

Unravelling nuclear natural variation under  
fluctuating light conditions reveals the complexity  
of photosynthesis



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## Abstract

Light intensities outside do not only change between days, but also within a day. Plants have to adapt to these changes by finding a balance between protection to photodamage and high photosynthesis efficiency. Non photochemical quenching (NPQ) is one of the mechanisms in plants that protects the

plant to high light intensity. Recently, the presence of natural variation for NPQ was demonstrated in *Arabidopsis thaliana*. The physiology of NPQ has not yet been completely unravelled and evolution might have developed different mechanisms to do photoprotection, therefore it is an interesting trait to investigate. The nuclear genome of Ely induces higher NPQ values under high light intensity. In a biparental population between the Ely and Col-0 accession, a QTL on chromosome 2 was shown to have a large effect size on NPQ. In this thesis, this QTL was the basis in understanding the genetics and physiology of NPQ. A near isogenic line (NIL) for this QTL was developed and homozygous lines with a recombination in the QTL region were used for fine mapping of the gene of interest. This reduced the size of the QTL from 250,000 bp to 25,709 bp. Nine candidate genes are located in this QTL, of which two genes (PMM and CPFTSY) have previously been shown to be involved in photosynthesis pathways. A system was built in which different light patterns could be programmed. This system was used to study the long-term effect of the QTL in different environments on physiology. The QTL on chromosome 2 resulted in a decrease in dry weight of 17% and 12% in respectively a constant and slowly fluctuating light environment. A highly fluctuating light pattern resulted in an increase of 5% in dry weight. This revealed the complexity of photosynthesis phenotyping and how photosynthesis behaves dynamically under different light conditions. Acclimation to specific light fluctuations influences NPQ measurements, which means that the manner of measuring NPQ influences the results and makes it a complicated phenotype.

## Introduction

Feeding the continuously growing world population will be a challenge in the future. In 2050, there will be 9.7 billion people on earth, which requires increased food production by the agricultural sector (FAO, 2018). However, climate change and intensive agriculture have put pressure on the availability of natural resources and arable land (FAO, 2018). These developments demand more efficient food production. Yet, research showed that yield improvements of important crops is stagnating (S. P. Long & Ort, 2010; Ray et al., 2012). Yield improvement was originally driven by classical breeding focussed on plant architecture and light capture (S. P. Long & Ort, 2010; Ray et al., 2012). Even though photosynthesis is the primary driver of yield, little increase in crop yield has been achieved by increasing photosynthetic efficiency (Zhu et al., 2010).

Photosynthesis consists of two core mechanisms, the light reaction and the dark reaction (Calvin-Benson cycle). The Calvin-Benson cycle is independent of light and uses the energy produced by the light reaction to fixate CO<sub>2</sub> in carbohydrates. In the light reaction (Figure 1), light energy is converted to ATP and NADPH by multiple proteins that are located in the thylakoid membrane. The Light Harvesting Complex (LHC) consists of many antenna pigments e.g., chlorophyll A, that catch photons from sunlight and transport them to the Reaction Centre (RC) of the photosystem the LHC belongs to. In photosystem II (PSII), the energy from photons is used to split water molecules and consequently excite electrons to a higher energy state. In the electron transport chain (ETC), the energy state of the electron gradually decreases and at the same time H<sup>+</sup> protons are transported across the membrane to create a membrane potential. This transport of H<sup>+</sup> protons is performed by the transmembrane proteins plastoquinone (PQ) and the cytochrome complex (Cyt b<sub>6</sub>f). ATP synthase makes use of this membrane potential to create energy in the form of ATP. The electron will be again excited, but then in photosystem I (PSI), with the energy of photons captured by the LHC of PSI. The energy of the excited electron is used to produce NADPH, or the electron will be transported back to either PQ or Cyt b<sub>6</sub>f.

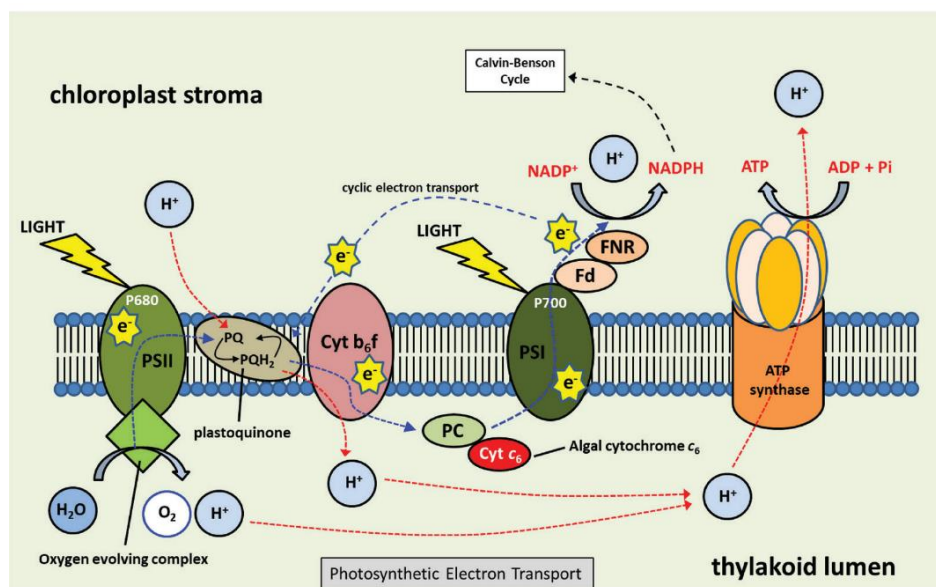


Figure 1 Schematic representation of the light reaction (Simkin et al., 2019).

The efficiency of PSI is already close to 100% (Croce & van Amerongen, 2020). However, currently the maximum efficiency of PSII ( $\phi$ PSII) is only 85% under optimal conditions. With suboptimal conditions, such as high light, the efficiency of PSII decreases rapidly (Croce & van Amerongen, 2020). Can we increase this efficiency and consequently increase crop yield? Besides the inefficiencies in PSII, yield can potentially be increased with >40% by improving overall photosynthesis efficiency (Simkin et al.,

2019). Many energy losses in the photosynthetic pathway lead to a difference between light energy captured by leaves and the consequently produced biomass (Zhu *et al.*, 2010). Only 4.6% and 6.0% of the solar energy is converted to biomass for C3 and C4 plants respectively (Zhu *et al.*, 2010). Part of the energy captured from sun light is lost in the form of heat due to photochemical inefficiency, which accounts for 6.6% of the losses (Zhu *et al.*, 2010). Leaves produce this heat to protect themselves for photodamage, this phenomenon is called non-photochemical quenching (NPQ) (Zhu *et al.*, 2010).

All day long, leaves experience different light intensities by changing weather conditions and shade due to movement of other leaves (Ruban *et al.*, 2012). If plants catch more sunlight than they can process, the excessive energy is dissipated in the form of heat (Simkin *et al.*, 2019). This phenomenon is called NPQ and prevents the formation of toxic reactive oxygen species (ROS) and photodamage to the RC under high intensity light (Goss & Lepetit, 2015; Ohad *et al.*, 1984). NPQ is triggered by sudden high light intensities and induces conformational changes of antenna proteins which results in a closed state (Horton *et al.*, 1991; Schansker *et al.*, 2011). Consequently, the antenna protein is not able to receive any protons until the RC is available again for new electrons to bring pigments to an excited state (Schansker *et al.*, 2011). This process of relaxation of the antenna pigments is relatively slow and can decrease carbon uptake with 17-32% (Zhu *et al.*, 2004). Induction of NPQ is relatively fast, compared to relaxation (Kromdijk *et al.*, 2016). Therefore, enhancing the relaxation rate of NPQ seems an opportunity to increase photosynthesis efficiency (Kromdijk *et al.*, 2016; Murchie & Niyogi, 2011).

It is clear that photosynthesis is subjected to many fluctuations, this raises questions how the photoprotective mechanism works and how PSII perceives fluctuations in light intensity. When light intensity suddenly increases, proton concentration in the thylakoid lumen will increase because more water molecules are split and more protons are transported across the membrane by proteins in the ETC. This induces a  $\Delta pH$  across the thylakoid membrane. Consequently, antenna pigments transition to a quenched state, called energy dependent quenching (qE). This transition is induced by two different mechanisms, increasing the concentration of the PSII protein subunit S (PsbS) and activation of the xanthophyll cycle (Simkin *et al.*, 2019) (Figure 2). Under high- or fast fluctuating light, higher PsbS levels provide protection of the photosystems, but can also decrease  $\phi PSII$  (Kromdijk *et al.*, 2016; Xiao-Ping Li *et al.*, 2000). Higher amounts of PsbS result in faster response of NPQ, thus faster induction and relaxation of qE (Hubbart *et al.*, 2012; Li *et al.*, 2002; Zia *et al.*, 2011). The precise underlying mechanism remains unrevealed (Croce & van Amerongen, 2020). The xanthophyll cycle response is slightly slower and is regulated by the enzymes violaxanthin de-epoxidase (VDE) and zeaxanthin epoxidase (ZEP) (Giimore, 1997; Horton *et al.*, 1991; Kromdijk *et al.*, 2016; Xiao-Ping Li *et al.*, 2000). VDE converts violaxanthin to zeaxanthin and ZEP vice versa. Binding of H<sup>+</sup> protons and zeaxanthin to antenna pigments can lead to conformational changes, which results in quenched state pigments in the LHC. A higher amount of zeaxanthin results in faster induction and slower relaxation of NPQ (Nilkens *et al.*, 2010; Pérez-Bueno *et al.*, 2008). De-epoxidation of violaxanthin, which induces heat dissipation, is faster than epoxidation of zeaxanthin, which results in relaxation of NPQ (Giimore, 1997; Horton *et al.*, 1991; Kromdijk *et al.*, 2016; Xiao-Ping Li *et al.*, 2000). By making epoxidation more efficient, the speed of NPQ relaxation might be increased, which results in less unnecessary heat loss under fluctuating light conditions.

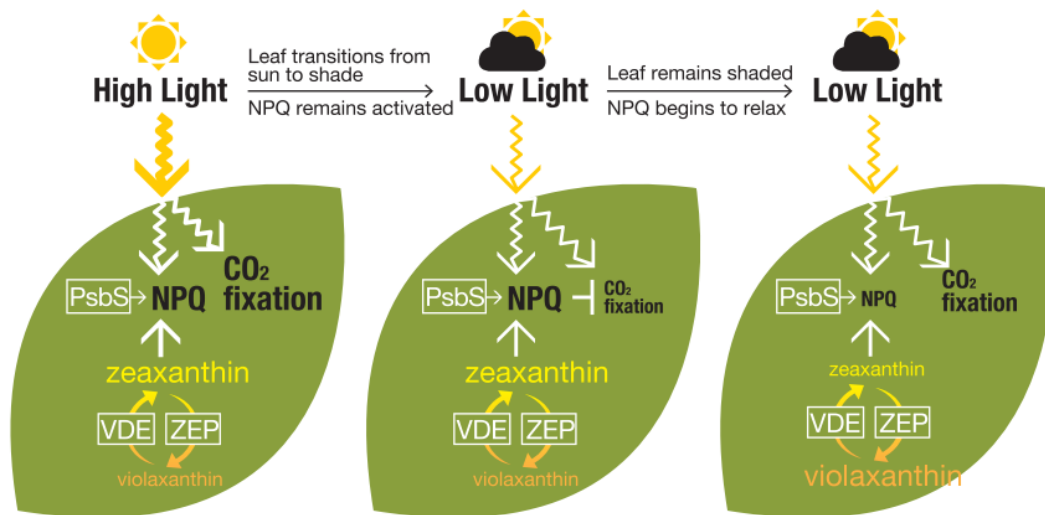


Figure 2 NPQ mechanism in relation to CO<sub>2</sub> fixation, triggered by changing light intensities. Two different mechanisms influence NPQ formation, (1) PsbS and the (2) xanthophyll cycle. (1) Higher amounts of PsbS increase NPQ induction and relaxation, which is independent of PsbS gene expression. (2) The xanthophyll cycle is regulated by VDE, which converts violaxanthin to zeaxanthin. Zeaxanthin leads to conformation changes of the antenna pigments, which result in a quenched state that blocks excitation of pigments. PsbS reacts faster to changing light intensities than the xanthophyll cycle (Kromdijk et al., 2016).

Previously, Kromdijk et al. (2016) investigated the potential of increasing photosynthesis efficiency by accelerating NPQ relaxation and induction in tobacco (*N. benthamiana*). Overexpression of VDE, ZEP and PsbS led to an increase in NPQ recovery after transition from high light to low light. This faster NPQ relaxation rate was correlated with an increase in CO<sub>2</sub> fixation of 14%, which provides evidence that NPQ the mechanism influences photosynthesis efficiency and assimilation. Kiss et al. (2008), Xiao-Ping Li et al. (2002) and Xiao-Ping Li et al. (2000) showed that *A. thaliana thaliana* lines deficient in PsbS or overexpressing PsbS differ in NPQ response. These studies all show that PsbS and the xanthophyll cycle are involved in NPQ relaxation, however they only show the effect of genes that are already known to be involved in NPQ. Nature might have developed additional mechanisms, genes or alleles being involved in NPQ that are not yet described in literature. Therefore, evaluating natural variation of NPQ seems a promising tool to increase our knowledge on the mechanisms behind NPQ and other mechanisms that potentially influence photosynthesis efficiency. Finding and studying genes that are not yet linked to NPQ will explain more about the process than is currently understood. In order to improve the efficiency, it is important that photosynthesis parameters are accurately measured, Box 1 explains the phenomenon of NPQ and how it can be measured.

#### Box 1: Photosynthesis and NPQ measurements

Photons absorbed by pigments in plant leaves can have three different destinies: (1) photochemical quenching (qP), (2) dissipation in the form of heat and (3) fluorescence (Zaman et al., 2018). By calculating the rate constants of these destinies, we are able to analyse how the different yield components, or efficiencies, relate to each other.

Energy used for photosynthesis is used by PSII and PSI. Since the efficiency of PSI is close to 100%, efficiency of photosynthesis (efficiency of qP) can be approximated by  $\Phi_{PSII}$ .

Heat dissipation can be either active or passive. Energy loss by dissipation consists of NPQ (active heat dissipation) and NO (in the form of fluorescence and passive heat dissipation). The passive form of heat dissipation is mainly high when the plant is not able to prevent damage to the photosystem by NPQ under high light (Schansker et al., 2011). The efficiencies of active and passive dissipation are  $\Phi_{NPQ}$  and  $\Phi_{NO}$ , respectively.

The efficiencies, or yields, of these destinies add up to 1 since the efficiencies are calculated as a ratio of the total rate. When the efficiency of either PSII, NPQ or NO increases, another will be reduced. This leads to the following formula:

$$\Phi_{NPQ} + \Phi_{NO} + \Phi_{PSII} = 1 \quad (1)$$

The different efficiencies can be calculated by different fluorescence parameters, as explained in (Fluorescence measurements Robin, page 29).

NPQ is the ration between  $\Phi_{NPQ}$  and  $\Phi_{NO}$ , which is described by the following formula:

$$NPQ = \frac{\Phi_{NPQ}}{\Phi_{NO}} \quad (2)$$

Many genes that influence photosynthesis are located on the nuclear genome, the nucleotype. However, the chloroplast genome also influences photosynthesis related traits. The chloroplast genome is part of the plasmotype, which consists of the genome of the organelles. Research on photosynthesis is complicated because epistatic effect can influence the photosynthesis phenotypes and it is difficult to separate the effect of the nucleus and chloroplasts. Furthermore, the chloroplast genome does not recombine and is always maternally inherited. There are several methods available to study the effect of photosynthetic genes, such as forward and reverse genetics, proteomics, transcriptomics, comparative genomics (Armbruster et al., 2011). Another powerful method is phenotyping of cybrid. Cybrids are lines that inherited only the parental nuclear DNA and the maternal plasmotype (mitochondrial and chloroplast) DNA. To study natural variation of the plasmotype, Flood *et al.* (2020) made a panel of cybrids of all nucleotype-plasmotype combinations of 7 different *A. thaliana* accessions (Bur, C24, Col-0, Ler-0, Sha, WS-4 and Ely). This was achieved by using maternal haploid-inducer (HI) lines, which resulted in abortion of the maternal nuclear genome before or during meiosis, and consequently haploid cybrid offspring was obtained (Figure 3). By whole genome duplication or restitutional meiosis, these haploid plants were able to produce diploid cybrid offspring. By photosynthesis phenotyping of different nucleotype-plasmotype combinations, the effect of natural variation on different nucleotypes, plasmotypes and epistatic effects could be analysed. Interesting nucleotypes or plasmotypes were identified to study further.

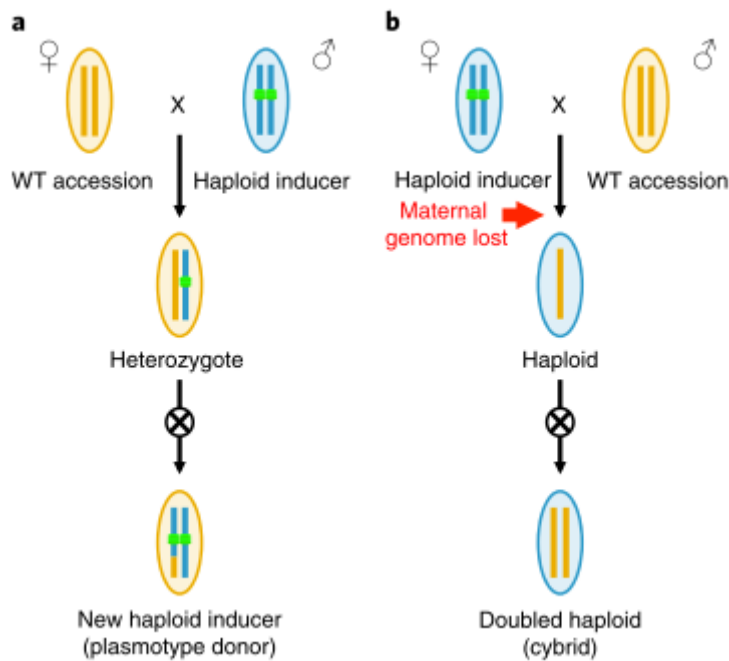


Figure 3 Generation of cybrids. a) Generation of new haplo-inducer lines for the lines (all except for Col) that did not yet contain the GFP-tagged CENH3/ HRT12 mutant. A WT (female) and HI (male) were crossed to obtain a heterozygous F1. The F1 was selfed and lines homozygous for the GFP-tagged CENH3/ HRT12 mutant were selected from the F2. These lines could be used as a new HI line for making cybrids. The ovals represent the plasmotype and the vertical bars represent the nucleotype. Yellow is maternally, and blue is paternally inherited DNA. Green circles on the bars represent the chromosomes with a GFP-tagged CENH3/ HRT12 mutation. b) Generation of cybrids by crossing a female HI with a male WT accession. The F1 consists of haploid lines, all containing the maternal plasmotype and paternal nucleotype. Stable diploid (double haploid) plants were obtained by spontaneous genome duplication or restitutional meiosis.

One of the accessions in the cybrid panel is Ely, which has a large effect mutation in the chloroplast *PsbA* gene (El-Lithy et al., 2005). Due to this mutation, triazine cannot bind to the QB binding site, which results in triazine resistance. QB is a binding site of the D1 protein of PSII to which plastoquinone binds for photosynthesis. Due to the mutation in *PsbA*, there will be no competition for QB by triazine and plastoquinone anymore, which results in triazine resistance. However, the mutation also reduces the binding efficiency of plastoquinone to Q<sub>B</sub> and consequently  $\Phi$ PSII (El-Lithy et al., 2005; Gronwald, 1994, as cited in Flood et al., 2016). Almost all accessions collected across the whole UK that contain the *psbA* mutation appear to have the exact same nucleotide. This specific nucleotide is only found in Ely and present in all Ely accessions collected across the UK. Besides the reduced PSII efficiency of the plasmotype, the Ely nucleotide showed higher levels of NPQ and q<sub>E</sub> (Flood et al., 2020). Altogether, this raises many questions, which nuclear gene is or which genes are responsible for the higher levels of NPQ and q<sub>E</sub>? Is the Ely nucleotide beneficial without the mutation in *PsbA*? Are there other genotypes with a mutation in *PsbA* that do not show a higher NPQ phenotype? How did the Ely nucleotide spread and why is the *psbA* mutation always found together with the Ely nucleotide?

All organisms on earth strive for passing on their genetics to the next generation by producing offspring. Natural selection, or adaptive evolution, is based on presence of genetic variation between individuals, heritability of traits and a difference in fitness between alleles. All accessions that were found to have a mutation in *PsbA* happen to contain the Ely nucleotide, this leaves us with two theories of the spread of the Ely nucleotide. The first possibility is that the Ely nucleotide developed by mutations during the period that triazine was applied to railways and spread throughout the whole railway population. The second scenario is that the Ely nucleotide was already present in the population before the triazine application and did not negatively affect its fitness. During the

application of triazine, there was selection on triazine resistance, which resulted in an increase of the fitness of accessions with the *PsbA* mutation. Next to triazine resistance, this mutation affects photosynthesis negatively and Ely might need for an alteration in the photosynthesis pathway to be viable. This second scenario seems more likely since the period of application of triazine was only about 35 years (Flood et al., 2016), which is probably not long enough for a mutation to fixate in a population. From this scenario it can be hypothesised that the Ely nucleotype increases the fitness of accessions with a mutation in *PsbA*. However, the effect of the Ely nucleotype without presence of the mutation in *PsbA* is unknown. Therefore, it is interesting to understand the effect of the Ely nucleotype on the photosynthesis pathway.

To further investigate the influence of the Ely nucleotype on photosynthesis phenotypes, QTL mapping of several photosynthesis related phenotypes was performed (Theeuwes and Logie, unpublished data). A doubled haploid (DH) population was created, which originates from crosses between cybrids with Ely nucleotype and Columbia (Col) plasmotype (E-C) and Col nucleotype and plasmotype (C-C). From this data two major QTLs were found for NPQ, positioned on chromosome 2 (chr2) and chromosome 4 (chr4) (Figure 4 and Figure 5). Previously, a large QTL for qE was already found on chromosome 2 by Jung and Niyogi (2009). However, the region they defined covers 37 cM, which is much larger than the QTL of 1.1 cM, as defined by Theeuwes and Logie. The effects seem to be additive, because lines with the Ely genotypes for one of the QTLs on chromosome 2 and 4, as well as the combination of the two, showed an increase in NPQ (Figure 5). The results do not show a significant difference between lines that have Ely on chromosome 2 combined with Col on chromosome 4 and lines with the Ely genotype on both QTLs. Less lines were phenotyped with the Ely genotype on both QTLs. Including more lines might give the opportunity to conclude whether the QTLs show epistasis or additivity. Furthermore, it will decrease the number of genes within the QTL that currently includes 38 genes for the QTL on chromosome 2.

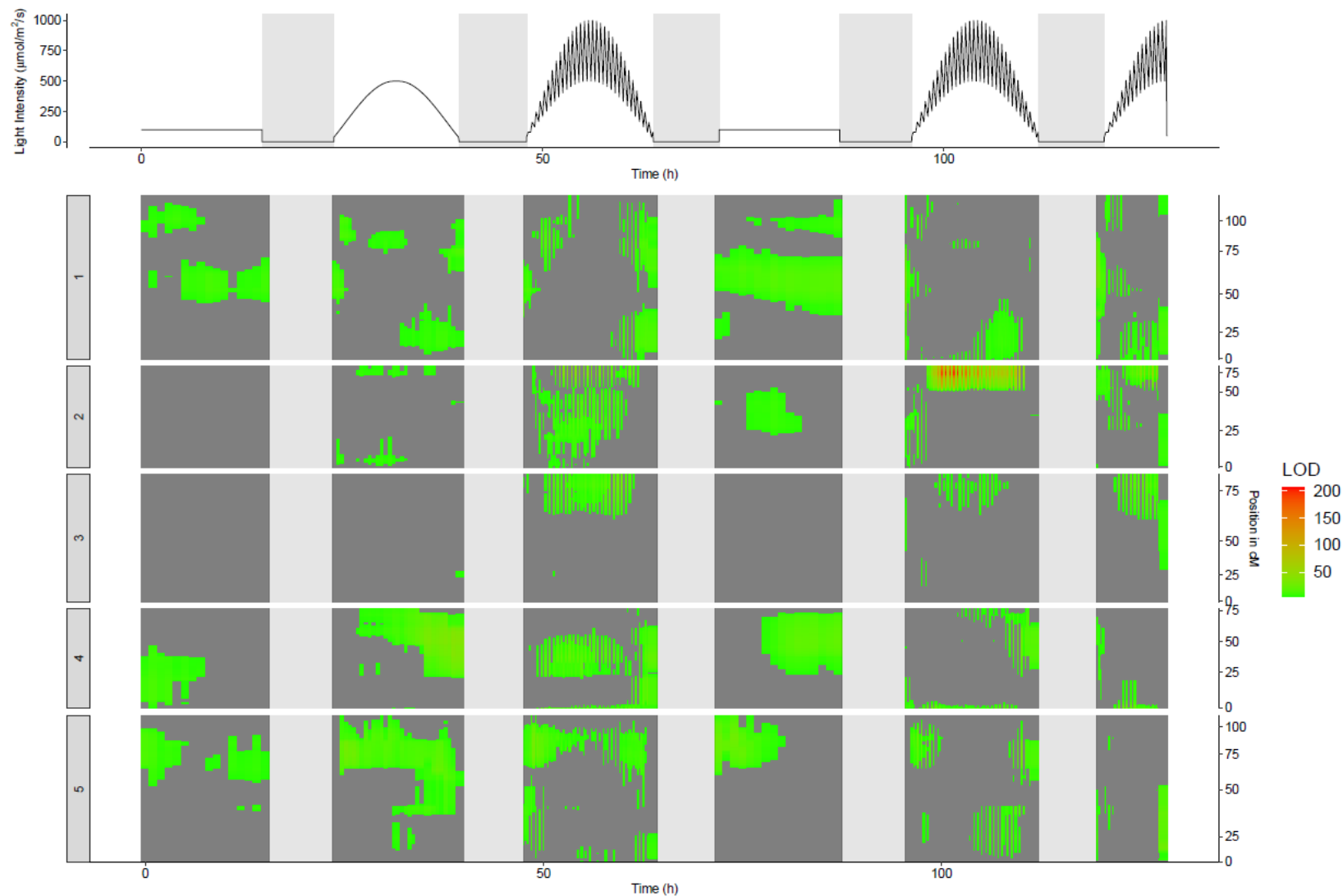


Figure 4 QTL mapping of NPQ by use of DH lines of a cross between C-C and E-C. The lines were phenotyped in the DEPI (Dynamic Environmental Photosynthetic Imager) chambers at Michigan State University. On the x-axis time in hours is shown. (a) Fluctuating light intensity during measurements in  $\mu\text{mol/m}^2/\text{s}$ . Measurements have been performed over 5 and half a day, grey areas are the nights when the light is turned off. During the days of fluctuating light, blocks of 20 minutes low light are alternated with 10 minutes of high light. (b) On the left y-axis the 5 different chromosomes are shown, on the right y-axis the position on the chromosome is shown in cM. In the legend the LOD score based on Multiple QTL Mapping (MQM) is shown. Grey areas are positions that do not show a significantly different phenotype between the Col and Ely nucleotype. (Theeuwes and Logie, Unpublished, 2019).

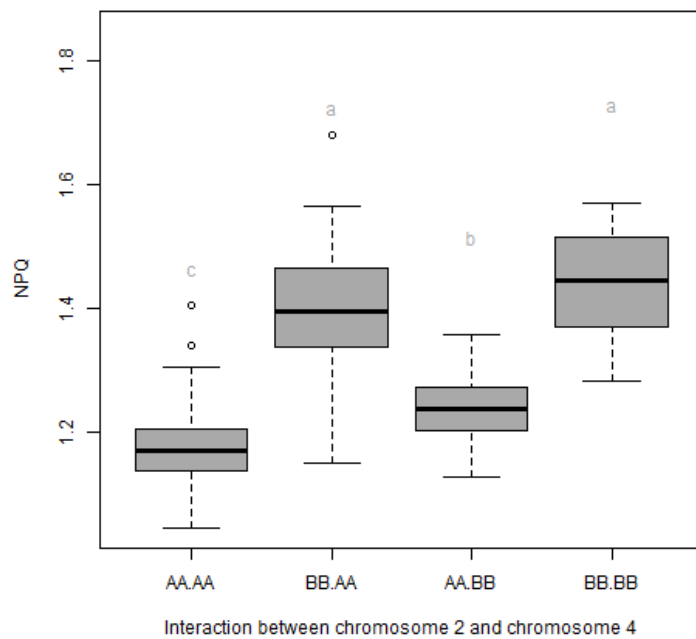


Figure 5 NPQ data for QTL on chromosome 2 and chromosome 4. The first two letters show the alleles for chromosome 2, the second two letters show the alleles for chromosome 4. AA means homozygosity for the Col allele, BB means homozygosity for the Ely allele. Significance differences are shown by different letters, tested with Tukey. Both QTLs show an increase in NPQ, as well as a significant interaction ( $p < 0.0001$ ) between the two QTLs. (Theeuwes, unpublished data).

## Aim

The main aim of this thesis is fine mapping of the NPQ QTL on chromosome 2 and to produce a NIL to study the physiology behind the QTL. By doing this, the natural variation present between the Ely and Col is used to unravel one of the mechanisms of NPQ. Fine mapping was performed to identify the gene in the QTL on chromosome 2 that is responsible for the high NPQ phenotype of the Ely nucleotype. The size of the QTL was reduced by extending the QTL analysis with lines that were previously not phenotyped and by fine mapping. The QTL was expected to be reduced to 10 kb, based on the number of recombinants expected for the 3,000 plants that were screened. The candidate genes identified in the QTL region were analysed for genomic differences between Ely and Col with Illumina and Oxford Nanopore Technology (ONT) sequencing data. Four differentially expressed genes between Ely and Col in the original defined QTL region on chromosome 2 were analysed for involvement in NPQ by allelic complementation. One of the genes, CPFTSY, is a likely candidate as this gene was previously shown to be involved in NPQ. At the same time, the dominant allele for the QTL on chromosome 2 was determined to study the phenotypic effect of the genetic inheritance of the Col and Ely allele. Besides the genetics, the physiological effect of the QTL on chromosome 2 was researched. The QTL was expected to be involved in NPQ relaxation, because the QTL also appeared in the QTL analysis of the fast mechanism of NPQ, qE. To study the physiology of the NPQ gene on chromosome 2, I produced a near isogenic line (NIL) with the QTL on chromosome 2 as an Ely introgression in a background of Col. The effect of different light treatments on both NPQ as well as biomass were analysed. In all of this we want to generate an overall insight in how to break down the complexity of photosynthesis phenotypes and the effect of the underlying genetics. To do so, want to investigate the potential of a NIL in breaking down this complexity.

## Material and methods

### High throughput phenotyping platforms

#### Dynamic environmental photosynthesis imager (DEPI)

The dynamic environmental photosynthesis imager is located at Michigan State University, United States of America (Cruz et al., 2016). It is a growth cabinet in which a dynamic growing environment can be programmed. High light intensity up to 15,000  $\mu\text{mol}/\text{m}^2/\text{s}$  can be emitted by the LEDs in the ceiling. The LEDs can fluctuate light up to every 0.5 second. Cameras can monitor photosynthesis parameters and growth-related phenotypes over time. All plants can be measured by the multiple cameras at the exact same time. It is also possible to emit near-infrared wavelengths.

#### Robin PSI PlantScreen™ system (Robin)

The Robin is a stand-alone phenotyping system from Photon Systems Instruments, Czech Republic (<https://plantphenotyping.com/products/plantscreen-sc-system/#details>). Trays that fit 20 *A. thaliana* plants are manually placed in the system and all plants can be measured in one time. Photosynthesis and growth parameters can be measured with cameras in the ceiling. Actinic as well as far-red light can be applied, and cameras can measure different fluorescence parameters. The system is designed for minute like measurements but measuring protocols can be adjusted for longer measuring regimes.

### Analysis of data from DEPI

#### Including late flowering lines in the QTL analysis to define the size of the QTL

##### Plant material

All 450 lines from the DH population were used for the experiment.

##### Statistical analysis

The photosynthesis phenotypes were calculated from different fluorescence parameters (Fluorescence measurements Robin, page 29). The best linear unbiased estimates (BLUEs) of these parameters were calculated by maximum likelihood (ML). With the R/qtl package, a scanone and a MQM analysis were performed for QTL mapping of 2533 different phenotypes (FvFm at 6 time points, growth, NPQ, NPQt,  $\Phi\text{PSII}$ ,  $\Phi\text{NO}$ ,  $\Phi\text{NPQ}$ , qE, qEt, qL at 231 time points and qI and qIt at 224 time points). 478 genetic markers were used with a distance between two markers of 250 kb. For the MQM analysis a co-factor was included every 3 markers. The LOD threshold was calculated with a Bonferroni correction with a multiple correction of 3,011, which is the sum of the number of phenotypes and markers.

A qualitative analysis of NPQ was performed, by plotting lines that recombine in the region of the QTL next to their NPQ values.

#### Epistatic interaction by scantwo

Epistatic interactions between different genotypic positions were examined by a scantwo analysis from the R-package R/qtl which was performed for NPQ on time point 101.5214. The EM algorithm was used as a method to calculate the MLE of the different photosynthesis parameters.

### NPQ relaxation and induction

##### Plant material

Three different *A. thaliana* genotypes were used C-C (Col nucleotype and plasmotype), E-C (Ely nucleotype and Col plasmotypes) and DH6.

## Replications

For each growing condition 8 completely randomised blocks (CRBs) of 20 plants each were grown, of which 6 blocks were used for the analysis. The blocks of plants grown in the climate chamber contained 10 C-C and 10 E-C replications. The blocks in the tunnel contained 8 C-C, 8 E-C and 4 DH6 replications.

## Growing conditions

Seeds were pre-sowed on a petri dish with filter paper and put in the cold room at 4 °C for 3 days, to ensure homogeneous germination. The seeds were transferred to the tissue culture and sown after 1 day.

*A. thaliana* was grown in two different environments: (1) climate chamber and (2) a tunnel outside.

- (1) Seeds were sown on rockwool blocks in a climate chamber at a light intensity of 200  $\mu\text{mol}/\text{m}^2/\text{s}$ . The day length was 16 hours, day temperature was 20 °C and night temperature was 18 °C. Humidity was 70%. Plants were watered two times a week with Hyponex for 10 minutes.
- (2) Seeds were sown in pots with a mixture of 50% soil and 50% sand. To be assured of proper germination and development of at least one plant, 3 seeds were sown in the pots outside. To prevent for border effects, which were shown in earlier experiments in the tunnel and probably caused by uneven watering, a border of C-C plants was included. Outside weather conditions influenced the growing condition in the tunnel because no extra light treatment was applied. Watering was only done in dry periods. The plants were grown from 24-09-2020 to 14-01-2021 at 51°59'22.7"N and 5°39'41.6"E.

## Robin measurement

Fluorescence parameters were measured in the Robin after 20 days for plant grown in the climate chamber and after 56 days for plants grown in the tunnel.

NPQ relaxation and induction of the 6 blocks from each environment were measured every 30 seconds at different time intervals after turning from high to low light and vice versa. The measurement protocol can be found in the appendix (1. Robin measuring protocols, 1: NPQ relaxation and induction). To be able to correct for differences between the blocks, NPQ was measured during the last high light period before the measurements. The measurement protocol can be found in the appendix (1. Robin measuring protocols, 2: 12 cycles of alternating high and low light intensity).

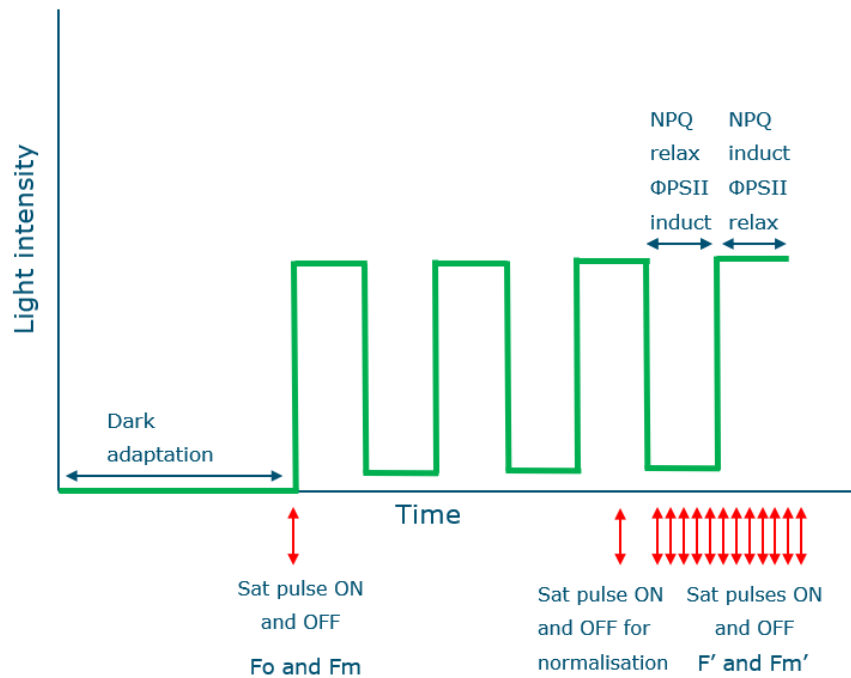


Figure 6 Measurement protocol Robin. After a dark adaptation period of 30 minutes,  $F_o$  and  $F_m$  were calculated by 8 measurements, respectively before and after a saturating light pulse. High and low light intensity, respectively  $1000 \mu\text{mol}/\text{m}^2/\text{s}$  and  $100 \mu\text{mol}/\text{m}^2/\text{s}$  were alternated to trigger NPQ with time intervals of 5 min. In the third high light period, a saturating light pulse was applied for NPQ normalisation, to correct for differences between blocks that are measured at different time intervals. During NPQ relaxation and induction,  $F'$  and  $F_m'$  were measured every 30 seconds for a period of 5 minutes.

### Statistical analysis

The data generated in the experiment consists of many fluorescence measurements. Multiple measurements, which were performed rapidly after each other were used to calculate the average value of a parameter. An R-script was used to calculate the different fluorescence parameters,  $F_o$ ,  $F_m$  and multiple  $F_o'$  and  $F_m'$  parameters. These parameters were used to calculate the photosynthesis phenotypes (Fluorescence measurements Robin, page 29). The data was analysed for each growing environment separately.

A linear model (LM) was used to calculate the least square estimators (LSE) of the genotypes:

$$\text{Photosynthesis phenotype} = \text{Genotype} + \text{Normalised NPQ} + \varepsilon$$

Compensation for differences between blocks was done by including the NPQ normalisation measurement that was applied just before changing from high light to low light as a regression parameter. A Tukey post hoc test was used to compare the different genotypes with  $\alpha = 0.05$ .

### Effect of different growing environments on dry weight

#### Plant material

Three different *A. thaliana* genotypes were used C-C, E-C and DH6. For the plants grown in the tunnel, the plants are the same as for the previous experiment of NPQ induction and relaxation.

#### Replications

For each growing condition 8 CRBs of 20 plants each were grown, of which 6 blocks were used for the analysis. The blocks contained 8 C-C, 8 E-C and 4 DH6 replications.

### Growing conditions

Seeds were pre-sown on a petri dish with filter paper and put in the cold room at 4 °C for 3 days, to ensure homogeneous germination. The seeds were transferred to the tissue culture and sown after 1 day.

*A. thaliana* was grown in two different environments: (1) climate chamber, (2) greenhouse and (3) a tunnel outside.

- (1) Seeds were sown on rockwool blocks in a climate chamber at a light intensity of 200  $\mu\text{mol}/\text{m}^2/\text{s}$ . The day length was 16 hours, day temperature was 20 °C and night temperature was 18 °C. Humidity was 70%. Plants were watered two times a week with Hyponex for 10 minutes.
- (2) Seeds were sown on rockwool blocks in the greenhouse at a light intensity of minimal 125  $\mu\text{mol}/\text{m}^2/\text{s}$ . Light intensity depended on the intensity of sunlight. The day length was 16 hours, day temperature was 20 °C and night temperature was 18 °C. Humidity was 70%. Plants were watered approximately 3 times a week with Hyponex.
- (3) Seeds were sown in pots with a mixture of 50% soil and 50% sand. To be assured of proper germination and development of at least one plant, 3 seeds were sown in the pots outside. To prevent for border effects, which were shown in earlier experiments in the tunnel and probably caused by uneven watering, a border of C-C plants was included. Outside weather conditions influenced the growing condition in the tunnel because no extra light treatment was applied. Watering was only done in dry periods. For weather data: plants were grown from 24-09-2020 to 14-01-2021 at 51°59'22.7"N and 5°39'41.6"E.

### Dry weight

Dry weight of the shoots was harvested after 22 days for the climate chamber and greenhouse and after 112 days for the tunnel. The shoots were harvested and put in a stove at 60 °C. The dry weight was measured with an analytical balance.

### Statistical analysis

Dry weight was analysed by a Linear Mixed Model (LMM), using blocks as a random effect and calculating BLUEs of the different genotypes with REML.

$$\text{Dry weight} = \text{Genotype} + (1|\text{Block}) + \varepsilon$$

### Development of NILs

Prior to this work a DH population was generated by a cross between C-C and E-C. One of the DH lines was used to produce the NILs. NILs were produced with the Ely QTL in a C-C background. The strategy used to develop the NIL was to select a DH line (DH6) with the Ely genotype for the QTL on chromosome 2 and mostly Col genotype for the other positions. The DH line was backcrossed to C-C until only the introgression of the QTL was an Ely genotype. After selfing, homozygous lines were selected, and different NILs were obtained.

### Genotypic analysis

KASP markers were used to assess from which parent the chromosome segments were inherited. For each SNP, 2 forward (one for each parent) and 1 backward primer were developed. An overview of all KASP primers used in this thesis can be found in the appendix (S 1).

**Plant material**

DH6 is the line that was selected to produce a NIL of the chromosome 2 QTL. This line only contains two C-C chromosome segments (start of chromosome 1 and 2) next to the segment of the QTL of interest. C-C was used for backcrossing. After the first generation, the progeny of the crosses was used.

**Growing conditions**

Seeds were pre-sowed on a petri dish with filter paper and put in the cold room at 4 °C for 2 days, to ensure homogeneous germination. The seeds were transferred to the tissue culture and after 1 day sowed on rock wool blocks.

Plants were grown in the greenhouse at a light intensity of minimal 125  $\mu\text{mol}/\text{m}^2/\text{s}$ . Light intensity depended on the intensity of sunlight. The day length was 16 hours, day temperature was 20 °C and night temperature was 18 °C. Humidity was 70%. Plants were watered approximately 3 times a week with Hyponex.

**Crossing scheme**

The following crossing scheme was used to develop the NILs (Figure 7). In the F1 the plants were checked to be true F1s. In the F2 lines were selected that were heterozygous for the QTL region and homozygous C-C for the rest of the genome. In the F3, 523 NILs were found, which was a bit less than expected based on mendelian segregation. In this generation recombinants were also selected. In a region of 250 kb, 57 recombinants were obtained. The progeny of these recombinants was screened for homozygosity for the recombination.

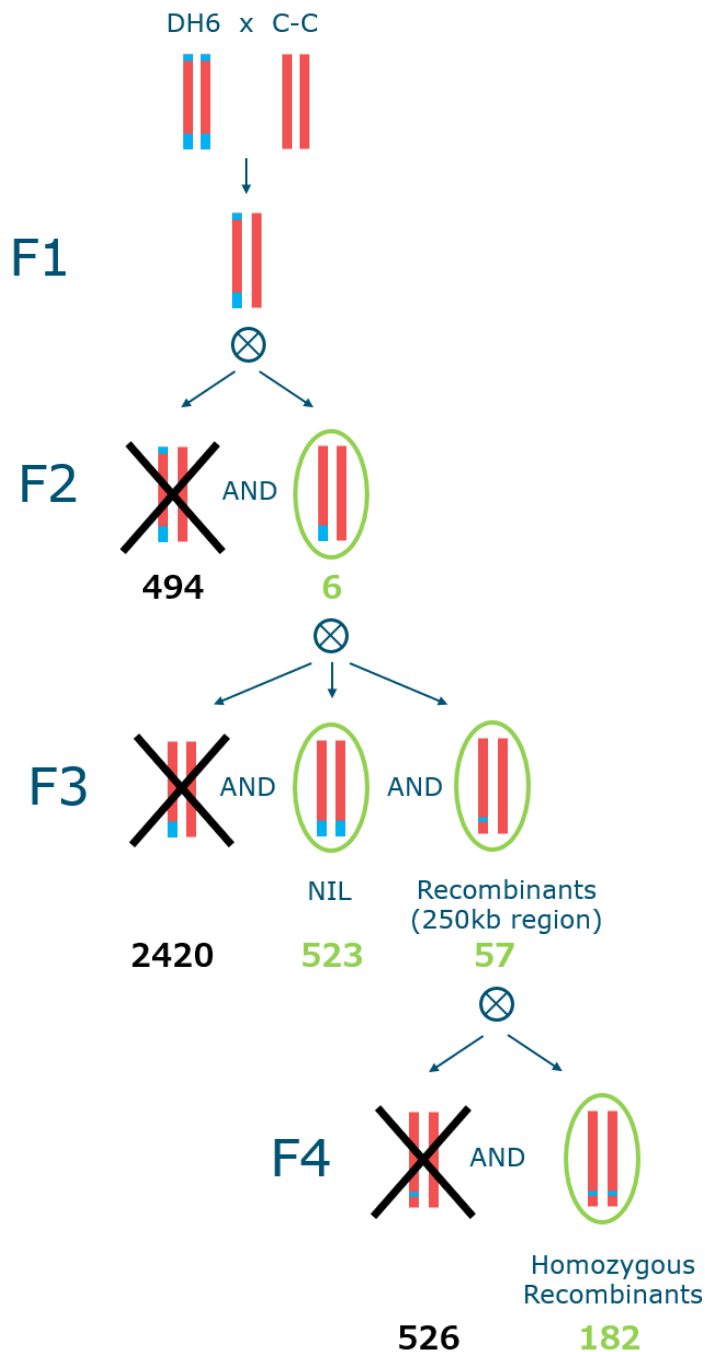


Figure 7 Crossing scheme of development of NILs. Chromosomes represent chromosome number 2. Other chromosomes were left out of the scheme for simplification. Numbers indicate the number of plants; plants of green numbers were selected for the next generation or for physiological experiments.

## Dominance of the alleles

### Plant material

The lines measured were C-C, F1 (from Col x Ely), E-C, NIL, hetero NIL (heterozygous for the introgression in a C-C background), DH6 and DH6 x C-C. By comparing C-C, the NIL and the hetero NIL or by comparing C-C, DH6 and DH6 x C-C it can be concluded what the effect is of both alleles.

### Growing conditions

Seeds were pre-sowed on a petri dish with filter paper and put in the cold room at 4 °C for 2 days, to ensure homogeneous germination. The seeds were transferred to the tissue culture and after 1 day sowed on rock wool blocks.

Plants were grown in the greenhouse at a light intensity of minimal 125  $\mu\text{mol}/\text{m}^2/\text{s}$ . Light intensity depended on the intensity of sunlight. The day length was 16 hours, day temperature was 20 °C and night temperature was 18 °C. Humidity was 70%. Plants were watered approximately 3 times a week with Hyponex.

17 days after sowing, the plants were transferred to the Fluctor. Explanation of the Fluctor can be found in The Fluctor (page 21) Growing conditions are stated in the experiment with the NILs (NILs in the Fluctor, page 28).

### Replications

Seven different CRBs of 20 plants each were measured. All genotypes were represented three times, except for the F1 (Col x Ely), which was represented twice.

### Robin measurement

Fluorescence parameters were measured in the Robin after 21 days.

The measuring protocol that used had a duration of 30 minutes with 5-minute alterations of high and low light. High light intensity was 1000  $\mu\text{mol}/\text{m}^2/\text{s}$  and low light was either 100 or 500  $\mu\text{mol}/\text{m}^2/\text{s}$ . After each 5-minute period, a far-red period of 20 seconds was applied to be able to calculate NPQt,  $\Phi\text{NOt}$ ,  $\Phi\text{NPQt}$ , qEt and qIt. The measurement protocol can be found in the appendix (1. Robin measuring protocols, 2: 12 cycles of alternating high and low light intensity).

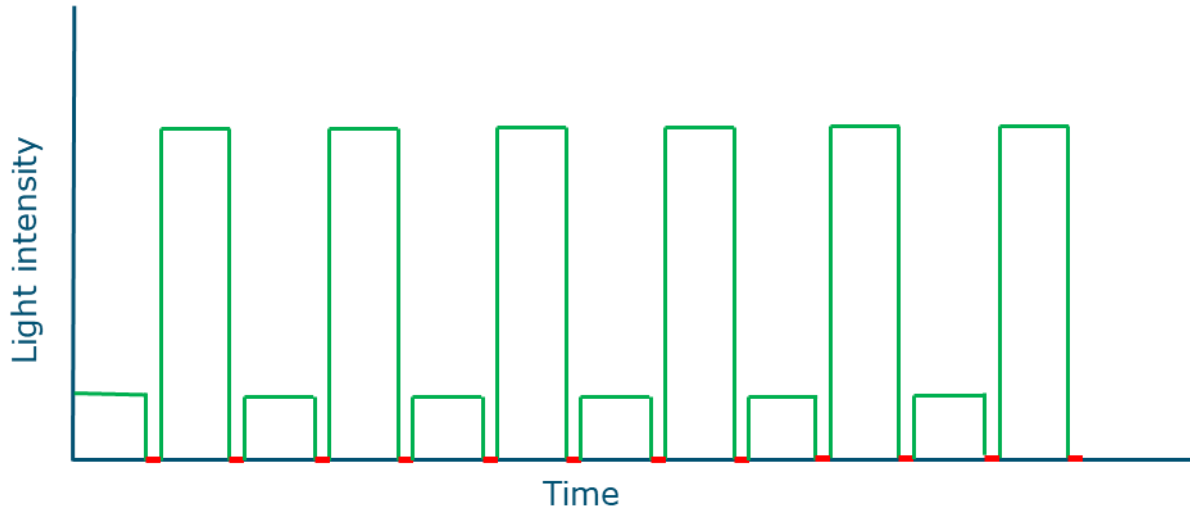


Figure 8 Robin measuring protocol for determining the dominant allele. 12 cycles of 5-minute alternations of low and high light. High light intensity was 1000  $\mu\text{mol}/\text{m}^2/\text{s}$  and low light was either 100  $\mu\text{mol}/\text{m}^2/\text{s}$ . A far red period was applied after each 5-minute period to be able to calculate NPQt,  $\Phi\text{NOt}$ ,  $\Phi\text{NPQt}$ , qEt and qIt.

### Statistical analysis

An R-script was used to calculate the different fluorescence parameters,  $F_o'$ ,  $F_m'$ ,  $F'$  and  $F_m''$ . From these parameters NPQt,  $\Phi\text{NOt}$ ,  $\Phi\text{NPQt}$ ,  $\Phi\text{PSII}$ , qEt and qIt were calculated (Fluorescence measurements Robin, page 29). An LMM was used to calculate the MLE of the photosynthesis parameters. The block number was used as a random term, to correct for the dependency of measurements on the block. A Tukey post hoc test was used to compare the different genotypes.

$$\text{Photosynthesis phenotype} = \text{Genotype} + (1|\text{Block}) + \varepsilon$$

## Reproduce DEPI phenotype

### Plant material

The genotypes that were used in the experiment are C-C, E-C and DH6.

### Growing conditions

Seeds were pre-sowed on a petri dish with filter paper and put in the cold room at 4 °C for 3 days, to ensure homogeneous germination. The seeds were transferred to the tissue culture and after 1 day sowed on rock wool blocks.

Plants were grown in a climate chamber at a light intensity of 200  $\mu\text{mol}/\text{m}^2/\text{s}$ . The day length was 16 hours, day temperature was 20 °C and night temperature was 18 °C. Humidity was 70%. Plants were watered two times a week with Hyponex for 10 minutes.

### Replications

Seven replicates of C-C and DH6 were used and six replicates of E-C in one CRB of 20 plants.

### Robin measurement

Fluorescence parameters were measured in the Robin after 19 days.

The third, fourth and fifth day of the DEPI treatment were simulated with two extra fluctuating days (Figure 9). The fluctuation consists of alternating high and low light periods in which the high light period lasts for 10 minutes and the low light period for 20 minutes. After each period, a far-red period was applied to be able to calculate NPQt,  $\Phi\text{NOt}$ ,  $\Phi\text{NPQt}$ , qEt and qIt. At the start of each day  $F_o$  and  $F_m$  were measured. For the measurement, many different protocols were used. The protocol for the FvFm measurement at the start of the day and the first protocol can be found in the appendix (2. Robin measuring protocols, 3: FvFm measurement DEPI treatment and 4: First 3 cycles of the DEPI treatment). The other protocols only differ in light intensity values.

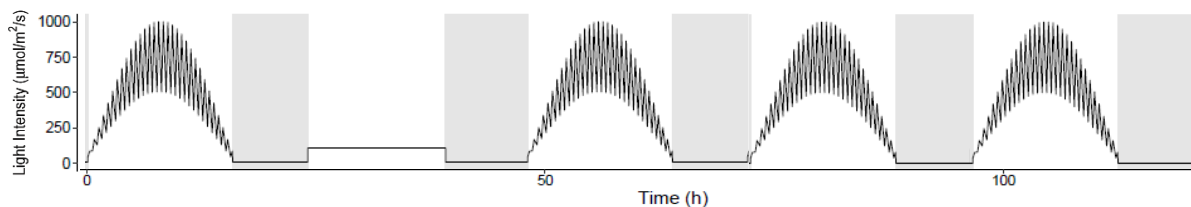


Figure 9 Light treatment during measurement in de Robin to reproduce the DEPI phenotype.

### Statistical analysis

The photosynthesis phenotypes were calculated from different fluorescence parameters (Fluorescence measurements Robin, page 29). A LM was used to calculate the LSE of the genotypes for each photosynthesis parameter. A Tukey post hoc test was used to compare the different genotypes.

$$\text{Photosynthesis phenotype} = \text{Genotype} + \varepsilon$$

## The Fluctor

The Fluctor is a climate chamber in which three compartments are separated and each has their own light treatment (Figure 10):

1. DEPI treatment: simulation of the light treatment used in DEPI during a fluctuating day (Figure 11a). Light intensity values can be found in the appendix (S 2). Low light periods were 20 minutes long and high light periods 10 minutes. The average light intensity was  $438 \mu\text{mol}/\text{m}^2/\text{s}$ .
2. Constant: a constant light intensity which is the average light intensity of the DEPI system ( $438 \mu\text{mol}/\text{m}^2/\text{s}$ ).
3. Maize: light intensity measured inside a maize canopy of a day with many fluctuations, during summer (Figure 11b). A light sensor was attached to one of the leaves to measure the effect of both shading of leaves and clouds. Average light intensity was  $418 \mu\text{mol}/\text{m}^2/\text{s}$

The plants were on a 16-hour day rhythm.

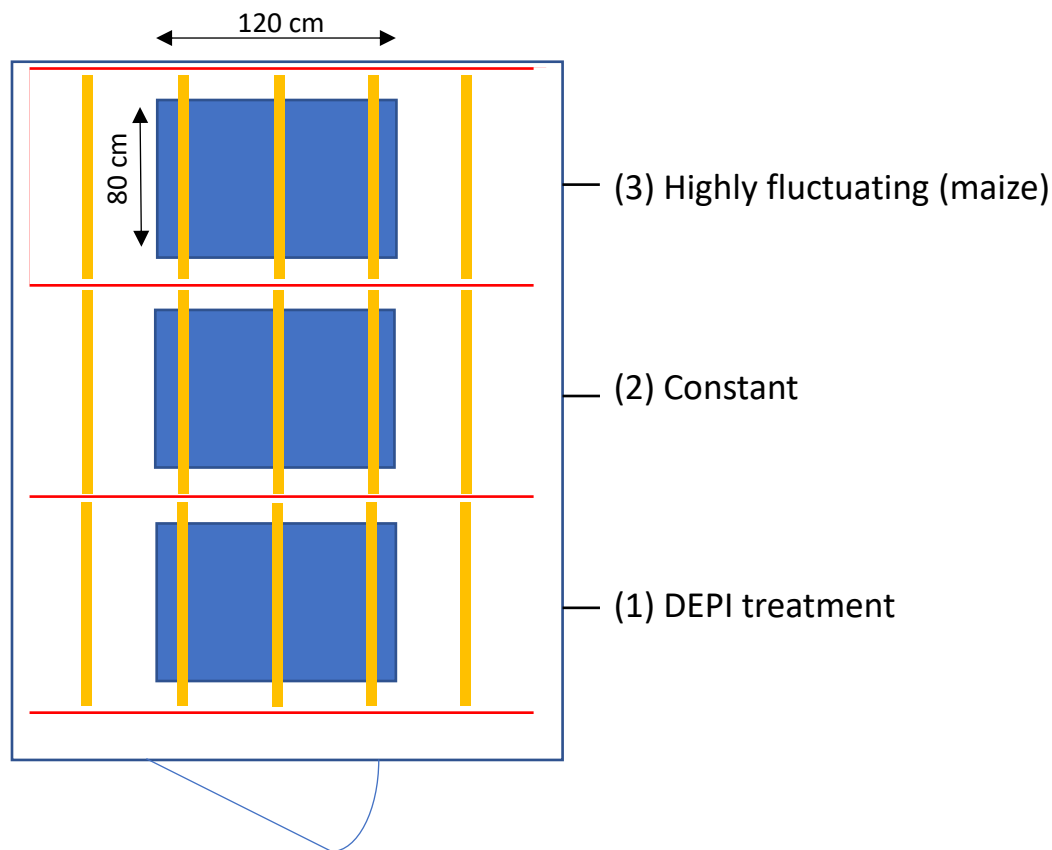


Figure 10 Set up of the Fluctor in the climate chamber. Three different light treatments that were separated from each other by white plastic (red lines). The yellow lines are the different LEDs. In each treatment the growing area of plants is 80 cm x 120 cm.

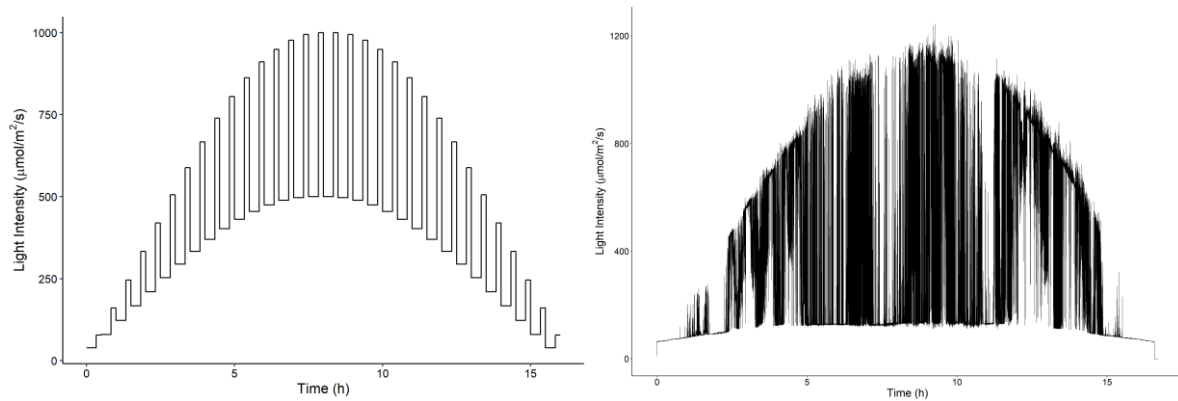


Figure 11 Light intensity for a) the DEPI treatment and b) the maize treatment.

## Programming

The five different LED modules (Fluence VYPR modules) of one light treatment used the same power supply. The light intensity of the LEDs was determined by the current that flows through the LED circuit (Figure 13). If the circuit of the LEDs is fully closed (maximum flow of current), the LEDs are fully dimmed. The closure of the circuit is controlled by an optocoupler, which has a small LED inside. If this LED is on, the circuit on the other side (of the LED) will be closed and the LEDs will be dimmed. This optocoupler can switch on and off in very short time. The ratio of on/off will determine the current flow through the circuit. An ESP 32 pyboard is able to control this current by a variable duty cycle (Pulse With Modulation, PWM). Two pins on the pyboard, the ground (GND) and one of the General Purpose Input/Outputs (GPIOs) control the current by changing the duty cycle very quickly (Figure 12). Every LED is connected to a different GPIO.

The reason that the optocoupler is in between both circuits and that the LEDs are not directly connected to the ESP32 is because the power supply of the LEDs and the ESP32 is different. The ESP32 works with a potential difference of 3.3 V, and the potential difference of the LEDs is 10V. Connecting the LEDs directly to the ESP32 could potentially damage the LEDs.

The current flow through the circuits and therefore the light intensity of the LEDs is controlled by the ESP32. A python script on the EPS32 determines the PWM values of the duty cycle over time. By changing this PWM value very fast, highly fluctuating light intensities can be programmed. The scrips for the different can be found in the appendix (2. Python scripts for Fluctor).

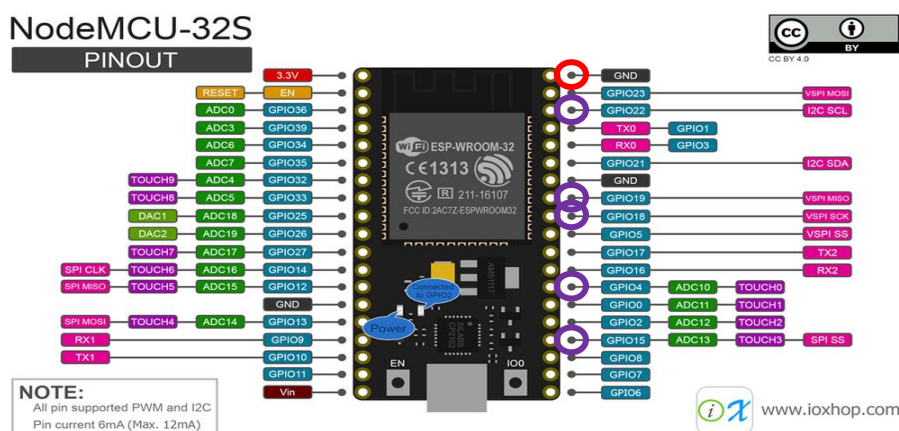


Figure 12 The ESP32 that was used to programme the lights of the Fluctor. In the red circle the ground pin is shown. This pin is used to close the circuit of all lamps. The pins with the purple circles indicate the different GPIOs that were used for dimming the different lamps.

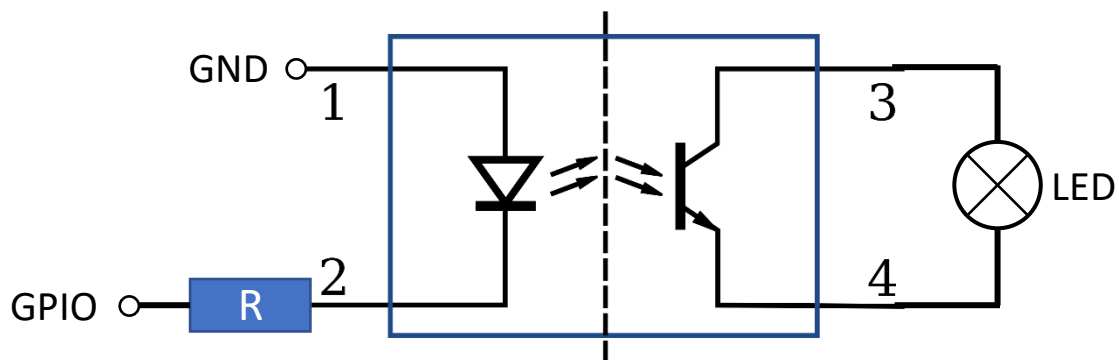


Figure 13 The circuit between the fluence modules and the ESP32. GND is the ground and GPIO is the General Purpose Input/Output. In the blue square the optocoupler is indicated with the LED that transfers the electrical signal between the two closed circuits. Each optocoupler is connected to a different fluence module and a different GPIO, but to the same ground. The  $R$  is a resistance.

### Calibration of fluence modules

The Fluence modules are programmed by PWM values that dim the Fluence modules with a value from 0 to 1023. A PWM value of 0 means that the fluence modules are on full capacity and 1023 means that the fluence modules are turned off. To convert light intensity to PWM values, light intensity was measured at different PWM values. By plotting these values, a conversion formula was calculated (Figure 14). At very high PWM values, the fluence modules were turned off. Light intensities of below  $48 \mu\text{mol}/\text{m}^2/\text{s}$  were therefore not possible to program.

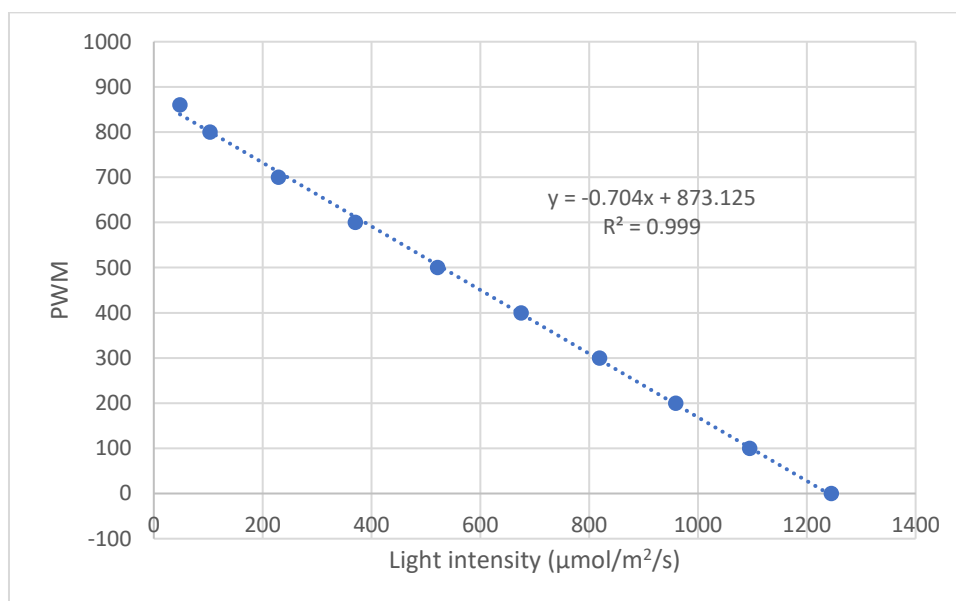


Figure 14 Calibration of fluence modules. The light intensity was measured at different PWM values.

To make sure that the light distribution was homogeneous over the whole growing area, the light intensity was measured at different positions in the growing area. Because each treatment contained five Fluence modules, the modules were distributed at positions which made sure that light intensities over the whole growing area differed maximally 5%.

## Fine mapping

### Plant material

The homozygous recombinants produced during the development of the NILs were phenotyped. In a region of 300 kb, 57 recombinants were found. Before the recombinants were phenotyped, the QTL region was already reduced to 50 kb. In this region, 17 recombinants were found. It was decided to phenotype these 17 lines and to include a few lines outside this region to verify the correctness of the reduction of the QTL.

16 days after sowing, the plants were sampled for KASP genotyping.

### Plant material

The genotypes that were used in the experiment are C-C, E-C and DH6.

### Growing conditions

Seeds were pre-sowed on a petri dish with filter paper and put in the cold room at 4 °C for 2 days, to ensure homogeneous germination. The seeds were transferred to the tissue culture and after 1 day sowed on rock wool blocks.

Plants were grown in the greenhouse at a light intensity of minimal 125  $\mu\text{mol}/\text{m}^2/\text{s}$ . Light intensity depended on the intensity of sunlight. The day length was 16 hours, day temperature was 20 °C and night temperature was 18 °C. Humidity was 70%. Plants were watered approximately 3 times a week with Hyponex.

17 days after sowing, the plants were transferred to the Fluctuator. Growing conditions are stated in the experiment with the NILs (NILs in the Fluctuator).

### Robin measurement

Fluorescence parameters were measured in the Robin after 19 days.

The NPQ phenotype of the NIL could not be triggered by the measurement protocol that was used in the Robin. Therefore, the recombinants were put in the Fluctuator for 1.5 days before phenotyping. In the Robin, NPQt,  $\Phi\text{PSII}$ ,  $\Phi\text{NOt}$ ,  $\Phi\text{NPQt}$ , qEt and qIt were measured.

The measuring protocol that used had a duration of 30 minutes with 5-minute alterations of high and low light (Figure 15). High light intensity was 1000  $\mu\text{mol}/\text{m}^2/\text{s}$  and low light was either 100 or 500  $\mu\text{mol}/\text{m}^2/\text{s}$ . After each 5-minute period, a far-red period of 20 seconds was applied to be able to calculate NPQt,  $\Phi\text{NOt}$ ,  $\Phi\text{NPQt}$ , qEt and qIt. Measurement protocol can be found in the appendix (2. Robin measuring protocols, 5: First 6 cycles of alternating low and high light).

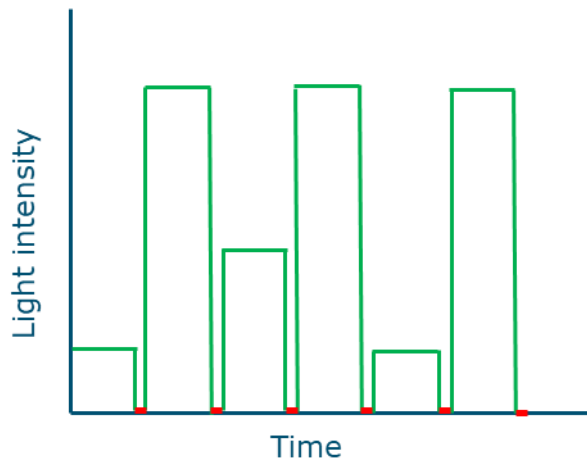


Figure 15 Robin measuring protocol for determining the dominant allele. 6 cycles of 5-minute alternations of low and high light. High light intensity was 1000  $\mu\text{mol}/\text{m}^2/\text{s}$  and low light was either 100 or 500  $\mu\text{mol}/\text{m}^2/\text{s}$ . A far red period was applied after each 5-minute period to be able to calculate NPQt,  $\Phi\text{NOt}$ ,  $\Phi\text{NPQt}$ , qEt and qIt.

### Statistical analysis

The photosynthesis phenotypes were calculated from different fluorescence parameters (Fluorescence measurements Robin, page 29). BLUEs for the different recombinants were calculated by a LMM that included the block effects as a random effect.

$$\text{Photosynthesis phenotype} = \text{Genotype} + (1|\text{Block}) + \epsilon$$

A Tukey post hoc test was used to compare the differences between genotypes, with  $\alpha = 0.05$ . The BLUEs were used in a MQM analysis by use of the R/qtl package, including 18 marker positions and a co-factor every 3 markers. The LOD threshold was calculated per phenotype and was based on a permutation test with 1000 permutations.

### Analysing SNP data in QTL region of fine mapping

The database that was used for analysing SNPs and INDELs between Col and Ely was a SnpEff database. The database was built with VCF files with differential variant calling and the functional effects of SNPs and INDELs were determined by the genome, transcriptome and proteome of TAIR10.1 (Flood et al., 2020). The VCF files were based on Illumina sequencing data. The SNPs and INDELs in the QTL region were selected, only if this modification was considered to have a moderate or high impact.

### Structural variation analysis

For this analysis, a *de novo* assembly was used in which scaffolds were built with contigs of Oxford Nanopore Technology (ONT) data. The Minlon data contains longer reads but is more error prone. The *de novo* assembly was improved with Illumina sequencing data. The Ely scaffold was plotted against the Col genotype of TAIR10.1 to visualise the structural differences. We zoomed in into the region of the QTL to check for structural differences.

## Mapping the Robin phenotype

### Plant material

13 different DH lines were selected for this experiment (Figure 16). This selection was based on that the lines were Col for the QTL of chromosome 2. Furthermore, a variation in Ely segments should be covered by the different DH lines. DH6 was also included as a control to check if the QTL on chromosome 2 was triggered by the measuring protocol. Both parents, C-C and E-C were also included. Eight CRBs, consisting of 20 plants were measured. These blocks all consisted of one replicate of each DH line and 3 C-C and 4 E-C plants.

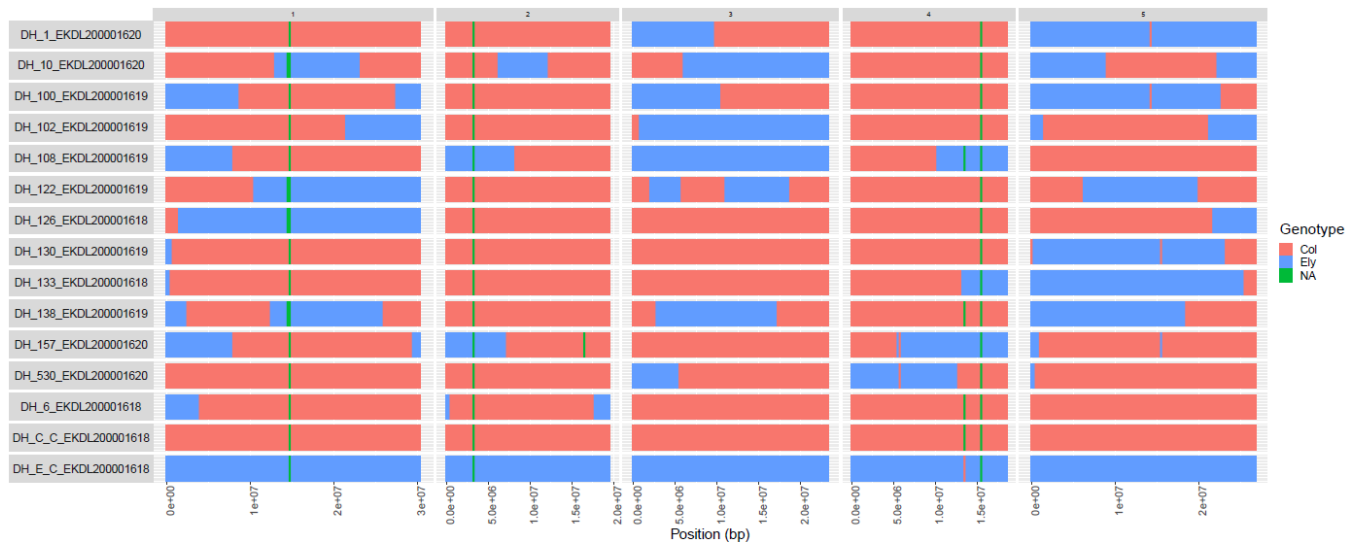


Figure 16 DH lines selected for the experiment to find the chromosome segment that was responsible for the high NPQt phenotype that was measured before. Blue segments are from Ely and red segments from Col.

### Growing conditions

Seeds were pre-sowed on a petri dish with filter paper and put in the cold room at 4 °C for 3 days, to ensure homogeneous germination. The seeds were transferred to the tissue culture and after 1 day sowed on rock wool blocks.

Plants were grown in the greenhouse at a light intensity of minimal 125  $\mu\text{mol}/\text{m}^2/\text{s}$ . Light intensity depended on the intensity of sunlight. The day length was 16 hours, day temperature was 20 °C and night temperature was 18 °C. Humidity was 70%. Plants were watered approximately 3 times a week with Hyponex.

### Robin measurement

Fluorescence parameters were measured in the Robin after 21 days.

The same measuring protocol was used as for the dominance experiment, which is a 60-minute protocol with 12 cycles of 5-minute alternating high and low light intensity (Figure 8Figure 15). The photosynthesis parameters that were calculated are NPQt,  $\Phi\text{PSII}$ ,  $\Phi\text{NOt}$ ,  $\Phi\text{NPQt}$ , qEt and qIt. The measurement protocol can be found in the appendix (2. Robin measuring protocols, 2: 12 cycles of alternating high and low light intensity).

### Statistical analysis

The photosynthesis phenotypes were calculated from different fluorescence parameters (Fluorescence measurements Robin, page 29). BLUEs for the different lines were calculated by an LMM that included the block effects as a random effect.

$$\text{Photosynthesis phenotype} = \text{Genotype} + (1|\text{Block}) + \varepsilon$$

A Tukey post hoc test was used to compare the differences between genotypes, with  $\alpha = 0.05$ . These BLUEs were used in a scanone analysis of the R/qtl package, including 478 markers with an interval of 250 kb. The LOD threshold was calculated per phenotype and was based on a permutation test with 1000 permutations.

## Allelic complementation

### Plant material

Four different T-DNA lines were selected because of differential expression, based on RNAseq data. The four different T-DNA lines were knock-out lines of CPFTSY, DUF295, CAPRICE and CYCLIC NUCLEOTIDE-GATED CHANNEL 12 (CNGC12).

By crossing a NIL of the QTL with each T-DNA line, lines were obtained with one knock-out allele of the investigated gene and one allele from Ely. The T-DNA line was also crossed to Col which resulted in lines with one knock-out allele and one Col allele. Next to these lines, the T-DNA line, C-C, E-C, NIL and hetero NIL were measured. 24 CRBs of 20 plants were used in this experiment. Each block contained two plants of C-C, E-C, NIL and hetero NIL and one plant of each T-DNA line and the crosses (T-DNA x Col and T-DNA x NIL).

### Growing conditions

Seeds were pre-sowed on a petri dish with filter paper and put in the cold room at 4 °C for 3 days, to ensure homogeneous germination. The seeds were transferred to the tissue culture and after 1 day sowed on rock wool blocks.

Plants were grown in a climate chamber at a light intensity of 200  $\mu\text{mol}/\text{m}^2/\text{s}$ . The day length was 16 hours, day temperature was 20 °C and night temperature was 18 °C. Humidity was 70%. Plants were watered two times a week with Hyponex for 10 minutes.

20 days after sowing, the plants were transferred to the Fluctor. Growing conditions are stated in the experiment with the NILs (NILs in the Fluctor).

### Robin measurement

Fluorescence parameters were measured in the Robin after 23 or 24 days.

The blocks were put in the Fluctor for 2.5 or 3.5 days before phenotyping. NPQt,  $\Phi\text{PSII}$ ,  $\Phi\text{NOt}$ ,  $\Phi\text{NPQt}$ , qEt and qIt were measured with the same protocol as was used for fine mapping (Figure 15). This is a protocol with six cycles of alternating high and low light intensities. Measurement protocol can be found in the appendix (2. Robin measuring protocols, 5: First 6 cycles of alternating low and high light).

### Statistical analysis

The photosynthesis phenotypes were calculated from different fluorescence parameters (Fluorescence measurements Robin, page 29). BLUEs for the different lines were calculated by an LMM that included the block effects as a random effect.

$$\text{Photosynthesis phenotype} = \text{Genotype} + (1|\text{Block}) + \varepsilon$$

Two different analyses were performed:

1. For each T-DNA line separately; the T-DNA line, the cross with Col and the NIL, the NIL, the hetero NIL, C-C and E-C were compared. This analysis could conclude if the T-DNA line is a knockout line of the gene of interest.
2. C-C, E-C, the NIL and the hetero NIL were compared to be able to investigate which of the alleles is dominant.

For both analyses, a Tukey post hoc test was performed, with  $\alpha = 0.05$ .

## NILs in the Fluctor

### Plant material

In each treatment three genotypes were grown; C-C, E-C and the NIL. In total 12 CRBs with 7 C-C, 7 NILs and 6 E-C plants were measured, 4 in each growing environment.

### Growing conditions

Seeds were pre-sowed on a petri dish with filter paper and put in the cold room at 4 °C for 2 days, to ensure homogeneous germination. The seeds were transferred to the tissue culture and after 1 day sowed on rock wool blocks.

Plants were sown in a climate chamber with three different light treatments:

1. DEPI treatment: simulation of the light treatment used in DEPI during a fluctuating day. Light intensity values can be found in the appendix (S 2). Low light periods were 20 minutes long and high light periods 10 minutes.
2. Constant: a constant light intensity which is the average light intensity of the DEPI system (438  $\mu\text{mol}/\text{m}^2/\text{s}$ ).
3. Maize: light intensity measured inside a maize canopy of a day with many fluctuations, during summer. A light sensor was attached to one of the leaves to measure the effect of both shading of leaves and clouds. Average light intensity was 418  $\mu\text{mol}/\text{m}^2/\text{s}$ .

The day length was 16 hours, day temperature was 20 °C and night temperature was 19 °C. Humidity was 70%. Plants were watered two times a week with Hyponex for 10 minutes.

### Robin measurement

Fluorescence parameters were measured in the Robin after 25 days.

NPQ<sub>t</sub>,  $\Phi\text{PSII}$ ,  $\Phi\text{NOt}$ ,  $\Phi\text{NPQt}$ , qEt and qIt were measured with the same protocol as was used for fine mapping (Figure 15). This is a protocol with six cycles of alternating high and low light intensities. Measurement protocol can be found in the appendix (2. Robin measuring protocols, 5: First 6 cycles of alternating low and high light).

### Dry weight

Dry weight was measured after 25 days. The shoots were harvested and put in a stove at 60 °C. The dry weight was measured with an analytical balance.

### Statistical analysis

The photosynthesis phenotypes were calculated from different fluorescence parameters (Fluorescence measurements Robin, page 29). BLUEs both the photosynthesis parameters as well as for the dry weight were calculated for the different genotypes for each growing environment

separately by a LMM model. The different blocks were used as a random effect to correct for dependency of observations.

$$\text{Photosynthesis phenotype} = \text{Genotype} + (1|\text{Block}) + \varepsilon$$

$$\text{Dry weight} = \text{Genotype} + (1|\text{Block}) + \varepsilon$$

Two different statistical tests were done, one that compared the different genotypes within a growing environment and one that included a genotype x growing environment interaction. For both analyses a Tukey post hoc test was performed, with  $\alpha = 0.05$ . The statistical differences for the interaction between genotype and environment were calculated with the following models:

$$\text{Phenotype} = \text{Genotype} + \text{Growing environment} + (\text{Genotype} * \text{Growing environment}) + \varepsilon$$

$$\text{Dry weight} = \text{Genotype} + \text{Growing environment} + (\text{Genotype} * \text{Growing environment}) + \varepsilon$$

#### Fluorescence measurements Robin

The parameters can be easily measured and consist of the wavelengths that are not used in PQ or NPQ and therefore re-emitted by the pigments (Baker, 2008). The different parameters are measured by switching a measuring and actinic light on and off and giving saturating light pulses (Figure 17). The measuring light is weak irradiance of  $<1 \mu\text{mol}/\text{m}^2/\text{s}$  and used to measure fluorescence of leaves in the dark.  $F_0$  is the measurement of a dark-adapted leave (about 20 minutes), at this moment the RC of PSII will be fully oxidized and able to receive new electrons. The dark-adapted leave will be exposed to a very short saturating light flash of about 1 second. By this flash, all RCs of PSII will be closed (fully reduced) and NPQ is not yet triggered. The measured fluorescence is the maximum fluorescence ( $F_m$ ) as there is absence of both photochemical quenching mechanisms. The actinic (photosynthetic) light will be turned on, photosynthesis is induced, and consequently NPQ will slowly start up and reduce fluorescence to a steady state ( $F'$ ). A new saturating light pulse will increase fluorescence ( $F_m'$ ). The RCs are fully reduced and the presence of NPQ results in the decreased value of  $F_m'$  compared to  $F_m$ . Subsequently, the actinic light will be turned off and  $F_0'$  can be measured. In the beginning, NPQ is still present and causes  $F_0'$  to be lower than  $F_0$ . Slowly, NPQ starts to relax and fluorescence yield will return to a steady state (Harbinson, 2013).

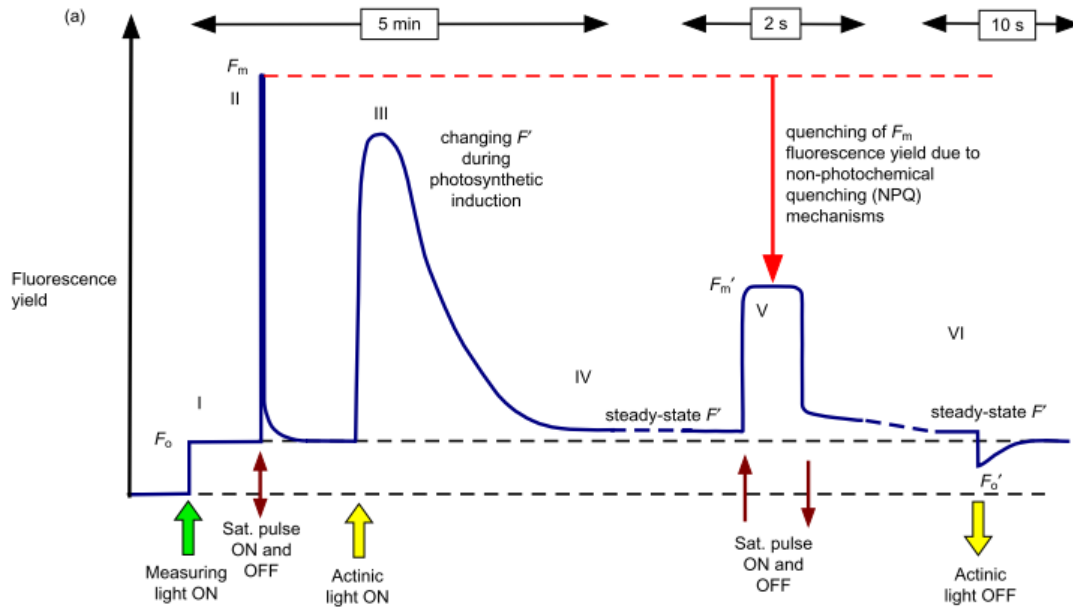


Figure 17 Measurement of fluorescence parameters (Harbinson, 2013).

To be able to separate NPQ in  $NPQ_F$  (fast NPQ, or  $q_E$ ) and  $NPQ_S$  (slow NPQ, or  $q_I$ ) another parameter  $F_m''$  should be calculated.  $F_m''$  can be measured with a saturating pulse after a period of far-red light. To calculate many of photosynthesis parameters, the measurement of  $F_m$  is necessary. This is measured after a period of dark adaptation. During long measurements it can be that the  $F_m$  measured in the beginning is not accurate in the end due to chloroplast and leave movement. Therefore, I might calculate  $NPQ_t$ ,  $\Phi NPQ_t$  and  $\Phi NO_t$  with the  $F_m''$  value.

The photosynthesis parameters  $\Phi PSII$ ,  $\Phi NPQ$ ,  $\Phi NO$ ,  $NPQ$ ,  $NPQ_F$  (or  $q_E$ ) and  $NPQ_S$  (or  $q_I$ ) can be calculated with the fluorescence parameters by the following equations (2, 3, 4, 5, 6 and 7) (Klughammer & Schreiber, 2008):

$$\Phi PSII = \frac{F_m' - F'}{F_m'} \quad (2)$$

$$\Phi NPQ = \frac{F'}{F_m'} - \frac{F'}{F_m} \quad (3)$$

$$\Phi NO = \frac{F'}{F_m} \quad (4)$$

$$NPQ = \frac{F_m - F_m'}{F_m'} \quad (5)$$

$$q_E = \frac{F_m}{F_m'} - \frac{F_m}{F_m''} \quad (6)$$

$$q_I = \frac{F_m - F_m''}{F_m''} \quad (7)$$

$$NPQ_t = \frac{-4.88}{\frac{F_m'}{F_o'} - 1} - 1 \quad (8)$$

$$q_L = \frac{F_m' - F'}{F_m' - F_o'} * \frac{F_o'}{F'} \quad (9)$$

$$\Phi NO_t = \frac{1}{NPQ_t + 1 + q_L * 4.88} \quad (10)$$

$$\Phi NPQ_t = 1 - \Phi NO_t + \Phi PSII \quad (11)$$

## Results

### Defining the size of the QTL on chromosome 2

The QTL analysis of different photosynthesis phenotypes on the biparental DH population was performed again because extra DH lines were measured in the DEPI system. These lines are late flowering lines that were not yet phenotyped in the DEPI system. The QTL map of NPQ is shown below (Figure 18). When comparing this QTL map (Figure 18) to the QTL map that was made from the smaller data set (Figure 4), it can be concluded that the maps are quite similar. However, including the late flowering lines results in some extra QTLs or a change in the size of the QTL. The strict Bonferroni threshold was used during data analysis, which corrected for the sum of the number of different phenotypes and the number of markers. This means that QTLs with a LOD score higher than the LOD threshold are very significant and are called with high confidence. The QTL maps show that many QTL are involved in NPQ response under different light conditions. To see if the increase of NPQ by the different QTLs was always caused by E-C genes, or whether C-C genes also cause an increase in NPQ, the LOD scores are displayed as positive and negative values. A positive LOD score means that the BLUEs of C-C is higher than the BLUEs of E-C, a negative value indicates a higher BLUEs for E-C. This QTL map shows that differences in NPQ values are caused by many QTLs, which cause lower as well as higher NPQ values for E-C compared to C-C. The QTL maps for other photosynthesis phenotypes show different QTLs under different light conditions. To illustrate this the plot of phiNPQ is added to the appendix (S 3). The QTL on the end of chromosome 2 at the 5<sup>th</sup> day shows a high LOD score of around 200. The highest LOD score for this QTL was obtained under low light at 101.5214 hours after the start of the experiment. In the QTL map of qE, the same QTL appears as for NPQ (S 4). This suggests that the QTL might be involved in the fast mechanism of NPQ.

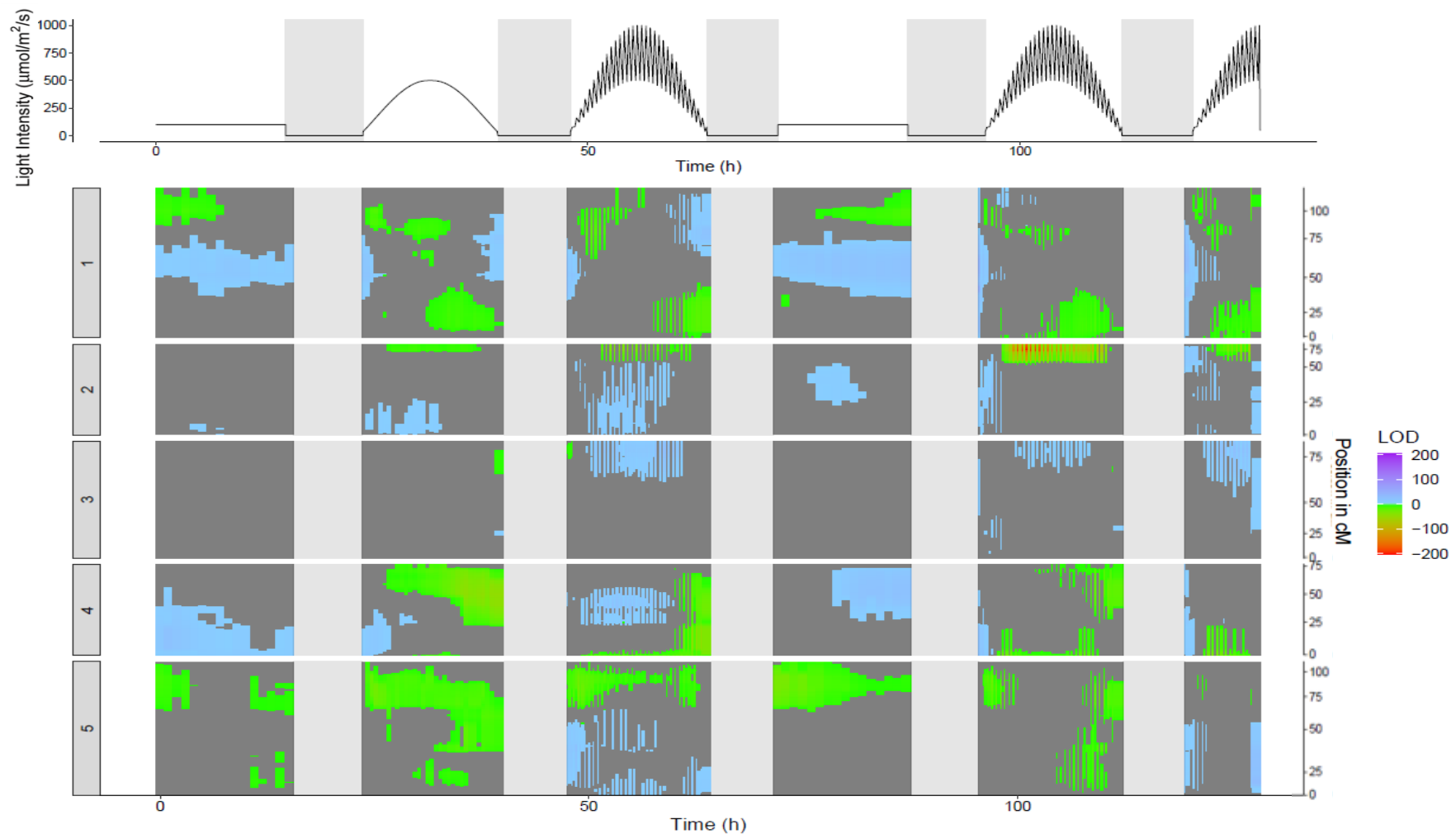


Figure 18 QTL analysis of NPQ on biparental DH population, extended with late flowering lines. The upper plot shows the light treatment over 5.5 days. The lower plot is the QTL plot with on the left y-axis the chromosome numbers and on the right y-axis the position on the chromosome in cM. On the x-axis the time is equal to the time in the plot of the light intensity and the QTLs are therefore appearing under this light condition. Grey areas are not significant QTLs. The legend shows positive and negative LOD scores. A positive LOD score indicates a higher BLUEs for C-C than E-C, a negative LOD scores vice versa. The threshold is a LOD score of 4.776 ( $p < 1.7e-05$ ) and the QTL analysis was based on an MQM analysis, with markers every 250,000 bp.

Based on the QTL mapping approach with a Bonferroni threshold the identified QTL on chromosome 2 has a width of 250 kb. A qualitative analysis can reduce the size of the QTL by using observations of individual recombinants within the previously defined QTL and compare the recombination position with the NPQ values. Lines with a recombination within the range of the QTL defined by the MQM analysis were selected. From this subset, representative lines with extreme NPQ values at timepoint 101.5214 were selected. The resolution of the genotypic data used for the qualitative analysis was 25,000 bp. If more than 50% of the SNPs in the window of 25,000 bp was from for example C-C, this whole region was determined as C-C. This means that the window where the parent changes from one to the other, the first half of the 25,000bp region between the two markers belongs to the first parent and the second half of the region to the other parent. Based on line DH 37, the QTL starts at 18,812,500 bp and from line DH 23 and 362 it can be concluded that the QTL ends at 18,862,500 (Figure 19). This leaves us with a QTL size of 50,000 bp. The qualitative analysis was performed on the genomic data with markers every 25,000 bp. The qualitative analysis could potentially be performed on the 10,000 bp genomic data, however in some regions there were not enough SNPs present to identify if the region was inherited from C-C or E-C. Furthermore, fine mapping will give more detail about the exact size and position of the QTL and therefore the identified size was sufficient at this state.

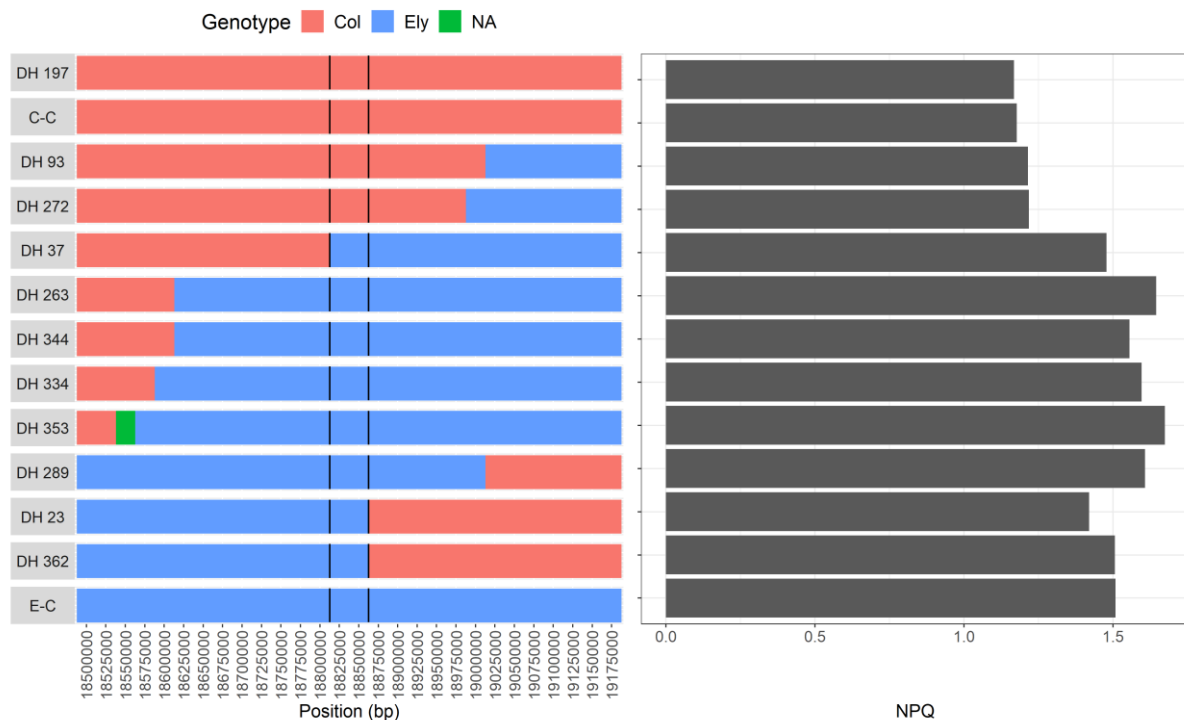


Figure 19 Qualitative analysis on NPQ at timepoint 101.5214 of a selection of DH lines to define the region of the QTL. On the left side a plot is shown with the genotype data of the selected lines in the region of the QTL. On the right side a plot is shown with the NPQ values at timepoint 101.5214 per line. The black lines in the left plot show the position of the QTL, based on the qualitative analysis.

To investigate if next to the QTL on chromosome 2, other QTLs have a significant effect on NPQ values at timepoint 101.5214, a separate QTL map was made for this phenotype (Figure 20). The QTL map shows in total four QTLs with an MQM LOD score above the Bonferroni LOD threshold. The two QTL with the highest LOD scores are on chromosome 2 at 74.61 cM (18,500,000 bp) and on chromosome 4 at 0 cM with a LOD score of respectively 202.55 and 12.37. The other two QTLs are found at chromosome 1 at 5.8 cM (500,000 bp) and 80.1 cM (22,250,000 bp) of 6.23 and 5.90, respectively. An effect size plot shows that the QTL is not only highly significant, but also results in a large absolute effect (Figure 21). The QTLs on chromosome 2 and 4 show significant interaction ( $p = 0.0009$ ) and a

negative epistatic effect. The QTL on chromosome 2 increases NPQ with 18.8% and 13.3% with respectively a Col or Ely allele for the QTL on chromosome 4. When looking at the effect of the QTL on chromosome 2 alone, the effect of the Ely allele is 17.3% and the difference between the genotypes E-C and C-C is 28.1%. From this we can conclude that the NPQ difference between C-C and E-C are not fully caused by the QTL on chromosome 2. The fast component of NPQ, qE, results in an increase of 34.4% for the Ely allele compared to Col, while qI, the slower mechanism of NPQ, only increases with 2.3% for Ely compared to Col on chromosome 2. This suggests that the increase of NPQ is linked to an increase of qE.

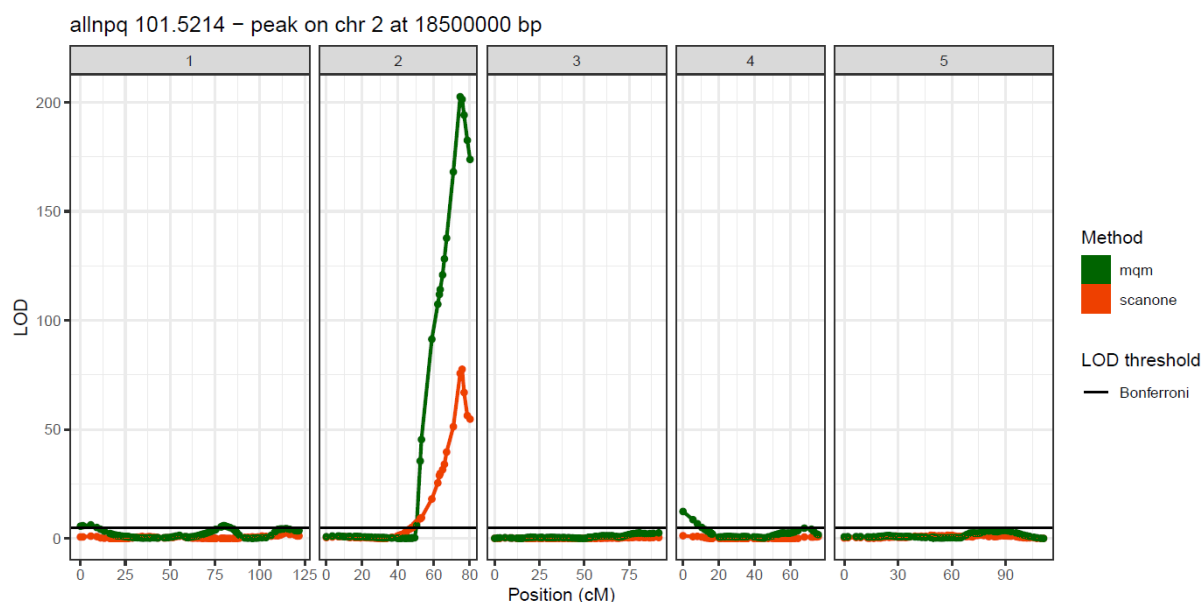


Figure 20 QTL map at timepoint 101.5214. Four QTLs have a LOD score from the MQM analysis above the Bonferroni LOD threshold of 4.776 ( $p < 1.67e-05$ ).

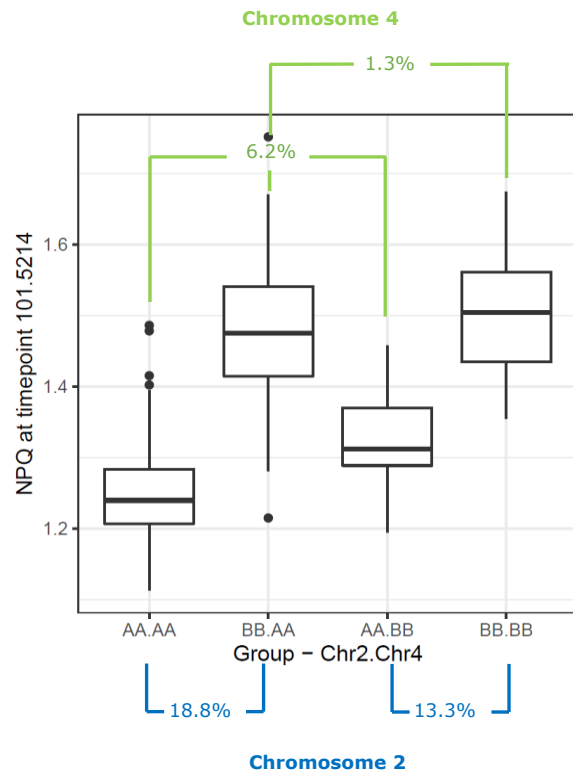


Figure 21 Effect size plot of the QTL on chromosome 2 and 4. On the x-axis is the interaction between the different alleles on the two positions shown. AA means homozygous for the Col allele and BB means homozygous for the Ely allele. The first two letters represent the genotype at the QTL on chromosome 2 and the second two letters the QTL on chromosome 4. The interaction between the two QTLs was significant ( $p = 0.0009$ ), which implies an epistatic interaction.

QTL maps only show additive effects of alleles and by manually analysing a combination of 2 QTLs (as was done in Figure 21), epistatic effects can be identified. However, large and multi epistatic interactions will likely not be identified. To investigate epistatic interactions in high through-put, a scantwo analysis was performed for NPQ on timepoint 101.5214 (Figure 22). In the right lower triangle, the result of a scanone of the full model, with additive and epistatic effects, is displayed (similar to Figure 20). The LOD score of the scanone analysis (Figure 22) is lower than the MQM analysis (Figure 20), due to higher statistical power. The additive effect of the QTL on chromosome 2 is shown to be significant. In the upper left triangle, the result of the epistasis model is shown. The epistatic interaction of the QTL on chromosome 2 and 4, which was already demonstrated in the effect size plot (Figure 21) also appears on the scantwo plot (Figure 22). This interaction has a p-value of 0.0009, which correlates with a LOD score of 3.05. In the scantwo plot this interaction is also shown in the upper-left triangle. Besides this interaction, other epistatic effects were found, of which some are even more significant. For example, the interaction between the end of chromosome 3 and 5 is significant, while the separate effect of both positions was not significant in the QTL plot (Figure 20).

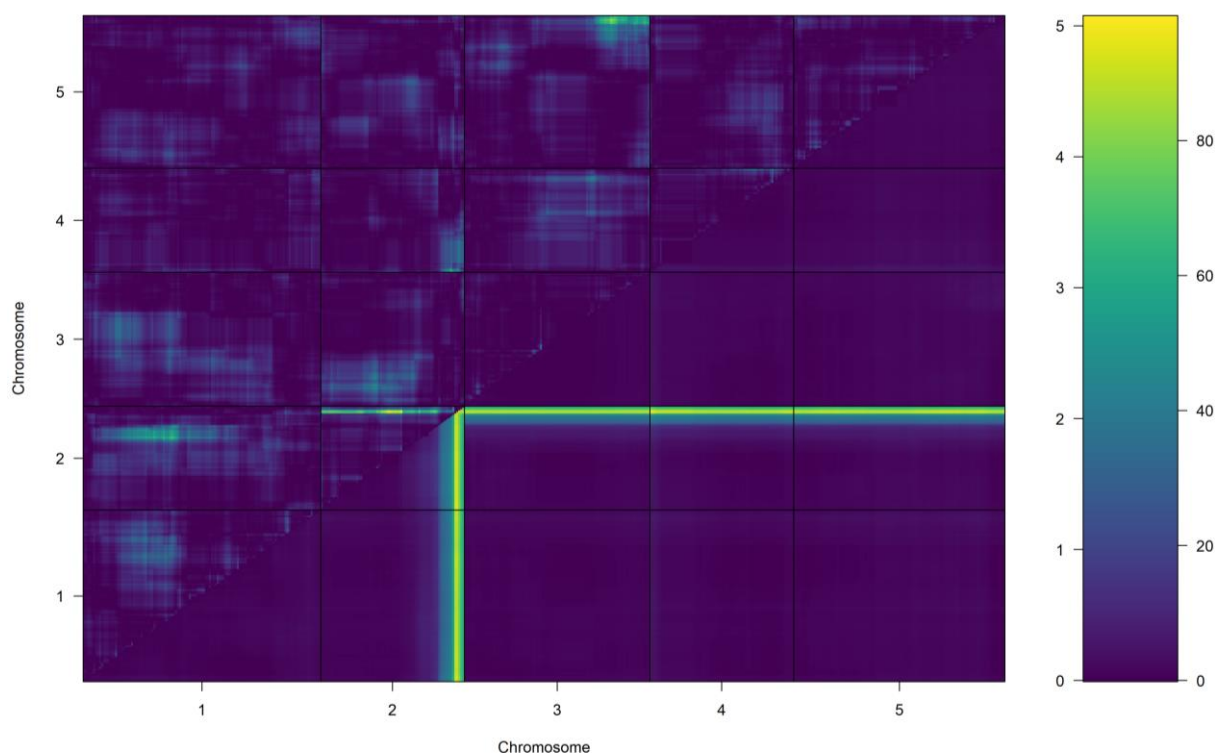


Figure 22 Scantwo plot which tests for additive and epistatic effects for NPQ QTLs at timepoint 101.5214. Left-upper triangle is the plot for the epistasis model and right lower triangle is a plot of the full model (additivity and interaction). Legend on the left represents epistasis LOD scores and right shows full model LOD scores.

### NPQ relaxation and induction

As described earlier in the results, the QTL on chromosome 2 seems to be involved in the fast component of NPQ, because the same QTL is shown for qE. Justine Drouault investigated the difference in relaxation of NPQ between E-C and C-C and concluded that NPQ relaxation of E-C is faster than of C-C. It was decided to repeat this experiment with plants grown in a climate chamber and to include NPQ induction in the experiment. After 25 minutes of alternating high and low light every 5 minutes, the light was switched from high light to low light and NPQ relaxation was measured with intervals of 5 seconds. After 5 minutes, the light intensity was increased and NPQ induction was measured under high light intensity.

A statistical difference was observed for NPQ relaxation at some of the timepoints in the first 100 seconds (Figure 23a). This data is a summary of 6 different blocks that were measured every 30 seconds. The first measurement of the different blocks was either at  $t=0, 5, 10, 15, 20$  or  $25s$ . The differences between the genotypes are probably caused by a batch effect, because it differs between timepoints if NPQ of C-C or E-C is higher. For NPQ induction, NPQ of C-C and E-C follow the same pattern and no significant differences are found for the first phase (Figure 23b). The genotypes that were grown in constant conditions in a climate chamber do not show consistent differences in NPQ relaxation or induction. Since a growing environment can influence phenotypes, it was decided to grow plants outside in a tunnel to see if the fluctuating condition triggers a difference in NPQ relaxation and/or induction.

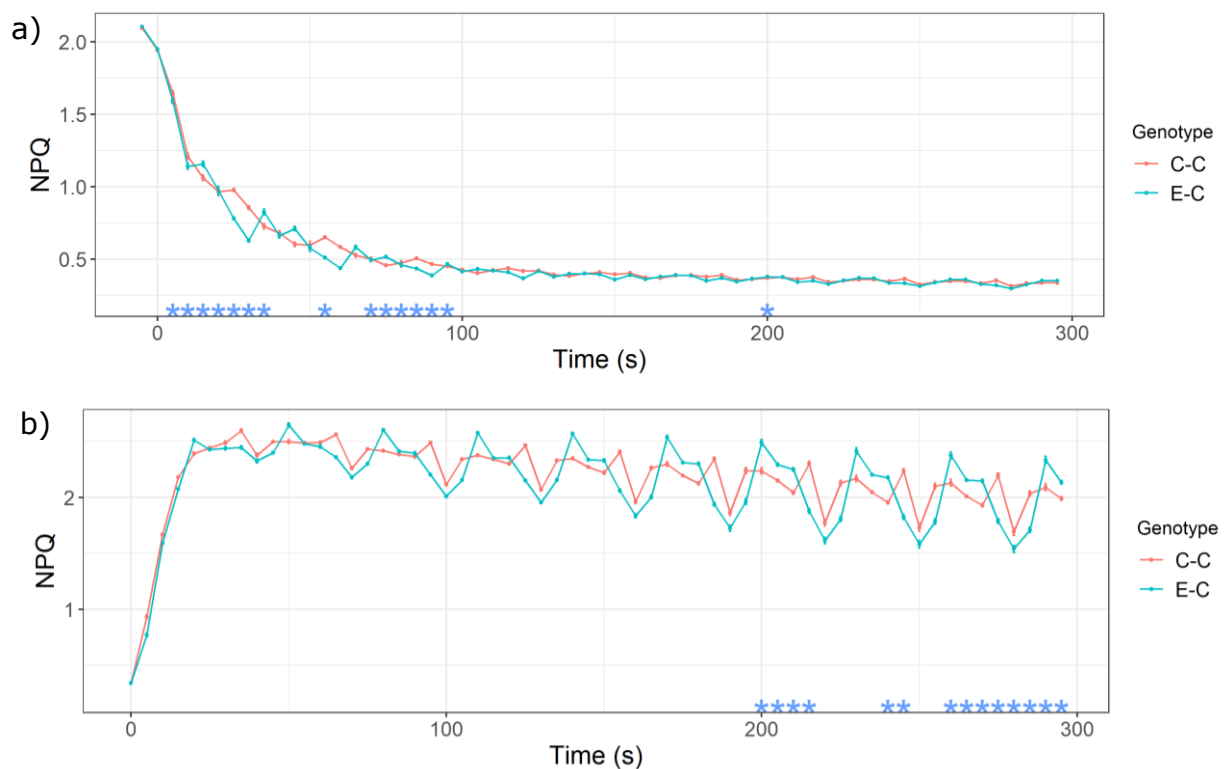


Figure 23 NPQ relaxation (a) and induction (b) of plants grown in a climate chamber. The plants were subjected to five cycles of 5 minutes alternating high and low light before the measurement of successive relaxation and induction. C-C and E-C lines were measured in 6 different batches with measurements every 30 seconds. The first measurement of each batch was at a different time interval after the light was turned off, to be able to have a measurement every 5 seconds. Blue stars indicate a statistical difference between C-C and E-C and green stars indicate a statistical difference between DH6 and C-C. For each block,  $n = 10$  for both genotypes. Error bars indicate the standard errors.

At the time of the experiment, a NIL of the QTL on chromosome 2 was not yet available and therefore DH6 was included in the experiment as a near NIL to isolate the effect of the QTL. The incentive was to confirm that the QTL on chromosome 2 is involved in the NPQ phenotype that was measured in the DEPI system. The results show that during NPQ relaxes faster for E-C than C-C (Figure 24a). In the beginning NPQ is higher for E-C compared to C-C and already after 10 seconds NPQ is lower. After 20 seconds there is no significant difference between C-C and E-C anymore. However, NPQ of E-C is still lower until 100 seconds after turning from high to low light. DH6 shows higher initial NPQ values than C-C, however the NPQ value of DH6 does not become lower than of C-C. NPQ values for induction are significantly different between E-C and C-C for the first 15 seconds (Figure 24b). The differences are not very big in the beginning, but NPQ of E-C is lower than C-C and after 10 seconds NPQ is higher. NPQ induction is therefore faster for E-C than C-C. DH6 does not show a significant difference for the first 50 seconds. It seems that the growing environment has an influence on the measured NPQ phenotype of C-C and E-C and that a fluctuating environment results in a significant difference in NPQ between C-C and E-C.

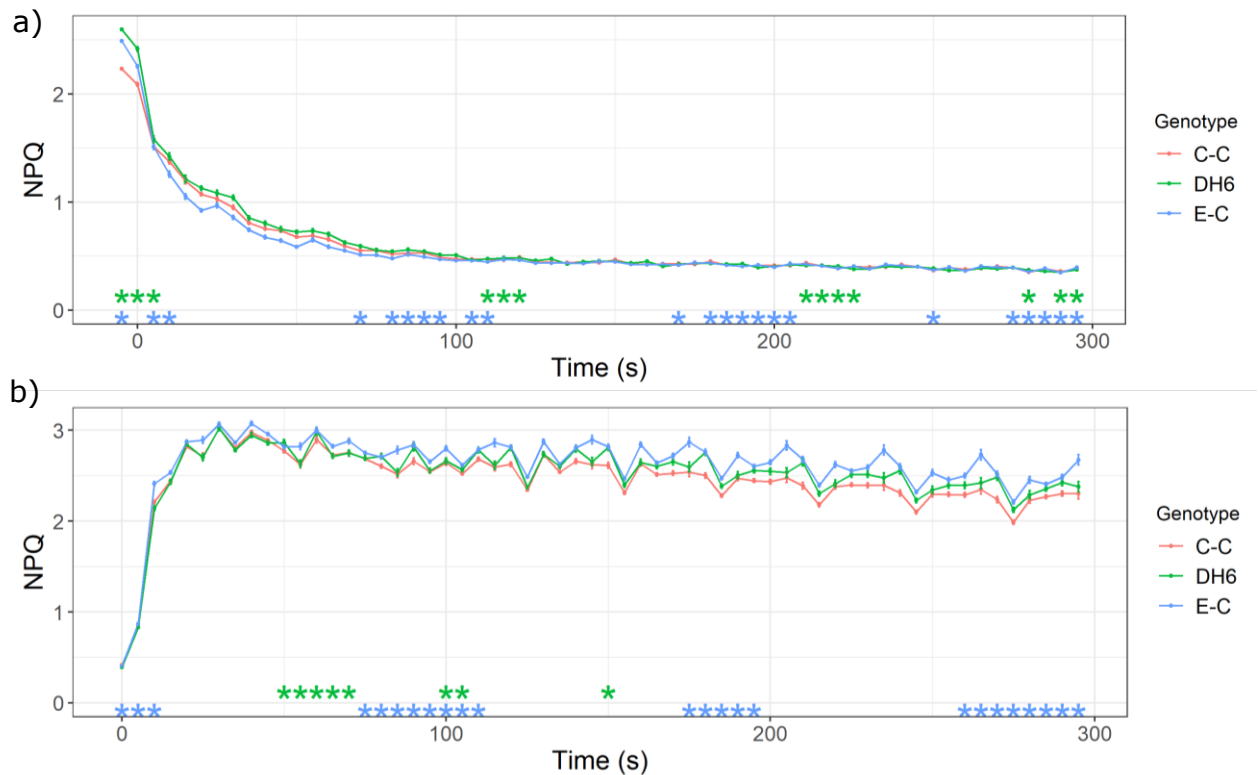


Figure 24 NPQ (a) relaxation and (b) induction for plants grown in a tunnel outside. DH6 is a near NIL of the chromosome 2 QTL as Ely in a C-C background. The plants were subjected to five cycles of 5 minutes alternating high and low light before the measurement of successive relaxation and induction. C-C and E-C lines were measured in 6 different batches with measurements every 30 seconds. The first measurement of each batch was at a different time interval after the light was turned off, to be able to have a measurement every 5 seconds. Blue stars indicate a statistical difference between C-C and E-C and green stars indicate a statistical difference between DH6 and C-C. For each block,  $n = 8$  for C-C and E-C and  $n = 4$  for DH6. Error bars indicate the standard errors.

The effect of the growing environment seems to have an influence on NPQ relaxation and induction. However, many QTLs influence photosynthesis related traits and NPQ measurements are artificial since the measuring protocol might influence the measured phenotype. Still, it was interesting to identify if the growing environment, next to its influence on NPQ, also influences dry weight. Eventually, the goal is to improve overall plant performance, of which dry weight is an important factor. To identify the effect of growing condition and genotype on dry weight, the three different genotypes, C-C, E-C and DH6, were grown in three different growing environments, a climate chamber, greenhouse and tunnel outside. E-C is the only genotype that is statistical different from other genotypes in the greenhouse and the tunnel. In the climate chamber, under constant light conditions, no difference between the genotypes is observed. The light condition in the greenhouse is supposed to be relatively constant because the lamps were the main source of irradiance. This is because the plants were grown in the autumn when the intensity of the sunlight was often low, and the lamps were turned on and emitted a constant light intensity. In the greenhouse a small decrease in dry weight of E-C compared to C-C and DH6 was shown. This might be due to some fluctuations in light intensity on sunny days. However, there are many genetic differences between E-C and the other genotypes which makes it difficult to draw conclusions about the (genetic) origin of this difference.

In the tunnel an opposite effect was observed, since the dry weight of E-C was higher than the dry weight of C-C and DH6. The plants that were grown in the tunnel experienced fluctuating outside weather conditions. Part of the plants showed a stressed phenotype (Figure 26), which was probably caused by low temperatures. The plants were harvested in January, when temperatures dropped

drastically. The stress phenotypes were scored on a scale from 0 to 2 with 2 being very stressed. The average scores were 1.9, 1.3 and 0.4 for C-C, DH6 and E-C respectively. The dry weight of plants grown in the tunnel is higher than of the plants grown in the climate chamber and the greenhouse. This is because the plants grown in the tunnel were growing for a longer period, as short day length causes later flowering. In the tunnel, the effect of the QTL on chromosome 2 seems to have a negative effect on biomass production. The average dry weight of DH6 shows a trend of lower biomass compared to C-C, however due to the stress phenotype and because the difference is not significant, no conclusions can be drawn based on this data.

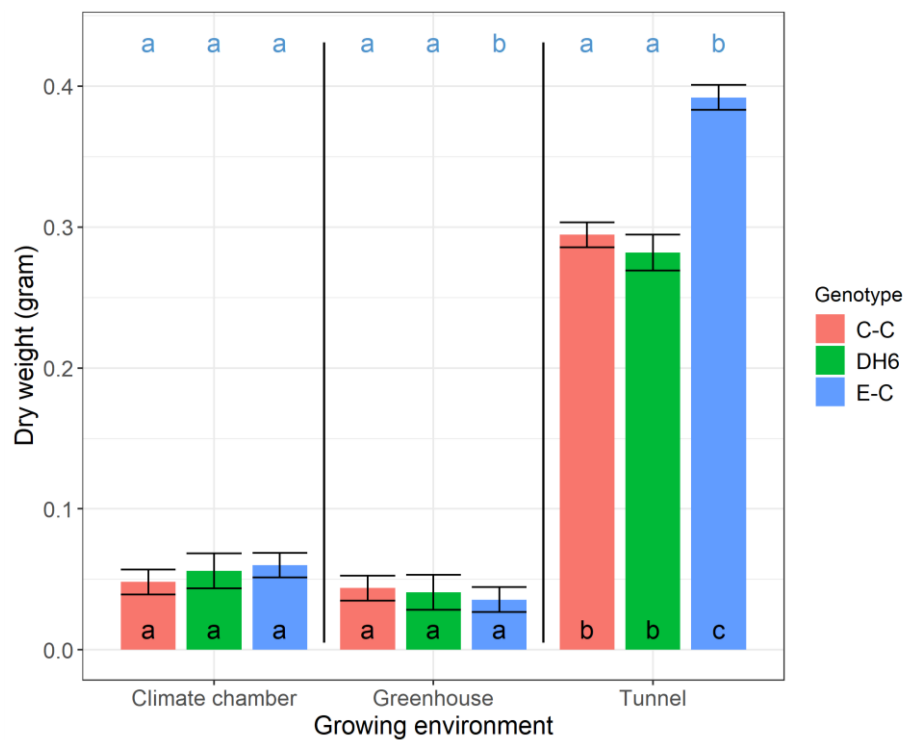


Figure 25 Dry weight of E-C, C-C and DH6 grown in different growing environments. DH6 is a near NIL of the chromosome 2 QTL as Ely in a C-C background. Blue letters on the top show if the genotypes are significantly different within one growing environment. Black letters on the bottom indicate significant differences with including a genotype x growing environment interaction. Both statistical analyses are based on a Tukey post hoc test, with  $\alpha = 0.05$ . For C-C and E-C,  $n = 48$  and for DH6  $n = 24$ . Error bars indicate the standard errors.



Figure 26 Plants grown in tunnel, just before harvesting for measuring the dry weight. From left to right C-C, DH6 and E-C. C-C and DH6 seem to experience stress, this might be due to the cold.

### Development of a NIL

It was decided to develop a NIL to investigate the physiological effect of the QTL on chromosome 2. In the experiment with the DH population that was phenotyped in the DEPI system, epistatic effects were shown to influence NPQ phenotypes (Figure 22). A NIL can isolate the effect of a single QTL. It was hypothesised that the QTL has an effect of relaxation or induction based on previous experiments, however repetition of this experiment could not confirm this. New physiological experiments should shine a light on the environmental condition in which the QTL influences NPQ. During production of the NIL recombinants within the QTL region were also selected to be able to perform fine mapping. For development of the NILs and recombinants, the progeny of the F1 from DH6 and C-C was sown, and lines were selected that were heterozygous for the QTL on chromosome 2 and homozygous Col for the rest of the genome. Six out of 500 plants were selected and their progeny was sown. One out of four was expected to be a NIL (homozygous Ely for the QTL in a Col background), however only 523 out of 2420 appeared to be homozygous Ely for the introgression. Eight of these lines were selected to harvest the seeds for physiological experiments. 57 recombinants were selected in the region of the QTL, of which the progeny was sown to select for homozygous recombinants. These homozygous recombinants were phenotyped to fine map the gene that is responsible for a higher NPQ phenotype caused by the Ely allele.

The NIL was used to identify which of the two alleles on chromosome 2 is dominant and to check if a difference in NPQ between the NIL and C-C could be measured. The measuring protocol used in the Robin was a simplification of the fluctuating day in DEPI, where high and low light intensities alternate. The protocol consisted of cycles of 5 minutes with alternating low and high light intensity. Since only 20 plants could be measured at one time, the protocol was only one hour long, and high and low light intensities were extreme with the aim of triggering the phenotype in a short period. The results of the 12<sup>th</sup> cycle show that the NIL does not differ significantly from C-C in NPQt values (Figure 27). This means that it was not possible to measure the effect of the QTL on chromosome 2 with this measuring protocol. However, there is a difference between C-C and E-C, which means that the effect that is measured with this protocol is caused by a different QTL than the one on chromosome 2. E-C and the NIL respectively have a 16.9% and 0.8% higher NPQt value compared to C-C. The hetero NIL (which is heterozygous for the QTL on chromosome 2 and homozygous C-C for the other chromosome segments) was included to identify the allele that is dominant. Because the results do not show a different NPQ phenotype for the NIL, hetero NIL and C-C in the Robin, no conclusion can be drawn about dominance of the alleles. Most other photosynthesis phenotypes showed similar patterns (S 5).

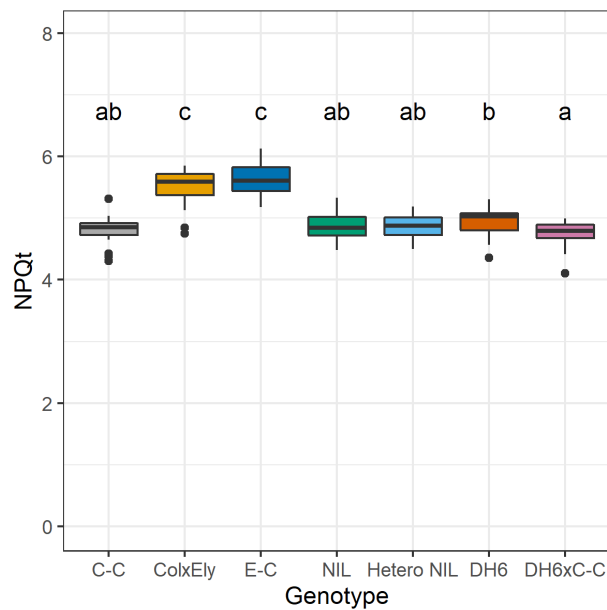


Figure 27 NPQt for different genotypes after 12 fluctuations of 5 minutes with alternating low and high light. The black letters on the top indicate the significantly different groups for a tukey post hoc test for comparing different genotypes, with  $\alpha = 0.05$ . 21 replicates were used for all genotypes, except for Col x Ely, for which  $n = 14$ .

It was not possible to measure a difference in NPQt between C-C and the NIL in the Robin as was expected based on the data of the DEPI system. The NIL contains the large effect size QTL on chromosome 2 and was therefore expected to show a higher NPQt value compared to C-C. In a new experiment, E-C, C-C and DH6 were measured in the Robin over multiple days with a protocol that simulates the fluctuating light treatment of DEPI. This was done, to check if the phenotyped that was measured in the DEPI system could be reproduced in the Robin. We wanted to do fine mapping in the Robin, but if the difference in NPQ between C-C and the NIL cannot be measured in the Robin, it is impossible to perform fine mapping in the Robin. It was tested if mimicking the DEPI light treatment in the Robin would result in an NPQ difference between C-C and DH6. In the Robin, the treatment of DEPI was mimicked for a fluctuating day, followed by a low light day and 3 fluctuating days, to see how the different genotypes behaved over time with a DEPI light treatment. DH6 was included and not the NIL because the NIL was not yet available at the time of the experiment. NPQt was used instead of NPQ, because NPQt is more informative if a long protocol is used. NPQ uses a dark-adapted measurement at the start of the day. During the day, many factors can influence the value of the measurement after dark adaptation, such as leaf and chlorophyll movement. NPQ values are therefore less accurate if there is much time between a particular measurement and the measurement after dark adaptation. Higher NPQt values were measured for DH6 compared to C-C (Figure 28). From this it can be concluded that when using the DEPI light treatment, it is possible to measure the higher NPQ phenotype for the NIL that was expected from the data of the DEPI system. Over the days the NPQt values decreased, this is probably because of adaptation of the plants to fluctuating light. The potential of photosystem II was calculated by FvFm at the start of each day, which is normally around 0.83. The average FvFm of all plants decreased over the days (Table 1). This was probably caused due to a low average temperature inside the Robin of 14.9°C.

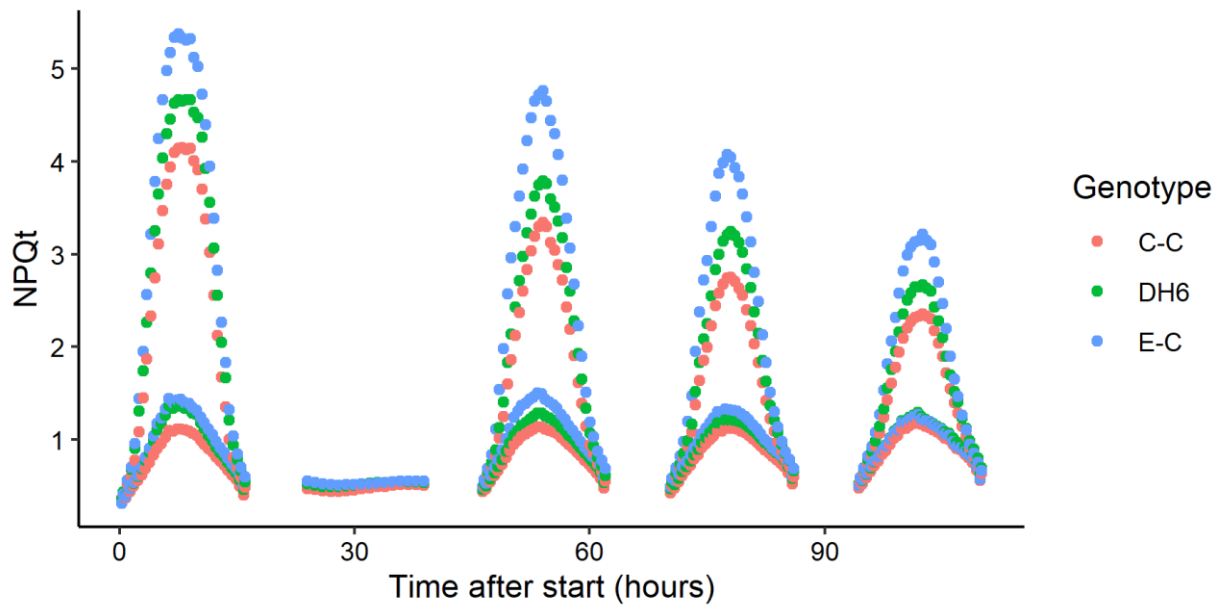


Figure 28 NPQt measurements over 5 days with light fluctuations based on the DEPI treatment. The first day was a fluctuating day, followed by a day of low light intensity and 3 fluctuating days. Fluctuations were based on alternating low and high light periods, of which the low light periods were 20 minutes long and the high light periods 10 minutes.

Table 1 Average FvFm values for 5 days.

Day 1	Day 2	Day 3	Day 4	Day 5
0.808291	0.757767	0.770711	0.753507	0.745513

These results showed that it is possible to trigger a higher NPQt value of DH6 compared to C-C by extended fluctuation of light. For this reason, the Fluctor was build. This is a system in a climate chamber in which LEDs are programmed to fluctuate in the same pattern as the lamps in the DEPI system. Five different optocouplers, that control the current flowing through the circuit, were connected to 5 different pins of the ESP32 pyboard (Figure 29a). The script on the pyboard uses a text file with values to dim the LEDs based on pulse-width modulation (PWM). The LEDs are divided equally over the width of the climate chamber and light intensity differs less than 5% between different positions in the growing area. The light intensity was measured to check if the measured light intensity (Figure 29d) was similar to the programmed intensity (Figure 29c). For higher light intensities there is some decrease in intensity over time, this is probably due to an increased temperature of the LEDs, which results in lower efficiency. The overall pattern correctly simulates the treatment from the DEPI system.

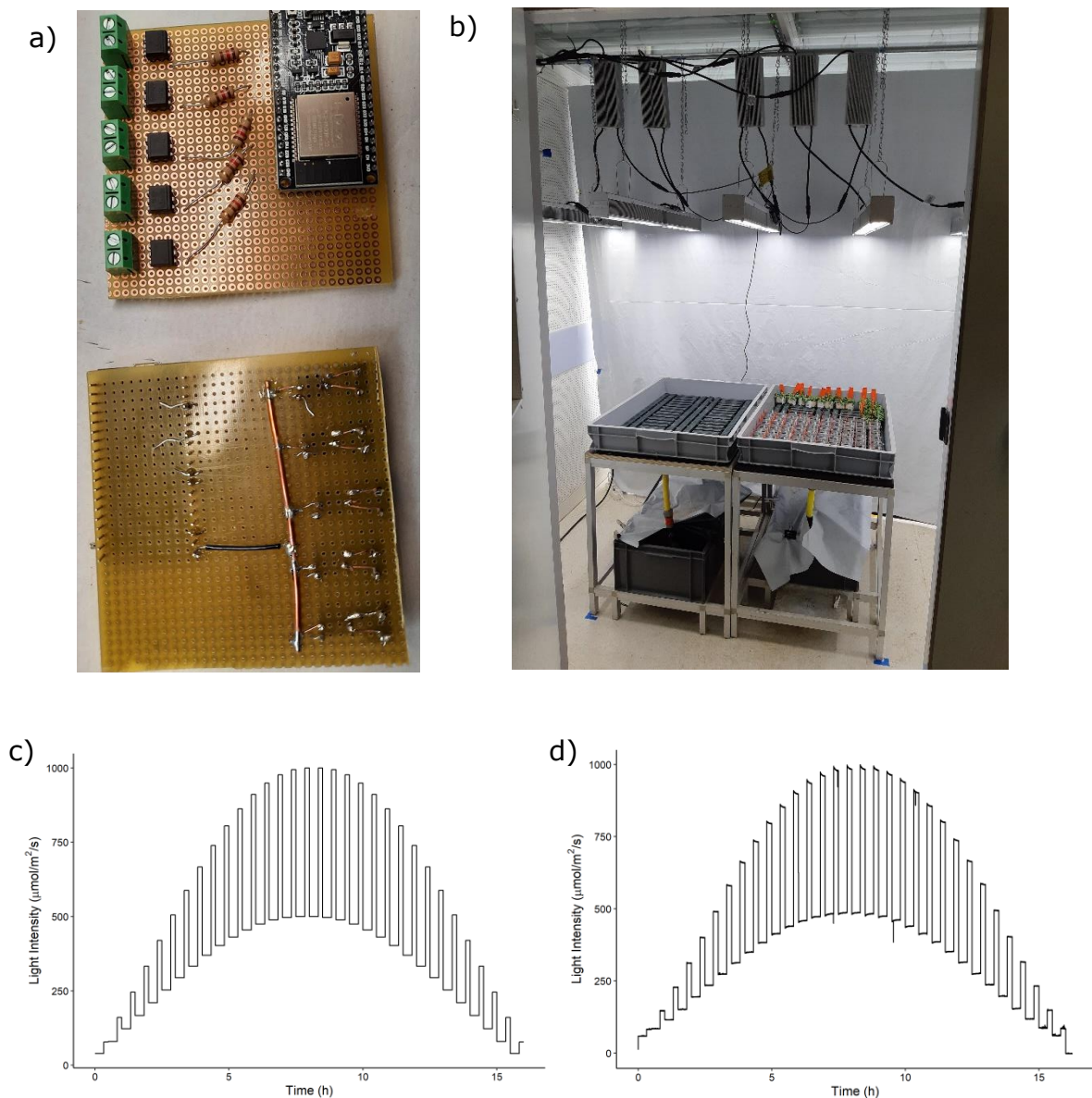


Figure 29 Fluctor set-up. a) The pyboard is programmed to dim the five different light modules that are connected to the different optocouplers. b) The system in the climate chamber, five different lamps are divided equally over the whole climate chamber. c) The programmed light fluctuation pattern. d) The measured light fluctuations inside the climate chamber.

### Investigating the genetics behind the QTL on chromosome 2

The higher NPQ value for Ely at the QTL on chromosome compared to C-C can be caused by large structural differences or by one (or more) gene(s). To identify large structural variation in the region of the QTL on chromosome 2, Tom Theeuwes used Oxford Nanopore Technology (ONT) sequencing for a *de novo* assembly of the Ely accession. The *de novo* assembly was improved with Illumina reads. Chromosome wide, there is not a lot of variation between the Col and Ely nucleotide (Figure 30a). Zooming into the QTL region shows that no structural differences exist between both lines (Figure 30b).

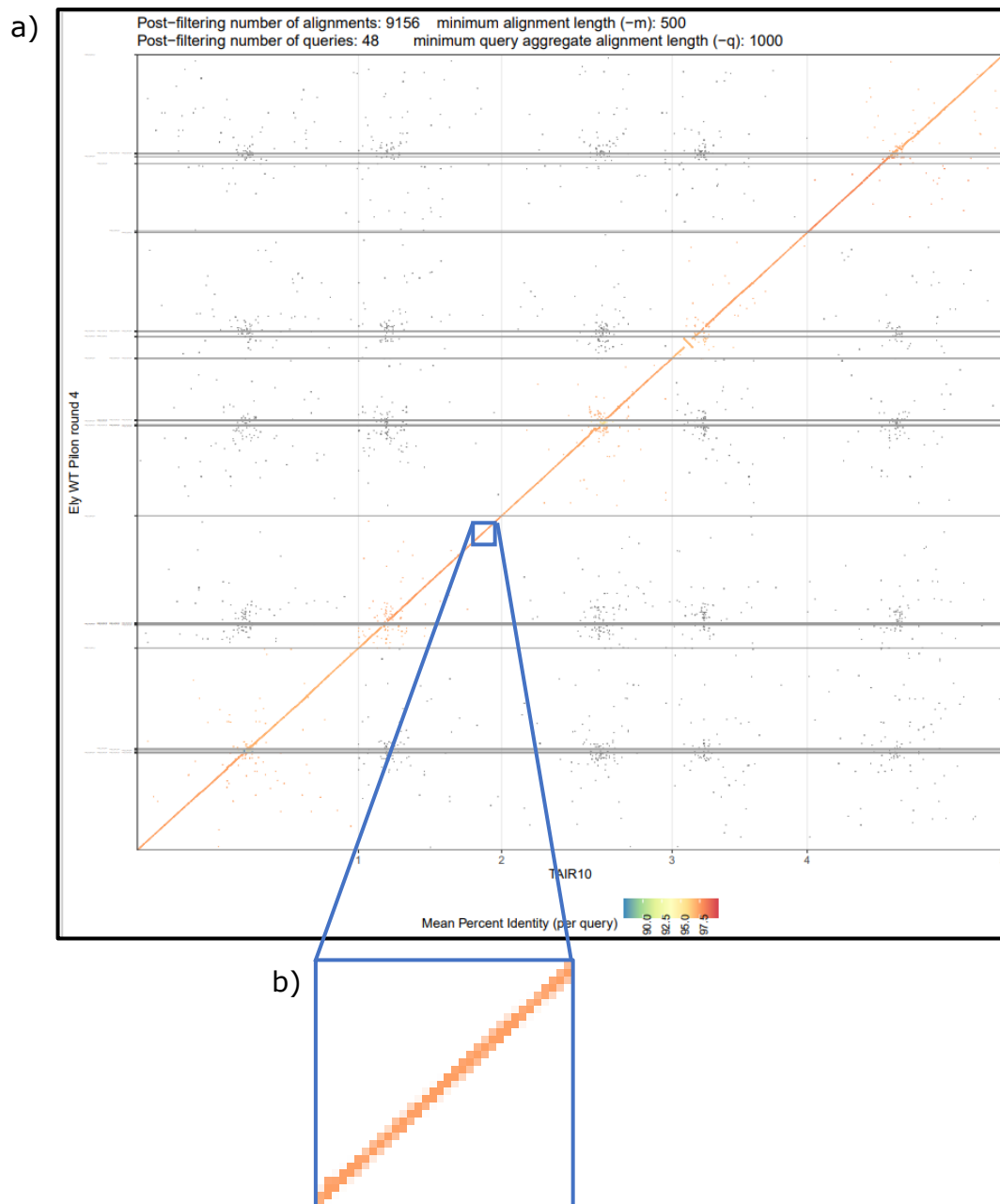


Figure 30 a) Analysis of structural variation between the Ely and Col nucleotide. The Col genome, based on TAIR10.1 was plotted on the x-axis. The de novo alignment of Ely, based on Minion data was plotted on the y-axis. b) Zoom in of structural variation for the QTL region.

Since the higher NPQ value for the Ely allele on chromosome 2 is not caused by a large structural difference, the effect is probably caused by a gene. For this reason, fine mapping of the gene of interest was performed. The Fluctor was built to be able to measure the difference in NPQ caused by the Ely and Col allele on chromosome 2, as was measured in the DEPI system. Different lines that recombined within the QTL were measured to reduce the size of the QTL. To obtain these lines, 3000 plants were screened for a recombination within the QTL region in the F3 (Figure 7). Markers at a distance of 250 kb were used and 57 recombining lines were selected. Since the F3 was still heterozygous for the recombination, the F4 was sown, and the same markers used to check for homozygosity of the

recombination. The homozygous recombinants were transferred to the Fluctor 1.5 or 2.5 days before measuring. The recombinants were measured with 5-minute cycles of alternating low and high light intensities. The QTL map of the 4<sup>th</sup> cycle only shows significant LOD scores for phiNOt, but not for NPQt (Figure 31). Since phiNOt is a component of NPQ, it was decided to use this phenotype for further analyses.

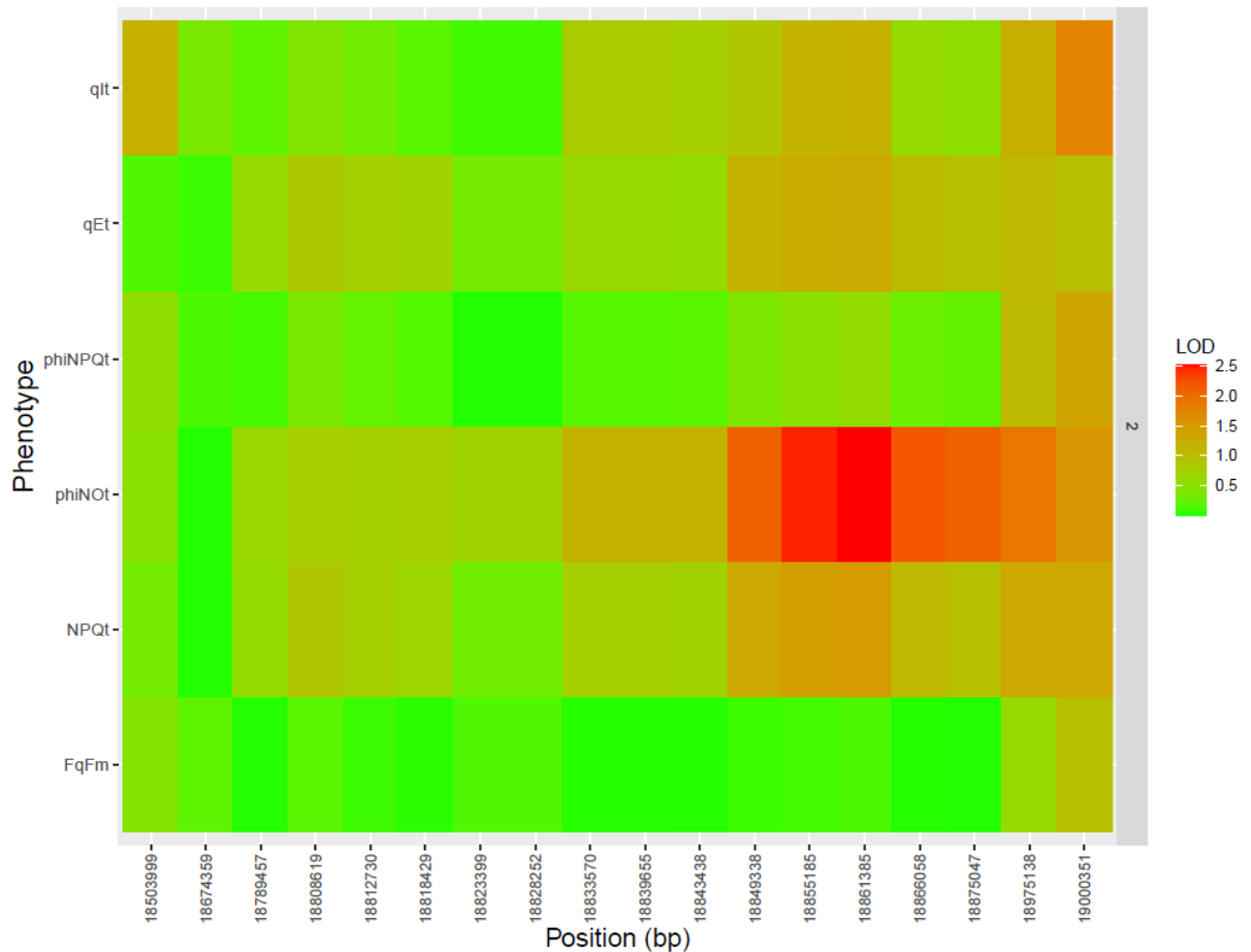


Figure 31 QTL map of fine mapping with homozygous recombinants in the F4 at the 4<sup>th</sup> cycle of fluctuating light with 5-minute intervals. The marker positions are on the x-axis and the 6 different photosynthesis phenotypes on the y-axis. There were only significant marker positions for phiNOt, that exceeded the LOD score threshold that was calculated by a permutation test.

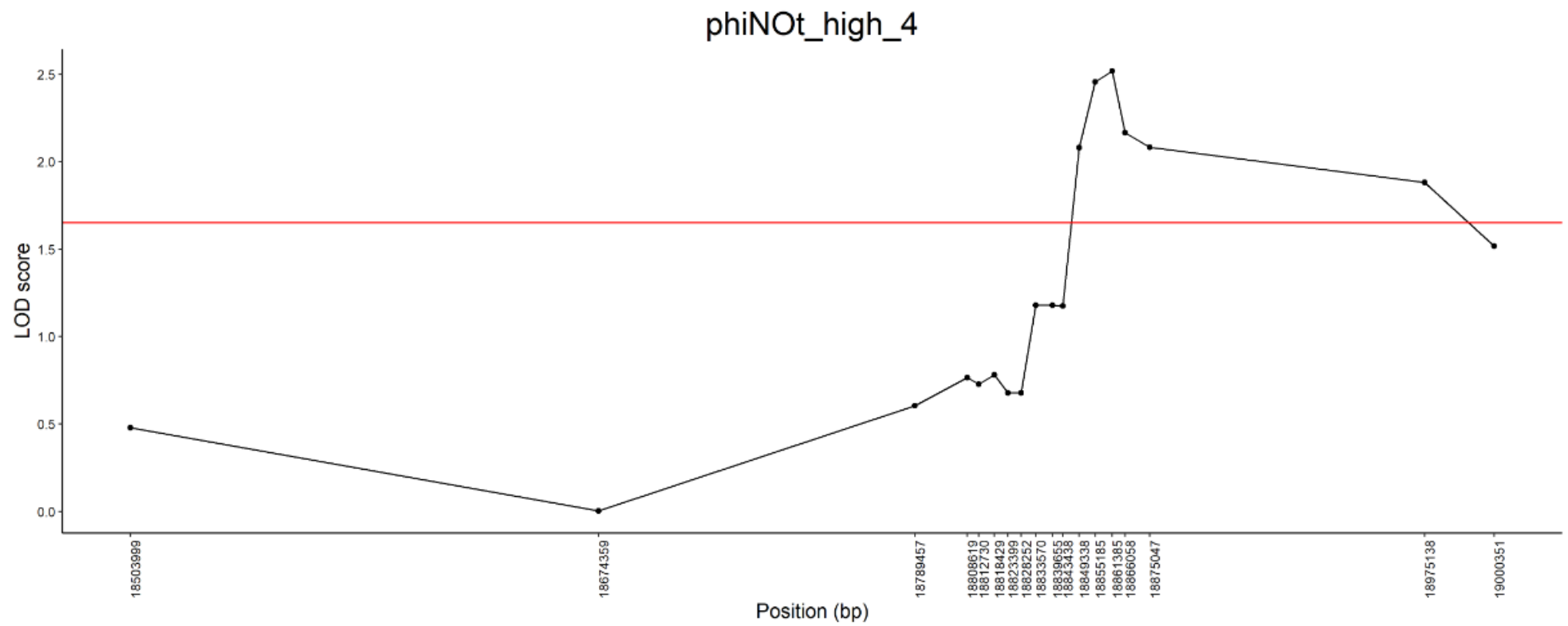


Figure 32 QTL map of phiNOt at the 4<sup>th</sup> cycle of fluctuating light with 5-minute intervals. The markers used for the analysis are shown on the x-axis. The LOD threshold (1.56) was based on a permutation test.

As the plants were pre-treated in the Fluctor, but afterwards transported to the Robin, 4 cycles of fluctuating light were used to trigger the response to changing light intensities. The QTL map of phiN<sub>OT</sub> at the 4<sup>th</sup> cycle of fluctuating light shows that 6 markers have a LOD score of above the threshold of 1.56 (Figure 32). This threshold was based on a test with 1000 permutations. The two marker positions with the highest LOD scores are 18,855,185 and 18,861,385. One of the markers (18,975,138) is just above the LOD threshold is outside the QTL region that was previously defined based on the data from the DEPI system. For this reason and because markers close to the gene of interest automatically have higher LOD scores due to linkage, this marker was excluded from the QTL region. By fine mapping, the QTL region is therefore reduced to the region starting 18,849,338 bp and ending at 18,875,047 bp, which is a size of 25,709 bp. The theoretical resolution that could be obtained with fine mapping is 3,041 bp, because 17 different recombinants were selected in a region of 48,655 bp.

A qualitative analysis based on the NPQ phenotype of the recombinants was performed in an attempt to narrow down the region of the QTL. However, this was difficult due to low heritability values (7.6% for phiN<sub>OT</sub> at the 4<sup>th</sup> cycle) and because some recombinant lines only had one observation. Including all the recombinant lines shows that it is difficult to reduce the size of the QTL by a qualitative analysis (S 6). Therefore, a selection of lines was chosen for the qualitative analysis, consisting of lines that recombine within the defined QTL region of 25,709 bp (Figure 33). Based on line 3\_E05 it would be plausible that the left border of the QTL is at a position of 18,846,388. And the right border was defined by line 25\_F04 to be 18,863,722. This reduces the size of the QTL to 17,334 bp. However, only one replicate was measured for both lines and the observations are less reliable as the heritability of the experiment was low. Therefore, we continue with the QTL size of the fine mapping of 25,709.

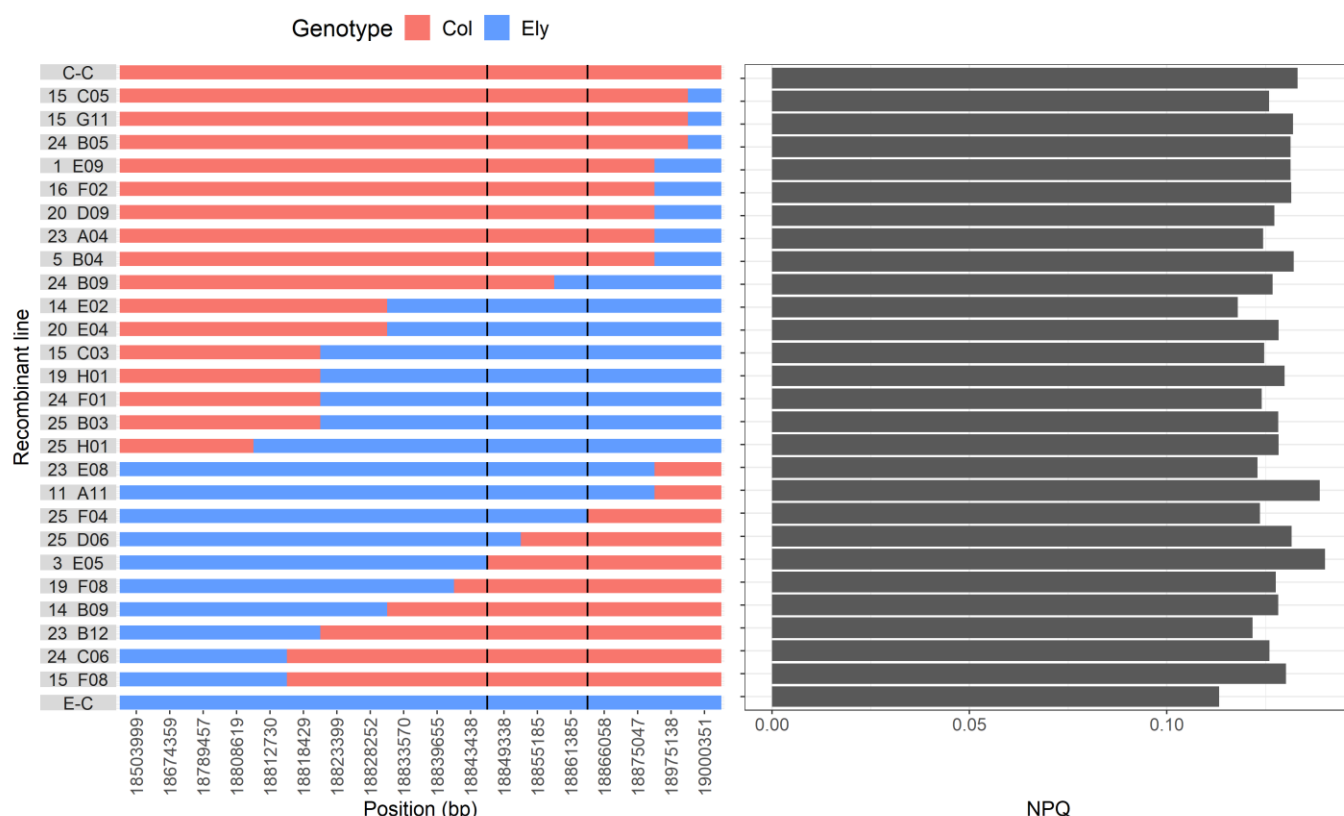


Figure 33 Qualitative analysis of a selection of recombinants. On the left side a plot is shown with the genotype data of the selected lines in the region of the QTL. On the right side a plot is shown with the NPQ values per line. The black lines in the left plot show the position of the QTL, based on the qualitative analysis.

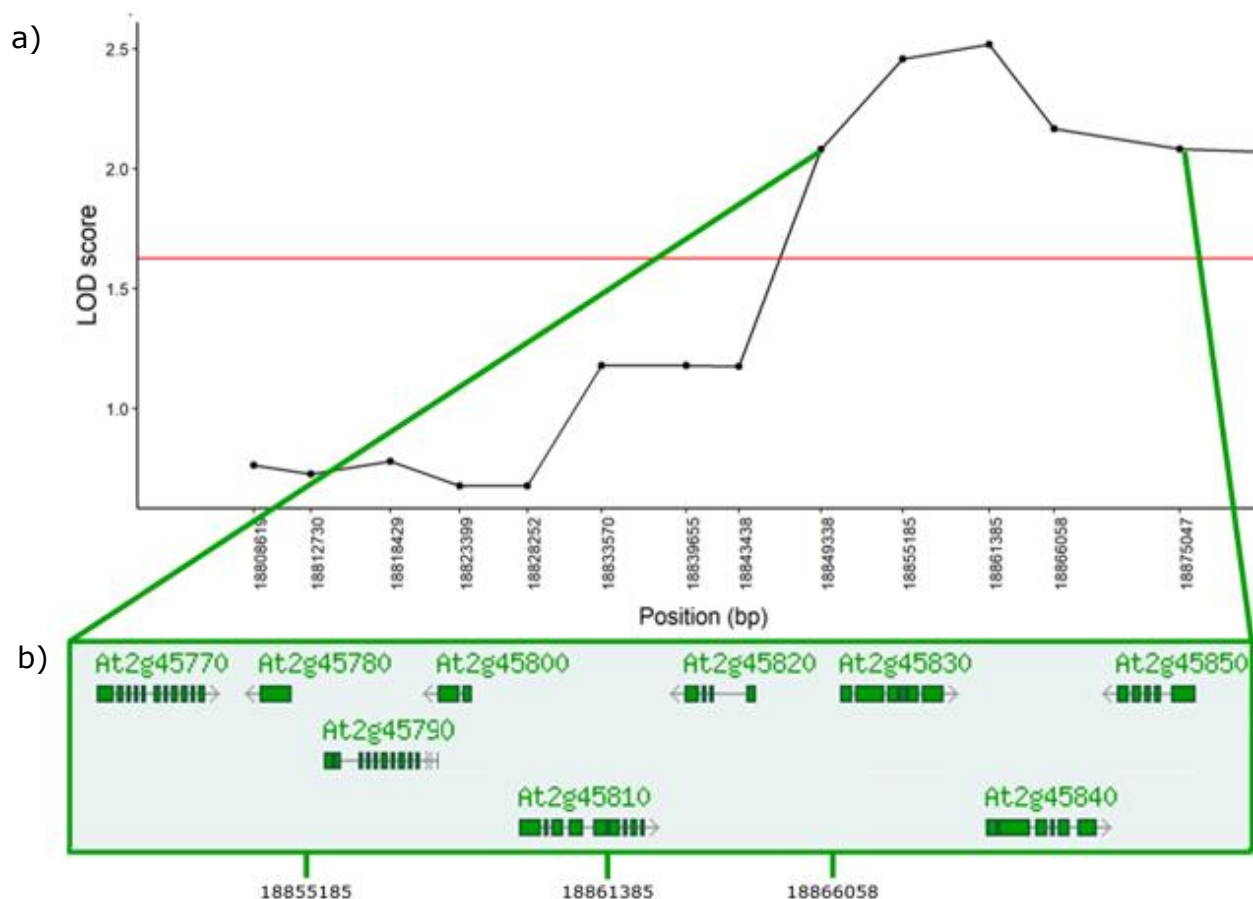


Figure 34 a) Zoom in of QTL map of *phiNOt* at the 4<sup>th</sup> cycle of fluctuating light with 5-minute intervals. The LOD threshold (1.56) was based on a permutation test. Only markers inside the QTL region defined with the data of the DEPI system are shown. b) These genes are found inside the QTL interval defined by fine mapping. This information is based on the genome browser *Arabidopsis* 1,001 Genomes.

Table 2 Candidate genes in QTL region of position 18,849,338 to 18,875,047. Expression in leaves was based on functional annotation information from TAIR about the plant structures in which the genes are expressed.

Position (bp)	Gene	Name	Function	Expressed in leaves
18,851,088 – 18,853,741	At2g45770	CPFTSY	LHCP integration into isolated thylakoids	Yes
18,854,555 – 18,855,184	At2g45780		Unknown	No
18,855,675 – 18,858,018	At2g45790	PHOSPHO-MANNOMUTASE	Cytoplasmic phosphomannomutase	Yes
18,857,941 – 18,859,278	At2g45800	PLIM2A	Regulates actin cytoskeleton organization.	No
18,859,472 – 18,862,970	At2g45810	RNA HELICASE 6	DEA(D/H)-box RNA helicase family protein	Yes
18,862,953 – 18,864,741	At2g45820	REMORIN 1.3	Control plasmodesmata aperture and functionality	Yes
18,865,923 – 18,868,542	At2g45830	DTA2	Downstream target of AGL15 2	No
18,869,153 – 18,871,786	At2g45840	DUF821	O-glucosyltransferase rumi-like protein	No
18,871,479 – 18,873,972	At2g45850	AHL9	Hook motif DNA-binding family protein	Yes

In the 25.7 kb region, nine different genes are located (Figure 34 and Table 2). Five of these genes are expressed in the leaves. This was concluded from functional annotation information from TAIR on the plant structure in which the genes are expressed. Variant calling data between Col and Ely based on Illumina sequencing data was used to identify SNPs and INDELs in the QTL region (Table 3). Only variants with a high or moderate impact were selected. Low impact variants are synonymous SNPs and excluded because synonymous SNPs are not expected to induce a functional change of the protein. Four moderate impact SNPs and one INDEL were found in the QTL region, all in different genes. To estimate if the SNP or INDEL has an effect on protein function, the PROVEAN score is given. The PROVEAN score not only predicts the effect of single amino acid substitutions, but also the effect of multiple SNPs, insertions and deletions. This method aligns the original query sequence and the altered query to the sequence of protein homologs. The PROVEAN score can be positive as well as negative and a value below -2.5 expects that a substitution has a deleterious effect on protein function. None of the substitutions are predicted to have a deleterious effect (Table 3).

*Table 3 SNPs in QTL region. The reference genotype (Ref) is the Col genotype, the alternative genotype (Alt) is the Ely genotype. Only high and moderate impact SNPs were selected. The change in amino acids (AA) and the difference in property is shown for each modification.*

Position (bp)	Ref	Alt	Type	Impact	Gene/protein	AA change	AA properties	PROVEAN score
18,851,326	C	T	Non synonymous	MODERATE	CPFTSY	Pro → Ser AA 27	Ser: polar Pro: non-polar, cyclic structure	0.146 Neutral
18,859,982	ACCTCAG	A	Disruptive in frame deletion	MODERATE	RH6	Gln and Pro del AA 53/54		-0.360 Neutral
18,866,715	T	C	Non synonymous	MODERATE	DTA2	Phe → Leu AA 97	Both non-polar	0.378 Neutral
18,871,417	G	A	Non synonymous	MODERATE	DUF821	Ser → Asn AA 501	Both polar	2.611 Neutral
18,873,394	A	G	Non synonymous	MODERATE	AHL9	Ser → Pro AA 22	Ser: polar Pro: non-polar, cyclic structure	-0.031 Neutral

Low impact variants were not selected from the variant calling dataset because they are not expected to affect protein function. However, two relatively large deletions were found upstream of two genes. These deletions were probably not scored as a modification with a moderate or high impact, because they are located outside the open reading frame. The promotor region can be up to 1000 bp upstream of the gene and therefore these deletions can be located in the promotor regions of the genes. These modifications will not change the protein itself but might influence gene expression levels. SnpEff was probably not able to correctly estimate the effect of deletions in promotor regions. The two deletions that were found are:

- CPFTSY: a 35 bp deletion, located 337 bp upstream of the start codon.
- PHOSPHO-MANNOMUTASE (PMM): a 26 bp deletion, located 201 bp upstream of the start codon.

### Allelic complementation

An allelic complementation experiment was performed to verify if one of the selected genes in the experiment is responsible for the difference in NPQ between E-C and C-C. Due to the long duration of an allelic complementation experiment, different T-DNA lines were selected for the experiment at the start of this research project. Genes were chosen within QTL region that was defined in the original QTL analysis performed by Tom Theeuwes. Within this region, genes were selected for one of the two following reasons; (1) involvement in photosynthesis, which was based on previous functional annotation (TAIR) or (2) differential expression between Ely and Col based on data from Flood *et al.* (2020). The selection consists of four different genes (Table 4). The T-DNA lines were back crossed to Col and to the NIL. The underlying principle of a complementation test is that the mutant phenotype will be recovered if a T-DNA line of the gene of interest is crossed with the parent. If the T-DNA line is a mutant line of a different gene than the causal gene, backcrossing will have no effect on the measured phenotype.

Table 4 Genes included in the allelic complementation test for the QTL on chromosome 2.

Gene	Name	Function
AT2G45770	CPFTSY	LHCP integration into thylakoids
AT2G45940	DUF295	Hypothetical protein
AT2G46410	CAPRICE	Hair-cell differentiation
AT2G46450	CYCLIC NUCLEOTIDE-GATED CHANNEL 12	Defence response, ion channel activity

### DUF295

The phenotype of the mutant line of DUF295 is not statistically different from the NIL and the progeny of a cross between the mutant and the NIL (Figure 35). For C-C and the cross between C-C and the NIL there is also no significant difference with the mutant line. However, there is an absolute decrease in NPQt for C-C compared to the mutant. The cross between Col and the mutant shows a NPQt value in between these lines. The other photosynthesis phenotypes showed patterns similar to NPQt (S 7).

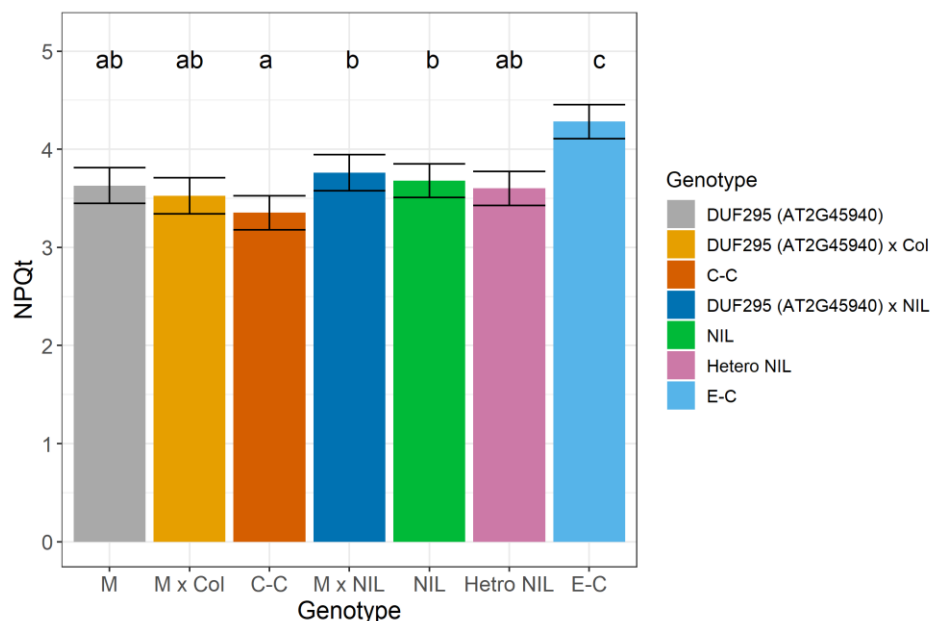


Figure 35 NPQt values of the allelic complementation experiment of DUF for the 6th cycle of 5-minute low and light light alternations. M is the mutant line of DUF295. The black letters on the top indicate the significantly different groups for a tukey post hoc test for comparing different genotypes, with  $\alpha = 0.05$ . 24 replicates were used for the mutant and the crosses. For the other genotypes  $n = 48$ . Error bars indicate the standard errors.

## CYCLIC NUCLEOTIDE-GATED CHANNEL 12

The T-DNA line of CNGC12 does not show significant differences with one of the parents (C-C and NIL) or the progeny of the crosses (Figure 36). The phenotype of the crosses is almost equal to the mutant, which means that both the Col as well as the Ely allele had no effect on the phenotype. The other photosynthesis phenotypes showed patterns similar NPQt (S 8). We can exclude CNGC12 from the candidate genes.

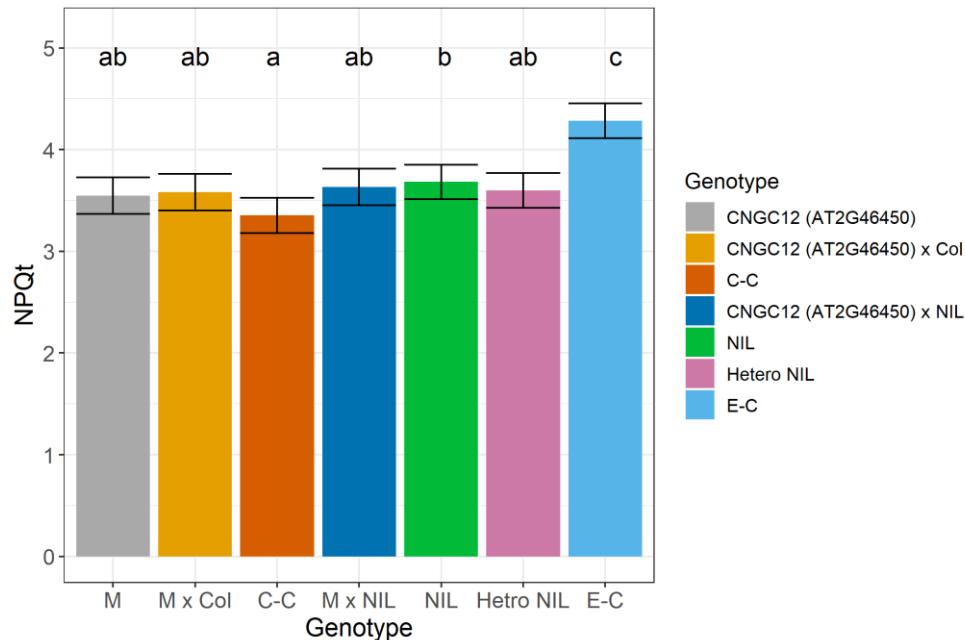


Figure 36 NPQt values of the allelic complementation experiment of CNGC12 for the 6th cycle of 5-minute low and light light alternations. M is the mutant line of CNGC12. The black letters on the top indicate the significantly different groups for a tukey post hoc test for comparing different genotypes, with  $\alpha = 0.05$ . 24 replicates were used for the mutant and the crosses. For the other genotypes  $n = 48$ . Error bars indicate the standard errors.

## CPFTSY

The T-DNA line, the NIL and the NIL crossed to Col show almost equal phenotypes with values of 3.66, 3.68 and 3.67 respectively (Figure 37). C-C is statistically different from the NIL and CPFTSY x NIL, but not from the mutant. CPFTSY x Col shows a NPQt value between the mutant and C-C but is not significantly different from both lines. The other photosynthesis phenotypes showed patterns similar NPQt (S 9).

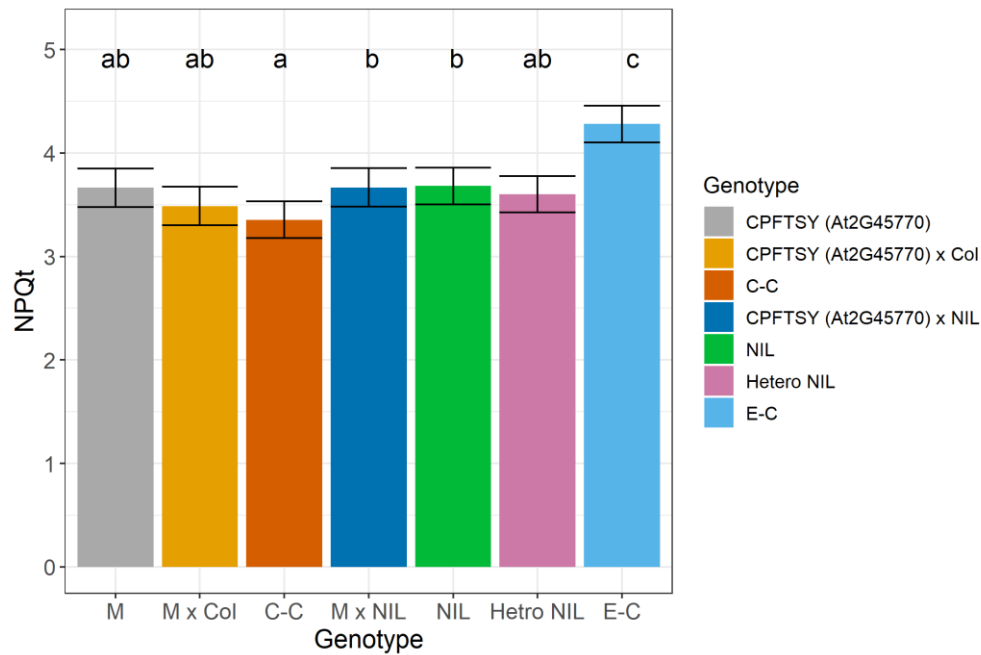


Figure 37 NPQt values of the allelic complementation experiment of CPFTSY for the 6th cycle of 5-minute low and light light alternations. M is the mutant line of CPFTSY. The black letters on the top indicate the significantly different groups for a tukey post hoc test for comparing different genotypes, with  $\alpha = 0.05$ . 24 replicates were used for the mutant and the crosses. For the other genotypes  $n = 48$ . Error bars indicate the standard errors.

#### CAPRICE

C-C is statistically different from the NIL, the mutant and the cross of the mutant and the NIL (Figure 38). The NPQt value of the cross between the mutant and Col is in between the value of the mutant and C-C. The other photosynthesis phenotypes showed patterns similar NPQt (S 10).

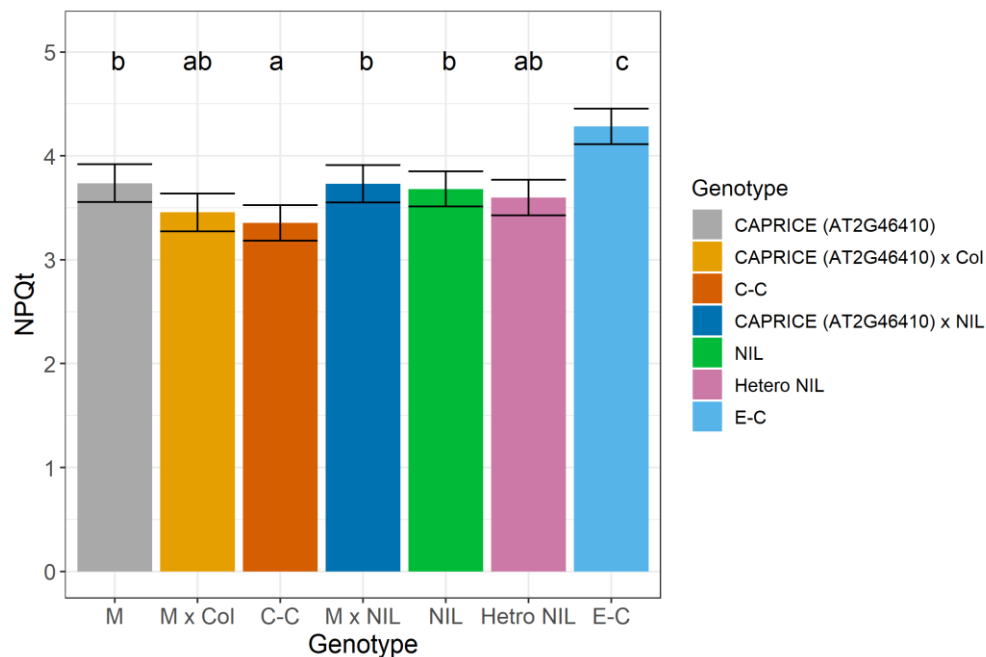


Figure 38 NPQt values of the allelic complementation experiment of CAPRICE for the 6th cycle of 5-minute low and light light alternations. M is the mutant line of CAPRICE. The black letters on the top indicate the significantly different groups for a tukey post hoc test for comparing different genotypes, with  $\alpha = 0.05$ . 24 replicates were used for the mutant and the crosses. For the other genotypes  $n = 48$ . Error bars indicate the standard errors.

All mutants show elevated NPQt values compared to C-C. Only slight differences could be observed, but no significant differences. Strikingly, the heritability of all allelic complementation experiments was above 0.7, which is substantially higher compared to previous experiments. In this experiment the number of replicates per line was 24 or 48.

Because of the higher heritability of the allelic complementation experiment, the dominance of the alleles can be determined. The NIL and the hetero NIL were both significantly different from C-C (Figure 39). This means that the phenotype caused by the chromosome 2 QTL was triggered by the treatment in the Fluctator. E-C is significantly different from all other lines. Since the genotype of E-C differs a lot from the other lines, no further conclusions can be drawn for the phenotypic effect of genes. The hetero NIL and the NIL are not significantly different in NPQt value, and only genotypically differ for the QTL on chromosome 2. From this result we can say that the Ely allele is dominant over the Col allele, because the hetero NIL only has one Ely allele and shows the same phenotype as the NIL, which has two Ely alleles. The increase in NPQt from C-C to the NIL is 9.7%, which is the effect of homozygosity at the chromosome 2 QTL. From the QTL analysis based on the DH lines measured in the DEPI system, an increase of 25.3% was found. When comparing E-C to C-C, the effect size is 27.7%. Most other photosynthesis phenotypes show the same pattern as NPQt (S 11).

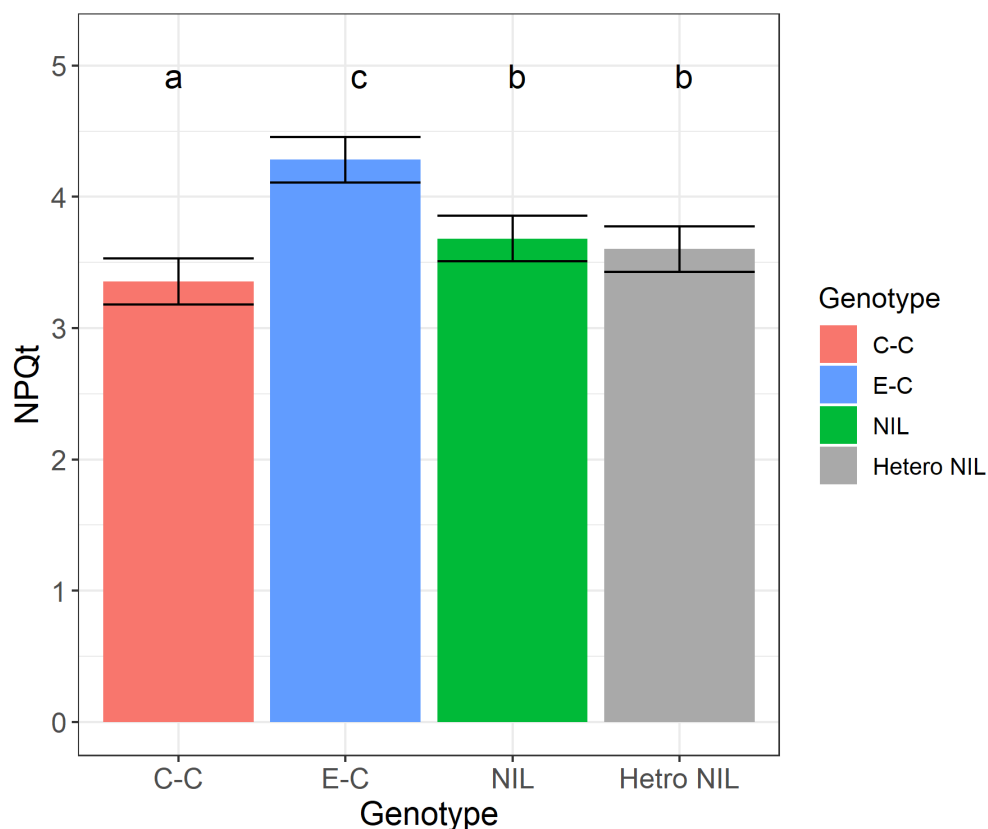


Figure 39 NPQt values for C-C, E-C, NIL and hetero NIL. The black letters on the top indicate the significantly different groups for a tukey post hoc test for comparing different genotypes, with  $\alpha = 0.05$ . For all genotypes  $n = 48$ . Error bars indicate the standard errors.

### The effect of the QTL on chromosome 2 in different growing environments

From the experiment in which C-C, E-C and DH6 were grown in three different growing environments, it became clear that the condition in which plants are grown influences the phenotype in the long term. The QTL on chromosome 2 might only have an effect on phenotype after a prolonged exposure to specific light treatments. Therefore, three different light conditions were programmed in the Fluctor to investigate what the effect is of fluctuating light and the duration of fluctuations. The three different light conditions were a constant light intensity, a fluctuating environment with slow fluctuations (simulation of DEPI fluctuation) and a very fast fluctuating light intensity. For all growing environments, the day length was 16 hours. The light intensity in the constant growing environment was constantly  $438 \mu\text{mol}/\text{m}^2/\text{s}$ . An intensity of  $438 \mu\text{mol}/\text{m}^2/\text{s}$  was chosen, because this was the average light intensity of the DEPI fluctuating light treatment. The light intensity in the DEPI treatment is the same as the fluctuating day that is used in the DEPI chambers. Hereafter, this treatment is referred to as the “DEPI growing environment”. The fluctuating day of the DEPI treatment of MSU was included because the highest effect size is seen for the QTL on chromosome 2 during light fluctuation. The maize growing environment is a highly fluctuating light pattern, based on light intensity measurements inside a maize canopy. The measurement was performed during the late summer and a day with highly fluctuating light intensities was chosen. A light sensor was attached to one of the leaves to measure the effect of both shading of leaves and clouds. Hereafter, this treatment is referred to as the “maize growing environment”. The maize growing environment was included because of the correlation between NPQ and qE, the fast response to changing light intensities, in the data from MSU and because the effect size of qE was even larger than of NPQ.

Within the different growing environments, C-C and the NIL are not significantly different in NPQt value (Figure 40). The effect sizes of the NIL compared to C-C were respectively 1.3%, 4.3% and -3.3% for the constant, DEPI and maize growing environments. E-C is significantly higher in each growing condition. The effect sizes of E-C compared to C-C were respectively 43.0%, 31.9% and 65.9% for the constant, DEPI and maize growing environments. When comparing the genotypes between the constant and the DEPI growing environment, NPQt values are higher in the constant growing environment than in the DEPI growing environment. The NPQt values in the maize growing environment are a bit lower for C-C and the NIL compared to the constant growing environment. However, the average light intensity differed between these light treatments and therefore we cannot conclude if this is because of the difference in fluctuation or in average light intensity. Most other photosynthesis phenotypes show the same pattern as for NPQt (S 12).

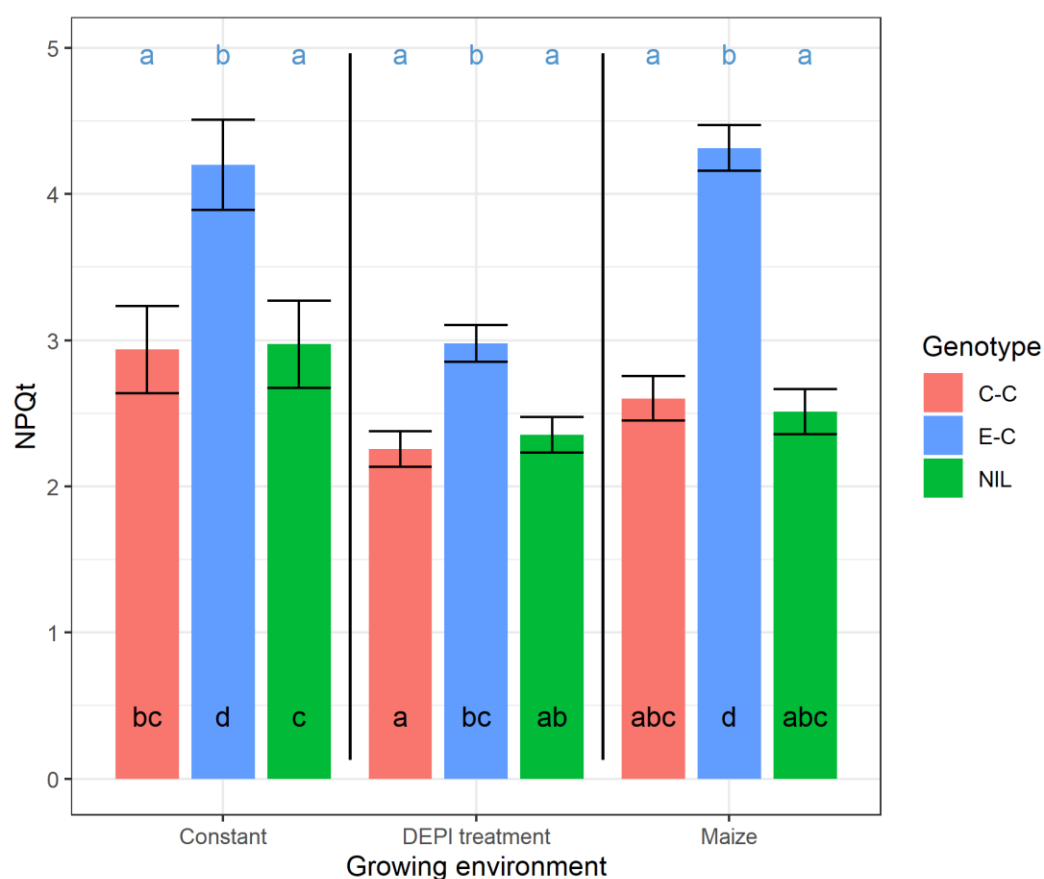


Figure 40 NPQt values for C-C, E-C and the NIL in different growing environments. The constant environment is a constant light intensity of 430 and the DEPI growing environment is the same light treatment as was used in DEPI experiments. The light intensity in the maize growing environment is based on measurements outside during a highly fluctuating day. Blue letters on the top indicate the significantly different groups for a tukey post hoc test for genotypes within a growing environment. The black letters on the bottom indicate the significantly different groups for a tukey post hoc test for interaction between genotypes and growing environments.

The patterns for the dry weight of different genotypes is similar for the constant and DEPI growing environment (Figure 41). However, in the DEPI growing environment C-C shows a trend towards a lower value, and E-C and the NIL towards a higher value compared to constant light. These differences are not significant, but there is a trend that E-C and the NIL perform relatively better under fluctuating conditions versus constant than C-C. The dry weight in the maize growing environment is the lowest for all genotypes. The average light intensity was lower in the maize growing environment, namely 418  $\mu\text{mol}/\text{m}^2/\text{s}$ , compared to 438  $\mu\text{mol}/\text{m}^2/\text{s}$ . In all growing environments, C-C has the highest dry weight, except for the maize growing environment. The NIL obtained the highest biomass, although it is not significantly different from C-C. The biomass of the NIL is 5% higher than the biomass of C-C.

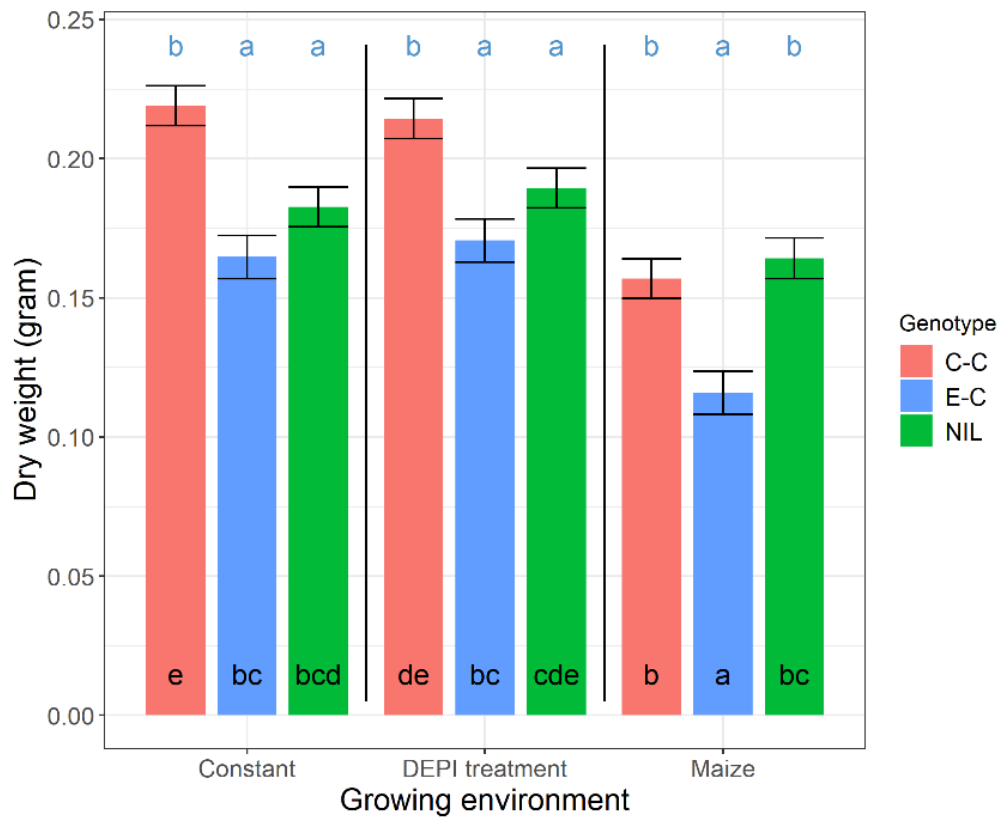


Figure 41 Dry weight of C-C, E-C and NIL in different growing environments. The constant environment is a constant light intensity of 430 and the DEPI growing environment is the same light treatment as was used in DEPI experiments. The light intensity in the maize growing environment is based on measurements outside during a highly fluctuating day. Blue letters on the top indicate the significantly different groups for a tukey post hoc test for genotypes within a growing environment. The Black letters on the bottom indicate the significantly different groups for a tukey post hoc test for interaction between genotypes and growing environments.

#### Mapping the phenotype as was measured in the Robin with the dominance experiment

An experiment was performed in which the dominance of the alleles of the QTL on chromosome 2 was investigated (Figure 27). These plants were grown under a constant light intensity and consequently measured in the Robin with 12 cycles of 5-minute alternating low and high light intensity. No difference in NPQ was observed between C-C and the NIL but there was a difference between E-C and C-C. This means that a different gene than the one responsible for the effect of the QTL on chromosome one causes the different phenotype. It can partly be explained because of the epistatic interaction between the Ely alleles on chromosome 2 and 4. However, this cannot fully explain the difference between E-C and C-C. Therefore, I setup an experiment to identify which QTL causes the difference in NPQ between C-C and E-C for the measurement with the 12-cycle protocol in the Robin. Due to the limited capacity of the Robin it was not possible to include all DH lines and therefore I selected 13 DH lines from the DH population. DH lines were only selected if the QTL on chromosome 2 was Col and the total set of DH lines had to represent both E-C and C-C alleles at the other chromosome segments.

The QTL map of the DH lines does not show significant QTLs at the 12<sup>th</sup> measuring cycle under high light (Figure 42). However, the beginning of chromosome 3 does show a trend towards significantly different qE values. The BLUEs of the NPQt values for the different DH lines are all not significantly different from each other (Figure 43). This is probably due to the low heritability of the traits. The heritability values were between 0% and 9%.

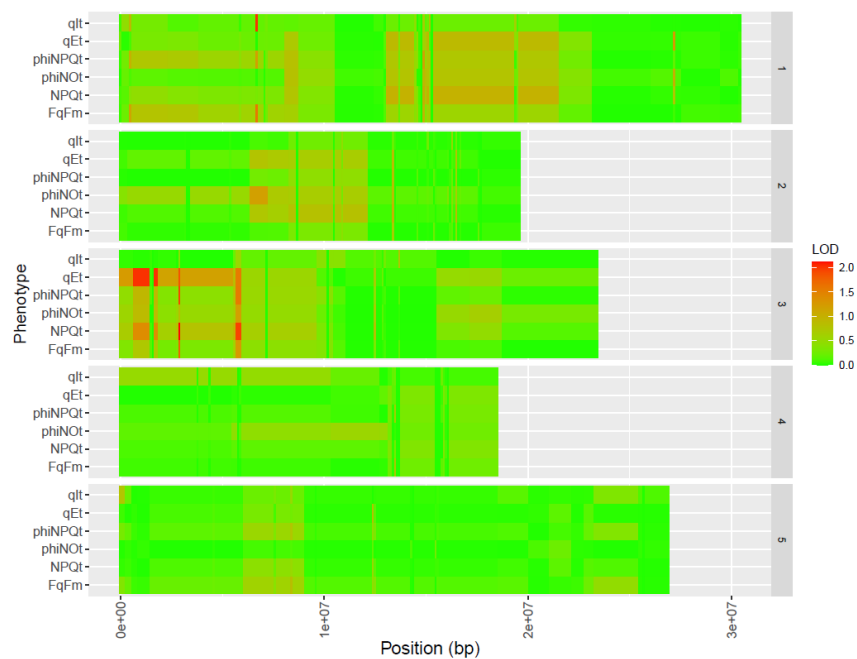


Figure 42 QTL map to map the Robin phenotype. Scanone QTL mapping was performed on the data of the 12<sup>th</sup> cycle with 5-minute interval alternating low and high light. 13 DH lines and C-C and E-C were included in the analysis. Six different photosynthesis phenotypes were mapped. No QTLs exceed the LOD threshold that was calculated based on a permutation test.

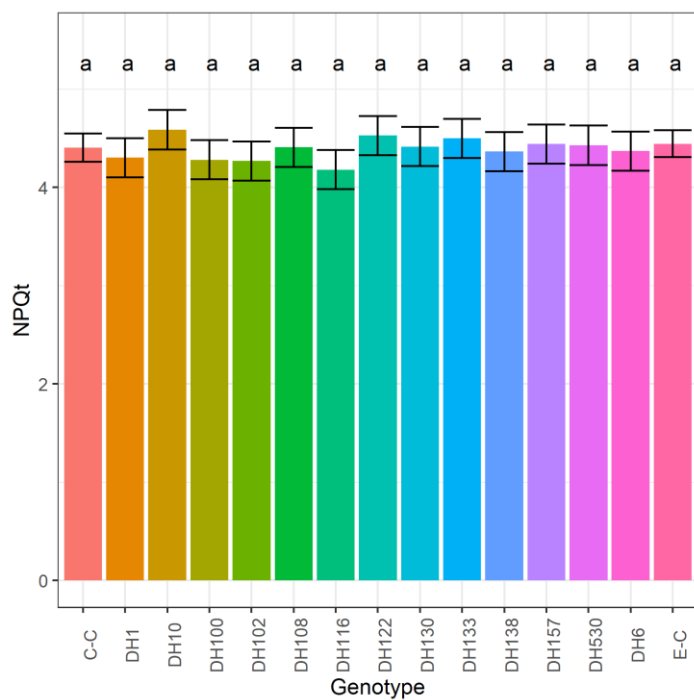


Figure 43 BLUEs of NPQt values for the different DH lines at the 12th cycle of 5-minute interval alternating low and high light.

## The effect of growing environment and acclimation on NPQ

In all the photosynthesis phenotyping experiments NPQ values differed for the same genotype because the measurement of NPQ is influenced by many different factors. The light intensity was either constant, slowly fluctuating or highly fluctuating. Besides the effect of the growing environment, I researched the effect of acclimation. Table 5 shows a summary of the differences in NPQ between E-C and C-C and between the NIL and C-C for the experiments performed during this thesis. With these values, the effect of the chromosome 2 QTL on NPQ could be examined as well as the fraction of the E-C phenotype that is explained by the QTL on chromosome 2. For the DEPI phenotyping data, there is only an effect of the allele on chromosome 2 measured, because a NIL was not available when the experiment was performed.

*Table 5 Effect size of NPQ of E-C and the NIL compared to C-C in percentage, using different growing environments, measuring protocols and phenotyping machines. Acclimation in the Fluctor means that the plants were sown in the Fluctor and grown there during the whole growing period up until just before measuring in the Robin. Short acclimation in the Fluctor was done by growing plants under a constant light intensity and exposing the plants for 2 or 3 days to the DEPI treatment in the Fluctor just before measurement in the Robin.*

Effect size NPQ	E-C vs C-C (%)	NIL vs C-C (%)	Effect of the Ely allele on chromosome 2 (%)	Fraction of E-C phenotype explained by QTL
Robin (dominance, 12 cycles)	16.9	0.8		4.8
DEPI (NPQ)	28.1		17.3	61.7
Fluctor constant (acclimation)	43.0	1.3		2.9
Fluctor DEPI (acclimation)	31.9	4.3		13.5
Fluctor Maize (acclimation)	65.9	-3.3		
Fluctor DEPI allelic complementation (short acclimation)	27.7	9.7		35.2

## Discussion

Photosynthesis is a complex trait because many genes and environments affect different photosynthesis phenotypes. To break down this complexity, the aim of this thesis was to study the natural variation for photosynthesis by a biparental population. This experiment resulted in many photosynthesis related QTLs. The potential of developing a NIL was investigated for identifying the effect of single photosynthesis genes. A NIL should be able to isolate one of the genetic effects on a photosynthesis phenotype to be able to study the physiology behind the genetics. Besides this, the gene of interest can be isolated to be studied in other experiments. One of the physiological questions that I wanted to answer was under which light conditions the high NPQ phenotype of the QTL on chromosome 2 will be observed. Next to the research on the physiological effect of the QTL, the gene was fine mapped by selecting and phenotyping recombinant lines.

As photosynthesis traits are influenced by nuclear as well as chloroplast genetics, epistatic interactions between the two make it complicated to study the effects separately. There is less variation between different plasmotypes because mutation, which only occur sporadically, is the main source of variation. Plasmotypes do not recombine during meiosis because it is maternally inherited. Therefore, plasmotypes are relatively conserved, which makes it interesting to study the adaptation of the nucleotype to plasmotype mutations. To study both epistatic interactions and nuclear adaptation to plasmotype mutations, Flood et al. (2020) studied cybrids, developed with inducer lines. By comparing the different combinations of nucleotypes and plasmotypes, the effects of both can be studied separately. One of the lines included in this cybrid panel was Ely, which has a mutation in the chloroplast gene *PsbA*. This mutation in *PsbA* results in lower NPQ caused by the Ely chloroplast compared to other chloroplasts, however the Ely nucleus counter acts this with a higher NPQ. To study if this nuclear effect has evolved from an adaptation to the chloroplast mutation a DH population was made by (Theeuwes and Logie, unpublished). This population was phenotyped in the DEPI system because plants could be measured at the exact same time with high throughput and in an artificial environment. This made it possible to measure the response of many plants to a controlled changing environment. Besides, the nuclear effect of Ely on NPQ was previously shown in DEPI and we wanted to change the way of phenotyping.

The DH population was used to map the high NPQ phenotype of Ely. First of all, this showed that many QTLs are involved in NPQ and other photosynthesis phenotypes and that the Ely alleles of these QTLs result in high as well as low NPQ values. Secondly, a highly significant QTL was found on chromosome 2, which also has a large effect size. Little previous research has been performed on the genetics behind NPQ under fluctuating light conditions. A GWAS study found several QTLs, among which *PsbS*, that was already shown to be involved in the mechanism of NPQ (Rungrat et al., 2019). By making use of a DH population, we increased the statistical power for identification of QTLs because of balanced allele frequencies. This made it possible to find more significant QTLs with a higher confidence. Biparental populations have been used before to study NPQ, Jung and Niyogi (2009) studied NPQ with an F2 mapping population and found a QTL on chromosome 2. However, this QTL is 37 cM, which is almost half the chromosome. It might be the same gene of interest underlying the QTL that we found, but it can also be a completely different gene. The QTL defined by Tom Theeuwes was already much smaller with a size of 1.1 cM. Jung and Niyogi (2009) did not study this QTL further to find candidate genes and the physiology of the gene of interest in relation to NPQ. There is still a lot unknown about how NPQ works, and which different mechanisms are involved in NPQ and how these mechanisms interact with each other. By further fine mapping of the gene of interest the physiology can be studied by reverse genetics. This has the potential to determine the pathway(s) that are involved in NPQ.

Why are we even looking at NPQ? In photosynthesis, energy absorbed from photons can have different destinations and these destinations are in competition with each other. One of the destinations is photochemistry, which results in energy production that can be used by the plant to grow. NPQ is the loss of energy in the form of heat, which means that this energy is not used by the plant. Since we want plants to be more productive to be able to feed the ever-growing world population, we want to reduce the amount of energy lost in the process of photosynthesis. This means we have to reduce the amount of energy lost in the form of heat, however NPQ is also useful in protecting plants from damage by high light intensities. Preventing damage to photosystems will increase the photosynthesis efficiency under fluctuating conditions because it reduces damage to the photosynthetic apparatus. To improve photosynthesis efficiency, it is important to understand the mechanism of protection by NPQ and the way that the plant perceives environmental stresses and consequently induces NPQ. Previously, Kromdijk et al. (2016) showed that the NPQ response can be accelerated and consequently increases dry matter production. However, no screening has been performed to find other response mechanisms to fluctuating light intensities.

### NPQ induction and relaxation

The QTL on chromosome 2 that was found by QTL mapping with the DH population was also found for qE, which is the fast mechanism involved in NPQ. This suggests that the QTL on chromosome 2 is involved in the first phase after a change in light intensity, which is relaxation and/or induction of NPQ. Although we do not know if the QTL found by Jung and Niyogi (2009) is the same as the QTL found for the DH population, they showed that their QTL was also involved in qE. Justine Drouault showed that the parents, E-C and C-C, differed in speed of NPQ relaxation. Previously, not only differences in NPQ relaxation, but also in induction were shown (Hubbart et al., 2012; Li et al., 2002; Zia et al., 2011). It was decided to redo this experiment and next to relaxation, I also measured NPQ induction. Interestingly, there were no consistent differences in the speed of both relaxation and induction between the parental lines. These plants were grown under constant conditions in a climate chamber and the QTL was not found in DEPI for days with constant light conditions the hypothesis was that the high NPQ phenotype was not triggered due to lack of fluctuating light. Therefore, it was decided to measure plants that were grown under fluctuating light conditions outside. The experiment showed that C-C and E-C plants grown in the tunnel did differ for both NPQ relaxation and induction. To investigate if the QTL on chromosome 2 was responsible for this difference, a near NIL, DH6, was included in the experiment. DH6 did not show a significant difference in NPQ relaxation or induction compared to C-C, while E-C did differ from C-C. At the beginning of the NPQ relaxation, few significant differences were found, however, NPQ of DH6 was always higher than C-C and not lower as expected with faster relaxation. From this result, it can be concluded that the QTL on chromosome 2 is not responsible for the difference in NPQ relaxation or induction between E-C and C-C. There are two possible explanations. There might be another fast mechanism, apart from relaxation or induction, that is influenced by the QTL on chromosome 2, or the difference in relaxation and induction is caused by an epistatic interaction between two genes. This should be studied further. Furthermore, it also remains unclear if relaxation and induction is induced by the same mechanism. There was also a small defect in the measurement of induction, which might have influenced the results. This is because NPQ induction was measured directly after NPQ relaxation. For this experiment, six different blocks were measured every 30 seconds, with the first measurement of each block five seconds after the other. Therefore, the time between the last saturating pulse used for the measurement of NPQ relaxation and the switch from low to high light differed between the blocks. This could have influenced the later measurements during induction. Justine Drouault showed larger differences between C-C and E-C

during relaxation. It is proposed to repeat the experiment and measure relaxation and induction in separate experiments.

#### The effect of different growing environments

We have seen that NPQ induction and relaxation behaves differently for plants that were grown under different environmental conditions. This raised the question whether dry weight is also affected by differences in growing conditions and if this difference is caused by the QTL on chromosome 2. Therefore, C-C, E-C and DH6 were grown in three different environments. In the climate chamber the light intensity is constant, in the greenhouse relatively constant and in the tunnel highly fluctuating. The dry weight of DH6 did not differ from C-C in any of the growing environments, which means that there was no effect of the QTL on chromosome 2 on dry weight in all growing environments. E-C does show a difference in dry weight for the tunnel and the greenhouse compared to the other genotypes. Many nucleotypic effects can underly this difference. When comparing dry weight over the different growing environments, the dry weight in the tunnel is higher for all genotypes. The plants in the tunnel were grown for a longer period with shorter day lengths. This resulted in a longer vegetative phase before bolting and the plants were harvested later. The vegetative phase for *A. thaliana* is relatively short compared to crops. Therefore, it would be interesting to study the effect of a longer vegetative growing phase by growing *A. thaliana* with shorter day lengths in the greenhouse and climate chamber. Under these conditions there will be potentially more differences in dry weight. In previous research overexpression of *VDE*, *Psbs* and *ZEP* was shown to increase tobacco dry weight with 15% (Kromdijk et al., 2016). However, dry weight is a snapshot measurement and does not evaluate development over time. In the Robin, leaf area can be measured, which has previously been shown to highly correlate with dry weight (Poorter & Remkes, 1990). Measuring leaf area over time can give more insight in the development of biomass production. It would also be interesting to look into the relationship between the shoot and the root biomass. This would give a more precise idea about the total amount of biomass produced, however this is a destructive measurement and can only be done once.

#### The complexity of photosynthesis phenotyping

As mentioned before, photosynthesis is a complex process. The QTL mapping data from the DEPI system shows that many different genes underly different photosynthesis parameters. This research shows that next to the light protocol used for the measurement, the growing environment also has an effect on photosynthesis phenotypes. The QTL on chromosome 2 was mapped based on measurements in the DEPI system, but measurements in the Robin with an adapted measuring protocol, did not show higher NPQ for DH6 compared to C-C. This gives an extra dimension to the complexity of a genotype x environment interaction. The way of measuring and the environment before the measurement also has an influence on the phenotype. Keller et al. (2019) also noticed that most photosynthesis research is performed under constant conditions, while these are not representative of the outside growing conditions in which most crops are grown. They established the new photosynthesis parameters,  $Fr2/F_v$  and  $Fr2'/F_q'$ , which account for fluctuating light conditions. Rascher & Nedbal (2006) also stressed the importance of understanding photosynthesis under fluctuating light conditions as this represents natural outside conditions better. It is important to simulate specific natural conditions because we showed that the measuring protocol has an effect on the results, and we eventually want our crops to grow better under natural conditions. High-throughput phenotyping platforms, such as DEPI, can contribute to a better understanding of responses to acclimation of different light fluctuations. Next to the interaction of genotype x environment x acclimation, photosynthesis response can be measured with different phenotypes. For

example, NPQ, qE and  $\Phi$ PSII. Therefore, we should include another parameter to our interaction which leaves us with an interaction term of genotype x environment x acclimation x phenotype.

The complexity of phenotyping photosynthesis and the influence of acclimation can be illustrated by different experiments in this research. For example, no difference between DH6 and C-C was measured with a relatively short measuring protocol of 12 cycles of alternating low and high light in the Robin. However, when the fluctuating light treatment of a whole day as in DEPI was applied during measurement in the Robin, this difference was present. The Robin can only measure a tray of 20 plants at a time, which means that the DEPI treatment cannot be used for measuring in a high throughput fashion. For this reason, the Fluctor was build. This is a climate chamber, in which 3 separate light conditions can be programmed. There is full control of light intensity within a range of intensities and sub second level control. The difference between the short and long phenotyping protocol is one of the many comparisons that we made from the different experiments in this study related to the effect of acclimation and the measuring protocol that was used. To illustrate the effects, Table 5 summarises all previously mentioned effect sizes of E-C, the NIL or the Ely allele on chromosome 2 for different acclimations and measuring protocols. As we see, both the whole Ely nucleotype as well as the QTL on chromosome 2 respond differently for different growing environments. Furthermore, the part of the E-C phenotype that was caused by the QTL on chromosome 2 differed between environments. For example, in the maize growing environment in the Fluctor, the E-C nucleotype increased NPQ most, but the NIL resulted in a decrease in NPQ. The effect size of the Ely allele on chromosome 2 in DEPI explained more than half of the phenotype of E-C. This is the highest fraction of all growing environments. The NIL will be soon measured in the DEPI system, which will answer the question if this was due to epistatic interactions or a highly controlled way of measuring many plants simultaneously.

To break down the complexity of photosynthesis it is useful to know which genes underly different photosynthesis phenotypes. To eliminate the potential effect of Ely alleles of other genes, it was decided to develop a NIL for the QTL on chromosome 2. NILs are shown to be more useful for mapping than a RIL, because it eliminates epistatic effects and masking effects of major QTLs (Keurentjes et al., 2007). The NIL was developed by selfing of the selected plants in each generation. It would have been more efficient to backcross the selected plants in the F1 to C-C. By doing this, less plants should have been screened in the F2. To produce a NIL from DH6, only two Ely chromosome segments had to be replaced with Col. Backcrossing to C-C would significantly speed up the production of a NIL from a DH line of which more chromosome segments should be substituted by the other parent. In the F3 of a cross between DH6 and C-C a NIL, with a homozygous Ely introgression for the QTL on chromosome 2 in a background of C-C. This NIL was used for physiological experiments. During development of the NIL also heterozygous recombinants in the QTL were selected in the F3. In the next generation homozygous recombinants were selected for fine mapping.

### Fine mapping

The recombinants that were selected during the development of the NIL were used for fine mapping. The theory behind fine mapping is that the recombinant lines recombine in different positions in the QTL. After phenotyping the recombinants, the genotype and the phenotype of the lines can be used to determine which exact QTL region is responsible for the phenotype. This can reduce the number of candidate genes significantly. Prior to fine mapping, the size of the QTL was 50,000 bp. 17 of the recombinants were found to recombine within this region. This leaves us with a theoretical resolution of 3,041 bp. The distance between the markers differs, but the two closest markers are at a distance of 3,783. To achieve the theoretical resolution, more markers should be tested.

Because the Robin has a limitation of 20 plants, the measuring protocol for fine mapping could not be very long because this reduces the number of plants that can be measured. The DEPI treatment in the Fluctor was shown to increase the differences in NPQ between the NIL and C-C compared to no priming in the Fluctor before measuring in the Robin. Therefore, it was decided to acclimate the recombinants in the DEPI treatment in the Fluctor before being measured in the Robin. QTL mapping of the different phenotypes of the recombinants for the QTL on chromosome 2 only showed significant differences between Col and Ely markers for the photosynthesis phenotype phiNOt. NPQ is the ratio of phiNPQ and phiNO, which means that NPQ is partly determined by phiNO (Kramer et al., 2004). phiNOt is an approximation of phiNO and it was therefore decided to continue the fine mapping with phiNOt (Tietz et al., 2017). Later on, the relevance of NPQ and its components phiNO and phiNPQ will be discussed. The data showed 6 markers with a LOD score above the threshold. One of these markers was positioned outside the QTL region of 50,000 bp. This was one of the reasons to exclude this marker for the fine mapping region. The other reason is that markers close to the gene of interest will automatically show higher LOD scores due to linkage. This leaves us with a QTL size of 25,709 bp, which is almost half the size of the previously defined QTL.

There are two ways to optimize the fine mapping:

1. More marker data can define the position of the recombination in the recombinant with higher precision.
2. Higher heritability of the photosynthesis traits by a controlled environment and a prolonged measuring protocol.

Heritability of the photosynthesis phenotypes was low. When referring to heritability, we mean repeatability. In more detail, this is how much of the variance is explained by the genotype and therefore how high observations over different experiments correlate (Falconer & Mackay, 1996). For example, the heritability of phiNOt at the 4<sup>th</sup> measuring cycle was only 7.6%. Low heritability was observed for multiple experiments that were performed in the Robin. The phenotyping experiments in DEPI showed much higher heritability, 54% for NPQ and 70% for qE. It is expected that the movement of plants to the Robin and the short duration of the measuring protocol resulted in low heritability. To measure the plants, I had to transport them from a climate room to the Robin. The plants were sometimes exposed to low outside temperatures and besides, plants are not used to being moved. Results from the 4<sup>th</sup> measuring cycle were used for fine mapping. However, some recombinants were measured for 8 cycles. These results show that heritability increases with the number of cycles. The data from the DH population that was phenotyped in the DEPI system also shows increasing heritability values over time. Higher LOD scores might have been observed with a prolonged measuring protocol. The homozygous recombinants will soon be phenotyped in the DEPI system at MSU, which is a controlled environment, and the plants will be measured over multiple days. I expect that results with higher heritability will be obtained and fine mapping will reduce the number of candidate genes.

### Candidate genes

With the currently available data, a qualitative analysis was performed. Two different lines were used to identify the left and right border of the QTL. This reduced the number of candidate genes in the QTL with three. However, the differences between the recombinant lines were not large and both conclusions were drawn based on only one observation. Because of low confidence I decided that the size of the QTL was not reduced by the qualitative analysis. This leaves us with nine candidate genes within a region of 25,709 bp. A *de novo* assembly of Ely was recently performed with ONT data.

Developments of ONT make it easier and cheaper to make *de novo* assemblies. This makes it possible to compare genotypes of different accessions without a reference. The QTL was screened for structural variation between Ely and Col with the *de novo* assembly of Ely. No large structural variation was observed. Therefore, the candidate genes in the QTL region were analysed for genotypic differences with a SnpEff dataset based on Illumina sequencing data. SNPs and INDELs with moderate or high impact effects on functionality were selected. In five different genes, CPFTSY, RH6, DTA2, DUF821 and AHL9, a moderate impact SNP or INDEL was found. With PROVEAN a prediction was made for the effect of the substitution on protein function. No substitution was predicted to have a deleterious effect on protein function.

Both CPFTSY and AHL9 had a serine to proline substitution. Proline is an imino acid, because the side group is connected to the backbone twice. Proline is unable to form a helix conformation and often induces kinks into  $\alpha$ -helices (Barnes & Gray, 2003). Next to this potential effect on protein structure, serine is polar and proline is non-polar. Polar AAs are often found on the outside of a protein and non-polar AAs on the inside. However, due to the small size, serine is often found within tight turns on the protein surface and able to form a hydrogen bond between the backbone and the hydroxyl oxygen side chain (Barnes & Gray, 2003). The non-synonymous substitution in DTA2 changes the AA from a phenylalanine to a leucine. Both AAs are non-polar, however phenylalanine is aromatic and leucine is aliphatic. Both proteins are often found in hydrophobic cores (Barnes & Gray, 2003). DUF821 has a substitution of serine to asparagine (Barnes & Gray, 2003). Both AAs are polar and are relatively small, therefore they are not expected to have a large effect on protein function.

Next to the moderate impact SNPs and INDELs, there are two deletions that were scored as a modifier. One of the deletions is a 35 bp deletion, 337 bp upstream of CPFTSY. The other deletion is 26 bp and 201 bp upstream of PMM. The promotor region of a gene can be up to 1000 bp upstream of the gene. These deletions will not alter the protein structure but can potentially affect gene expression. Therefore, it would be interesting to do either RNAseq or RT-qPCR on C-C and the NIL before and after a fluctuating light treatment. Interestingly, these deletions in the promotor regions were not called as moderate or high impact deletions by SnpEff, although they can affect gene expression and therefore protein abundancy. From this it can be concluded that SnpEff cannot always correctly predict the impact of modifications in the promotor region.

### The dominant allele

An allelic complementation experiment was performed in which a high number of replicates were used. The NIL, hetero NIL, C-C and E-C were included in this experiment. Because of high heritability of this experiment, the dominance of the allele on chromosome 2 could be determined. It was concluded that the Ely allele for chromosome 2 is dominant. The NIL and the hetero NIL show a higher NPQt phenotype compared to C-C. The NIL is homozygous Ely and the hetero NIL is heterozygous. Since there is no significant difference in NPQt between the NIL and hetero NIL, we can conclude that the Ely allele is dominant. The same data shows that E-C has a higher NPQt than the NIL and the hetero NIL. This means that the QTL on chromosome 2 cannot fully explain the phenotype of E-C. Other QTLs or epistatic effect will influence the phenotype of E-C, next to the QTL on chromosome 2. It was shown that the QTL on chromosome 4 has a negative epistatic effect on NPQ. Next to these two QTLs, three other QTLs were found in the QTL analysis that might explain the higher NPQ phenotype of E-C. Development of NILs of these QTLs and lines that contain combinations of QTLs will explain more about additive and epistatic effects of these QTLs. To check if more epistatic interactions might cause the higher NPQ phenotype for E-C, a scan two analysis was performed. Multiple epistatic interactions were found besides the interaction between the QTLs on chromosome 2 and 4. The analysis was only

performed on timepoint 101.5214. An efficient way of analysing these epistatic interactions through time should be found. Manually analysing all 2533 phenotypes would be too laborious, however these epistatic interaction analyses could be very insightful. Analysing the genetics of a trait by GWAS populations often results in “missing heritability”. This means that only a minor part of the heritability is explained by the genotype. One of the main causes of “missing heritability” is probably the presence of epistatic interactions (Zuk et al., 2012). In a GWAS populations alleles are often not equally represented within the population, therefore there is lack of statistical power to identify the effect of epistatic interactions. A DH population is very powerful to research epistatic interactions because it is assumed that the different alleles, as well as all possible interactions are balanced within the population. Furthermore, there can also be 3-way or higher-level interactions between genes that can influence the phenotype. These higher-level interactions can also be determined by phenotyping a DH population.

### Allelic complementation

At the start of this thesis, 4 different genes were selected for the allelic complementation experiment. This selection was based on differential expression of genes in the QTL region, detected by RNAseq. The theory behind allelic complementation is that if the T-DNA line is a knock-out of the gene of interest, crossing a mutant line with the parent would recover the phenotype of the mutant. Allelic complementation is more difficult if the allele of the researched genotype is dominant, which is the case for the QTL on chromosome 2 (Turner, 2014; Weigel, 2012). The only gene included in the allelic complementation experiment that is within the QTL region that was defined with fine mapping is CPFTSY. The phenotype of the NIL is almost equal to the mutant line. This suggests that the Ely allele is a knock-out of the gene. The Ely allele was shown to be dominant over the Col allele. Combining both conclusions would mean that the knock-out is dominant. This is seldomly seen and invalidated by the phenotype of the T-DNA line x Col (Meinke, 2013). If the knock-out would be dominant, this genotype would have the same phenotype as the T-DNA line, which is not the case. Another possibility is that the Ely allele is dominant, but not a knock-out. Coincidentally, the same phenotype arises from a knock-out as from the Ely allele. The intermediate phenotype of the T-DNA line x Col could be due to a dosage effect of the knock-out and the functional Col allele. However, most lines are not significantly different in NPQt, therefore it is difficult to conclude if CPFTSY is the gene in the QTL that is responsible for the high NPQt phenotype.

If we look at the other knock-out lines that were used for the allelic complementation experiment, for CAPRICE as well as for DUF295, similar patterns are seen as for CPFTSY. These genes are not within the QTL region and therefore not expected to cause the high NPQt phenotype. However, knocking out these genes also shows elevated NPQt values. This suggests that the knock-outs of these genes have an effect on NPQt, even if this gene might not be involved in an NPQt pathway. As these results do not seem logical, a few shortcomings in this allelic complementation experiment are described. First of all, the T-DNA lines were not checked for gene expression. It could be that the knock-out was not efficient and the phenotype of the T-DNA line does not correlate with a real knock-out line. Furthermore, many T-DNA lines were shown to have structural variation compared to Col that could influence the phenotype of the mutant (Pucker et al., 2021). One of the options mentioned for the allelic complementation experiment for CPFTSY is that the Ely allele could be a knock-out. To check this, gene expression of CPFTSY should be also checked for in the NIL. Because of the many uncertainties around T-DNA lines, it would be better to induce a knock-out of the gene by CRISPR. CRISPR can also be useful to check if the Ely allele is a knock-out, by comparing a CRISPR induced knock-out of CPFTSY in the NIL, a knock-out in Col and the unchanged NIL and Col. Another shortcoming is the small differences

between the phenotypes. In the Robin, the NPQ phenotype is not triggered to the same extent as in the DEPI chambers. The lines that were included in the allelic complementation experiment of CPFTSY will soon be phenotyped in the DEPI system. This will probably result in larger differences, which might confirm if the mutant phenotype is the same as the Ely phenotype. In allelic complementation experiments, often multiple genotypes are included to check if the phenotype of two different genotypes with the same phenotype is caused by the same gene (A. D. Long et al., 1996; Weigel, 2012). However, there are no other nucleotypes known to have the same effect on the NPQ phenotype as the Ely nucleotype.

### Genes involved in photosynthesis

After fine mapping, the region of the QTL still includes nine candidate genes. Two genes have previously been shown to be involved in photosynthesis. However, the other genes are equally important as a candidate gene. Picking candidate genes is prone to biases and therefore the other genes cannot be excluded (Baxter, 2020). To get to a functional understanding of the genetics behind a phenotype, it is important to know the gene responsible for a phenotype. It is important to reduce the number of candidates, for example by fine mapping, to a number that is feasible to research separately. In this way human bias can be eliminated. Without being biased in excluding the 7 other genes, I would still like to discuss the function of PMM and CPFTSY, which were shown to be involved in photosynthesis pathways in previous research. CPFTSY is involved in the PSII repair cycle and PMM is involved in ascorbate biosynthesis.

Studies with *cpftsy* mutants showed that plants lacking CPFTSY have a reduced photosynthesis related protein content (Asakura et al., 2008; Walter et al., 2015). Walter *et al.* (2015) found out that *cpftsy* mutants result in an impaired replacement of damaged D1 proteins. It is suggested that translating ribosome binding to the thylakoid membrane is hindered, which results in reduced insertion of D1 into the thylakoid membrane during PSII repair. Asakura *et al.* (2008) determined protein concentration of photosynthesis related proteins in *cpftsy* mutants. They found that LHC concentration was reduced to 10-33% compared to the wild type concentration. Also, cytochrome f (Cyt f) concentration, which is a subunit of cytochrome b6f, was reduced to 10% and reduction in PSI and PSII concentration was observed. All these proteins are involved in the light harvesting process of photosynthesis or in the ETC. As was suggested before, the Ely allele could be a knock-out, which would result in less protein production. Consequently, the Ely allele result in less LHCs, which in turn means lower light capture under high light intensity. The RC of PSII will therefore have a lower chance of damage, which might be an advantage under fluctuating conditions. It does seem that Ely performs relatively better under fluctuating conditions compared to C-C. However, to prevent bias in picking candidate genes, the QLT region should be further decreased and all candidate genes that will be left should be investigated separately. If fine mapping of the recombinants in the DEPI system results in CPFTSY being a likely candidate gene, it would be interesting investigate photosynthesis related protein concentrations. If repairment of PSII is impaired due to the ribosome binding to the thylakoid membrane, RNAseq data would not give explain the difference in phenotype. However, translation is hindered and therefore protein concentration differences might give more insight in the differences. Protein concentrations of the NIL and C-C should be compared before and after a fluctuating light treatment.

PMM is shown to be involved in ascorbate biosynthesis, this pathway influences photosynthesis (Ntagkas et al., 2018). Ascorbate is an antioxidant and a scavenger of ROS, which is produced during high light intensities and can result in photodamage (Akram et al., 2017). High light intensities and higher photosynthesis rates correlate with accumulation of ascorbate (Ntagkas et al., 2018). Eskling *et al.* (1997) showed that ascorbate is a cosubstrate of VDE, which causes de-epoxidation of violaxanthin

to zeaxanthin. Zeaxanthin increases fast changes in NPQ (Kromdijk et al., 2016). As ascorbate can protect plants to photodamage and is shown to be involved in de-epoxidation of violaxanthin to zeaxanthin, PMM might be involved in a different NPQ phenotype.

### The physiology behind the gene of interest

To investigate how our, still unknown, gene of interest behaves under different light conditions, physiological experiments were performed in which the NIL was included. Both parents, C-C and E-C, and the NIL were measured after being grown in the Fluctor with three different treatments. The first treatment was a constant light intensity of  $438 \mu\text{mol}/\text{m}^2/\text{s}$ , the second was a simulation of the light treatment in the DEPI system with an average light intensity of  $438 \mu\text{mol}/\text{m}^2/\text{s}$ . The third one was a highly fluctuating light treatment, simulating a light intensity in the canopy of a maize field outside. The average light intensity was somewhat lower,  $418 \mu\text{mol}/\text{m}^2/\text{s}$ . The plants were measured in the Robin with a DEPI-like measuring protocol with alternating high and low light periods of 5 minutes. Over the different growing environments, no difference in patterns was observed for the genotypes. E-C shows higher NPQt values and there is no difference between C-C and the NIL. By comparing the constant treatment and the DEPI treatment, it can be concluded that all genotypes show lower NPQt values for the DEPI treatment. This means that we see acclimation of NPQt to fluctuating light. The NPQt values of the maize treatment are not significantly different from the constant treatment. However, it is not possible to directly compare the treatments, since the average light intensity was somewhat lower for the maize treatment. Since the measurement was performed with a DEPI-like measuring protocol, it seems that growing plants in a DEPI treatment primes that plants for fluctuating light. The maize treatment does not seem to (fully) prime the plants and therefore, the type of fluctuation also influences the phenotype by acclimation. As in all light treatments, the NIL is not significantly different from C-C, which means that the QTL on chromosome 2 does not influence the NPQt values in these particular environments. If NPQt is not affected by the QTL, the question rises whether the QTL does influence biomass. The biomass of the different genotypes under constant light and the DEPI treatment are not statistically different. What we do see is that, contradictory to NPQt values, the QTL on chromosome 2 does influence biomass. The QTL has a negative effect on biomass because the NIL has lower dry weight compared to C-C in the constant and DEPI growing environment. However, when looking at the maize treatment, a different pattern is observed. The NIL does not have a lower dry weight than C-C, but a small increase of 5% is observed. This means that under constant or slow fluctuating light conditions that QTL has a negative effect on biomass production, but a positive effect under highly fluctuating light.

From the observations of NPQt and dry weight, it can be concluded that NPQt is not directly correlated to dry weight. NPQt of the NIL and C-C are equal over all growing environment, however dry weight is different. Next to that, the QTL on chromosome 2 only influences dry weight under highly fluctuating conditions. NPQt measurements seem highly dependent on the measuring protocol that is used and does not directly correlate with plant performance. Plants grown in these three different growing environments should be measured with three different measuring protocols. A measurement under constant light conditions, slowly fluctuating light conditions and fast fluctuating light conditions. From this data acclimation to certain light conditions can be investigated. The fact that acclimation to fluctuating light influences NPQt is in coherence with the observations in the DEPI system. The QTL on chromosome 2 is already present at the first day of fluctuating light, however it only becomes highly significant at the second day of fluctuation. One of the discrepancies in this experiment is the high average light intensity. In the DEPI system, the plants are grown at a light intensity of  $200 \mu\text{mol}/\text{m}^2/\text{s}$  for 21 days, before being measured for 5.5 days. In the Fluctor the plants were exposed to an average

of 438  $\mu\text{mol}/\text{m}^2/\text{s}$  for the whole growing period. Therefore, this experiment should be repeated and light intensities of the different treatments should be converted to a lower average intensity. This would also make it possible to draw conclusions from maize treatment with more certainty. The average intensity of the maize treatment was lower because the highest measured light intensity in the canopy was higher than the LEDs in the Fluctor could obtain. Therefore, all light intensity values were converted to a lower intensity.

### Future perspectives

All of these efforts reveal the huge potential in using natural genetic variation for both understanding and improving photosynthesis. In this work a few contextual points moved to the foreground and are important to address in future research.

As we have seen in multiple experiment, there is a lot of variation in photosynthesis traits, which highly depend on adaptation to different circumstances. Next to adaptations in the life of one particular plant, evolution is also based on adaptations, for example adaptation to mutations. PsbA can be taken as an example of a mutation to which plants have been adapted. Ely was an accession with a mutation in PsbA, which impaired the rate of photosynthesis. The Ely nucleotide may have evolved and adapted to both the environment and the reduced rate of photosynthesis caused by the mutation. Recently, another accession, Huntly, was found to have a mutation in PsbA. Although Huntly has a mutation in PsbA, it does not show the same NPQ phenotype as Ely. Thus, Huntly might have adapted to the mutation of PsbA in a different manner than Ely. As nuclear adaptation to selection on organelle has a role in evolution and variation of the nuclear genome, it would be interesting to sequence Huntly and compare the genotype of both accessions (Flood et al., 2016). This could give more insight in the origin of the high NPQ phenotype of Ely and the adaptation to reduced photosynthesis efficiency.

NPQ itself is a difficult parameter to understand in the context of photosynthesis research. As NPQ is the ratio of  $\phi\text{NPQ}$  and  $\phi\text{NO}$ , higher or lower NPQ values can be influenced by either  $\phi\text{NPQ}$  or  $\phi\text{NO}$ , or both. In terms of improving photosynthesis efficiency, it would therefore be more interesting to look into the effect on  $\phi\text{NPQ}$  and  $\phi\text{NO}$  separately instead of NPQ. The QTL found on chromosome 2 is highly significant, which means that this QTL has a real effect on NPQ. However, this effect should be translated to a function within the photosynthesis pathway in which a ratio might not be informative enough. Furthermore, in photosynthesis phenotyping NPQ as well as NPQt are used. NPQt is an approximation of NPQ for which dark adaptation is not required. If it is possible to dark adapt plants before the measurement, without interfering with acclimation of plants, NPQ is the preferred parameter. However, sometimes NPQt might even be a better estimation than NPQ. For example, if the time between dark adaptation and the measurement of the fluorescence under saturating light is very large. Leaf and chlorophyll movement over the day will not be corrected for with NPQ, however NPQt can correct for it. Therefore I opt to use all the components in future analyses, instead of focussing on NPQ solely.

One of the questions that remain after this thesis is why and when NPQ will benefit plant performance. Photosystems can be damaged due to high light intensities if too many photons are captured by the LHCs of one photosystem. Plants can cope with high light intensities in multiple ways. Extra photosystems can be made and integrated into the thylakoid membrane. This is a costly process, which only results in a benefit under high light conditions. The other possibility is to dissipate the energy in the form of heat, but then light use efficiency reduces. Under constant high light intensity, it is more beneficial to produce more photosystems and increase the rate of photosynthesis. However, if light fluctuates, the costs might be higher than the benefits. Light conditions differ between places on the

earth, but also within a canopy. Upper leaves will receive more constant light intensities, whereas lower leaves experience more fluctuating light conditions by shading of other leaves. For plant performance it would be best to adapt lower leaves in the canopy to fluctuating light and canopy leaves to more constant conditions. Plants should also be adapted to the climate they grow in. If the underlying genetic mechanisms of plant responses to fluctuating light conditions is further understood, tissue specific gene expression could potentially increase plant performance. Promoters could induce gene expression differences in within different plant parts. In this thesis it was already shown that the QTL on chromosome 2 increases biomass under highly fluctuating conditions. The gene responsible for this phenotype would be interesting to express in leaves inside the canopy.

Before being able to alter photosynthesis pathways in plants, the pathways should be further understood. As has been shown NPQ phenotypes depend on light treatments. Most photosynthesis research has been done under constant light conditions. However, light conditions in the field are rather dynamic. Currently, more and more photosynthesis research is performed under fluctuating light conditions. (Demmig-Adams *et al.* (2012) showed that deep-shade-grown and light fluctuation acclimated plants response differently to sudden high light intensities. Deep-shade plants show low levels of NPQ under high light intensity and slow recovery of FvFm. These plants are not used to high light intensity and therefore slow in their response. NPQ is not used for protection against photodamage and therefore reduces the potential photosynthesis efficiency, FvFm. Plants that are acclimated to high light fluctuations show higher levels of NPQ and less photodamage. Stress-tolerant species were shown to respond the same as acclimated plants. Intrinsic PSII efficiency was lower, because of higher NPQ under high light conditions. Külheim *et al.* (2002) also showed that NPQ, and mainly the fast component qE, increases fitness under fluctuating field conditions. They also showed that for this fitness advantage, tolerance to fluctuation of light intensity is more important than tolerance to high light intensity. Acclimation to fluctuations in light intensity was also researched by Alter *et al.* (2012). This research investigated the effect of long and short fluctuating light conditions, which is similar to the experiment in the Fluctor with the DEPI and maize growing environment. They found out that the process of photoprotection and carbon fixation highly depends on duration, frequency and intensity of light fluctuations. This is in accordance with the conclusion of the experiment in the Fluctor. Alter *et al.* (2012) also showed that there is difference in responses to short sunflecks by seven *A. thaliana* accessions. This means that there is variation for the ability to use the energy of short sunflecks and therefore a potential for improvement. The increase of biomass of the NIL compared to C-C in the highly fluctuating maize treatment is in accordance with their conclusion. Alter *et al.* (2012) also showed that simulated long and short sunflecks induce different responses. Acclimation to short sunflecks induces an increase in NPQ over time when plants are exposed to high light intensities. However, acclimation to long sunflecks increase the ETC rather than NPQ. Acclimation to long sunflecks is therefore more prone to damage by high light fluctuations. Alter *et al.* (2012) used short sunflecks of 20 seconds and long sunflecks of 40 minutes. However, there are many more possible durations of sunflecks and many combinations of short and long sunflecks. This makes researching the effect of fluctuating light complicated. It would be interesting to identify patterns observed in phenotypic responses to fluctuating light conditions. Machine learning is increasingly used to find patterns in complex datasets. Libbrecht and Noble (2015) describe applications of machine learning in genetic and genomic datasets. Machine learning can be useful in genomic prediction of certain genotypes, genes or alleles.

As high-throughput phenotyping gets more easily available, increasing amounts of data become available (Peng *et al.*, 2020; Yang *et al.*, 2017). Both Peng *et al.* (2020) and Yang *et al.* (2017) developed algorithms to identify relations between phenotypes and genotypes in dynamic environments. (Zhu *et al.*, 2010) explained that models can increase the understanding of photosynthesis and find potential

improvements. Training models with data of photosynthesis parameters has potential to increase our understanding of acclimation and photosynthetic responses to different fluctuating light conditions. Modelling another complex trait, transpiration efficiency, has previously been shown to assist in increasing yield in cereals (Chenu et al., 2018). In my opinion, machine learning can benefit genetic photosynthesis research by both forward and reverse genetics. We have showed that NILs have potential to improve understanding of the effect of genes on photosynthesis phenotypes. By exposing NILs to different light fluctuations and by finding patterns in these responses forward genetics can give insight in effects of genes under different circumstances. Phenotyping the DH population showed that there is a lot of variation in photosynthesis phenotypes under different conditions. Machine learning could be used in reverse genetics by finding patterns in genetic diverse populations and identify genes that are responsible for certain phenotypes.

## Conclusions

In this study we aimed to use a QTL mapping approach to reveal novel physiological processes. The dynamic process of photosynthesis is influenced by a genotype x environment x acclimation x phenotype interaction. A population derived from a biparental cross showed the large variation for photosynthesis. It was shown that a NIL is useful in breaking down the complexity of photosynthesis by using the variation that exist. In the future, physiology of photosynthesis can be further unravelled by making different bi- or multiparental populations.

We can conclude that there are 9 candidate genes in the QTL on chromosome 2, of which, two genes (CPFTSY and PMM) have previously been shown to be related to photosynthesis. Both genes have a deletion in the promotor region in Ely that can potentially influence gene expression.

We suggest that the QTL on chromosome 2 has a positive influence on dry weight under fast fluctuating light with an increase of 5%. It was shown that the effect of the QTL depends on the growing environment, as dry weight was negatively affected by the QTL under constant and slow fluctuating condition. NPQ was shown to depend on acclimation to constant, slow fluctuating and highly fluctuating conditions.

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## Appendix

### 1. Robin measuring protocols

#### 1: NPQ relaxation and induction

TS=50ms

include default.inc ;Includes standard options, do not remove it !

include light.inc ;Includes standard options, do not remove it !

;NPQ relaxation protocol

;Extended version 02102020

;Tom & Sanne

\_\_LightA=0

\_\_LightB=0

\_\_Lightintensity =<17,75>

Shutter=3

Sensitivity=1

Super=100

;FAR=20

dark\_period = 1800s;#### standard setting: 1800s ####

end\_dark\_period = dark\_period

;\*\*\* F0 Measurement \*\*\*\*\*

F0Duration=20s;

F0measurement =1s

a1=end\_dark\_period

a3=a1+F0Duration

<a1,a1+F0measurement..a3>=>mfmsub

<a1>=>checkPoint,"startFo"

<a1+F0Duration>=>checkPoint,"endFo"

;\*\*\* Saturating Pulse & Fm Measurement \*\*\*\*\*

SatPulseDuration=800ms;#### standard setting: 800ms ####

PreSatPulse =800ms

<a1,a1+(F0Duration/20)..a3-TS>=>mfmsub

<a1>=>checkPoint,"startFo"

<a1+PreSatPulse-TS>=>checkPoint,"endFo"

<a3+2\*TS>=>SatPulse(SatPulseDuration)

<a3+2\*TS>=>mpulse2

<a3+2\*TS>=>checkPoint,"startFm"

<a3+ SatPulseDuration >=>checkPoint,"endFm"

;\*\*\* Light fluctuation 2000/200 uE \*\*\*\*\*

```

Fluct_light_period = 180s ;#### standard setting: 300s ####
Fluct_light_measurement= 30s;#### standard setting: 10s ####
Fluct_low_intensity = 16.5 ;#### standard setting: 100mMol/s ####
Fluct_high_intensity = 77.6 ;#### standard setting: 50mMol/s ####

a4=a3+2s
a5=a4+Fluct_light_period
a6=a5+Fluct_light_period
a7=a6+Fluct_light_period
a8=a7+Fluct_light_period

<a4-TS>=>SI_Act2(Fluct_high_intensity)
<a4>=>act2(Fluct_light_period)
<a4+TS,a4+Fluct_light_measurement..a4+Fluct_light_period>=>mfmsub

<a5-TS>=>SI_Act2(Fluct_low_intensity)
<a5>=>act2(Fluct_light_period)
<a5 +TS,a5+Fluct_light_measurement..a5+Fluct_light_period>=>mfmsub

<a6-TS>=>SI_Act2(Fluct_high_intensity)
<a6>=>act2(Fluct_light_period)
<a6 +TS,a6+Fluct_light_measurement..a6+Fluct_light_period>=>mfmsub

<a7-TS>=>SI_Act2(Fluct_low_intensity)
<a7>=>act2(Fluct_light_period)
<a7+TS,a7+Fluct_light_measurement..a7+Fluct_light_period>=>mfmsub

<a8-TS>=>SI_Act2(Fluct_high_intensity)
<a8>=>act2(Fluct_light_period)
<a8 +TS,a8+Fluct_light_measurement..a8+0.5*Fluct_light_period-TS>=>mfmsub
<a8+0.5*Fluct_light_period+PreSatPulse+SatPulseDuration+2*TS,a8+0.5*Fluct_light_period+PreSatP
ulse+Fluct_light_measurement..a8+Fluct_light_period>=>mfmsub

;*****Measuring relaxation *****
Relax_act_light_period=300s;#### standard setting: 300s ####
Relax_act_light_time_interval = 30s;#### standard setting: 30s ####
Relax_act_light_intensity = 13;#### standard setting: 50 mMol/s ####
Batch = 0s

a9=a8+Fluct_light_period+Batch+TS
a10=a9+Relax_act_light_time_interval
a11=a10+Relax_act_light_time_interval
a12=a11+Relax_act_light_time_interval
a13=a12+Relax_act_light_time_interval
a14=a13+Relax_act_light_time_interval
a15=a14+Relax_act_light_time_interval
a16=a15+Relax_act_light_time_interval
a17=a16+Relax_act_light_time_interval

```

a18=a17+Relax\_act\_light\_time\_interval

a20=a18+Relax\_act\_light\_time\_interval

<a9-Batch-2\*TS>=>SI\_Act2(Relax\_act\_light\_intensity)

<a9-Batch-2\*TS>=>act2(Relax\_act\_light\_period)

b9=a9+PreSatPulse

b10=a10+PreSatPulse

b11=a11+PreSatPulse

b12=a12+PreSatPulse

b13=a13+PreSatPulse

b14=a14+PreSatPulse

b15=a15+PreSatPulse

b16=a16+PreSatPulse

b17=a17+PreSatPulse

b18=a18+PreSatPulse

;Normalise NPQ measurement

<a8+0.5\*Fluct\_light\_period,a8+0.5\*Fluct\_light\_period+(PreSatPulse/8)..a8+0.5\*Fluct\_light\_period+PreSatPulse-TS>=>mfmsub

<a8+0.5\*Fluct\_light\_period+PreSatPulse+TS\*2>=>SatPulse(SatPulseDuration)

<a8+0.5\*Fluct\_light\_period+PreSatPulse+TS\*2>=>mpulse2

<a9,a9+(PreSatPulse/8)..b9-TS>=>mfmsub

<a9>=>checkPoint,"startFo\_Lss"

<a9+PreSatPulse-TS>=>checkPoint,"endFo\_Lss"

<b9+TS\*2>=>SatPulse(SatPulseDuration)

<b9+TS\*2>=>mpulse2

<b9+TS\*2>=>checkPoint,"startFm\_Lss"

<b9 + SatPulseDuration >=>checkPoint,"endFm\_Lss"

;<b9+2\*TS>=>checkPoint,"timeVisual"

<a10,a10+(PreSatPulse/8)..b10-TS>=>mfmsub

<a10>=>checkPoint,"startFo\_Lss\_2"

<a10+PreSatPulse-TS>=>checkPoint,"endFo\_Lss\_2"

<b10+TS\*2>=>SatPulse(SatPulseDuration)

<b10+TS\*2>=>mpulse2

<b10+TS\*2>=>checkPoint,"startFm\_Lss\_2"

<b10 + SatPulseDuration >=>checkPoint,"endFm\_Lss\_2"

;<b10+2\*TS>=>checkPoint,"timeVisual"

<a11,a11+(PreSatPulse/8)..b11-TS>=>mfmsub

<a11>=>checkPoint,"startFo\_Lss\_3"

<a11+PreSatPulse-TS>=>checkPoint,"endFo\_Lss\_3"

<b11+TS\*2>=>SatPulse(SatPulseDuration)

<b11+TS\*2>=>mpulse2

<b11+TS\*2>=>checkPoint,"startFm\_Lss\_3"

<b11 + SatPulseDuration >=>checkPoint,"endFm\_Lss\_3"

```

;<b11+2*TS>=>checkPoint,"timeVisual"

<a12,a12+(PreSatPulse/8)..b12-TS>=>mfmsub
<a12>=>checkPoint,"startFo_Lss_4"
<a12+PreSatPulse-TS>=>checkPoint,"endFo_Lss_4"
<b12+TS*2>=>SatPulse(SatPulseDuration)
<b12+TS*2>=>mpulse2
<b12+TS*2>=>checkPoint,"startFm_Lss_4"
<b12 + SatPulseDuration >=>checkPoint,"endFm_Lss_4"
;<b12+2*TS>=>checkPoint,"timeVisual"

<a13,a13+(PreSatPulse/8)..b13-TS>=>mfmsub
<a13>=>checkPoint,"startFo_Lss_5"
<a13+PreSatPulse-TS>=>checkPoint,"endFo_Lss_5"
<b13+TS*2>=>SatPulse(SatPulseDuration)
<b13+TS*2>=>mpulse2
<b13+TS*2>=>checkPoint,"startFm_Lss_5"
<b13 + SatPulseDuration >=>checkPoint,"endFm_Lss_5"
;<b13+2*TS>=>checkPoint,"timeVisual"

<a14,a14+(PreSatPulse/8)..b14-TS>=>mfmsub
<a14>=>checkPoint,"startFo_Lss_6"
<a14+PreSatPulse-TS>=>checkPoint,"endFo_Lss_6"
<b14+TS*2>=>SatPulse(SatPulseDuration)
<b14+TS*2>=>mpulse2
<b14+TS*2>=>checkPoint,"startFm_Lss_6"
<b14 + SatPulseDuration >=>checkPoint,"endFm_Lss_6"
;<b14+2*TS>=>checkPoint,"timeVisual"

<a15,a15+(PreSatPulse/8)..b15-TS>=>mfmsub
<a15>=>checkPoint,"startFo_Lss_7"
<a15+PreSatPulse-TS>=>checkPoint,"endFo_Lss_7"
<b15+TS*2>=>SatPulse(SatPulseDuration)
<b15+TS*2>=>mpulse2
<b15+TS*2>=>checkPoint,"startFm_Lss_7"
<b15 + SatPulseDuration >=>checkPoint,"endFm_Lss_7"
;<b15+2*TS>=>checkPoint,"timeVisual"

<a16,a16+(PreSatPulse/8)..b16-TS>=>mfmsub
<a16>=>checkPoint,"startFo_Lss_8"
<a16+PreSatPulse-TS>=>checkPoint,"endFo_Lss_8"
<b16+TS*2>=>SatPulse(SatPulseDuration)
<b16+TS*2>=>mpulse2
<b16+TS*2>=>checkPoint,"startFm_Lss_8"
<b16 + SatPulseDuration >=>checkPoint,"endFm_Lss_8"
;<b16+2*TS>=>checkPoint,"timeVisual"

<a17,a17+(PreSatPulse/8)..b17-TS>=>mfmsub

```

```

<a17>=>checkPoint,"startFo_Lss_9"
<a17+PreSatPulse-TS>=>checkPoint,"endFo_Lss_9"
<b17+TS*2>=>SatPulse(SatPulseDuration)
<b17+TS*2>=>mpulse2
<b17+TS*2>=>checkPoint,"startFm_Lss_9"
<b17 + SatPulseDuration >=>checkPoint,"endFm_Lss_9"
;<b17+2*TS>=>checkPoint,"timeVisual"

```

```

<a18,a18+(PreSatPulse/8)..b18-TS>=>mfmsub
<a18>=>checkPoint,"startFo_Lss_10"
<a18+PreSatPulse-TS>=>checkPoint,"endFo_Lss_10"
<b18+TS*2>=>SatPulse(SatPulseDuration)
<b18+TS*2>=>mpulse2
<b18+TS*2>=>checkPoint,"startFm_Lss_10"
<b18 + SatPulseDuration >=>checkPoint,"endFm_Lss_10"
;<b18+2*TS>=>checkPoint,"timeVisual"

```

```

;***** Measureing induction*****

```

```

Induce_act_light_period=300s;#### standard setting: 300s ####
Induce_act_light_time_interval = 30s;#### standard setting: 30s ####
Induce_act_light_intensity = 77.6;#### standard setting: 1000 mMol/s ####

```

```

a21=a20+Induce_act_light_time_interval
a22=a21+Induce_act_light_time_interval
a23=a22+Induce_act_light_time_interval
a24=a23+Induce_act_light_time_interval
a25=a24+Induce_act_light_time_interval
a26=a25+Induce_act_light_time_interval
a27=a26+Induce_act_light_time_interval
a28=a27+Induce_act_light_time_interval
a29=a28+Induce_act_light_time_interval
a30=a29+Induce_act_light_time_interval

```

```

<a20-2*TS-Batch>=>SI_Act2(Induce_act_light_intensity)
<a20-2*TS-Batch>=>act2(Induce_act_light_period)

```

```

b20=a20+PreSatPulse
b21=a21+PreSatPulse
b22=a22+PreSatPulse
b23=a23+PreSatPulse
b24=a24+PreSatPulse
b25=a25+PreSatPulse
b26=a26+PreSatPulse
b27=a27+PreSatPulse
b28=a28+PreSatPulse
b29=a29+PreSatPulse
b30=a30+PreSatPulse

```

```

<a20,a20+(PreSatPulse/8)..b20-TS>=>mfmsub
<a20>=>checkPoint,"startFo_Lss_12"
<a20+PreSatPulse-TS>=>checkPoint,"endFo_Lss_12"
<b20+TS*2>=>SatPulse(SatPulseDuration)
<b20+TS*2>=>mpulse2
<b20+TS*2>=>checkPoint,"startFm_Lss_12"
<b20 + SatPulseDuration >=>checkPoint,"endFm_Lss_12"
;<b20+2*TS>=>checkPoint,"timeVisual"

```

```

<a21,a21+(PreSatPulse/8)..b21-TS>=>mfmsub
<a21>=>checkPoint,"startFo_Lss_13"
<a21+PreSatPulse-TS>=>checkPoint,"endFo_Lss_13"
<b21+TS*2>=>SatPulse(SatPulseDuration)
<b21+TS*2>=>mpulse2
<b21+TS*2>=>checkPoint,"startFm_Lss_13"
<b21 + SatPulseDuration >=>checkPoint,"endFm_Lss_13"
;<b21+2*TS>=>checkPoint,"timeVisual"

```

```

<a22,a22+(PreSatPulse/8)..b22-TS>=>mfmsub
<a22>=>checkPoint,"startFo_Lss_14"
<a22+PreSatPulse-TS>=>checkPoint,"endFo_Lss_14"
<b22+TS*2>=>SatPulse(SatPulseDuration)
<b22+TS*2>=>mpulse2
<b22+TS*2>=>checkPoint,"startFm_Lss_14"
<b22 + SatPulseDuration >=>checkPoint,"endFm_Lss_14"
;<b22+2*TS>=>checkPoint,"timeVisual"

```

```

<a23,a23+(PreSatPulse/8)..b23-TS>=>mfmsub
<a23>=>checkPoint,"startFo_Lss_15"
<a23+PreSatPulse-TS>=>checkPoint,"endFo_Lss_15"
<b23+TS*2>=>SatPulse(SatPulseDuration)
<b23+TS*2>=>mpulse2
<b23+TS*2>=>checkPoint,"startFm_Lss_15"
<b23 + SatPulseDuration >=>checkPoint,"endFm_Lss_15"
;<b23+2*TS>=>checkPoint,"timeVisual"

```

```

<a24,a24+(PreSatPulse/8)..b24-TS>=>mfmsub
<a24>=>checkPoint,"startFo_Lss_16"
<a24+PreSatPulse-TS>=>checkPoint,"endFo_Lss_16"
<b24+TS*2>=>SatPulse(SatPulseDuration)
<b24+TS*2>=>mpulse2
<b24+TS*2>=>checkPoint,"startFm_Lss_16"
<b24 + SatPulseDuration >=>checkPoint,"endFm_Lss_16"
;<b24+2*TS>=>checkPoint,"timeVisual"

```

```

<a25,a25+(PreSatPulse/8)..b25-TS>=>mfmsub
<a25>=>checkPoint,"startFo_Lss_17"

```

```

<a25+PreSatPulse-TS>=>checkPoint,"endFo_Lss_17"
<b25+TS*2>=>SatPulse(SatPulseDuration)
<b25+TS*2>=>mpulse2
<b25+TS*2>=>checkPoint,"startFm_Lss_17"
<b25 + SatPulseDuration >=>checkPoint,"endFm_Lss_17"
;<b25+2*TS>=>checkPoint,"timeVisual"

```

```

<a26,a26+(PreSatPulse/8)..b26-TS>=>mfmsub
<a26>=>checkPoint,"startFo_Lss_18"
<a26+PreSatPulse-TS>=>checkPoint,"endFo_Lss_18"
<b26+TS*2>=>SatPulse(SatPulseDuration)
<b26+TS*2>=>mpulse2
<b26+TS*2>=>checkPoint,"startFm_Lss_18"
<b26 + SatPulseDuration >=>checkPoint,"endFm_Lss_18"
;<b26+2*TS>=>checkPoint,"timeVisual"

```

```

<a27,a27+(PreSatPulse/8)..b27-TS>=>mfmsub
<a27>=>checkPoint,"startFo_Lss_19"
<a27+PreSatPulse-TS>=>checkPoint,"endFo_Lss_19"
<b27+TS*2>=>SatPulse(SatPulseDuration)
<b27+TS*2>=>mpulse2
<b27+TS*2>=>checkPoint,"startFm_Lss_19"
<b27 + SatPulseDuration >=>checkPoint,"endFm_Lss_19"
;<b27+2*TS>=>checkPoint,"timeVisual"

```

```

<a28,a28+(PreSatPulse/8)..b28-TS>=>mfmsub
<a28>=>checkPoint,"startFo_Lss_20"
<a28+PreSatPulse-TS>=>checkPoint,"endFo_Lss_20"
<b28+TS*2>=>SatPulse(SatPulseDuration)
<b28+TS*2>=>mpulse2
<b28+TS*2>=>checkPoint,"startFm_Lss_20"
<b28 + SatPulseDuration >=>checkPoint,"endFm_Lss_20"
;<b28+2*TS>=>checkPoint,"timeVisual"

```

```

<a29,a29+(PreSatPulse/8)..b29-TS>=>mfmsub
<a29>=>checkPoint,"startFo_Lss_21"
<a29+PreSatPulse-TS>=>checkPoint,"endFo_Lss_21"
<b29+TS*2>=>SatPulse(SatPulseDuration)
<b29+TS*2>=>mpulse2
<b29+TS*2>=>checkPoint,"startFm_Lss_21"
<b29 + SatPulseDuration >=>checkPoint,"endFm_Lss_21"
;<b29+2*TS>=>checkPoint,"timeVisual"

```

## 2: 12 cycles of alternating high and low light intensity

TS=50ms

include default.inc ;Includes standard options, do not remove it !

include light.inc ;Includes standard options, do not remove it !

; Written by Sanne Put as a fast fluctuating light protocol

\_\_LightA=0

\_\_LightB=0

\_\_LightIntensity=<25.1>

Shutter=3

Sensitivity=1

Super=100

FAR=20

;general parameters

total\_period\_duration = 270s

pulse\_duration = 800ms

first\_act\_measurements = 120s

second\_act\_measurements = 10s

f\_measurements = 400ms

dark\_period = 4s

FR\_period = 16s

FR\_light\_period = 5s

dark\_FR\_measurements = 1s

;cycle \_1 - periods

start\_period\_1 = 0s

end\_period\_1 = start\_period\_1 + total\_period\_duration

first\_act\_end\_1 = end\_period\_1 - 20s

fp\_start\_1 = first\_act\_end\_1 - 2s;start for F' measurements

fmp\_end\_1 = first\_act\_end\_1 + 1s;start for 20s Act light period

<start\_period\_1>=>act2(total\_period\_duration) ;set Act light for the given period

<start\_period\_1>=>SI\_Act2(21.6);set Act light intensity during period

<first\_act\_end\_1>=>SatPulse(pulse\_duration);duration of saturating pulse

<first\_act\_end\_1>=>mpulse2;measure Fm'

;cycle \_1 - measurements

<start\_period\_1, start\_period\_1 + first\_act\_measurements .. fp\_start\_1 - TS>=>mfmsub ;general F' measurments, just for monitoring, no calculations done on this

<fp\_start\_1, fp\_start\_1 + f\_measurements .. first\_act\_end\_1 - TS>=>mfmsub;higher intensity F' measurments for F'

<fmp\_end\_1, fmp\_end\_1 + second\_act\_measurements .. end\_period\_1 - TS>=>mfmsub;F' measurements to have light relax, before a potential dark/FR relaxation

```

;Take images
<fmp_end_1+TS>=>checkPoint,"startFt_Lss"
<end_period_1>=>checkPoint,"endFt_Lss"

<first_act_end_1+TS>=>checkPoint,"startFm_Lss"
<first_act_end_1+pulse_duration-TS>=>checkPoint,"endFm_Lss"

;Addition _1 - dark/FR relaxation
start_dark_period_1 = end_period_1 + TS
start_FR_period_1 = start_dark_period_1 + dark_period
end_FR_period_1 = start_FR_period_1 + FR_period
<start_FR_period_1>=>FAR(FR_light_period)
<end_FR_period_1>=>SatPulse(pulse_duration)
<end_FR_period_1>=>mpulse2
end_1 = end_FR_period_1 + 1s

<start_dark_period_1, start_dark_period_1 + dark_FR_measurements ..
end_FR_period_1>=>mfmsub

;cycle _2 - periods
start_period_2 = end_1
end_period_2 = start_period_2 + total_period_duration
first_act_end_2 = end_period_2 - 20s
fp_start_2 = first_act_end_2 - 2s;start for F' measurements
fmp_end_2 = first_act_end_2 + 1s;start for 20s Act light period
<start_period_2 - TS>=>act2(total_period_duration) ;set Act light for the given period
<start_period_2 - TS>=>SI_Act2(77.9);set Act light intensity during period
<first_act_end_2>=>SatPulse(pulse_duration);duration of saturating pulse
<first_act_end_2>=>mpulse2;measure Fm'

;cycle _2 - measurements
<start_period_2, start_period_2 + first_act_measurements .. fp_start_2 - TS>=>mfmsub ;general F'
measurments, just for monitoring, no calculations done on this
<fp_start_2, fp_start_2 + f_measurements .. first_act_end_2 - TS>=>mfmsub;higher intensity F'
measurments for F'
<fmp_end_2, fmp_end_2 + second_act_measurements .. end_period_2 - TS>=>mfmsub;F'
measurements to have light relax, before a potential dark/FR relaxation

;Addition _2 - dark/FR relaxation
start_dark_period_2 = end_period_2 + TS
start_FR_period_2 = start_dark_period_2 + dark_period
end_FR_period_2 = start_FR_period_2 + FR_period
<start_FR_period_2>=>FAR(FR_light_period)
<end_FR_period_2>=>SatPulse(pulse_duration)
<end_FR_period_2>=>mpulse2
end_2 = end_FR_period_2 + 1s

```

```
<start_dark_period_2, start_dark_period_2 + dark_FR_measurements ..  
end_FR_period_2>=>mfmsub
```

```
;cycle _3 - periods
```

```
start_period_3 = end_2
```

```
end_period_3 = start_period_3 + total_period_duration
```

```
first_act_end_3 = end_period_3 - 20s
```

```
fp_start_3 = first_act_end_3 - 2s;start for F' measurements
```

```
fmp_end_3 = first_act_end_3 + 1s;start for 20s Act light period
```

```
<start_period_3 - TS>=>act2(total_period_duration) ;set Act light for the given period
```

```
<start_period_3 - TS>=>SI_Act2(21.6);set Act light intensity during period
```

```
<first_act_end_3>=>SatPulse(pulse_duration);duration of saturating pulse
```

```
<first_act_end_3>=>mpulse2;measure Fm'
```

```
;cycle _3 - measurements
```

```
<start_period_3, start_period_3 + first_act_measurements .. fp_start_3 - TS>=>mfmsub ;general F'  
measurments, just for monitoring, no calculations done on this
```

```
<fp_start_3, fp_start_3 + f_measurements .. first_act_end_3 - TS>=>mfmsub;higher intensity F'  
measurments for F'
```

```
<fmp_end_3, fmp_end_3 + second_act_measurements .. end_period_3 - TS>=>mfmsub;F'  
measurements to have light relax, before a potential dark/FR relaxation
```

```
;Addition _3 - dark/FR relaxation
```

```
start_dark_period_3 = end_period_3 + TS
```

```
start_FR_period_3 = start_dark_period_3 + dark_period
```

```
end_FR_period_3 = start_FR_period_3 + FR_period
```

```
<start_FR_period_3>=>FAR(FR_light_period)
```

```
<end_FR_period_3>=>SatPulse(pulse_duration)
```

```
<end_FR_period_3>=>mpulse2
```

```
end_3 = end_FR_period_3 + 1s
```

```
<start_dark_period_3, start_dark_period_3 + dark_FR_measurements ..  
end_FR_period_3>=>mfmsub
```

```
;cycle _4 - periods
```

```
start_period_4 = end_3
```

```
end_period_4 = start_period_4 + total_period_duration
```

```
first_act_end_4 = end_period_4 - 20s
```

```
fp_start_4 = first_act_end_4 - 2s;start for F' measurements
```

```
fmp_end_4 = first_act_end_4 + 1s;start for 20s Act light period
```

```
<start_period_4 - TS>=>act2(total_period_duration) ;set Act light for the given period
```

```
<start_period_4 - TS>=>SI_Act2(77.9);set Act light intensity during period
```

```
<first_act_end_4>=>SatPulse(pulse_duration);duration of saturating pulse
```

```
<first_act_end_4>=>mpulse2;measure Fm'
```

```
;cycle _4 - measurements
```

```
<start_period_4, start_period_4 + first_act_measurements .. fp_start_4 - TS>=>mfmsub ;general F'  
measurments, just for monitoring, no calculations done on this
```

<fp\_start\_4, fp\_start\_4 + f\_measurements .. first\_act\_end\_4 - TS>=>mfmsub;higher intensity F'  
 measurments for F'  
 <fmp\_end\_4, fmp\_end\_4 + second\_act\_measurements .. end\_period\_4 - TS>=>mfmsub;F'  
 measurements to have light relax, before a potential dark/FR relaxation

;Addition \_4 - dark/FR relaxation  
 start\_dark\_period\_4 = end\_period\_4 + TS  
 start\_FR\_period\_4 = start\_dark\_period\_4 + dark\_period  
 end\_FR\_period\_4 = start\_FR\_period\_4 + FR\_period  
 <start\_FR\_period\_4>=>FAR(FR\_light\_period)  
 <end\_FR\_period\_4>=>SatPulse(pulse\_duration)  
 <end\_FR\_period\_4>=>mpulse2  
 end\_4 = end\_FR\_period\_4 + 1s

<start\_dark\_period\_4, start\_dark\_period\_4 + dark\_FR\_measurements ..  
 end\_FR\_period\_4>=>mfmsub

;cycle \_5 - periods  
 start\_period\_5 = end\_4  
 end\_period\_5 = start\_period\_5 + total\_period\_duration  
 first\_act\_end\_5 = end\_period\_5 - 20s  
 fp\_start\_5 = first\_act\_end\_5 - 2s;start for F' measurements  
 fmp\_end\_5 = first\_act\_end\_5 + 1s;start for 20s Act light period  
 <start\_period\_5 - TS>=>act2(total\_period\_duration) ;set Act light for the given period  
 <start\_period\_5 - TS>=>SI\_Act2(21.6);set Act light intensity during period  
 <first\_act\_end\_5>=>SatPulse(pulse\_duration);duration of saturating pulse  
 <first\_act\_end\_5>=>mpulse2;measure Fm'

;cycle \_5 - measurements  
 <start\_period\_5, start\_period\_5 + first\_act\_measurements .. fp\_start\_5 - TS>=>mfmsub ;general F'  
 measurments, just for monitoring, no calculations done on this  
 <fp\_start\_5, fp\_start\_5 + f\_measurements .. first\_act\_end\_5 - TS>=>mfmsub;higher intensity F'  
 measurments for F'  
 <fmp\_end\_5, fmp\_end\_5 + second\_act\_measurements .. end\_period\_5 - TS>=>mfmsub;F'  
 measurements to have light relax, before a potential dark/FR relaxation

;Addition \_5 - dark/FR relaxation  
 start\_dark\_period\_5 = end\_period\_5 + TS  
 start\_FR\_period\_5 = start\_dark\_period\_5 + dark\_period  
 end\_FR\_period\_5 = start\_FR\_period\_5 + FR\_period  
 <start\_FR\_period\_5>=>FAR(FR\_light\_period)  
 <end\_FR\_period\_5>=>SatPulse(pulse\_duration)  
 <end\_FR\_period\_5>=>mpulse2  
 end\_5 = end\_FR\_period\_5 + 1s

<start\_dark\_period\_5, start\_dark\_period\_5 + dark\_FR\_measurements ..  
 end\_FR\_period\_5>=>mfmsub

```

;cycle _6 - periods
start_period_6 = end_5
end_period_6 = start_period_6 + total_period_duration
first_act_end_6 = end_period_6 - 20s
fp_start_6 = first_act_end_6 - 2s;start for F' measurements
fmp_end_6 = first_act_end_6 + 1s;start for 20s Act light period
<start_period_6 - TS>=>act2(total_period_duration) ;set Act light for the given period
<start_period_6 - TS>=>Sl_Act2(77.9);set Act light intensity during period
<first_act_end_6>=>SatPulse(pulse_duration);duration of saturating pulse
<first_act_end_6>=>mpulse2;measure Fm'

;cycle _6 - measurements
<start_period_6, start_period_6 + first_act_measurements .. fp_start_6 - TS>=>mfmsub ;general F'
measurments, just for monitoring, no calculations done on this
<fp_start_6, fp_start_6 + f_measurements .. first_act_end_6 - TS>=>mfmsub;higher intensity F'
measurments for F'
<fmp_end_6, fmp_end_6 + second_act_measurements .. end_period_6 - TS>=>mfmsub;F'
measurements to have light relax, before a potential dark/FR relaxation

;Addition _6 - dark/FR relaxation
start_dark_period_6 = end_period_6 + TS
start_FR_period_6 = start_dark_period_6 + dark_period
end_FR_period_6 = start_FR_period_6 + FR_period
<start_FR_period_6>=>FAR(FR_light_period)
<end_FR_period_6>=>SatPulse(pulse_duration)
<end_FR_period_6>=>mpulse2
end_6 = end_FR_period_6 + 1s

<start_dark_period_6, start_dark_period_6 + dark_FR_measurements ..
end_FR_period_6>=>mfmsub

;cycle _7 - periods
start_period_7 = end_6
end_period_7 = start_period_7 + total_period_duration
first_act_end_7 = end_period_7 - 20s
fp_start_7 = first_act_end_7 - 2s;start for F' measurements
fmp_end_7 = first_act_end_7 + 1s;start for 20s Act light period
<start_period_7 - TS>=>act2(total_period_duration) ;set Act light for the given period
<start_period_7 - TS>=>Sl_Act2(21.6);set Act light intensity during period
<first_act_end_7>=>SatPulse(pulse_duration);duration of saturating pulse
<first_act_end_7>=>mpulse2;measure Fm'

;cycle _7 - measurements
<start_period_7, start_period_7 + first_act_measurements .. fp_start_7 - TS>=>mfmsub ;general F'
measurments, just for monitoring, no calculations done on this
<fp_start_7, fp_start_7 + f_measurements .. first_act_end_7 - TS>=>mfmsub;higher intensity F'
measurments for F'

```

<fmp\_end\_7, fmp\_end\_7 + second\_act\_measurements .. end\_period\_7 - TS>=>mfmsub;F'  
measurements to have light relax, before a potential dark/FR relaxation

;Addition \_7 - dark/FR relaxation  
start\_dark\_period\_7 = end\_period\_7 + TS  
start\_FR\_period\_7 = start\_dark\_period\_7 + dark\_period  
end\_FR\_period\_7 = start\_FR\_period\_7 + FR\_period  
<start\_FR\_period\_7>=>FAR(FR\_light\_period)  
<end\_FR\_period\_7>=>SatPulse(pulse\_duration)  
<end\_FR\_period\_7>=>mpulse2  
end\_7 = end\_FR\_period\_7 + 1s

<start\_dark\_period\_7, start\_dark\_period\_7 + dark\_FR\_measurements ..  
end\_FR\_period\_7>=>mfmsub

;cycle \_8 - periods  
start\_period\_8 = end\_7  
end\_period\_8 = start\_period\_8 + total\_period\_duration  
first\_act\_end\_8 = end\_period\_8 - 20s  
fp\_start\_8 = first\_act\_end\_8 - 2s;start for F' measurements  
fmp\_end\_8 = first\_act\_end\_8 + 1s;start for 20s Act light period  
<start\_period\_8 - TS>=>act2(total\_period\_duration) ;set Act light for the given period  
<start\_period\_8 - TS>=>SI\_Act2(77.9);set Act light intensity during period  
<first\_act\_end\_8>=>SatPulse(pulse\_duration);duration of saturating pulse  
<first\_act\_end\_8>=>mpulse2;measure Fm'

;cycle \_8 - measurements  
<start\_period\_8, start\_period\_8 + first\_act\_measurements .. fp\_start\_8 - TS>=>mfmsub ;general F'  
measurments, just for monitoring, no calculations done on this  
<fp\_start\_8, fp\_start\_8 + f\_measurements .. first\_act\_end\_8 - TS>=>mfmsub;higher intensity F'  
measurments for F'  
<fmp\_end\_8, fmp\_end\_8 + second\_act\_measurements .. end\_period\_8 - TS>=>mfmsub;F'  
measurements to have light relax, before a potential dark/FR relaxation

;Addition \_8 - dark/FR relaxation  
start\_dark\_period\_8 = end\_period\_8 + TS  
start\_FR\_period\_8 = start\_dark\_period\_8 + dark\_period  
end\_FR\_period\_8 = start\_FR\_period\_8 + FR\_period  
<start\_FR\_period\_8>=>FAR(FR\_light\_period)  
<end\_FR\_period\_8>=>SatPulse(pulse\_duration)  
<end\_FR\_period\_8>=>mpulse2  
end\_8 = end\_FR\_period\_8 + 1s

<start\_dark\_period\_8, start\_dark\_period\_8 + dark\_FR\_measurements ..  
end\_FR\_period\_8>=>mfmsub

;cycle \_9 - periods  
start\_period\_9 = end\_8

```

end_period_9 = start_period_9 + total_period_duration
first_act_end_9 = end_period_9 - 20s
fp_start_9 = first_act_end_9 - 2s;start for F' measurements
fmp_end_9 = first_act_end_9 + 1s;start for 20s Act light period
<start_period_9 - TS>=>act2(total_period_duration) ;set Act light for the given period
<start_period_9 - TS>=>SI_Act2(21.6);set Act light intensity during period
<first_act_end_9>=>SatPulse(pulse_duration);duration of saturating pulse
<first_act_end_9>=>mpulse2;measure Fm'

;cycle_9 - measurements
<start_period_9, start_period_9 + first_act_measurements .. fp_start_9 - TS>=>mfmsub ;general F'
measurments, just for monitoring, no calculations done on this
<fp_start_9, fp_start_9 + f_measurements .. first_act_end_9 - TS>=>mfmsub;higher intensity F'
measurments for F'
<fmp_end_9, fmp_end_9 + second_act_measurements .. end_period_9 - TS>=>mfmsub;F'
measurements to have light relax, before a potential dark/FR relaxation

;Addition_9 - dark/FR relaxation
start_dark_period_9 = end_period_9 + TS
start_FR_period_9 = start_dark_period_9 + dark_period
end_FR_period_9 = start_FR_period_9 + FR_period
<start_FR_period_9>=>FAR(FR_light_period)
<end_FR_period_9>=>SatPulse(pulse_duration)
<end_FR_period_9>=>mpulse2
end_9 = end_FR_period_9 + 1s

<start_dark_period_9, start_dark_period_9 + dark_FR_measurements ..
end_FR_period_9>=>mfmsub

;cycle_10 - periods
start_period_10 = end_9
end_period_10 = start_period_10 + total_period_duration
first_act_end_10 = end_period_10 - 20s
fp_start_10 = first_act_end_10 - 2s;start for F' measurements
fmp_end_10 = first_act_end_10 + 1s;start for 20s Act light period
<start_period_10 - TS>=>act2(total_period_duration) ;set Act light for the given period
<start_period_10 - TS>=>SI_Act2(77.9);set Act light intensity during period
<first_act_end_10>=>SatPulse(pulse_duration);duration of saturating pulse
<first_act_end_10>=>mpulse2;measure Fm'

;cycle_10 - measurements
<start_period_10, start_period_10 + first_act_measurements .. fp_start_10 - TS>=>mfmsub ;general
F' measurments, just for monitoring, no calculations done on this
<fp_start_10, fp_start_10 + f_measurements .. first_act_end_10 - TS>=>mfmsub;higher intensity F'
measurments for F'
<fmp_end_10, fmp_end_10 + second_act_measurements .. end_period_10 - TS>=>mfmsub;F'
measurements to have light relax, before a potential dark/FR relaxation

```

```

;Addition _10 - dark/FR relaxation
start_dark_period_10 = end_period_10 + TS
start_FR_period_10 = start_dark_period_10 + dark_period
end_FR_period_10 = start_FR_period_10 + FR_period
<start_FR_period_10>=>FAR(FR_light_period)
<end_FR_period_10>=>SatPulse(pulse_duration)
<end_FR_period_10>=>mpulse2
end_10 = end_FR_period_10 + 1s

<start_dark_period_10, start_dark_period_10 + dark_FR_measurements ..
end_FR_period_10>=>mfmsub

;cycle _11 - periods
start_period_11 = end_10
end_period_11 = start_period_11 + total_period_duration
first_act_end_11 = end_period_11 - 20s
fp_start_11 = first_act_end_11 - 2s;start for F' measurements
fmp_end_11 = first_act_end_11 + 1s;start for 20s Act light period
<start_period_11 - TS>=>act2(total_period_duration) ;set Act light for the given period
<start_period_11 - TS>=>SI_Act2(21.6);set Act light intensity during period
<first_act_end_11>=>SatPulse(pulse_duration);duration of saturating pulse
<first_act_end_11>=>mpulse2;measure Fm'

;cycle _11 - measurements
<start_period_11, start_period_11 + first_act_measurements .. fp_start_11 - TS>=>mfmsub ;general
F' measurments, just for monitoring, no calculations done on this
<fp_start_11, fp_start_11 + f_measurements .. first_act_end_11 - TS>=>mfmsub;higher intensity F'
measurments for F'
<fmp_end_11, fmp_end_11 + second_act_measurements .. end_period_11 - TS>=>mfmsub;F'
measurements to have light relax, before a potential dark/FR relaxation

;Addition _11 - dark/FR relaxation
start_dark_period_11 = end_period_11 + TS
start_FR_period_11 = start_dark_period_11 + dark_period
end_FR_period_11 = start_FR_period_11 + FR_period
<start_FR_period_11>=>FAR(FR_light_period)
<end_FR_period_11>=>SatPulse(pulse_duration)
<end_FR_period_11>=>mpulse2
end_11 = end_FR_period_11 + 1s

<start_dark_period_11, start_dark_period_11 + dark_FR_measurements ..
end_FR_period_11>=>mfmsub

;cycle _12 - periods
start_period_12 = end_11
end_period_12 = start_period_12 + total_period_duration
first_act_end_12 = end_period_12 - 20s
fp_start_12 = first_act_end_12 - 2s;start for F' measurements

```

```

fmp_end_12 = first_act_end_12 + 1s;start for 20s Act light period
<start_period_12 - TS>=>act2(total_period_duration) ;set Act light for the given period
<start_period_12 - TS>=>SI_Act2(77.9);set Act light intensity during period
<first_act_end_12>=>SatPulse(pulse_duration);duration of saturating pulse
<first_act_end_12>=>mpulse2;measure Fm'

;cycle_12 - measurements
<start_period_12, start_period_12 + first_act_measurements .. fp_start_12 - TS>=>mfmsub ;general
F' measurments, just for monitoring, no calculations done on this
<fp_start_12, fp_start_12 + f_measurements .. first_act_end_12 - TS>=>mfmsub;higher intensity F'
measurments for F'
<fmp_end_12, fmp_end_12 + second_act_measurements .. end_period_12 - TS>=>mfmsub;F'
measurements to have light relax, before a potential dark/FR relaxation

;Addition_12 - dark/FR relaxation
start_dark_period_12 = end_period_12 + TS
start_FR_period_12 = start_dark_period_12 + dark_period
end_FR_period_12 = start_FR_period_12 + FR_period
<start_FR_period_12>=>FAR(FR_light_period)
<end_FR_period_12>=>SatPulse(pulse_duration)
<end_FR_period_12>=>mpulse2
end_12 = end_FR_period_12 + 1s

<start_dark_period_12, start_dark_period_12 + dark_FR_measurements ..
end_FR_period_12>=>mfmsub

```

### 3: FvFm measurement DEPI treatment

TS=50ms

include default.inc ;Includes standard options, do not remove it !

include light.inc ;Includes standard options, do not remove it !

; Written by Sanne Put as a fast fluctuating light protocol

\_\_LightA=0

\_\_LightB=0

\_\_LightIntensity=<25.1>

Shutter=3

Sensitivity=1

Super=100

FAR=20

;general parameters

period\_Fv\_Fm = 60s

pulse\_duration = 800ms

first\_act\_measurements = 60s

second\_act\_measurements = 10s

f\_measurements = 200ms

;FvFm measurment (after dark adaptation during night)

start\_period\_FvFm = 0s

end\_period\_FvFm = start\_period\_FvFm + period\_Fv\_Fm

first\_act\_end\_FvFm = end\_period\_FvFm - 20s

fp\_start\_FvFm = first\_act\_end\_FvFm - 2s;start for F' measurements

fmp\_end\_FvFm = first\_act\_end\_FvFm + 1s;start for 20s Act light period

<start\_period\_FvFm>=>act2(period\_Fv\_Fm) ;set Act light for the given period

<start\_period\_FvFm>=>SI\_Act2(0);set Act light intensity during period

<first\_act\_end\_FvFm>=>SatPulse(pulse\_duration);duration of saturating pulse

<first\_act\_end\_FvFm>=>mpulse2;measure Fm'

; measurements

<start\_period\_FvFm, start\_period\_FvFm + first\_act\_measurements .. fp\_start\_FvFm - TS>=>mfmsub

;general F' measurments, just for monitoring, no calculations done on this

<fp\_start\_FvFm, fp\_start\_FvFm + f\_measurements .. first\_act\_end\_FvFm - TS>=>mfmsub;higher intensity F' measurments for F'

#### 4: First 3 cycles of the DEPI treatment

TS=50ms

include default.inc ;Includes standard options, do not remove it !

include light.inc ;Includes standard options, do not remove it !

; Written by Sanne Put as a fast fluctuating light protocol

\_\_LightA=0

\_\_LightB=0

\_\_LightIntensity=<25.1>

Shutter=3

Sensitivity=1

Super=100

FAR=20

;general parameters

period\_Fv\_Fm = 60s

total\_period\_duration1 = 1120s

total\_period\_duration2 = 640s

pulse\_duration = 800ms

first\_act\_measurements = 60s

second\_act\_measurements = 10s

f\_measurements = 200ms

Act\_intensity\_period\_1 = 12.4

Act\_intensity\_period\_2 = 15.0

Act\_intensity\_period\_3 = 15.1

dark\_period = 4s

FR\_period = 16s

FR\_light\_period = 5s

dark\_FR\_measurements = 1s

;FvFm measurment (after dark adaptation during night)

start\_period\_FvFm = 0s

end\_period\_FvFm = start\_period\_FvFm + period\_Fv\_Fm

first\_act\_end\_FvFm = end\_period\_FvFm - 20s

fp\_start\_FvFm = first\_act\_end\_FvFm - 2s;start for F' measurements

fmp\_end\_FvFm = first\_act\_end\_FvFm + 1s;start for 20s Act light period

<start\_period\_FvFm>=>act2(period\_Fv\_Fm) ;set Act light for the given period

<start\_period\_FvFm>=>Sl\_Act2(0);set Act light intensity during period

<first\_act\_end\_FvFm>=>SatPulse(pulse\_duration);duration of saturating pulse

<first\_act\_end\_FvFm>=>mpulse2;measure Fm'

; measurements

```

<start_period_FvFm, start_period_FvFm + first_act_measurements .. fp_start_FvFm - TS>=>mfmsub
;general F' measurments, just for monitoring, no calculations done on this
<fp_start_FvFm, fp_start_FvFm + f_measurements .. first_act_end_FvFm - TS>=>mfmsub;higher
intensity F' measurments for F'
<fmp_end_1, fmp_end_1 + second_act_measurements .. end_period_1 - TS>=>mfmsub;F'
measurements to have light relax, before a potential dark/FR relaxation

;FvFm - Take images
<fmp_end_1+TS>=>checkPoint,"startFt_Lss"
<end_period_FvFm>=>checkPoint,"endFt_Lss"

<first_act_end_FvFm+TS>=>checkPoint,"startFm_Lss"
<first_act_end_FvFm+pulse_duration-TS>=>checkPoint,"endFm_Lss"

;cycle _1 - periods
start_period_1 = end_period_FvFm
end_period_1 = start_period_1 + total_period_duration1
first_act_end_1 = end_period_1 - 20s
fp_start_1 = first_act_end_1 - 2s;start for F' measurements
fmp_end_1 = first_act_end_1 + 1s;start for 20s Act light period
<start_period_1>=>act2(total_period_duration1) ;set Act light for the given period
<start_period_1>=>SI_Act2(Act_intensity_period_1);set Act light intensity during period
<first_act_end_1>=>SatPulse(pulse_duration);duration of saturating pulse
<first_act_end_1>=>mpulse2;measure Fm'

;cycle _1 - measurements
<start_period_1, start_period_1 + first_act_measurements .. fp_start_1 - TS>=>mfmsub ;general F'
measurments, just for monitoring, no calculations done on this
<fp_start_1, fp_start_1 + f_measurements .. first_act_end_1 - TS>=>mfmsub;higher intensity F'
measurments for F'
<fmp_end_1, fmp_end_1 + second_act_measurements .. end_period_1 - TS>=>mfmsub;F'
measurements to have light relax, before a potential dark/FR relaxation

;Addition _1 - dark/FR relaxation
start_dark_period_1 = end_period_1 + TS
start_FR_period_1 = start_dark_period_1 + dark_period
end_FR_period_1 = start_FR_period_1 + FR_period
<start_FR_period_1>=>FAR(FR_light_period)
<end_FR_period_1>=>SatPulse(pulse_duration)
<end_FR_period_1>=>mpulse2
end_1 = end_FR_period_1 + 1s

<start_dark_period_1, start_dark_period_1 + dark_FR_measurements ..
end_FR_period_1>=>mfmsub

;Take images
<fmp_end_1+TS>=>checkPoint,"startFt_Lss"

```

```

<end_period_1>=>checkPoint,"endFt_Lss"

<first_act_end_1+TS>=>checkPoint,"startFm_Lss"
<first_act_end_1+pulse_duration-TS>=>checkPoint,"endFm_Lss"

;cycle _2 - periods
start_period_2 = end_1
end_period_2 = start_period_2 + total_period_duration2
first_act_end_2 = end_period_2 - 20s
fp_start_2 = first_act_end_2 - 2s;start for F' measurements
fmp_end_2 = first_act_end_2 + 1s;start for 20s Act light period
<start_period_2>=>act2(total_period_duration2) ;set Act light for the given period
<start_period_2>=>SI_Act2(Act_intensity_period_2);set Act light intensity during period
<first_act_end_2>=>SatPulse(pulse_duration);duration of saturating pulse
<first_act_end_2>=>mpulse2;measure Fm'

;cycle _2 - measurements
<start_period_2, start_period_2 + first_act_measurements .. fp_start_2 - TS>=>mfmsub ;general F'
measurments, just for monitoring, no calculations done on this
<fp_start_2, fp_start_2 + f_measurements .. first_act_end_2 - TS>=>mfmsub;higher intensity F'
measurments for F'
<fmp_end_2, fmp_end_2 + second_act_measurements .. end_period_2 - TS>=>mfmsub;F'
measurements to have light relax, before a potential dark/FR relaxation

;Addition _2 - dark/FR relaxation
start_dark_period_2 = end_period_2 + TS
start_FR_period_2 = start_dark_period_2 + dark_period
end_FR_period_2 = start_FR_period_2 + FR_period
<start_FR_period_2>=>FAR(FR_light_period)
<end_FR_period_2>=>SatPulse(pulse_duration)
<end_FR_period_2>=>mpulse2
end_2 = end_FR_period_2 + 1s

<start_dark_period_2, start_dark_period_2 + dark_FR_measurements ..
end_FR_period_2>=>mfmsub

;Take images
<fmp_end_2+TS>=>checkPoint,"startFt_Lss"
<end_period_2>=>checkPoint,"endFt_Lss"

<first_act_end_2+TS>=>checkPoint,"startFm_Lss"
<first_act_end_2+pulse_duration-TS>=>checkPoint,"endFm_Lss"

;cycle _3 - periods
start_period_3 = end_2
end_period_3 = start_period_3 + total_period_duration1
first_act_end_3 = end_period_3 - 20s

```

```

fp_start_3 = first_act_end_3 - 2s;start for F' measurements
fmp_end_3 = first_act_end_3 + 1s;start for 20s Act light period
<start_period_3>=>act2(total_period_duration1) ;set Act light for the given period
<start_period_3>=>SI_Act2(Act_intensity_period_3);set Act light intensity during period
<first_act_end_3>=>SatPulse(pulse_duration);duration of saturating pulse
<first_act_end_3>=>mpulse2;measure Fm'

;cycle_3 - measurements
<start_period_3, start_period_3 + first_act_measurements .. fp_start_3 - TS>=>mfmsub ;general F'
measurments, just for monitoring, no calculations done on this
<fp_start_3, fp_start_3 + f_measurements .. first_act_end_3 - TS>=>mfmsub;higher intensity F'
measurments for F'
<fmp_end_3, fmp_end_3 + second_act_measurements .. end_period_3 - TS>=>mfmsub;F'
measurements to have light relax, before a potential dark/FR relaxation

;Addition_3 - dark/FR relaxation
start_dark_period_3 = end_period_3 + TS
start_FR_period_3 = start_dark_period_3 + dark_period
end_FR_period_3 = start_FR_period_3 + FR_period
<start_FR_period_3>=>FAR(FR_light_period)
<end_FR_period_3>=>SatPulse(pulse_duration)
<end_FR_period_3>=>mpulse2
end_3 = end_FR_period_3 + 1s

<start_dark_period_3, start_dark_period_3 + dark_FR_measurements ..
end_FR_period_3>=>mfmsub

;Take images
<fmp_end_3+TS>=>checkPoint,"startFt_Lss"
<end_period_3>=>checkPoint,"endFt_Lss"

<first_act_end_3+TS>=>checkPoint,"startFm_Lss"
<first_act_end_3+pulse_duration-TS>=>checkPoint,"endFm_Lss"

```

## 5: First 6 cycles of alternating low and high light

TS=50ms

include default.inc ;Includes standard options, do not remove it !

include light.inc ;Includes standard options, do not remove it !

; Written by Sanne Put as a fast fluctuating light protocol

\_\_LightA=0

\_\_LightB=0

\_\_LightIntensity=<25.1>

Shutter=3

Sensitivity=1

Super=100

FAR=20

;general parameters

total\_period\_duration = 280s

pulse\_duration = 800ms

first\_act\_measurements = 60s

second\_act\_measurements = 10s

f\_measurements = 200ms

dark\_period = 4s

FR\_period = 16s

FR\_light\_period = 5s

dark\_FR\_measurements = 1s

PreSatPulse = 800ms

SatPulseDuration = 800ms

;cycle \_1 - periods

start\_period\_1 = 0s

end\_period\_1 = start\_period\_1 + total\_period\_duration

first\_act\_end\_1 = end\_period\_1 - 20s

fp\_start\_1 = first\_act\_end\_1 - 2s;start for F' measurements

fmp\_end\_1 = first\_act\_end\_1 + 1s;start for 20s Act light period

<start\_period\_1>=>act2(total\_period\_duration) ;set Act light for the given period

<start\_period\_1>=>SI\_Act2(16.5);set Act light intensity during period, 100 umol

<first\_act\_end\_1>=>SatPulse(pulse\_duration);duration of saturating pulse

<first\_act\_end\_1>=>mpulse2;measure Fm'

;cycle \_1 - measurements

<start\_period\_1, start\_period\_1 + first\_act\_measurements .. fp\_start\_1 - TS>=>mfmsub ;general F' measurements, just for monitoring, no calculations done on this

```

<fp_start_1, fp_start_1 + f_measurements .. first_act_end_1 - TS>=>mfmsub;higher intensity F'
measurments for F'
<fmp_end_1, fmp_end_1 + second_act_measurements .. end_period_1 - TS>=>mfmsub;F'
measurements to have light relax, before a potential dark/FR relaxation

```

```

;Addition _1 - dark/FR relaxation
start_dark_period_1 = end_period_1 + TS
start_FR_period_1 = start_dark_period_1 + dark_period
end_FR_period_1 = start_FR_period_1 + FR_period
<start_FR_period_1>=>FAR(FR_light_period)
<end_FR_period_1>=>SatPulse(pulse_duration)
<end_FR_period_1>=>mpulse2
end_1 = end_FR_period_1 + 1s

```

```

<start_dark_period_1, start_dark_period_1 + dark_FR_measurements ..
end_FR_period_1>=>mfmsub

```

```

;Take images
<fmp_end_1+TS>=>checkPoint,"startFt_Lss"
<end_period_1>=>checkPoint,"endFt_Lss"

```

```

<first_act_end_1+TS>=>checkPoint,"startFm_Lss"
<first_act_end_1+pulse_duration-TS>=>checkPoint,"endFm_Lss"

```

```

;cycle _2 - periods
start_period_2 = end_1
end_period_2 = start_period_2 + total_period_duration
first_act_end_2 = end_period_2 - 20s
fp_start_2 = first_act_end_2 - 2s;start for F' measurements
fmp_end_2 = first_act_end_2 + 1s;start for 20s Act light period
<start_period_2 - TS>=>act2(total_period_duration) ;set Act light for the given period
<start_period_2 - TS>=>Sl_Act2(77.6);set Act light intensity during period 1000 umol
<first_act_end_2>=>SatPulse(pulse_duration);duration of saturating pulse
<first_act_end_2>=>mpulse2;measure Fm'

```

```

;cycle _2 - measurements
<start_period_2, start_period_2 + first_act_measurements .. fp_start_2 - TS>=>mfmsub ;general F'
measurments, just for monitoring, no calculations done on this
<fp_start_2, fp_start_2 + f_measurements .. first_act_end_2 - TS>=>mfmsub;higher intensity F'
measurments for F'
<fmp_end_2, fmp_end_2 + second_act_measurements .. end_period_2 - TS>=>mfmsub;F'
measurements to have light relax, before a potential dark/FR relaxation

```

```

;Addition _2 - dark/FR relaxation
start_dark_period_2 = end_period_2 + TS
start_FR_period_2 = start_dark_period_2 + dark_period
end_FR_period_2 = start_FR_period_2 + FR_period
<start_FR_period_2>=>FAR(FR_light_period)

```

```

<end_FR_period_2>=>SatPulse(pulse_duration)
<end_FR_period_2>=>mpulse2
end_2 = end_FR_period_2 + 1s

<start_dark_period_2, start_dark_period_2 + dark_FR_measurements ..
end_FR_period_2>=>mfmsub

;Take images
<fmp_end_2+TS>=>checkPoint,"startFt_Lss"
<end_period_2>=>checkPoint,"endFt_Lss"

<first_act_end_2+TS>=>checkPoint,"startFm_Lss"
<first_act_end_2+pulse_duration-TS>=>checkPoint,"endFm_Lss"

;cycle_3 - periods
start_period_3 = end_2
end_period_3 = start_period_3 + total_period_duration
first_act_end_3 = end_period_3 - 20s
fp_start_3 = first_act_end_3 - 2s;start for F' measurements
fmp_end_3 = first_act_end_3 + 1s;start for 20s Act light period
<start_period_3>=>act2(total_period_duration) ;set Act light for the given period
<start_period_3>=>SI_Act2(43.7);set Act light intensity during period, 500 umol
<first_act_end_3>=>SatPulse(pulse_duration);duration of saturating pulse
<first_act_end_3>=>mpulse2;measure Fm'

;cycle_3 - measurements
<start_period_3, start_period_3 + first_act_measurements .. fp_start_3 - TS>=>mfmsub ;general F'
measurments, just for monitoring, no calculations done on this
<fp_start_3, fp_start_3 + f_measurements .. first_act_end_3 - TS>=>mfmsub;higher intensity F'
measurments for F'
<fmp_end_3, fmp_end_3 + second_act_measurements .. end_period_3 - TS>=>mfmsub;F'
measurements to have light relax, before a potential dark/FR relaxation

;Addition_3 - dark/FR relaxation
start_dark_period_3 = end_period_3 + TS
start_FR_period_3 = start_dark_period_3 + dark_period
end_FR_period_3 = start_FR_period_3 + FR_period
<start_FR_period_3>=>FAR(FR_light_period)
<end_FR_period_3>=>SatPulse(pulse_duration)
<end_FR_period_3>=>mpulse2
end_3 = end_FR_period_3 + 1s

<start_dark_period_3, start_dark_period_3 + dark_FR_measurements ..
end_FR_period_3>=>mfmsub

;Take images
<fmp_end_3+TS>=>checkPoint,"startFt_Lss"
<end_period_3>=>checkPoint,"endFt_Lss"

```

```

<first_act_end_3+TS>=>checkPoint,"startFm_Lss"
<first_act_end_3+pulse_duration-TS>=>checkPoint,"endFm_Lss"

;cycle _4 - periods
start_period_4 = end_3
end_period_4 = start_period_4 + total_period_duration
first_act_end_4 = end_period_4 - 20s
fp_start_4 = first_act_end_4 - 2s;start for F' measurements
fmp_end_4 = first_act_end_4 + 1s;start for 20s Act light period
<start_period_4 - TS>=>act2(total_period_duration) ;set Act light for the given period
<start_period_4 - TS>=>SI_Act2(77.6);set Act light intensity during period 1000 umol
<first_act_end_4>=>SatPulse(pulse_duration);duration of saturating pulse
<first_act_end_4>=>mpulse2;measure Fm'

;cycle _4 - measurements
<start_period_4, start_period_4 + first_act_measurements .. fp_start_4 - TS>=>mfmsub ;general F'
measurments, just for monitoring, no calculations done on this
<fp_start_4, fp_start_4 + f_measurements .. first_act_end_4 - TS>=>mfmsub;higher intensity F'
measurments for F'
<fmp_end_4, fmp_end_4 + second_act_measurements .. end_period_4 - TS>=>mfmsub;F'
measurements to have light relax, before a potential dark/FR relaxation

;Addition _4 - dark/FR relaxation
start_dark_period_4 = end_period_4 + TS
start_FR_period_4 = start_dark_period_4 + dark_period
end_FR_period_4 = start_FR_period_4 + FR_period
<start_FR_period_4>=>FAR(FR_light_period)
<end_FR_period_4>=>SatPulse(pulse_duration)
<end_FR_period_4>=>mpulse2
end_4 = end_FR_period_4 + 1s

<start_dark_period_4, start_dark_period_4 + dark_FR_measurements ..
end_FR_period_4>=>mfmsub

;Take images
<fmp_end_4+TS>=>checkPoint,"startFt_Lss"
<end_period_4>=>checkPoint,"endFt_Lss"

<first_act_end_4+TS>=>checkPoint,"startFm_Lss"
<first_act_end_4+pulse_duration-TS>=>checkPoint,"endFm_Lss"

;cycle _5 - periods
start_period_5 = end_4
end_period_5 = start_period_5 + total_period_duration
first_act_end_5 = end_period_5 - 20s
fp_start_5 = first_act_end_5 - 2s;start for F' measurements
fmp_end_5 = first_act_end_5 + 1s;start for 20s Act light period

```

```

<start_period_5>=>act2(total_period_duration) ;set Act light for the given period
<start_period_5>=>SI_Act2(16.5);set Act light intensity during period, 500 umol
<first_act_end_5>=>SatPulse(pulse_duration);duration of saturating pulse
<first_act_end_5>=>mpulse2;measure Fm'

;cycle _5 - measurements
<start_period_5, start_period_5 + first_act_measurements .. fp_start_5 - TS>=>mfmsub ;general F'
measurments, just for monitoring, no calculations done on this
<fp_start_5, fp_start_5 + f_measurements .. first_act_end_5 - TS>=>mfmsub;higher intensity F'
measurments for F'
<fmp_end_5, fmp_end_5 + second_act_measurements .. end_period_5 - TS>=>mfmsub;F'
measurements to have light relax, before a potential dark/FR relaxation

;Addition _5 - dark/FR relaxation
start_dark_period_5 = end_period_5 + TS
start_FR_period_5 = start_dark_period_5 + dark_period
end_FR_period_5 = start_FR_period_5 + FR_period
<start_FR_period_5>=>FAR(FR_light_period)
<end_FR_period_5>=>SatPulse(pulse_duration)
<end_FR_period_5>=>mpulse2
end_5 = end_FR_period_5 + 1s

<start_dark_period_5, start_dark_period_5 + dark_FR_measurements ..
end_FR_period_5>=>mfmsub

;Take images
<fmp_end_5+TS>=>checkPoint,"startFt_Lss"
<end_period_5>=>checkPoint,"endFt_Lss"

<first_act_end_5+TS>=>checkPoint,"startFm_Lss"
<first_act_end_5+pulse_duration-TS>=>checkPoint,"endFm_Lss"

;cycle _6 - periods
start_period_6 = end_5
end_period_6 = start_period_6 + total_period_duration
first_act_end_6 = end_period_6 - 20s
fp_start_6 = first_act_end_6 - 2s;start for F' measurements
fmp_end_6 = first_act_end_6 + 1s;start for 20s Act light period
<start_period_6 - TS>=>act2(total_period_duration) ;set Act light for the given period
<start_period_6 - TS>=>SI_Act2(77.6);set Act light intensity during period 1000 umol
<first_act_end_6>=>SatPulse(pulse_duration);duration of saturating pulse
<first_act_end_6>=>mpulse2;measure Fm'

;cycle _6 - measurements
<start_period_6, start_period_6 + first_act_measurements .. fp_start_6 - TS>=>mfmsub ;general F'
measurments, just for monitoring, no calculations done on this
<fp_start_6, fp_start_6 + f_measurements .. first_act_end_6 - TS>=>mfmsub;higher intensity F'
measurments for F'

```

<fmp\_end\_6, fmp\_end\_6 + second\_act\_measurements .. end\_period\_6 - TS>=>mfmsub;F'  
measurements to have light relax, before a potential dark/FR relaxation

;Addition \_6 - dark/FR relaxation

start\_dark\_period\_6 = end\_period\_6 + TS

start\_FR\_period\_6 = start\_dark\_period\_6 + dark\_period

end\_FR\_period\_6 = start\_FR\_period\_6 + FR\_period

<start\_FR\_period\_6>=>FAR(FR\_light\_period)

<end\_FR\_period\_6>=>SatPulse(pulse\_duration)

<end\_FR\_period\_6>=>mpulse2

end\_6 = end\_FR\_period\_6 + 1s

<start\_dark\_period\_6, start\_dark\_period\_6 + dark\_FR\_measurements ..

end\_FR\_period\_6>=>mfmsub

;Take images

<fmp\_end\_6+TS>=>checkPoint,"startFt\_Lss"

<end\_period\_6>=>checkPoint,"endFt\_Lss"

<first\_act\_end\_6+TS>=>checkPoint,"startFm\_Lss"

<first\_act\_end\_6+pulse\_duration-TS>=>checkPoint,"endFm\_Lss"

## 2. Python scripts for Fluctor

### 1: Constant light treatment

```
# Main for starting the 5 PWM output, can be controlled by REPLD
import esp32
import machine
import time
import os

pin4 = machine.Pin(4, machine.Pin.OUT)
pin15 = machine.Pin(15, machine.Pin.OUT)
pin18 = machine.Pin(18, machine.Pin.OUT)
pin19 = machine.Pin(19, machine.Pin.OUT)
pin22 = machine.Pin(22, machine.Pin.OUT)

pwm4 = machine.PWM(pin4)
pwm15 = machine.PWM(pin15)
pwm18 = machine.PWM(pin18)
pwm19 = machine.PWM(pin19)
pwm22 = machine.PWM(pin22)

pwm4.freq(2000)
pwm15.freq(2000)
pwm18.freq(2000)
pwm19.freq(2000)
pwm22.freq(2000)

for i in range(1, 7):
    pwm4.duty(1023)
    pwm15.duty(1023)
    pwm18.duty(1023)
    pwm19.duty(1023)
    pwm22.duty(1023)

    time.sleep(3600) # setting to start while loop at 1:00, press boot at 19:00

while True:

    for i in range(1,17):
        pwm4.duty(564) # 438 umol/m2/s
        pwm15.duty(564)
        pwm18.duty(564)
        pwm19.duty(564)
        pwm22.duty(564)

        time.sleep(3600) # day of 16 hours
        print("one hour past, ", i, "hour(s) in total")

    for i in range(1,9):
        pwm4.duty(1023)
        pwm15.duty(1023)
        pwm18.duty(1023)
        pwm19.duty(1023)
        pwm22.duty(1023)

        time.sleep(3600) # night of 8 hours
        print("one hour past, ", i, "hour(s) in total")
```

## 2: DEPI light treatment

*#Main for starting the 5 PWM output, can be controlled by REPLD*

```
import machine
import time
import os
```

```
def read_chunks(file_object):
```

*"""Lazy function (generator) to read a file piece by piece.  
Default chunk size: 1k."""*

```
    while True:
```

```
        data = file_object.readline()
```

```
        if not data:
```

```
            break
```

```
        yield int(data)
```

```
pin4 = machine.Pin(4,machine.Pin.OUT)
```

```
pin15 = machine.Pin(15,machine.Pin.OUT)
```

```
pin18 = machine.Pin(18,machine.Pin.OUT)
```

```
pin19 = machine.Pin(19,machine.Pin.OUT)
```

```
pin22 = machine.Pin(22,machine.Pin.OUT)
```

```
pwm4 = machine.PWM(pin4)
```

```
pwm15 = machine.PWM(pin15)
```

```
pwm18 = machine.PWM(pin18)
```

```
pwm19 = machine.PWM(pin19)
```

```
pwm22 = machine.PWM(pin22)
```

```
pwm4.freq(2000)
```

```
pwm15.freq(2000)
```

```
pwm18.freq(2000)
```

```
pwm19.freq(2000)
```

```
pwm22.freq(2000)
```

```
for i in os.listdir():
```

```
    if ".txt" in i:
```

```
        filename = i
```

```
for i in range(1, 10):
```

```
    pwm4.duty(1023)
```

```
    pwm15.duty(1023)
```

```
    pwm18.duty(1023)
```

```
    pwm19.duty(1023)
```

```
    pwm22.duty(1023)
```

```
    time.sleep(3420) #setting to start while loop at 1:00, press boot at 15:30
```

```
while True:
```

```
    count = 0
```

```
    with open(filename, "r") as f:
```

```
        for piece in read_chunks(f):
```

```
            count += 1
```

```
            if (count % 2) == 1: # if odd number --> Low Light
```

```
                print("The pwm value is: " + str(piece))
```

```
                print(count)
```

```
                pwm4.duty(piece)
```

```
                pwm15.duty(piece)
```

```
                pwm18.duty(piece)
```

```
                pwm19.duty(piece)
```

```
                pwm22.duty(piece)
```

```

        time.sleep(1137) ## #seconds Low Light
    elif (count % 2) == 0: # if even number --> high Light
        print("The pwm value is: " + str(piece))
        print(count)
        pwm4.duty(piece)
        pwm15.duty(piece)
        pwm18.duty(piece)
        pwm19.duty(piece)
        pwm22.duty(piece)
        time.sleep(663) ## #seconds high Light 667

for i in range(1,9):
    pwm4.duty(1023)
    pwm15.duty(1023)
    pwm18.duty(1023)
    pwm19.duty(1023)
    pwm22.duty(1023)

time.sleep(3600) # night of 8 hours
print("one hour past, ", i, "hour(s) in total")

```

### 3: Fluctuating maize treatment

*# Main for starting the 5 PWM output, can be controlled by REPLD*

```

import esp32
import machine
import time
import os

def read_chunks(file_object):
    """Lazy function (generator) to read a file piece by piece.
    Default chunk size: 1k."""
    while True:
        data = file_object.readline()
        if not data:
            break
        yield int(data) # Read 4 bytes but yield 3 (remove space)

pin4 = machine.Pin(4, machine.Pin.OUT)
pin15 = machine.Pin(15, machine.Pin.OUT)
pin18 = machine.Pin(18, machine.Pin.OUT)
pin19 = machine.Pin(19, machine.Pin.OUT)
pin22 = machine.Pin(22, machine.Pin.OUT)

pwm4 = machine.PWM(pin4)
pwm15 = machine.PWM(pin15)
pwm18 = machine.PWM(pin18)
pwm19 = machine.PWM(pin19)
pwm22 = machine.PWM(pin22)

pwm4.freq(2000)
pwm15.freq(2000)
pwm18.freq(2000)
pwm19.freq(2000)
pwm22.freq(2000)

for i in os.listdir():
    if ".txt" in i:
        filename = i

```

```

for i in range(1, 8):
    pwm4.duty(1023)
    pwm15.duty(1023)
    pwm18.duty(1023)
    pwm19.duty(1023)
    pwm22.duty(1023)

    time.sleep(3600) #setting to start while loop at 1:00, press boot at 18:00

while True:

    with open(filename, "r") as f:
        for piece in read_chunks(f):
            print("The pwm value is: " + str(piece))
            pwm4.duty(piece)
            pwm15.duty(piece)
            pwm18.duty(piece)
            pwm19.duty(piece)
            pwm22.duty(piece)
            time.sleep(0.1544) # 0.16 second at certain light intensity

    for i in range(1,9):
        pwm4.duty(1023)
        pwm15.duty(1023)
        pwm18.duty(1023)
        pwm19.duty(1023)
        pwm22.duty(1023)

        time.sleep(3600) # night of 8 hours
        print("one hour past, ", i, "hour(s) in total")

```

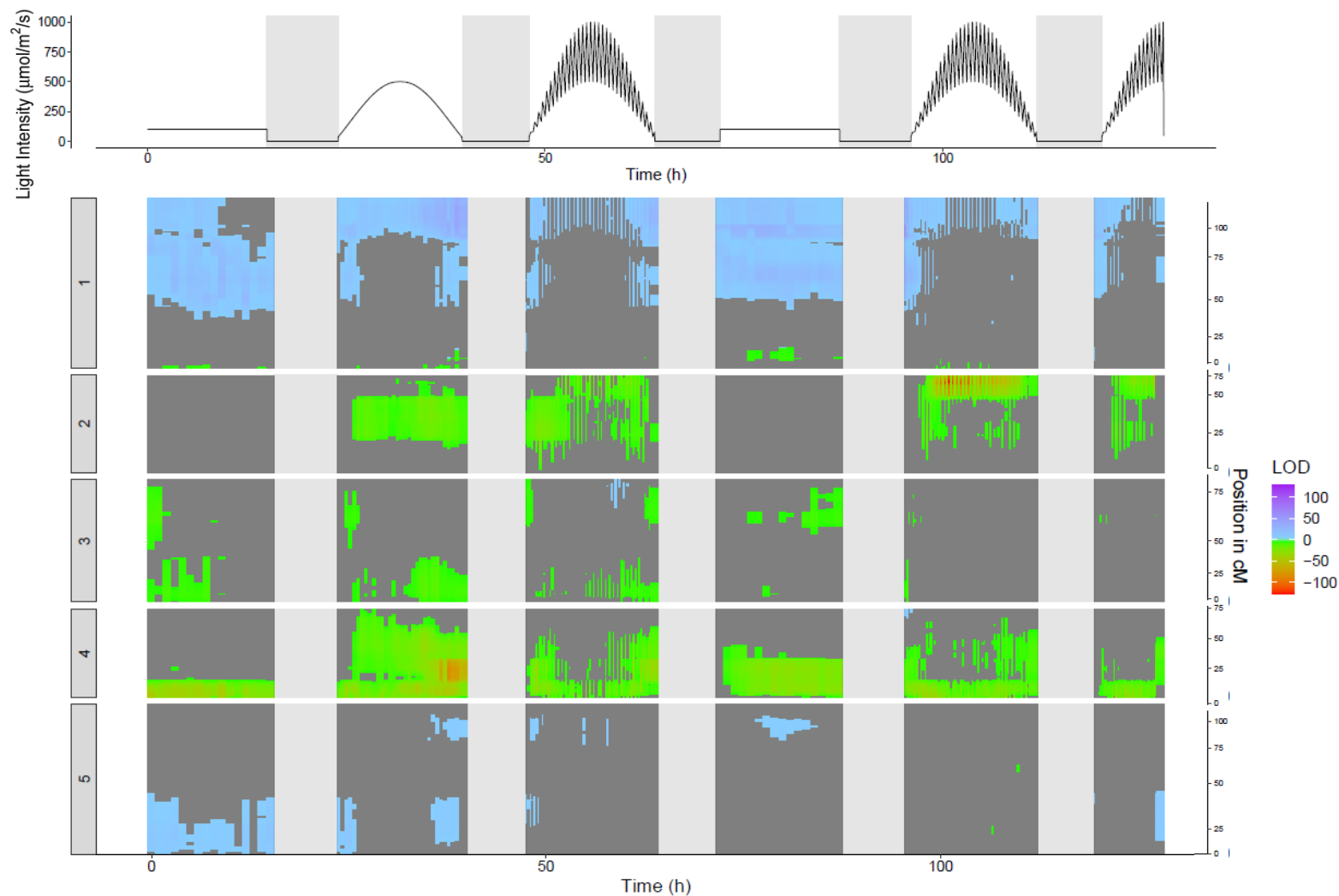
S 1 KASP primers used for genotyping. FW1 is the forward primer of Col and FW 2 the forward primer of Ely. RV is the reverse primer.

Name	Chr	Position (bp)	Primer	Sequence
1_892	1	892	FW 1	GAAGGTGACCAAGTTCATGCTATGTGTAAACGTGTGTCGATCATAATTTTA
1_892	1	892	FW 2	GAAGGTCGGAGTCAACGGATTGTGTAAACGTGTGTCGATCATAATTTTC
1_892	1	892	RV	GTCGTAGATATTAGGTAATCTGTAAGTCAA
1_3600341	1	3600341	FW 1	GAAGGTGACCAAGTTCATGCTGTACTTAAAAGAGATGGTGAACATTCATAT
1_3600341	1	3600341	FW 2	GAAGGTCGGAGTCAACGGATTACTTAAAAGAGATGGTGAACATTCATAG
1_3600341	1	3600341	RV	AGAGTATGGACAATGGTGACTACAGAAA
2_17603	2	17603	FW 1	GAAGGTGACCAAGTTCATGCTGGATCGTTGGCTAGGACTTGGA
2_17603	2	17603	FW 2	GAAGGTCGGAGTCAACGGATTGGATCGTTGGCTAGGACTTGGT
2_17603	2	17603	RV	CTTATGGCCAGCTCCTTCTCACTT
2_24794	2	24794	FW 1	GAAGGTGACCAAGTTCATGCTGATAATCGATGCATAGCCTAAAGCTAA
2_24794	2	24794	FW 2	GAAGGTCGGAGTCAACGGATTATAATCGATGCATAGCCTAAAGCTAC
2_24794	2	24794	RV	GCCGGTTCTGTTTGTGAAAAAGAAAGAT
2_290715	2	290715	FW 1	GAAGGTGACCAAGTTCATGCTACAACGTTAAGTGATTATGATAATTAGAAATAC
2_290715	2	290715	FW 2	GAAGGTCGGAGTCAACGGATTACAACGTTAAGTGATTATGATAATTAGAAATAT
2_290715	2	290715	RV	GAACATTTAAAAGTGGCGCATATGCAATTT
2_17680399	2	17680399	FW 1	GAAGGTGACCAAGTTCATGCTCGTTAAAAACCATGATCAGTTTTGAGTAA
2_17680399	2	17680399	FW 2	GAAGGTCGGAGTCAACGGATTCTGTTAAAAACCATGATCAGTTTTGAGTAT
2_17680399	2	17680399	RV	GACTGCTCTATGATTATCATGTAGTTTGAT
2_17850242	2	17850242	FW 1	GAAGGTGACCAAGTTCATGCTCGTTAAAAACCATGATCAGTTTTGAGTAA
2_17850242	2	17850242	FW 2	GAAGGTCGGAGTCAACGGATTCTGTTAAAAACCATGATCAGTTTTGAGTAT
2_17850242	2	17850242	RV	GACTGCTCTATGATTATCATGTAGTTTGAT
2_18503999	2	18503999	FW 1	GAAGGTGACCAAGTTCATGCTTTGATTTTCGTTTCCAGACTATTTTCTTC
2_18503999	2	18503999	FW 2	GAAGGTCGGAGTCAACGGATTCTTTGATTTTCGTTTCCAGACTATTTTCTTT
2_18503999	2	18503999	RV	GCGATTATTATAAECTCGGAACAGCCAT
2_18674359	2	18674359	FW 1	GAAGGTGACCAAGTTCATGCTGCAGCTTCCTCCGGATATTTCT
2_18674359	2	18674359	FW 2	GAAGGTCGGAGTCAACGGATTGCAGCTTCCTCCGGATATTTCC
2_18674359	2	18674359	RV	CTCTTGAAGCTGATTGGAATGCCAAATTT
2_18791560	2	18791560	FW 1	GAAGGTGACCAAGTTCATGCTGCGCCATTTGTTTGGCCGAG
2_18791560	2	18791560	FW 2	GAAGGTCGGAGTCAACGGATTGCGCCATTTGTTTGGCCGAT
2_18791560	2	18791560	RV	CGTCTTTGGAAGCGGCGAGCTT
2_18808619	2	18808619	FW 1	GAAGGTGACCAAGTTCATGCTGATCAAACCTCTCTATGCATCATGAA
2_18808619	2	18808619	FW 2	GAAGGTCGGAGTCAACGGATTGATCAAACCTCTCTATGCATCATGAT
2_18808619	2	18808619	RV	ATTGAACAACCTCGAAGCTTCTAAACGGTT
2_18823399	2	18823399	FW 1	GAAGGTGACCAAGTTCATGCTATCTGTAGGAGCCTTTGATGATCTG
2_18823399	2	18823399	FW 2	GAAGGTCGGAGTCAACGGATTAAATCTGTAGGAGCCTTTGATGATCTA
2_18823399	2	18823399	RV	TATATGCAACGGAAGAATATTGCCATTGTT
2_18839659	2	18839659	FW 1	GAAGGTGACCAAGTTCATGCTCAGAGGCATTGCGAATTTTAATTGATCA
2_18839659	2	18839659	FW 2	GAAGGTCGGAGTCAACGGATTAGAGGCATTGCGAATTTTAATTGATCG
2_18839659	2	18839659	RV	TTTCGATTGGAATACCTTTGGGAGAGAT
2_18855185	2	18855185	FW 1	GAAGGTGACCAAGTTCATGCTGATAAGAAAATCTCGCACTAAGGAGAA
2_18855185	2	18855185	FW 2	GAAGGTCGGAGTCAACGGATTATAAGAAAATCTCGCACTAAGGAGAG
2_18855185	2	18855185	RV	AAGATCCTCAACGTAAAGAGGATTCGATT
2_18875047	2	18875047	FW 1	GAAGGTGACCAAGTTCATGCTGTAGTTTATGATATCTGTGAGTTGGTTG

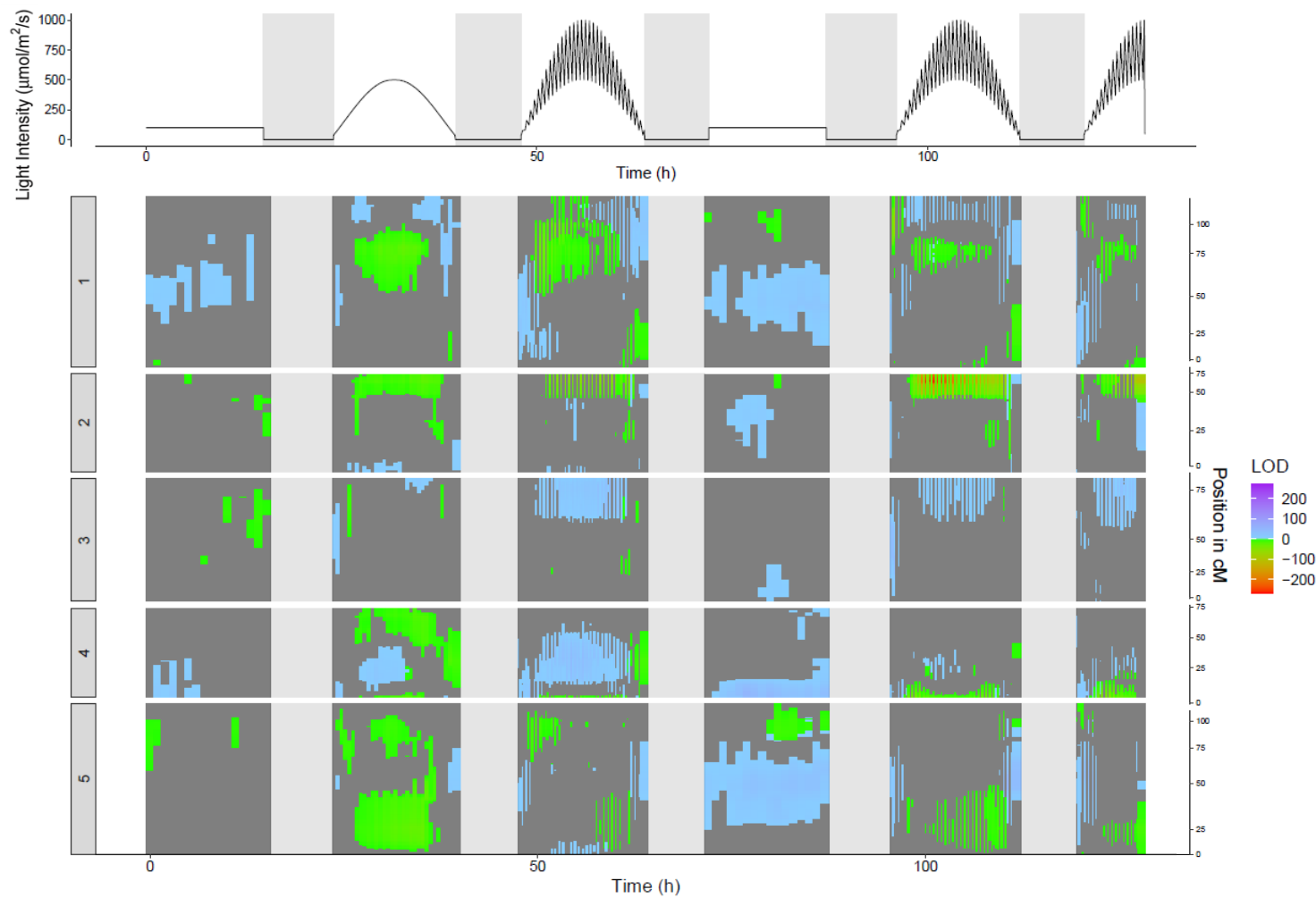
2_18875047	2	18875047	FW 2	GAAGGTCGGAGTCAACGGATTAGTAGTTTATGATATCTGTGAGTTGGTTT
2_18875047	2	18875047	RV	CTTTCTTCTTCTTCCCACTTCCATCAA
2_18975138	2	18975138	FW 1	GAAGGTGACCAAGTTCATGCTATATGATATAGAAGAGATAGGGCTTTTAC
2_18975138	2	18975138	FW 2	GAAGGTCGGAGTCAACGGATTGATATGATATAGAAGAGATAGGGCTTTTAA
2_18975138	2	18975138	RV	GGAAGTAATATCGACATGGGTAGACTTAT
2_19000351	2	19000351	FW 1	GAAGGTGACCAAGTTCATGCTTCGAATTCGCGATTAAATTTCTGGG
2_19000351	2	19000351	FW 2	GAAGGTCGGAGTCAACGGATTGTTTCGAATTCGCGATTAAATTTCTGGA
2_19000351	2	19000351	RV	TGAAGAAGAAGAAGCTTAAACAGAGAGAAA
2_19054489	2	19054489	FW 1	GAAGGTGACCAAGTTCATGCTACTTGAGAGATTTACTATCTCAATAGTTC
2_19054489	2	19054489	FW 2	GAAGGTCGGAGTCAACGGATTACTTGAGAGATTTACTATCTCAATAGTTT
2_19054489	2	19054489	RV	CAAAACAAGAATAGTTCATCATCATTCCCTA
2_19055629	2	19055629	FW 1	GAAGGTGACCAAGTTCATGCTCAAGCAAATGATTGAATACAACACTATTTGC
2_19055629	2	19055629	FW 2	GAAGGTCGGAGTCAACGGATTCTCAAGCAAATGATTGAATACAACACTATTTGT
2_19055629	2	19055629	RV	GTTTTTCAGGGCATATTACATCAGACTGAA
2_19694796	2	19694796	FW 1	GAAGGTGACCAAGTTCATGCTACGTCATCAATAGTCTGTCCACCA
2_19694796	2	19694796	FW 2	GAAGGTCGGAGTCAACGGATTTCGTCATCAATAGTCTGTCCACCG
2_19694796	2	19694796	RV	ATCAAGGTTGCAAACAGAGAAGCTTCAAA

*S 2 Light intensities DEPI treatment.*

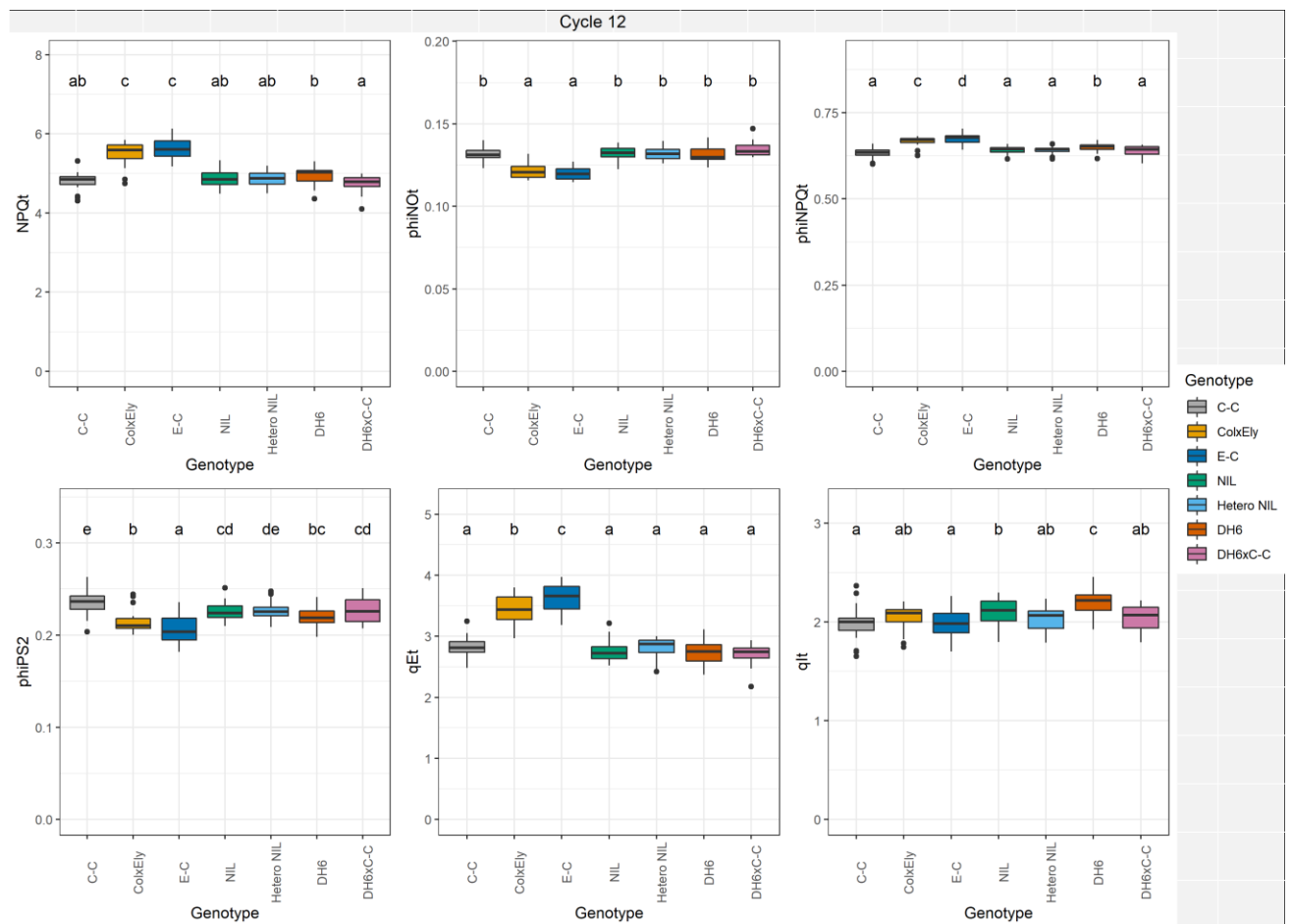
Cycle	Time (min)	Light intensity ( $\mu\text{mol}/\text{m}^2/\text{s}$ )		Cycle	Time (min)	Light intensity ( $\mu\text{mol}/\text{m}^2/\text{s}$ )
1	20	39		33	20	500
2	10	78		34	10	1000
3	20	80		35	20	497
4	10	161		36	10	994
5	20	123		37	20	489
6	10	246		38	10	977
7	20	167		39	20	475
8	10	333		40	10	949
9	20	210		41	20	455
10	10	420		42	10	911
11	20	253		43	20	431
12	10	506		44	10	862
13	20	294		45	20	402
14	10	588		46	10	805
15	20	333		47	20	370
16	10	667		48	10	739
17	20	370		49	20	333
18	10	739		50	10	667
19	20	402		51	20	294
20	10	805		52	10	588
21	20	431		53	20	253
22	10	862		54	10	506
23	20	455		55	20	210
24	10	911		56	10	420
25	20	475		57	20	167
26	10	949		58	10	333
27	20	489		59	20	123
28	10	977		60	10	246
29	20	497		61	20	80
30	10	994		62	10	161
31	20	500		63	20	39
32	10	1000		64	10	78



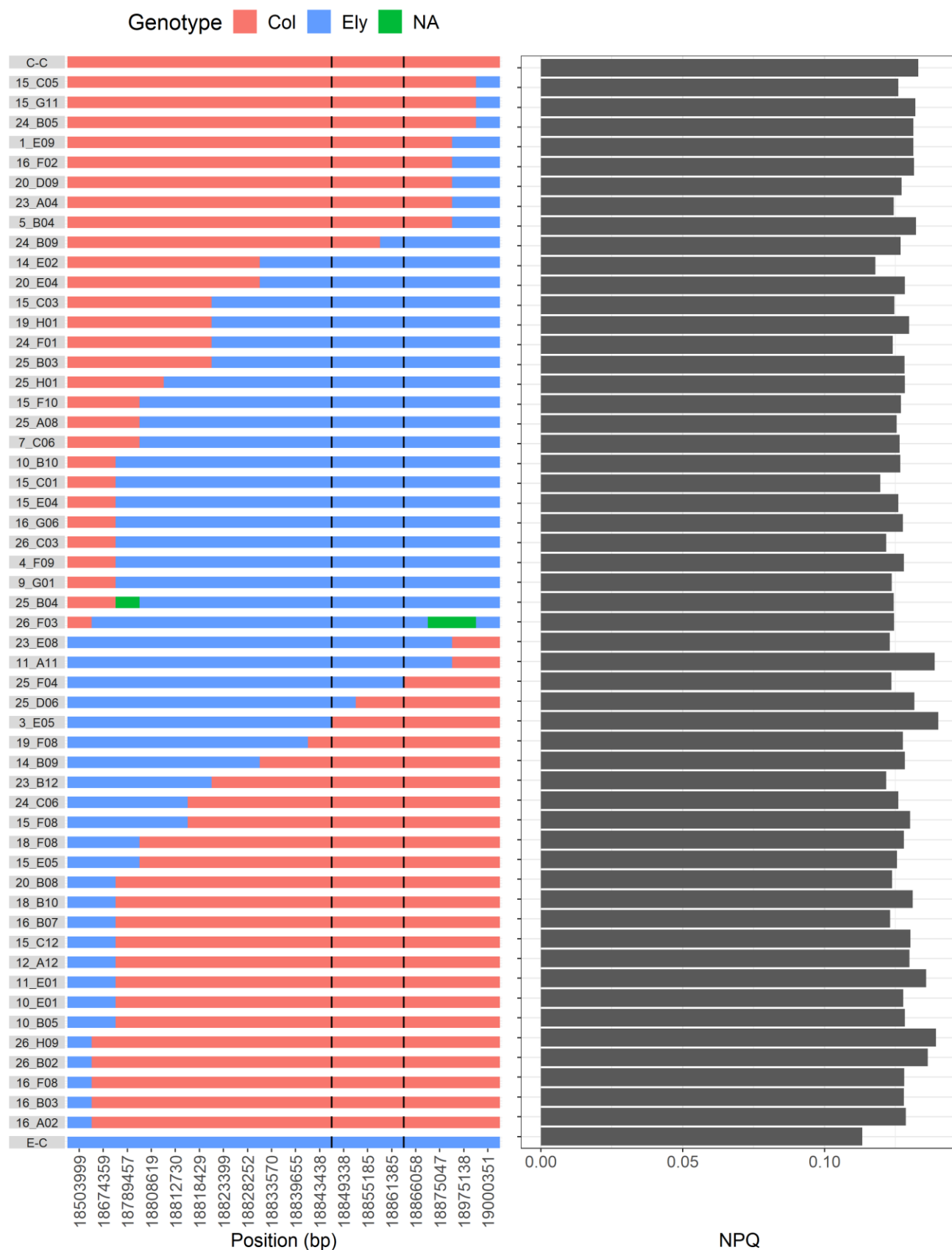
*S 3 QTL analysis of phiNPQ on biparental DH population, extended with late flowering lines. The upper plot shows the light treatment over 5.5 days. The lower plot is the QTL plot with on the left y-axis the chromosome numbers and on the right y-axis the position on the chromosome in cM. On the x-axis the time is equal to the time in the plot of the light intensity and the QTLs are therefore appearing under this light condition. Grey areas are not significant QTLs. The legend shows positive and negative LOD scores. A positive LOD score indicates a higher BLUEs for C-C than E-C, a negative LOD scores vice versa. The threshold is a LOD score of 4.776 ( $p < 1.7e-05$ ) and the QTL analysis was based on an MQM analysis, with markers every 250,000 bp.*



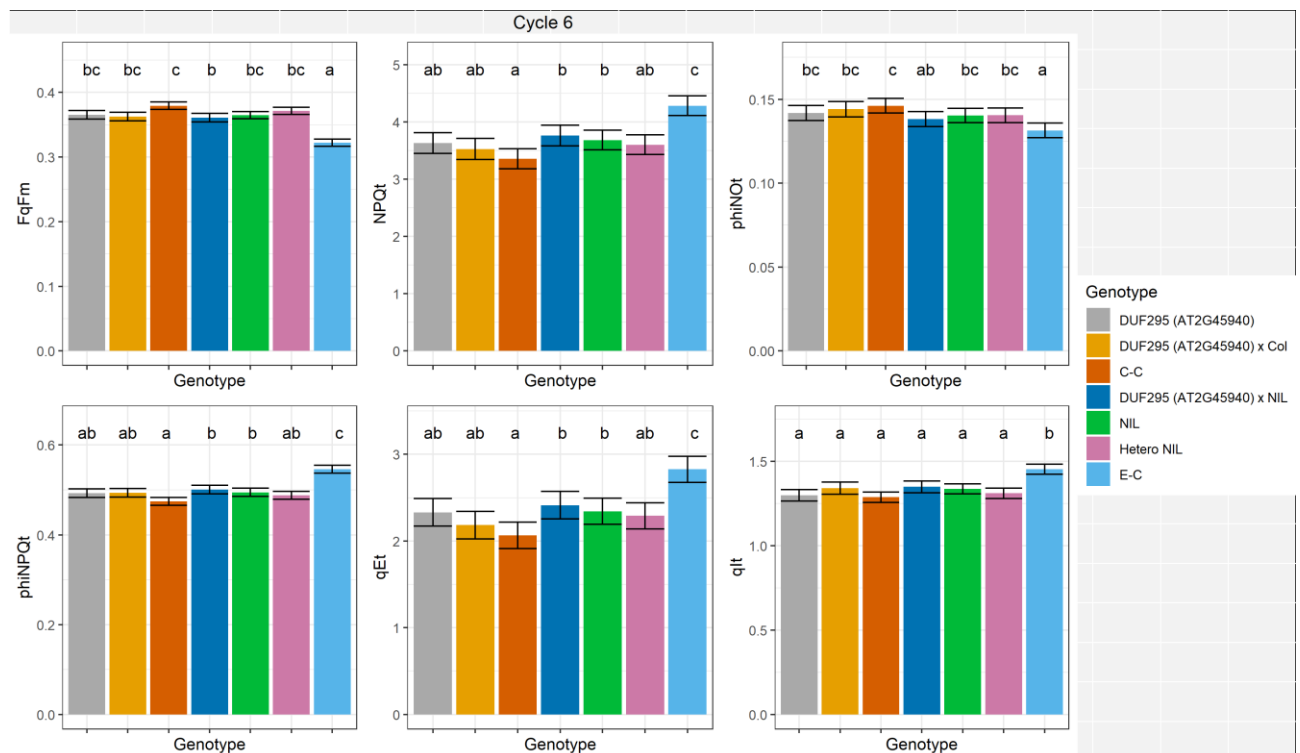
*S 4 QTL analysis of qEsv on biparental DH population, extended with late flowering lines. The upper plot shows the light treatment over 5.5 days. The lower plot is the QTL plot with on the left y-axis the chromosome numbers and on the right y-axis the position on the chromosome in cM. On the x-axis the time is equal to the time in the plot of the light intensity and the QTLs are therefore appearing under this light condition. Grey areas are not significant QTLs. The legend shows positive and negative LOD scores. A positive LOD score indicates a higher BLUEs for C-C than E-C, a negative LOD scores vice versa. The threshold is a LOD score of 4.776 ( $p < 1.7e-05$ ) and the QTL analysis was based on an MQM analysis, with markers every 250,000 bp.*



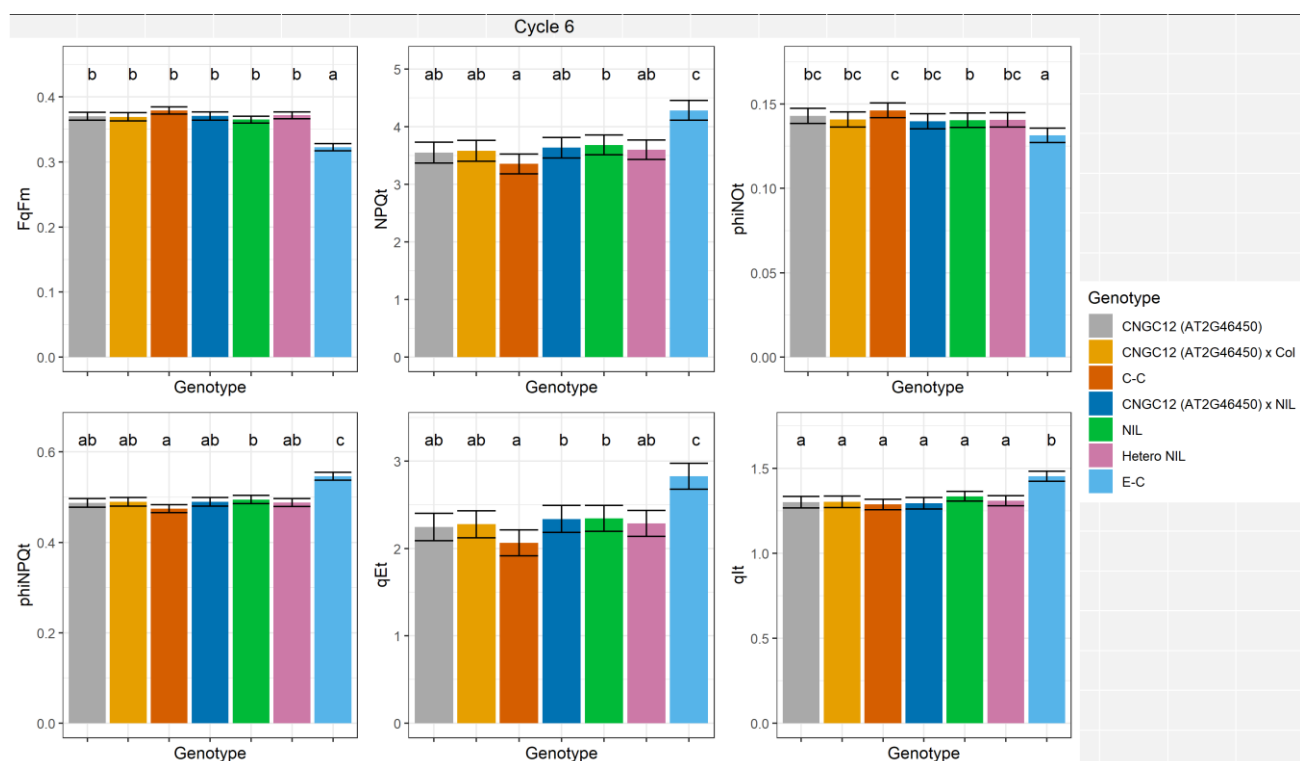
*S 5 NPQt for different genotypes after 12 fluctuations of 5 minutes with alternating low and high light. Letters show the result of a Tukey post hoc test.*



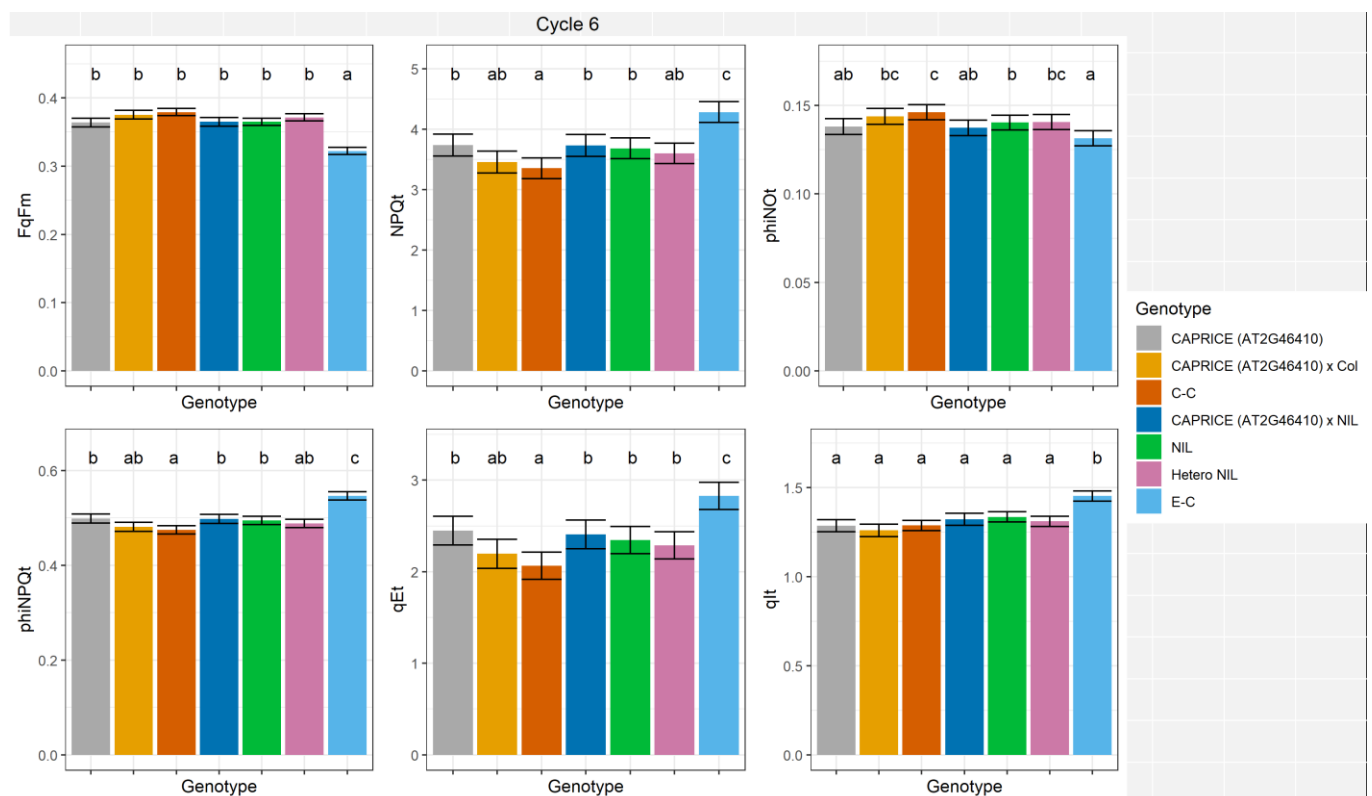
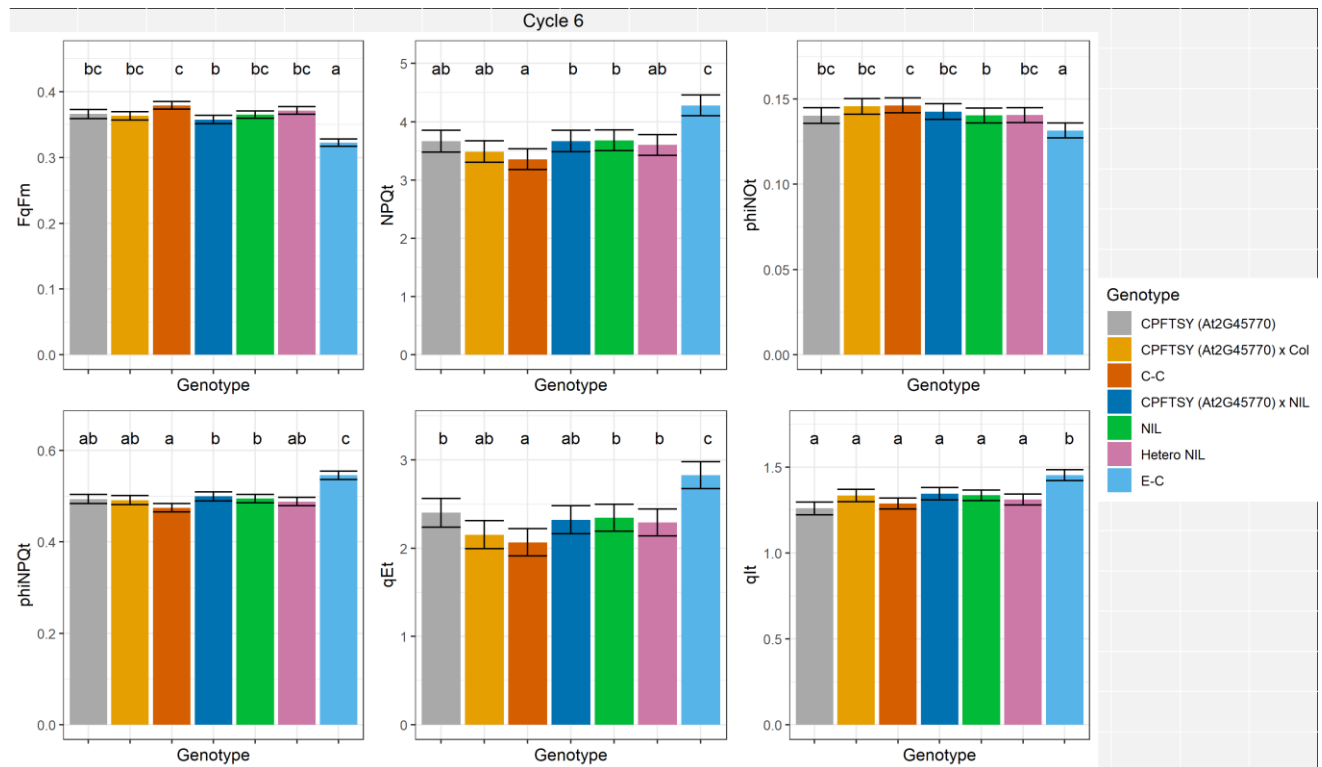
S 6 Qualitative analysis with all recombinant lines included. On the left side a plot is shown with the genotype data of the selected lines in the region of the QTL. On the right side a plot is shown with the NPQ values per line. The black lines in the left plot show the position of the QTL, based on the qualitative analysis.

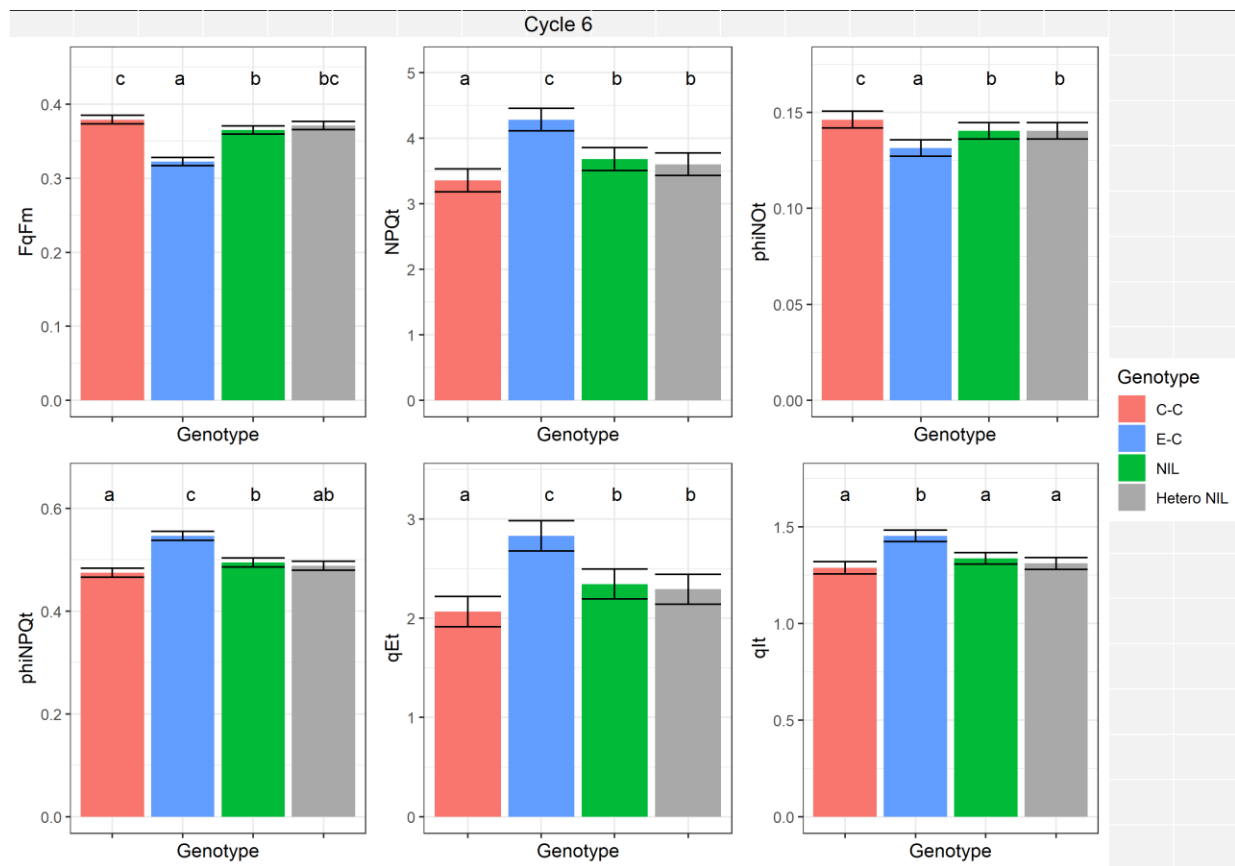


*S 7 Photosynthesis phenotypes of the allelic complementation experiment of DUF for the 6<sup>th</sup> cycle of 5-minute low and light light alternations.*

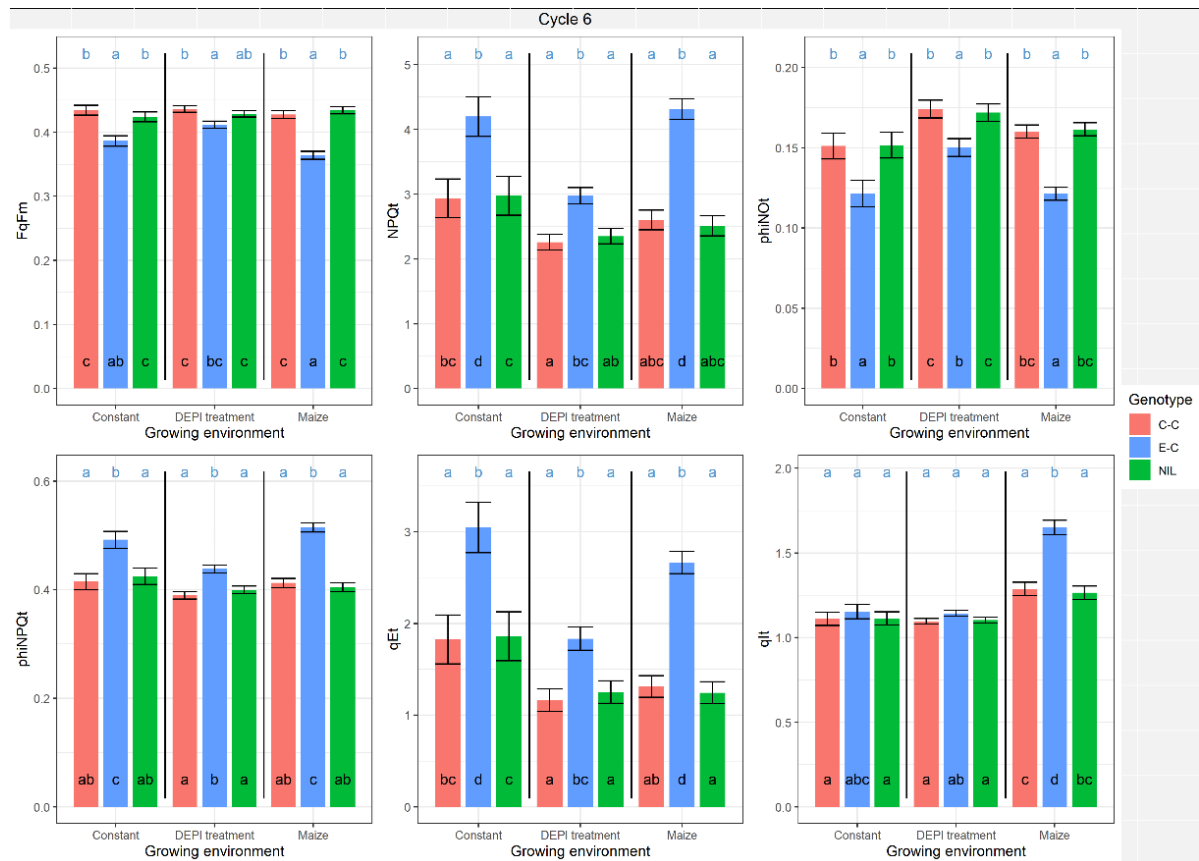


*S 8 Photosynthesis phenotypes of the allelic complementation experiment of CNGC12 for the 6<sup>th</sup> cycle of 5-minute low and light light alternations.*





*S 11 Photosynthesis phenotypes for C-C, E-C, NIL and hetero NIL. The letters on the top indicate the significantly different groups for a tukey post hoc test for the different genotypes.*



*S 12 Photosynthesis phenotypes for C-C, E-C and the NIL in different growing environments. The constant environment is a constant light intensity of 430 and the DEPI treatment is the same light treatment as was used in DEPI experiments. The light intensity in the maize growing environment is based on measurements outside during a highly fluctuating day. Blue letters on the top indicate the significantly different groups for a tukey post hoc test for genotypes within a growing environment. The black letters on the bottom indicate the significantly different groups for a tukey post hoc test for interaction between genotypes and growing environments.*