LHCSR3 affects de-coupling and re-coupling of LHCII to PSII during state transitions in *Chlamydomonas reinhardtii*

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Photosynthetic organisms have to tolerate rapid changes in light intensity, which is facilitated by non-photochemical quenching (NPQ) and involves modification of energy transfer from light-harvesting complexes (LHC) to the photosystem reaction centres. NPQ includes dissipating excess light energy to heat (qE) and the reversible coupling of LHCII to photosystems (state transitions/qT), which are considered separate NPQ mechanisms. In the model alga *Chlamydomonas reinhardtii* the LHCSR3 protein has a well characterised role in qE. Here, it is shown in the *npq4* mutant, deficient in LHCSR3, that energy coupling to photosystem II (PSII) more akin to qT is also disrupted, but no major differences in LHC phosphorylation or LHC compositions were found in comparison to wild-type cells. The qT of wild-type cells possessed two kinetically distinguishable phases, with LHCSR3 participating in the more rapid (<2 min) phase. This LHCSR3-mediated qT was sensitive to physiological levels of H2O2, which accelerated qE induction, revealing a way that may help *C. reinhardtii* tolerate a sudden increase in light intensity. Overall, a clear mechanistic overlap between qE and qT is shown.

Achieving photosynthetic efficiency under naturally fluctuating light intensities requires mechanisms that can rapidly switch between highly efficient light absorption and dissipation of excess-absorbed light energy. Otherwise over-excited reaction centres lead to the elevated formation of potentially damaging reactive oxygen species (ROS) and photoinhibition1–3. Non-photochemical quenching is a collective name for the mechanisms that regulate energy transfer to the photosystem reaction centres, thereby protecting from photoinhibition.

The most rapidly inducible component of NPQ is qE, which is regulated by the pH of the thylakoid lumen. A low pH leads to protonation of a LHC-type protein triggering the switch between light harvesting and excess light-energy dissipation4–9. In higher plants this LHC-type protein is PsbS10, whereas in *Chlamydomonas reinhardtii* Light-Harvesting-Complex-Stress-Related-3 (LHCSR3) is involved11. *Arabidopsis thaliana* or *C. reinhardtii* mutants deficient in PsbS or LHCSR3, respectively, are both referred to as *npq4* and have severely diminished qE under excess light10,11. Using *npq4* and dissipaters of the trans-thylakoid pH gradient (ΔpH) it has been shown that qE protects from ROS production and photoinhibition under excess light12–14. Expression of the gene coding for LHCSR3 (previously referred to as LI818) follows diurnal cycles in day/night grown photoautotrophic cells15, and can be rapidly up-regulated when cells are subjected to high light under ambient CO211, conditions that lead to excess light absorption and a need for qE.

State transitions (qT) are the reversible associations of LHCs, mainly LHCII, with PSII and PSI. Transitioning from state I, where LHCII is coupled to PSII, to state II is regulated by a thylakoid-bound kinase, which phosphorylates LHC proteins when the plastoquinone (PQ) pool becomes reduced. It is an NPQ mechanism that is much more active in algae than higher plants16. In *C. reinhardtii* up to 80% of LHCII de-couples energy transfer from PSII in state II, with PSI able to use 20% of this light energy17,18. STN7, the higher plant analogue of Stt7 in *C. reinhardtii*19, has H2O2-sensitive thiol groups20 that are likely conserved in Stt7, as Stt7-mediated LHC phosphorylations are decreased by H2O221. Hydrogen peroxide is a ROS produced in the chloroplast under high light22. Reduction of cysteines 68 and 73 of Stt7 are required for Stt7 kinase activity23, explaining why H2O2 that

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subunit of PSII (PsbA) is shown as loading control.

Results

Between the light intensities of 250–2000 μmol quanta m$^{-2}$ s$^{-1}$ the qE of npq4 was six times lower than in wild-type cells, a deficiency due to the absence of the light-inducible LHCSR3 protein (Fig. 1). To compare the qT of npq4 and wild-type cells chlorophyll fluorescence at a temperature of 77 K was measured after various light treatments. Chlorophyll excitation at 77 K leads to emission peaks centred at 685 and 715 nm, corresponding to PSII and PSI, respectively, thereby indicating the location of the mobile fraction of LHCII. Dark adapting pre-state I when LHCII couples energy transfer to PSII. Transitioning from state I (under high light) to state II (due to dark) and back to state I (due to light) was accompanied by a major increase then progressive decreases in LHC phosphorylation of wild-type cells in state I or state II (Fig. 2c). Other explanations for a decrease in fluorescence from PSII could be due to a higher Stt7 activity preventing cells from attaining state I. However, no major differences in LHC phosphorylation were observed between npq4 and wild-type cells in state I or state II (Fig. 2c). Other explanations for a decreased fluorescence from PSII could be photo inhibition, smaller antenna size and higher qE, but the similar maximum quantum yield of PSII ($F_{m}/F_{m}$) after pre-high light treatment indicated limited differences in photo inhibition (Table 1), the insignificantly different maximum chlorophyll fluorescence ($F_{m}$) measured in the presence of DCMU showed equal antenna size (Table 1) and qE is not higher in npq4 (Fig. 1). Therefore, a lack of fully achieving state I in npq4 could be explained by an absence of LHCSR3 restricting energy coupling to PSII. It is important to note that while traditionally a transition to state II assumed that most LHCII migrated to PSI this is now considered unlikely. The definition of state II used here is that LHCII has disassociated energy transfer from PSII regardless of any association to PSI.

At room temperature chlorophyll fluorescence is primarily from PSII. By following changes $F_{m}$, qE can be measured in vivo by increases and decreases in $F_{m}$ that correspond to transitioning to state I and state II, respectively. Cells were placed in state I with far-red light until $F_{m}$ no longer increased. After switching off the far-red light and placing cells in darkness, $F_{m}$ decreased as cells transitioned to state II. The greatest decrease in $F_{m}$ occurred during the first minute and in npq4 this decrease was 59% less than in wild-type (Fig. 3). Therefore, the...
absence of LHCSR3 affected measurements of qT in mechanism completely separate to qE (i.e. in darkness). It was observed that npq4 has a slightly higher chlorophyll a:b ratio and a larger size of the xanthophyll cycle pool than wild-type cells (Table 1), indicating there are some minor differences in the LHC composition of npq4, as noted previously11, but the PSII antenna size was equal (Table 1). Cells were treated as in (a) and phosphorylated proteins were detected by immunoblotting with phospho-threonine antibody. (c) Levels of thylakoid protein phosphorylation (Phos.) of wild-type and npq4 in high light (HL) and after a subsequent 4 min of darkness (Dark). Supplementary Figure S6 shows an uncropped blot. The D1 subunit of PSII (PsbA) blotted from the same membrane is shown as a loading control.

Figure 2. Characterisation of qT in wild-type and npq4. (a) Low-temperature (77 K) chlorophyll fluorescence emission spectra (ex: 440 nm) of wild-type in state I after 2 h high light pre-treatment (HL; green), in state II after a subsequent 5 min dark (red dotted) and then re-transitioning to state I by exposure to 3 min (blue) and 5 min (yellow) high light, and a further 10 min far-red light (black dashed). The changes in npq4 are shown below wild-type and all spectra were normalised at 715 nm. (b) Modification in the level of phosphorylation of thylakoid proteins in wild-type during transitioning between states I and II. Cells were treated as in (a) and phosphorylated proteins were detected by immunoblotting with phospho-threonine antibody. (c) Levels of thylakoid protein phosphorylation (Phos.) of wild-type and npq4 in high light (HL) and after a subsequent 4 min of darkness (Dark). Supplementary Figure S6 shows an uncropped blot. The D1 subunit of PSII (PsbA) blotted from the same membrane is shown as a loading control.
Deciphering chlorophyll fluorescence for measuring qE in *C. reinhardtii* can be problematic because of the large overlap of qT. For example, qE apparently decreased to negative values during a quenching analysis because $F_{m}'$ ($F_{m}$ measured in the light) increased above $F_{m}^{\circ}$ ($F_{m}$ measured in the dark) (Fig. 4). This can be explained by cells transitioning from state II to state I when they are transferred from dark to light, as shown by 77 K chlorophyll fluorescence in Fig. 2a. In low light-acclimated wild-type cells, which only accumulated low levels of LHCSR3 (Fig. 1), there was an absence of the rapid light-induced rise in $F_{m}^{\circ}$ of wild-type (black), npq4 (grey) or stt7-7/stt7–9 (white) during 0–1 min or 2–4 min in the absence (solid) or presence (white diagonal-stripe) of 0.1 mM DCMU. % from WT’ was calculated as (npq4-WT)/WT × 100 and ‘*’ corresponds to a significant difference (P < 0.05), n = 4 ± SD.

Table 1. The differences in pigment composition and chlorophyll fluorescence parameters of wild-type and npq4 cells after the high light pre-treatment. WT = wild-type, ‘Total V AZ’ = total xanthophyll pool of violaxanthin, antheraxanthin and zeaxanthin calculated on a mol basis and expressed as molx100:mol total chlorophyll, as for lutein. ‘Chl. a:b’ = ratio of chlorophyll a:b calculated on a mol basis, ‘$F_{r}/F_{m}$’ = maximum quantum yield of PSII, ‘$F_{o}$ (DCMU)’ = Fo measured in cultures at 10 μg mL$^{-1}$ chlorophyll in the presence of 10 μM DCMU, % from WT’ was calculated as (npq4-WT)/WT × 100 and ‘*’ corresponds to a significant difference (P < 0.05), n = 4 ± SD.

|          | WT | SD  | npq4 | SD  | % from WT |
|----------|----|-----|------|-----|-----------|
| Lutein   | 15.06 | 1.32 | 16.03 | 0.89 | +6.4      |
| Total V AZ | 9.09 | 0.43 | 10.31 | 0.77 | +13.4*    |
| Chl. a:b | 2.49  | 0.11 | 2.70  | 0.08 | +8.5*     |
| $F_{r}/F_{m}$ | 0.63  | 0.01 | 0.61  | 0.01 | −3.8*     |
| Fo (DCMU) | 0.100 | 0.004 | 0.107 | 0.003 | +7.0      |

Figure 3. The state I to II transition of qT separated into the LHCSR3-mediated and Stt7 kinase-mediated components and effects of H$_2$O$_2$. High light-treated cells were subjected to far-red light to fully induce state I ($F_{m}^{\circ}$). State II conditions were activated by placing cells in darkness and the decrease in $F_{m}^{\circ}$ was followed (see inset) in wild-type (WT; squares), npq4 (circles), stt7-7 (downward triangles) or stt7–9 (upward triangles). The bar chart shows decrease rates in $F_{m}^{\circ}$ of wild-type (black), npq4 (grey) or stt7-7/stt7–9 (white) during 0–1 min or 2–4 min in the absence (solid) or presence (white diagonal-stripe) of 0.1 mM H$_2$O$_2$ added 1 min before measurements. Data was normalised to $F_{m}^{\circ}$ at 0 min. Different letters indicate significant differences (P < 0.05), n = 4 ± SD.
(Supplementary Figure S4), \( F'_m \) of \( stt7-7 \) did not decrease by the time \( qE \) had been induced in wild-type or \( stt7-9 \) (Fig. 5). Therefore, although \( qE \) was LHCSR3 dependent (i.e. absent in \( npq4 \)) there was further control mediated by Stt7 only apparent in the non-leaky \( stt7-7 \). When the quenching analysis of wild-type cells was made in the presence of catalase the light-induced transition to state I was delayed\(^2\), as was the induction of \( qE \) (Fig. 6). The same slowing of \( qE \) induction from the addition of catalase was also observed with \( stt7-9 \), but not \( stt7-7 \) that was unable to induce \( qE \) during the analysis (Supplementary Figure S4).

**Discussion**

The NPQ mechanisms of \( qE \) and \( qT \) have each been characterised under distinct conditions, leading to the notion of completely unique processes. For example, \( qT \) has been investigated in conditions such as anoxia in the dark\(^{17,19,21}\), which is far away from the excess light required for inducing \( qE \). However, Tokutsu and Minagawa\(^5\) showed that the majority of LHCSR3 in high light-treated \( C. reinhardtii \) was associated with detached LHCII, a situation that could have derived from either \( qE \) or \( qT \). Moreover, others have shown LHCSR3 attached to the PSI-supercomplex\(^{1,2,23}\), fitting with a role for LHCSR3 in \( qT \). As discussed below, our data would fully support a role for LHCSR3 in energy coupling of LHCII to PSII as part of \( qT \).
Placing *C. reinhardtii* from high-light or far-red light into darkness activates chlororespiration, a reduction of the PQ pool and transition to state II13,27. Two kinetically separate phases were evident in wild-type cells during this transition, with *npq4* retarded in the initial rapid decrease of $F_{m}^\circ$ (Fig. 3). However, the later linear and slower decrease of $F_{m}^\circ$ from 2–4 min, absent in *stt7* mutants, was equally present in *npq4* and wild-type alongside equally phosphorylated LHCs after 4 min dark in *npq4* and wild-type (Fig. 2c). In summary, *npq4* was inhibited in the rapid de-coupling of energy to PSII during a transition to state II, revealing the involvement of LHCSR3, but *npq4* was not affected in the slower de-coupling of energy that was attributable to Stt7 kinase.

A role for LHCSR3 in energy coupling to PSII during transition to state I was also explored. Exposing dark-treated cells in state II to actinic light induces transition to state I13, as shown by changes in chlorophyll fluorescence at 77 K (Fig. 2a). A light-induced transition to state I can be called the “S” (semi-steady state) to “M” (maximum) rise when using the so-called O-J-I-P-S-M nomenclature28. Here, it was observed during a quenching analysis that the increase in $F_{m}'$ of wild-type cells possessed two kinetically separate phases; a rapid initial increase partially present in *stt7* mutants, but totally absent in *npq4* (Fig. 5), and a second slower increase over several minutes (Fig. 4c) that occurred alongside a decrease in LHC phosphorylation (Fig. 2b), explaining its absence in *stt7* mutants (Fig. 5). A lack in the rapid $F_{m}'$ increase early in the quenching analysis by *npq4* confirms an involvement of LHCSR3, and also explains why a high light pre-treatment to induce LHCSR3 was required to see this phenomenon (Supplementary Figure S2). This also explains why the rapid increase in $F_{m}'$ was also observed in

![Figure 5. The NPQ phases during dark to light exposure separated into the LHCSR3-mediated and Stt7 kinase-mediated components.](image)

**Figure 5.** The NPQ phases during dark to light exposure separated into the LHCSR3-mediated and Stt7 kinase-mediated components. Wild-type (squares), *npq4* (circles), *stt7–9* (upward triangles) and *stt7–7* (downward triangles) were pre high light-treated and then dark-adapted for 15 min inducing state II conditions. Subsequently, cells were treated with 474μmol quanta m$^{-2}$ s$^{-1}$, as indicated by the white bar on the X-axis, to induce state I before qE became induced. Data are normalised to $F_{m}^\circ$ at 0 min. The dashed red arrows of $F_{m}'$ changes in wild-type cells indicate 1) rapid LHCSR3-dependent qE, 2) rapid LHCSR3-involved qT transition to state I, 3) slower Stt7-mediated qT transition to state I, and 4) LHCSR3- and Stt7-dependent qE of state I cells (see text for details).

![Figure 6. H2O2 accelerates the induction of qE.](image)

**Figure 6.** H$_2$O$_2$ accelerates the induction of qE. Wild-type cells were pre high light-treated and then dark-adapted for 15 min in the presence (closed squares) or absence (open circles) of 500 U mL$^{-1}$ catalase. Cells were subsequently treated with 474μmol quanta m$^{-2}$ s$^{-1}$ to induce qE. Significant differences ($P < 0.05$) in the presence or absence of catalase at each interval are indicated by *.
It is known that stt7–9 is a leaky mutant with residual Stt7 activity. However, measuring qT by shifting far-red light-treated cells to dark showed stt7–7 and stt7–9 behaved very similar (Fig. 3). After all, despite its leaky nature, stt7–9 cannot phosphorylate the LHClI protein LHCBM5 in state II conditions and cannot perform Stt7-mediated qT. The differences in the behaviour of stt7–7 and stt7–9 during the quenching analysis are therefore only apparent under actinic light. Stromal residues of the LHCSR3 N-terminal (Ser-26, Ser-28, Thr-32, Thr-33, and Thr-39), can be phosphorylated in wild-type and stt7–9, but not in a non-leaky Stt7-deficient mutant. We suggest that Stt7 phosphorylation of LHCSR3 is involved in the LHCSR3-mediated and light-dependent qT, which could explain the smaller increase in $F_m$ early during the quenching analysis by stt7–7 compared to stt7–9. It is tempting to speculate that such LHCSR3 phosphorylations may also explain the deficiency of qE in stt7–7. However, a difference in the phosphorylation level of LHCB4, or other proteins that occur in stt7–9, with a non-leaky Stt7 mutant, may also be responsible.

In summary, the rapid and slower decreases of $F_m$ during a transition to state II (Fig. 3 inset) kinetically mirrored the rapid and slower increases in $F_m$ during transition to state I (Fig. 4c). With the use of npq4, stt7–7 and stt7–9 we are able to deduce that LHCSR3 is required for the more rapid transitions of qT, while only Stt7 kinase activity was involved in the slower transitions. Furthermore, Stt7-mediated phosphorylations are also involved in qE.

Previously, it has been shown that STN7 kinase of Arabidopsis has $H_2O_2$-sensitive exposed thiol groups and that LHCl phosphorylations mediated by Stt7 kinase in C. reinhardtii were inhibited by $H_2O_2$. It is now apparent that the more rapid transition to state II, which involves LHCSR3, is more sensitive to $H_2O_2$ than the slower transition to state II, which involves only Stt7 kinase (Fig. 3). Furthermore, the involvement of LHCSR3 explains why measurements of npq4 were much less influenced by $H_2O_2$ (Fig. 3). An explanation to why LHCSR3-mediated qT is particularly sensitive to $H_2O_2$ could be that a smaller change in phosphorylation levels leads to a larger level of regulation than LHClI phosphorylation, which merits further investigation. A transition in the reverse direction was also sensitive to $H_2O_2$, as shown by the delayed transition to state I in wild-type cells treated with catalase. This phenomenon can be explained by $H_2O_2$ slowing Stt7 kinase activity, which accelerates the transition to state I. As this effect was seen after removing $H_2O_2$, rather than by its addition, this level of regulation is clearly operational under standard lab conditions and with physiological levels of $H_2O_2$. Transitions to state I during a sudden increase in light intensity has been previously described as a mechanism that facilitates qE induction by increasing light absorption by PSII. Here we showed that catalase delayed the onset of qE by approximately 2.5 min in wild-type (Fig. 6) and stt7–7 (Supplementary Fig. 4), both of which can phosphorylate LHCSR3. In conclusion, $H_2O_2$ production in the chloroplast can benefit C. reinhardtii by adjusting to a rapid increase in light intensity through a process involving Stt7 and LHCSR3, and potentially Stt7-mediated phosphorylation of LHCSR3.

**Materials and Methods**

**Strains and Growth Conditions and high light pre-treatments.** *Chlamydomonas reinhardtii* wild type (wild-type) strain T222 (in the 137C background), stt7–9 (a leaky mutant with an estimated 6-fold decrease in Stt7 kinase activity) and stt7–7 (a totally Stt7-deficient mutant, J-D. Rochaix, personal communication) were gifts from J-D. Rochaix, University of Geneva. npq4 [CC-4614] was purchased from the Chlamydomonas Centre (www.chlamycollection.org). Cultures were initiated in Tris-Acetate-Phosphate media (TAP) and adjusted by increasing light absorption by PSII. Here we showed that catalase delayed the onset of qE by approximately 2.5 min in wild-type (Fig. 6) and stt7–9 (Supplementary Fig. 4), both of which can phosphorylate LHCSR3. In conclusion, $H_2O_2$ production in the chloroplast can benefit *C. reinhardtii* by adjusting to a rapid increase in light intensity through a process involving Stt7 and LHCSR3, and potentially Stt7-mediated phosphorylation of LHCSR3.

**Photosynthetic pigments.** Carotenoids were measured from 10 mg of lyophilized cells extracted in 1 mL of ice-cold acetone by shaking (TissueLyser II, Qiagen, Düsseldorf, Germany) at 30 Hz for 2 min with two 2 mm glass beads before centrifugation at 26,000 g for 45 min. Ten μl of the supernatant was injected using an Agilent 1100 HPLC system equipped with a LiChrospher 100 RP-18 (5 μm) column (Agilent Technologies, Santa Clara, California, USA). Peak identity and quantification was made against individual standards with absorbance at 440 nm. Total chlorophyll and chlorophyll a and b were measured according to in 80% acetone.

**Photosynthetic Measurements.** Pulse amplitude-modulated (PAM) chlorophyll fluorescence measurements were made with a PAM 2500 (Walz GmbH, Effeltrich, Germany). Maximum chlorophyll fluorescence ($F_m$) was measured with a 200 ms saturating pulse. Cultures of 1.5 mL were constantly stirred with a magnetic bar during measurements. For measuring the light dependency of qE induction cells were first allowed to recover from high light for at least 1 h and then treated with far-red light to fully achieve maximum $F_m$ ($F_m^\prime$). Increasing intensities of light were provided for 1 min intervals after which $F_m$ ($F_m$ under actinic light) was measured in order to calculate qE via $(F_m^\prime − F_m)/F_m'$. For measuring the state II to I transition, cells were treated with far-red light to achieve state I, as observed when $F_m$ no longer increased (typically 10 min when saturating pulses were kept 90 s apart). Induction of state II was measured by following the decrease in $F_m$ after the far-red light was switched off.

$H_2O_2$ was added 1 minute before measurements from a stock of 100 μM. For splitting the qE and qT responses.
cells were dark-adapted for various times, as stated in the Figure legend, before treating at a light intensity of 474 μmol quanta m⁻² s⁻¹. The Fₔ₋ was measured every 30 s. To observe the effects of nigericin the cell wall-less wild-type strain (cw15) was used, because nigericin can enter its cells and the strain possesses typical LHC phosphorylation patterns and can accumulate LHCSR3 (Roach, unpublished). Nigericin was used at 10 μM from a 10 mM stock dissolved in methanol. To assess PSII antenna size via F₄₋ cells were first treated for 1 min in the dark with 10 μM 3-(3,4-dichlorophenyl)-1,1-dimethyleurea (DCMU) from a 10 mM stock dissolved in methanol.

Chlorophyll fluorescence emission between 650–750 nm was measured at 77 K with a Cary Eclipse fluorescence spectrometer (Varian, Mulgrave, Australia) at an excitation of 440 nm (5 nm slit width) in samples capillary-loaded in glass Pasteur pipettes and immediately frozen in N₂(l) before measurement.

Analysis of LHCSR3, PsbA and three-phosphorylated protein levels. Total cellular proteins were extracted in 2% SDS in 100 mM TRIS-HCl, pH 6.8, containing a protease inhibitor cocktail (Complete Mini, Roche Diagnostics, Switzerland). Proteins were quantified using the bicinchoninic acid assay (Sigma-Aldrich, St Louis MO, USA), loaded at 20 μg protein/sample and separated by PAGE using 12% acrylamide gels at 40 mA for 1.5 h. For western blotting separated proteins were transferred to nitrocellulose membranes at 40 mA.

Roche Diagnostics, Switzerland). Proteins were quantified using the bicinchoninic acid assay (Sigma-Aldrich, St Louis MO, USA), loaded at 20 μg protein/sample and separated by PAGE using 12% acrylamide gels at 40 mA for 1.5 h. For western blotting separated proteins were transferred to nitrocellulose membranes at 40 mA/gel for 1 h, which were subsequently blocked in 5% fat-free milk powder before incubating with the LHCSR3 (Agrisera, Sweden) or anti-phospho-threonine antibody (Cell Signalling Technologies, USA) at 1:10,000 dilution for 1.5 h. For western blotting separated proteins were transferred to nitrocellulose membranes at 40 mA/gel for 1 h, which were subsequently blocked in 5% fat-free milk powder before incubating with the LHCSR3 (Agrisera, Sweden) or anti-phospho-threonine antibody (Cell Signalling Technologies, USA) at 1:10,000 dilution or PsbA antibody (Agrisera, Sweden) at 1:25,000 dilution. The peroxidase-coupled antibodies were visualised with enhanced chemiluminescence (Amersham, GE Healthcare, UK) and light sensitive film (Amersham, GE Healthcare, UK).

Statistics. Significant differences at P < 0.05 were calculated using IBM SPSS (v.21) and one-way ANOVA with Tukey’s post-hoc test.

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Author Contributions
T.R. conceived and designed the experiments. T.R. and C.S.N. conducted the experimental work and analysed the data. T.R. wrote the paper.

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