Vibrational Spectroscopy 65 (2013) 36–43

Contents lists available at SciVerse ScienceDirect

Vibrational Spectroscopy

journal homepage: www.elsevier.com/locate/vibspec

A multivariate statistical analysis approach to highlight molecular processes in plant cell walls through ATR FT-IR microspectroscopy: The role of the α-expansin *PhEXPA1* in *Petunia hybrida*

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ARTICLE INFO

Article history:
Received 25 January 2012
Received in revised form 9 November 2012
Accepted 13 November 2012
Available online 29 November 2012

PACS:
78.30 Jw
02.50 Sk

Keywords:
Mid-Infrared
FT-IR microspectroscopy
ATR
Multivariate statistical analysis
PCA
Heatmaps
Spectral analysis
Cell wall
Petunia
Expansin

ABSTRACT

Mid-infrared ATR FT-IR microspectroscopy is applied in conjunction with multivariate statistical analysis on petal samples of *Petunia hybrida* from wild-type and from two transgenic lines in which the *PhEXPA1* expansin gene expression was down-regulated and up-regulated, respectively. New insights are given on the role of expansin in the rearrangement of the cell wall polymer network. Measurements are done without any previous sample treatment to preserve the native cell wall structure and natural biological variability. An original combination of data analysis techniques is proposed to highlight molecular processes in plant cells, based on an automated spectra selection procedure, Principal Component Analysis, Wilcoxon rank sum test and heat map data representation.

1. Introduction

Fourier Transform Infrared (FT-IR) spectroscopy/microspectroscopy in the mid-infrared represents a rapid, easy to use and accurate non-destructive technique for the structural and molecular characterization of biological systems, which allows to determine the global composition of a given sample in one single experiment, including information on functional groups or bonds in the biochemical components such as proteins, lipids, nucleic acids and carbohydrates of the whole cell, cell wall and membranes [1]. Since the pioneer work of Naumann and coworkers, it is often used in conjunction with multivariate statistical analysis and pattern recognition methodologies when handling a very high number of spectra is required to account for biological variability [2–5].

FT-IR spectroscopy has also been quite extensively used in studies on plant cell walls, which contain a great variety of macromolecular compounds, including cellulose, hemicellulose and pectins [6–9].

In this work, we explore the potentialities of mid-infrared FT-IR microspectroscopy to highlight molecular processes in plant cell walls through an original combination of multivariate data analysis techniques.

An interesting and still open question is related to the role of expansins in cell wall metabolism and particularly in cell expansion. Expansins are cell wall proteins known to be involved in the control of cell enlargement and wall loosening [10–12], but the precise mechanism of action is still poorly defined. It appears that expansins could bind non-crystalline polysaccharides and enhance the hydrolysis of hydrogen bonds between cellulose microfibrils and cross-linking glycans, thus allowing cellulose fibres to slide relative to each other and facilitate cell expansion [13].

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http://dx.doi.org/10.1016/j.vibspec.2012.11.010

PhEXPA1 is the first expansin gene isolated from *Petunia hybrida* and it shows a high expression level in petals [14]. Petunia represents one of the most attractive and useful plant model systems since it offers several advantages among which the possibility to easily obtain transgenic plants and its well-characterized morphology suitable to investigate the role of expansin. Recently, two transgenic petunia lines, in which *PhEXPA1* expression was down-regulated, "antisense line", or up-regulated, "overexpressing line", were generated to study the *PhEXPA1* function in petal morphogenesis [14–16]. These previous results showed that expansin has a direct role in the determination of final organ size. It directly affects the size, but not the number, of petal cells, modifying the cell wall polymer composition, probably reflecting its complex role in cell wall metabolism.

Herein, we apply FT-IR microspectroscopy in Attenuated Total Reflection (ATR) mode to give new insights into the cell wall polymer rearrangement in petunia transgenic plants. Thanks to the advantages offered by ATR FT-IR spectroscopy, measurements are done directly on the hydrated petal samples of wild-type, down-regulated and up-regulated transgenic plants without any previous treatment, with the aim of preserving the native cell wall. To enhance spectral differences among the three plant types beyond the natural biological variability and facilitate the extraction of information concerning the underlying molecular processes, we propose an original and statistically reliable method based on an automatic spectra selection procedure combined with Wilcoxon rank-sum test and Principal Component Analysis together with a graphical heat map representation of the data.

2. Experimental

P. hybrida (var Mitchell) plants were genetically modified following the method fully described in [14,15]. Two lines of transgenic plants were generated: antisense (T−) and overexpressing (T+), for which the expression of *PhEXPA1* gene in petal was 4-fold down-regulated and 20-fold up-regulated compared with non-transgenic plants, respectively.

Twelve petal samples from six different flower limbs (two samples for each flower limb) were randomly chosen for the wild-type as well as for each of the two transgenic lines. Raw petals were directly put on the microscope sample holder without any preparation, in order to preserve the native cell wall structure. In fact, water can strongly affect cell wall properties: e.g. hydrated walls exhibits greater flexibility and extensibility than non-hydrated ones [17]. On the other side, sample treatment could alter cell wall structure by creating artefacts such as cross-bridges among cellulose microfibrils [18].

Mid-infrared spectra were acquired on the petal surface in ATR mode using a Vertex 70 Bruker spectrometer coupled to a Hyperion 3000 vis/IR microscope equipped with a photoconductive MCT detector and a 20× Germanium ATR-crystal objective. The ATR technique was chosen since it is intrinsically more sensitive to modifications in the plant cell wall due to the reduced photon beam penetration depth [3]. For each petal sample, at least 10 point by point spectra were acquired in the 4000–900 cm^{-1} range at 4 cm^{-1} resolution on a 100 μm × 100 μm area by co-adding 64 scans (27 s of acquisition time).

A typical single point absorbance spectrum is reported in Fig. 1 to show the quality of the original acquired experimental data previous to any further data treatment.

3. Data treatment and analysis

Absorbance spectra were corrected for the wavelength dependence of penetration depth inside the sample. Data analysis was performed in the 1800–900 cm^{-1} wavenumber range, where we found the most significative differences among spectra. The single point absorbance spectra were baseline corrected by the rubber-band method and area normalized in this range using the Bruker OPUS 6.0 software. For each plant type, an average spectrum was also obtained, as shown in Fig. 2.

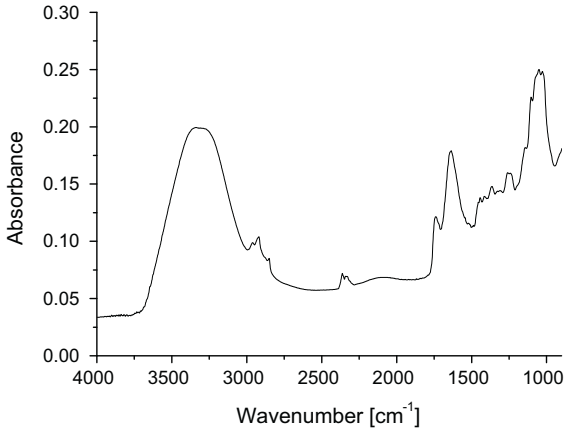

Fig. 1. An experimental absorbance spectrum obtained on the petal sample of a wild-type plant.

Subsequent multivariate analysis combined three main steps, as described in the following. Toward this aim, the R software environment [19] was utilized using the available statistical packages (stats, amap, gplots [20,21]).

3.1. PCA and Wilcoxon rank-sum test

To evaluate the statistical meaning with respect to the underlying populations of the spectral differences among the three plant types visible in the average spectra, we applied a two-step approach on the original set of spectra and, afterwards, on the spectra selected as described in the next section. We followed the method of first performing a Principal Component Analysis (PCA) on the single point spectra of WT vs. T−, WT vs. T+and T− vs. T+and then a two sample Wilcoxon rank-sum test [22,23], equivalent to the Mann–Whitney *U* test, on the scores of the corresponding first Principal Components (PC1).

The PCA analysis was carried out by separately mean-centering each feature vector, corresponding to a single spectral channel across all samples. PCA [24] produces score and loading plots in an

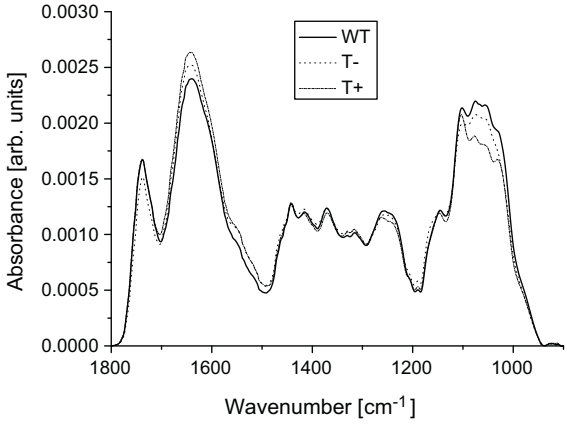

Fig. 2. Average absorbance spectra for wild-type (solid) plants and for the overexpressing (dashed) and antisense (dotted line) transgenic lines. Averages have been calculated over all the collected spectra.