Chapter

Photosynthesis in Plants Undergoing Silencing

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Abstract

RNA silencing shares common features among different eukaryotes. However little is known about the metabolic consequences of this mechanism relate to the (plant) cell homeostasis. Here, we probe the chlroroplast bioenergetics in transgenic plants undergoing silencing. An increased capacity for non-photochemical energy quenching followed by a limiting photosystem II functionality characterize the photosynthesis of silenced cells compared to non-silenced ones. These alterations are accompanied by a significant up-regulation of photosystem I, providing evidence for active cyclic electron flow in silencing conditions. The biological significance of our results is discussed related to possible energetic inter-communication between photosynthesis and RNA silencing.

Keywords: ATP, cyclic electron flow, photosynthesis, photosystem I, RNA silencing mechanism

1. Introduction

RNA-mediated gene silencing pathways are responsible for the regulation of plant development, buffering of the genome integrity and for the effective response of plant organism to various stressful a/−biotic factors (reviewed by [1]). Currently, it has been identified a positive effect of light intensity on the induction and spread of spontaneous RNA silencing in transgenic *N. benthamiana* plants, indicating possible implication of photosynthesis in this phenomenon [2]. The objective of this study is to investigate the impact of the silencing signal on the plant's energetic repertoire. For that reason a detailed photosynthetic characterization was performed in transgenic tissues that are characterized by a dynamic transition from non-silenced to silenced state. We tried to characterize possible mechanistic aspects of the interplay between RNA silencing and light intensity in plants [3], via biophysical, biochemical and molecular data. This piece of work could promote the understanding of the crosstalk between RNA silencing mechanism and photosynthesis in transgenic plants. Therefore, we believe that our experimental findings would supply a creative motivation for further investigation in the area of other RNA-mediated gene silencing pathways, concerning the plant physiology.

2. Methods

2.1 Plant material

N. benthamiana transgenic lines that were used in this study are described in Kotakis et al. [2]. More specifically, GFP transgenic lines (line 6.4 and 5.1.1) that initiate local as well as systemic silencing spontaneously and the NIB-transgenic line 20-1A1 that undergoes also silencing, were used. Plants were grown as described in Kotakis et al. [2], under a white (PAR: 400–700 nm) light intensity of 130 μmol m^{-2} s^{-1}. Systemically fully silenced leaves from non-silenced ones; they are termed 'silenced' or non-silenced' respectively inside the text, were discriminated phenotypically as described in [2]. All data are derived from experiments in line 6.4, otherwise the specific plant line used is cited inside the text. The constitutively GFP-expressing line 16c was used for agroinfiltration of a hairpin *GFP* construct in order to induce systemic silencing as described in Kościańska et al. [4].

2.2 *In vivo* chlorophyll fluorescence measurements

The parameters studied in the light curves were quantified as described in Lichtenthaler et al. [5] using a PAM-210 fluorometer (Waltz, Germany). The fluorescence data concerning maximum photosynthetic performance [6] were obtained using a Handy-PEA fluorometer (Hansatech, UK) by consequent OJIP-analysis with Biolyser 4.0 software. Estimation of the relative plastoquinone (PQ) pool reduction as well as DCMU treatment were performed according to Toth et al. [7]. The capacity for state transitions and cyclic electron flow in examining leaf types was calculated according to Bellafiore et al. [8] and Munne-Bosch et al. [9], respectively.

2.3 Thylakoids isolation and polarography

Isolation of functional thylakoids from leaves and linear electron flow rate (ETR) estimation were performed according to Casazza et al. [10]. Activity of photosystem (PS) I & PSII was estimated as described in Romanowska et al. [11].

2.4 Determination of adenylates and reducing equivalents

ATP and NADPH production from thylakoid membranes were estimated as described in Casazza et al. [10]. ATP and ADP content in leaves were extracted and quantified by HPLC as described in Andronis and Roubelakis-Angelakis [12].

2.5 Putrescine quantification

Putrescine extraction from leaves and following quantification were performed in accordance to Navakoudis et al. [13].

2.6 Immunoblotting

Phosphorylation of LHCII was determined by western blotting as described in Bellafiore et al. [8].

2.7 Quantitative real-time PCR analysis

The mRNA levels of chloroplastic *PSaA* (GI:81238323) and *PSbA* (GI:76559634) genes that encode reactions centers of PSI and PSII respectively, were quantified by qPCR analysis. RNA extraction and qPCR was performed as previously described in Kotakis et al. [2] and the sequence of primer pairs used, is given in **Table A1**.

2.8 Method of sampling, repetition of experiments and statistics

For the *in vivo* measurements 5 leaves of similar growth, angle and distance of their surface to the light source, from corresponding different plants were used in each category studied. For all the biochemical and molecular approaches, the experiments were repeated at least 3 times in a pooled material from 5 different plants. All the plots represent average ± standard deviations. Significance of differences in the various parameters plotted with bars, was assessed by One-way ANOVA ($P < 0.05$), (SPSS 17.0, SPSS Inc., USA). Single asterisks denote statistically significant differences between wt tissue and either one of non-silenced or silenced tissue. The double asterisks denote statistically significant differences between non-silenced tissue and silenced tissue. In the **Figures 2E** and **3B**, the presence of asterisks denotes statistical significance of the difference displaying, compared to the zero-change level and in the case of **Figure A3B**, they stand for statistical significance between the two treatments.

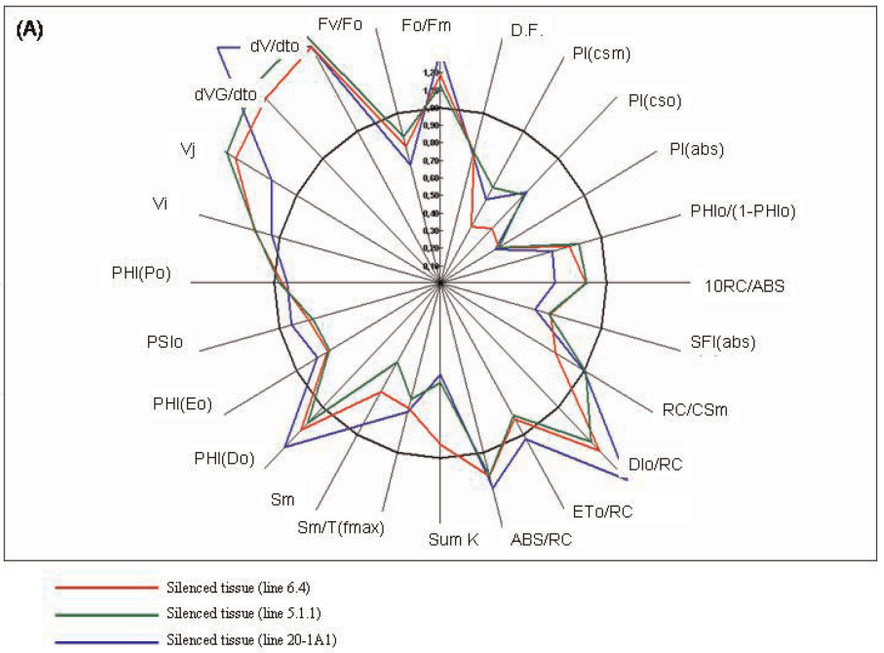
The etymology of some abbreviations used in the text, is provided in **Table A2**.

3. Results and discussion

3.1 Photosynthesis is differentiated in tissues undergoing silencing

In this study, firstly we attempted to correlate basic bioenergetic parameters that characterize the photosynthetic process, with the RNA silencing conditions. Towards this aim we used chlorophyll a fluorescence induction kinetics as a probe for quantifying PSII behavior in transgenic *N. bethanmiana* plants that are functionally related to the presence of the RNA silencing signal. Non-silenced transgenic tissues as well as wild-type (wt) samples were served as controls. At least two different transgenic lines (three for the silenced- and two for the non-silenced state) were used in this study in order to eliminate possible masking in our results from factors such as accidental insertional effects in genomic loci related to the plant physiology and specificities due to the transgene nature. The data obtained by this analysis denoted that similar bioenergetic profiles (**Figures 1A** and **A1A**) characterize the transgenic lines studied with an active RNA silencing mechanism and though quite different in comparison to the corresponding controls. Also, it has to be emphasized that two different transgenic lines related to non-silenced state shared a high degree of similarity in such type of measurements (**Figure A1B**). According to these data sets, it seems that the photosynthesis is remarkably differentiated in comparison to either non-silenced or wt conditions. More specifically, a limiting potential for reduction of PSII acceptor side is reported in silencing conditions as it can be confirmed by a diminishing gradient of the maximum chlorophyll a fluorescence plateau from the non-silenced to silenced state (**Figure 1B**).

Next, we decided to continue our study, determining the steady-state photosynthesis, since the preliminary experiments had revealed similar bioenergetic principles, independently the transgenic line studied. For this purpose, we chose one


(A)
Fv/Fo
Fo/Fm
D.F.
Pl(csm)
Pl(cso)
Pl(abs)
PHIo/(1-PHIo)
10RC/ABS
SFI(abs)
RC/CSm
DIo/RC
ETo/RC
ABS/RC
Sum K
Sm/T(fmax)
Sm
PHI(Do)
PHI(Eo)
PSIo
PHI(Po)
Vi
Vj
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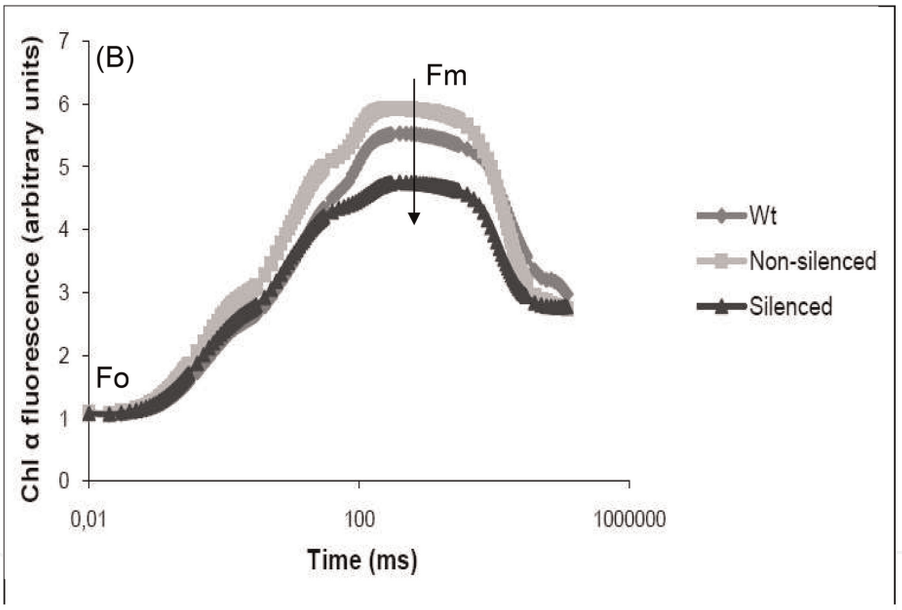
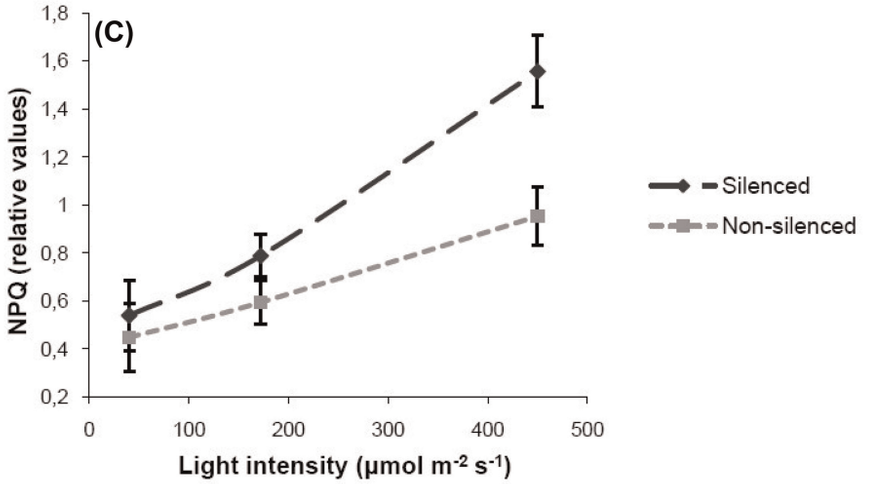
dV/dto
Silenced tissue (line 6.4)
Silenced tissue (line 5.1.1)
Silenced tissue (line 20-1A1)


(B)
Fm
Fo
Chl α fluorescence (arbitrary units)
Time (ms)
0,01
100
1000000
Wt
Non-silenced
Silenced


(C)
NPQ (relative values)
Light intensity (µmol m-2 s-1)
Silenced
Non-silenced

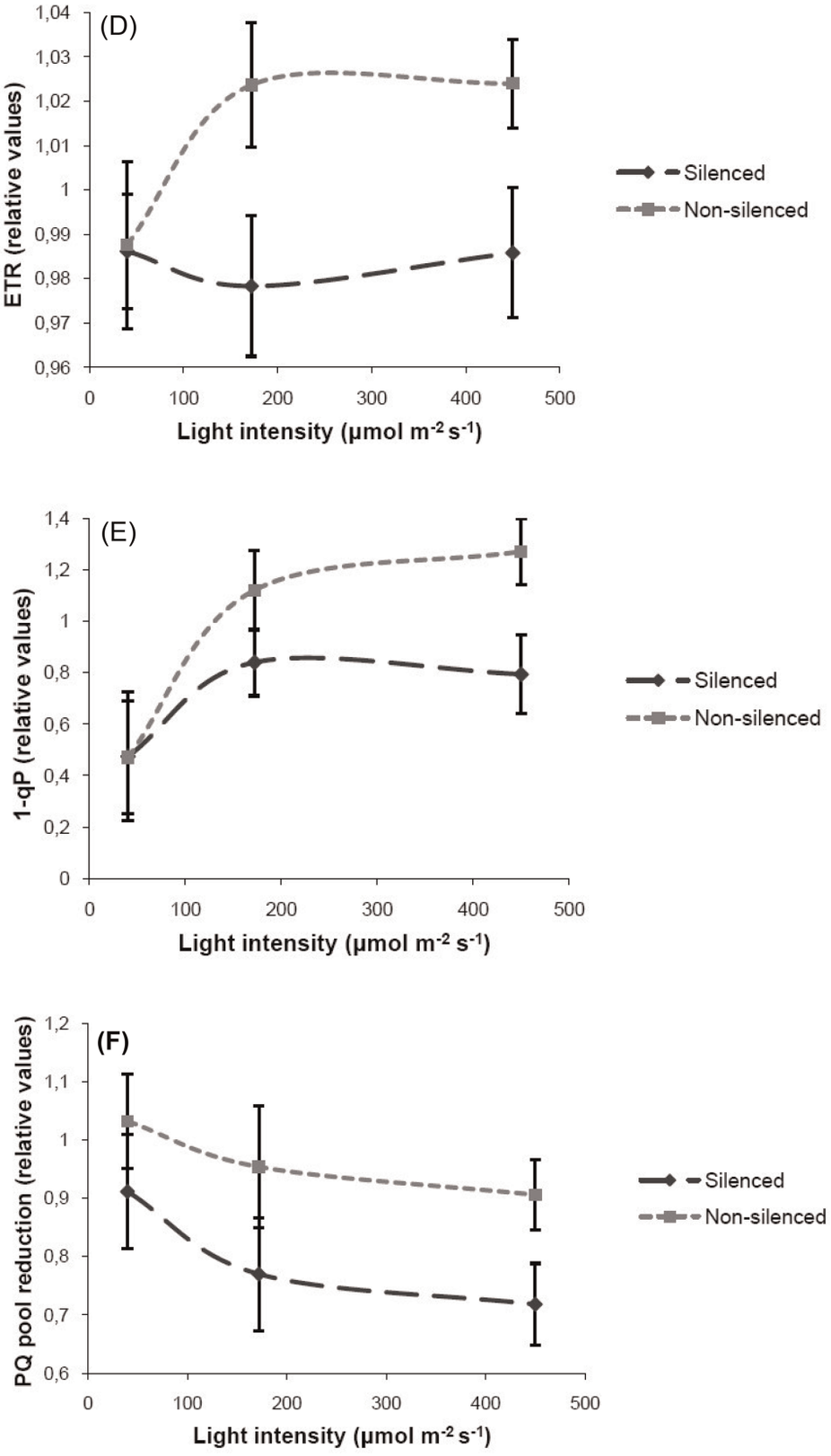


Figure 1.
The photosynthetic function of cells where silencing is established, is altered in comparison to non-silenced cells. A: Bioenergetic profiles are displaying basic parameters of maximum photosynthetic acclimation in the examining tissues. The control condition (non-silenced tissue) is presented as circle. B: Representative chlorophyll fluorescence induction curves, normalized by the Fo. The time in the x-axis is presented in a logarithmic scale. The arrow depicts the Fm plateau. Light curves for C: NPQ, D: ETR, E: 1-qP and for F: PQ pool reduction, in different light intensities. Transgenic leaf values normalized over corresponding wt values in the parameters of light curves. NPQ; non-photochemical quenching, ETR: Electron transport rate, 1-qP; excitation pressure of PSII.

transgenic line that has advantage to display silenced and non-silenced plant areas at the same time, with a high frequency in the initiation and spread of silencing events [2]. As we can conclude from the respective light curves, silenced tissues are characterized by a deficient PSII energy perception as indicated by up to 67% higher capacity for NPQ formation than the non-silenced ones (**Figure 1C**). Furthermore, the coherent utilization of light energy absorbed by PSII is considerably limiting, regarding the linear electron flow, (**Figure 1D**) in silencing conditions. Next, the corresponding PSII excitation pressure levels remain quite low (**Figure 1E**), most probably due to the inefficient generation of reduced PQ pool that was found to follow concomitant trace (**Figure 1F**), accordingly to the low electron flow rates. Taken together the above data, we can point out that the redox potential of chloroplasts that drives the linear electron flow to provide both ATP and NADPH is not efficient in silenced cells.

3.2 PSI plays a significant role in chloroplast bioenergetics of silenced cells

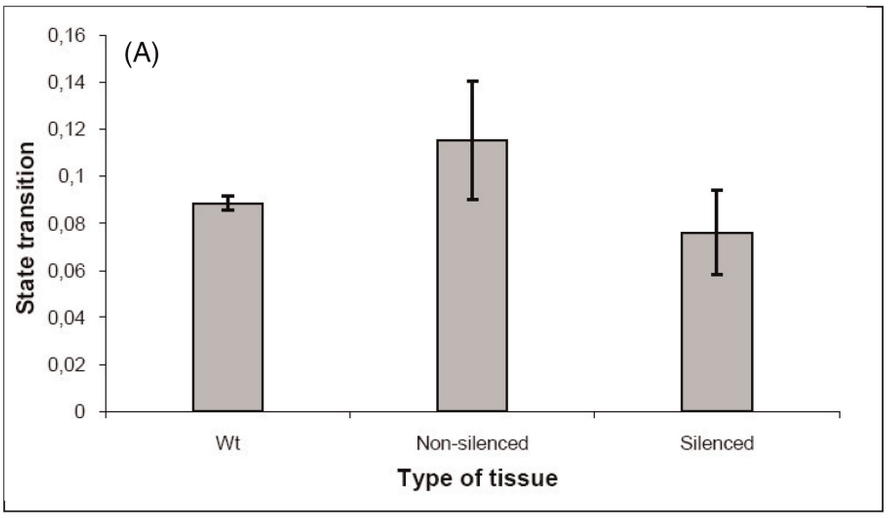
Drawing the outline up to now, the chemiosmotic capacity of the photosynthetic apparatus in silenced cells is significantly limiting because the availability of intermediate electron carriers at PSII acceptor side was found decreased compared to the non-silenced cells. The last is shown also by the statistically lower ETR, even normalized in a chlorophyll basis (**Figure A2**). By contrast it is arisen that possible adequate sinks of electrons may be managed as dissipating valves in the electron transport chain, when the redox poise of plastoquinone was reduced by inhibiting the electron flow at the Q_B binding site. Particularly, not only the reduced PQ pool remained low but also the 'silenced' values were maintained at significant high levels compared to the almost close to zero, 'non-silenced' ones (**Figure 2C**). So, we make the hypothesis of the probable existence of a strong source of electrons donation and it is proposed the switch to a different mode of electron flow (e.g. PSI-alternative route of electrons via PQ recycling) [14].

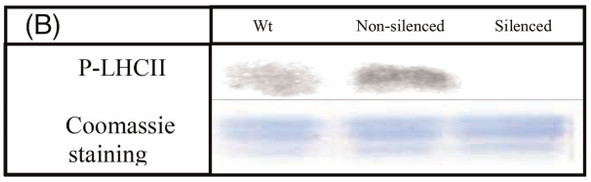
Then, we decided to resolve the composition and function of the thylakoid membrane in depth. Mechanisms such as state transitions and photosystem stoichiometry adjustment were needed to be elucidated by biochemical and molecular means, since both responses are functionally coupling with the redox state of the plastoquinone pool (reviewed by [15]) that found modified in silenced cells. The silenced tissues show a decreased, though not statistically significant, capacity for the migration of LHCII from state 1 to state 2 (**Figure 2A**), in comparison to the non-silenced ones. The tendency for light utilization preferably not by PSII in non-silenced cells, is accompanied by a strong phosphorylation of LHCII antenna (**Figure 2B**). These findings show an active state transition mechanism in cells that are prepared to perceive the silencing signal. On the other hand, the complete lack of corresponding signal in silencing conditions (**Figure 2B**), maybe underline that this type of regulation is not sufficient and/or inactivated since it is related to short-term time scales of response (reviewed by [16]) and LHCII kinase is activated by a specific range of PQ redox state [17], respectively. In this case, a second level of long-term regulation such as the photosystem stoichiometry adjustment is possibly needed. Actually, this organization strategy is reinforced as it is reflected by an approximate 1.5 fold increment to the PSI/PSII activity (**Figure 2D**) in the thylakoid membranes of tissues undergoing silencing in comparison to non-silenced ones. This functional PSI-enrichment in the chloroplasts of plant cells that are characterized by an metabolically active RNA silencing mechanism is entailed by a transcriptional up-regulation of the *PSaA* gene over 5-fold in the silenced tissues, with

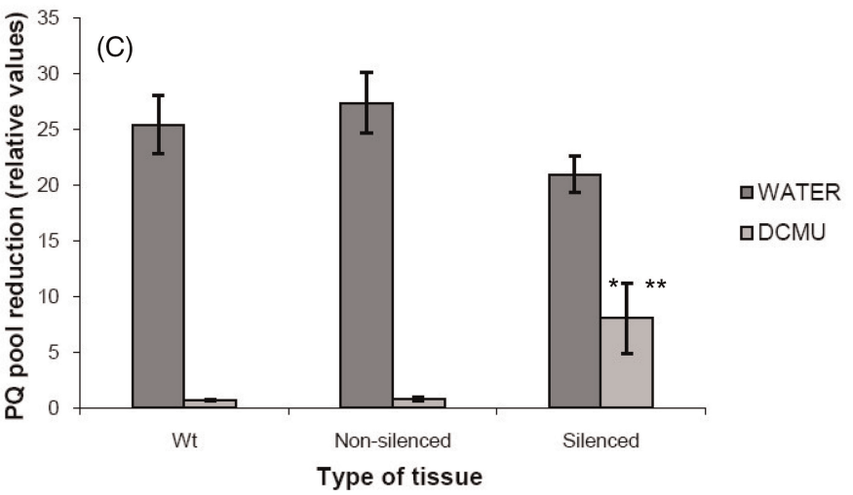
the mRNAs of *PSbA*, to be remained almost zero-changed (**Figure 2E**). The modified stoichiometry of photosystems even in the transcriptional level, comes to substantiate the rest body of evidence for a significant PSI involvement in the chloroplast bioenergetics, when the RNA silencing signal has been established.

3.3 Evidence for ATP requirement via cyclic electron flow in chloroplasts of cells, where silencing signal is established

The decreased PQ pool in reduced form, can be justified by the transcriptional PS stoichiometry [18] found when RNA silencing mechanism is activated. A switch to cyclic electron flow is quite possible that is compensating for the inefficient operation of the linear electron flow of chloroplasts there-in, since the first mode of flow is driven by the PSI alone, and it provides a mechanism whereby ATP production can be increased







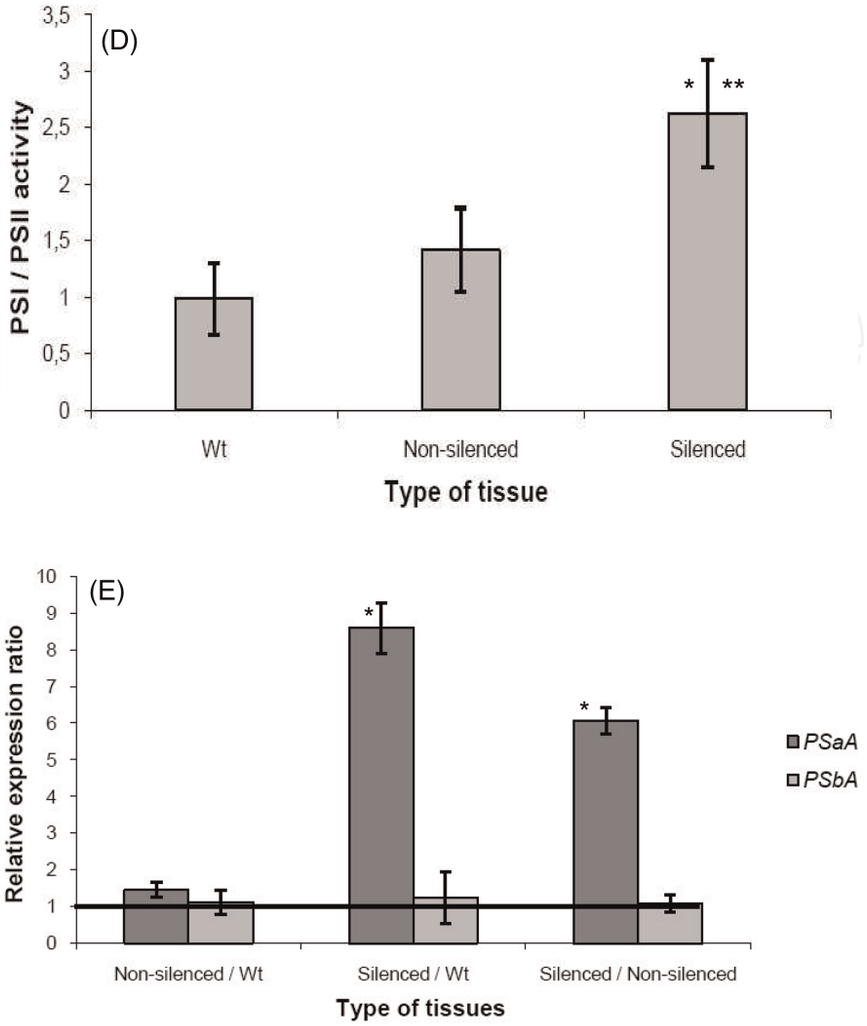


Figure 2.
Evidence for a significant PSI involvement in plant cells with the signature of RNA silencing. A: Capacity for transition from state 1 to state 2 and B: Phosphorylation levels of LHCII among the photosynthetic tissues studied. C: Quantification of the steady-state PQ pool in control (water) and oxidized (DCMU) conditions for the PSII acceptor side. D: Relative photosystem's activity (PSI/PSII) in thylakoid membranes of the corresponding leaf tissues. E: Relative expression ratio of PS genes, between the different leaf types examined. The horizontal line designates the zero-change level, (ratio one).

relative to NADPH (reviewed by [19]). Quantifying the capacity for cyclic electron flow, a statistical higher difference is exhibited in silenced tissues compared to the non-silenced ones (**Figure 3A**). This regulatory adaptation confirms that the metabolic conditions inside the silenced cells impose the necessity for proper adjustment of the ATP/NADPH ratio that is reported to follow more than 2-fold increment (**Figure 3B**). We further note the slightly increased ATP/NADPH ratio of non-silenced tissues in comparison to wt ones (**Figure 3B**) that may reflect the energetic requirements of the plant cell metabolism in order to sustain the transgene expression in a constitutive manner. The pattern of the altered chloroplastic contribution is clearly manifested in the ATP/ADP ratio of silenced cells (**Figure 3C**), validating the augmentation of ATP there-in. From all the above, it emerges that the mode of electron flow in the chloroplast of cells undergoing RNA silencing is different compared to cells that restore an energetic budget towards a continuous transgene expression. As a consequence, we could speculate that cyclic electron flow around PSI is accelerated in this type of tissues, providing additional ATP molecules in cells where RNA silencing is maintained.

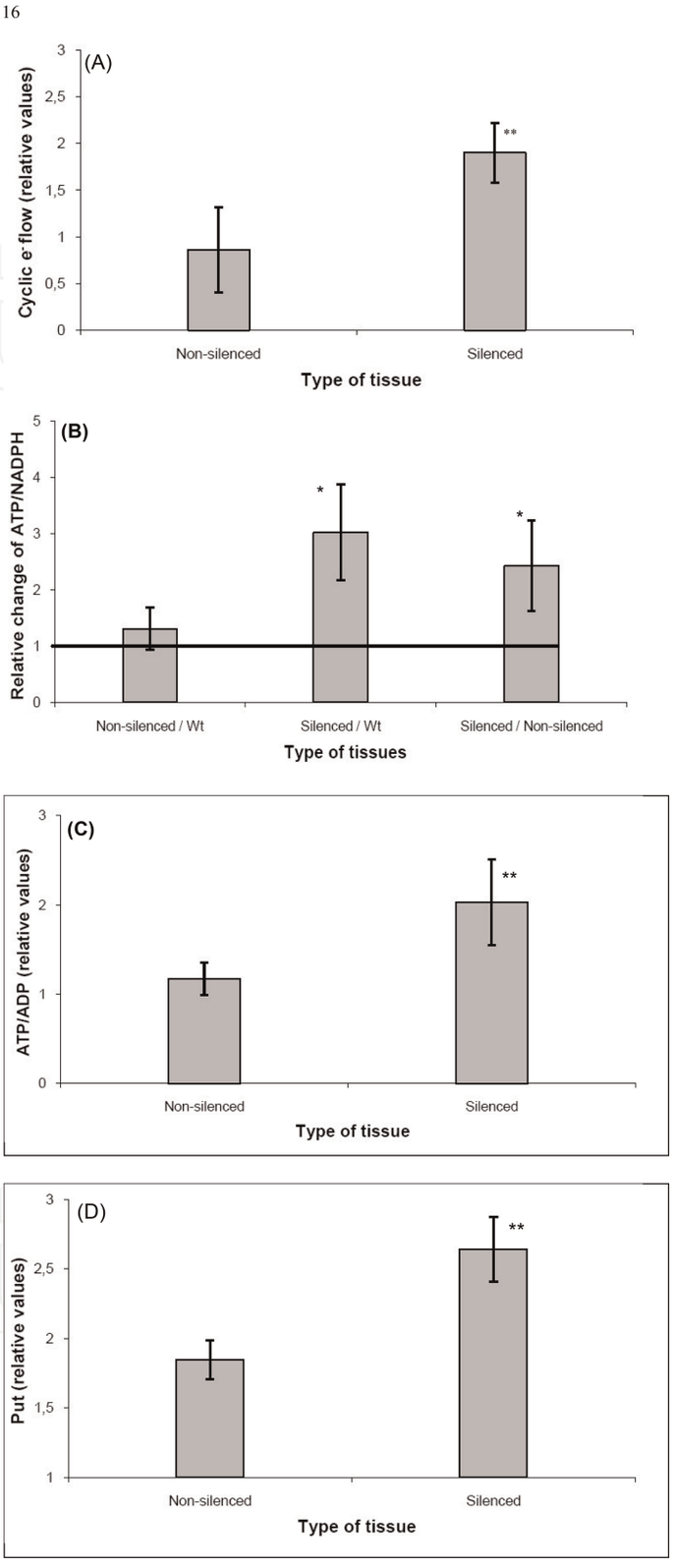


Figure 3.
The adenylate status is redistributed in tissues undergoing silencing, via an active cyclic electron flow. A: Capacity for cyclic electron flow and B: Relative change of chloroplastic ATP/NADPH ratio, between the different plant types, examined. The horizontal line designates the zero-change level. C: ATP/ADP ratio and D: Put content, in fully silenced leaf tissues and non-silenced ones. Data normalized over corresponding wt values in A, C and D.

Hence, an interplay between silencing and photosynthesis is addressed, taking also into account the differential response of RNA silencing to variations of light intensity [2]. To testify this notification, we tried to simulate HL-conditions by using the polyamine Put. The role of this biogenic amine is known currently, concerning the regulation of the photo-adaptive status of photosynthetic apparatus [13] via an increased chloroplastic ATP pool [20]. Based on the last, the content of Put was quantified in the examining tissues. Fully silenced leaves were found to contain higher levels of Put, compared to the non-silenced ones (**Figure 3D**). This result is in agreement with the rearrangement of the energy charge in plant cells' in silenced state. After that, non-silenced leaf tissues were treated by Put, creating an ATP-fuelled environment inside the corresponding cells. Daily monitoring of the silencing initiation and spread events was followed in the plant individuals studied. The scores obtained by Put-treated samples were higher (**Figure A3A**) and followed by earlier initiated phenotypes (**Figure A3B**) compared to the short-range silencing and systemic signal appearance, respectively in control conditions. The data derived by these time kinetics suggest that silencing performance could efficiently be regulated by bioenergetic manipulation, strengthening the possibility for inter-communication between silencing and photosynthesis.

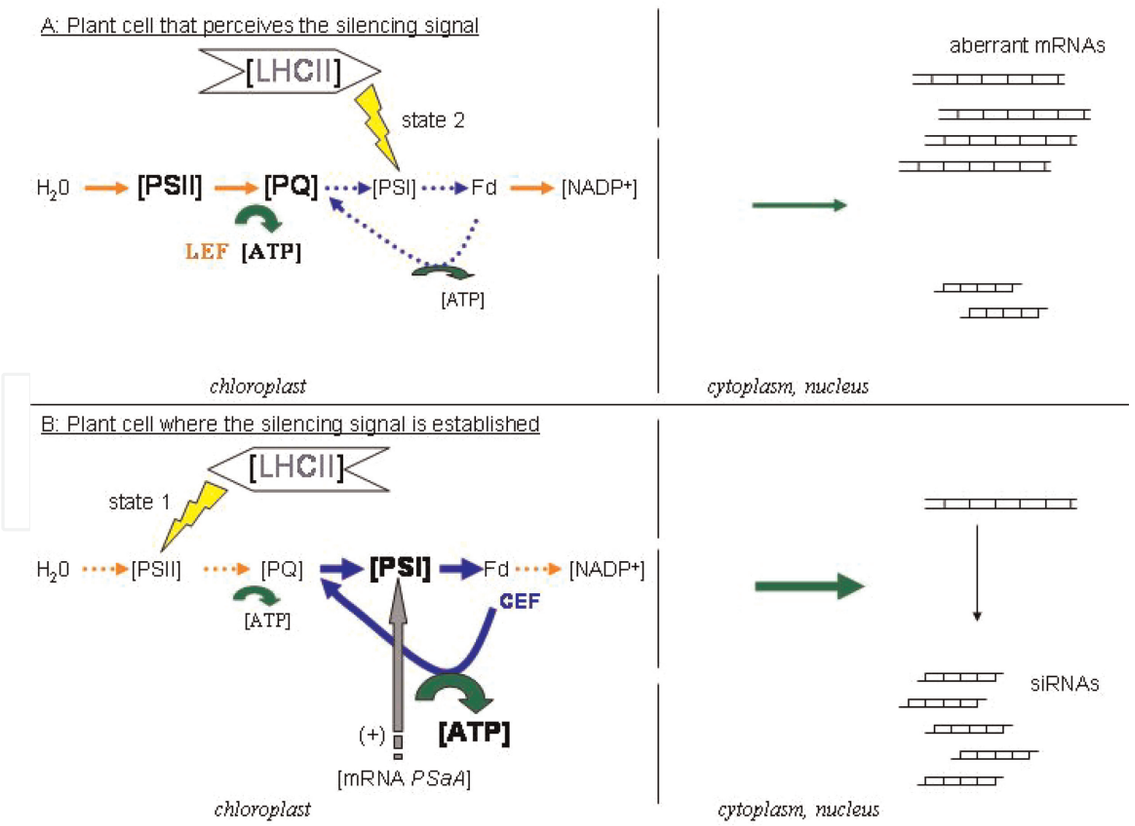


Figure 4.
Mechanistic implication of photosynthetic apparatus in the RNA silencing processing. Schematic representation of basic photosynthetic components as characterized in the dynamic transition of plant cells from the A: Non-silenced to B: Silenced state. Orange and blue arrows stand for the linear (LEF) and cyclic electron flow (CEF), respectively. The green ones stand for the ATP generation. The relative size of letters and arrows that depicted in the diagram, as well as the graphical (dis)- continuity of some arrows is indicative of their functional role inside the cell.

By conclusion, the structure and function of the photosynthetic machinery are differentiated in tissues undergoing silencing compared to tissues where silencing signal has not been established yet. The photosynthetic mechanism under silencing conditions is described by an enhancement of the non-photochemical quenching process of the energy captured by the light-harvesting antenna and a subsequent increased PSI/PSII activity. The PQ redox state act as a signal for the LHCII transition in state 2, in non-silenced cells (**Figure 4A**) and for a transcriptional up-regulation of *PSaA* gene in silenced ones. As a result, the onset of the cyclic electron flow is engaging and additional ATP molecules are likely provided in cell metabolism where silencing conditions are supported (**Figure 4B**). Taking into consideration our results, we envision that the ATP requirement of the biochemical pathway of RNA silencing mechanism, may be responsible for the changes observed. The ATP-dependent processing of dsRNA into siRNAs [21, 22] and the enhanced RISC activity in the presence of ATP [21] are some mechanistic aspects for an effective silencing mechanism. There is also evidence for a role of ATP in unwinding of siRNA duplex as well as in maintaining 5′ phosphate groups in a functional siRNA molecule [23].

It is claimed that plants grown under HL conditions, are characterized by higher availability of ATP pools in comparison to low light grown plants (reviewed by [24]). Hence, we are tempting to speculate that one part of these energetic equivalents is related with the increased frequency of silencing events as well as with an effective and conductive silencing signal, concerning the overall RNA silencing perception found in HL conditions [2]. Thus, cyclic electron flow is considered to constitute a protective mechanism against redox imbalances (e.g. stress conditions), acting synergistically with the antioxidant machinery for restoring the energetic and redox status of the plant cell (reviewed by [25]). So, it cannot be excluded that such micro-environmental conditions could probably be linked with the dynamic transition from a strong transgene expression to siRNAs 'burst' and it remains to be investigated.

3.4 Core finding and perspective

We report evidence that chloroplast bioenergetics is altered remarkably in silencing conditions and activation of transgene silencing imposes the photosystem I involvement in chloroplast energetics. The data propose bioenergetic manipulation as potential means of regulating silencing efficiency.

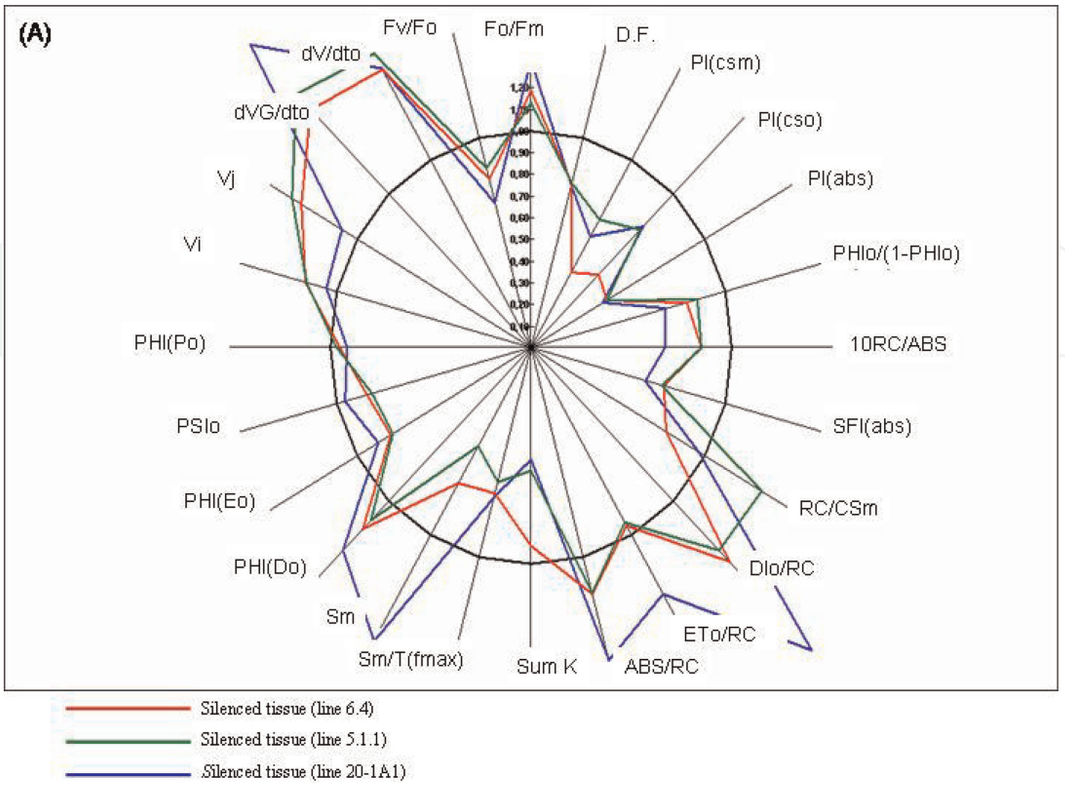
A. Appendix

Primer ID	Primer sequence 5′-3′
PSaA-FOR	GCTCTAGATGGCAGGGCTACTAGGA
PSaA-REV	CGAATTCAATGGTGATGGGCAATA
PSbA-FOR	GCTCTAGATTGACGGCAACTTCTGT
PSbA-REV	CGAATTCCCAAGGTCGCATACCCA

Table A1.
List of primer sequences used in quantitative real-time PCR assays.

Abbreviation	Etymology
DCMU	3-(3′,4′-dichlorophenyl) 1, 1′-dimethyl urea
Fo	Initial fluorescence value
Fm	Maximum fluorescence value
Fd	Ferredoxin
GFP	Green Fluorescent Protein
HL	High Light
HPLC	High-Performance Liquid Chromatography
LHCII	Light-Harvesting Complex II
NIB	*Plum Pox Virus* polymerase
PAR	Photosynthetically Active Radiation
PAM	Pulse Amplitude Fluorometry
Put	Putrescine
Q_B	Quinone B
RISC	RNA-Induced-Silencing-Complex
siRNA	Short-interfering RNA

Table A2.
List with some abbreviations that are cited inside the text and their corresponding etymology.



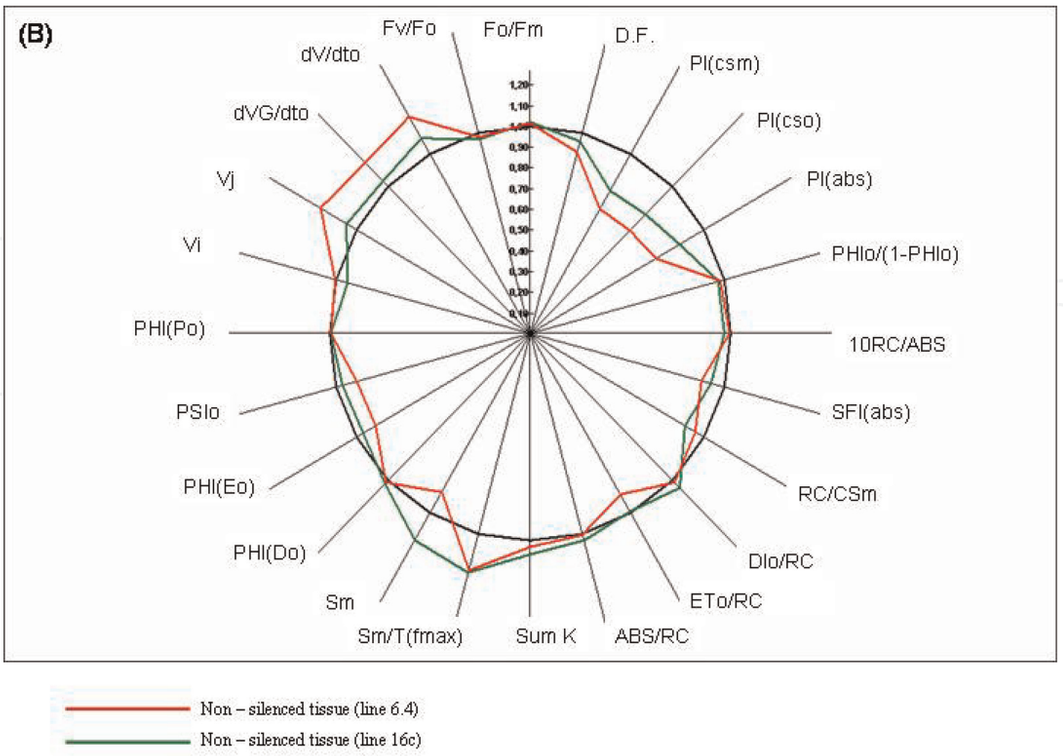


Figure A1.
A, B: Representative radar plots are denoting basic bioenergetic parameters of photosynthesis, in the examining tissues. The control condition (wt tissue) is presented as circle.

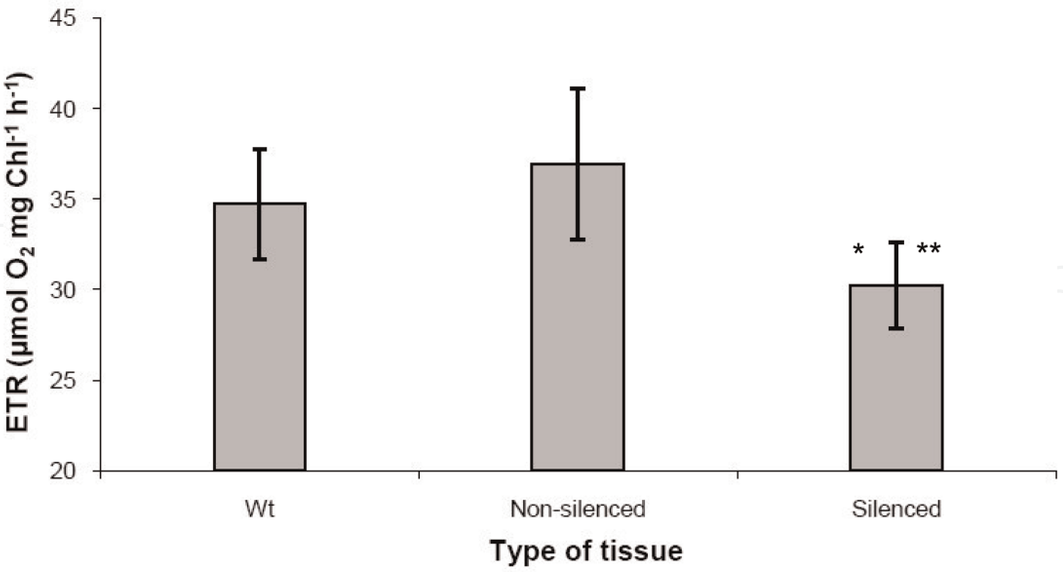


Figure A2.
In vitro *estimation of the linear electron flow in thylakoid membranes from the plant categories studied. Values normalized over wt ones. ETR: Electron Transport Rate, Chl: chlorophyll; h: hour.*

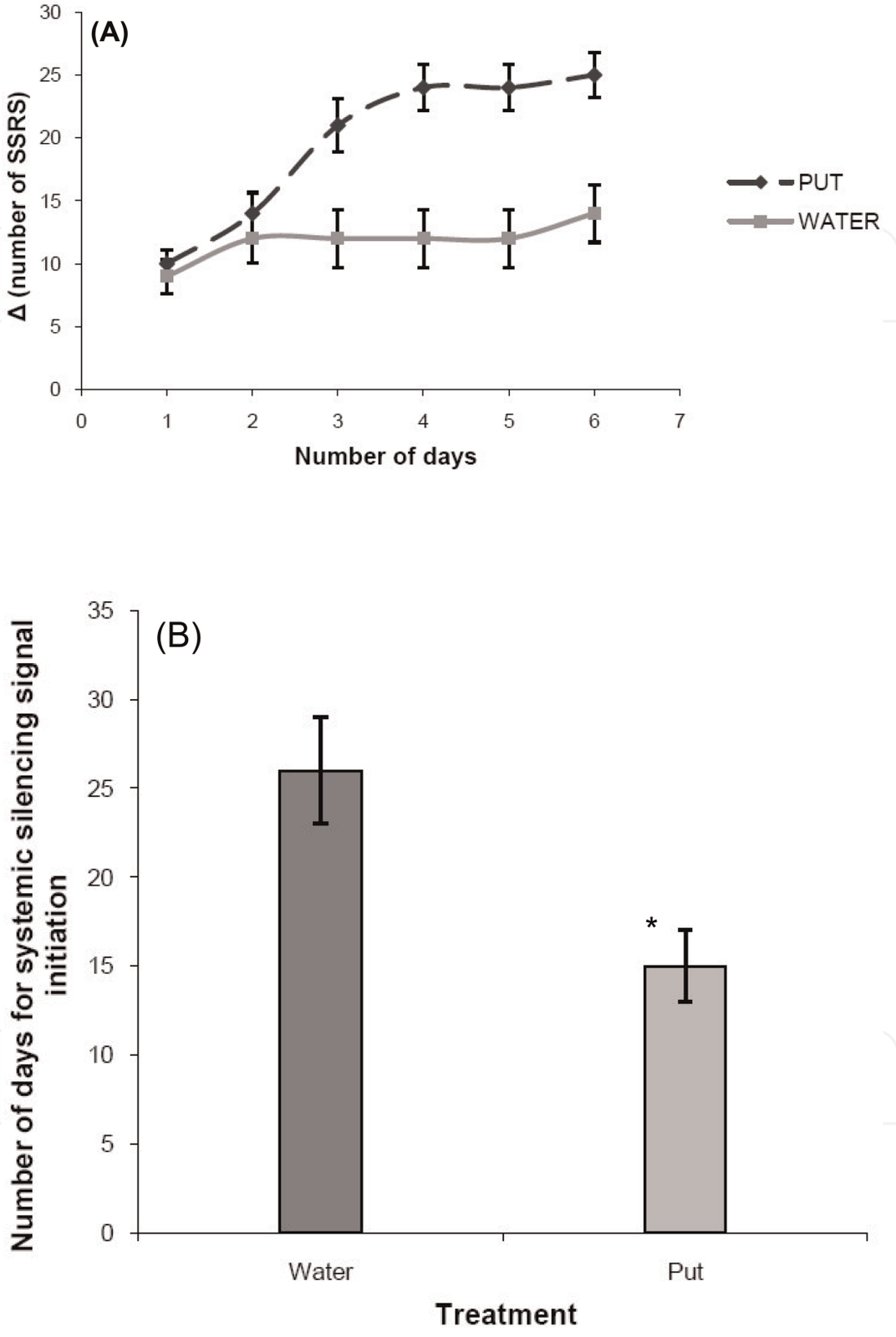


Figure A3.
A Daily monitoring for the number of short-range silencing spread (SSRS) events. B: Number of days for the systemic silencing signal onset in plants that express GFP (line 16c), after agroinfiltration with a hairpin GFP construct. Treatment of tissues studied with either 1 mM Put or water, was performed for 5 days, prior the beginning of the monitoring. The data presented in A are sum of SSRS events from the total surface of leaves per plant individual, examined for each treatment. The initial number of spots before the beginning of monitoring was considered and it was subtracted (Δ) from the new appearing ones during the time of monitoring. Similar method of sampling in the case of B, was followed.

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