O_2$ formation by direct quenching of $^3Chl^*$ and/or by deactivation of $^1O_2$ (Triantaphylidès and Havaux, 2009). Direct quenching of $^3Chl^*$ requires physical proximity and is

Abbreviations: β-Car, β-carotene; β-CC, β-cyclocitral; Chl, chlorophyll; dhA, dihydroactinidiolide; GC–MS, gas-chromatograph–mass-spectrometer; LOCs, light-weight oxygenated compounds; LOXs, lipoxygenase chain oxidation products; Lut, lutein; Neo, neoxanthin; PTR-TOF-MS, proton-transfer reaction time-of-flight mass-spectrometer; RH, relative humidity; ROS, reactive oxygen species; SPME, solid-phase microextraction; VCCPs, volatile carotenoid cleavage products; Vio, violaxanthin; Zea, zeaxanthin.

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unlikely to occur in the reaction center because of the distance between β-carotene (β-Car) and Chl. Thus, the main function of β-Car in reaction centers is to quench the $^1O_2$ generated by ^{3}Chl* (Telfer, 2002). Quenching of $^1O_2$ by β-Car may occur through a physical or chemical interaction, the latter involving the oxidation of β-Car (Ramel et al., 2012a). Oxidation of β-Car by $^1O_2$ generates an array of different cleavage products (apocarotenoids), most of them aldehydes of varying chain length (Ramel et al., 2012a). Among these compounds, those with short chain length are volatile (VCCPs): β-cyclocitral (β-CC, C10), β-ionone, α-ionone (both C13) and dihydroactinidiolide (dhA, C11) (Ramel et al., 2012a).

Two of the VCCPs, β-CC and dhA, have been identified recently as being involved in the transcriptional modulation of a large set of genes, most of them responsive to $^1O_2$ (Ramel et al., 2012b; Shumbe et al., 2014). These changes in gene expression are associated with an enhancement of tolerance to photooxidative stress. This observation, together with the fact that the generation of volatile β − CC, dihydroactinidiolide and other VCCPs is faster in plants exposed to high light (Ramel et al., 2012b), has led researchers to propose their role as stress-signaling molecules. β-CC has been also considered as a candidate for retrograde (chloroplast control over nuclear gene expression) signaling, capable of crossing cell membranes and diffusing from its site of formation in chloroplasts to other organelles, thanks to its lipid-soluble and volatile character (Ramel et al., 2013; Estavillo et al., 2012). A likely mechanism for the regulatory effect of β-CC is through its reaction with thiol groups of proteins leading to regulation of gene expression through the activation of multiple transcription factors (Havaux, 2013).

When released into the atmosphere, all these volatile apocarotenoids add to the blend of inducible volatiles produced by plants (Holopainen, 2004). Apart from their potential role in stress-signaling, β-CC and other VCCPs have been shown to play significant roles in biotic interactions as allelochemicals (Ikawa et al., 2001; Kato-Noguchi and Seki, 2010) or chemical attractants to pollinators (Simkin et al., 2004a; Guédot et al., 2008), grazer repellents (Jüttner et al., 2010) and olfactory signals to birds with carotenoid-colored plumage (Senar et al., 2010). Recent studies also suggest that thanks to their ability to overcome restrictions imposed by vascular system, VCCPs may be involved in long-distance signaling (Carmody et al., 2016).

Overall, VCCPs, especially β-CC, are firm candidates for being involved in stress signaling and acclimation. Since these molecules are lipid-soluble and volatile, they may diffuse through membranes, escape the chloroplast and assist in communication between different organelles within the cell. For the same reason, they can leave the cell, and eventually the photosynthetic organ. Once in the atmosphere, uptake of these compounds by surrounding organisms is possible depending upon the physico-chemical characteristics of the interaction (Niinemets et al., 2004, 2014). Considering the implications of the bidirectional exchange of signaling molecules such as β-CC, in the present study, we hypothesized that photosynthetic organisms under stress conditions can behave as emitters of β-CC and other VCCPs that can further be involved in long-distance signaling. To address this point we have used the foliose lichen *Lobaria pulmonaria* as the model system. We have chosen such a model and not a vascular plant to simplify the pathway between the thylakoids and the open atmosphere. Previous studies have detected the presence of β-CC in dried leaves (Nezhadali and Nezhadali Bagham, 2011) or in leaf extracts (Priestap et al., 2003; Ramel et al., 2012b; Shibamoto et al., 2007). In the present study, we demonstrate that β-CC and other VCCPs can be emitted *in vivo* from an intact photosynthetic organism.

2. Methods

2.1. Sampling and preservation of Lobaria pulmonaria thalli

Lobaria pulmonaria is a tripartite symbiosis formed by a fungus and two photobionts: a cyanobacterium *Nostoc* and a green alga *Dyctiochloropsis*. Among them, the second is quantitatively the dominant in terms of total biomass and it forms a continuous photosynthetic layer, while *Nostoc* only occurs in isolated cephalodia (Schofield et al., 2003; Cornejo and Scheidegger, 2013 Cornejo and Scheidegger, 2013). As a consequence, the photosynthetic responses observed in this species are mostly generated by the green algal layer. *Lobaria pulmonaria* is a threatened species in several western and central European countries, where is considered as an old-growth forest indicator. This is not the case of northern Spain where *L. pulmonaria* is frequent in most beech and oak forests. In the present study, to affect as less as possible sampled populations, the collection of thalli was limited to a small number of specimens and when possible, the specimens were collected from recently fallen trees or branches. The possibility of using pure algal cultures was rejected because of possible interactions of volatiles with the growth media, and their artificial character that impedes any extrapolation to intact photosynthetic tissues.

Thalli of *L. pulmonaria* were collected in an holm oak (*Quercus ilex*) forest in northern Spain (lat 42°52′N long 3°4′W, elevation 800 m a.s.l). Samples were dried under room conditions, and once dried, stored a maximum of two weeks at a relative humidity (RH) of less than 10% and air temperature of 4 °C until use. No loss of vitality (measured as the dark-adapted maximum chlorophyll fluorescence yield F_v/F_m) was detected after this period in reactivated specimens. After storage, the thalli were re-moistened in contact with a moist paper tissue and preconditioned for 48 h at 100% RH, 23 °C and dim light (12 h day/night), similarly to Gauslaa et al. (2012). For online volatile emissions, intact thalli were used, while for pigment determination, 12 mm discs were cut from the thalli.

2.2. Stress treatments

First, the emission of VCCPs was checked by qualitative assays by enclosing the intact thalli in a 10 × 10 cm ovenproof polyethylene terephthalate bag (Stewart-Jones and Poppy, 2006; Niinemets et al., 2011). Prior to trapping of volatiles, the bag with thalli was conditioned at 20 °C (control) and 33 °C (moderate heat stress treatment) for 20 min under a quantum flux density of 1000 μmol m^{-2} s^{-1}.

For quantitative analyses, heat stress was induced by immersing each intact thallus into a bath of water for five minutes as described in Copolovici et al. (2012). Four temperature treatments were used: 23 °C (control), 37 °C, 46 °C and 51 °C (heat stress). These temperatures were chosen as 40 °C is the temperature threshold at which heat-induced damage, i.e. enhanced cellular ion leakage, is elicited in *L. pulmonaria* thalli (Shirazi et al., 1996). Immediately after the heat treatment, the thalli were incubated for 10 min beneath a sun simulation lamp (SOL 500, UV-A + VIS + IR (320–3000 nm), Dr. Hoenle, Germany) supplying a quantum flux density of ca. 350 μmol m^{-2} s^{-1}. For pigment analysis, five discs were exposed at each temperature following the same protocol described before and collected after 10 min of light exposure. Samples were frozen in liquid nitrogen and stored at −80 °C until analysis. Additionally, and for comparative purposes, a parallel set of samples was subjected to an intensive wounding (approximately a total of 0.5 m of parallel linear cuts per sample performed with a razor blade).