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Chlorophyll fluorescence of *Nicotiana tabacum* expressing the green fluorescent protein

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Abstract

Evidence that the green fluorescence protein (GFP) develops a significant toxicity in plants has not been found, but it may represent a source of free radicals as a consequence of its fluorescence. In addition, green light is known to trigger the acclimatisation of the photosynthetic system towards a shady environment. Moreover, the light-harvesting system may acclimate to an increased availability of green light. Each of these effects may be induced by the GFP. Therefore, the hypothesis was tested, whether transformation of *Nicotiana tabacum* cv. Bursan to express the GFP could affect chlorophyll fluorescence parameters. The analysis revealed a significantly lower absorption of energy per excited cross section in GFP-transformed tobacco, a lower number of active reaction centres per excited cross section, a larger absorption and trapped energy flux leading to the reduction of the primary quinone electron acceptor of PSII per reaction centre, and a lower variable fluorescence.

Additional key words: low-light stress; OJIP transient; steady-state procedure; toxicity of GFP.

Introduction

The green fluorescent protein (GFP) is a protein composed of 238 amino acid residues (26.9 kDa) that results in green fluorescence when exposed to light in the blue to ultraviolet range (Tsien 1998). It is characterized by a major excitation peak at 395 nm and a minor one at 475 nm. Excitation at 395 nm results in emission peaking at 509 nm, whereas excitation at 475 nm corresponds to a maximum at 503 nm (Tsien 1998, BioTek Instruments 2006).

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Abbreviations: ABS/CS_0 – absorption of energy per excited cross section of a photosynthesizing sample (CS_0) in the dark-adapted state ≈ F_0 – minimum fluorescence when all reaction centres are open at the onset of fluorescence induction; ABS/CS_m – absorption of energy per excited cross section of a photosynthesizing sample (CS_m) at maximum fluorescence ≈ F_m – maximum fluorescence when all reaction centres are closed; ABS/RC – absorption flux of antenna Chls per reaction centre (RC); BAP – 6-benzylaminopurine; Chl – chlorophyll; CS_0 – cross section of a photosynthesizing sample in the dark-adapted state; CS_m – cross section of a photosynthesizing sample at maximum fluorescence; ET_0/CS_m – electron transport flux further than Q_A^- per CS_m; ET_0/RC – electron transport flux further than Q_A^- per RC; F_0' – minimum chlorophyll fluorescence in the light-adapted state; F_m' – maximum fluorescence under actinic light; F_t – stationary fluorescence; F_v – variable fluorescence = $F_0 - F_m$; F_v' – variable fluorescence in the light-adapted state = $F_0' - F_m'$; GFP – green fluorescence protein; IAN – 3-indolylacetonitrile; MS medium – Murashige-Skoog medium; NPQ – Stern-Volmer nonphotochemical quenching parameter; PAM – pulse-amplitude modulation; PI_{ABS} – performance index for energy conservation of photons absorbed by PSII to the reduction of intersystem electron acceptors; PI_{Total} – performance index for energy conservation from photons absorbed by PSII to the reduction of PSI end acceptors; Q_A – the primary quinone electron acceptor of PSII; q_L – estimate of the fraction of open PSII reaction centres; q_P – photochemical quenching coefficient; RC – reaction centre; RC/CS_m – number of active reaction centres per excited cross section; RE_0/CS_m – electron flux reducing end electron acceptors at the PSI acceptor side per CS_m; RE_0/RC – electron flux reducing end electron acceptors at the PSI acceptor side per RC; R_{Fd} – fluorescence decrease ratio; TR_0/CS_m – trapped energy flux leading to Q_A reduction per CS_m; TR_0/RC – trapped energy flux leading to Q_A reduction per RC; δ_{R0} – probability with which an electron from the intersystem electron carriers is transferred to reduce end electron acceptors at the PSI acceptor side; φ_{E0} – quantum yield for electron transport; φ_{P0} – maximum quantum efficiency of PSII; φ_{PSII} – actual photochemical quantum yield; φ_{R0} – quantum yield for the reduction of end electron acceptors at the PSI acceptor side; ψ_0 – probability (at t = 0 μs) that a trapped exciton moves an electron into the electron transport chain beyond Q_A.
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GFP is frequently used as a proof-of-concept assuring a successful genetic transformation process by expressing the corresponding gene. In comparison to other marker genes it does not require addition of any exogenous substrates or enzymes. The GFP is produced soon after a successful transformation of cells so that early growth stages of callus or recovered transgenic plants can be monitored *in vivo* without the necessity to destructively analyse the transformed plant material. However, using the GFP as a marker is also associated with distinct limitations, first of all its low level of expression in plants (Malabadi *et al*. 2008). Some authors suggest a significant toxicity of the GFP in plants, especially, when the expression level is high, while many other researchers concluded in more recent studies, summarized by Stewart (2001) and Malabadi *et al*. (2008), that the GFP is not cytotoxic in plants. Here, the question is addressed, whether the GFP itself might affect photosynthesis parameters assessed with chlorophyll (Chl) fluorescence measurements, another standard method to detect plant stress influencing the light reactions of photosynthesis. Two different strategies for the assessment of Chl fluorescence are here examined, the 'OJIP' Chl transient and the saturation pulse measurement in combination with the steady-state fluorometry measured with the *PAM-2500* (*Heinz Walz GmbH*, Effeltrich, Germany).

Fast dynamic changes in Chl *a* fluorescence occur, when dark-adapted leaves are exposed to a saturating light pulse, this induction being known as Kautsky effect. From the fluorescence rise, which is polyphasic, information about the efficiency of electron transport through PSII can be extracted (Stirbet *et al*. 2018). Here, fluxes at the onset of fluorescence induction are designated by the subscript '0', whereas the fluorescence levels are determined based on specific times during the transient, *i.e*., for OJIP: $F_O = F_{0ms}$, $F_J = F_{2.5ms}$, $F_I = F_{40ms}$, and $F_P = F_m$. Strasser *et al*. (2010, 2004, 2000) developed the so-called 'OJIP'-analysis, offering simple equations for energy fluxes for light absorption (ABS), trapping (TR) of excitation energy and electron transport (ET, RE) as well as quantum yields and efficiencies of energy absorption by PSII antenna pigments, energy trapping and intersystem electron acceptors to the end electron acceptor at PSI acceptor side by analysing fluorescence induction transients that occur during the application of a 0.6-s saturation pulse to dark-adapted leaf samples.

In case of the steady state measurements, leaf samples are illuminated at a predetermined light intensity for *ca.* 5 min, frequently after dark adaptation and a subsequent saturation pulse, when a 'classical' steady-state procedure is applied (Schreiber *et al*. 1986). The Chl *a* fluorescence in the steady state is affected by the redox state of the electron transport chain, especially of Q_A, the formation of a transthylakoid ΔpH, xanthophyll cycle activity, antenna size changes, photoinhibition, and/or the activation of ferredoxin $NADP^+$ reductase (Kalaji *et al*. 2014). With the classical quenching analyses, it is possible to distinguish between photochemical quenching (q_P) and nonphotochemical quenching (NPQ) under a certain light regime, which is selected by the scientist with respect to the examined hypothesis.

The present study reports on the results of Chl fluorescence analyses using the 'OJIP' Chl transient and the steady-state fluorometry of genetically modified tobacco plants expressing the GFP in order to test the hypothesis that the GFP does not affect Chl fluorescence measurements.

Materials and methods

***Agrobacterium*-mediated transformation with tobacco leaf disks**: Leaf disks were obtained from four-week-old plantlets of tobacco (*Nicotiana tabacum* cv. Bursan) grown *in vitro*. These explants were transformed with *Agrobacterium tumefaciens* strain ABI containing a binary plasmid pMON30069 with the neomycin phosphotransferase gene and the green fluorescent protein gene driven by the P-e35S promoter from cauliflower mosaic virus (Troczyńska *et al*. 2001). After 24-h co-incubation with *Agrobacterium*, the explants were dried on sterile filter paper and transferred to MS1 medium with 1.8 mg dm^{-3} of 6-benzylaminopurine (BAP) and 0.2 mg dm^{-3} of 3-indolylacetonitrile (IAN). After 7 d, the explants were rinsed with sterile water, dried, and transferred onto shoot-inducing medium MS2 with 1.8 mg(BAP) dm^{-3}, 0.2 mg(IAN) dm^{-3}, 200 mg(kanamycin) dm^{-3} for selection of transformed cells, and 400 mg(timentin) dm^{-3} to control the growth of *Agrobacterium* (*see* text table below). Regenerated shoots were separated from the original explants and transferred to the elongation and rooting medium MS3 without growth regulators, but supplemented with antibiotics. On some shoots anthocyanin formation, partial loss of Chl or even complete bleaching was observed, although the presence of the GFP protein was detectable in green sectors when present. This was interpreted in line with Troczyńska *et al*. (2001) as indicative of a chimeric nature of these transformants and these shoots were discarded. GFP expression of the remaining shoots was checked in the leaf epidermis and subepidermal tissues using a *Nicon Eclipse E600* fluorescence microscope. The epidermis of leaves was isolated and the fluorescence was observed after excitation at a wavelength of 470–490 nm at a 400× magnification. Green fluorescence was best seen in stomatal cells due to their low Chl content, whose intense fluorescence masked GFP fluorescence.

Media components	Media MS1	MS2	MS3
Nicotinic acid [mg dm^{-3}]	1.5	1.5	1.5
Pyridoxine [mg dm^{-3}]	0.5	0.5	0.5
Biotin [mg dm^{-3}]	0.25	0.25	0.25
Thiamine [mg dm^{-3}]	0.5	0.5	0.5
Folic acid [mg dm^{-3}]	0.5	0.5	0.5
Glycine [mg dm^{-3}]	2.0	2.0	2.0
6-benzylaminopurine [mg dm^{-3}]	1.8	1.8	-
3-indolylacetonitrile [mg dm^{-3}]	2.0	2.0	-
Kanamycin [mg dm^{-3}]	-	200	200
Timentin [mg dm^{-3}]	-	400	400
Sucrose [mg dm^{-3}]	30	30	30

Genetically modified and rooted plantlets were transferred to plastic pots (4-cm diameter) containing autoclaved organic-mineral substrate and for two weeks were covered by polyethylene bags that were progressively opened in order to adapt the plantlets to the lower humidity of the growth chamber. The genetically modified plants were allowed to produce seeds. These seeds were cultivated in plastic pots with potting soil, in addition to seeds of non-modified tobacco plants cv. Bursan, at 22–24/20°C under a 16/8-h day/light regime and an air humidity of approximately 85%. Light was generated by cool white fluorescent lamps (*TLD36 W/840*, *Philips*) and the light intensity was kept low at PAR of 90 μmol(photon) m^{-2} s^{-1}, measured with the light sensor of the *PAM-2500* (*Heinz Walz GmbH*, Effeltrich, Germany). Plants were watered with tap water when needed. Completely expanded leaves of these plants were used for the experiments at growth stage 1108 according to the *CORESTA* classification (2009), after assuring the expression of the green fluorescence protein in the genetically modified tobacco plants.

Chl *a* fluorescence using the 'OJIP' transient and steady-state procedures: The youngest completely developed leaves either from genetically modified or control plants were used. The photosynthetic capacity of leaves was assessed using Chl fluorescence measurements with the *PAM-2500* (*Heinz Walz GmbH*, Effeltrich, Germany). For the 'OJIP' analyses and saturation pulse measurements in combination with the steady-state procedure, leaves were dark-adapted in an accordingly designed box. Recommended times for dark adaptation vary from 15 min (Kalaji *et al*. 2014) to 20–25 min (Kalaji *et al*. 2017a) to up to 30 min (Kalaji *et al*. 2017b). Dark adaptation for 15–20 min ensures the relaxation of the transthylakoid pH difference, inactivation of the ferredoxin NADP reductase, and realignment of the chloroplasts within the cell, while zeaxanthin-dependent quenching, the release of part of the PSII antenna, and the recovery from photoinhibition need considerably longer time (Kalaji *et al*. 2017a). Here, a period in the upper range (30 min) was applied in order to ensure a complete dark adaptation of leaves already acclimated to a low light intensity. The measurement of the Kautsky induction curves started with a modulated measuring beam with a photon irradiance of 0.1 μmol(photon) m^{-2} s^{-1} to assess dark fluorescence (F_0). Measuring light was supplied with red LEDs, maximum emission at 630 nm, FWHM (full width half max) 20 nm (1-μs pulses at 200 Hz modulation frequencies for the determination of F_0). Then, a saturating pulse of red light was applied [red LEDs, maximum emission at 630 nm, FWHM 15 nm, PAR of 10,000 μmol(photon) m^{-2} s^{-1}] and measurements were collected every 10 μs. As a consequence, the measured curves had to be smoothed using the method of the simple moving average with the number of values used for the calculation of the mean values depending on the time of measurements (*see* text table below). A logarithmic time scale was used to visualize the measurements of the 'OJIP' fluorescence rise (Kalaji *et al*. 2014). The origin 'O' (F_0) was at the onset of fluorescence induction (t = 0). The 'J' step was taken at 2.5 ms after illumination and the 'I' step at 40 ms, while the 'P' step (F_m) was taken at either 150, 200, or 250 ms, depending on which value was the highest (Fig. 1). The derived Chl fluorescence indices characterising energy absorption by the PSII antenna pigments, energy trapping leading to the reduction of Q_A, further electron transport to so-called intersystem electron acceptors, and the reduction of end acceptors at PSI electron acceptor side were calculated in line with Strasser *et al*. (2010) and Stirbet *et al*. (2018). The procedure of indices calculation is well established and described in several publications; however, differences exist for the calculation of M_0, the initial slope (in ms^{-1}) of the O–J fluorescence rise. It is calculated here as $M_0 = (\Delta V/\Delta t)_0 \approx 4(F_{0.3ms} - F_0)/F_v$.

Time interval [ms]	Number of averaged values
0–0.001	1
0.002–0.100	2
0.101–0.500	5
0.501–0.600	15
0.601–0.775	21
0.776–3.500	25
3.501–6.301	51
6.311–10.000	101
10.001–16.000	301
16.001–100.000	501
100.001–125.000	4,501
125.001–300.000	up to 5,999

For the saturation pulse and steady-state fluorescence procedure, measurements started with a modulated measuring beam to determine F_0, too. Then a saturating pulse of red light (10,000 μmol m^{-2} s^{-1}; *see* above) to measure maximum fluorescence (F_m) was applied. Actinic red light [red LEDs with a maximum emission at 630 nm, FWHM 15 nm, PAR of 925 μmol(photon) m^{-2} s^{-1}] was switched on and saturation pulses were applied every 20 s for 5 min in order to follow the response of the light reactions of photosynthesis to the applied actinic light intensity by measuring F_m' and F_t, the fluorescence under the actinic light. The shapes of the response curves indicated that by the time of the last measurement an almost steady state was reached, so that the last value was used for further calculations. Dark fluorescence of the light-adapted leaf (F_0') was calculated in line with Oxborough and Baker (1997). From these values variable fluorescence ($F_v = F_m - F_0$), maximum quantum efficiency of PSII photochemistry (φ_{P0}), the efficiency of the water-splitting complex on the donor side of PSII, sometimes referred to as photosynthetic integrity (F_v/F_0), the quantum efficiency of PSII electron transport in the light-adapted state (φ_{PSII}), nonphotochemical quenching (NPQ), photochemical quenching (q_P), the estimate of the fraction of open PSII reaction centres (q_L), the maximum efficiency of PSII photochemistry in the light if all centres were open [$F_v'/F_m' = (F_m' - F_0')/F_m'$], and the fluorescence decrease ratio (R_{Fd}) were calculated in line with Bilger and Björkman (1994), Lichtenthaler *et al*. (2005), and Murchie and Lawson (2013).

Statistical analyses: For the 'OJIP' analyses, 20 measurements for transformed and control leaves each were performed, for the steady-state fluorescence measurements 10 each. In addition, 20 saturation pulse measurements each were executed to determine F_0 and F_m in order to calculate F_v, φ_{P0}, and F_v/F_0. The derived parameters of the 'OJIP' transient, steady-state procedures, and saturation pulse measurements were calculated and tested for outliers applying the *Grubbs* test (https://contchart.com/outliers.aspx). In case of an outlier identified, the complete measurement was eliminated from further analyses, resulting in 17 accepted 'OJIP' measurements for control and 18 for transformed plants as well as 10 accepted steady-state fluorescence measurements for control and 9 for transformed plants. Moreover, all of the 20 saturation pulse measurements of dark-adapted control leaves and 19 of transformed ones were accepted. For the final comparative analyses of parameters, the measurements were tested for normal distribution applying the *Kolmogorov-Smirnov* and *Shapiro-Wilk* tests of the *SPSS* program (*IBM® SPSS® Statistics version 24*). In case of a normal distribution, the variances were compared by *F*-tests and, based on the results, *t*-tests were performed either using the conventional *t*-test or, in case of unequal variances, the *Welch-Satterthwaite* method as implemented in the *Excel* version of *Microsoft Office 2013*. In case one of the normality tests did not confirm a normal distribution of measurements, the *Mann-Whitney-U*-test of the *SPSS* program was applied to test for significant differences.

Results

The measurements of the fast Chl *a* transient ('OJIP' curve) revealed few significant differences between control and GFP-expressing tobacco leaves (Table 1). The absorbed photon flux per excited cross section of PSII, ABS/CS_m (or F_m), and the maximum trapped exciton flux per cross section of PSII, TR_0/CS_m (or F_v), were significantly smaller in transformed plants. Within the electron transport chain differences between the variants diminished, so that

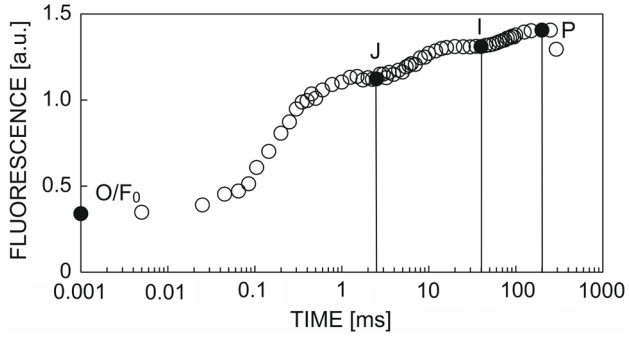

Fig. 1. Fast chlorophyll *a* fluorescence transient ('OJIP' curve) of a dark-adapted GFP-expressing tobacco leaf at *ca*. 10,000 μmol(photon) m^{-2} s^{-1}. Minimum fluorescence (F_0) at the origin 'O' was taken at the onset of fluorescence induction (t = 0), but is indicated in the figure for convenience at t = 0.001, because log(0) is not defined. The 'J' step set at 2.5 ms, the 'I' step at 40 ms, while the 'P' step (or F_m) was taken at either 150, 200, or 250 ms, depending on which value was highest (here 200 ms).

electron transport flux further than Q_A^- (ET_0/CS_m) and the flux reducing end electron acceptors at the PSI acceptor side per excited cross section of PSII (RE_0/CS_m) did not differ significantly. The density of reaction centres per excited cross section (RC/CS_m) and the absorption flux of antenna Chls per reaction centre (ABS/RC) were significantly lower and higher, respectively, in GFP-expressing tobacco. The probability that a PSII Chl molecule functions as a reaction centre ($Chl_{RC}/Chl_{total} = \gamma_{RC}$) was also significantly reduced in leaves that expressed the GFP. When electron fluxes are expressed per reaction centre (RC), the significances resemble those when expressed per excited cross section of PSII (CS_m): differences between control and GFP-expressing leaves were significant for TR_0/RC, but not for ET_0/RC and RE_0/RC. In addition, efficiencies of electron transport (φ_{P0}, ψ_0, φ_{E0}, δ_{R0}, φ_{R0}) and the absolute (PI_{ABS}) and the total performance index (PI_{Total}) for energy conversion of photons absorbed by PSII to the reduction of PSI end acceptors were not significantly different.

In independent saturation pulse measurements, the significant difference between control and GFP-plants for variable fluorescence (F_v) was confirmed; dark fluorescence (F_0), maximum fluorescence of a dark-adapted leaf (F_m), maximum quantum efficiency of PSII photochemistry (φ_{P0}), and the efficiency of the water-splitting complex on the donor side of PSII (F_v/F_0) were not significantly different (Fig. 2). The significance for F_v can be explained by differences in F_m, because the significance of the here applied *Mann-Whitney-U*-test was only slightly larger than 5%. It may be added that, in case all measurements of the 'OJIP', Chl transient and the saturation pulses were lumped, not only F_m and F_v were significantly smaller in GFP-expressing tobacco leaves, but also φ_{P0} ($P_{t\text{-test}} = 0.047$) and F_v/F_0 ($P_{Mann\text{-}Whitney\text{-}U\text{-test}} = 0.038$).

Differences in actual photochemical quantum yield (φ_{PSII}), nonphotochemical quenching (NPQ), photochemical quenching (q_P), the estimate of the fraction of open PSII reaction centres (q_L), the maximum efficiency of PSII photochemistry in the light if all centres were open (F_v'/F_m'), and the fluorescence decrease ratio (R_{Fd}) were not significantly different between variants with probabilities ranging between 0.37 for φ_{PSII} and 0.81 for NPQ.

Discussion

The hypothesis was formulated that the Chl fluorescence of tobacco plants transformed to express GFP is not different from nontransformed control plants. The present study revealed small but reproducible differences between both variants and as a consequence the hypothesis is rejected. The significant differences in the maximum fluorescence between control and transformed tobacco leaves may be interpreted as differences in the photosynthetic capacity of the leaves, assuming that neither the Mehler reaction nor any other process is involved. In this respect, the maximum fluorescence is interpreted as the absorption of energy per excited cross section of a photosynthesizing sample (CS) at the corresponding time (ABS/CS_m).

Energy absorption flux may not only be expressed per cross section, but also per reaction centre (ABS/RC) and

Table 1. Chlorophyll (Chl) fluorescence parameters derived from the measurements of the fast Chl *a* transient ('OJIP' curve) of tobacco leaves expressing the GFP compared with control leaves. * indicates a significance $P<0.05$, ** a significance $P<0.01$, x that this is a lower bound of the true significance, KS: *Kolmogorov-Smirnov*, SW: *Shapiro-Wilk*, MW: *Mann-Whitney-U*, a.u.: arbitrary unit.

Parameter	Control	GFP	$P_{KS\text{-test}}$	$P_{SW\text{-test}}$	$P_{F\text{-test}}$	$P_{t\text{-test}}$	$P_{MW\text{-test}}$
$ABS/CS_0 = F_0$ [a.u.]	0.318 ± 0.043	0.309 ± 0.049	0.200	0.643	0.587	0.574	-
$ABS/CS_m = F_m$ [a.u.]	1.369 ± 0.050	1.269 ± 0.185	0.063	0.063	0.000**	0.039*	-
$TR_0/CS_m = F_v$ [a.u.]	1.051 ± 0.076	0.960 ± 0.160	0.200x	0.740	0.005**	0.040*	-
ET_0/CS_m [a.u.]	0.330 ± 0.102	0.274 ± 0.054	0.200x	0.964	0.012*	0.054	-
RE_0/CS_m [a.u.]	0.072 ± 0.019	0.065 ± 0.023	0.200x	0.525	0.510	0.297	-
RC/CS_m [a.u. ms]	0.356 ± 0.047	0.301 ± 0.062	0.200x	0.689	0.305	0.005**	-
ABS/RC [ms^{-1}]	3.894 ± 0.431	4.273 ± 0.338	0.200x	0.716	0.330	0.006**	-
γ_{RC} [a.u.]	0.206 ± 0.018	0.190 ± 0.012	0.200x	0.195	0.103	0.006**	-
TR_0/RC [ms^{-1}]	2.978 ± 0.253	3.222 ± 0.247	0.200x	0.687	0.925	0.007**	-
ET_0/RC [ms^{-1}]	0.916 ± 0.241	0.919 ± 0.136	0.200x	0.532	0.024*	0.964	-
RE_0/RC [ms^{-1}]	0.201 ± 0.041	0.212 ± 0.054	0.200x	0.567	0.274	0.525	-
φ_{P0} [a.u.]	0.767 ± 0.035	0.755 ± 0.034	0.200x	0.690	0.935	0.297	-
ψ_0 [a.u.]	0.311 ± 0.086	0.285 ± 0.035	0.200x	0.286	0.001**	0.260	-
φ_{E0} [a.u.]	0.240 ± 0.072	0.215 ± 0.029	0.200x	0.465	0.001**	0.197	-
δ_{R0} [a.u.]	0.240 ± 0.094	0.235 ± 0.073	0.177	0.016*	0.311	0.840	0.947
φ_{R0} [a.u.]	0.053 ± 0.014	0.050 ± 0.013	0.200x	0.708	0.662	0.506	-
PI_{ABS} [a.u.]	0.447 ± 0.245	0.302 ± 0.092	0.048*	0.006**	0.000**	-	0.069
PI_{Total} [a.u.]	0.124 ± 0.060	0.093 ± 0.035	0.200x	0.035*	0.035*	0.079	0.156

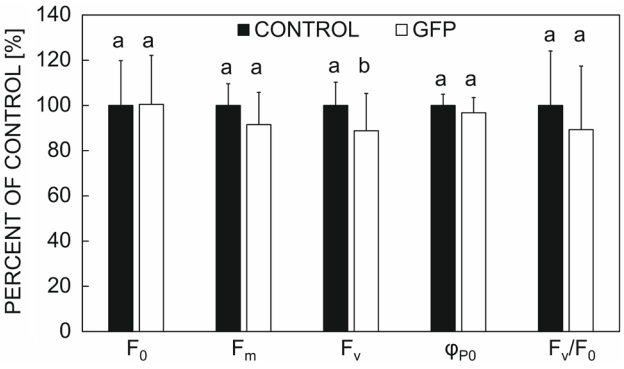

Fig. 2. Comparison of dark fluorescence (F_0), maximum fluorescence (F_m), variable fluorescence (F_v), maximum quantum efficiency of PSII photochemistry (φ_{P0}), and F_v/F_0 of a dark-adapted leaf based on a saturation pulse measurement in GFP-expressing and control tobacco leaves. The mean value of the control leaves is set 100%. Error bars indicate the standard deviation.

ABS/RC is larger in GFP-expressing tobacco plants, in contrast to ABS/CS_m, which is significantly smaller. There are two possible explanations for the increase in ABS/RC: (*1*) an increase in apparent antenna size or (*2*) a decrease in active RC (Falqueto *et al*. 2017). The probability that a PSII Chl molecule functions as a RC (γ_{RC}) is significantly smaller in transformed plants, implying that a decrease in the amount of active RC may have contributed to the increase of ABS/RC. The density of active reaction centres per excited cross section (RC/CS_m) is also significantly reduced when the GFP is expressed. A decrease of RC/CS_m is sometimes even interpreted as an indicator of photoinhibition when co-occurring with a decrease of φ_{P0} (Cheng *et al*. 2016). However, φ_{P0} was not significantly different between the variants in case the measurements of the 'OJIP' Chl transient and the saturation pulses were separately evaluated. Nevertheless, when lumped, the lower φ_{P0} of GFP-expressing tobacco became significant.

The results of the present study may be explained in three different ways, which are not excluding each other. The first approach refers to Kalaji *et al*. (2012), who demonstrated using the example of two Syrian barley landraces that not only high but also low light intensities may induce stress to the photosynthetic system. The here studied tobacco plants were adapted to a low light intensity of 90 μmol(photon) m^{-2} s^{-1} and as a consequence the light-harvesting system was optimised in order to use most of the absorbed light. Plants growing under low light intensities are expected to have a larger antenna size than those growing under high light intensities (Kalaji *et al*. 2012). If it is adopted that stress in the photosynthetic system is always associated with the generation of reactive oxygen species, then also tobacco growing at 90 μmol(photon) m^{-2} s^{-1} in principle must cope with oxidative stress. However, under low light intensities, the antioxidative system is characterized by a lower capacity to scavenge reactive oxygen species (Logan *et al*. 1998, Kalaji *et al*. 2012). It has been shown that reactive oxygen species inactivate the repairing mechanisms of PSII, especially by suppressing the *de novo* synthesis of the D1 protein of the reaction centre, and also the synthesis of a large amount of other proteins (Kalaji *et al*. 2012 with references). Malabadi *et al*. (2008) mentioned the assumption that the GFP may represent a source of free radicals as a consequence of its fluorescence. They argued that photons from fluorescence

could create free radicals and induce oxidative damage, but convincing evidence that the GFP was toxic in plants was not found (Stewart 2001). Nevertheless, Liu *et al*. (1999) revealed an association between the GFP and apoptosis in mammalian cells. There is, hence, a realistic possibility that the expression of the GFP could increase the oxidative stress level in the transformed tobacco plants, however, at a rather low level, because otherwise a toxic effect would have become evident in the earlier experiments (summary in Stewart 2001 and Malabadi *et al*. 2008). Considering that the inactivation of the protein-synthesis machinery might be a specific target of reactive oxygen species, it is well possible that the synthesis of chlorophyll-protein complexes, especially of the D1 proteins of reaction centres, are affected. Noteworthy, the synthesis is not directly affected by the expression of the GFP, but by a low capacity to scavenge reactive oxygen species under low light intensity, which is intensified by GFP-generated free radicals. All of the chlorophyll-protein complexes are associated with the light-harvesting complex and the electron transport chain in thylakoid membranes and, hence, a reduced absorption of energy per excited cross section (ABS/CS_m) together with a decrease in the number of active reaction centres per excited cross section (RC/CS_m) are a logical consequence of this scenario.

Noteworthy, the differences between GFP-expressing and control tobacco faded out within the electron transport chain indicated by the lack of significant differences in electron transport flux further than Q_A^- (ET_0/CS_m, ET_0/RC) and electron flux reducing end electron acceptors at the PSI acceptor side (RE_0/CS_m, RE_0/RC). This result is well in line with the observed lack of a yield decrease or growth retardation in GFP-expressing plants in field experiments (Elliott *et al*. 1999, Lawton *et al*. 2000, Li *et al*. 2001, Zhou *et al*. 2004) indicating that any effect of the GFP on photosynthesis does not impair the plant's vitality. The weak effect of the GFP on the light reactions of photosynthesis is also confirmed by the insignificant differences between the studied tobacco variants in PI_{ABS}, PI_{Total}, and efficiencies of electron transport (φ_{P0}, ψ_0, φ_{E0}, δ_{R0}, φ_{R0}). In addition, the results of the steady-state fluorometry, which did not reveal any significances for φ_{PSII}, NPQ, q_P, q_L, F_v'/F_m', and R_{Fd} between controls and GFP-expressing tobacco, correspond well with this interpretation. The steady-state fluorometry refers to the relatively stable photosynthetic activity that is maintained during the present measurement for 5 min at a light intensity of 925 μmol(photon) m^{-2} s^{-1}, which was ten times higher than the original ambient level of PAR, 90 μmol(photon) m^{-2} s^{-1}. It could be expected that such a tenfold increase of light intensity would reflect an unbalanced relationship between light and dark reactions of photosynthesis: light harvesting would be rather efficient, while the dark reactions of photosynthesis may not be able short-term to exploit the available amount of ATP and reduction equivalents.

Although maximum fluorescence in the light-adapted state (F_m') was not significantly smaller in transformed tobacco than that in controls, it accounted for only 92.8% of the value of controls. For comparison, F_m of transformed plants was 91.5% smaller than that in controls when assessed with the fast Chl *a* transient ('OJIP' curve) and F_m was 92.7% smaller when assessed with the saturation pulse method immediately before steady-state fluorometry on the same plants used to measure F_m', respectively, indicating that the observed effect was detectable at PAR of 925 μmol(photon) m^{-2} s^{-1} as well. As a consequence of a more efficient light absorption in control plants, a higher NPQ might be expected, assuming that light utilization for photosynthetic processes would be similar in both tobacco variants, but with values of 0.53 for GFP-expressing leaves and 0.55 for control leaves, the NPQs were about similar. As a matter of fact, the actual photochemical quantum yield (φ_{PSII}) reflected the differences in the tobacco variants, with φ_{PSII} of transformed tobacco representing only 88.0% of the yield of controls. By contrast, the 'vitality index' R_{Fd} was rather similar between GFP-expressing and control leaves, being 97.0% the value of controls in the transformed variant. Although it is pointed out by Roháček (2002) that the interpretation of R_{Fd} is controversial, because it requires knowledge of principal Chl fluorescence parameters, it has been used successfully in ecophysiological studies as a measure of photosynthetic activity or has been linked to CO_2 assimilation rates (Lichtenthaler *et al*. 1984, 2000; Lichtenthaler 1990). If interpreted in the latter respect, the R_{Fd} values correspond well with the lack of any effect of GFP on yield and growth of plants in field experiments.

The second possibility to interpret the present results refers to the theoretical possibility that the expression of the GFP could also have a positive effect on the photosynthetic efficiency of leaves. When exposed to light in the blue to ultraviolet range, the GFP emits light in the cyan–green range with maxima between *ca.* 500 and 510 nm (Tsien 1998). This light may at least partly be absorbed by the five xanthophylls, such as lutein, neoxanthin, violaxanthin, zeaxanthin, and antheraxanthin, in the light-harvesting complex, which absorb mainly in the range of 400–500 nm, but this range may be extended up to 520 nm as in the case of lutein and zeaxanthin (Ruban *et al*. 2001, Keşan *et al*. 2016). However, it has to be kept in mind that some xanthophylls transfer energy to reaction centres with a very low efficiency, *e.g.*, lutein with an efficiency of 0.7 and neoxanthin with an efficiency of 0.09 (Akimoto and Mimuro 2005, Akimoto *et al*. 2005).

Because green light is only weakly absorbed by Chl and other leaf pigments, green light can penetrate further into the leaf than red or blue light (Rappaport *et al*. 2007, Terashima *et al*. 2009). The progressive light absorption of red and blue light leads to a decreasing light intensity in deeper cell layers of a leaf, which is expected to be all the more pronounced as the exciting wavelength is strongly absorbed. As a consequence, any additional green light emitted by a light source such as the GFP is more probably absorbed by chloroplast pigments in lower layers of a leaf. Based on these considerations, Terashima *et al*. (2009) concluded that green light, when added to red and blue light, should increase leaf photosynthesis to a larger extent than would additional red or blue light.

For the expression of the GFP in transformed tobacco leaves these considerations would lead to the conclusion that, first of all, blue light would be transformed to green

light. If light intensity is high, the red and blue light portion of any white light source would be preferentially absorbed by the chloroplasts in the upper adaxial part of the leaf and excessive light energy would be dissipated as heat. The green light portion of the white light would pass the upper layers and drive photosynthesis in the lower cell layers, where light saturation would not occur (Sun *et al*. 1998, Nishio 2000). At low, nonsaturating light intensities, *e.g.*, at PAR of 90 μmol(photon) m^{-2} s^{-1} as in the growth chamber of the present experiment, any effect of a light transformation from blue to green by the GFP may be small. The difference, if any, would merely represent the position within the leaf, where a light quantum would be used for photosynthesis, because the probability to pass the upper cell layers of a leaf is higher for a green light quantum emitted by the GFP. However, it can be expected that even this small change in light distribution within a leaf results in an acclimation of the light-harvesting system.

In the *PAM-2500* (*Heinz Walz GmbH*) the saturation pulse is red light, which is absorbed almost exclusively by the upper cell layers, but not by the GFP. If in transformed and control leaves the dark reactions of photosynthesis are similar and light harvesting of the entire leaf is acclimated to the photosynthesis level at a low, nonsaturating light intensity of white light, then relatively less reaction centres could be expected in the upper cell layers of GFP-expressing leaves when compared to controls, whereas for lower cell layers, it would be just the opposite. Applying a saturation pulse of red light would only characterize light reactions of photosynthesis in the upper cell layers and may, as a consequence, result in a lower maximum fluorescence due to less reaction centres per excited cross section in tobacco leaves expressing the GFP. This interpretation could also explain the observed results and would not assume reactive oxygen species or any other kind of radical stress to be involved as a consequence of low light stress.

The third possibility to interpret the present results considers the general sensitivity of plants to green light, which is provided by a group of photoreceptors, among them cryptochromes, phytochromes, and phototropins due to their broad absorption spectrum that tails into the green portion of the spectrum (Golovatskaya and Karnachuk 2015). Responses to green light occur typically under low-light intensities and contribute to growth acclimation and fine-tuning of light responses. Here, green light is said to sense in general shade and for tobacco it has been reported that green light may reduce the stomatal opening (Wang and Folta 2013). The latter authors also mentioned that a set of light-induced plastid genes including *psaA* (photosystem I P700 Chl *a* apoprotein A1), *psbD* (photosystem II D2 protein), and *rbcL* (ribulose-1,5-bisphosphate carboxylase large chain) were downregulated by a green light pulse relative to dark levels. This downregulation could affect light energy absorption in GFP-expressing tobacco leaves per excited cross section (ABS/CS_m) by reducing the number of active reaction centres per excited cross section (RC/CS_m). However, the impairment of the synthesis of Rubisco should also affect the dark reactions of photosynthesis, but this effect might not have been detectable in leaves acclimated to PAR of 90 μmol(photon) m^{-2} s^{-1}. If green light recognises shading and induces acclimation responses, then the expression of the GFP should modify leaf physiology towards an environment of less light intensity, for instance by increasing the antenna size, which should manifest itself in larger values for ABS/RC.

In summary, three mechanisms are here discussed that may have contributed to the measured differences in Chl fluorescence parameters between plants expressing the GFP and controls. Firstly, it was assumed that the GFP contributed to the radical stress level in leaf cells, thus lowering the radical scavenging capacity of leaves, which resulted in an impairment of light harvesting. Secondly, it was suggested that leaves acclimated their light-harvesting system to the increased availability of green light, and thirdly, it was pointed out that green light could potentially downregulate light-induced plastid genes encoding components of the photosynthetic system and trigger the acclimatisation of the photosynthetic system towards a shady environment. It will be the subject of further studies to elucidate, whether the slight modification of the photosynthetic system is caused by a single of these processes or by a combination of two or three of the mechanisms discussed. However, evidence has been presented that Chl fluorescence parameters may be modified in plants expressing the GFP at least under certain environmental conditions.

References

Akimoto S., Mimuro M.: Excitation relaxation dynamics of carotenoids probed by ultrafast fluorescence spectroscopy. – In: Wada N., Mimuro M. (ed.): Recent progress of bio/chemiluminescence and fluorescence analysis in photosynthesis. Pp 213-214. Research Signpost, Serala 2005.

Akimoto S., Yokono M., Ohmae M. *et al*.: Ultrafast excitation-relaxation dynamics of lutein in solution and in the light-harvesting complex II isolated from *Arabidopsis thaliana*. – J. Phys. Chem. B **109**: 12612-12619, 2005.

Bilger W., Björkman, O.: Relationships among violaxanthin deepoxidation, thylakoid membrane conformation, and non-photochemical chlorophyll fluorescence quenching in leaves of cotton (*Gossypium hirsutum* L.) – Planta **193**: 238-246, 1994.

BioTek Instruments: Excitation and Emission of Green Fluorescent Proteins. Tech Note, Winooski, Vermont 05404-0998 USA, 2006. *Available at*: https://www.biotek.com/resources/docs/FL600_Excitation_and_Emission_of_Green_Fluorescent_Proteins_Tech_Note.pdf. *Accessed*: 6 June 2018.

Cheng D.-D., Zhang Z.-S., Sun X.-B. *et al*.: Photoinhibition and photoinhibition-like damage of the photosynthetic apparatus in tobacco leaves induced by *Pseudomonas syringae* pv. *tabaci* under light and dark conditions. – BMC Plant Biol. **16**: 29, 2016.

CORESTA – Cooperation Centre for Scientific Research Relative to Tobacco: A scale of coding growth in tobacco crops. – CORESTA Guide No. 7. Pp. 15. *Available at*: https://www.coresta.org/scale-coding-growth-stages-tobacco-crops-29211.html, 2009.

Elliott A.R., Campbell J.A., Dugdale B.R. *et al.*: Green fluorescent protein facilitates rapid *in vivo* detection of genetically

transformed plant cells. – Plant Cell Rep. **18**: 707-714, 1999.
Falqueto A.R., da Silva Jr R.A., Gomes M.T.G. *et al*.: Effects of drought stress on chlorophyll *a* fluorescence in two rubber tree clones. – Sci. Hortic.-Amsterdam **224**: 238-243, 2017.
Golovatskaya I.F., Karnachuk R.A.: Role of green light in physiological activity of plants. – Russ. J. Plant Physl+ **62**: 727-740, 2015.
Kalaji H.M., Carpentier R., Allakhverdiev S.I., Bosa K.: Fluorescence parameters as early indicators of light stress in barley. – J. Photoch. Photobio. B **112**: 1-6, 2012.
Kalaji H.M., Goltsev V.N., Żuk-Gołaszewska K. *et al*.: Chlorophyll Fluorescence. Understanding Crop Performance – Basics and Applications. Pp. 222. CRC Press, Boca Raton, 2017b.
Kalaji H.M., Schansker G., Brestič M. *et al.*: Frequently asked questions about chlorophyll fluorescence, the sequel. – Photosynth. Res. **132**: 13-66, 2017a.
Kalaji H.M., Schansker G., Ladle R.J. *et al.*: Frequently asked questions about *in vivo* chlorophyll fluorescence: practical issues. – Photosynth. Res. **122**: 121-158, 2014.
Keşan G., Litvín R., Bína D. *et al.*: Efficient light-harvesting using non-carbonyl carotenoids: Energy transfer dynamics in the VCP complex from *Nannochloropsis oceanica*. – BBA-Bioenergetics **1857**: 370-379, 2016.
Lawton R., Winfield S., Daniell H. *et al*.: Expression of green-fluorescent protein gene in sweet potato tissues. – Plant Mol. Biol. Rep. **18**: 139a-139i, 2000.
Li Z., Jayasankar S., Gray D.J.: Expression of a bifunctional green fluorescent protein (GFP) fusion marker under the control of three constitutive promoters and enhanced derivatives in transgenic grape (*Vitis vinifera*). – Plant Sci. **160**: 877-887, 2001.
Lichtenthaler H.K.: Applications of chlorophyll fluorescence in stress physiology and remote sensing. – In: Steven M.D., Clark J.A. (ed.): Applications of Remote Sensing in Agriculture. Pp. 287-305. Butterworth-Heinemann, Stoneham 1990.
Lichtenthaler H.K., Babani F., Langsdorf G., Buschmann C.: Measurement of differences in red chlorophyll fluorescence and photosynthetic activity between sun and shade leaves by fluorescence imaging. – Photosynthetica **38**: 521-529, 2000.
Lichtenthaler H.K., Buschmann C., Knapp M.: How to correctly determine the different chlorophyll fluorescence parameters and the chlorophyll fluorescence decrease ratio R_{Fd} of leaves with the PAM fluorometer. – Photosynthetica **43**: 379-393, 2005.
Lichtenthaler H.K., Meier D., Buschmann C.: Development of chloroplasts at high and low light quanta fluence rates. – Israel J. Bot. **33**: 185-194, 1984.
Liu H.S., Jan M.S., Chou C.K. *et al*.: Is green fluorescent protein toxic to the living cells? – Biochem. Bioph. Res. Co. **260**: 712-717, 1999.
Logan B.A., Demmig-Adams B., Adams III W.W., Grace S.C.: Antioxidants and xanthophyll cycle-dependent energy dissipation in *Cucurbita pepo* L. and *Vinca major* L. acclimated to four growth PPFDs in the field. – J. Exp. Bot. **49**: 1869-1879, 1998.
Malabadi R.B., Teixeira da Silva J.A., Nataraja K.: Green fluorescent protein in the genetic transformation of plants. – Transgenic Plant J. **2**: 86-109, 2008.
Murchie E.H., Lawson T.: Chlorophyll fluorescence analysis: A guide to good practice and understanding some new applications. – J. Exp. Bot. **64**: 3983-3998, 2013.
Nishio J.N.: Why are higher plants green? Evolution of the higher plant photosynthetic pigment complement. – Plant Cell Environ. **23**: 539-548, 2000.
Oxborough K., Baker N.R.: Resolving chlorophyll *a* fluorescence images of photosynthetic efficiency into photochemical and non-photochemical components – calculation of q_P and F_v'/F_m' without measuring F_0'. – Photosynth. Res. **54**: 135-142, 1997.
Rappaport F., Béal D., Joliot A., Joliot P.: On the advantages of using green light to study fluorescence yield changes in leaves. – BBA-Bioenergetics **1767**: 56-65, 2007.
Roháček K.: Chlorophyll fluorescence parameters: the definitions, photosynthetic meaning, and mutual relationships. – Photosynthetica **40**: 13-29, 2002.
Ruban A.V., Pascali A.A., Robert B., Horton P.: Configuration and dynamics of xanthophylls in light-harvesting antennae of higher plants. Spectroscopic analysis of isolated light-harvesting complex of photosystem II thylakoid membranes. – J. Biol. Chem. **276**: 24862-24870, 2001.
Schreiber U., Schliwa U., Bilger W.: Continuous recording of photochemical and non-photochemical chlorophyll fluorescence quenching with a new type of modulation fluorometer. – Photosynth. Res. **10**: 51-62, 1986.
Stewart Jr C.N.: The utility of green fluorescent protein in transgenic plants. – Plant Cell Rep. **20**: 376-382, 2001.
Stirbet A., Lazár D., Kromdijk J., Govindjee: Chlorophyll *a* fluorescence induction: Can just a one-second measurement be used to quantify abiotic stress responses? – Photosynthetica **56**: 86-104, 2018.
Strasser R.J., Srivastava A., Tsimilli-Michael M.: The fluorescence transient as a tool to characterize and screen photosynthetic samples. – In: Yunus M., Pathre U., Mohanty P. (ed.): Probing Photosynthesis: Mechanisms, Regulation and Adaptation. Pp. 445-483. Taylor & Francis, London 2000.
Strasser R.J., Tsimilli-Michael M., Qiang S., Goltsev V.: Simultaneous *in vivo* recording of prompt and delayed fluorescence and 820-nm reflection changes during drying and after rehydration of the resurrection plant *Haberlea rhodopensis*. – BBA-Bioenergetics **1797**: 1313-1326, 2010.
Strasser R.J., Tsimilli-Michael M., Srivastava A.: Analysis of the chlorophyll *a* fluorescence transient. – In: Papageorgiou G.C., Govindjee (ed.): Chlorophyll *a* Fluorescence: A Signature of Photosynthesis. Advances in Photosynthesis and Respiration. Pp. 321-362. Springer, Dordrecht 2004.
Sun J., Nishio J.N., Vogelmann T.C.: Green light drives CO_2 fixation deep within leaves. – Plant Cell Physiol. **39**: 1020-1026, 1998.
Terashima I., Fujita T., Inoue T. *et al*.: Green light drives leaf photosynthesis more efficiently than red light in strong white light: revisiting the enigmatic question of why leaves are green. – Plant Cell Physiol. **50**: 684-697, 2009.
Troczyńska J., Flasiński S., Drozdowska L.: [*Agrobacterium tumefaciens*-mediated transformation of rapeseed with green fluorescent protein gene.] – Oilseed Crops **22**: 569-578, 2001. [In Polish]
Tsien R.Y.: The green fluorescent protein. – Annu. Rev. Biochem. **67**: 509-544, 1998.
Wang Y., Folta K.M.: Contributions of green light to plant growth and development. – Am. J. Bot. **100**: 70-78, 2013.
Zhou X., Chandrasekharan M.B., Hall T.C.: High rooting efficiency and functional analysis of GUS and GFP expression in transgenic *Medicago truncatula* A17. – New Phytol. **162**: 813-822, 2004.

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