Monitoring and visualising plant cuticles by confocal laser scanning microscopy

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Abstract — The present work shows the visualisation of phenolics and flavonoids of plant cuticles by confocal laser scanning microscopy (CLSM). Selected isolated fruit and leaf cuticles were monitored on the basis of autofluorescent phenolics and flavonoids which, in most cases, permitted us to obtain three-dimensional images of the cuticular membranes. The utility of this technique in investigations of cuticular translocation and diffusion of exogenous applied chemicals and cuticle degradation has also been explored. © 1999 Éditions scientifiques et médicales Elsevier SAS

Confocal laser scanning microscopy / cuticle / cutin / flavonoids / phenolic compounds

CLSM, confocal laser scanning microscopy

1. INTRODUCTION

Aerial surfaces of higher plants are covered with cuticle, an extracellular, non-living, lipid covering forming the interface between the plant and its environment [6]. The cuticles of most plants have an intrinsic fluorescence emission that is mainly due to the presence in this complex biopolymer of flavonoids and cinnamic acids. Thus, the presence of flavonoids in cuticles of tomato and pepper fruits has been previously reported [1, 7, 11, 13]. Flavonoids are a class of phenolic compounds of low molecular weight that are widely distributed in the plant kingdom. They exhibit a diverse spectrum of biological functions and play an important role in the interaction between plants and the environment [15].

During fruit ripening, specific flavonoids are biosynthesised and transported from epidermal cells to the different components or parts of the cuticular membrane [9]. In fact, a major difference between mature green and ripe growth stages in tomato fruits is the presence of the flavonoids naringenin and chalconaringenin in the cuticular membrane of ripe fruits [10, 11]. Both phenolics are present in cutin (5–10 % of total weight) and epicuticular waxes (10–40 % of total weight) of tomato fruits [1, 7]. On the other hand, variable quantities of simple phenolic acids, commonly coumaric (their o-, m-, and p-derivatives) and ferulic acids, are often released from other isolated plant cuticles during the de-esterification of cutin [7, 12]. These compounds are bound to the cutin polyester, being liberated and identified only following treatment with reagents that cleave ester linkages.

Recent developments in microscopic techniques, such as confocal laser scanning microscopy (CLSM), are providing the opportunity to study tissue localisation of phenolic compounds in plant tissues in a more precise way [8]. CLSM can serve in the visualisation of chemicals and macromolecular structures using their specific fluorescence properties on the basis of their molecular absorption and emission behaviour [4].

The ability of CLSM to create three-dimensional images is an important feature for plant biologists. Hutzler et al. [8] have recently demonstrated that this technique can give information on the subcellular localisation of phenolic compounds, in particular whether or not the signal is derived from the vacuole or the cell wall. In this short paper, we present three-dimensional images of plant cuticles isolated from selected fruits and leaves using the autofluorescence present in the plant cuticular membranes. Possible and future applications of this technique, taking
into account the molecular barrier properties of this lipophilic membrane, are also presented.

2. RESULTS AND DISCUSSION

2.1. Autofluorescence and tri-dimensional images of isolated cuticles

Some authors [3, 5] have observed under the ultraviolet microscope a strong fluorescence of some plant cuticles, especially localised on the cell wall of the epidermal cells. Figure 1 shows a set of micrographs corresponding to the autofluorescence of some isolated fruit cuticles after excitation at 488 nm. All pictures were taken from the inner surface of the corresponding cuticle sample. Phenolics present strong autofluorescence in solution after UV excitation, whereas in solid samples and because of their molecular self-aggregation, they show a low quantum yield of fluorescence when the samples are excited at higher wavelengths. However, the fluorescence emission is sufficient to obtain good images. Figure 1 A and B shows the autofluorescence of cuticles isolated from mature and green fruit peppers, respectively. Fluorescence of the green pepper isolate is confined to the cutinised cell wall of this cuticle (figure 1 B), whereas the matured isolated fruit cuticle showed a weak additional autofluorescence localised around the cuticular region delimited by the cell wall, i.e. the cuticular matrix, mainly the cutin (figure 1 A).

Figure 1 C and D demonstrates one of the most important advantages of CLSM compared to conventional optical and fluorescence microscopy. The ability to scan in the z direction perpendicular to the system axis allows the study of the distribution of the fluorescence in a three-dimensional manner. Using this approach, spatial information on the cuticle sample can be obtained from the fluorescence signal. These facts have permitted the obtention of new images of plant cuticles. From these images, it can be deduced that, since the x-z scans of the isolated cuticle passes across the thickness of the sample, the weak homogeneously distributed fluorescence recorded between the remaining cutinised cell walls demonstrates the presence of phenolics in the cutin of the green pepper cuticle (figure 1 C). This particular fluorescence was practically zero in the case of the green pepper isolated cuticle (figure 1 D) indicating the absence of phenolics in the cutin and cuticular matrix of this cuticle.

CLSM also provided real images of the thickness of the isolated cuticles. Figure 1 E and F shows the autofluorescence of transversal sections of the isolates. In spite of the fact that only the fluorescence of the sample was recorded, the average thickness measured for both types of cuticles agree well (approximately 30 µm) with that obtained by transmission electron microscopy and optical microscopy [6]. In ripe pepper fruit cuticle, the fluorescence, which indicates the distribution of phenolics, forms a thick layer without discontinuities (figure 1 E), whereas the green pepper fruit cuticle sample shows severe discontinuities localised between the region marked by the remaining cell walls encrusted into the cuticular matrix (figure 1 F). This observations confirm what is evident in figure 1 C and D.

In order to extend the study to other types of plant cuticles, we carried out similar studies on cuticles isolated from mature apple and tomato fruits. Figure 1 G and H shows the spatial distribution of the autofluorescence of these cuticles. They indicate two cuticles with a fluorescence pattern similar to that described in figure 1 C. Both types of fruit cuticles contain significant amounts of phenolics in their cutin matrices [7] that can justify their respective autofluorescence.

Isolated cuticles from leaves were also studied. For this communication, we have selected two classes of leaf cuticles with different autofluorescence patterns. Figure 2 A shows the autofluorescence of an isolated cuticle from expanded leaves of Clivia miniata. This is a type of cuticle with a very weak intrinsic fluorescence. The images indicate that the presence of phenolic compounds was restricted to the cutinised cell wall. The three-dimensional image of this leaf cuticle yielded a very dark picture (data not shown). On the other hand, the CLSM analysis obtained for the Citrus aurantium isolates was completely different as is seen

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Figure 1. Autofluorescence of selected isolated cuticles obtained by CLSM, viewed from the inner surface. The two-dimensional projections from 20–24 sections of mature (A) and green (B) isolated pepper fruit cuticles in the red-colour mode after blue laser excitation at 488 nm. Three-dimensional representations of fluorescence intensity of the mature and green cuticle isolates are shown in C and D, respectively. These pictures are three-dimensional projections from 20–24 sections. E and F. Fluorescence intensity of a view corresponding to the thickness (see localisation of the different axis) at the front of the same isolated pepper fruit cuticles. Three-dimensional images corresponding to cuticles isolated from apple fruit and ripe tomato fruit are shown in G and H, respectively.

Figure 2. Autofluorescence of selected isolated cuticles obtained by CLSM after different treatments, viewed from the inner surface. Autofluorescence of isolated C. miniata (A) and C. aurantium (B) leaf cuticles. C. Three-dimensional image of the isolated Citrus cuticle. Experimental conditions as in figure 1. The autofluorescence and the corresponding three-dimensional image of an isolated green pepper fruit cuticle after paraquat deposition and translocation are shown in D and E, respectively. The effect of hydroxyl radicals generated by a Fenton chemical reaction (see Methods) on an isolated mature fruit pepper cuticle is shown in F (an optical section of autofluorescence) and G (three-dimensional reconstruction).
in figure 2 B. This cuticle, isolated from the adaxial part of the leaf, presented numerous stomata which showed a high intensity of fluorescence, in conjunction with a fluorescence pattern distributed across the cuticular membrane (figure 2 B). This is particularly evident in the corresponding three-dimensional image (figure 2 C).

2.2. CLSM as a tool for monitoring cuticle deposition and degradation

Foliar penetration through the cuticle is a wide field of study including research in foliar uptake of nutrients, growth regulators and pesticides [2, 14], but the mechanisms by which substances penetrate plant cuticles has not yet been well documented. In this sense, CLSM can help to understand the preferential movement and diffusion of certain substances. Cuticular translocation might be achieved using chemicals with a strong or medium intrinsic fluorescence, in a spectral region distinct from the autofluorescence of the corresponding cuticle, and working with isolated cuticles with very low or very localised fluorescence. In order to test this approach, small drops of a micromolar paraquat aqueous solution were deposited on the morphological outer surface of isolated green pepper cuticles. Paraquat, a powerful herbicide, has a strong fluorescence under blue light excitation (maximum wavelength absorption in methanol, 510 nm; in a more hydrophobic environment, the absorption shifts to shorter wavelengths) which could be monitored during its cuticular translocation. After 2–3 h of deposition of the chemical on the cuticle surface, the summed fluorescence (endogenous compounds plus paraquat) of the pepper fruit cuticle appeared as is shown in figure 2 D and E. If we compare these pictures with the micrographs represented in figure 1 B and D, respectively, we can conclude that the chemical diffuses across the cuticular membrane showing a homogeneous ground fluorescence covering the whole surface. These data indicate that plant cuticles behave like homogeneous membranes towards paraquat. There is no indication of the existence of preferential pathways or sites for the diffusion of the herbicide investigated here.

Another application of CLSM concerning plant cuticles involves environmental factors producing cuticular degradation. Specifically, our laboratory is studying the effect of reactive oxygen species on plant cuticular components during pathogen-cuticle interactions. One of the more reactive chemical species generated during the first events of the infection process is the hydroxyl radical (OH-). It has been postulated that this radical can produce a set of different reactions including lipid oxidation, and that certain phenolics, mainly flavonoids, can act as molecular scavengers of this reactive ion [16, 17]. The effect that this radical species has on the different cuticular components [16], mainly waxes and cutin, is unknown. Figure 2 F and G shows, respectively, the autofluorescence and the three-dimensional image after the treatment of mature isolated pepper fruit cuticle with an OH- generating solution. Pictures clearly show that cuticular morphology appeared very degraded, the thickness of the cuticle was reduced and that the autofluorescence suffered a strong quenching. The first observation can be a direct effect of cutin cleavage via radical reactions and the second, cuticle thinning, could be a structural consequence of the reaction of phenolics and flavonoids with the OH- radical.

In combination with ultrastructural studies and structural analysis of the phenolic compounds, CLSM can help to improve our understanding of this unique and fascinating biopolymer, the cuticle, by giving a global and even dynamic view of its processes.

3. CONCLUSION

CLSM has been shown to be an useful tool to obtain three-dimensional images of isolated fruit and leaf cuticles on the basis of autofluorescent phenolics and flavonoids. Despite the need of further research, the technique may be used to investigate the cuticular uptake of selected chemicals deposited on the outer surface of plant cuticles and to obtain important qualitative information on cuticle reactivity and degradation.

4. METHODS

4.1. Plant material and cuticular isolation

Cuticles of ripe tomato (Lycopersicon esculentum L.), green and mature pepper (Capsicum annuum L.) and apple (Malus pumila L.) fruits and bitter orange (Citrus aurantium L.) and Clivia minata Reg. leaves were enzymatically isolated using standard procedures after incubations of the plant tissues in a mixture of cellulase and pectinase [13].

4.2. Cuticular treatments

Small drops (0.5 μL) of freshly prepared 10 μM paraquat (Sigma) aqueous solution were put on the
outer surface of green pepper isolated cuticles. The cuticles were kept at 25 °C for 15 h in a small closed chamber (relative humidity 95 %) until imaging. On the other hand, small pieces of isolated mature pepper cuticles were exposed to a Fenton reagent mixture consisting of 10 mM H₂O₂ and 0.1 mM FeCl₃ aqueous solutions. This mixture produces hydroxyl radicals.

4.3. Confocal laser scanning microscopy and image acquisition and processing

The CLSM analyses were performed on a Leica TCS NT confocal laser scanning microscope (Leica, Heidelberg, Germany) and images were processed using the Solid algorithm which is a direct composition with diffuse gradient shading (Leica TCS software). In this work, small pieces of isolated fruit cuticles were put on microscope slides under a coverslip and the autofluorescence of the tissue was studied by CLSM. Generally, an excitation beam splitter DD488/568 was used. For visualisation of multispectral image data, the selected channel was associated with a colour value on the display system, red in this case, using a band pass filter (BP) of 515–565 nm.

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