Natural variation in phosphorylation of photosystem II proteins in *Arabidopsis thaliana*: is it caused by genetic variation in the STN kinases?

Pádraic J. Flood¹,²,³, Lan Yin³,⁻¹, Andrei Herdean³, Jeremy Harbinson², Mark G. M. Aarts¹ and Cornelia Spetea³

¹Laboratory of Genetics, and ²Horticulture Production Chains Group, Wageningen University, Wageningen 6708 PB, The Netherlands
³Department of Biological and Environmental Sciences, University of Gothenburg, Gothenburg 40530, Sweden

Reversible phosphorylation of photosystem II (PSII) proteins is an important regulatory mechanism that can protect plants from changes in ambient light intensity and quality. We hypothesized that there is natural variation in this process in *Arabidopsis (Arabidopsis thaliana)*, and that this results from genetic variation in the STN7 and STN8 kinase genes. To test this, *Arabidopsis* accessions of diverse geographical origins were exposed to two light regimes, and the levels of phospho-D1 and phospho-light harvesting complex II (LHCII) proteins were quantified by western blotting with anti-phosphothreonine antibodies. Accessions were classified as having high, moderate or low phosphorylation relative to Col-0. This variation could not be explained by the abundance of the substrates in thylakoid membranes. In genotypes with atrazine-resistant forms of the D1 protein, low D1 and LHCII protein phosphorylation was observed, which may be due to low PSII efficiency, resulting in reduced activation of the STN kinases. In the remaining genotypes, phospho-D1 levels correlated with STN8 protein abundance in high-light conditions. In growth light, D1 and LHCII phosphorylation correlated with longitude and in the case of LHCII phosphorylation also with temperature variability. This suggests a possible role of natural variation in PSII protein phosphorylation in the adaptation of *Arabidopsis* to diverse environments.

1. Introduction

Plants require light energy to drive photosynthesis. Their photosynthetic machinery is profoundly affected by changes in irradiance, which can be in both the intensity and in the spectral quality of light, and can occur across a range of temporal scales, from seconds (light flecks) to months (seasonal changes). Plants have developed diverse response mechanisms to adjust and protect their photosynthetic machinery in the face of such fluctuations (for recent review, see [1]). Short-term responses involve a dynamic reorganization of photosynthetic complexes, whereas long-term responses involve changes in the chloroplast and nuclear gene expression, resulting in altered levels of the photosynthetic machinery to optimize and sustain photosynthesis. Both types of responses are induced by changes in the redox state of the photosynthetic electron transport chain, and are mediated through a complex network of reactions involving protein kinases and phosphatases [1]. Plants which have a compromised ability to respond to both short- and long-term light fluctuations show fitness costs in nature [2,3]. In this study, we aimed to quantify the natural genetic variation in these responses, both to document the extent of this variation, and to gain some initial insights into the selective forces which may be acting on these processes.

Photosystems II (PSII) and I (PSI) are connected in series through the electron transport chain which includes the plastoquinone (PQ) pool, the cytochrome b₅₆ (Cytb₅₆) complex and plastocyanin. Changes in the light environment may lead to...
more reduction or oxidation of the PQ pool, initiating signalling processes that drive changes in the organization and composition of the photosynthetic machinery. The process of state transitions is used by algae and also plants, to correct the redox state of the PQ pool. In the case of a reduced PQ pool, plastocyanin (PQH$_2$) docks to the Q$_i$ site of Cyt$b$_6f [4]. This event leads to the activation of a protein kinase that phosphorylates several proteins of the light harvesting complex II (LHClI). Upon phosphorylation, the mobile part of LHClI is displaced from PSII to PSI, thus re-equilibrating the cross sections of the antennae of PSI and PSII and their respective light excitation. The process is reversible, as overexcitation of PSI causes the oxidation of the PQ pool, deactivation of the kinase, dephosphorylation of LHClI proteins by a phosphatase and the return of LHClI to PSII. Using molecular genetic approaches, the LHClI kinase was identified in Arabidopsis and named STN7 [5].

Of the two photosystems, PSII is more susceptible to photo-inactivation, and undergoes a repair cycle to replace its reaction centre D1 protein [6]. In the plant thylakoid membrane, PSII is mostly present as PSII–LHClI dimeric supercomplexes located in the appressed (grana) membranes. However, the repair of photoinactivated PSII complexes and the assembly of new ones occur through the monomeric form of PSII in the non-appressed (stroma) thylakoid membranes. PSII core protein phosphorylation in general, and D1 phosphorylation in particular, has been suggested to facilitate disassembly of photoinactivated PSII complexes and is thought to play a role in the regulation of PSII repair [7]. The kinase involved in PSII core protein phosphorylation was identified in Arabidopsis and named STN8 [8,9].

To elucidate the substrate specificity of STN7 and STN8 kinases, thylakoid protein phosphorylation patterns of wild-type Arabidopsis plants and stn7 and stn8 mutant lines have been monitored by different approaches, including western blot analyses with different anti-phosphothreonine antibodies and mass spectrometric analyses. The STN7 kinase is involved in phosphorylation of LHClI, CP29, CP26 and TSP9 proteins, whereas the STN8 kinase phosphorylates PSII core D1, D2, PsbH and to some extent CP43 proteins (for reviews, see [10,11]). Besides the PSII core proteins, STN8 has additional targets, including the chloroplast calcium-sensing protein CAS and named STN8 [8,9].

As outlined above, both the STN7 and STN8 kinases play essential roles in the response to changes in ambient light, by influencing LHClI distribution between PSI and PSII and facilitating protein repair, respectively. A recent review compares such responses across a wide phylogenetic spectrum [28]; however, very little is known about the intraspecific variation in these processes [29]. Such within-species variation drives natural selection and may represent different adaptive strategies to photosynthetic regulation within a species. Arabidopsis has a wide geographical distribution, ranging from Tanzania to...
with the observed variation in PSII protein phosphorylation. As the geographical and climatic origin of the genotype correlate the protein or transcript level of the respective kinase, as well, we have investigated whether variation in and we used the levels in the standard accession Col-0 as reference. Furthermore, we have investigated whether variation in the protein or transcript level of the respective kinase, as well as the geographical and climatic origin of the genotype correlate with the observed variation in PSII protein phosphorylation.

Norway and from Portugal to Japan. As such it occupies a wide range of light environments, and it may have adopted different strategies to cope with this environmental diversity. Here, we screen 16 Arabidopsis genotypes composed of 13 diverse accessions from throughout the natural range of Arabidopsis as well as a backcross, the stn8 mutant and a hybrid between two of these accessions. We have analysed thylakoid protein extracts by western blotting with anti-phosphothreonine antibodies, and we used the levels in the standard accession Col-0 as reference. Furthermore, we have investigated whether variation in the protein or transcript level of the respective kinase, as well as the geographical and climatic origin of the genotype correlate with the observed variation in PSII protein phosphorylation.

2. Material and methods

(a) Plant growth and light treatment

Arabidopsis thaliana plants were grown for 34 days in a chamber at 100 μmol photons m⁻² s⁻¹ (GL) using a 10/14 h day/night cycle. After 14 h of darkness, plants were exposed for 3 h to GL and subsequently transferred for 3 h to HL (600 μmol photons m⁻² s⁻¹). Leaf material corresponding to 2–3 g bulked from five to six plants was harvested, frozen immediately in liquid nitrogen and stored at −80 °C until thylakoid isolation.

A total of 16 Arabidopsis genotypes were included in this study (table 1). We used 13 naturally occurring accessions from a range of geographical locations. In addition, a previously characterized stn8 mutant line lacking a functional STN8 kinase [15] was used. One F1 hybrid was included (Tsu-0 × Ws-4) to test for the presence of a dominant phenotype, and because pilot experiments identified both parental accessions as extremes. The atrazine-resistant Ely accession was included because it has compromised PSII functionality or other factors. Western blots with anti-D1 and anti-Lhcb2 antibodies indicated the level of corresponding substrate for the phosphorylation reaction (figure 1a). The levels of the STN8 and STN7 kinases were assessed in samples from thylakoid preparations using specific antibodies, and control western blots to verify the identity of the corresponding cross-reacting bands with the STN8 and STN7 antibodies. Two Chl loadings are shown to indicate the linearity of the immunodetected signal. Any possible variation in phosphorylation could be caused by variation in the amount of substrate, kinase involved or other factors. Western blots with anti-D1 and anti-Lhcb2 antibodies recognized PSII-phospho-proteins, but with different affinities (figure 1a). The Zymed antibody recognized the phospho-D1 protein best, whereas the Cell Signaling antibody was found most suitable for quantification of phospho-LHCII proteins. Two Chl loadings are shown to indicate the linearity of the immunodetected signal. Any possible variation in phosphorylation could be caused by variation in the amount of substrate, kinase involved or other factors. Western blots with anti-D1 and anti-Lhcb2 antibodies indicated the level of corresponding substrate for the phosphorylation reaction (figure 1a). The levels of the STN8 and STN7 kinases were assessed in samples from thylakoid preparations using specific antibodies, and control western blots with CP43 protein were used to correct the amount of protein loaded (figure 1b). The stn8 double mutant [33] was used to verify the identity of the corresponding cross-reacting bands with the STN8 and STN7 antibodies. Two Chl loadings are shown to indicate the linearity of the immunodetected signal for the three antibodies.

(b) Thylakoid isolation and protein analysis

Thylakoid membranes were isolated from frozen leaves as previously described [31] with the modification that 10 mM NaF (a general phosphatase inhibitor) was included in all isolation buffers. Chlorophyll (Chl) was extracted in 80% (v/v) acetone and the concentration was determined according to Porra et al. [32]. Thylakoid proteins were separated by SDS–PAGE in 14% (w/v) acrylamide gels containing 6 M urea followed by electrotransfer and immunoblotting with various antibodies. The following antibodies were used: anti-D1, anti-Lhcb2, anti-STN8 and anti-STN7 from Agrisera (Umeå, Sweden), anti-phosphothreonine antibodies from Cell Signaling (New England BioLabs, UK) and Zymed (Invitrogen, Carlsbad, CA, USA), and anti-CP43 from our laboratory. Western blots were analysed using a Fusion FX-7 imager (Vilbert Lourmat, France) and quantified using Multi Gauge software. Col-0 was used as internal standard, making it possible to compare different Western blots.

As shown by representative blots for Col-0, both anti-phospho-Thr antibodies recognized PSII-phospho-proteins, but with different affinities (figure 1a). The Zymed antibody recognized the phospho-D1 protein best, whereas the Cell Signaling antibody was found most suitable for quantification of phospho-LHCII proteins. Two Chl loadings are shown to indicate the linearity of the immunodetected signal. Any possible variation in phosphorylation could be caused by variation in the amount of substrate, kinase involved or other factors.

Western blots with anti-D1 and anti-Lhcb2 antibodies recognized the phospho-CP43 protein best, whereas the Cell Signaling antibody was found most suitable for quantification of phospho-LHCII proteins. Two Chl loadings are shown to indicate the linearity of the immunodetected signal. Any possible variation in phosphorylation could be caused by variation in the amount of substrate, kinase involved or other factors. Western blots with anti-D1 and anti-Lhcb2 antibodies indicated the level of corresponding substrate for the phosphorylation reaction (figure 1a). The levels of the STN8 and STN7 kinases were assessed in samples from thylakoid preparations using specific antibodies, and control western blots with CP43 protein were used to correct the amount of protein loaded (figure 1b). The stn8 double mutant [33] was used to verify the identity of the corresponding cross-reacting bands with the STN8 and STN7 antibodies. Two Chl loadings are shown to indicate the linearity of the immunodetected signal for the three antibodies.

Table 1. The names, stock numbers, abbreviations, geographical origin and habitat of all genotypes used in this study (source: TAIR, www.arabidopsis.org). n.a., not applicable.

<table>
<thead>
<tr>
<th>stock number</th>
<th>accession name</th>
<th>abbreviated name</th>
<th>country</th>
<th>latitude (°)</th>
<th>longitude (°)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CS76113</td>
<td>Columbia-0</td>
<td>Col-0</td>
<td>n.a.</td>
<td>n.a.</td>
<td>n.a.</td>
</tr>
<tr>
<td>SALK060869</td>
<td>stn8-1 (in Col-0)</td>
<td>stn8</td>
<td>n.a.</td>
<td>n.a.</td>
<td>n.a.</td>
</tr>
<tr>
<td>CS76227</td>
<td>Shakdara</td>
<td>Sha</td>
<td>Tadjiikistan</td>
<td>38.35</td>
<td>68.48</td>
</tr>
<tr>
<td>CS76106</td>
<td>C24</td>
<td>C24</td>
<td>Portugal</td>
<td>41.25</td>
<td>−8.45</td>
</tr>
<tr>
<td>CS76105</td>
<td>Burren-0</td>
<td>Bur-0</td>
<td>Ireland</td>
<td>52.9</td>
<td>−9</td>
</tr>
<tr>
<td>CS28595</td>
<td>Palermo</td>
<td>Pa-2</td>
<td>Italy</td>
<td>38.07</td>
<td>13.22</td>
</tr>
<tr>
<td>CS76192</td>
<td>Martuba</td>
<td>Mt-0</td>
<td>Libya</td>
<td>32.34</td>
<td>22.46</td>
</tr>
<tr>
<td>CS76210</td>
<td>Perm</td>
<td>Per-1</td>
<td>Russia</td>
<td>58.00</td>
<td>56.31</td>
</tr>
<tr>
<td>CS76100</td>
<td>Borky</td>
<td>Bor-4</td>
<td>Czech</td>
<td>49.40</td>
<td>16.23</td>
</tr>
<tr>
<td>CS76109</td>
<td>Canary Island</td>
<td>Can-0</td>
<td>Spain</td>
<td>29.21</td>
<td>−13.48</td>
</tr>
<tr>
<td>CS28780</td>
<td>Tushima</td>
<td>Tsu-0</td>
<td>Belarus</td>
<td>34.43</td>
<td>136.31</td>
</tr>
<tr>
<td>n.a.</td>
<td>Tsu-0 × Ws-4</td>
<td>Tsu × Ws-4</td>
<td>n.a.</td>
<td>n.a.</td>
<td>n.a.</td>
</tr>
<tr>
<td>CS5390</td>
<td>Wassilewskija-4</td>
<td>Ws-4</td>
<td>Belarus</td>
<td>52.3</td>
<td>30</td>
</tr>
<tr>
<td>CS76164</td>
<td>Landsberg erecta</td>
<td>Ler-1</td>
<td>Poland</td>
<td>52.71</td>
<td>15.23</td>
</tr>
<tr>
<td>n.a.</td>
<td>((Ely × Ler) × Ler)BC6</td>
<td>ELB</td>
<td>n.a.</td>
<td>n.a.</td>
<td>n.a.</td>
</tr>
<tr>
<td>CS28631</td>
<td>PHW-31 (Ely)</td>
<td>Ely</td>
<td>England</td>
<td>52.39</td>
<td>0.26</td>
</tr>
</tbody>
</table>

As shown by representative blots for Col-0, both anti-phospho-Thr antibodies recognized PSII-phospho-proteins, but with different affinities (figure 1a). The Zymed antibody recognized the phospho-D1 protein best, whereas the Cell Signaling antibody was found most suitable for quantification of phospho-LHCII proteins.
Figure 1. Representative western blots of thylakoid proteins isolated from Col-0. The plants were illuminated for 3 h with growth light (GL, 150 μmol m⁻² s⁻¹) and subsequently transferred for 3 h to high light (HL, 600 μmol m⁻² s⁻¹). Thylakoid membranes were isolated in the presence of NaF and the proteins were separated by gel electrophoresis. (a) The phosphorylated PSI proteins were immunodetected with anti-phospho-Thr antibodies from Zymed and Cell Signaling. Control blots with anti-D1 and Lhcb2 antibodies are also shown. (b) The levels of STN8 and STN7 protein kinases are shown in parallel with control CP43 blots. Thylakoids isolated from the stn7 × stn8 double mutant were used as a control for specificity of the anti-STN8 and STN7 antibodies. Two chlorophyll loadings (μg per lane) are shown to demonstrate the linearity of the immunodetected signal from each antibody used.

The selected accessions were analysed by the assay described above using conditions optimized for Col-0. The levels of various immunodetected proteins were determined and expressed relative to those in Col-0 (see the electronic supplementary material, tables S1 and S2). The genotypes were classified as displaying high (80–120%), moderate (40–80%) or low (less than 40%) levels of immunodetected proteins.

(c) RNA isolation and transcript analysis
RNA was isolated from frozen leaves as described in [34]. One fully expanded leaf was taken from three plants after 3 h of GL or HL treatment. The RNA concentration was measured using a NanoDrop 2000, and the volume adjusted with Millipore water to obtain a final concentration of 0.25 μg μl⁻¹. RNA (1 μg) was used for cDNA synthesis. Equal volumes of cDNA were used in all subsequent qPCRs. Eight reference genes [35,36] were tested on all samples (see the electronic supplementary material, table S3) and seven of them were found stable enough for use in further analysis. Primer sequences are listed in the electronic supplementary material, table S3. A normalization factor was calculated from the seven reference genes, which was used to calculate relative transcription levels.

(d) Statistical analysis
Scatterplots were created in GraphPad software (La Jolla, CA, USA). Best-fit lines were applied, and the correlation coefficient (r²) and its significance (two-tailed p-value) were calculated with the same software. Correlations were considered significant at p ≤ 0.05.

3. Results
(a) D1 protein phosphorylation in growth- and high-light conditions
Large differences in D1 protein phosphorylation were observed in GL in the 16 genotypes ranging between 4% and 100% of the phosphorylation level found in Col-0 (figure 2a). D1 protein level in the studied accessions ranged between 70% and 120% of Col-0 (see the electronic supplementary material, table S1), and cannot explain the large variation observed in phosphorylation. Notably, the relative level of the STN8 kinase varied largely among accessions (78–192%; figure 2a). Col-0 had the highest phosphorylation level, which was approx. 20% higher than that of the next highest genotype (Tsu-0; 79%, electronic supplementary material, table S1). Both genotypes also displayed high levels of the STN8 kinase. Four accessions displayed low levels of phosphorylation, but high levels of STN8 kinase. Five accessions displayed low D1 protein phosphorylation (Bur-0, Pa-2, Mt-0, Bor-4, Can-0 and Ely) although they had high STN8 levels. Sha, Ler-1 and ELB had very high STN8 levels (more than 120% of Col-0), but low D1 phosphorylation. Thus, there was no correlation between the STN8 protein level and the level of D1 phosphorylation, though stn8, which had the lowest level of phospho-D1, lacked any STN8 (figure 2b). The Tsu × Ws-4 hybrid and the ELB backcross were moderate or lower than their parents in the D1 phosphorylation levels.
D1 phosphorylation in HL ranged between 2% and 117% of Col-0 (figure 2b). As in GL, there was little variation in D1 protein level (87–114%), whereas STN8 abundance showed greater variation (32–121%) among accessions (see the electronic supplementary material, table S2). Under HL conditions there was a much tighter grouping of genotypes than in GL, with the exception of stn8, Ely and ELB showing low D1 phosphorylation (compare figure 2a with b). There was a general upward trend in D1 phosphorylation, and Col-0, while still high, was no longer an outlier. Six accessions displayed high phosphorylation levels and also high STN8 levels (Col-0, Bur-0, Mt-0, Per-1, Bor-4 and Tsu × Ws-4; the electronic supplementary material, table S2 and figure 2b). Another three displayed moderate phosphorylation levels and also moderate STN8 levels (Sha, C24 and Ws-4). The stn8 mutant displayed residual D1 phosphorylation. The remaining genotypes displayed either high levels of phospho-D1 despite moderate STN8 kinase levels (Pa-2, Tsu-0 and Ler-1) or moderate levels of phospho-D1 and low kinase levels (Can-0). Ely and ELB were found low phosphorylation accessions despite high STN8 protein levels, suggesting that kinase abundance was not limiting. No significant correlation was obtained between phospho-D1 level and STN8 abundance if all accessions were included. Nevertheless, a weak but significant correlation was obtained if Ely and ELB were excluded from the analysis (figure 2b).

When comparing GL with HL conditions, the phosphorylation of the D1 protein increased by 70%, whereas the STN8 level remained stable in Col-0 (see the electronic supplementary material, figure S1a,b). The other accessions also displayed increased levels of phospho-D1, but the level of the STN8 protein either decreased (ELB, Pa-2, Ler-1, Can-0, Tsu-0, Ws-4 and Tsu × Ws-4), remained quite stable (C24, Bur-0, Per-1, Bor-4 and Ely) or even increased (Mt-0) upon transfer from GL to HL. All genotypes showed reduced transcription of STN8 in HL with the exception of Ely, C24, Bur-0, Pa-2, Sha and Mt-0, which either showed an increase or no difference in transcription (see the electronic supplementary material, figure S1c).

(b) LHCII protein phosphorylation in growth- and high-light conditions

Phosphorylation of LHCII proteins in GL varied between 7% and 102% of Col-0 (figure 3a). With the exception of Ely and ELB, the level of Lhcb2 showed limited variation, whereas STN7 protein levels varied between 70% and 213% of Col-0 (see the electronic supplementary material, table S1 and figure 3a). There were seven high phosphorylation genotypes (Col-0, stn8, Sha, Mt-0, Per-1, Tsu-0, Tsu × Ws-4), seven moderate (C24, Bur-0, Pa-2, Bor-4, Can-0, Ws-4 and Ler-1) and two low accessions (Ely and ELB). All high phosphorylation accessions displayed high levels of STN7 kinase, including Col-0 and the stn8 mutant. Accessions displaying moderate levels of LHCII phosphorylation accumulated STN7 at either high or very high (more than 120%) levels, indicating that other factors limited the kinase activity. Tsu × Ws-4 displayed similar and high phospho-LHCII as Tsu-0 although it had much higher levels of STN7 (213% versus 70%). One striking observation was that both genotypes with the atrazine-resistant cytoplasm, Ely and ELB, showed Lhcb2 levels of 143% and 126%, respectively, relative to Col-0, and yet showed extremely low levels of LHCII phosphorylation. The reduced phosphorylation levels in these two genotypes cannot be explained by the abundance of the kinase, which was 71% and 163% relative to Col-0, and indicate that the STN7 kinase was not properly activated.

Phosphorylation of LHCII proteins in HL varied between 5% and 107% relative to Col-0, whereas Lhcb2 and STN7 protein levels varied between 89–123% and 58–179% relative to Col-0, respectively. Col-0, Sha, C24, Bur-0, Pa-2, Mt-0, Tsu-0, Tsu × Ws-4 and Ler-1 were high phosphorylation accessions, Per-1, Bor-4 and Ws-4 were moderate, and Can-0, Ely and ELB were low phosphorylation accessions (see the electronic supplementary material, table S2). As in GL conditions, the level of kinase did not appear to correlate with the level of LHCII phosphorylation (figure 3b). Genotypes with high phosphorylation, including Col-0 and the stn8 kinase, displayed high to very high levels of STN7 kinase. In addition, the moderate and the low phosphorylation accessions displayed high STN7 levels, indicating that factors related to activation rather than to the amount of kinase may limit the phosphorylation reaction.
When comparing GL with HL, LHCII phosphorylation decreased by 30% in Col-0, whereas STN7 abundance remained stable (see the electronic supplementary material, figure S2a,b). With few exceptions (Ler-1, C24 and Bur-0), phospho-LHCII also decreased in the other accessions, whereas STN7 abundance varied between the two light regimes. The STN7 transcript level in GL was comparable among genotypes (see the electronic supplementary material, figure S2c). Upon transfer to HL, the STN7 transcript abundance decreased in all genotypes except ELB and Mt-0. The extent of reduction in transcript abundance varied considerably, with Col-0, C24, Per-1 and Ely showing a much more pronounced reduction than the other accessions. Interestingly, the stn8 mutant did not show the same response as Col-0 wild-type, retaining relatively higher expression of STN7 in HL.

4. Discussion

(a) Variation in PSII protein phosphorylation and factors involved

The reversible and differential phosphorylation of PSII proteins is dependent on the interplay between the STN7 and STN8 kinases. This process has been intensively studied in the standard laboratory accession Col-0 and stn mutants in the Col-0 background. A recent report compared phosphorylation levels in Col-0 with those in Ler-0 and Ws-4, found that Ws-4 displayed 50% lower phospho-D1 and attributed this difference to 50% less STN8 kinase than in the other two accessions [23]. Here, we report on the occurrence of variation in D1 and LHCII protein phosphorylation ranging between approximately 5% and 120% in Arabidopsis accessions of diverse geographical origins, when expressed relative to Col-0. The large differences observed in GL in this set of accessions were not caused by variation in the amount of substrate or STN7 and STN8 protein levels. In HL, the levels of D1 phosphorylation correlated with the STN8 kinase levels, indicating that kinase abundance can be a limiting factor for phosphorylation under these conditions.

Among the genotypes we analysed, some resembled the standard laboratory accession Col-0 in phosphorylation levels and were classified as high accessions. However, the other accessions displayed moderate or even low phosphorylation levels. Why would Arabidopsis accessions have variable phosphorylation of PSII proteins? Is this an adaptive mechanism facilitating survival and reproduction across the range of environmental conditions where Arabidopsis naturally occurs? In support of this hypothesis, we found a significant correlation between latitude and both D1 and LHCII phosphorylation in GL conditions (figure 4a,b). This suggests that there may be some form of selective pressure that correlates with latitude.

In order to test this, climate data were obtained from the WorldClim database [37] (http://www.worldclim.org/). Bioclimatic variables 4 (temperature seasonality) and 7 (temperature annual range) correlated significantly with phospho-LHCII in GL conditions (figure 4c,d). While there was some correlation between these climatic variables and phospho-D1, it was not found to be significant. This correlation between temperature variability and PSII protein phosphorylation in GL conditions is interesting and may be the outcome of a photoprotective mechanism similar to that observed in evergreen trees which must maintain functioning leaves in very cold conditions [38]. Interestingly, there was no correlation between protein phosphorylation and longitude in HL conditions, which could be due to a stronger, more geographically uniform, selective pressure in the HL response. This makes sense considering the damage an inappropriate response to HL can cause. To better understand the relationship between phosphorylation and the natural habitat, many more accessions will need to be investigated from a wide range of environments.

In line with published data [17,23], we show that HL-treated plants contained more phospho-D1, whereas GL-treated plants displayed a higher extent of LHCII phosphorylation. The amount of kinase involved could be one mechanism to regulate enzyme activity, as indicated by the significant correlation between phospho-D1 levels and STN8 abundance in HL (figure 2b). In the case of Arabidopsis STN7 or its Chlamydomonas homologue Stn7, it has been suggested that their amounts are regulated by the redox status of the electron transport chain, by phosphorylation and by transcript abundance [24,25]. In our panel, we had two genotypes, Ely and ELB, that displayed reduced LHCII phosphorylation under both GL and HL conditions but high STN7 protein levels (figure 3). These genotypes are atrazine-resistant owing to a deficient binding of quinones in the Qa pocket, and as a result have reduced PSII efficiency [30]. Therefore, they are likely to have a more oxidized PQ pool especially at limiting irradiances. Although this requires experimentation, we use as support of our assumption the fact that at low, light-limiting irradiances Chl b deficient barley mutants displayed a more oxidized PQ pool than the wild-type owing to diminished PSII activity relative to PSI activity [39]. However, the unaffected abundance of STN7 protein relative to Col-0 in our study indicates that the redox state did not alter STN7 expression level. Therefore, the observed reduced level of LHCII phosphorylation is most likely due to reduced kinase activity.

(b) Regulation of D1 protein phosphorylation

The factors regulating the amount of STN8 kinase in the membrane have not yet been investigated. D1 protein phosphorylation requires the presence of the STN8 kinase, because phospho-D1 is hardly detected in the stn8 mutant, and what remains is most likely due to a partial replacement by STN7 or other yet unknown kinases [8]. This potential redundancy between STN7 and STN8 is illustrated by the much higher STN7 transcript and also protein levels in the stn8 mutant in HL when compared with Col-0. The mechanism by which this difference is mediated is not clear and may be either direct or indirect. This may be due to the absence of functional STN8 protein stimulating additional STN7 transcription and translation under HL conditions. However, the stn8 mutant displayed unaltered LHCII phosphorylation levels, suggesting that the STN8 kinase does not play any role in this process. STN8 levels in Col-0 did not change upon transfer from GL to HL conditions in line with Wunder et al. [26].

All studied accessions displayed reduced D1 phosphorylation relative to Col-0 in GL despite high levels of the D1 substrate and STN8 kinase. One cause could be a poor activation of the STN8 kinase in GL, which could be related to the redox state of the PQ pool, as in the case of STN7. In support of this possibility is the low phosphorylation in Ely and ELB. As previously discussed with reference to STN7, owing to low PSII efficiency, the PQ pool may be more oxidized which results in reduced activation of the kinase. ELB...
contains Ler-1 nuclear DNA but the organellar DNA of Ely, and as such allows us to compare the phosphorylation level of PSII proteins in the same nuclear background. At both HL and GL, ELB displayed half the phosphorylation level of D1 as that found in Ler-1 (figure 2), thus resembling Ely in the deficient activation of STN8. Because ELB is effectively identical to Ler-1 as far as the nuclear genome is concerned, the difference reflects the strong effect of the cytoplasm on the level of D1 phosphorylation. In all accessions except Ely and ELB, a weak but significant correlation was found in HL between phospho-D1 level and STN8 abundance. This indicates that, under these conditions, the abundance of STN8 may be either limiting or plays a regulatory role in D1 phosphorylation.

(c) Regulation of LHCII protein phosphorylation

The phosphorylation of LHCII proteins enables the excitation and redox balance between PSII and PSI under low irradiance. This process requires the STN7 kinase, which is activated by a reduced state of the PQ pool under these light conditions [5,22]. Upon exposure to HL, the kinase is inactivated by a thioredoxin-mediated reduction of disulfide bonds [22]. In our study, many accessions displayed moderate LHCII phosphorylation in GL, whereas the genotypes with atrazine-resistant forms of D1, Ely and ELB displayed only residual phosphorylation levels, while at the same time showing much higher levels of Lhcb2 protein than Col-0 (see the electronic supplementary material, table S1). There may be a common cause with D1 phosphorylation, namely the inability to fully reduce the PQ pool, and thus to activate the kinase. ELB resembles Ely in low levels of LHCII protein phosphorylation, thus in the deficient activation of STN7. The reduction in PSII efficiency in these genotypes [30] may result in both increased antennae size and reduced phosphorylation in order to increase PSII light-absorption relative to that of PSI under light-limiting conditions.

In our experimental conditions, STN7 levels did not change upon a shift from GL to HL in Col-0 despite a decrease in LHCII phosphorylation levels (see the electronic supplementary material, figure S2), indicating that STN7 is regulated at activity rather than at protein level. This observation is in contrast to a recent report about downregulation of STN7 at both protein and transcript levels [26]. The reason for this discrepancy could be the distinct light regimes used in this study or other yet unknown factors. However, the protein levels did change in other accessions (see the electronic supplementary material, figure S2), indicating that STN7 is regulated at transcript abundance decreased, but did not correlate with the abundance of the STN7 protein (see the electronic supplementary material, figure S2). This is in contrast to a recent

Figure 4. (a) Scatterplots comparing the levels of (a) phospho-D1 (p-D1) and (b–d) phospho-LHCII (p-LHCII) in growth light (GL) relative to geographical (a,b) and climatic factors (c,d). A significant correlation was found for both p-D1 (a) and p-LHCII levels (b). Vertical dashed lines delimit longitude for European accessions (from −14° to 30°). Per-1, Sha and Tsu-0 are labelled, because they are non-European accessions. A significant correlation was found between the levels of p-LHCII and the temperature seasonality (c) and the temperature annual range (d). Correlation of p-D1 with the parameters in (c,d) was low, but a trend was visible, however not significant \( r^2 = 0.23, p = 0.128 \) and \( r^2 = 0.24, p = 0.123 \), respectively. Temperature data were obtained from the WorldClim database (http://www.worldclim.org/). The phosphorylation data in all panels are expressed relative to Col-0 and are means of two to three technical replicates ± s.d. Ely is labelled because it was excluded from regression analysis in all panels. Horizontal dashed lines delimit high, moderate and low phosphorylation levels in all panels.
study which showed that the accumulation of the STN7 protein was controlled at the level of transcript abundance [23]. However, that study was performed on Col-0 wild-type and mutants in the Col-0 background, and based on our results it appears that Col-0 does not show a typical level of transcription for STN7.

The picture that emerges from these primary studies is that of highly diverse levels of PSI1 protein phosphorylation in nature, in which kinase activation may play a central role at least under GL conditions. It is likely that under HL conditions PSI1 core protein phosphorylation is, in addition, regulated by STN8 protein abundance in the thylakoid membrane. Longitudinal and temperature variability may be involved at least under GL conditions in variation of PSI1 protein phosphorylation. In conclusion, the significant variation found in both traits highlights our lack of understanding of the role these processes play in plant performance in nature. Using knockout mutants, it has been shown that a complete absence of the STN7 kinase, and to a lesser extent the STN8 kinase, results in reduced fitness. This fitness cost is much more pronounced in the double mutant [3], once again illustrating that there is some degree of functional redundancy. A further conclusion from this work is that Col-0 appears to be an outlier accession. It operates at the phenotypic extreme for this trait and as such is most likely not representative of thylakoid protein phosphorylation in Arabidopsis. Based on an analysis of both STN7 and STN8 sequences in the many re-sequenced Arabidopsis accessions as found in the ‘1001 Genomes’ website (http://www.1001genomes.org/), it appears unlikely that the diversity of phosphorylation phenotypes observed is due to sequence variation in the kinase genes themselves. It is much more likely that the observed variation is due to variation elsewhere in the process, be that upstream signalling or downstream dephosphorylation rates. In order to identify the genes responsible for such variation, genetic mapping studies using either recombinant inbred line populations or genome wide association mapping panels could be undertaken [40]. However, currently, the main limiting factor to such a study is not the availability of suitable genetic material but rather the ability to screen the necessary number of individuals (more than 200 genotypes would be required), in sufficient replicates to allow for detection of the genetic loci involved, and even more to identify the causal sequence variation [41]. Identification of such genes is likely to provide us with additional insights into the regulation of the photosynthetic process and the selective pressures acting upon this trait. Such knowledge will not only be of use to fundamental research, but is also likely to provide new avenues to crop improvement whereby the photoprotective processes can be optimized for different agricultural or climatic conditions [29,42].

Acknowledgements. We thank Prof. Maarten Koornneef (Max Planck Institute for Plant Breeding, Cologne, Germany/Wageningen University) for initiating collaboration between the two laboratories. We thank Drs Ross Alexander and Bas Dekkers (Wageningen University) for advice on qPCR analysis and Dr Joost van Heerwaarden (Wageningen University) for help with climate data.

Funding statement. This work was supported by funding from the Swedish Research Council and the Olle Engkvist Foundation (to C.S.), the Netherlands Organization of Scientific Research sections Earth and Life Sciences (NWO-AW), the Technological Top Institute Green Genetics and the BioSolar Cells research programme (to P.F., J.H. and M.G.M.A.).

References


