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Comparison of Photoacoustic Signals in Photosynthetic and Nonphotosynthetic Leaf Tissues of Variegated *Pelargonium zonale*

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Abstract Green-white variegated leaves of *Pelargonium zonale* were studied using the photoacoustic method. Our aim was to characterize photosynthetically active green tissue and nonphotosynthetically active white tissue by the photoacoustic amplitude signals. We observed lower stomatal conductance and higher leaf temperature in white tissue than in green tissue. Besides these thermal differences, significantly higher absorbance in green tissue was based on chlorophyll and carotenoids which were absent in white tissue. However, optical properties of epidermal layers of both tissues were equal. The photoacoustic amplitude of white tissue was over four times higher compared to green tissue, which was correlated with lower stomatal conductance. In addition, at frequencies >700 Hz, the significant differences between the photoacoustic signals of green and white tissue were obtained. We identified the photoacoustic signal deriving from photosynthetic oxygen evolution in green tissue, using high intensity of red light modulated at 10 Hz. Moreover, the photoacoustic amplitude of green tissue increased progressively with time which corresponded to the period of induction of photosynthetic oxygen evolution. For the first time, very high frequencies (1 kHz to 5 kHz) were applied on leaf material.

Keywords *Pelargonium zonale* · Photoacoustic method · Photosynthesis · Stomatal conductance · Thermal imaging · Variegated plants

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1 Introduction

In plants, light energy absorbed by photosynthetic pigments, carotenoids and chlorophylls (*chls*), is divided between the following competitive processes: photochemistry, thermal dissipation and chlorophyll fluorescence [1]. The main photosynthetic pigments, *chl a* and *chl b*, absorb in blue (absorbance maximum around 430 nm and 453 nm) and red spectral region (absorbance maximum around 642 nm and 662 nm), while carotenoids have an absorbance maximum in 440 nm to 480 nm range [2].

The photoacoustic (PA) signal from leaves is a combination of photothermal (PT) and photobaric (PB, photosynthetic oxygen evolution) contributions at a given modulation frequency [3]. The PT contribution presents an acoustic wave produced by a periodic heat flow from the leaf, while the PB contribution is produced by the periodic O_2 evolution. Important contributor to PT in green leaves is nonradiative de-excitation of photosynthetic pigments, carotenoids and *chls* [4–6]. In addition, the PT component of the PA signal may be affected by light scattering inside the PA cell, and by internal light scattering within the leaf, which alters the temperature distribution in the sample [7].

Photoacoustic technique can provide information on energy transfer processes within the pigment systems, photochemical energy storage, the extent and dynamics of the gas evolution and uptake in the leaves, and quantum yield of photosynthesis [3–6,8–11]. The study of photosynthesis *in vivo* and *in situ* using the PA technique is a fruitful field and has been extensively explored by research group of Professor Vargas using the so-called "Open Photoacoustic Cell" (OPC) [3,10,12,13], but also in conventional PA cells on pieces of detached leaves [14–18].

The advantage of PA technique is detection of signals at different depths of the sample by modulating the frequency of excitation light; the maximum depth of the solid sample from where the heat emission is detected decreases with an increase of modulation frequency [6,16,17]. However, unlike solid materials, leaves consist of layers differing in thermal (i.e., conductance, density) and optical properties (i.e., transparency, scattering), which specifically determine the PA signal at a given frequency. At low modulation frequencies ($<$100 Hz), the PA signal is related to O_2 evolution, while at higher frequencies ($>$200 Hz) the PT component becomes more dominant, since the O_2 evolution is a relatively slow process and the PB contribution is damped [3,6]. Taking this into account, the frequency dependence of the PA signal has been applied to monitor stress-induced alterations on photosynthetic apparatus, such as heavy metal excess [17,18] or water stress [14]. In addition, changing the modulation frequency, intensity and spectral region of excitation light allows pigment analysis in different leaf layers [15,16].

Variegated *Pelargonium zonale* is a suitable model system to examine "source–sink" interactions in relation to photosynthesis within the same leaf, providing identical morphological characteristics and microenvironment conditions. The same model has been used for studying differences in sugar composition and phenolic metabolism [19], as well as nitrogen metabolism [20,21] in photosynthetically active (green tissue—GT) and photosynthetically nonactive leaf tissues (white tissue—WT).

Here we monitored the PA signals of intact green and white areas of the same leaf: (i) using low and high intensities of white and red light; (ii) over a wide range of

modulation frequencies (10 Hz, and from 50 Hz to 5000 Hz) and (iii) in real time (up to 30 min). Our aim was to differentiate and characterize the parameters of the PA signal deriving from photosynthetic activity by comparing the PA response of GT and WT of variegated leaves.

2 Materials and Methods

2.1 Plant Material and Growth Conditions

The variegated *Pelargonium zonale* cv. "Ben Franklin" plants were obtained from Fir Trees Pelargonium (Stokesley, Nort Yorkshire, UK). This cultivar is a periclinal chimera with white margins and green centre of the leaf (Fig. 1). Green leaf tissue (GT) has functional chloroplasts with well-organized thylakoids, while mesophyll cells of white tissue (WT) have smaller plastids without thylakoids and starch granules [19]. Six months prior to the experiment, the plantlets were propagated vegetatively. The soil used for propagation was Klasman-Potgrond H (Klasmann-Deilmann GmbH, Geeste, Germany). Plants were grown under 300 $\mu mol \cdot m^{-2} \cdot s^{-1}$ of photosynthetically active radiation (400 nm to 700 nm) under 14/10 h day/night photoperiod, 26/18 °C day/night temperature and relative humidity of 60 % to 70 %.

2.2 Pigment Extraction

Green and white leaf tissues (four leaves per plant, from four different plants) were powdered in liquid nitrogen, and pigments were extracted in chloroform (1:25, w/v) overnight on a shaker at 4 °C. Following centrifugation, the samples were reextracted and the supernatants combined. The absorbance spectra of chloroform extracts were recorded at 25 °C using a temperature-controlled spectrophotometer (Shimadzu, UV-160, Kyoto, Japan).

Fig. 1 Photos of representative variegated *P. zonale* plants

2.3 Measurements of Stomatal Conductance

Direct readout of stomatal conductance was done using a porometer (AP4, Delta-T, Cambridge, U.K.). Stomatal conductance was recorded on GT and WT from ten leaves per plant, from ten plants in total, under light and dark conditions.

2.4 Thermal Imaging

Thermal images were obtained using an infrared camera FLIR T335 (FLIR Systems, Wilsonville, OR, USA). Infrared thermal images were taken on seven fully expanded leaves from seven plants between 9:30 a.m. and 10:30 a.m. (2 h after onset of light) and in the dark. Infrared digital images were subsequently analysed for temperature determination using the FLIR Quick Report 1.2 SP2 software. The software provides the function of measuring spot, line and area. In this study, a polygon area was drawn to measure the overall leaf temperature.

2.5 Photoacoustic Apparatus and Procedure

The used experimental setup consisted of an Optical & Acoustical part (optical source, PA cell) and Signal processing & Control unit (modulator, lock-in amplifier and computer) and was recently schematically presented by Todorović et al. [22]. The so-called OPC with transmission detection configuration was used to minimize the PA cell acoustic (detection) volume, and increase the PA response [3,10]. The miniature electret microphone had a cylindrical aluminium body with small circular holes as the sound inlets. The samples (leaf discs of GT and WT, 10 mm in diameter) together with the sample holder were immediately mounted onto a front surface of the microphone with two o-rings which enabled the sample to be acoustically close to the cell. Only a small part of the sample surface (smaller than 15 %) was used for "fixing". About 30 s after mounting, the samples were excited by an electronically modulated high-power LED on one side, and the PA response was detected on the opposite side, as described by Todorović et al. [22]. The PA cell was designed to minimize the possible parasitic acoustic signal from the light that reached the membrane of the microphone, since more than 90 % of the transmitted light would be reflected from the front surface of aluminium microphone body.

The PA signals of GT and WT were measured under different conditions:

(a) PA signal versus frequency—the recordings with modulated white LED (wavelength 400 nm to 700 nm) started after the signal reached a steady state (approximately 2 min), and lasted 15 min. The repeatability of the measurement was checked using segments from three different leaves. In order to check the stability of the PA signal and exclude the effect of leaf drying, we performed two consecutive measurements of the same sample. The frequency spectrum of the PA signal was measured during 15 min (first measurement), then the measurement of the PA signal continued, without opening the PA cell, for the next 15 min (the

second measurement). There was no significant change in leaf disc relative water content during the first and the second measurement;

(b) PA signal versus time—the PA signals versus time in the GT and WT were measured using red light (LED, wavelength 640 ± 20 nm), at 10 Hz modulation frequency, and under two optical intensities: low, 10 W· m^{-2}, and high, 100 W· m^{-2}, (L10 and L100, respectively);

(c) PA signal under different wavelengths (white light and red light)—two modulated optical excitation sources were used: white LED and red LED.

Before PA measurements, the plants were kept at low light (100 μmol· m^{-2}· s^{-1}). Three discs of each tissue type from three different plants were used for each measurement.

2.6 Microscopic Analysis

Optical microscopic examination of isolated epidermal strips from both leaf tissues was performed using Olympus BX41 light microscope equipped with Olympus C7070 camera (Olympus Optical Co., Hamburg, Germany). Chlorophyll autofluorescence within the chloroplasts was detected with mercury lamp and blue filter (Olympus).

2.7 Statistical Analysis

Significant differences between GT and WT stomatal conductance and leaf temperature were tested using Mann–Whitney *U* test with IBM SPSS statistics software (Version 20.0, SPSS Inc., Chicago, USA). Significance threshold value was set at 0.05.

3 Results and Discussion

Absorption spectra of GT chloroform extracts had three absorbance maximums, at 432 nm, 457 nm and 665 nm, deriving from both, *chl a* and *chl b* (Fig. 2). In addition, absorption peak at 331 nm and in 250 nm to 260 nm range were observed, deriving from phenolic compounds. We have previously reported that the total content of hydroxycinnamates and flavonoids, which absorb in the spectral range between 300 nm and 400 nm, was similar in both tissues under optimal light conditions [19]. Thus, higher absorbance in this region in GT originated from *chl*, which was about 37-fold higher compared to WT (Fig. 2). This was in accordance with the absence of thylakoid structures and proteins associated with photosynthetic processes in white cell plastids [19].

Besides differences in optical properties (optical absorption and spectral characteristics), GT and WT differed in their thermal properties. The results obtained by thermal imaging showed 2 °C higher temperature in WT than in GT under optimal light conditions, while this difference was less pronounced in dark-adapted plants (Fig. 3). Leaf temperature depends on transpiration (related to stomatal conductance),

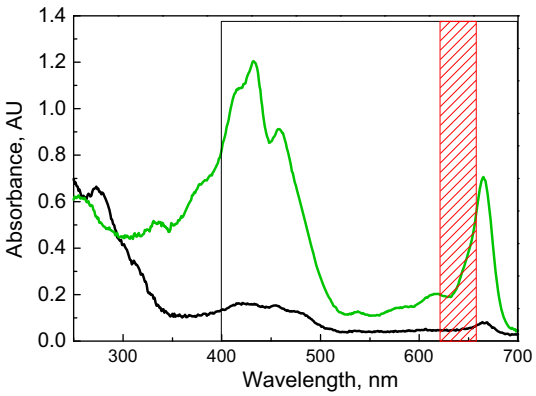
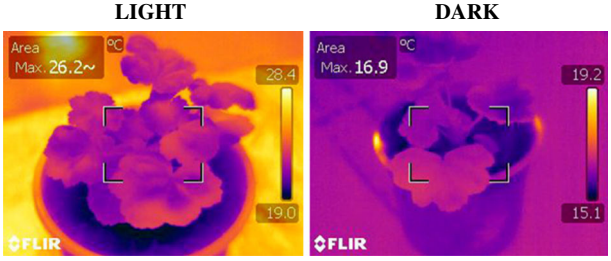


Fig. 2 Absorption spectra of white tissue extract (*black*) and *green* tissue extracts (*green*). For better presentation, green extracts were five times diluted. Spectral ranges of white and red light are marked with *white square* and *red patterned square*, respectively (Color figure online)



Green	20.6 ±.10	16.5 ± 0.1
White	22.5 ± 0.1	17.1 ± 0.1

Fig. 3 Thermal images of *P. zonale* leaves at 300 µmol· m^{-2}· s^{-1} and in the dark ($n = 7$) showing higher leaf temperature (°C) in white, compared with green leaf tissue. Significant differences ($P < 0.001$) were observed between the two tissues under both light conditions (according to Mann–Whitney U test) (Color figure online)

Table 1 Stomatal conductance (g_s, mmol·m^{-2}· s^{-1}) of green tissue (GT) and white tissue (WT) of *P. zonale* leaves determined at light (300 µmol·m^{-2}· s^{-1}) and in the dark ($n = 10$)

	GT	WT
Light	579.7 ± 77.8**	158.5 ± 19.7
Dark	326.1 ± 47.3*	106.1 ± 10.2

Significant differences between the two tissues according to Mann–Whitney U test are marked. *$P < 0.05$, **$P < 0.01$

photosynthesis, and other metabolic processes [23,24]. We observed more than three-fold lower stomatal conductance in WT compared to GT under both light and dark conditions (Table 1), which significantly contributed to higher temperature in WT. Similarly, higher temperature related to lower stomatal conductance were previously described in Arabidopsis plants [25,26].

In order to further characterize the two leaf tissues, the PA signals of leaf discs were recorded over a wide range of frequencies (from 50 Hz to over 5000 Hz) under white light (Fig. 4). We performed two consecutive measurements of the PA signals (0 min

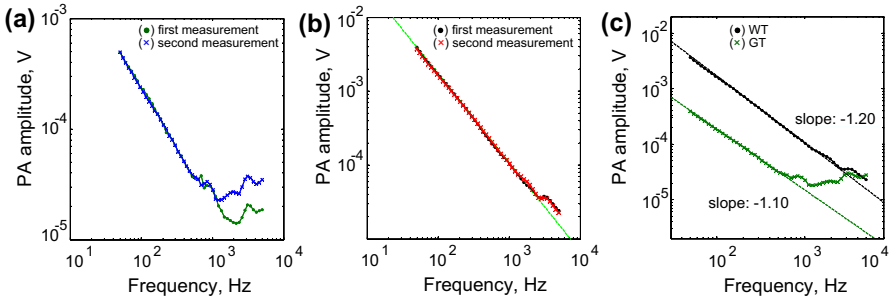


Fig. 4 The amplitude of the PA signal versus the modulation frequency. The first and the second measurement: (a) of green leaf tissue and (b) of white leaf tissue; (c) the comparison of the PA signals of the second measurements of green tissue (GT) and white tissue (WT). The PA amplitudes present mean values of three leaf segments of both tissues. The measuring conditions: white LED; optical intensity level L10 (Color figure online)

to 15 min, and 15 min to 30 min) to test for the sample stability (Fig. 4a, b). After 15 min, the second PA signal below 700 Hz was practically the same for both types of tissues, and therefore, in this region it could be considered stable, i.e. it depended only on the frequency. However, only at frequencies above 700 Hz in GT the difference between the two measurements became more apparent. We suggest that photosynthetic induction due to biochemical and stomatal limitations may be responsible for obtained differences between the first and the second measurements in green leaf tissue [11,27].

Although the leaf samples were cut from the plant, potential 'stress' effects during the measurements were limited to the disc edge, and not to the central, active surface. The significant artefact effect on the PA signal, such as leaf drying, was noted only after more than 30 min from the start of measurement (relative water content decreased by about 15 %), similarly as obtained by Havaux and colleagues [9]. In addition, it should be noted that leaves are highly scattering material, and the recorded PA amplitudes are higher than for the solid samples with the same absorption [7]. Considering that GT and WT originated from the same leaf, there should be no differences in light scattering between the two tissues.

The PA amplitude in both GT and WT decreased with increasing frequency. The slope of the averaged PA amplitude curve (log–log) is defined as $amp = C \cdot f^s$, where C is a constant, f is the frequency and s is the slope. The slopes of the PA amplitudes of both samples were similar for frequencies <700 Hz: for GT it was $s = -1.10$, and for WT it was $s = -1.20$ (Fig. 4c). This behaviour of the PA signal was characteristic for the PT component of the PA signal as observed in tobacco leaves, in which the amplitude of PT signal linearly decreased with increasing frequency [9]. The comparison of GT and WT samples showed that the PA amplitude of WT is over four times greater than that of GT at frequencies below 700 Hz. In another study with green and white leaves, Pereira et al. [12,13] demonstrated that the total PA signal was almost threefold higher in white maize leaves compared to green ones. This was attributed to higher light transparency of white leaves, resulting in illumination of microphone membrane, which may have additionally contributed to the PA signal. However, in our measurement system, the light that passed throughout the sample could not reach the membrane of the microphone, since more than 90 % of the transmitted light would

be reflected from the front surface of aluminium microphone body. Therefore, we excluded the possible contribution of a parasitic PA signal.

Interestingly, the linear decrease of the PA signal in GT stopped above 700 Hz, and the signal intensity became equal with WT at 5000 Hz (Fig. 4c). This change indicated the existence of a different mechanism of PA signal generation in GT at high modulation frequencies. These results could not be directly compared with literature data, since the PA spectra of green leaves at such high frequency (5000 Hz) have not been published so far to our knowledge. At high modulation frequencies (>500 Hz), the PA signal is influenced by the absorption properties of the epidermis alone [15,28]. In order to compare the optical properties of epidermal layers of both leaf tissues, we analysed their epidermal strips (Fig. 5). Since epidermises of both leaf tissues of *P. zonale* had similar optical properties (number and size of stomata and *chl* content), differences in PA signals above 700 Hz might be related solely to their differential thermal properties (Table 1; Fig. 3).

We compared the PA signals of GT and WT after normalization (PA amplitude of GT was divided with PA amplitude of WT) (Fig. 6). Furthermore, to distinguish the signals deriving from photosynthesis, we used red light of different intensities (L10 and L100) modulated by low frequency (10 Hz) which allows mesophyll tissue analysis (Fig. 7). Under L10, the PA signals from both tissues were similar, while under L100, the PA signal of WT was higher than GT, and it was unchanged over

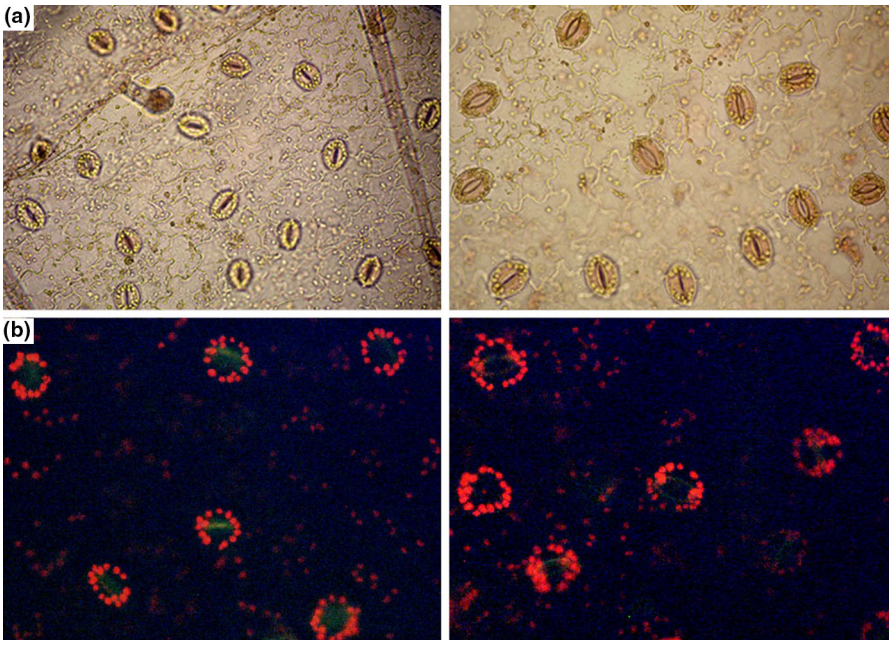


Fig. 5 Micrographs of adaxial epidermal strips of green (*left*) and white (*right*) leaf segments of *P. zonale*. (a) Photos observed by light microscopy (×10 magnification) after staining with 0.01 % neutral red (3-amino-7-dimethylamino-2-methylphenazine hydrochloride) at pH 5.6. (b) Autofluorescence of *chls* in chloroplasts of stomata guard cells (×20 magnification) (Color figure online)

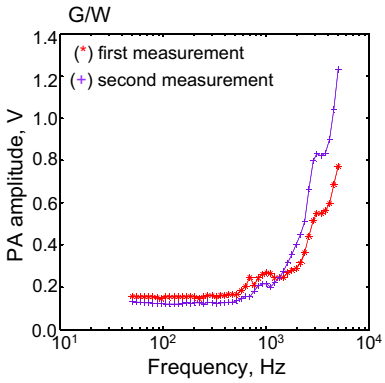


Fig. 6 The normalized PA amplitude (green tissue/white tissue, G/W, amplitude ratio) versus the modulation frequency (the measuring conditions: LED white; optical intensity level L10; the first and the second measurement) (Color figure online)

time. The use of high intensity of red light at low frequency enabled monitoring photosynthetic oxygen evolution in GT compared to WT, as it was demonstrated for green and white maize leaves [12,13]. In accordance with the degree of O_2 evolution, the PA signal of GT was higher at L100 (0.007 AU) compared to L10 (0.0038 AU). Photosynthetic rate is directly proportional to light intensity, until it becomes constant and maximal CO_2 assimilation is achieved [29]. Analysis of light-response curve of green tissue of variegated *P. zonale* showed that light intensity of 300 $\mu mol \cdot m^{-2} \cdot s^{-1}$ enabled maximal photosynthetic efficiency (Fig. 8). At the higher level of excitation, the PA amplitude of GT increased progressively with time, until it reached a maximum level and the steady PA signal was achieved. Bults et al. [11] showed a similar PA signal increase in excited dark-adapted tobacco leaves, and explained this response as a period of induction of photosynthetic oxygen evolution.

In WT, the PA signal was enhanced almost 10 times with increase of excitation light intensity (Fig. 7), which might derive from tissue heat release. In contrast to GT samples, the PA signal of WT at L100 modulated at 10 Hz was constant over time. The

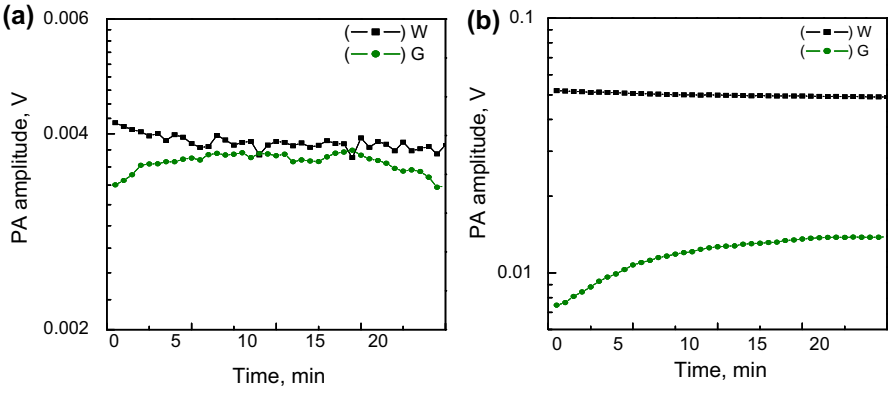


Fig. 7 The amplitude of the PA signal versus time in the green (G) and white (W) leaf tissue (LED red, 640 ± 20 nm, at modulation frequency 10 Hz) for two optical intensities: (a) L10, 10 $W \cdot m^{-2}$; and (b) L100, 100 $W \cdot m^{-2}$ (Color figure online)

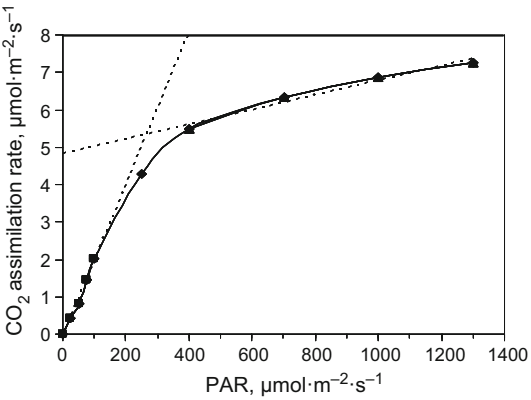


Fig. 8 The CO_2 assimilation rate as a function of photosynthetically active radiation intensity (25 μmol· m^{-2}· s^{-1} to 1300 μmol· m^{-2}· s^{-1})

PA signals of leaf discs depigmented in ethanol recorded under the same conditions were similar for WT and GT, decreasing slowly and reaching a steady state during 20 min (not shown). In this way, we excluded the possible effect of structural differences (i.e., leaf thickness) on PA signals of white and green leaf areas and confirmed that in GT, photosynthesis predominantly influenced the behaviour of the PA signal.

4 Conclusion

The photoacoustic signals of leaves were measured for the first time in the high frequency range (from 1000 Hz to 5000 Hz). Distinct frequency dependence of PA signals of both chlorophyllous and nonchlorophyllous tissues was observed above 700 Hz. Increased PA signal under high red light intensity at 10 Hz was mostly attributed to O_2 evolution in green tissue, while in white tissue it was related to heat release and significantly lower stomatal conductance. Further work should be focused on characterization of PA signal originated solely from photosynthetic activity, by correlating the PA signals obtained under saturating background light with simultaneously monitored photosystem II fluorescence parameters. The future goal should be quantification of specific PA signals related to photosynthetic electron transfer.

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References

1. N.R. Baker, Annu. Rev. Plant Physiol. **59**, 89 (2008)
2. A.R. Wellburn, J. Plant Physiol. **144**, 307 (1994)
3. A.C. Pereira, M. Zerbelto, G.C. Silva, H. Vargas, W.J. da Silva, G. de Neto, N. Cella, L.C.M. Miranda, Meas. Sci. Technol. **3**, 931 (1992)
4. C. Buschmann, H. Prehn, in *Modern Methods of Plant Analysis*, vol. 11 ed. by H.-F. Linskens, J.F. Jackson (Physical Methods in Plant Sciences), (Springer, Berlin, 1990), p. 148
5. H.J. Hou, T.P. Sakmar, Sensors **10**, 5642 (2010)
6. C. Buschmann, H. Prehn, H.K. Lichtenthaler, Photosynth. Res. **5**, 29 (1984)

7. P. Helander, I. Lundström, D. McQueen, J. Appl. Phys. **51**, 3841 (1980)
8. S.K. Herbert, T. Han, T.C. Vogelmann, Photosynth. Res. **66**, 13 (2000)
9. M. Vargas-Luna, L. Madueño, G. Gutiérrez-Juárez, J. Bernal-Alvarado, M. Sosa, J.L. González-Solís, S. Sánchez-Rocha, V. Olalde-Portugal, J.J. Alvarado-Gil, P. Campos, Rev. Sci. Instrum. **74**, 706 (2003)
10. W.J. da Silva, L.M. Prioli, A.C.N. Magalhaes, A.C. Pereira, H. Vargas, A.M. Mansanares, N. Cella, L.C.M. Miranda, J.J. Alvarado Gil, Plant Sci. **104**, 177 (1995)
11. G. Bults, B. Horwltz, S. Malkin, D. Cahen, Biochim. Biophys. Acta **679**, 452 (1982)
12. A.C. Pereira, L.M. Príoli, W.J. da Silva, G.O. de Neto, H. Vargas, N. Cella, J.J. Alvarado Gil, Plant Sci. **96**, 203 (1994)
13. A.C. Pereira, J.J. Alvarado-Gil, O. Zelaya, H. Vargas, N. Cella, J. Phys. IV **4**, C7–527 (1994)
14. M. Havaux, O. Canaani, S. Malkin, Plant Physiol. **82**, 827 (1986)
15. C. Buschmann, Anal. Sci. Suppl. **17**, s334 (2002)
16. M. Havaux, K. Kloppstech, Planta **213**, 953 (2001)
17. A. Baryla, P. Carrier, F. Franck, C. Coulomb, C. Sahut, M. Havaux, Planta **212**, 696 (2001)
18. G. Ouzounidou, Plant Sci. **113**, 229 (1996)
19. M. Vidović, F. Morina, S. Milić, A. Albert, B. Zechmann, T. Tosti, J.B. Winkler, S. Veljović-Jovanović, Plant Physiol. Biochem. **93**, 44 (2015)
20. G. Tcherkez, F. Guérard, F. Gilard, M. Lamothe, C. Mauve, E. Gout, R. Bligny, Funct. Plant Biol. **39**, 959 (2012)
21. C. Abadie, M. Lamothe, C. Mauve, F. Gilard, G. Tcherkez, Funct. Plant Biol. **42**, 543 (2015)
22. D.M. Todorović, M.D. Rabasovic, D.D. Markushev, M. Sarajli, J. Appl. Phys. **116**, 053506 (2014)
23. H.G. Jones, Plant Cell Environ. **22**, 1043 (1999)
24. K. Omasa, K. Takayama, Plant Cell Physiol. **44**, 1290 (2003)
25. L. McAusland, P.A. Davey, N. Kanwal, N.R. Baker, T. Lawson, J. Exp. Bot. **64**, 4993 (2013)
26. J.M. Costa, O.M. Grant, M.M. Chaves, J. Exp. Bot. **64**, 3937 (2013)
27. O. Urban, M. Košvancová, M.V. Marek, H.K. Lichtenthaler, Tree Physiol. **27**, 1207 (2007)
28. E.M. Nagel, C. Buschmann, H.K. Lichthenthaler, Physiol. Plant. **70**, 427 (1987)
29. C.H. Foyer, J. Neukermans, G. Queval, G. Noctor, J. Harbinson, J. Exp. Bot. **63**, 1637 (2012)