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Raman, AFM and SNOM high resolution imaging of carotene crystals in a model carrot cell system

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ABSTRACT

Three non-destructive and complementary techniques, Raman imaging, Atomic Force Microscopy and Scanning Near-field Optical Microscopy were used simultaneously to show for the first time chemical and structural differences of carotenoid crystals. Spectroscopic and microscopic scanning probe measurements were applied to the released crystals or to crystals accumulated in a unique, carotenoids rich callus tissue growing in vitro that is considered as a new model system for plant carotenoid research. Three distinct morphological crystal types of various carotenoid composition were identified, a needle-like, rhomboidal and helical. Raman imaging using 532 and 488 nm excitation lines provided evidence that the needle-like and rhomboidal crystals had similar carotenoid composition and that they were composed mainly of β-carotene accompanied by α-carotene. However, the presence of α-carotene was not identified in the helical crystals, which had the characteristic spatial structure. AFM measurements of crystals identified by Raman imaging revealed the crystal topography and showed the needle-like and rhomboidal crystals were planar but they differed in all three dimensions. Combining SNOM and Raman imaging enabled indication of carotenoid rich structures and visualised their distribution in the cell. The morphology of identified subcellular structures was characteristic for crystalline, membraneous and tubular chromoplasts that are plant organelles responsible for carotenoid accumulation in cells.

1. Introduction

Carotenoids are secondary metabolites playing crucial functions in photosynthesis and photooxidative protection in plants [1]. Their biological roles, such as prevention of cancer, cardiovascular disease, and macular degeneration, have been mainly attributed to their antioxidant properties [2]. The most important carotenoids for human health are those of pro-vitamin A activity, including α- and β-carotenes. They are accumulated in carrot root in large amounts making this vegetable one of the most important source of pro-vitamin A in human diet [3]. In green tissues, carotenoids are synthesised in chloroplasts where they are associated with membranous structures. In photosynthetically inactive tissues, chromoplasts are main organelles containing carotenoids either in lipid-dissolved, liquid-crystalline or crystalline phases, and different chromoplast types may coexist in the cell [4]. Crystalline chromoplasts are common in tomato fruit and carrot root although composed of different carotenes, mainly lycopene and β-carotene, respectively, and they can be identified using optical microscopy [5,6].

Despite carotenoids are minor components in plant cells, their detection can be achieved by using resonance Raman spectroscopy with a laser excitation in the visible region, where the wavenumber of the excitation line coincides with an electronic transition of the individual carotenoid [7]. Four Raman vibrational modes are considered as key markers of carotenoids, i.e. ν_1 at 1500–1530 cm^{-1} assigned to the C═C stretching vibrations, ν_2 at 1150–1160 cm^{-1} due to the C—C stretching vibrations, ν_3 at ca.1000 cm^{-1} assigned to the in-plane rocking vibration of the CH_3 groups attached to the conjugated chain coupled with the in-plane bending vibration of the adjacent C—H bonds, and ν_4 at ca. 960 cm^{-1} assigned to the C—H out of plane wagging vibration coupled with the C═C out-of-plane torsional twists of the carbon backbone [8]. Moreover, Raman spectroscopy combined with confocal microscopy, i.e. Raman imaging method, can be used as a 2D scanning probe of high resolution and high specificity to study carotenoids in situ [7].

Crystalline chromoplasts released from a carrot root are postulated to be not chemically uniform and presumably may contain both β-carotene and α-carotene as suggested by UV/Vis spectroscopic measurements [9]. Raman imaging applied to carotenoid crystals when they were still present in intact plant cells provided further information

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on their composition. Planar measurements and the resulted 2D chemical maps allowed identification of individual crystals and delivered evidence that they are composed of a mixture of carotenoid molecules, predominantly β-carotene accompanied by α-carotene, that are homogeneously distributed throughout the crystal. However, contribution of other carotenoid, i.e. lutein, has not been excluded due to the fact that lutein is present in the carrot root and its Raman spectrum is similar to that of α-carotene [10,11].

Other microscopic imaging techniques, atomic force microscopy (AFM) and scanning near-field optical microscopy (SNOM), can complement information derived from Raman measurements. Both, AFM and SNOM, can be considered as non-invasive scanning probe techniques allowing characterization of morphology, topography and physical properties of biological samples [12,13]. AFM provides information on sample topography with a nanometric resolution based on the interaction of the probe tip with the probed surface [13–15]. Moreover, it may deliver information on the stiffness of the probed object that is defined as the relationship between the applied force and the depth of indentation. AFM has been used in biological research; in plant material especially for visualization and analysis of the cell wall [16–19].

A scanning approach combined with a classical optical microscopy have led to the development of SNOM, which offers simultaneous acquisition of topographical and optical images of a sample. The use of SNOM in transmission mode is limited to thin samples, from which the signal acquisition is gathered from the layer of approx. 100 nm or less, and requires a well-trained person to operate the instrument. The main advantage of SNOM is high lateral resolution of optical images, which is below the diffraction limit (~λ/2), not achievable in far-field microscopy governed by the Abbe criterion. The optical resolution of SNOM is typically in the range of 50–200 nm and depends on the size of the aperture of the used probe (in the aperture SNOM mode) or on the dimension of the tip (in the scattering-type SNOM), as well as on a laser wavelength. SNOM is used to study both material and biological samples [12,20–22], however, plant material was rarely used [23–26].

In this work, we applied all three complementary techniques, Raman imaging, AFM and SNOM, to reveal differences in carotenoid crystals accumulating in carrot cells. We used carrot callus tissue grown on mineral media in vitro that can be considered as a new model system for carotenoid research. This tissue was established by us in a long term culture and selected for its high ability for carotenoid biosynthesis and accumulation. It is a unique, feasible biological material that can be easily propagated, available year round, and exposed to stress factors if needed. Morphological variation of carotenoid crystals observed in this tissue have suggested possible differences in their chemical composition that might be important for understanding carotenoid biogenesis in living organisms. Thus, for the first time, results of complementary and non-invasive scanning probe techniques utilized for carotene measurements are shown and they have delivered new evidence for structural and chemical differences among morphologically distinct crystals. Moreover, we applied SNOM and Raman imaging to indicate carotene cellular localization and subcellular structures associated with carotenoids in an intact cell.

2. Experimental

2.1. Materials

Carotenoid rich tissue of dark orange callus clone derived from a root of the doubled haploid carrot line DH1/7 [27] was used. It contained a similar total amounts of carotenoids as the root from which it was derived i.e., 2120 μg g^{-1} dry weight (DW) of total carotenoids, including 2015 μg g^{-1} DW β-carotene, and was poor in α-carotene and lutein (98 μg g^{-1} DW and 5 μg g^{-1} DW, respectively). Callus was maintained on Petri dishes in vitro filled with BI mineral medium (Gamborg B5 macro- and microelements with B5 vitamins; Duchefa, Haarlem, The Netherlands) supplemented with 1 mg l^{-1} 2,4-dichlorophenoxyacetic acid, 0.0215 mg l^{-1} kinetin and 30 g l^{-1} sucrose, pH 5.8 at 26 °C in the dark. Callus transfers to fresh medium was done every month taking care to select only dark orange clumps.

2.2. Sample Preparation

Samples of intact cells were obtained according to the previously published protocol [11]. Essentially, small tissue aggregates were squashed on a microscopic slide under cover slip, which was later removed just before measurements and after a deep freezing of the slide at −80 °C.

Purified carotenoid crystals released from the tissue were used for Raman and AFM measurements. To avoid crystal damage and to minimize the amount of debris occurring when standard tissue homogenization is done we isolated protoplasts from callus according to the protocol by [28]. Then protoplasts were washed in distilled water at 350 g for 5 min to release plastids. Protoplast-derived chromoplast fraction was extracted using centrifugation in a sucrose gradient as described before by [29] with following modifications. Chromoplasts were purified from the pellet by centrifugation in the sucrose gradient (50%, 30%, 17%, w/v, in the extraction buffer consisting of 50 mM HEPES, 2 mM EDTA, 330 mM sorbitol, 5 mM 2-mercaptoethanol, pH 7.5) at 26,000 g for 45 min at 4 °C. Intact chromoplasts located between the 50/30% sucrose gradient layers were collected and washed in the extraction buffer at 5000 g for 5 min. Carotenoid crystals were released from chromoplasts by washing repeated three times in distilled water at 20,000 g for 3 min each, and they were finally stored at 4 °C in the dark until slide preparation using a smear technique.

2.3. Imaging

Raman imaging, as well as AFM and SNOM analyses were done using a Confocal Raman Imaging system WITec alpha 300. The Raman measurements were performed with the application of the 100× air objective (Olympus, MPlan FL N, NA = 0.9) whilst for AFM and SNOM techniques the 20× air objective (Zeiss, EC EPIPLAN 20×/0.4) was applied.

2.4. Raman Spectroscopy Imaging

Samples were measured by using laser excitation wavelengths of 532 and 488 nm. Using the 532 nm laser, the laser power was set to ca. 20 mW, the integration time was 0.1 s per spectrum, and the resolution was 5–6 steps/1 μm with one Raman spectrum recorded per step. Using the 488 nm laser, the laser power was set to 10 μW, integration time was 0.3 s and the resolution was 2 steps/1 μm. The imaging with poorer resolution than using the 532 nm laser was necessary to avoid the photo- and thermal degradation of the samples.

Data matrices were evaluated using the WITec Project software applying background subtraction using a polynomial of degree 2 and the automatic procedure for cosmic rays removal. The Raman images were obtained based on the band integration or band position.

2.5. Atomic Force Microscopy (AFM)

AFM measurements were performed both in AC and PFM (Pulse Force Mode) modes with Force Modulation probes (k = 2.8 N/m, WITec). The image resolution was about 10–15 pixels/1 μm. The AC mode was used to provide information about sample topography whilst the PFM mode provide the additional information about its stiffness.

2.6. Scanning Near-field Optical Microscopy (SNOM)

SNOM measurements were performed using the 532 nm laser in the contact mode in transmission configuration of the microscope. Standard