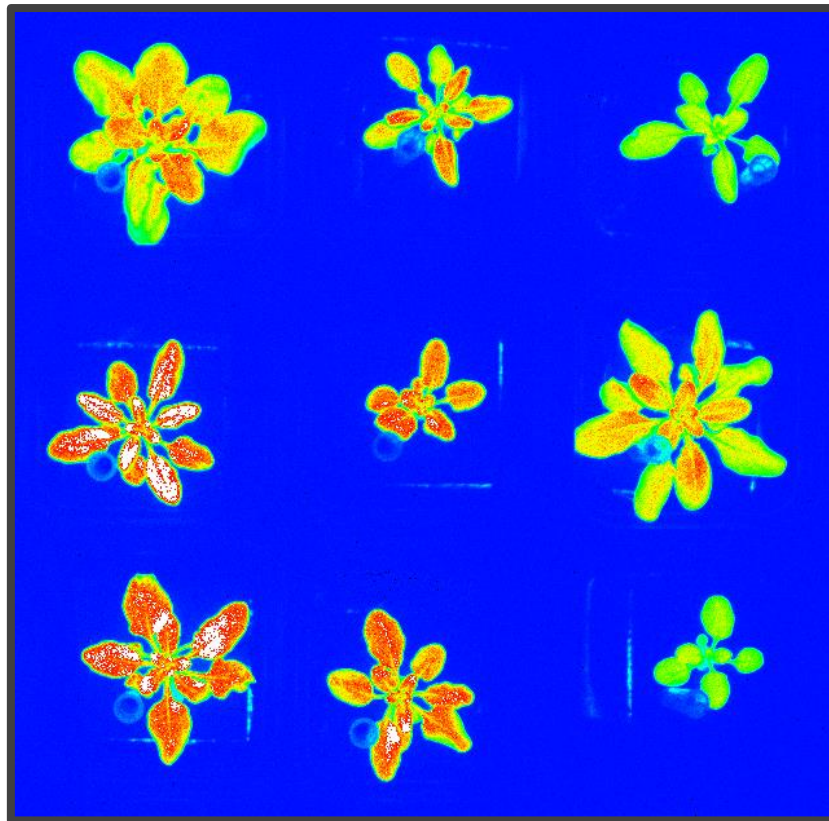# Preliminary exploration of the NPQ phenotype of the Ely nucleotype

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Contents

Summary

As sessile organisms, plants are exposed to changing environments continuously. Since they do not have the luxury to evade these changes they are dependent on their ability to acclimate to dynamic environments. This general plasticity is dependent on the available genetic variation of the plant composed of the nuclear genome and the plasmotype genome (mitochondrial and chloroplastic genomes). One such combination in *Arabidopsis thaliana*, an Ely nucleotype with a Columbia plasmotype (E-C) was previously shown to have a higher non-photochemical quenching (NPQ) than the Columbia nucleotype with a Columbia plasmotype (C-C). NPQ is a mechanism that the plant uses to protect itself from damage caused by excess light and is tightly linked with photosynthesis efficiency. In this thesis I set up a protocol to phenotype NPQ and other photosynthesis related traits in a new phenotyping platform the Robin in Wageningen. Once that worked I phenotyped C-C plants, E-C plants and plants from a doubled haploid population which I created from a cross between C-C and E-C (the LUX population). I was able to measure the difference in NPQ in E-C compared to C-C that was previously observed. Furthermore I show that exposure to fluctuating light increases the E-C NPQ phenotype and that the increase in NPQ is probably caused by a quick mechanism involved in NPQ called qE. Besides I started phenotyping the LUX population and I show that the LUX population has variation for NPQ, qE, qI, φNPQ, φNO and φPSII, which makes it very suitable to further photosynthesis research.

Introduction

The human population is expected to grow to 10 billion individuals by 2050. In order to feed our world population adequately our current crops need to produce 60% more biomass by 2050 than they did in 2006 (Alexandratos & Bruinsma, 2012). Breeders can make better-performing crops by selecting for traits that make the crop more suitable for the environment they grow in. These traits include resistances against pests, ease of mechanical harvesting, drought tolerance or salt tolerance (Loboguerrero et al., 2018). Natural variation in the traits of interest is essential for a breeder, because they are dependent on natural variation of a certain trait to improve the trait (Gur & Zamir, 2004). The vast majority of plant traits are not directly related to yield increases because natural selection favours plants with high fitness, where yield does not necessarily increase fitness. As sessile organisms, plants are exposed to changing environments continuously. Since they do not have the luxury to evade these changes they are dependent on their ability to acclimate to dynamic environments. Consequentially, a lot of plant traits are geared towards this acclimation. Along those lines, breeders will find a lot of natural variation in acclimation traits; the variation that can be used for selection of robust and dynamic crops.

Crop yield is often modelled as depending on four different factors: 1) the level of light energy received per unit of arable area; 2) the efficiency at which the plant is able to intercept the light energy; 3) the efficiency at which a plant is able to convert intercepted light energy into biomass; 4) and the amount of biomass invested in the harvestable parts (Long, Marshall-Colon, & Zhu, 2015). Evidentially, the amount of sunlight is not something we have control over but we do over the other 3. Through the green revolution crops have increased drastically in light interception and partitioning of biomass to harvestable parts (factors 2 and 4), leading to yield increases up to 60%. That leaves factor 3: The energy conversion through photosynthesis, the process in which light energy and water are converted into carbohydrates and O_2. Indeed, photosynthesis has not directly been selected for in the past, so this factor still has the potential to be drastically improved by breeders. Currently our crops only fix about 5% of the total emitted sun light (Zhu, Long, & Ort, 2008). The rest is left unused for plant energy production. However, this energy has the potential to be fixed too, drastically increasing plant biomass in the process (Zhu et al., 2008).

Photosynthesis is the fixation of light energy into sugar. All plants, green algae and some bacteria do this. In plants it takes place in the thylakoids found in the chloroplast. Multiple steps occur during photosynthesis. The light independent phase (Calvin-Benson cycle) will not be discussed further in this report, but is essential for the transfer of energy bound in NADPH and ATP to sugars while converting CO_2. This allows the plant to store energy in

the form of sugar or starch. The light dependent part of photosynthesis is responsible for the storage of light energy into chemical bonds of NADPH and ATP. Light is captured by the photosynthetic pigments or light harvesting complexes (LHCs) of photosystems I and II (PSI and PSII) which are themselves located in the thylakoid membranes of the chloroplast. The energy then travels through the photosynthetic apparatus and is converted into ATP and NADPH, this is called photochemical quenching of the energy. The efficiency of PSI is considered to be optimal and has no effect on the level of photochemical quenching. However, PSII has sub optimal efficiency, and thus the amount of photochemically quenched light is also called PSII efficiency (Φ_{PSII}). Alternatively, light can be re-emitted through fluorescence (also by PSI) or transformed into heat in a constitutive manner (Φ_{NO}) or it can be non-photochemically quenched into heat by excess light induction (Φ_{NPQ}). Both Φ_{NO} and Φ_{NPQ} represent energy losses for the plant. Together these three efficiencies make up all the light that touches PSII LHCs (Eq. 1).

$$1 = \Phi_{PSII} + \Phi_{NO} + \Phi_{NPQ} \qquad \text{(Eq. 1)}$$

Φ_{PSII} represents the amount of excited states used in PSII. It is dependent on the fraction of electron acceptors (plastoquinone Q_a) free to accept an electron (q_L) (Eq. 2, Figure 1) (Kramer, Johnson, Kiirats, & Edwards, 2004).

$$\Phi_{PSII} = (1 - \Phi_{NO} - \Phi_{NPQ}) * q_L \qquad \text{(Eq. 2)}$$

Measurements regarding photosynthesis are performed by machines that are able to emit a controlled amount of light and are able to measure fluorescence emitted by the leaf. By performing these measurements in different actinic light intensities and in the absence or presence of saturating light flashes, we are able to calculate the fraction of all three factors (Φ_{PSII}, Φ_{NO} and Φ_{NPQ}) that contribute to light use. From these the non-photochemical quenching ratio (NPQ) is calculated. NPQ is the fraction of Φ_{NPQ} to Φ_{NO}. Thus NPQ is a
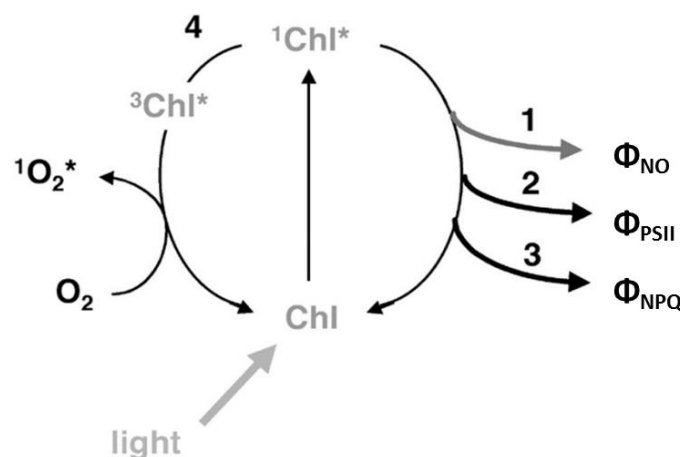

Figure 1. The different paths light energy can have in the chlorophyll. 1, the energy can be lost due to constitutive energy loss. 2, the energy can be used for photosynthesis and stored in chemical bounds. 3, the energy can be lost due to induced mechanisms. 4, the energy is used to create harmful reactive oxygen species (ROS). Adapted from Müller, Li, & Niyogi, 2001.

measure for the relative amount of energy loss due to high light induction compared to constitutive energy loss (Eq. 3).

When there is a low amount of light, φPSII simply operates at a lower rate. In contrast, when the plant gets a lot of light at once, this will excite many chlorophyll a (Chl*) molecules. This excess of excited states leads to the production of singlet oxygen ($^1O_2{}^*$) and triplet chlorophyll ($^3Chl^*$), both harmful reactive oxygen species (ROS) (Vass, 2011). In order to get rid of these excess excited states the energy is turned into harmless heat through φNPQ. However, changes in rate of φNPQ are relatively slow and a point will be reached when φNPQ is still quenching energy but there are not enough excited states left for maximal Φ_{PSII}. Consequentially φPSII decreases and less energy is fixed. The energy emitted through φNPQ prevents the production ROS. NPQ plays a crucial role in the regulation of photosynthesis, but protecting the plant against ROS formation comes at the cost of limiting the amount of photosynthetic energy that is fixed (Ruban, 2016). To successfully increase photosynthesis traits in crops, it is crucial to have insights on the physiology and genetics of NPQ.

Kromdijk et al (2016) have shown that plants genetically engineered to boost fast relaxation of NPQ, are more robust than wild-type plants, which leads to a 15% higher yield. In order to increase the energy conversion of the plant it would be beneficial to reduce NPQ. However in order to do this properly, without extensive damage through ROS, we will need detailed knowledge on how NPQ works and which genes are involved. Currently we know that many different quenching ("q") mechanisms contribute to NPQ (Croce, Van Grondelle, Van Amerongen, & Van Stokkum, n.d.; Liguori, Periole, Marrink, & Croce, 2015; Müller, Li, & Niyogi, 2001; Ruban, 2016). Some reduce the amount of captured light (indirect quenching) and others dissipate the energy through NPQ (direct quenching). To decrease photon supply the plant will move its chloroplasts in the cell with the aim to capture less light (qM)(Cazzaniga, Dall' Osto, Kong, Wada, & Bassi, 2013; Wada, Kagawa, & Sato, 2003). Another way to lower photon supply is by detaching the LHCs from PSII to connect to PSI. In state transition (qT), the LHCs still capture light but are unable to transfer the energy to PSII (Tikkanen, Grieco, & Aro, 2011). Direct energy quenching also occurs. For short term responses, the plant does two things in parallel; energy dependent quenching (qE) and zeaxanthin formation (qZ). When PSII is energy-saturated, the H+ concentration in the thylakoid lumen increases, thus lowering the lumen pH. This change in pH activates violaxin de-epoxi DASe (VDE), an enzyme that converts violaxanthin, a chloroplast pigment, into zeaxanthin (Li et al., 2000). Zeaxanthin then binds PsbS (PSII subunit S) in the LHCs and together they form quenching centres in the LHCs where the energy is dissipated into heat (Horton, Johnson, Perez-Bueno, Kiss, &

Ruban, 2008; Müller et al., 2001). When the plant needs long term protection against ROS accumulation, it will photoinhibit itself, through unknown mechanisms (qI)(Müller et al., 2001). How all these mechanisms relate to each other and contribute to NPQ is still not understood.

To study the genetics underlying any plant trait it is important to know that the plant genome constitutes of three genomes; the nuclear genome (the nucleotype), the chloroplast genome and the mitochondrial genome. While the nuclear genome codes 99.62% of the total genome, the chloroplast and mitochondrial genome (the plasmotype) are crucial for processes such as respiration and photosynthesis. In contrast to the nuclear genome which undergoes recombination, the plasmotype is uniparentally inherited and it is not subject to recombination. However, the plasmotype still significantly contributes to phenotype difference by interacting with nuclear genes (Flood et al., 2019). For a well-functioning cell it is imperative that there is good communication and coordination between the nucleotype and the plasmotype. Natural variation in the plasmotype and nucleotype causes difference in the communication and coordination of the plasmo- and nucleotype, which impacts different *Arabidopsis thaliana* strains differently. For more information on how these cyto-nuclear interactions can be studied, see box 1.

Using the cybrid method, Flood and Theeuwen made all possible nucleotype-plasmotype combinations for 7 different *Arabidopsis thaliana* accessions, producing 49 different lines (Flood et al., 2019). One of the seven lines is *Arabidopsis thaliana* line Ely (CS6088 at ABRC). This line has been described and studied due to its resistance to atrazine, a commonly used herbicide (El-Lithy et al., 2005). The resistance is acquired through a
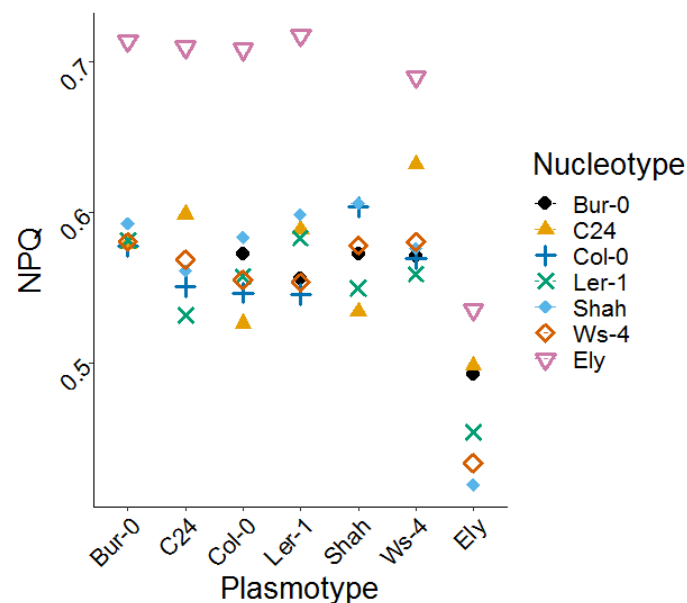

Figure 2. NPQ of cytoswaps from Flood et al. The Ely nucleotype clearly causes an increase in NPQ regardless of the plasmotype (Flood et al., 2019).

mutation in the *PsbA* gene located in the Ely chloroplast. The consequence of this mutation is that there is a lower photosynthetic efficiency for plants with an Ely plasmotype (El-Lithy et al., 2005; Flood et al., 2019, 2016). While phenotyping the 49 line from the panel many different interactions between the nucleotype and plasmotype were found, amongst which an additive effect of the Ely nucleotype on NPQ (Figure 2). This additive effect of the nucleotype could indicate a compensatory mechanism for the mutation in *PsbA*.

Box1. Ways to separate nuclear and plasmotype effect

Several methods to try and do this have been developed (Joseph et al., 2013; Roux et al., 2016; Tang et al., 2014). One of these methods produced so called cytolines (Roux et al., 2016). They used a marker assisted backcross design. Hybrids of 2 distinct lines were backcrossed three times with one of the parents and then selected based on markers that showed on of the parental genotypes. Although these lines were shown to be homozygous for the 384 SNPs genotyped, total homozygosity was not proven. This made it hard to show whether difference were due to the genome combination or some accidental heterozygosity (Roux et al., 2016).

Now, with the use of haploid inducer lines with a GFP-tagged CENTROMERE HISTONE 3 protein in a *cenh3/htr12* mutant background (Ravi & Chan, 2010), the plasmotype can be inherited without any of the maternal nuclear genes (Flood et al., 2019). The fraction of actually haploid plants from this cross is around 1/5, the rest of the progeny will be either aneuploid or diploid. After selection of the haploid progeny the plants will, by chance, self-fertilize and produce seeds that will be diploid. These lines are called cybrids. This method only requires one cross and a selfing round, and is thus, much quicker than the method proposed by (Roux et al., 2016). Besides, homozygosity of the genome is guaranteed.

Phenotyping photosynthesis is based on measuring changes in chlorophyll fluorescence. The fluoresce of chlorophyll changes according to the excitation of the chlorophyll molecule. So fluctuations in the chlorophyll fluorescence are used to determine the scale of change in the photosynthetic apparatus (Baker, 2008).

Measuring chlorophyll fluorescence is done by use of two light variables; actinic light and saturating flashes. Actinic light mimics an all surround light source such as the sun, when exposed to actinic light, not all chlorophyll molecules will be excited and fluorescence will not be maximal. The saturating flashes are used to measure chlorophyll fluorescence when all chlorophyll molecules are excited, in this way we measure the maximal amount of chlorophyll fluorescence possible in the specific actinic light conditions. These will differ depending on the actinic light intensities due to the amount of φNPQ occurring to protect the plant. In practice, we establish a window with a minimal possible chlorophyll fluorescence (F_0) in the dark and a maximal amount of chlorophyll fluorescence (F_M) in

dark with saturating flash. After which chlorophyll fluorescence is measured in different light conditions (F_p) and during saturating light flashes while exposed to light (F_{MP}). The F_0 and F_M values create a context in which the F_p and F_{MP} values can be analysed (Murchie & Lawson, 2013). After each saturating flash actinic light would stay on for one minute and then plant would be put in the dark and exposed to far red light, after flashing the plant again the F_{MPP} are obtained (Tietz, Hall, Cruz, & Kramer, 2017).

The aim of this thesis is first and foremost to phenotype φPSII, φNPQ, φNO, NPQ, qE and qI in the Robin, our phenotyping platform in Wageningen. Once a working protocol has been established I will continue on by phenotyping the differences between E-C and C-C. these should be the same as previously measured in the DEPI system in Michigan. Lastly, I will start phenotyping the doubled haploid population that I will make during my thesis. In order to understand what this effect on photosynthesis could mean for plant functioning and ultimately how to make better performing crops.

Material and Methods

Plant material and cultivation

Plant material was generated by first stratifying seeds at 4 °C for 4 days in 0.6 mL water soaked filter paper in small petri dish or in 1mL in big petri dish. Then the Petri dishes were moved to a growth chamber for germination of the seeds in a climate room with a 16/8h day/night rhythm at 24°C.

Once germinated the seeds were transferred to pre-soaked Rockwool (www.grodan.com) either in a growth chamber with a 16/8h day/night rhythm at 20°C during the day and 18°C during the night. Growing lights in the chamber were set at 200 µmol m^{-2} s^{-1} photon irradiance and 70% relative humidity. Plants were watered Monday, Wednesday and Friday with hyponex growth mix (pH =6.5)(recipe in appendix). Or seedlings were transferred to a greenhouse (51°59'17.6"N 5°39'52.6"E) with a natural day/night rhythm at 21°C during the day and 19°C during the night. Plants were watered everyday with water if grown on soil, hyponex if grown on rockwool.

Doubled haploid and F2 population

Previously, F1 seeds resulting from a Col x Ely cross, with Col as the maternal contributor, had been harvested. These F1 plants were genotyped with kompetitive allele specific PCR (KASP) with markers for both parents, to confirm they were true hybrids. These plants were grown on soil as mentioned previously and once flowering were used as pollen donor on Col CENH3 mutants (Ravi & Chan, 2010). Resulting seeds were harvested and sown as described. 10 days after sowing, selection for haploids amongst these plants started. Balance and symmetry of the rosette as well as small rounded leaves and small flowers

Figure 3. Haploid selection. The left side picture shows big flowers on top and small flowers on the bottom of the picture, the small flowers are a clear indicator of haploidy. On the right top side variation within the haploid plants in rosette and leaf size. Right bottom side indicates haploid plants with a toothpick and non-haploid plants without a toothpick.

were indicators of haploid genomes (Figure 3). Curled leaves, pointy leaves and big flowers were clear indicators of diploid or aneuploid genomes, these plants were discarded. In total 488 haploid plants were selected. While the haploid plants grow, a somatic duplication may occur leading to a doubled haploid genome before seed production. More often though, a doubled genome is achieved by the fusion of a balanced haploid ovule and a balanced haploid pollen. This yielded about 20 doubled haploid seeds on average on each plant. These seeds were collected and grown to generate the LUX population. The LUX population was sown and grown in soil in the greenhouse as previously described. Once matured the seeds were harvested and stored for further use. The previously mentioned F1 flowers were also allowed to self-cross, which resulted in the production of F2 seeds, referred to as the F2 population.

Phenotyping

For consistency of the experiments phenotyping was started at 09.00 on days that phenotyping was required. Plants from the climate chamber or greenhouse were transported to the PSI PlantScreen Module (Photon Systems Instruments, Czech Republic), also referred to as Robin, and put in trays specifically made for the Robin. To achieve the desired height, dry Rockwool blocks (www.grodan.com) were used under the plants in each slot in the tray. Protocol as previously mentioned was started using the PlantScreen scheduler (Photon Systems Instruments, Czech Republic) and RGB pictures from the top of the plants were included in the scheduling assistant after the chlorophyll fluorescence measurements. Data was extracted from the software using the Plant data analyser (Photon System Instruments, Czech Republic) and further handled using R 3.5.0 (2018-04-23) (www.r-project.org). R packages used were: Dplyr, Tidyr and ggplot2.

According to the capacity of the Robin, 20 plants were phenotyped simultaneously. To keep measurements consistent 1 batch (20 plants) was measured every day at the same time. Batch 1 was used to test the role of fluctuating light before phenotyping, batch 2 and 3 were used to phenotype LUX lines. Together all three batch served to monitor the effect of age on NPQ.
Batch 1: 4 C-C plants and 4 E-C plants grown in a climate chamber (inside), 4 C-C plants and 4 E-C plants grown in a greenhouse (outside). This batch was phenotyped on 10, 18 and 25 DAS
Batch 2: 4 plants each for LUX34, LUX40, LUX97 and LUX290, 2 C-C plants and 2 E-C plants grown in a climate chamber. This batch was phenotyped on 14 and 21 DAS
Batch 3: 4 plants each for LUX30, LUX39, LUX107 and LUX137, 2 C-C plant and 2 E-C plants grown in a climate chamber. This batch was phenotyped on 15 and 22 DAS

All photosynthesis parameters are measured after acclimation to 200, 400, 350, 700, 500, 1000 and 200 m^{-2} s^{-1} photon actinic light this order in all plants. Furthermore, age of the plants expressed in days after sowing (DAS) and prior exposure to fluctuating light were also taken into account.

Photosynthesis parameters

From the chlorophyll fluorescence data that was obtained the φPSII, φNO, φNPQ, NPQ, qE and qI were calculated (Harbinson, 2013). These calculations were based on the F_M, F_0, F_P, F_{MP} and F_{MPP} values (Figure 4).

φPSII $= (Fmp - Fp)/Fmp$

φNO $= Fp/Fm$

φNPQ $= (Fp/Fmp) - (Fp/Fm)$

NPQ $= (Fm/Fmp) - 1$

qE $= (Fm/Fmp) - (Fm/Fmpp)$

qI $= (Fm - Fmpp)/Fmpp$

Results

All the data produced during the phenotyping can be found in the appendix. The most interesting and relevant graphs will be shown in this section. One of the main achievements of this thesis research was to be able to phenotype photosynthesis traits in the Robin Wageningen. Once I had established a working protocol for this, I set about to phenotype plants with an Ely nucleotype and a Colombia plasmotype (E-C), plants with a Colombia nucleotype and a Colombia plasmotype (C-C) and plants from a doubled haploid population resulting from a cross of E-C and C-C, called the LUX plants.

In this month-long experiment multiple variables were taken into account. First the experiment measures six different parameters: NPQ, qE, qI, φNPQ, φNO and φPSII. All in all this experiment yielded 5267 different data points.

Fluorcam protocol

To phenotype the photosynthesis traits in the Robin like it was done in the Dynamic environmental photosynthetic imaging system (DEPI), a protocol needed to be made for the Robin (Cruz et al., 2016; Flood et al., 2019). The DEPI system allows phenotyping of photosynthesis in plants for unlimited amount of time, in constant and fluctuating light up to 2500 µmol photons m^{-2} s^{-1} in a controlled environment. For the Robin no protocol like the one in the DEPI existed. I started by getting to understand the DEPI protocol and then try to recreate a similar but shorter protocol for the Robin. The Robin software limits the phenotyping time to two hours and has a maximal actinic light intensity of 1000 µmol photons m-2 s-1. These limitations meant that the experiments run in the Robin were
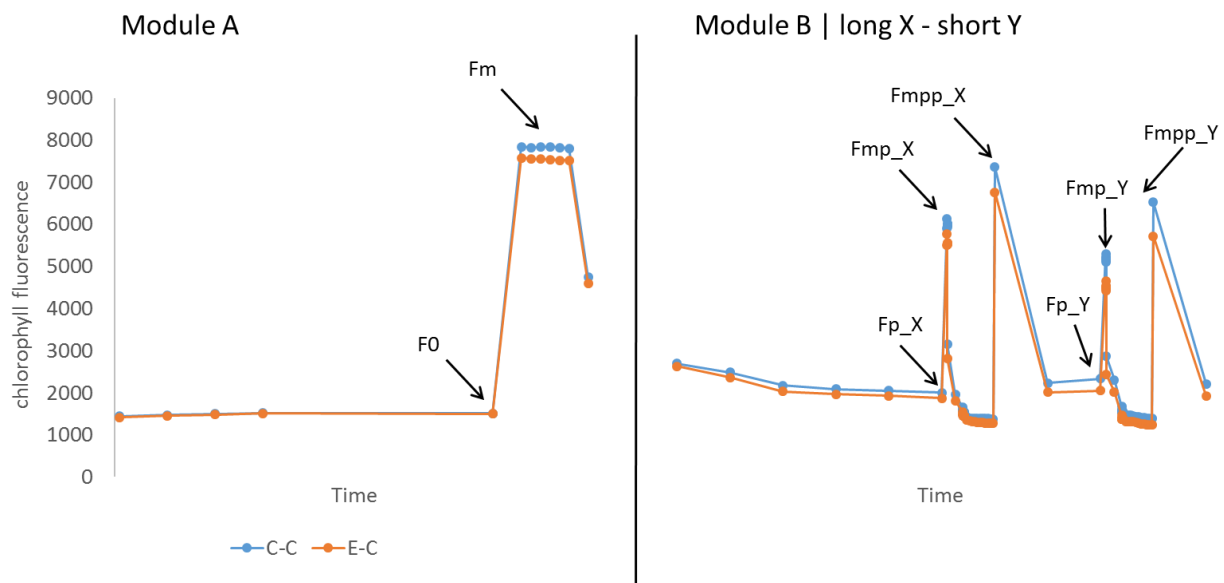

Figure 4. Chlorophyll fluorescence over time. Module A takes about 5 seconds, in which time F0 and F_M are measured. Module B consists of two parts, a short part at light intensity X and a short part at light intensity Y. the Long part takes 18 minutes and the short part takes 8 minutes. During each part the F_P, F_{MP} and F_{MPP} are measured.

shorter and challenged the plant less due to the lower maximal actinic light intensity. The experiment starts by putting the plants in the Robin. Before my protocol would start the plants stayed in the dark for an hour, to relax the photosystems so no photosynthesis stress remains in the plants and also to acclimate to the environment in the Robin. Then the protocol starts with a fixed module (module A), aimed at calculating the F_V/F_M value, followed by a second module of 30 minutes (module B) containing a long and a short variation. These are repeated however often the light intensity is changed during the protocol. These measurements are used to calculate φPSII, φNO, φNPQ, NPQ, qE and qI in those light intensities.

Module A is performed in the dark. First F_0 is measured by taking measurements (**mfmsub** command in protocol) every 500 ms for 2 seconds (Figure 4). Then a saturating pulse is applied for PulseDuration (=800 ms). The highest value measured is taken as the F_M value. Module B is used to fluctuate light intensities (X) and measure F_P_X, F_{MP}_X and F_{MPP}_X at the actinic light intensities (Figure 4). For this, first the actinic light is turned on at the desired intensity (<ALstart –TS>=> SI_Act2(X)) for a fixed period of time (ALdurationB). F_P_X is the last value that is measured during ALmeasure. Then a saturating light pulse is provided with <ALstart>=>SatPulse(PulseDurationp). The highest value measured during this pulse is F_{MP}_X. Actinic light is kept on for one more minute. Then the light is turned off and a Far red light is turned on to completely free up plastoquinone Qa, meaning that there is no NPQ anymore. Then the same saturating pulse is turned on again simultaneously with the actinic light. The measuring peak is called F_{MPP}_X.
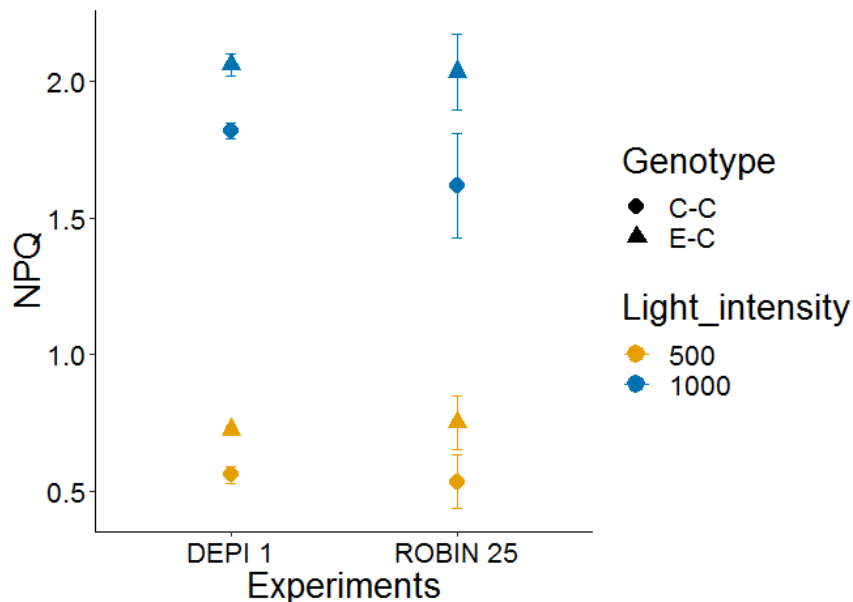

Figure 5. Comparison of results from the DEPI system in Michigan (DEPI 1) and the Robin in Wageningen (Robin 25). Plants were 25 DAS in both experiments. NPQ was measured after acclimation to 500 or 1000 µmol m^{-2} s^{-1} photon. Results from both experiments do not show significant differences between the different genotypes at a certain intensity. T-tests C-C at 500 µmol m^{-2} s^{-1} photon p= 0.61, C-C at 1000 µmol m^{-2} s^{-1} photon p= 0.08, E-C at 500 µmol m^{-2} s^{-1} photon p= 0.57, E-C at 1000 µmol m^{-2} s^{-1} photon p=0.70.

Since this protocol was made to reproduce the DEPI system day 3 protocol, short (8 minutes) and long (18 minutes) versions of module B exist (Cruz et al., 2016). The protocol used for this thesis looked like this: Dark period (1h) -> Module A -> Module B long (200 µmol m^{-2} s^{-1} photon irradiance) -> Module B short (400 µmol m^{-2} s^{-1} photon irradiance) -> Module B long (350 µmol m^{-2} s^{-1} photon irradiance) -> Module B short (700 µmol m^{-2} s^{-1} photon irradiance) -> Module B long (500 µmol m^{-2} s^{-1} photon irradiance) -> Module B short (1000 µmol m^{-2} s^{-1} photon irradiance) -> Module B long (200 µmol m^{-2} s^{-1} photon irradiance).

Reproduction of phenotyping data in the Robin

Once the protocol was working I checked whether the same genotypes yielded the same results as in the DEPI system. For this 5 E-C plants and 5 C-C plants were phenotyped in the Robin at 25 DAS (Robin 25 in Figure 5). Data is compared with the data obtained in the DEPI system in Michigan in 2014 at 25 DAS (DEPI1 in Figure 5) (Flood et al., 2019). Comparing the NPQ of the same genotype after acclimation to 500 or 1000 µmol m^{-2} s^{-1} photon irradiance between the experiments showed no significant differences (T-tests C-C at 500 µmol m^{-2} s^{-1} photon irradiance p= 0.61, C-C at 1000 µmol m^{-2} s^{-1} photon irradiance p= 0.08, E-C at 500 µmol m^{-2} s^{-1} photon irradiance p= 0.57, E-C at 1000 µmol m^{-2} s^{-1} photon irradiance p=0.70) (Figure 5).
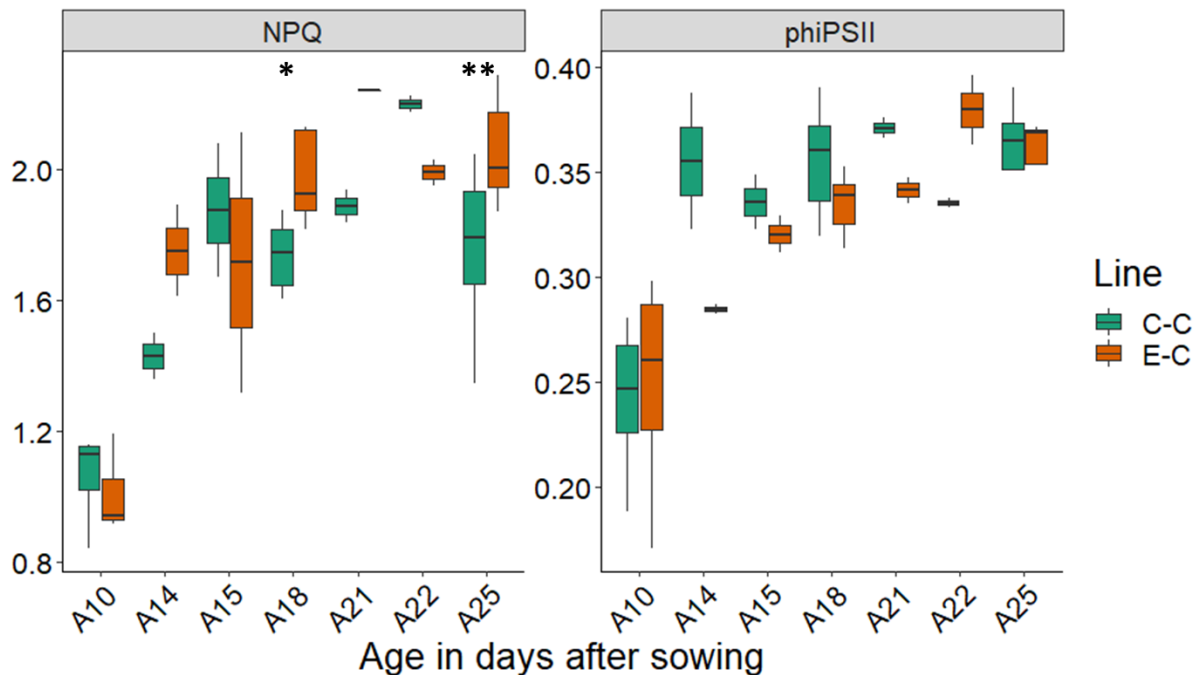

Figure 6. The amount of NPQ and φPSII for C-C and E-C at all ages phenotyped (A10= 10 DAS). All measurements after 1000 µmol m^{-2} s^{-1} photon actinic light in C-C and E-C grown in a climate chamber. C-C and E-C were significantly different from each other at 18 and 25 DAS for NPQ (T-test 18 DAS p=0.02, 25 DAS p=0.005)

Age effect on NPQ and φPSII

NPQ and φPSII after acclimation to 1000 µmol m^{-2} s^{-1} actinic light was measured on 10, 14, 15, 18, 21, 22 and 25 DAS in C-C and E-C plants grown in a climate chamber. Five plants each from batch 1 were measured on 10, 18 and 25 DAS, two plants from each genotype in batch 2 were measured on 14 and 21 DAS and two plants from each genotype from batch 3 were measured on 15 and 22 DAS. In batch 1 and 2 the E-C plants had higher NPQ than C-C plants and these differences were significant on 18 and 25 DAS (T-test 18 DAS p=0.02, 25 DAS p=0.005). In batch 3 E-C plants have a lower NPQ than C-C plants but this difference is not significant (Figure 6). NPQ values seem to stay the same after 18 DAS, whereas φPSII values already seem to stay constant after 15 DAS. φPSII does not differ between the genotypes.

Exposure to fluctuating light prior to phenotyping

In order to measure the effect of prior exposure to fluctuating light, 5 E-C plants and 5 C-C plants were grown in the greenhouse, these were compared with 5 C-C and 4 E-C grown in a climate chamber. Plants that grew up in the greenhouse were exposed to fluctuating lights caused by changes in sunlight-intensities. Differences between both genotypes for NPQ after acclimation to 1000 µmol m^{-2} s^{-1} photon irradiance actinic light became more pronounced when exposed to fluctuating light prior to phenotyping (T-test C-C vs E-C in
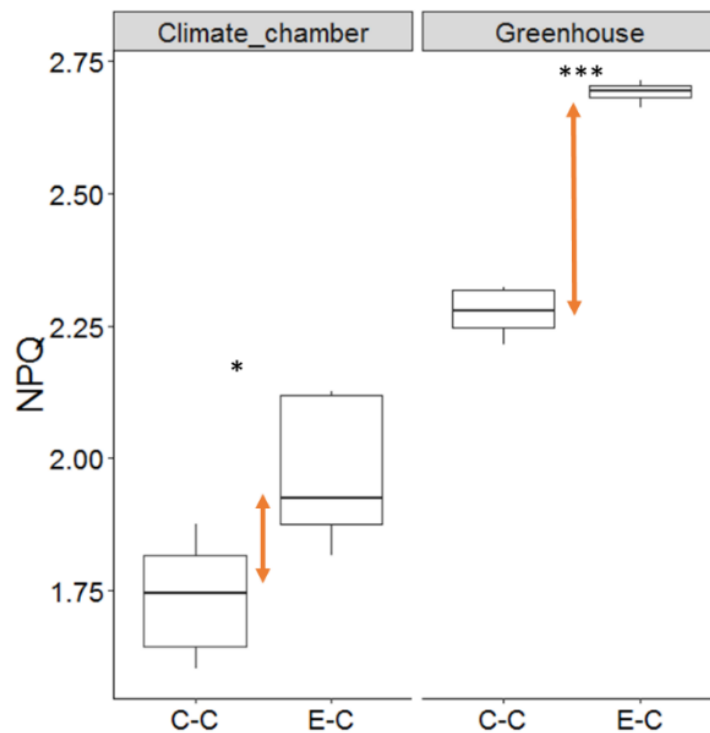

Figure 7. Comparison of NPQ after 1000 µmol m^{-2} s^{-1} photon actinic light acclimation in plants grown under steady light conditions and plants grown in greenhouse, thus exposed to fluctuating light. Plants are 18 DAS

climate chamber p= 0.02, C-C vs E-C in greenhouse p=2.2E-6) (Figure 7). NPQ was higher when grown in the greenhouse regardless of genotype.

Dissecting the type of NPQ response in Ely

All the data on NPQ, qE, qI, φNPQ, φNO and φPSII for E-C and C-C plants (except for the data obtained at 10 ,15 and 23 DAS) were analysed for correlation between them (Figure 8). The other data points were excluded because the plants were either too young or not showing the difference in NPQ. The correlation plot shows that NPQ is negatively correlated with φNO (Figure 8). Positively correlated with φPSII in low light and positively correlated with qI. Furthermore, φNO is negatively correlated with φPSII and qI. qE is not correlated
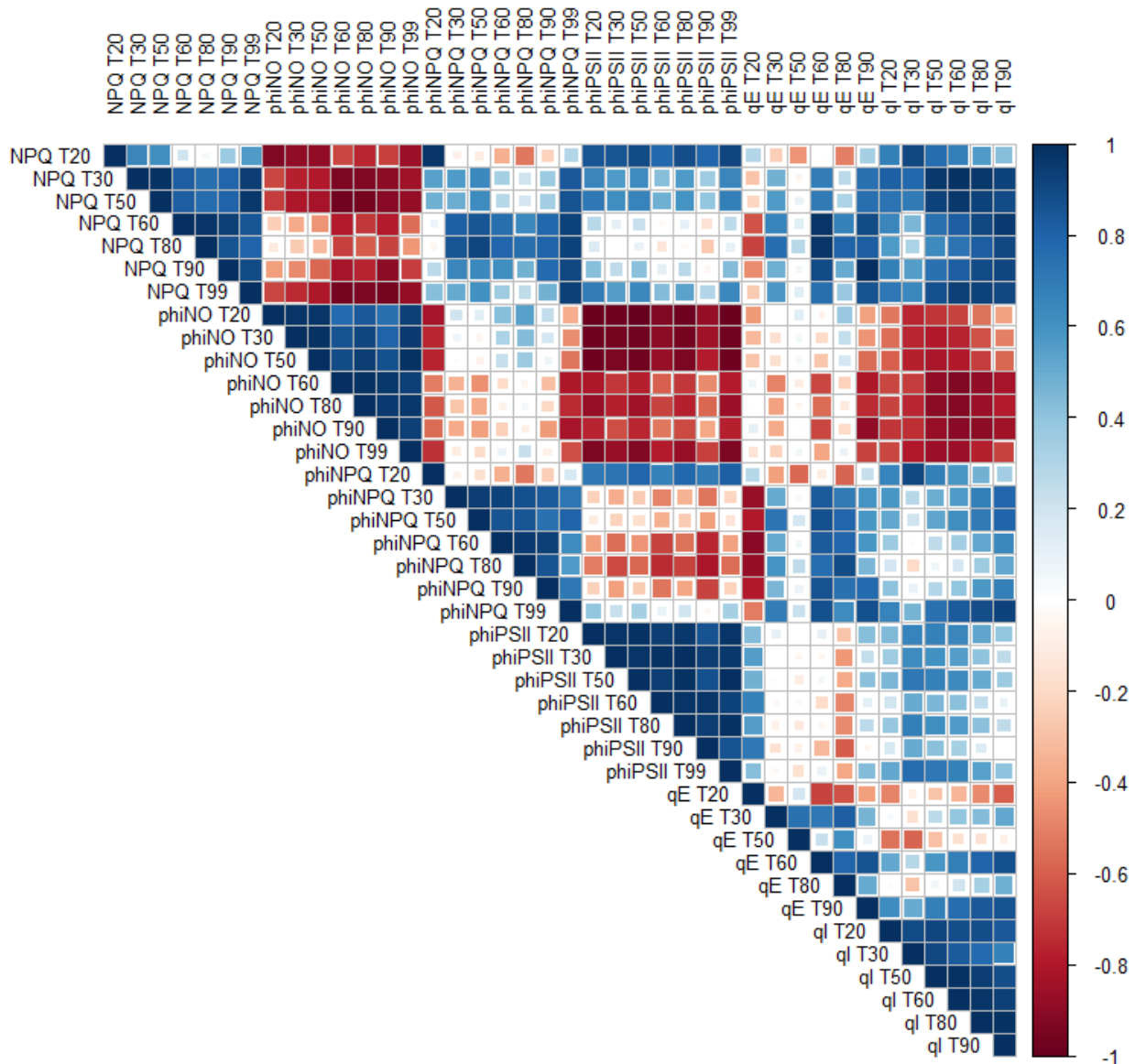

Figure 8. Correlation plot of all data points except measurements made on 10,15 and 23 DAS from E-C and C-C. Blue is a positive correlation and red a negative correlation. Size of squares indicate absolute value of correlation. phiNO is strongly negatively correlated with NPQ, phiPSII and qI. qE is not correlated with itself and NPQ and qI are positively correlated.
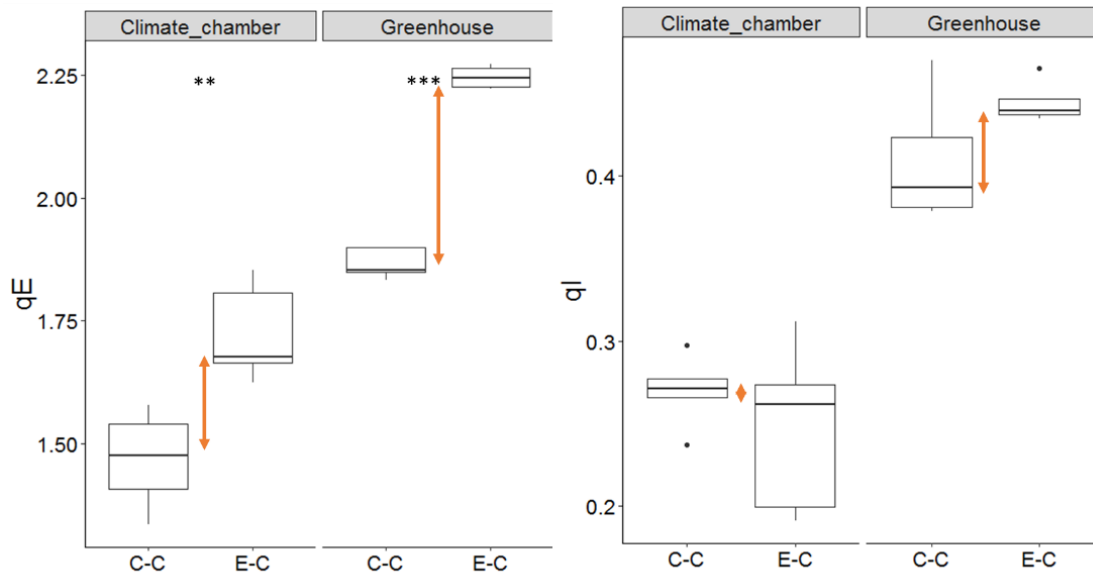
Figure 9. qE and qI values of C-C and E-C at 18 DAS after 1000 µmol m^{-2} s^{-1} photon actinic light acclimation. The comparison between the genotypes shows that qE is significantly higher in E-C plants than C-C plants. qI is equally as present in E-C plants as in C-C plants. Furthermore, plants previously exposed to fluctuating light in the greenhouse show bigger differences between the genotypes.

with itself at different light intensities. Besides, qE only shows strong correlation with other variables after acclimation to 1000 µmol m^{-2} s^{-1} photon irradiance actinic light.

Since NPQ is higher in E-C plants we looked at qE and qI, both of which contribute to φNPQ. Comparison between C-C and E-C grown in a climate chamber showed that qE has significantly higher values in E-C than in C-C (T-test, p=0.003) (Figure 9). Furthermore this difference in phenotype was increased when previously exposed to fluctuating light (T-test greenhouse p=2E-7). qI did not show significantly different values between C-C and
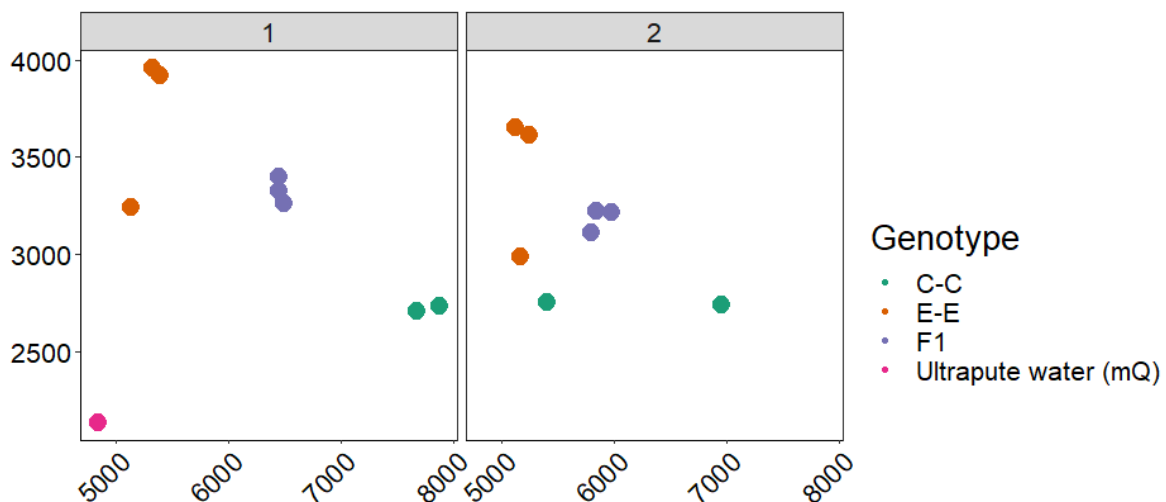

Figure 10. KASP marker results from 2 markers (1 and 2). Green dots indicate C-C, orange dots indicate the E-E. The F1 indicated with purple dots are both times inbetween both the C-C and E-E genotype. This indicates that the F1 were hybrids for these two markers.

E-C when grown in constant light or fluctuating light (T-test, Climate chamber p=0.41, Greenhouse p=0.11).

LUX doubled haploid population

To ensure that the LUX parents were really three F1 plants from a ColxEly cross, KASP markers were run (Figure 10). Segregation of the markers for C-C and E-C are visible for both primers and the F1 plants were neatly in between, indicating their hybrid nature. 480 different doubled haploid lines were obtained during this thesis; 300 were early flowering and 180 were late flowering. From these early flowering lines 8 were chosen for preliminary phenotyping in this thesis. These plants were grown in a climate chamber and phenotyped in batches during two days, some plants were 14 and 21 DAS (batch 2) and other were 15 and 22 DAS (batch 3). Heritability for the 6 phenotypes at all timepoints was calculated. Batch 2 has a higher heritability than plants from batch 3 (Figure 11).

φNPQ was significantly increased in seven out of the eight LUX lines compared to C-C at 14/15 DAS after acclimation to 1000 μmol m^{-2} s^{-1} photon irradiance actinic light (T-test LUX107 p=0.02, LUX39 p=0.02, LUX137 p=0.02, LUX30 p=0.02, LUX97 p=0.02, LUX34
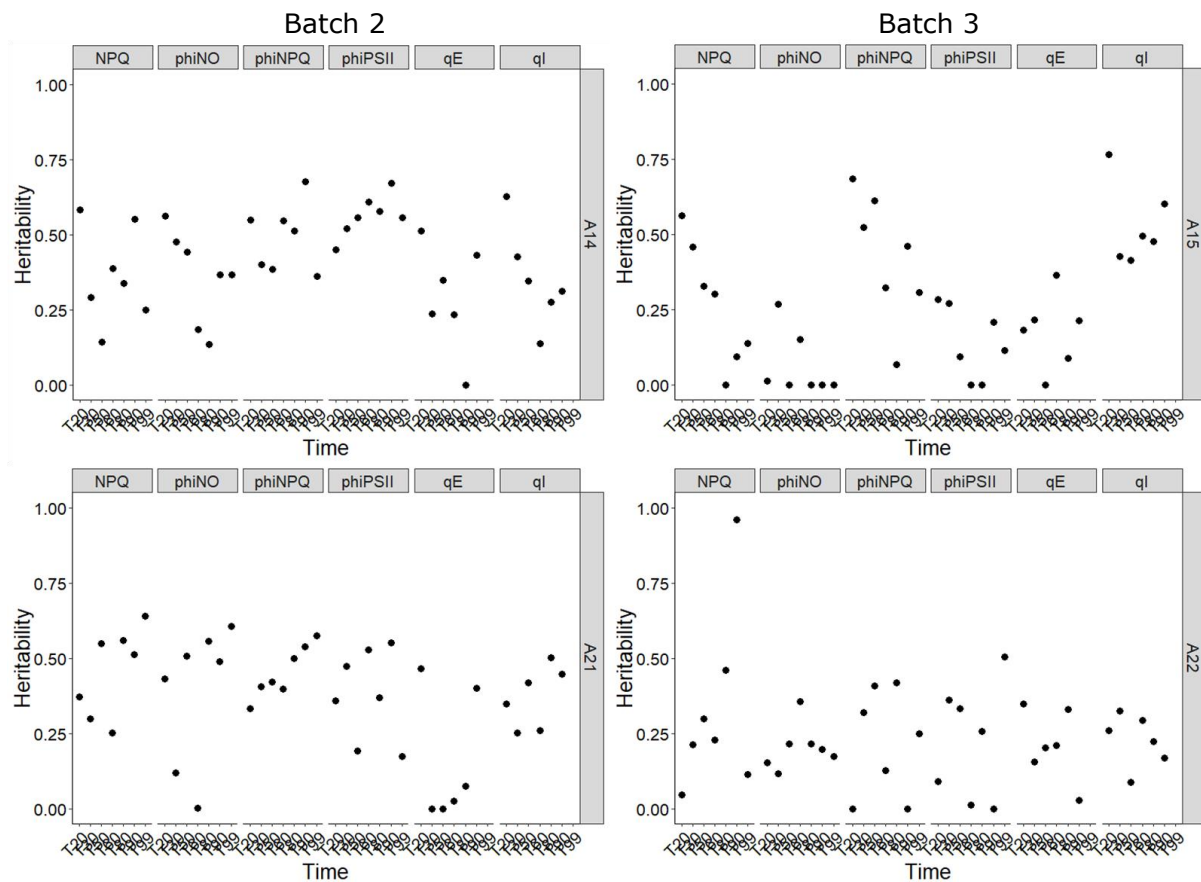

Figure 11. Heritability plots for all traits measured on all time points over the 8 LUX lines phenotyped. The plants that were phenotyped 14 DAS (A14) were also phenotyped at 21 DAS (A21). Plants phenotyped on 15 DAS (A15) were also phenotyped on 22 DAS (A22). Heritability in batch 2 (14 and 21 DAS) is higher than batch 3 (15 and 22 DAS).

p=0.03, LUX290 p=0.05) (Figure 12)**Error! Reference source not found.**. These same lines also has significantly lower φPSII (T-test LUX107 p=0.05, LUX39 p=0.04, LUX137 p=0.03, LUX30 p=0.03, LUX97 p=0.02, LUX34 p=0.02, LUX290 p=0.02). Furthermore, 4 of these plants also has a higher qI (T-test LUX39 p=0.01, LUX30 p=0.007, LUX97 p=0.02 and LUX34 p=0.03).

NPQ and qE values were also shown to be significant for LUX107, LUX39 and LUX137 (T-test LUX107 p=0.02, LUX39 p=0.04, LUX137 p=0.04) (Figure 13Figure 12). This was also the case for 500 µmol m^{-2} s^{-1} photon irradiance actinic light (Appendix).

The only significant values at the age of 21/22 DAS after acclimation to 1000 µmol m^{-2} s^{-1} photon irradiance actinic light were between C-C, E-C and LUX107. LUX107 has lower qI than C-C (T-test p=0.03) and E-C shows higher qI than C-C (t-test p=0.004). Interestingly
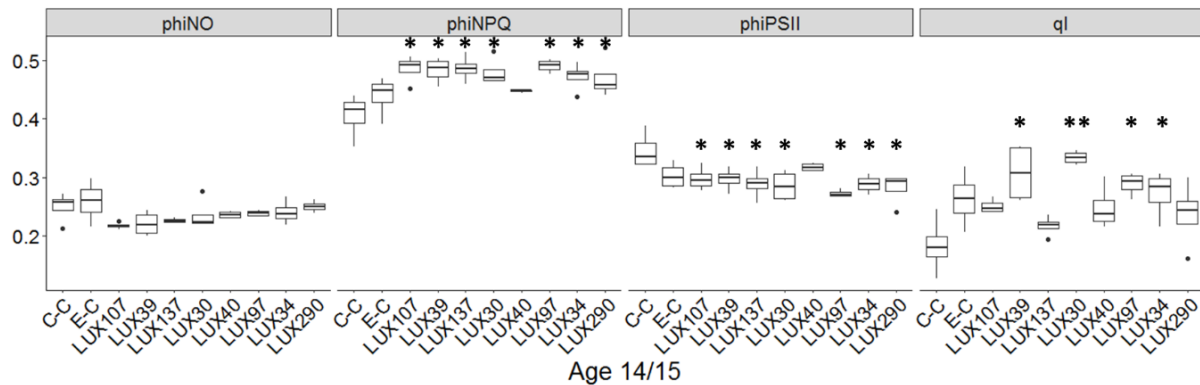

Figure 12. φNPQ and φPSII values for the 8 LUX lines after acclimation to 1000 µmol m^{-2} s^{-1} photon actinic light. E-C and LUX40 did not differ significantly from C-C. All the other were significantly higher (T-test φNPQ LUX107 p=0.02, LUX39 p=0.02, LUX137 p=0.02, LUX30 p=0.02, LUX97 p=0.02, LUX34 p=0.03, LUX290 p=0.05) (T-test φPSII LUX107 p=0.05, LUX39 p=0.04, LUX137 p=0.03, LUX30 p=0.03, LUX97 p=0.02, LUX34 p=0.02, LUX290 p=0.02). qI values were also significantly higher than C-C in LUX39, LUX30, LUX97 and LUX34 (T-test LUX39 p=0.01, LUX30 p=0.007, LUX97 p=0.02 and LUX34 p=0.03)
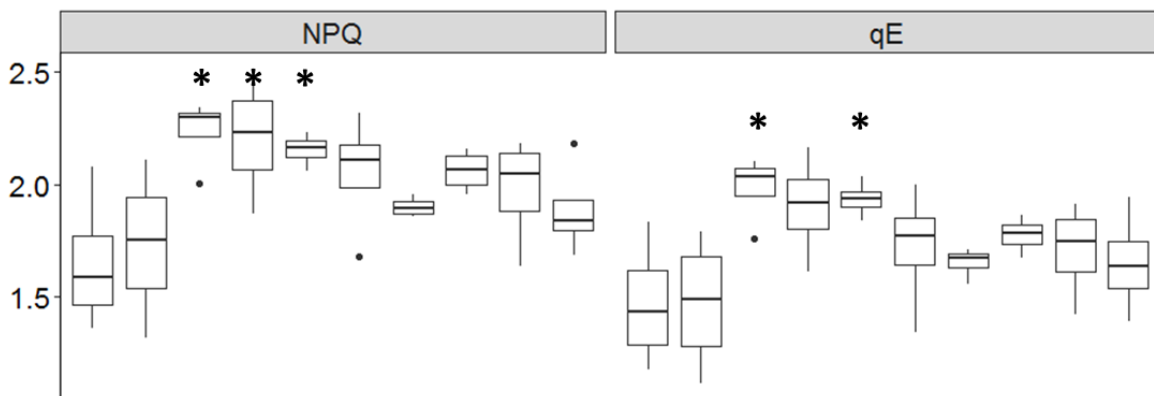

Figure 13. NPQ values for the 8 LUX lines after acclimation to 1000 µmol m^{-2} s^{-1} photon actinic light. NPQ was significantly higher in LUX170, LUX39 and LUX137 (T-test LUX107 p=0.02, LUX39 p=0.04, LUX137 p=0.04). qE was significantly higher for LUX107 and LUX 137 (T-test LUX107 p=0.03 and LUX137 p=-0.04).

much more variation was found earlier in the experiment: after acclimation to 700 µmol $m^{-2} s^{-1}$ photon irradiance actinic light. Both LUX137 and LUX97 had significantly higher NPQ and qE than C-C (T-test NPQ LUX137 p=0.01, NPQ LUX97 p=0.004, qE LUX137 p=0.03 and qE LUX97 p=0.005) (Figure 15).

Furthermore, E-C, LUX137,LUX30 and LUX97 have significantly less φNO than C-C (T-test E-X p=0.04, LUX137 p=0.008, LUX30 p=0.04, LUX97 p=0.02). LUX97 also showed significantly higher φNPQ than C-C (t-test p=0.006) and lower φPSII than C-C (T-test p=0.03). LUX290 and LUX107 had significantly lower qI values than C-C (T-test LUX107 =0.03 and LUX290 p=0.03) (Figure 14).
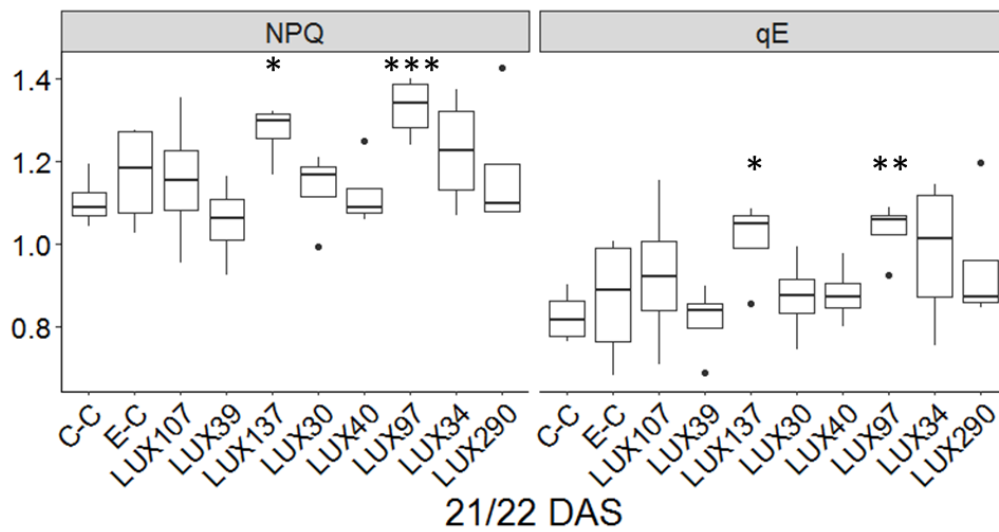
Figure 15. NPQ and qE values at 21/22 DAS after acclimation to 700 µmol $m^{-2} s^{-1}$ photon actinic light in the LUX lines. Both LUX137 and LUX97 were significantly higher than C-C for NPQ and qE (T-test NPQ LUX137 p=0.01, NPQ LUX97 p=0.004, qE LUX137 p=0.03 and qE LUX97 p=0.005).
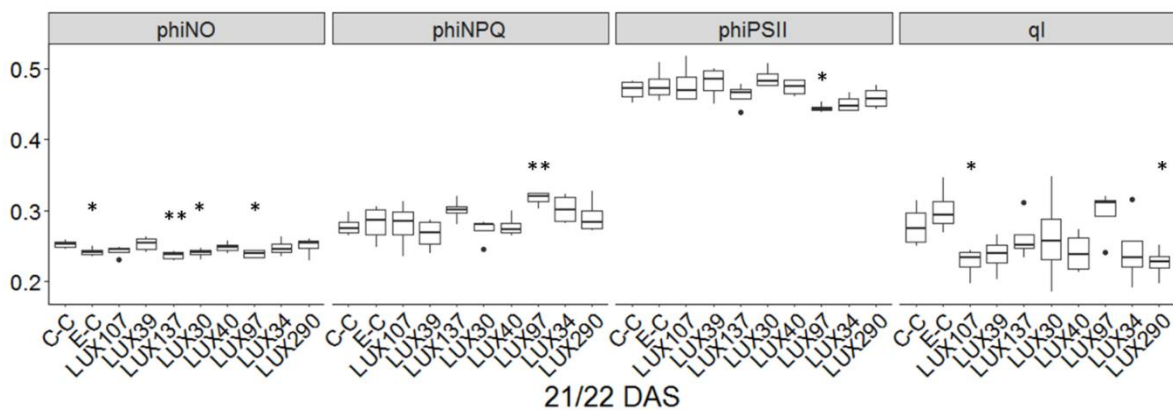
Figure 14. φNO, φNPQ, φPSII and qI values at 21/22 DAS after acclimation to 700 µmol $m^{-2} s^{-1}$ photon irradiance actinic light in the LUX lines. E-C, LUX137,LUX30 and LUX97 have significantly less φNO than C-C (T-test E-X p=0.04, LUX137 p=0.008, LUX30 p=0.04, LUX97 p=0.02). LUX 97 also showed significantly higher φNPQ than C-C (t-test p=0.006) and lower φPSII than C-C (T-test p=0.03). LUX290 and LUX107 had significantly lower qI values than C-C (T-test LUX107 p=0.03 and LUX290 p=0.03).

Discussion

## Reproduction

I was able to reproduce the photosynthesis data in the Robin that was previously obtained in the DEPI system with the C-C and E-C genotypes (Figure 5) (Flood et al., 2019). The differences and amount in NPQ found between C-C and E-C were similar in both phenotyping platforms despite the differences in protocol length. However, the variance in the data from the Robin is higher than the variance in the data from the DEPI. The higher variance in the Robin setup could be due to the stress caused by moving a plant towards the Robin. This stress would be similar to stress caused by exposure to wind, which could have an effect on photosynthesis. To my knowledge this has not been proven yet but, seems to be a likely explanation. In the DEPI the plants do not move and thus plants are more settled, which would explain why the DEPI data has less variation. Another factor that could lead to drastic variation is the plants circadian rhythm (van Bezouw, Keurentjes, Harbinson, & Aarts, 2019). However, plants were always phenotyped at 9 am, so this should not have played a role in this experiment.

Age effect

My experiments show that the age of the plants seems to affect the photosynthesis parameters I have measured (Figure 6). This age effect on photosynthesis was already suggested in the 60's (Flood et al., 2016; Sweet & Wareing, 1966). When the plants were 10 DAS they showed low φPSII and low NPQ, as the plants grew older the values increased and stayed more or less the same after 18 DAS. It seems as if young plants have lower maximum φPSII than older plants, which is well described in literature. Phenotyping *Arabidopsis thaliana* too early does not provide a good indication of photosynthesis traits in older plants. Even though genotypes have different speeds of development, using DAS as an approximation for development is the easy and straightforward way of determining plant age. I would not recommend to phenotype plants before 18 DAS.

Surprisingly, no increased NPQ was detected in E-C plants of batch 3, which were measured on 15 and 22 DAS (Figure 6). One might wonder whether the plants were really what they should be. However, the ambient temperature around the Robin fluctuated quite a bit from day to day and it could be that the E-C NPQ phenotype was not found in batch 3 because the plants were exposed to temperatures higher than 30°C during phenotyping. An experiment comparing results of phenotyping in different temperatures should indicate whether the excess heat was the issue with batch 3.

Fluctuating light

In this experiment I found that growth in a fluctuating environment increases the difference in NPQ between C-C and E-C. Especially the qE parameter showed larger differences between E-C and C-C plants. Since I wanted to look at the correlation of the E-C NPQ phenotype, I only used measurements that did show this difference in NPQ. In the correlation plot NPQ seems to be most correlated with qI, which hints at an important role for qI in NPQ in all plants (Figure 8). qE is also correlated to NPQ, moreover, qE does not correlate with φPSII, which would be expected since it is an energy trade-off. Furthermore, qE does not strongly correlate with itself, something the other parameters do. Low correlation indicates that both genotypes do not have the same behaviour.

qE is considered to be a fast mechanism to induce non-photochemical quenching (Ruban, Johnson, & Duffy, 2012). The combination of higher qE and higher NPQ would suggest that E-C plants quench more energy through qE than C-C plants, while keeping their φPSII at the same level as C-C plants (Figure 6). These findings implicate that E-C plants are capable of protecting themselves better in excess light than C-C plants, but this protection is not at the cost of photosynthesis efficiency. Whether the increase in qE is due to quicker changes in thylakoid lumen pH, higher abundance of violaxin and/or zeaxanthin or higher abundance of PSBS or a combination of these three factors cannot be determined from this dataset. For this more in depth phenotyping of qE is needed. More insights in the relaxation rate of qE and measuring ΔpH across the chloroplast membrane will already provide a better idea of what is going on in the E-C plants (Kromdijk et al., 2016; Ruban et al., 2012).

Increase in qE due to prior exposure to fluctuating light should also be investigated further by growing E-C and C-C plants in harsher light fluctuations than performed during this research in the greenhouse. This experiment would show how plastic this E-C NPQ phenotype is with regards to withstanding extreme dynamic light environments. Alternatively, the E-C and C-C plants could be phenotyped for longer than 2 hours with more fluctuation of actinic light and higher maxima of actinic light than 1000 µmol $m^{-2} s^{-1}$ photon irradiance. Both of these experiments would provide insights into the capability of E-C plants to withstand high light intensities, on the long term and on the short term respectively.

Considering that growth in a greenhouse is not very reproducible, we should start measuring photosynthesis parameters in plants that are grown in controlled dynamic environments. Fluctuating light is shown to have a very big impact on plant photosynthesis (Kaiser, Morales, & Harbinson, 2018). Therefore, research performed on plants grown in

controlled dynamic environments are very important. Furthermore results found in such research would be more applicable to real crop circumstances (Cruz et al., 2016; Fahlgren, Gehan, & Baxter, 2015).

In the Ely wildtype the increased NPQ in the Ely nucleotype is combined together with an Ely plasmotype mutation in the *PsbA* gene, which causes a bottleneck in the electron transport chain in PSII (El-Lithy et al., 2005). This mutation allowed Ely wildtype plants to survive strong selection caused by atrazine, a herbicide used in Great Britain on railroads to prevent weeds from growing. This mutation in *PsbA* leads to drastically lower φPSII, especially when the mutation is paired with other nucleotypes than the Ely nucleotype (Figure 2). However, this mutation let Ely plants grow and proliferate without competition while atrazine was used. It has been hypothesised before that the *PsbA* mutation has caused the whole nucleotype to hitchhike with the plasmotype, possibly due to a lack in outcrossing (Flood et al., 2016). However, the presence of an increased NPQ and qE in the nucleotype provides an explanation as to why the whole nucleotype was found in combination with the Ely plasmotype all across Great Britain. The Ely nucleotype potentially provides a fitness increase that compensates for the fitness decrease caused by the Ely plasmotype. However, as a *PsbA* mutation is a permanent handicap to photosynthesis we could contemplate why there is no significant increase in qI; the long term protection mechanism against excess light. The mutation causes the plant to have lower threshold for photosynthesis, meaning that NPQ is required to start at lower light intensities than in plants without the *PsbA* mutation. Consequentially, a long term increase in NPQ through qI would not be surprising. The fact that the whole genome hitchhiked is remarkable on its own. A possible explanation could be that the increase in qE is caused by many genes dispersed over the nuclear genome, underlying the need for the whole genome to stay together.

LUX

The doubled haploid population LUX consists of 488 lines. Of these, 300 plants were early flowering and 188 late flowering. From the population eight early flowering lines were preliminarily phenotyped during this thesis research. Four repeats of each line were measured. However, LUX 30, LUX39, LUX 107 and LUX137 were grown in batch 3 where the difference in NPQ phenotype was not observed. Additionally, the heritability of this batch is a lot lower than for the other batch (Figure 11). Probably some plants were wrongly labelled, leading to mistakes in the data analysis in batch 3. These results should be used with caution, keeping in mind that these results are preliminary.

Overall the eight lines from the LUX population that were phenotyped showed variation in φPSII, φNO, φNPQ, NPQ, qE and qI. No typical monogenetic or bigenetic inheritance patterns were observed, however, eight lines is a low number to see these patterns. I observe a wide range of phenotypes in these eight lines, which suggests that the E-C NPQ phenotype is dependent on multiple genes. Seven LUX lines showed higher φNPQ and lower φPSII than C-C at 14/15 DAS (Figure 12). These results make sense as these two parameters are in competition (Harbinson, 2013). The higher φNPQ and lower φPSII in the LUX plants can also be attributed to the fact that haploid plants produce less, but bigger seeds than cybrids: The LUX plants had more energy stored in their seeds meaning that they develop quicker than E-C and C-C which come from smaller seeds. From these seven lines, four seem to have higher φNPQ due to a higher qI mechanism and 2 due to a higher qE mechanism (Figure 12, Figure 13). Interestingly, only two of the lines show these same trends at 21/22 DAS (Figure 15, Figure 14). LUX137 completely switches from a higher qI at 14/15 DAS to a higher qE at 21/22 DAS. This could indicate a role for development in these mechanisms. However, LUX137 was part of the third batch of plants which did not show the difference in NPQ like the two other batches.

I was not expecting to find variation for qI in the LUX population as both parents C-C and E-C do not show differences in qI. The effect that this trait segregates in the progeny indicates that the amount qI is dependent on multiple genes. All in all this shows that the LUX population has a lot of variation within the lines for all photosynthesis traits, even when the parents do not show variation.

Conclusion

In my introduction I state that it would probably be beneficial to reduce NPQ, so as to increase photosynthesis efficient. From this research I conclude that a high NPQ and a high photosynthesis are not exclusive, as can be seen in E-C plants, which have high NPQ and equal phiPSII to C-C. This implies that we could have crops with high photosynthesis that are also well protected against excess light. However it is still worth pursuing the search for plants that are able to utilise this excess light instead of losing it to NPQ, increasing the maximal amount of photosynthesis possible. Which one of both options is the best for robust crops, has yet to be decided. I am of the opinion that it depends on the conditions the crop is grown in.

Furthermore in this research I find that qE is important for a higher NPQ in E-C. I also found that qE is even more present when the plant has been exposed to fluctuating light, instead of constant light. This finding underlies the importance of phenotyping plants grown in more realistic and dynamic conditions in order to understand the nature of

photosynthesis. Higher qE also indicates that the Ely nucleotype is likely to be more plastic and thus more robust in dynamic environments. To confirm this increase in plasticity further experiments are needed to test how plastic E-C plants are in dynamic environments.

The LUX population seems to be very diverse and will be a great tool to identify the genes causing the NPQ increase in the Ely nucleotype. This doubled haploid population will be further phenotyped in the DEPI. Results from this phenotyping round will hopefully also increase our understanding of the role of qE and qI in response to dynamic light conditions. Currently, qE is expected to contribute the most to NPQ in dynamic conditions. However, seeing that qI also varies in the 8 LUX lines already phenotyped and the strong correlations qI shows with the other photosynthesis traits, I am very eager to see what will result from the DEPI phenotyping. Maybe we will finally be able to understand the role of qI in NPQ in more detail than the brief explanation literature provides at this moment.

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Appendix:

Hyponex recipe

			Hyponex - standaard
EC	1,4		
NH4	1,70	mmol/L	Hoofdelementen in mmol/liter
K	4,13	mmol/L	Sporenelementen in micro-mol/liter
Na		mmol/L	pH wordt gesteld met: Kali loog en Zwavelzuur
Ca	1,97	mmol/L	
Mg	1,24	mmol/L	
NO3	4,14	mmol/L	
Cl		mmol/L	
SO4	3,14	mmol/L	
HCO3		mmol/L	
P	1,29	mmol/L	
Si		µmol/L	
Fe	21	µmol/L	Consists of 50% Fe-DTPA 3% and 50% Fe- EDDHSA 3%
Mn	3.4	µmol/L	
Zn	4.7	µmol/L	
B	14	µmol/L	
Cu	6.9	µmol/L	
Mo	0.5	µmol/L	

Fluorcam protocol

```
;
##############################################################################
#
TS=20ms
;DEPI day 3 in PSI Plantscreen
;Louise Logie

include default.inc ;Includes standard options, do not remove it !
include Light.inc ;Includes standard options, do not remove it !
__LightA=0
__LightB=0
__LightIntensity=<40,50,60,70,80,90,100>
```

```
Shutter=3
Sensitivity=1
Super=100
FAR=20

;#######################              duration              determination
###########################
DPeriod =0s ;1 hour darkness
F0duration=2s;
F0period = 500ms

ALdurationA = 1080s ;duration light period for long light block
ALdurationB = 480s;duration light period for shortlight block

ALmeasure = 200s;Measurements during actinic light exposure before Fmp
measuremnet
ALmeasure2 = 400ms;Fmp measurements during sat pulse
ALmeasure3 = 10s;ALmeasure for relaxation period
ALmeasure4 = 30s;Measurements during actinic light exposure after Fmp
measurement

Relaxduration = 120s ;already defined in ALstart definition
PulseDuration = 800ms;Fm saturating pulse
PulseDurationp = 2s ;duration Fm' and Fm'' sat pulse
FRPeriod = 1s;Fr light exposure for F0p measurements

;###########################              Module              a
#########################################
a1=DPeriod
a2=a1+F0duration
a3=a2+2s;
a4=a3+PulseDuration - mfmsub_length

<a1,a1+F0period..a1+F0duration>=>mfmsub ;5 measurements, 1 every second
<a1>=>checkPoint,"startFo"
<a2>=>checkPoint,"endFo"

<a3>=>SatPulse(PulseDuration)
<a3>=>mpulse2
<a3 + PulseDuration/2>=>checkPoint,"startFm"
<a4>=>checkPoint,"endFm"

;#######################  Module  B  point  determination  long-short
###########
ALstart1 = a4 + 120s
ALend1 = ALstart1 + ALdurationA - 60s
Actend1 = ALstart1 + ALdurationA
```

```
Fmpend1 = ALend1 + 2s
F0pend1 = Actend1 + 5s


ALstart2 = Actend1 + Relaxduration
ALend2 = ALstart2 + ALdurationB - 60s
Actend2 = ALstart2 + ALdurationB
Fmpend2 = ALend2 + 2s
F0pend2 = Actend2 + 5s
;-------------------------------------
ALstart3 = Actend2 + Relaxduration
ALend3 = ALstart3 + ALdurationA - 60s
Actend3 = ALstart3 + ALdurationA
Fmpend3 = ALend3 + 2s
F0pend3 = Actend3 + 5s


ALstart4 = Actend3 + Relaxduration
ALend4 = ALstart4 + ALdurationB - 60s
Actend4 = ALstart4 + ALdurationB
Fmpend4 = ALend4 + 2s
F0pend4 = Actend4 + 5s
;-------------------------------------
ALstart5 = Actend4 + Relaxduration
ALend5 = ALstart5 + ALdurationA - 60s
Actend5 = ALstart5 + ALdurationA
Fmpend5 = ALend5 + 2s
F0pend5 = Actend5 + 5s


ALstart6 = Actend5 + Relaxduration
ALend6 = ALstart6 + ALdurationB - 60s
Actend6 = ALstart6 + ALdurationB
Fmpend6 = ALend6 + 2s
F0pend6 = Actend6 + 5s
;-------------------------------------
ALstart7 = Actend6 + Relaxduration
ALend7 = ALstart7 + ALdurationB - 60s
Actend7 = ALstart7 + ALdurationB
Fmpend7 = ALend7 + 2s
F0pend7 = Actend7 + 5s


;#######################Light control #################
<ALstart1>=>act2(ALdurationA)
<ALstart2>=>act2(ALdurationB)
<ALstart3>=>act2(ALdurationA)
<ALstart4>=>act2(ALdurationB)
<ALstart5>=>act2(ALdurationA)
<ALstart6>=>act2(ALdurationB)
<ALstart7>=>act2(ALdurationB)
```

```
;<ALstart1>=>SatPulse(PulseDurationp)
<ALstart2>=>SatPulse(PulseDurationp)
<ALstart3>=>SatPulse(PulseDurationp)
<ALstart4>=>SatPulse(PulseDurationp)
<ALstart5>=>SatPulse(PulseDurationp)
<ALstart6>=>SatPulse(PulseDurationp)
<ALstart7>=>SatPulse(PulseDurationp)

;#####################Light intensity and control ##################
<ALstart1 - TS>=>SI_Act2(20) ;200uE
<ALstart2 - TS>=>SI_Act2(33) ;400uE
<ALstart3 - TS>=>SI_Act2(30) ;350uE
<ALstart4 - TS>=>SI_Act2(53) ;700uE
<ALstart5 - TS>=>SI_Act2(40) ;500uE
<ALstart6 - TS>=>SI_Act2(72) ;1000uE
<ALstart7 - TS>=>SI_Act2(20) ;200uE

<ALend1>=>SatPulse(PulseDurationp)
<ALend2>=>SatPulse(PulseDurationp)
<ALend3>=>SatPulse(PulseDurationp)
<ALend4>=>SatPulse(PulseDurationp)
<ALend5>=>SatPulse(PulseDurationp)
<ALend6>=>SatPulse(PulseDurationp)
<ALend7>=>SatPulse(PulseDurationp)

<Actend1>=>FAR(FRPeriod)
<Actend2>=>FAR(FRPeriod)
<Actend3>=>FAR(FRPeriod)
<Actend4>=>FAR(FRPeriod)
<Actend5>=>FAR(FRPeriod)
<Actend6>=>FAR(FRPeriod)

;######################### Module B long-short measurements 200/400
##########
<ALstart1, ALstart1 + ALmeasure .. ALend1>=>mfmsub ;actiniclight exposure
single int
<ALend1 + TS, ALend1 + ALmeasure2 .. Fmpend1>=>mfmsub;Fmp measurement
;<ALend1>=>checkPoint, "startFm_Lss1"
;<Fmpend1>=>checkPoint, "endFm_Lss1"
<Fmpend1 + TS, Fmpend1 + ALmeasure4 .. Actend1>=>mfmsub;actiniclight exposure
single int
<Actend1 + TS, Actend1 + ALmeasure2 .. F0pend1>=>mfmsub ;F0p with FR
measurement
<F0pend1 + TS, F0pend1 + ALmeasure3 .. ALstart2>=>mfmsub;dark relaxation
```

```
<ALstart2 + TS, ALstart2 + ALmeasure .. ALend2>=>mfmsub ;actiniclight
exposure double int.
<ALend2 + TS, ALend2 + ALmeasure2 .. Fmpend2>=>mfmsub;Fmp measurement
<Fmpend2 + TS, Fmpend2 + ALmeasure4 .. Actend2>=>mfmsub;actiniclight exposure
double int.
<Actend2 + TS ,Actend2 + ALmeasure2 .. FOpend2>=>mfmsub ;FOp with FR
measurement
<FOpend2 + TS, FOpend2 + ALmeasure3 .. ALstart3>=>mfmsub;dark relaxation


;######################### Module B long-short measurements 350/700
##########
<ALstart3 + TS, ALstart3 + ALmeasure .. ALend3>=>mfmsub ;actiniclight
exposure single int
<ALend3 + TS, ALend3 + ALmeasure2 .. Fmpend3>=>mfmsub;Fmp measurement
<Fmpend3 + TS, Fmpend3 + ALmeasure4 .. Actend3>=>mfmsub;actiniclight exposure
single in
<Actend3 + TS, Actend3 + ALmeasure2 .. FOpend3>=>mfmsub ;FOp with FR
measurement
<FOpend3 + TS, FOpend3 + ALmeasure3 .. ALstart4>=>mfmsub;dark relaxation


<ALstart4 + TS, ALstart4 + ALmeasure .. ALend4>=>mfmsub ;actiniclight
exposure double int.
<ALend4 + TS, ALend4 + ALmeasure2 .. Fmpend4>=>mfmsub;Fmp measurement
<Fmpend4 + TS, Fmpend4 + ALmeasure4 .. Actend4>=>mfmsub;actiniclight exposure
double int.
<Actend4 + TS, Actend4 + ALmeasure2 .. FOpend4>=>mfmsub ;FOp with FR
measurement
<FOpend4 + TS, FOpend4 + ALmeasure3 .. ALstart5>=>mfmsub;dark relaxation


;######################### Module B long-short measurements 500/1000
##########
<ALstart5 + TS, ALstart5 + ALmeasure .. ALend5>=>mfmsub ;actiniclight
exposure single int
<ALend5 + TS, ALend5 + ALmeasure2 .. Fmpend5>=>mfmsub;Fmp measurement
<Fmpend5 + TS, Fmpend5 + ALmeasure4 .. Actend5>=>mfmsub;actiniclight exposure
single int
<Actend5 + TS, Actend5 + ALmeasure2 .. FOpend5>=>mfmsub ;FOp with FR
measurement
<FOpend5 + TS, FOpend5 + ALmeasure3 .. ALstart6>=>mfmsub;dark relaxation


<ALstart6 + TS, ALstart6 + ALmeasure .. ALend6>=>mfmsub ;actiniclight
exposure single int
<ALend6 + TS, ALend6 + ALmeasure2 .. Fmpend6>=>mfmsub;Fmp measurement
;<ALend6>=>checkPoint, "startFm_Lss6"
;<Fmpend6>=>checkPoint, "endFm_Lss6"
<Fmpend6 + TS, Fmpend6 + ALmeasure4 .. Actend6>=>mfmsub;actiniclight exposure
single int
```

```
<Actend6 + TS, Actend6 + ALmeasure2 .. F0pend6>=>mfmsub ;F0p with FR
measurement
<F0pend6 + TS, F0pend6 + ALmeasure3 .. ALstart7>=>mfmsub;dark relaxation


;########################  Module  B  short  measurements  200
##################
<ALstart7 + TS, ALstart7 + ALmeasure .. ALend7>=>mfmsub ;acticlight
exposure single int
<ALend7 + TS, ALend7 + ALmeasure2 .. Fmpend7>=>mfmsub;Fmp measurement
<Fmpend7 + TS, Fmpend7 + ALmeasure3 .. Actend7>=>mfmsub;acticlight exposure
single int


; ######################## END ########################
```
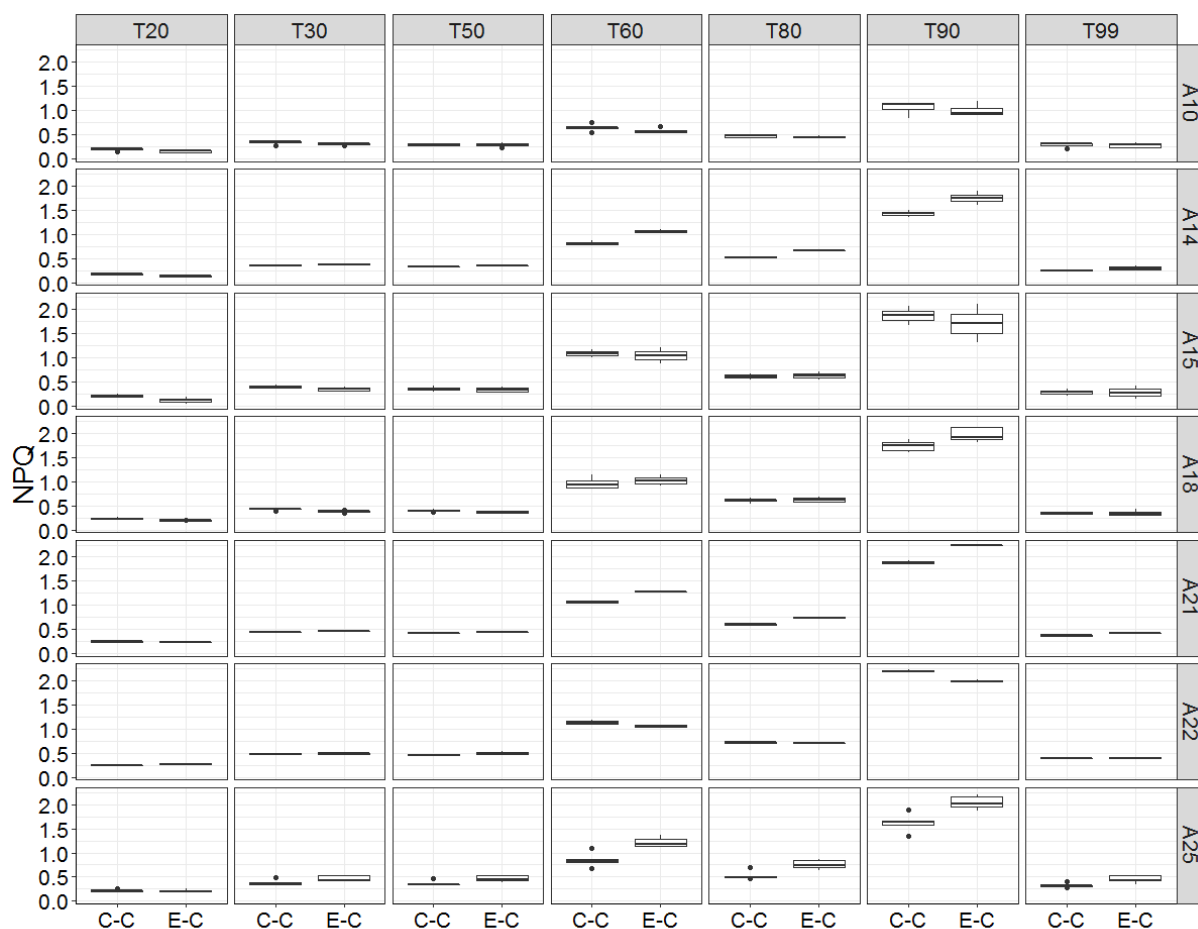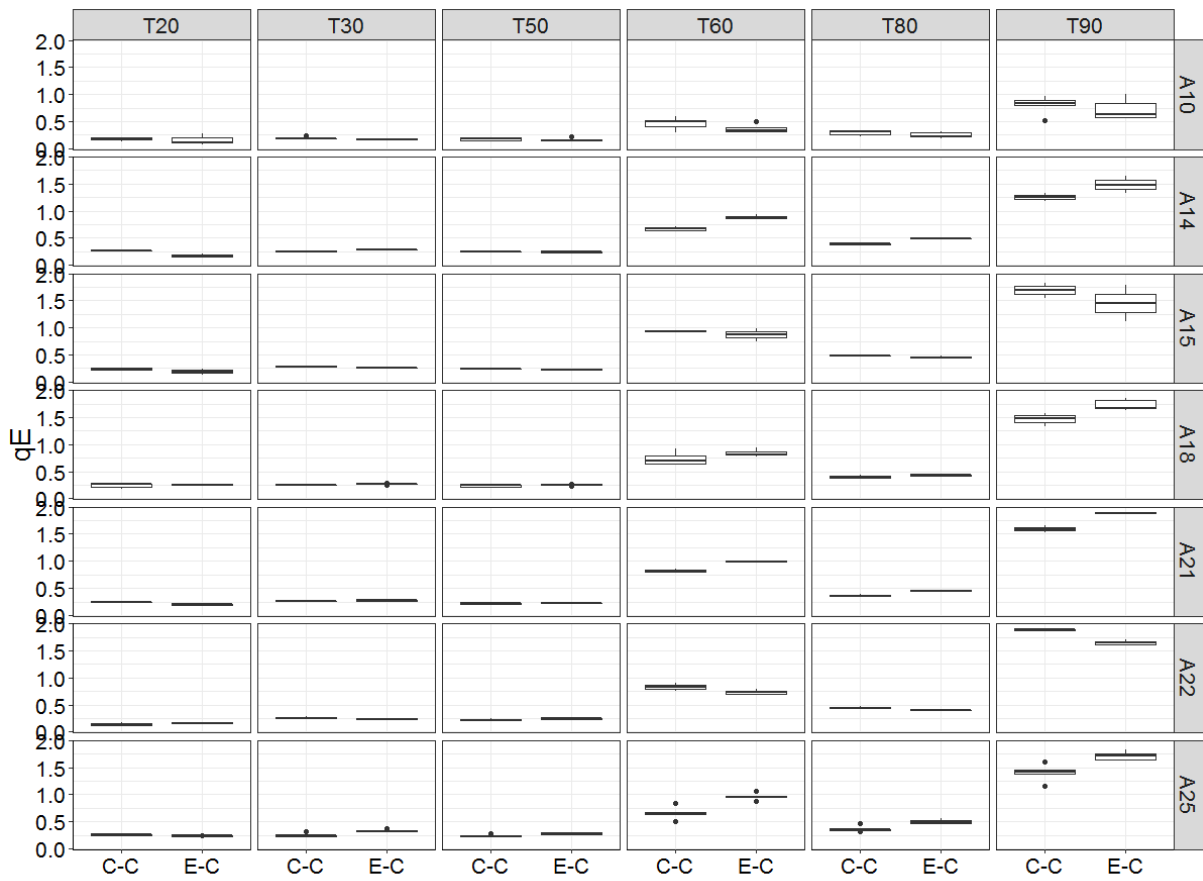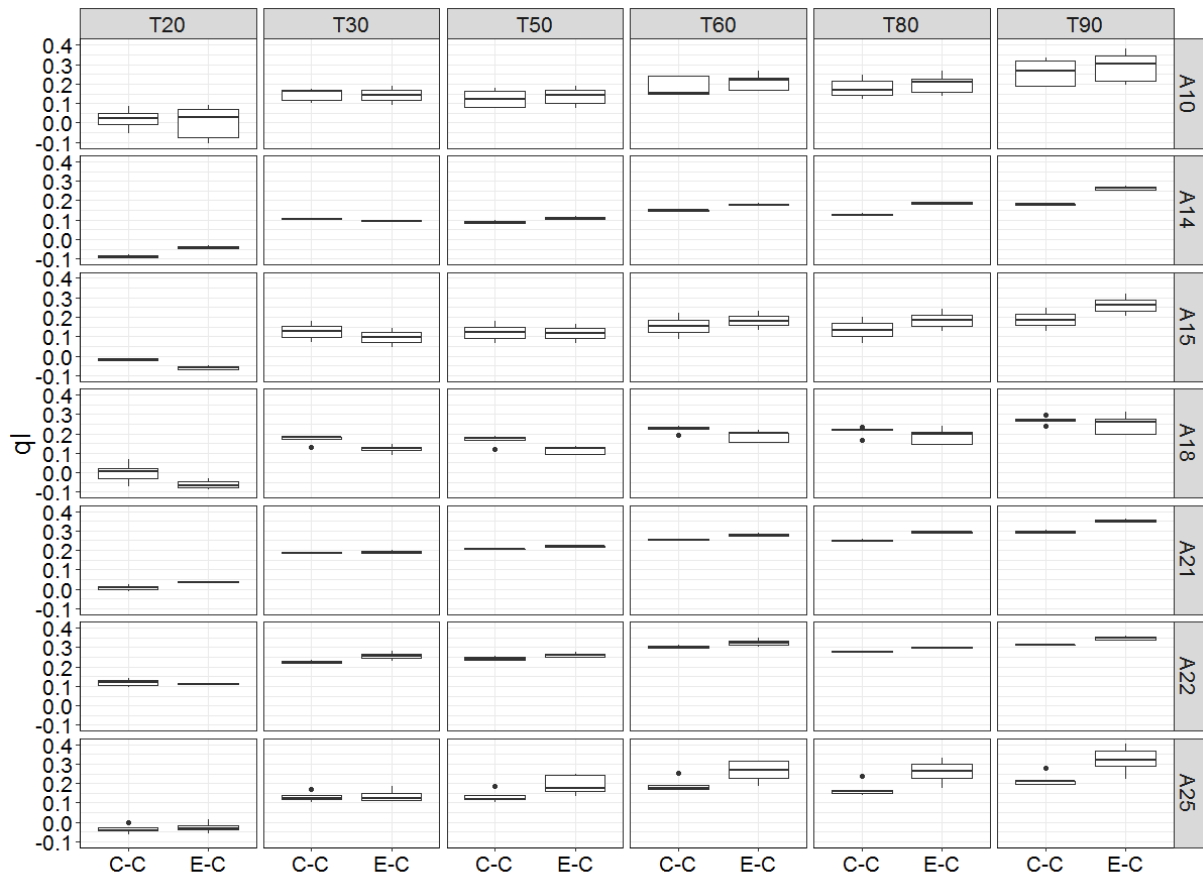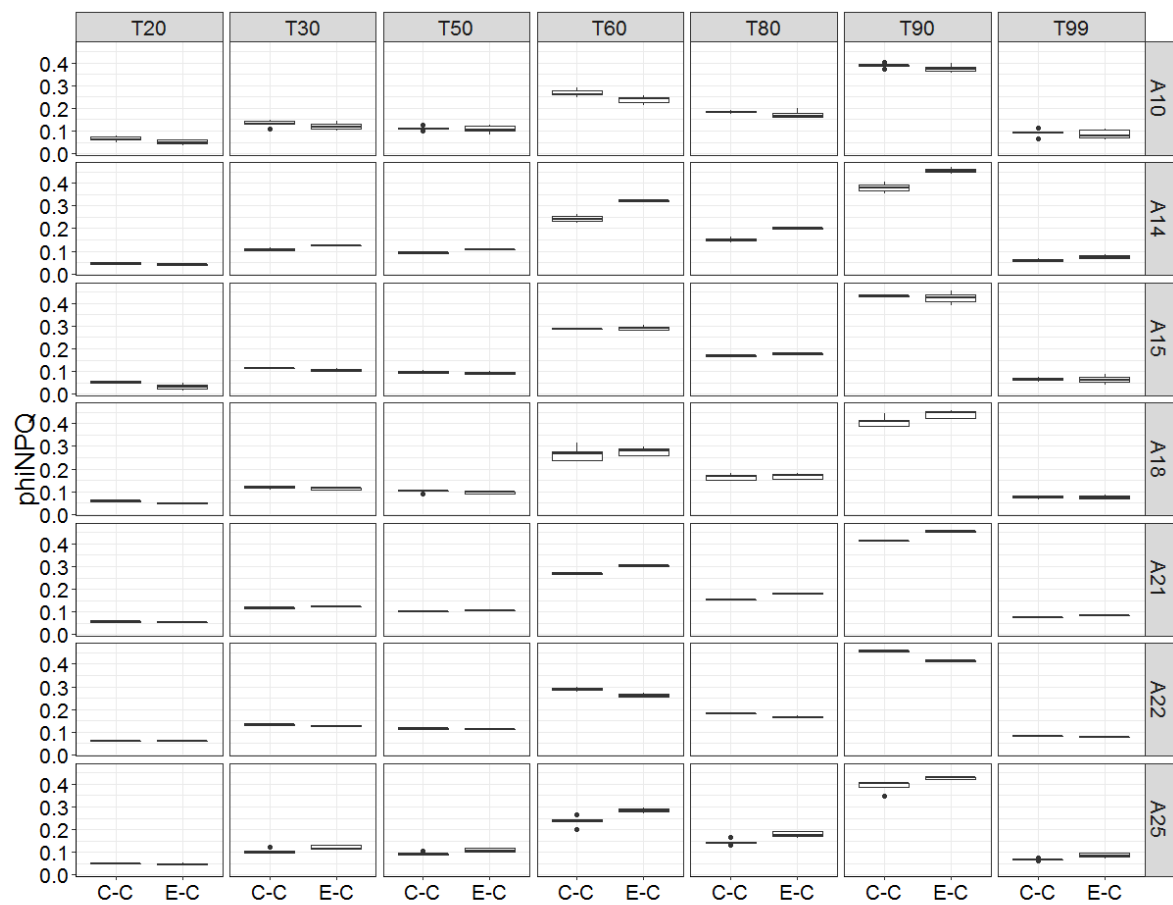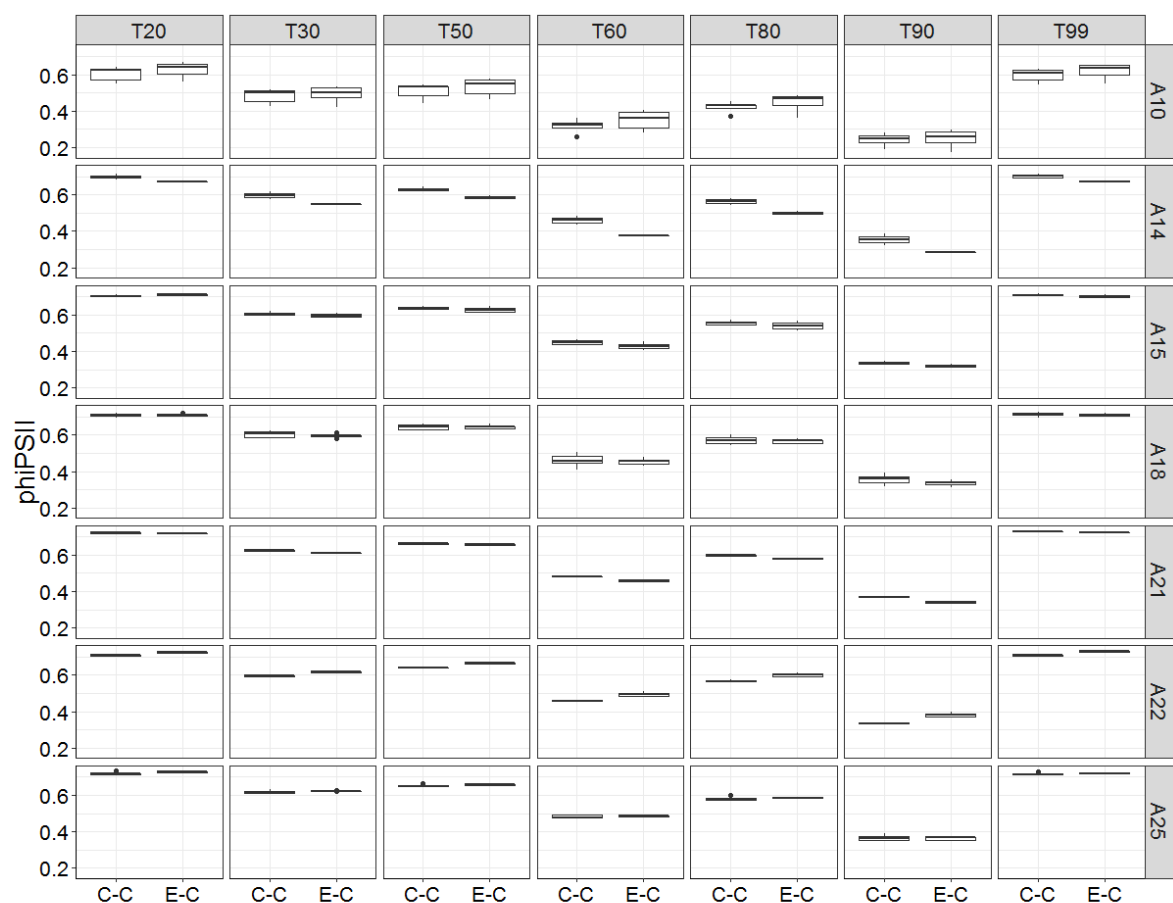
Climate chamber C-C and E-C

These graphs show the NPQ, qI, qE, φPSII, φNPQ and φNO of the C-C and E-C plants grown in the climate chamber. The columns depict the values after acclimation to 200, 400, 350, 700, 500, 1000 and 200 µmol m^{-2} s^{-1} photon irradiance respectively. The rows show the age in days after sowing (DAS); A10 stands for 10 DAS, A14 for 14 DAS and so forth.
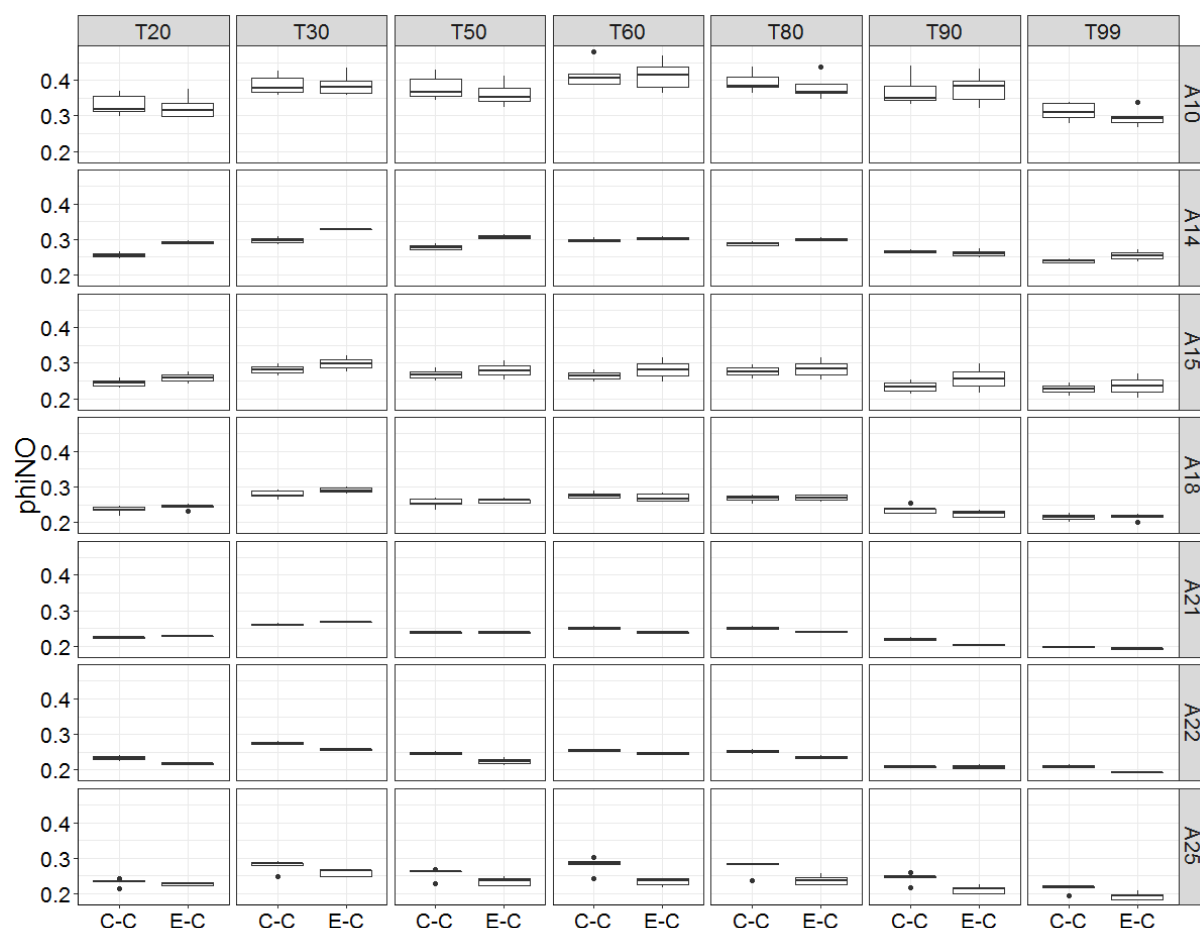
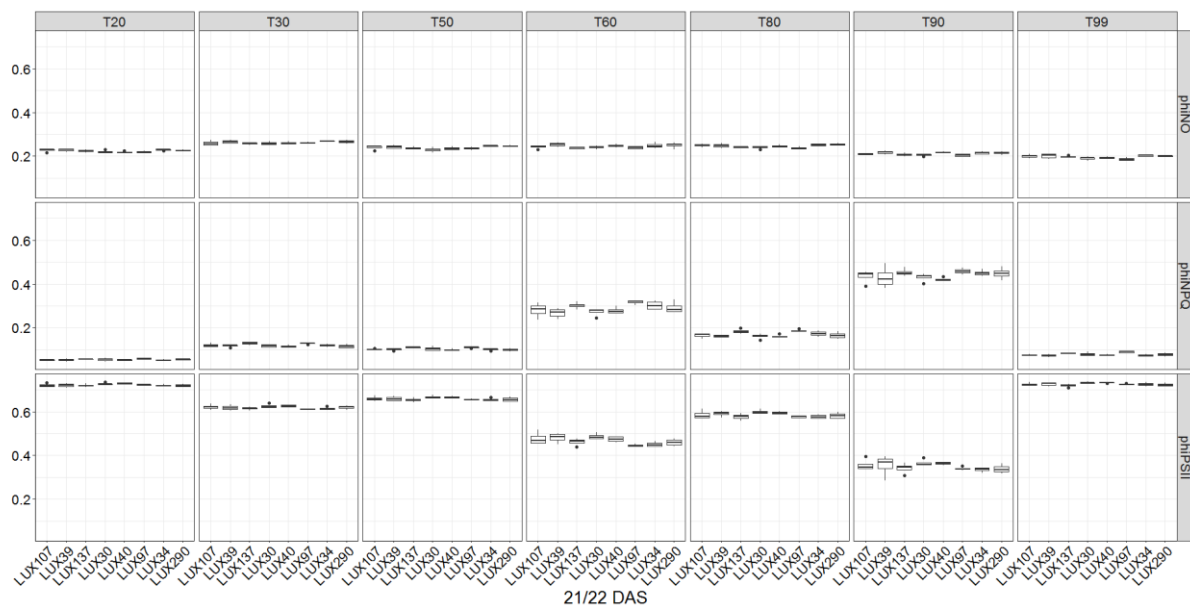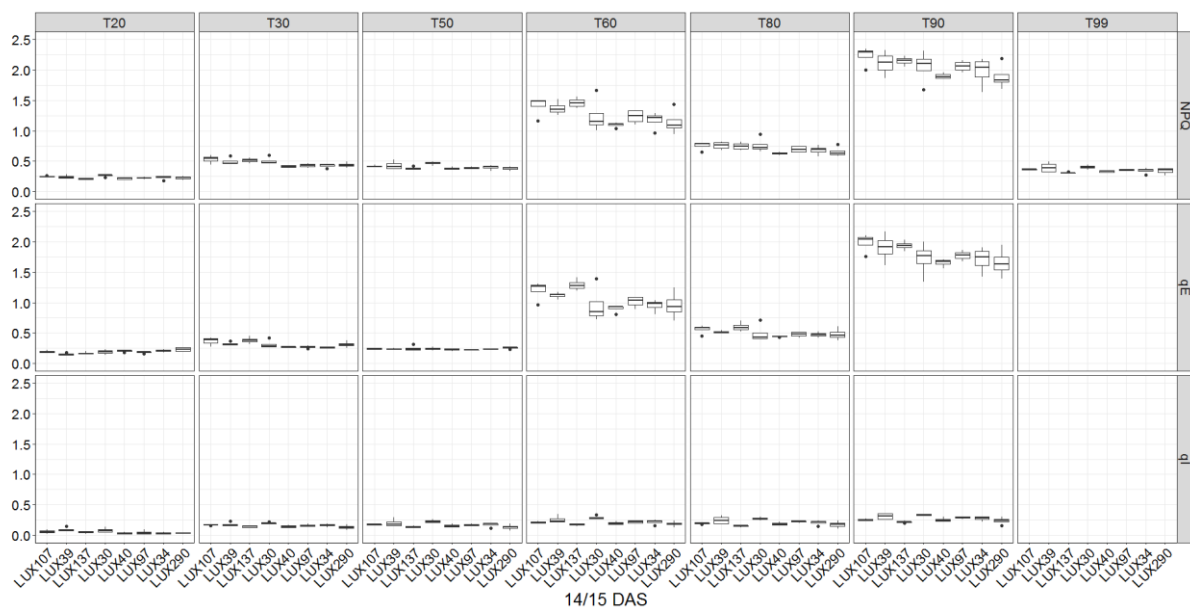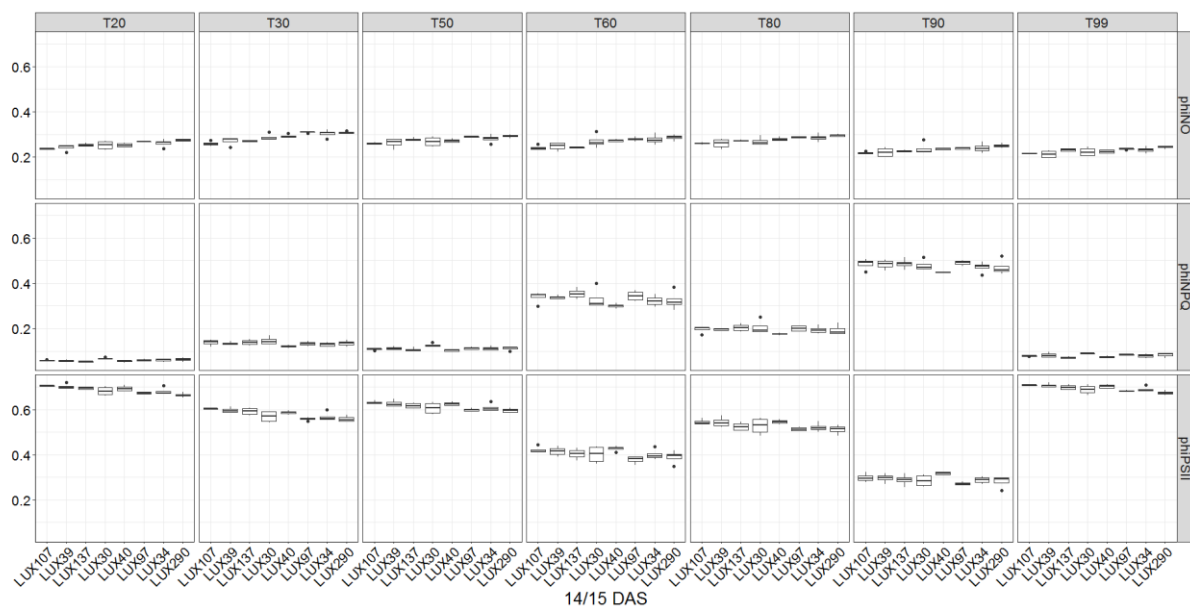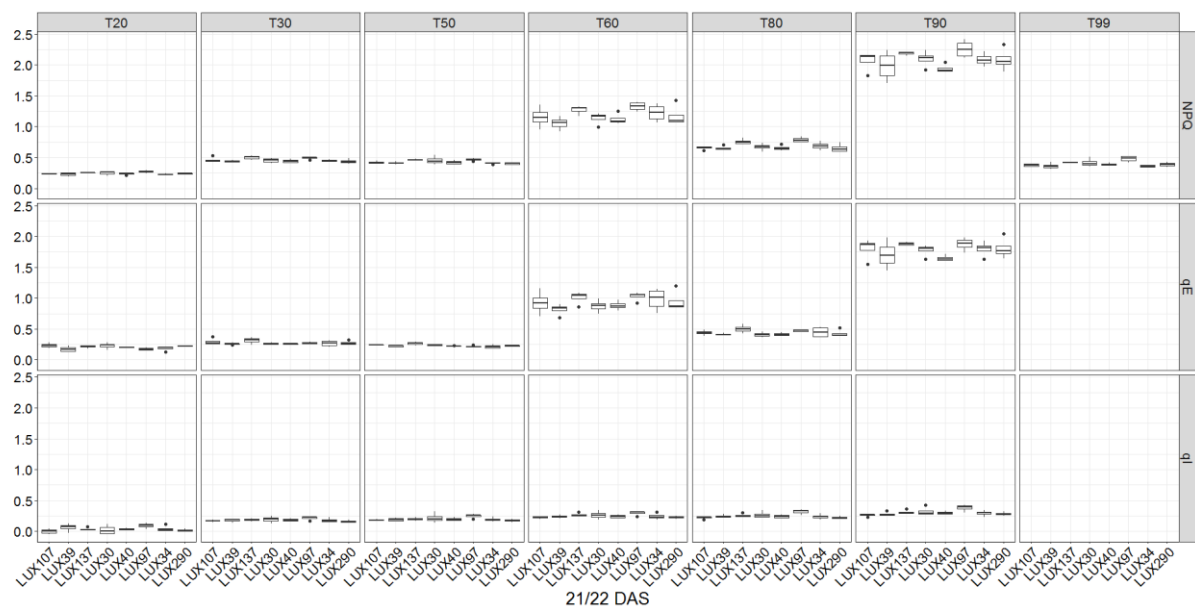## Climate chamber LUX

These graphs show the NPQ, qI, qE, φPSII, φNPQ and φNO of LUX plants grown in the climate chamber. The columns depict the values after acclimation to 200, 400, 350, 700, 500, 1000 and 200 µmol m^{-2} s^{-1} photon irradiance respectively. Age of plants is written on the x-axis. Since these plants were measured in two batches the plants were either 14 or 15 DAS and 21 or 22 DAS.

14/15 DAS

14/15 DAS

21/22 DAS

Greenhouse C-C and E-C

These graphs show the NPQ, qI, qE, φPSII, φNPQ and φNO of C-C and E-C plants grown in the greenhouse. The columns depict the values after acclimation to 200, 400, 350, 700, 500, 1000 and 200 µmol $m^{-2}\,s^{-1}$ photon irradiance respectively. Age of plants is written on the x-axis.