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Natural variation of photosynthetic efficiency in Arabidopsis thaliana accessions under low temperature conditions

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INTRODUCTION 1

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Abstract

Low, but non-freezing, temperatures have negative effects on plant growth and development. Despite some molecular signalling pathways being known, the mechanisms causing different responses among genotypes are still poorly understood. Photosynthesis is one of the processes that are affected by low temperatures. Using an automated phenotyping platform for chlorophyll fluorescence imaging the steady state quantum yield of photosystem II (PSII) electron transport (Φ_{PSII}) was measured and used to quantify the effect of moderately low temperature on a population of Arabidopsis thaliana natural accessions. Observations were made over the course of several weeks in standard and low temperature conditions and a strong decrease in Φ_{PSII} upon the cold treatment was found. A genome wide association study identified several quantitative trait loci (QTLs) that are associated with changes in Φ_{PSII} in low temperature. One candidate for a cold specific QTL was validated with a mutant analysis to be one of the genes that is likely involved in the PSII response to the cold treatment. The gene encodes the PSII associated protein PSB27 which has already been implicated in the adaptation to fluctuating light.

KEYWORDS

Arabidopsis, cold, GWAS, natural variation, photosynthesis

Low, but non-freezing, temperatures (between 5 and 15°C) negatively affect the growth and development of most plants (Mckersie & Leshem, 1994). Plants growing in moderate climates are often exposed to low temperatures at some stage of their life and have evolved strategies to adapt to such unfavourable temperature conditions. These strategies can comprise changes in enzyme activity, osmolyte accumulation, membrane fluidity (Upchurch, 2008; Zheng, Tian, Zhang, Tao, & Li, 2011) or more specific changes to photosynthesis and energy metabolism (Hüner et al., 2012). The effect of low temperature on plants depends not only on the temperature, but also on other environmental factors to which the plant is simultaneously exposed (Crosatti, de Laureto, Bassi, & Cattivelli, 1999; Huner, Öquist, & Sarhan, 1998; Waraich, Ahmad, Halim, & Aziz, 2012), especially light intensity (Franklin, Toledo-Ortiz, Pyott, & Halliday, 2014;

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Wanner & Junttila, 1999) and the developmental stage at cold exposure (da Cruz et al., 2013; Hatfield & Prueger, 2015; Nykiforuk & Johnson-Flanagan, 1997). Considering the complexity of environmental cues and the differences in sensitivity to cold, it is not surprising that there is ample natural genetic variation for the response to cold, both between (Atkin, Loveys, Atkinson, & Pons, 2006) and within species (Barah et al., 2013; Bravo et al., 2007; Brüggemann, van der Kooij, & van Hasselt, 1992; Sanghera, Wani, Hussain, & Singh, 2011). Although many molecular components of the cold signalling and adaptation pathway have been identified (Cheng et al., 2007; Chinnusamy, Zhu, & Zhu, 2007), the genetic differences that give rise to the natural variation in cold responses between species or varieties are largely unclear. For example, one common pathway for low temperature perception and induction of freezing tolerance is the "C-repeat/DRE-Binding Factor" (CBF)-"Inducer of CBF expression 1" (ICE1) regulon. This pathway has been well described for Arabidopsis thaliana (see review: Miura & Furumoto, 2013) and seems to be conserved in other species (Choi, Rodriguez, & Close, 2002; Jaglo et al., 2001). The cold-activated transcription factor ICE1 triggers the downstream transcription factor CBF3. The ICE1 and CBF transcription factors subsequently induce expression of cold responsive (COR) genes. These COR genes code for a variety of proteins needed for cold adaptation, for example those that cause osmotic changes, changes in membrane stability, or that act as chaperones (Plieth, Hansen, Knight, & Knight, 1999; Sangwan, Foulds, Singh, & Dhindsa, 2001; Usadel et al., 2008). However, the variation in CBF- and COR-genes identified so far cannot explain all the variation seen between different accessions in A. thaliana, at least in terms of their freezing tolerance (Gery et al., 2011; McKhann et al., 2008); similar studies in cold but non-freezing conditions are missing.

One possible option for determining the genetic regulation underlying variation in low temperature adaptation is to study the natural genetic variation in cold response between wild accessions of a species by genetic linkage or association mapping. Natural variation for freezing tolerance has already been studied for A. thaliana (Hannah et al., 2006; Mishra, Heyer, & Mishra, 2014) but not for cold, non-freezing temperatures. A. thaliana grows in diverse habitats and shows heritable differences stress responses among the different accessions (Alonso-Blanco et al., 2005; Lefebvre, Kiani, & Durand-Tardif, 2009). A HapMap population consisting of a diversity panel with \sim 360 accessions, representing the species-wide natural genetic variation, has been developed for Genome Wide Association Studies (GWAS) in A. thaliana (Li, Huang, Bergelson, Nordborg, & Borevitz, 2010). This population offers several interesting features for genetic analysis: it is based upon a publicly available set of accessions that have been genetically characterized and selected for diversity and minimal relatedness (Baxter et al., 2010; Horton et al., 2012). In addition, well-developed statistical tools for quantitative genetic analysis (Kruijer et al., 2015; Seren et al., 2012) as well as extensive genome information are available (TAIR www. arabidopsis.org; Araport www.araport.org) for A. thaliana. A SNP array with approximately 250,000 SNP makers (http://1001genomes.org/; Kim et al., 2007) at an average density of about one SNP per 500 bp (Atwell et al., 2010) provides a high genome coverage that enables the selection of a small number of candidate genes for loci identified in a GWAS. Since A. thaliana is predominantly self-fertilizing and lines have been propagated for several generations after collection, the accessions are reasonably assumed to be homozygous which means that replicate experiments with the same genotypes can be performed (Koornneef, Alonso-Blanco, & Vreugdenhil, 2004; Weigel & Mott, 2009). This enables the comparison of genotypic performance in different environmental conditions. Genes involved in the response to environmental changes have already been identified by using the A. thaliana HapMap population in GWAS. For example, Baxter et al. (2010) found strong evidence that variants of the HKT1 gene are involved in differences of leaf sodium accumulation, salt tolerance (Rus et al., 2006) and habitat distribution, while other studies provided candidate genes for the response to combined drought and herbivore stress (Davila Olivas et al., 2017) and a change in irradiance level (van Rooijen et al., 2017). The ability to compare the response of the HapMap population to different conditions has also allowed a multi-trait mapping for 11 single and combined stresses that revealed common underlying genetics (Thoen et al., 2017).

Considering the high genetic resolution of the A. thaliana HapMap population, the success of quantitative genetics studies depends mostly on the quality of the phenotypic data. The trait measured needs to be representative of the stress effect and the precision of its quantification needs to be high. In response to low, non-freezing temperature, differences in leaf biomass related traits, such as leaf size and other leaf morphological traits have been observed in A. thaliana (Armstrong, Logan, & Atkin, 2006; Gorsuch, Pandey, & Atkin, 2010). However, while these traits could be used for the quantification of the effect of cold, to allow the identification of differences between genotypes, their measurements need to be very precise, demanding high numbers of replicates, as the effect size is relatively small. This phenotyping is normally very labour intensive for large plant populations. In addition, measuring biomass-related traits is often destructive and therefore can only be assessed at one time point. A solution to this would be the use of image-based traits, for example the quantum yield of the photosystem II (PSII) electron transport under dark-adapted conditions (maximum quantum yield, F_v/F_m) or under steady-state illumination (the operating quantum yield of PSII, Φ_{PSII}). These are integrative, primary physiological traits that have been found to be highly responsive to different stresses (Baker & Rosenqvist, 2004; Maxwell & Johnson, 2000), including low temperature exposure (Gray, Hope, Qin, Taylor, & Whitehead, 2003; Hurry, Krol, Oquist, & Huner, 1992). F_v/F_m and Φ_{PSII} can be measured by chlorophyll fluorescence (CF) imaging, a fast technique that allows high-throughput phenotyping (Harbinson, Prinzenberg, Kruijer, & Aarts, 2012; Rungrat et al., 2016; van Bezouw, Keurentjes, Harbinson, & Aarts, 2019). It is non-invasive, allowing repeated measurements of the same plants, thus providing highly informative timecourse data. In a small rosette plant as A. thaliana, CF-imaging of the projected leaf area allows the measurement of nearly the whole rosette surface. The technique enables robust quantification of the relative quantum yield for electron transport by PSII (Φ_{PSII}), for which ample genetic variation has been found in A. thaliana (Flood et al., 2016; Mishra et al., 2014; van Rooijen, Aarts, &

Harbinson, 2015). Therefore, Φ_{PSII} is an ideal trait to screen large numbers of in *A. thaliana* for the quantitative genetic analysis of low temperature responses.

In this study the A. *thaliana* HapMap population has been employed in a GWAS to identify loci involved in natural variation of the response of photosynthesis to low temperature. Using an automated high-throughput phenotyping platform, the Phenovator (Flood et al., 2016), the response of all genotypes to a prolonged temperature decrease has been followed over time. Several loci specific to the low temperature condition that influence Φ_{PSII} are identified, and mutants were used to confirm the relevance of candidate genes residing at these loci.

2 | MATERIAL AND METHODS

2.1 | Plant material

Three or four replicates per genotype were evaluated for 347 lines of the A. thaliana HapMap population (Baxter et al., 2010) listed under N76309 at the Nottingham Arabidopsis Stock Centre, NASC; www. arabidopsis.info (Table S3). The T-DNA insertion mutant lines (Table S2) were also ordered from NASC and when needed tested for the homozygosity of the T-DNA insertion (full list of tested lines and primers in Table S2). Many of the tested T-DNA insertion lines have a Col-0 background, and in principle should be identical to N60000 (Col-0), apart from the mutated locus. In practice, however, propagation over several years in different laboratories can lead to accumulation of other mutations (Ossowski et al., 2010) or introgression of other parental alleles due to accidental cross pollination. To be able to evaluate the effect of different genetic backgrounds of Col-0 on Φ_{PSII} , three Col-0 lines (N60000, N907 and N76113) were characterized. No significant difference was detected in Φ_{PSII} between these lines in the cold or control temperatures (Figure S3). Therefore, all mutants with a Col-O background were compared to N60000. Only the mutants N851044 and N1001708 have the wild-type background Col-2 (N28170) and Col-4 (N933), respectively, and have been compared to these accessions. The mutant N866141 is in a Col-3 background but no phenotypic data on Col-3 could be obtained and in this study it is compared to Col-0. In the mutant analysis 12-24 replicate plants were evaluated per genotype, as not all mutants were characterized together but in two independent experiments (since only three replicates could be evaluated for N653579 in the initial experiment, the data from the quantitative complementation test with 103 replicates was used for Figure 3). For the complementation test the mutant line N653579 and Col-0 (N60000) were both crossed (as mother plants) to the accessions Catania (Ct-1; CS76114) and Siegen (Si-0; CS28739). The resulting F1 seeds were used for the complementation test. Phenotypic outliers were removed: as plants from one N653579 × Ct-1-cross and N653579 × Si-0-cross were similar to the mutant and plants from one Col-0 × Ct-1-cross were similar to Col-0, it is assumed that the crosses failed. For Ct-1 and Si-0, 13 and 19 replicates were evaluated respectively, 51 replicates for Col-0, 103 replicates for N653579, and 25-67 replicates were evalu-

2.2 | Growing conditions and photosynthetic phenotyping

ated for the different F1plants.

The A. thaliana seeds were placed on filter paper in a sealed petri dish and moistened with purified water. These dishes were kept at ${\sim}5^{\circ}\text{C}$ in the dark for stratification for 3-5 days. Afterwards they were sown on 4 cm × 4 cm × 4 cm rock-wool blocks (Grodan, Roermond, The Netherlands). For the Φ_{PSII} phenotyping experiment, the rock-wool blocks were placed in a grid system (Flood et al., 2016) and flooded three times per week for 5 min with nutrient solution for irrigation. The nutrient solution is composed of macronutrients, obtained from Yara Benelux B.V. fertilizers (Rotterdam-Vlaardingen, The Netherlands) and micronutrients from the Agrispoor product line of Horticoop B.V. (Bleiswijk, The Netherlands) to reach the following final elemental composition of dissolved ions: NH₄ 1.7 mM, K 4.13 mM, Ca 1.97 mM, Mg 1.24 mM, NO₃ 4.14 mM, SO₄ 3.14 mM, P 1.29 mM, Fe 21 µM (composed half-half of Fe-DTPA and Fe-EDDHSA), Mn 3.4 μM, Zn 4.7 μM, B 14 μM, Cu 6.9 μM, Mo 0.5 μM. The solution was adjusted with KOH or H₂SO₄ to a pH of 5.5, the final EC was 1.4. During the phenotyping experiment the plants were grown in a climate-controlled growth room that was set at a day length period of 12 hr (from 8 to 20 hr), a light intensity of 200 μ mol*m⁻²*s⁻¹, night and day temperatures of 21°C and a relative air humidity of \sim 70%. On day 14 after sowing (14 DAS) the air temperature was reduced, over the course of 2 hours after light onset, to approximately 5°C and this low temperature (cold) was kept day and night. The plants were grown for 7 days in the cold and on day 21 after sowing (21 DAS), the air temperature was raised again to 21°C. The light was supplied by light tubes and bulbs ("Master TL5 HO" and "Superlux Agro" from Philips, Eindhoven, The Netherlands). The light tubes were increasing and decreasing in intensity over the course of half an hour at the beginning and end of each light period. For seed propagation and crosses the plants were grown on rockwool blocks in a greenhouse with supplemental lighting (at a 16 hr light period). Those plants were irrigated with a diluted nutrient solution (relative composition see above) or tap water.

2.3 | Phenotyping

Over the course of the experiment three CF-images were evaluated per day with an automated camera system that was moving over the plants (Flood et al., 2016). To measure all plants took 47 min and the three measurements were started at 12 hr, 16.30/15.30 (for the mapping population/for the mutant experiments; termed "17 hr"), and at 18.30 (termed "19 hr"). CF images were evaluated for a period from 2 days before up to 2 days after the cold treatment (12 DAS-22 DAS).

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FIGURE 1 Response of Φ_{PSII} of the A. *thaliana* HapMap population to two changes in temperature between 21 and 5°C. The range of Φ_{PSII} within the population of 347 accessions is depicted in grey (defined by minimum and maximum individual values) for each time point over the course of 11 days measured at three time points per day. The average Φ_{PSII} values and standard deviation for Columbia-0 (CS76113; full, black line) and two accessions with extreme phenotypes in the cold Catania-1 (Ci-1; dashed line) and Siegen-0 (Si-0; dotted line) are highlighted. All plants were grown in control conditions (21°C) until 13 days after sowing (13 DAS), on 14 DAS the temperature was lowered to 5°C upon light onset and kept constant until 20 DAS. On 21 DAS at light onset, the temperature was raised again to 21°C. For the 12 hr-time point on the last day in control conditions (13 DAS) and the last cold day (20 DAS) the frequency distribution of Φ_{PSII} in the HapMap population is displayed

2.4 | Statistical analysis

As in van Rooijen et al. (2017), the GWAS analysis and heritability calculations were performed with a mixed model in R (Kruijer et al., 2015) using 199,589 SNPs with a minor allele frequency threshold of at least 0.05. The Bonferroni threshold for the analysis is 6.6012 (calculated as: -log10 [minimum allele frequency/number of tested genetic markers]). As phenotypic value, the average Φ_{PSII} for each accession was used and the GWAS analysis was performed for each individual time point covering the measurement time between 12 DAS and 22 DAS. SNPs with an association of $-\log_{10}(p)$ -value ≥ 3 were plotted in a heat map (custom R-script using the plot function in R version 3.3.2). Enlargements of the quantitative trait loci (QTLs) in Figure 2 were done with Preview (version 10.0, Apple Inc.) and EazyDraw (version 8.7.0, Dekorra Optics, LLC, Poynette, WI). Genetic regions were counted as candidate regions (named QTL 1 to 105, 90 or 94 in control, cold or recovery, respectively) if SNPs were significantly associated with the trait on at least three of the six time points in standard temperature (control and recovery, respectively) or at least 11 of the 21 time points in low temperatures. For selecting the candidate genes, a 30-kb window on each side of the associated SNPs was chosen; in case those regions overlapped they were counted as one QTL. The broad sense heritability (H^2) was calculated with the heritability package in R (Kruijer et al., 2015).

The difference between treatments was assessed with a Student's *t* test per genotype between control and low temperature conditions; differences between genotypes were tested per time point. For the assessment of how many genotypes of the HapMap had a reduced Φ_{PSII} upon cold treatment and how many recovered on 21DAS12h, plants were considered identical at a *p* > 0.05. Averages, standard deviation or error of the phenotypic values and *t*-tests were calculated with Microsoft Excel. When average values of Φ_{PSII} were formed over several (N_i) time points, the standard deviation was calculated from the individual standard deviations per time point (dx₁, dx₂, dx_i) as sqrt((dx₁² + dx₂² + dx_i²)/(N_i – 1)). The percentile change in Φ_{PSII} between control (C) and low temperature conditions (T) or between mutant (T) and wild type (C) were calculated as: ([C – T]/C)*100.

To test for the difference between the two pairs of F1 genotypes in the complementation test, N653579 × Si ("MS") versus N653579 × Ct ("MC") and Col × Si ("CS") versus Col × Ct ("CC"), a contrast was defined as (mean(CC) – mean(CS) – mean(MC) + mean(MS)). The standard error was calculated from the mean sum of squares (MSSq) of the residuals from a linear model explaining the Φ_{PSII} by the genotype, following the formula: sqrt(MSSq * $[1/N_{CC} + 1/N_{CS} + 1/N_{MC} + 1/N_{MS}])$



FIGURE 2 QTLs detected by association mapping on chromosome 1 and 5 in the Arabidopsis thaliana HapMap population. The heat map depicts the GWAS results of the association strength ($-\log_{10}(p)$) of the average Φ_{PSII} with the SNP information of the HapMap population (a). Each association of $a - \log_{10}(p) > 3$ at any given time point is indicated as a box at the physical genetic position (vertical axis) around the SNP. The GWAS analysis was performed for several time points at 12, 16, and 19 hr between 12 DAS and 22 DAS. Over the course of the experiment the temperature was changed: from 21°C (from 12 to 13 DAS) to 5°C (14 to 20 DAS), and back to 21°C (recovery; from 21 to 22 DAS). QTLs were defined as loci that were present on at least three time points in the control temperature conditions and 11 time points in the cold conditions. Of those, the QTLs #1, #70 and #90 in the cold condition and QTL #87 of the control condition are highlighted and shown in more detail (b-e). GWAS, Genome Wide Association Studies; QTL, quantitative trait locus [Colour figure can be viewed at wileyonlinelibrary.com]

in which N is the number of replicates per genotype group. The ratio of the contrast to the standard error was compared to the t-distribution and a p-value determined (two sided test). For Figure 4a a contrast with the absolute values is plotted: |mean(CC) - mean(CS)| - |mean(MC) mean(MS)|. Figures 3 and 4 were obtained with the package ggplot2 (version 3.0.0; Wickham, 2016) in R (version 3.5.1; R Core Team, 2018).

To test the relationship between the Φ_{PSII} and the environmental conditions at the place of origin of the accessions, climate data made available by the Bergelson Lab (http://bergelson.uchicago. edu/) was used (Hancock et al., 2011). A linear regression model was applied for 293 of the accessions used in the GWAS. The model, performed with the basic R package, was calculated to explain the Φ_{PSII} of at each time point by the following variables as additive factors: annual mean temperature ("bio1_meant"), mean monthly temperature range ("bio2_diur_rng"), maximal annual temperature range ("bio7_temp_annrange"), mean temperature of the warmest quarter ("bio10_meanT_warm"), mean temperature of the coldest quarter ("bio11_meanT_cold"), latitude, longitude, number of consecutive days below 4°C ("FROSTY_DAY"), number of consecutive days above 0°C ("FROST_FREE"). For all of those factors a Kendall correlation with the Φ_{PSII} was performed per experimental time point.

The gene enrichment analysis was done with the Database for Annotation, Visualization and Integrated Discovery (DAVID) 6.8 (https://david.ncifcrf.gov/home.jsp). An EASE Score threshold, an adopted Fisher Exact test to estimate the likelihood of the



FIGURE 3 Φ_{PSII} difference of five A. *thaliana* mutants compared to the Col-0 wild type. Mutants for the genes *PSB27* (At1g03600; mutants N653579 and N654830), a RING/U-box superfamily gene (At5g12310), *ASN2* (At5g65010) and *DGS1* (At5g12290) were characterized amongst others in a low temperature experiment. The Φ_{PSII} differences of the respective T-DNA mutants N653579, N654830, N661124, N543167 and N866141 are expressed as relative values compared to their genetic wild type (WT) background, Col-0. These relative values are calculated per time point over the course of the experiment: in the control condition before the cold treatment (12 and 13 DAS), during the cold treatment (14 DAS to 20 DAS) and in the control condition after the cold treatment (21 and 22 DAS). The time points of temperature change are indicated by arrows, an open arrow indicates the onset of the cold treatment and a filled arrow the raise of temperature at the end of the cold period. Three Φ_{PSII} measurements were taken per day in the light at ~12, 16 and 19 hr. The Φ_{PSII} values of the mutant N653579 and N866141 differ from those of Col-0 at all time points (p < 0.001). The mutant N661124 is different from Col-0 at 14 of 21 time points in the cold (p < 0.05) and N543167 at all time points in the cold (p < 0.05)

annotation term, of 0.05 was chosen (Huang, Sherman, & Lempicki, 2009a, 2009b).

SNP-based haplotype groups among 162 accessions for the *PSB27* gene (At1g03600) were determined with a Perl script (Kooke et al., 2016). The phenotypic association of the polymorphisms in *PSB27* were tested by grouping all accessions according to their allelic information per SNP and testing differences per genotype group and time point. The average values per genotype and time point were used. The parental accessions, Si-0 and Ct-1, were selected amongst their respective haplotype groups because of their high, respectively low, phenotypic values, seed availability, lack of vernalization requirement and relatively similar flowering time.

3 | RESULTS

The HapMap population in A. *thaliana* is a frequently used, genetically optimized population with a large genetic and phenotypic diversity (Atwell et al., 2010; van Rooijen et al., 2017), however the response of this population to low temperature has not been studied yet. The response to a temperature decrease and subsequent increase was monitored in this population by CF imaging. The plants were first raised for 2 weeks at regular temperatures for A. *thaliana* growth (21°C air temperature, day and night) by which time most plants had formed their second true leaf pair (control treatment). To simulate an extreme, sudden, cold spell, the air temperature was reduced to 5°C (day and night; cold treatment). Four hours after the temperature decrease, the quantum yield of photosystem II (Φ_{PSII}) of all genotypes was lower than in the control temperature (Figure 1). After 1 day at 5°C (15 days after sowing at 12 hr, 15DAS12h) more than 95% of all genotypes had a significantly lower Φ_{PSII} than on the same time of the

day before the temperature reduction (13DAS12h); those genotypes that did not show a statistically significant reduction in Φ_{PSII} still had at least a 15% lower Φ_{PSII} on average in the cold. So there is an overall trend of all lines for an immediately lower Φ_{PSII} in the cold. Comparing all measurements of Φ_{PSII} made on that day (15DAS) compared to the last day of the control treatment (13DAS), the average Φ_{PSII} of the accessions was decreased by 23.1% (Table 1), with differences in Φ_{PSII} reduction ranging from 11.6 to 46.6% depending on the genotype. The range of Φ_{PSII} over the whole population also changed; in control conditions it ranged from 0.494 to 0.732 (13DAS) while after 1 day of low temperature the Φ_{PSII} ranged from 0.194 to 0.66. After 1 week of cold, the air temperature was raised to 21°C again (recovery treatment). On the 12 hr-measurement of the day after the temperature increase (22 DAS), the $\Phi_{\mbox{\scriptsize PSII}}$ values had recovered to their pre-cold values for 97.7% the genotypes. Although the Φ_{PSII} of the remaining genotypes did not recover fully, their Φ_{PSII} values still had increased by 14-38% compared to the last day of the cold treatment.

To determine if there is a relationship between the Φ_{PSII} determined in this experimental setup and the environmental conditions in the original habitat of the accessions a linear regression was modelled. The position (in longitude and latitude) of the sampling of the accessions in the wild and the respective annual temperatures and temperature range at this position were used to explain the Φ_{PSII} at the different experimental time points. The linear regression models were significant (*p*-value <.001) for the time points in the cold (14 DAS to 20 DAS) with a R² of approximately 0.17 and an adjusted R² of approximately 0.15. The adjusted R² values were lower in the control (*p*-value <.05) and the recovery treatment, with approximately 0.02 and 0.03, respectively. Although for example, geographic position and the habitat-temperature range can be predictors of Φ_{PSII} (depending on the time point), the strongest predictive factor is the average

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 $\begin{array}{ll} \textbf{TABLE 1} & \text{Overview of the } \Phi_{\text{PSII}} \\ \text{values for the HapMap population} \end{array}$

	Average Φ_{PSII}	Min Ф _{РSII}	Max Ф _{РSII}	Reduction of Φ_{PSII} compared to control (%)	$\Phi_{PSII}H^2$
Last day control (21°C)	0.686 ± 0.024	0.494	0.732	-	0.18-0.22
Second cold day (5°C)	0.528 ± 0.07	0.194	0.66	23.1%	0.21-0.25
Seventh cold day (5°C)	0.56 ± 0.057	0.322	0.686	18.3%	0.15-0.18
Second day recovery (21°C)	0.695 ± 0.022	0.552	0.739	-1.3%	0.15-0.19

Note: The Φ_{PSII} values for the HapMap population were determined over 2 days of control temperature (21°C), 7 days of cold (5°C) treatment and two following days of recovery at 21°C. The average and standard deviation over three measurements per day in the respective condition, lowest and highest values within the population, the average Φ_{PSII} reduction in the cold compared to the control (21°C) in percent, and the ranges of broad sense heritability (H²) are given.





("Ct"; Ct-1) were crossed with the knock-out mutant N653579 ("M"). The genetic background of the mutant is the wild-type accession Columbia ("Col"; Col-0) which was also crossed with the two accessions Si-0 and Ct-1. To test the significance in difference of the average Φ_{PSII} between the F1 plants from the two pairs of crosses N653579 × Si ("MS") versus N653579 × Ct ("MC") and Col × Si ("CS") versus Col × Ct ("CC") the significance of the contrast, (mean(CC) – mean(CS)) – (mean(MC) – mean(MS)), was determined per time point. The absolute difference in mean Φ_{PSII} between those groups ([(CC-CS)] – [(MC-MS)]) ranges between –0.0136 and 0.0402 per time point ("abs. Contrast" depicted as triangles; a). The significance of the contrast is shown by dots, in case of a *p*-value below 0.05, the dots are larger with a border line. The average Φ_{PSII} per genotype (and 2*SE) is displayed for three specific time points 13DAS19h (Control), 14DAS19h (Cold) and 22DAS19h (Recovery; b). The Φ_{PSII} of all lines decreases in low temperature and there is a tendency for a higher Φ PSII in CS compared to other F1 plants in the cold which is apparent on time point 14DAS19h

temperature in the coldest quarter. This factor is a significant negative predictor of the Φ_{PSII} value, however only under cold temperature (*p* value below .01 or below .001); it is not predictive of Φ_{PSII} in the

21°C control temperature period (12 and 13 DAS) and also not on the second day of recovery (22 DAS). Correlation analysis of Φ_{PSII} with the individual habitat-factors tested in the linear model showed that

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at all time points in the cold there are significant negative correlations with the annual mean temperature (tau \sim -0.13), the average temperature in the coldest quarter (tau \sim -0.14) and the consecutive number of frost free days (tau \sim -0.12). Equally, at all time points in the cold there was a positive correlation between Φ_{PSII} and the consecutive number of days below 4°C in the accessions habitats (tau \sim 0.15).

The broad sense heritability of the Φ_{PSII} value varied depending on the time points; in the control treatment (at 21°C) it ranged between 0.18 and 0.22 and from 0.15 to 0.27 in the cold. These are sufficiently high heritability values to allow a GWAS approach (van Rooijen et al., 2017). An association analysis was performed per time point. An overview of all loci at log10(p) \ge 3 is shown in Figure S1. Further focus will be on chromosomes 1 and 5, containing two loci with a log₁₀(p)-value slightly above the very stringent Bonferroni threshold of 6.6; one at the control temperature (QTL # 87), the other at the low temperature (QTL # 70; Figure 2). The latter one exceeds the Bonferroni threshold at one single time-point (DAS20 19 hr). Because of the low number of highly significant associations, priority was given to those associations that were reoccurring over time rather than appearing occasionally at higher significance. We therefore selected SNPs with a p-value below 0.001 for at least half of the time-points within a given treatment period (i.e. at least three timepoints for each of the 21°C-treatments or 11 for the cold treatment). In the control treatment 105 SNPs were identified that meet these criteria; in the cold treatment 90 SNPs and in the recovery phase 94 SNPs were detected (Figure S1 and Table S1). Candidate genes were listed in a 3- kb region flanking each significantly associated SNP on either side. These lists of genes were compared across the three different conditions (control, cold, recovery) to determine if those candidate genes were common for the whole experiment or specific to one or two of the treatments. When comparing the genes in the control and cold, 1,415 genes were specific to the control condition and 1,309 genes were specific to the cold treatment. There were 638 genes specific to the recovery phase that were not present in any of the other two conditions. There are 207 genes common to all conditions, 711 that are common to the 21°C condition before (control) and after the cold treatment (recovery); and 374 and 341 genes that are common to the control and the cold treatment or to the cold and the recovery treatment, respectively. We focussed on the candidate genes specific to the cold treatment. A gene enrichment analysis shows that there is an overrepresentation of photosynthesis or chloroplast related genes. In the category 'cellular location' 2.5-16.4-fold enrichment was found for chloroplast photosystem II, chloroplast ribulose bisphosphate carboxylase complex and chloroplast thylakoid lumen (p-values between .012 and .4; Figure S2).

In order to validate any of the candidate genes in the regions covered by the QTLs, which may be responsible for the genetic variation displayed in Φ_{PSII} at low temperatures, 31 T-DNA insertion lines for 24 candidate genes were analysed. From the list of genes in the region covered by QTLs identified for Φ_{PSII} in the cold, those 1,309 that are unique to the cold treatment were to us of main interest. Further refinement of the candidate list was mainly done via the functional annotation in The Arabidopsis Information Resource database (TAIR, www.arabidosis.org) for genes that were involved in stress response, in signal transduction or in photosynthesis and associated metabolic functions (Table S2), and mutants were screened for phenotypic differences for Φ_{PSII} upon cold exposure, when compared to wild-type plants. Two prominent candidate genes for the highly significant cold-specific QTL #70, are At5g12310 and At5g12290. While the SNP with the highest association identifying this QTL is in At5g12310, encoding a predicted RING/U-box superfamily protein, the mutant, N661124, showed only a marginal increase in Φ_{PSII} compared to that of the wild type, on average 7% in the cold (p < .05 for 14 out of 21 time points in the cold; Figure 3). Instead, mutant N866141, corresponding to DGS1 (DIGALACTOLIPID-DEFICIENT MUTANT 1 SUPPRESSOR 1; At5g12290), had on average a 20% lower Φ_{PSII} than the wild type in the cold (significant difference of p < 7 $\times 10^{-5}$ at all time points in the cold). The mutant should be compared ideally to Col-3 and not Col-0, however, since Col-3 and Col-0 are phenotypically similar, we consider the difference in Φ_{PSII} sufficiently large to assume the DGS1 gene is involved in the cold response. The ASPARAGINE SYNTHETASE 2 gene (ASN2, At5g65010), was a candidate for the cold-treatment QTL #90. A mutant for this gene, N543167, had on average an 8% lower Φ_{PSII} in the cold treatment (at least p < 0.04 and for 13 time points even higher, p < 0.001). Finally, T-DNA insertion mutant N653579 for the PHOTOSYSTEM "B" (II) GENE 27 (PSB27, At1g03600, cold QTL #1), showed the strongest reduction in photosynthesis efficiency of all tested mutants; in control conditions the Φ_{PSII} of N653579 was on average 20% lower than that of Col-0 while in the cold it was on average 53% lower (p < 2 \times 10⁻³⁰). T-DNA insertion line N654830, containing another T-DNA insertion in the same gene, showed only a slight reduction in Φ_{PSII} , in the cold, compared to the wild type. However, since this insertion is in the presumed promotor region of the PSB27 gene, its function may be hardly affected by the mutation. N653579 carries a knock-out allele of the PSB27 gene that was described to have a role in light adaptation (Hou, Fu, Garcia, Buchanan, & Luan, 2015). PSB27 is also one of four genes found in the enriched cellular localisation categories "Chloroplast photosystem II/ thylakoid (/lumen)."

We further examined the genetic variation in PSB27, to validate that it indeed explains the variation in Φ_{PSII} associated with QTL #1. At gene At1g03600, an allelic difference at one non-synonymous SNP at position 899,112 (T or C, which leads to an amino acid change from alanine to a valine) is associated with a difference in phenotype at all of the time points in the cold (p-value ranging from 0.0184 to 0.0004 on the different time points). The accessions with a T-allele (79 out of 162 accessions) had an average Φ_{PSII} value of 0.686 ± 0.001 under standard temperature (21°C) and 0.55 ± 0.001 in the cold, while accessions with a C-allele (83 accessions) had an average Φ_{PSII} of 0.682 ± 0.001 and 0.534 ± 0.001, respectively. Finally, a quantitative complementation test (Mackay, 2001; Weigel, 2012) was performed to confirm that allelic changes at the PSB27 gene cause the phenotypic variation. Two accessions were selected to serve as parents in crosses to complement the mutant phenotype, Siegen-O (Si-O) and Catania-1 (Ct-1), with Si-0 representing the phenotypic class with the higher Φ_{PSII} (T at 899112) in the cold treatment and Ct-1 the group

with a lower Φ_{PSII} (C at 899112; Figure 1). For the complementation test, the different alleles under investigation are assessed in the heterozygote state to normalize the effect of genetic variation residing at other loci (Weigel, 2012). Thus, the accessions Si-O and Ct-1 were each crossed with the homozygous T-DNA insertion knock-out psb27-mutant N653579 as well as with the Col-0 wild type. If the difference in Φ_{PSII} of the F1s between the selected accessions and Col-0 is significantly different from the difference in Φ_{PSII} of the F1s between the same accessions and the KO mutant this difference can only be caused by the allelic variation of the gene under investigation. A difference between the absolute mean differences of the pairs shows that in the cold the difference between the F1 pairs in the Col-O background is larger than in the mutant background (Figure 4). In the cold, the absolute difference in Φ_{PSII} between Col \times Si ("CS") and Col × Ct ("CC") is between 0.01 and 0.04 larger than between N653579 × Si ("MS") and N653579 × Ct ("MC"). CS has on average a higher Φ_{PSII} than CC. However, the significance for this difference in contrast between the two pairs varies at a p-value between 0.03 and 0.9 (with a p-value below 0.05 on 8 out of 20 time points in the cold treatment). Therefore, only a tendency can be reported. This tendency supports, however, the proposition that natural variation at the PSB27 gene (At1g03600) has an influence on the Φ_{PSII} cold response and that it can be responsible for the observed variation attributed to QTL#1.

4 | DISCUSSION

In this study the effect of cold on natural accessions of *Arabidopsis thaliana* was examined by the means of CF imaging of photosynthesis efficiency. To ensure highly stable environmental conditions, the plants were grown in a closed growth room with controlled air temperature, light conditions, relative humidity and a hydroponic system that allowed the control of water and nutrient supply. These measures all contributed to reducing the variability and potentially disturbing effects of the environment on the genetics of the environmentally sensitive trait Φ_{PSII} . All genotypes were monitored over the complete duration of the experiment with three measurements made per day. These repeated measurements enabled us to identify persistent patterns over the course of time that are specific to the respective conditions, reducing the effect of errors that could come from individual observations.

The daily measurements allowed us also to visualize the progressive response of the plants to low temperature. Photosynthesis efficiency responded immediately to the lowering of the temperature in all genotypes. The severity of the impact of the temperature change on the absolute value of Φ_{PSII} depended on the genotype. After 1 day of low temperature exposure, the overall photosynthetic efficiency did not decrease further during 1 week of cold treatment. So the effect on light-use efficiency seems to be immediate and does not show any long-term effects in the - day cold exposure period. This is in agreement with the measurements of Strand, Hurry, Gustafsson, and Gardeström (1997) on leaves of Col-0 plants that were exposed

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to low temperatures. After three and 10 days at 5°C, the maximum quantum yield of photosystem II, F_v/F_m , stayed nearly the same (ca. one third of the value at the control growth temperature), and no further reduction was seen within the 7 days of cold. In our experiment, we increased the temperature after 1 week of cold exposure and observed a nearly full recovery of photosynthesis efficiency after 1 day. This implies that the plants were not permanently damaged by the low temperature. Such a recovery is consistent with the ecology of A. thaliana, which grows in temperate regions where exposure to low temperatures in the growing cycle is common. The species is known to have mechanisms to adapt to cold and even acclimate to freezing temperatures (Alonso-Blanco et al., 2005; Oakley et al., 2018; Schulz, Tohge, Zuther, Fernie, & Hincha, 2016). A. thaliana is shown to be chilling tolerant and after prolonged exposure to low temperatures a recovery of photosynthesis has been observed that is due to a change in the regulation of photosynthesis (Holaday, Mahan, & Payton, 2016; Strand et al., 1997). The linear regression model and the correlation analysis relating the Φ_{PSII} determined for accession in this experiment with the habitat environmental factors point to a general trend that accessions with low Φ_{PSII} under the cold conditions in this experiment come from habitats with shorter and milder winter periods. While much more work would be needed to confirm a selective advantage of certain genotypes in certain habitats, this observation suggests there may well be an adaptive mechanism in place that provides some accessions from colder climates with more efficient photosynthesis at mild (sub-zero) low temperature exposure than other accessions.

The photosynthetic efficiency measure we used, Φ_{PSII} , reflects not only on a local photosynthetic process but also the broader physiological state of the plants and responds to several environmental changes, including cold (Baker & Rosenqvist, 2004; Roháček, Soukupová, & Barták, 2008). It has already been used for genetic analysis (Fracheboud, Ribaut, Vargas, Messmer, & Stamp, 2002; Prinzenberg, Víguez-Zamora, Harbinson, Lindhout, & van Heusden, 2018; van Rooijen et al., 2017), but no genome wide association study was done for this trait to investigate low temperature responses. The Φ_{PSII} data of the HapMap population was used for a genetic analysis per time point and several associated loci were identified, both those common to all treatments and those that are specific to a treatment. Amongst the candidate genes, photosynthesis-related factors are found to be enriched for the response to cold, which is consistent with our quantification of the photosynthetic trait, Φ_{PSII} . In addition, Φ_{PSII} is very sensitive to low temperature exposure. It was hence not surprising, that candidate genes that are already described in photosynthetic adaptation are also candidate for the cold response of Φ_{PSII} . Protection of the photosynthesis apparatus, the primary energy acquiring process of the plant, against diverse and changeable environmental conditions is essential for plant survival. In lower temperatures, the overall metabolic activity of the plant is reduced. If photosynthesis efficiency is not downregulated, high excitation pressure and sink limitations in low temperature can lead to the enhanced production of reactive oxygen species (Juszczak, Cvetkovic, Zuther, Hincha, & Baier, 2016; Triantaphylidès et al., 2008) and photodamage WII FY

(Adam & Murthy, 2014; Krieger-Liszkay, 2005; Suzuki & Mittler, 2006; Wise, 1995). Several processes could play a role in photoprotection in the cold, like energy dissipation from the photosystem by non- photochemicalquenching (Kościelniak & Biesaga-Kościelniak,

2006; Müller, Li, & Niyogi, 2001; Oquist, Hurry, & Huner, 1993), changes in pigment content (Fracheboud et al., 2002; Huner, Elfman, Krol, & McIntosh, 1984; Schöner & Heinrich Krause, 1990), accumulation of osmolytes (DeRidder & Crafts-Brandner, 2008; Holmström, Somersalo, Mandal, Palva, & Welin, 2000) or changes in membrane fluidity and its associated proteins (Takami, Shibata, Kobayashi, & Shikanai, 2010). A fine-tuned regulation of photosynthesis and photoprotective mechanisms is necessary to prevent permanent damage to the photosystem, while still enabling sufficient photosynthetic operation for the production of photoassimilates. To allow this finetuning a multifactorial cold response of photosynthesis would be a likely strategy and a polygenic control of the photosynthetic response to cold is expected. Therefore, and not unexpectedly, many QTLs were identified, most with low association-strength. Only two loci exceeded the very conservative Bonferroni threshold. It illustrates the highly polygenic nature of the trait, which together with the modest broad sense heritability, strongly reduces the statistical power in GWAS to detect significant QTLs. The use of a stringent cut-off minimizes the detection of false positives but will also exclude many small-effect QTLs that are thought to be one of the main sources of missing heritability in quantitative genetic studies (Bloom, Ehrenreich, Loo, Lite, & Kruglyak, 2013). To overcome this, we gave more weight to the persistence of the association over time, rather than to the strength of the association, in the selection of candidate genes. No major, common genetic factor involved in the low temperature regulation of photosynthesis stands out in our study of the worldwide collection of genetic variants in A. thaliana. Instead there appears to be a much more multifactorial regulation, possibly reflecting the presence of different cold adaptation mechanisms, each with comparable representation in the germplasm. This is supported by the transcription analysis of 10 A. thaliana accessions which showed large natural variation in response to low temperature, with 75% of the differentially expressed genes to be genotype specific (Barah et al., 2013).

Genetic confirmation of multifactorial, allelic variation is not trivial, especially of small-effect loci. Studying knock-out mutants is one possible way to confirm the involvement of a candidate gene that was discovered by statistical association. However, natural allelic variation detected in a QTL study cannot always be proven by a mutant analysis, as for example, gene redundancy or genotype-specific pleiotropic effects may mask the mutant phenotype. Therefore, we decided to support the candidate gene study by another methodology. Complementation tests or the cloning and transformation of one allelic variant into a different genetic background is a way to look at functional differences compared to a loss-of-function effect. However, in these cases pleiotropic effects, heterosis or the genomic position of the transgene can also influence the phenotype and the outcome of the analysis. Nevertheless, due to the large number of available mutants for A. thaliana, albeit mostly in the Col-O background, a large number of candidate genes can be screened relatively easily. In combination

with a complementation test, this can provide sufficient proof to determine if an allelic variant is causal to the QTL effect. Our mutant study indicated the involvement of several genes in the photosynthesis phenotype. Two mutants that showed a significant and sizeable difference in Φ_{PSII} compared to the wild type are dgs1 and asn2, that are candidates for the QTLs #70 and #90 respectively. Both genes, DGS1 and ASN2, were previously also identified as candidates underlying QTLs involved in the adaptation of Φ_{PSII} to high-light stress conditions (van Rooijen et al., 2017). DGS1 is important for lipid remodelling, in this case the conversion of phospholipids to galactolipids, which is important for high-light stress response (van Rooijen, Harbinson, & Aarts, 2018). ASN2 is involved in nitrogen allocation and the mutant was shown to have a lower leaf nitrogen concentration and a pale, low-chlorophyll phenotype; however no effect on photosynthetic CO₂ assimilation was found under non-stressed growing conditions (Gaufichon et al., 2013). Both genes are strong candidates to be involved in the photosynthetic response to cold stress. Exposure to low temperatures can cause over-excitation of the photosystem. like high-light stress, and similar protective mechanisms can apply.

One candidate gene was confirmed to be underlying one of the QTLs. The Φ_{PSII} of the *psb27* mutant, corresponding to cold-QTL #1 on chromosome 1, was much lower than that of the wild type. The subsequent quantitative complementation test supports the influence of variation at PSB27 to affect Φ_{PSII} in the cold. Among the HapMap population, haplotypes with a thymine instead of a cytosine at SNP 899,112, which is in the PSB27 ORF, had a slightly (between 2 and 5%), but significantly (*p*-value < 0.05) higher Φ_{PSII} . In the complementation test, accessions Si-O and Ct-1, carrying the contrasting alleles, were crossed with the psb27-mutant. The F1s have a significantly higher Φ_{PSII} than the mutant. This indicates that both alleles are functional. which is expected of natural alleles of genes for which a knock-out mutant gives a strong fitness-decreasing phenotype. Based on the comparisons of the F1s between accessions, with the Col-0 \times Si-0 F1 resembling Si-O and Col-O × Ct-1 F1 resembling more Col-O than Ct-1, it could be that the dominance of the different tested PSB27-alleles is the following: the Si-O alleles (PSB27^{Si-O}) could be dominant over the other alleles with the mutant in the Col-O background (psb27^{Col-O}) being the least dominant (PSB27^{Si-0} > PSB27^{Col-0} > PSB27^{Ct-1} > $psb27^{Col-0}$). In the wild-type background, the F1 from the cross Col-0 \times Si-0 had a tendency for a higher Φ_{PSII} than the F1 from the cross to Ct-1. This is in accordance with the phenotypic difference observed between Si-O and Ct-1. There is no difference between the Si-O- and Ct-1- F1 plants in the mutant (N653579) background. As the effect in the heterozygote state shows a different tendency in a combination with a wild-type Col-0 than with a mutant (psb27-Col-0) background, the complementation test supports the conclusions from the association mapping that allelic variation of the PBS27 gene impacts Φ_{PSII} in the cold. The allelic effect of this locus on Φ_{PSII} may be too small to be consistently validated over all time points in the heterozygous background. Heterozygosity effects due to the mixed genetic backgrounds of the F1 plants may mask the gene effect. Furthermore, additional mutations or differences between the Col-O used for the mutant and for the F1crosses could also increase the phenotypic variability. Another mutation is

known to exist in N653579 (in the gene CP26), however in earlier studies this was shown not to influence Φ_{PSII} (Hou et al., 2015). CP26 is also not among the candidate genes identified in the GWAS analysis. Furthermore, we found another T-DNA insertion allele of PSB27 to also show a lower Φ_{PSII} in the cold, though with a much less drastic difference than seen in N653579. Taking together the results from the mutant analysis and the complementation test, PSB27 seems to influence photosynthetic responses to cold. PSB27 codes for a thylakoid lumen protein, associated with the PSII complex. In cyanobacteria, the PSB27 protein is involved in the repair and biogenesis of the PSII protein complex, transiently binding the photosystem II complex subunits D1 and CP47 (Cormann, Möller, & Nowaczyk, 2016; Liu, Huang, Chen, Gross, & Pakrasi, 2011; Liu, Roose, Cameron, & Pakrasi, 2011) and facilitating the assembly of the water oxidizing complex (Komenda et al., 2012; Roose & Pakrasi, 2008). Hou et al. (2015) studied the mutant N653579 and showed that the loss of the PSB27 protein caused an overall lower $\Phi_{\mbox{\scriptsize PSII}}$ growth retardation and pigment aberration in plants grown in fluctuating light when compared to the wild type. Though the exact function of PSB27 in PSII repair upon stress response is not confirmed in A. thaliana, there is a known involvement in fluctuating light adaptation. The temperature and high light stresses are connected. PSII can easily be damaged at low temperatures by high excitation pressure caused by light (Distelbarth, Nägele, & Heyer, 2013). Correspondingly, the photo-repair function of PSB27 may also contribute to the adaptation to low temperatures.

This study showed that the measurement of $\Phi_{\mbox{\scriptsize PSII}}$ in a diversity panel of A. thaliana suitable for GWAS is very effective in the identification of many genetic loci involved in photosynthesis response to cold. Environmental stability and especially the repeated measurements over time sufficiently repressed the non-genetic environmental effect on the phenotypic variation to allow the detection of these low-effect QTLs. To further study this genetic variation, and identify causal genes, a reduction in genetic complexity will be useful, for example, by examining biparental (Brachi et al., 2010), multiparent (such as MAGIC; Kover et al., 2009; Zheng, P Boer, & van Eeuwijk, 2014) or regional populations (Long et al., 2013). We assume that the genetic regulation in the A. thaliana HapMap population is complex and guided by different, multigenic pathways. Nevertheless, at least one candidate gene could be validated. This interdisciplinary approach, combining photosynthesis research and genetics, revealed a new molecular component, PSB27, to be of relevance in cold stress response.

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CONFLICT OF INTERESTS

The authors declare no conflicts of interest.

AUTHOR CONTRIBUTIONS

Aina E. Prinzenberg planned and supervised the experiments, performed the GWAS experiment, part of the mutant analysis and complementation test. Lucia Campos-Dominguez contributed to the mutant analysis and the complementation test. Willem Kruijer performed the GWAS analysis, made the corresponding heatmap and provided advice on the statistical analysis. Jeremy Harbinson and Mark G. M. Aarts initiated the project, assisted in project planning and advised on photosynthetic and genetic issues, respectively. Jeremy Harbinson did the enlargements for the Figure 2. All authors contributed to the writing of the manuscript.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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