Towards *in vivo* chemical imaging of epicuticular waxes


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Abstract

Epicuticular waxes, which are found on the outer surface of plant cuticles, are difficult to study *in vivo*. To monitor the growth, development and structural alterations of epicuticular wax layers, coherent anti-Stokes Raman scattering (CARS) might be used. CARS, as a Raman based technique, not only provides structural insight but also chemical information by imaging the spatial distribution of Raman-active vibrations. Here, we present a comparative study using CARS and scanning electron microscopy (SEM) to characterize the structure of epicuticular waxes. The ability of CARS to provide detailed structural information on the biologically important wax layer was detailed on the examples of *Prunus laurocerasus*, *Hoya carnosa* and *Monstera* sp. aff. *deliciosa* Liebm. We anticipate that the work presented will open a doorway for online monitoring of formation and alterations of epicuticular wax layers.

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The surface of plants has a variety of functions. Between the inner leaf area and the environment the exchange of air, nutrition and information is managed through the plant surface. The surface of land plant leaves is covered by epicuticular waxes. These epicuticular waxes reflect UV radiation, remove contaminations via the lotus effect, and disturb the
adhesion of insects (Jetter et al., 2001; Perkins et al., 2005). Epicuticular waxes consist of a variety of long-chain aliphatic compounds with different functional groups (Jetter et al., 2000). Furthermore, the hydrophobic properties of those compounds reduce waterloss via transpiration to the environment (Koch et al., 2008). In the case of *Prunus laurocerasus* the composition of epicuticular wax was shown to contain alkanes, alcohols, aldehyds, fatty acids and acetates with chain lengths between 20 and 36 carbon atoms (Jetter et al., 2000; Jetter et al., 2001). These waxes display a great diversity of structures (Barthlott et al., 1998), which are produced by self assembly (Koch et al., 2004; Koch et al. 2008). The roughness of the wax layer is enhanced, for example, by crystalloids and this increased roughness can lead to a better protection of the leaves with respect to contaminations (Barthlott et al., 1997) and an increased reflection of UV radiation (Koch et al., 2004). During leaf development epicuticular wax films change in composition as it was exemplified for *P. laurocerasus* (Jetter et al., 2001). The observation of dynamic changes of epicuticular wax layers with respect to their chemical composition and their structure with chemical, spatial and temporal resolution is of great importance in order to understand the molecular mechanisms behind the variety of biological functions performed by epicuticular waxes (Jetter et al., 2001).

The biological function of epicuticular waxes has been extensively described in the literature (Barthlott et al., 1998; Jetter et al., 2001; Perkins et al., 2005; Koch et al. 2008) and their chemical composition can be deciphered using chemical extraction methods and subsequent gas chromatography (GC) analysis (Jetter et al., 2000). Additionally, a recent study shows the potential of jointly using IR-imaging/MALDI-TOF analysis to determine the chemical composition and spatial distribution of extracted epicuticular waxes and carbohydrates on the leaf surface after extraction from the living plant (Fiedler et al., in preparation). In these studies the waxes are transferred to a specially prepared substrate suited both for IR-imaging and MALDI-TOF experiments. Both approaches, therefore, rely on wax-extraction prior to the experiment. Furthermore, MALDI-TOF constitutes a destructive technique, therefore MALDI-TOF will not be helpful imaging dynamical changes. A technique, which is in principle capable of imaging the wax distribution in living leaves, is Raman-based microscopy (Yu et al., 2008; Krafft et al. 2009; Krafft, Dietzek and Popp, 2009; Walter et al. 2010). Raman scattering provides chemical fingerprint information, can be recorded in back-scattering direction (epi-detection) and is generally not hampered by the presence of water in biological samples (Urlaub et al., 1998). Thereby it is of use to decipher the chemical composition of plant surfaces (Baranska et al., 2004; Schulz et al., 2004; Strehle et al., 2005; Strehle et al., 2006).
Particularly suited for monitoring not only the surfacial distribution of waxes but also recording their depth profile can be coherent anti-Stokes Raman microscopy (CARS-microscopy) – a technique, which in perspective will be even capable of deciphering wax transport and self-organisation processes in the formation of epicuticular wax layers in living leaves due to the high spatial and temporal resolution achievable with this technique. CARS (Schmitt et al., 1997), i.e. coherent anti-Stokes Raman scattering, refers to a non-linear variant of Raman-scattering that relies on the interaction of three photons with the sample (Cheng et al., 2002; Akimov et al., 2009; Bergner et al. 2009). In a four-wave-mixing process using typically two separately tunable laser pulses (providing so called pump and Stokes pulses), a signal is generated both in forward (in the following termed F-CARS) and backward (termed epi-CARS) direction (Volkmer et al., 2001). The generation of the CARS signal is based on the phase matching condition and the frequency matching condition, see Figure 1 for visualization. The wavevectors of all three excitation beams and the resulting signal have to fulfil the following equation [1]:

\[ \vec{k}_{AS} = \vec{k}_{p1} - \vec{k}_{S} + \vec{k}_{p2} \]

In this equation the subscript AS refers to the anti-Stokes shifted, i.e. blue-shifted CARS signal, while the subscript S refers to the Stokes laser beam. P1 and P2 refer to the pump laser beams 1 and 2. It can be shown that this equation, which is trivially met in non dispersive media, can be met even using a collinear beam geometry for microscopy if a strongly focusing, high numeric-aperture-objective (high NA) is employed. When the frequency difference between the pump, \( \omega_p \), and the Stokes pulses, \( \omega_S \), equals the energy of a Raman-active vibration, a vibrationally resonant CARS signal is generated. If – on the other hand – the difference \( \omega_p - \omega_S \) does not match the energy of a Raman active vibration, no such resonant CARS-signal is generated (Cheng and Xie, 2004). Due to the nonlinearity of the CARS-process the resulting signal is generated in a very confined sample volume only, i.e. only in the focal volume of the beams, where the field intensities are highest (Cheng and Xie, 2004). Thus, the spatial resolution of this technique is intrinsically below the Abbe-Limit of conventional linear light microscopy (Beeker et al., 2009). Therefore, CARS-microscopy offers good 3D-sectioning capabilities (Zumbusch et al., 2009). Due to the coherent nature of the CARS process the resultant signal strength is boosted compared to linear confocal Raman microscopy and, hence, CARS-microscopy enables fast image acquisition (Potma et al., 2001). However, in contrast to conventional Raman micro-spectroscopy (Baia et al., 2002;
single-mode CARS-microscopy yields images based on univariant results. Thereby, the information content to be obtained is limited to the spatial distribution of a single preselected Raman-active mode. However, as CARS allows for significantly faster image acquisition compared to Raman mapping, it opens the doorway to online imaging of physiological processes in living systems with chemical sensitivity.

The work presented here combines Raman spectroscopy and CARS microscopy to investigate the epicuticular wax layer of plants and, thus, constitutes a milestone on the way towards online, label-free and non-invasive imaging of wax-transport processes in living plants. In this paper we present CARS images recorded in forward and backward scattering direction and their correlation with scanning electron microscopic images, i.e. the gold standard for monitoring the spatial distribution of epicuticular waxes (Barthlott et al., 1998). Albeit our current microscope setup does not allow for in vivo measurements yet, the herein pursued approach illustrates the capability of CARS to capture the self-assembled structures of these biologically important substances. While this study focuses on an ex vivo proof-of-concept demonstration, later studies shall aim at the application of this experimental scheme to study the kinetics of wax-structure formation in living plants.

### Figure 1

A CARS signal ($\omega_{\text{AS}}$) is generated, if A the frequency difference between $\omega_p$ and $\omega_s$ equals one vibrational mode $\Omega_{\text{vib}}$ of the sample and B the wavevectors $\vec{k}_p$, $\vec{k}_s$ and $\vec{k}_{\text{AS}}$ follow the phase matching condition $\vec{k}_{\text{AS}} = \vec{k}_p - \vec{k}_s + \vec{k}_p$.

### Results and Discussion

Leaves of three different plants were chosen for the present proof-of-concept investigation of their epicuticular waxes by means of CARS-microscopy based on the different appearances of their epicuticular wax layers. The evergreen nature of all three plants provides samples all over the year. *Hoya carnosa* (Hoya) provides a distinct wax layer, due to its origin in tropical regions, which can easily be seen on the surface of its leaves even without additional optical magnification. Hoya serves as the starting point for the study at hand *Prunus laurocerasus* (Cherry laurel) has been investigated well in the past (Jetter et al., 2000; Jetter et al., 2001; Perkins et al., 2005; Fiedler et al., in preparation). Thus, it is the second plant to be investigated. Most of the *P. laurocerasus* material for this study was grown in the green house of the Max Planck Institute for Chemical Ecology. The third plant investigated here is *Monstera* sp. aff. *deliciosa* Liebm. (ceriman/ Swiss cheese plant), a creeping vine native to...
tropical rainforests of Central America. It exhibits an epicuticular wax layer that gives rise to an intense Raman signal. Samples of *H. carnosa* and *M. sp. aff. deliciosa* Liebm. were collected from indoor plants, grown at IPHT. Prior to microscopic characterization the waxes were prepared as described in section Materials and Methods.

In order to select the wavenumber shift best suited for recording CARS images, Raman spectra of the individual wax layers were taken prior to CARS-microscopic experiments. At Raman shifts of 2845 and 2880 cm\(^{-1}\) dominant vibrational Raman-active resonances are found for the CH\(_2\) stretching vibrations of the epicuticular wax layers for either of the plants. These vibrations correspond to the symmetric (\(\nu = 2845\) cm\(^{-1}\)) and the non-symmetric (\(\nu = 2880\) cm\(^{-1}\)) CH\(_2\)-streching modes and due to their large Raman-scattering cross-section the respective Raman shifts were selected for CARS microscopy.

Figure 2 comprises the Raman spectrum of the cryo-extracted adaxial epicuticular wax of *H. carnosa* (Fig. 2A) in addition to the CARS images obtained for the respective wax layer. For comparison of the structural information obtained by CARS imaging with a well established method Figure 2 additionally depicts the SEM image of the extracted wax layer. The adaxial epicuticular wax layer of *H. carnosa* leaves reveals an even structure on the top in the F-CARS image (Fig. 2B). Below this structure the silhouette of a cell-shaped structure is visible. As is highlighted by the F-CARS image of a leaf cross section (Fig. 2C), this structure originates from the distribution of the cuticle around the epidermal cells. Furthermore, the CARS images reveal that the layer of wax is slightly rough. These fissures are parallel to each other and more abundant in the area of the leaf veins. To compare the CARS images in a more quantitative manner to the gold-standard for wax characterization, i.e. SEM (see below), the diameters of the irregularly cell-shaped structures have been estimated to 17 – 32 \(\mu\)m with a mean of 26 \(\mu\)m. They have been determined by the use of two wax samples including 86 calculated diameters with a standard deviation of 3.5 \(\mu\)m. This second structure of the epicuticular wax layer gives a stronger Raman signal than the first one, caused by the increased thickness of this cell shaped structure. The epi-CARS image (Fig. 2D) was taken simultaneously with the F-CARS image. It shows detailed structural information and a higher resolution of the second wax layer revealing a non-uniform signal.

Figure 2 Epicuticular wax of *Hoya carnosa*: (A) Raman spectrum of the extracted wax layer in the CH\(_2\)-stretching region upon excitation at 532 nm. (B) F-CARS image at a relative wave number of 2870 cm\(^{-1}\). (C) shows the wax layer and the cuticula in a cross section (imaged at a relative wave number of 2795 cm\(^{-1}\)) (D) epi-CARS image at a relative wave number of 2870 cm\(^{-1}\).
cm\(^{-1}\) and (E) SEM- image, the inset shows the cell-outline shaped second layer more detailed on a sample prepared upside down.

The structural information obtained from CARS-microscopy was measured against the conventional technique SEM. The SEM-images (Fig. 2E) show the upper layer of the structure only: It shows a smooth surface without any particles sticking out off the plane. It is slightly fissured as it already seen in the F-CARS-microscopic image. The presence of cell-wall shaped silhouettes of the second layer in the CARS image can be clearly confirmed in the SEM image of the wax layer, which was prepared upside down, as it is shown in Figure 2E (inset). For quantitative comparison with the CARS images, the mean diameter of 33 cell-shaped structures in two samples is calculated to 24.5 μm with a standard deviation of 4.2 μm, which is coherent with the results found for CARS (26 μm). The fissures, which have been observed in F-CARS microscopy, can also be seen in SEM. The mean distance between two adjacent fissures was calculated to 22 μm with a standard deviation of 5.1 μm in the CARS measurements using 166 individual measures within two samples. The same distance is found to be 23 μm in the SEM images (based on 50 individual measures within a single sample). In SEM the standard deviation has been calculated to 6.1 μm. These results highlight the capabilities of CARS for the imaging of epicuticular wax layers using the chemical properties of the waxes for image generation. Additionally by the use of F-CARS both layers were imaged seperately. The size calculations showed the same results for both, SEM and CARS illustrating not only the capability of CARS for a qualitative but also a quantitative analysis of epicuticular wax structures.

To verify the potential of using CARS microscopy to characterize the epicuticular wax layers of plants, analogous results were obtained from independent investigations of two other plant-leaf surfaces, i.e. the abaxial surfaces of *P. laurocerasus* and *M. sp. aff. deliciosa* Liebm. leaves. The results for *P. laurocerasus* are summarized in Figure 3. The Raman spectrum of the CH\(_2\)-strecthing vibration of the cryo-extracted epicuticular wax layer can be seen in Figure 3 A. The spectrum reveals two pronounced Raman bands at 2840 and 2880 cm\(^{-1}\), respectively. The F-CARS microscopic image (Fig. 3B) recorded in resonance with the lower wave number peak shows a hilly structure, where the hills are arranged in an irregular pattern. The extracted epicuticular wax layer exhibits circular areas, the stomata, where no resonant CARS signal can be detected. This observation is due to the fact that stomata of *P. laurocerasus* are not covered with wax and therefore no wax could be extracted. The results of the Epi-CARS measurements are in line with the findings in forward direction, but it is noticeable, that they
show an inverted image. This is due to the sample preparation method. The structural characteristics of the epicuticular wax layer found in the CARS investigations are reproduced by the SEM investigation. Here, instead of stomata the surface of the specimen holder can be seen through the holes in the wax layer. Therefore, again the structure that was obtained by the use of CARS-microscopy is verified by the structure observed in SEM. Furthermore, the inset of Fig. 3D shows a SEM image of an intact leaf, i.e. after freeze drying of the intact leaf. While it is important to show the direct comparability of CARS and cryo-extracted SEM images in order to proof the potential of CARS microscopy in investigating epicuticular wax layers, the comparison of Figs. 3D and the inset shows that cryo-extraction does not significantly disturb the spatial distribution of waxes. We also acknowledge that SEM images of leaf surfaces of living plants have been reported in the literature (Danilatos, 1981), however, such experiments are not feasible with the equipment in our labs.

**Figure 3** Cryo-stripped abaxial epicuticular wax of *P. laurocerasus*: (A) Raman spectrum excited at 532 nm. (B) F-CARS image at a relative wave number of 2820 cm$^{-1}$. (C) Epi-CARS image at a relative wave number of 2846 cm$^{-1}$. (D) SEM-image the inset shows a SEM after freeze drying of the intact leaf.

Finally, Figure 4 summarizes the results of our joint Raman-CARS-SEM study applied to the abaxial epicuticular wax structures of *M. sp. aff. deliciosa* Liebm.. The Raman spectrum (Fig 4A) shows the two bands of the CH$_2$-streaching vibration and the fingerprint region of the Raman spectrum. In the F-CARS image (Fig. 4B) the adaxial epicuticular wax layer exhibits a large amount of plates, which are partially arranged in parallel and show a swirly pattern. The plates are found to be about 7.5 μm wide and 1 μm thick. The leaf vein can be seen on the right side of the CARS image by recognition of the epidermal structure, which can be identified by the rather coarse grain structures on the right hand side of the image (see highlighting by arrow). The epi-CARS image (Fig. 4C) shows the same structural properties, which were observed in F-CARS. Many plates can be seen in the SEM image (Fig. 4D), which cover the whole epicuticular wax layer as also seen in CARS microscopy. They were measured to be 7.5 μm wide and 1 μm thick, which is in agreement with the results from the CARS-microscopic images. The crystal size was determined from the CARS image of two different samples with in total 24 crystals taken into account, while the statistics of the SEM images have been done using 105 crystals in three samples. The qualitative analysis and the pattern-size estimates of CARS and SEM images shown for *M. sp. aff. deliciosa* Liebm. reveal that CARS is capable of resolving the same structural information on a length scale of larger than ~ 500 nm, i.e. larger than the spatial resolution obtained by CARS microscopy.
Conclusions

Here a proof-of-concept study is presented aiming at establishing CARS, i.e. coherent anti-Stokes Raman scattering microscopy as a tool for investigating the epicuticular wax layer of green plants. The results, i.e. the chemical images obtained by CARS microscopy, are cross-referenced to the standard technique for epicuticular wax characterization, i.e. scanning-electron microscopy. The results presented demonstrate the feasibility to monitor epicuticular waxes by means of CARS microscopy. Such an approach will be useful in studying the formation of the wax structures and their formation and adaption to environmental stress. In the study at hand CARS microscopy is corroborated by Raman microspectroscopy focussing on the CH$_2$-stretch region of the spectrum, which is particularly useful for CARS microscopy. The resultant Raman spectra reveal very similar features for the different plants under investigation. This result points to the fact that in this particular spectral region of the Raman spectrum chemical discrimination between different wax components is hardly possible. Therefore, in future it will be necessary to image the spatial distribution of Raman-active bands in the fingerprint region of the spectrum. The localization of the different wax compounds by the use of the chemical selectivity of CARS microscopy will open the doorway to online monitoring of chemical and structural changes in wax structures. Thereby, novel insight into the defence processes of plants might be obtained. The results presented here pave the way for CARS microscopy to be used as a potential route to *in-vivo* investigation on epicuticular wax layers, which are one important protective barrier of the surface of all plants.

Materials and Methods

Cryo-stripping of epicuticular waxes

The epicuticular way layers were isolated from the surface of the leaves using a modified cryo-stripping method (Ensikat et al., 2000). Compared to the cryo-stripping method, we used water as freezing liquid. By the use of water, the washing step after the extraction could be omitted. A droplet of deionised water was put on a glass slide, and the leaf was placed with the surface of interest towards the water. Subsequently a small metal plate on top of the piece
of leaf closed the arrangement. The whole array was now dipped into liquid nitrogen for ten seconds. Afterwards the sample was removed from the frozen droplet, and the wax layer remained on the frozen water. After thawing and drying, the waxes could be investigated by CARS-microscopy and SEM.

Scanning Electron Microscopy

For SEM-investigations cryo-stripped wax layers were transferred to specimen holders of aluminium or alternatively brass and air dried subsequently. Afterwards the samples were sputtered with platinum. Examination of the samples was performed with a field emission scanning electron microscope JSM6300F (Joel – Tokyo, Japan) by using secondary electron contrast under various tilting angles.

CARS Setup

The basic experimental setup used in this study was presented in Meyer et al., 2008. The MIRA HP oscillator (Coherent) producing the pulse trails for the experiment was operated in picosecond mode to achieve spectral resolution in the order of 20 cm⁻¹. One part of the output of the ps-oscillator at 831 nm was directly used for the Stokes-pulses, while a second part was used to pump an optical parametric oscillator (OPO – APE, Berlin, Germany). The output of the OPO was tuned between 671 and 674 nm in order to cover relative wavenumber shifts in the range between 2820 and 2890 cm⁻¹, thus allowing for imaging the spatial distribution of Raman active CH₂ vibrations (ν_s(CH₂) and ν_as(CH₂)). Both the pump and the Stokes beam were spatially and temporally overlapped by means of a dichroic beam splitter and an optical delay line, respectively, and subsequently coupled into a laser scanning microscope (LSM – Zeiss, Jena, Germany). The CARS signal was collected in forward and in epi direction. For image generation each pixel was averaged eight times. This protocol resulted in image acquisition times of only 2.56 μs per pixel.

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