Stress Responses of *Arabidopsis* Plants with a Varying Level of Non-photochemical Quenching

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Abstract

When light energy input exceeds the capacity for photosynthesis the plant need to dissipate the excess energy and this is done through non-photo-chemical quenching (NPQ). Photochemical quenching (photosynthesis), NPQ and fluorescence are three alternative faiths of excited chlorophylls. PsbS associates to photosystem II and is involved in NPQ.

The results presented in this thesis were generated on *Arabidopsis* plants and mainly based on wildtype Col-0 together with a mutant deficient in PsbS (*npq4*) and a transgene overexpressing PsbS (oePsbS). We connect light and herbivore stress and show that the level of PsbS influences the food preference of both a specialist (*Plutella*) and a generalist (*Spodoptera*) herbivore as well as oviposition of *Plutella*. Level of PsbS also affects both metabolomics and transcriptomics of the plant; up-regulation of genes in the jasmonic acid (JA) -pathway and amount of JA has been found in the *npq4* plants after herbivory.

Since many experiments were performed in field we have also characterized the field plant and how it differs from the commonly used lab plant. We have also studied the natural variation of NPQ in *Arabidopsis* plants both in the field and the lab. The results show surprisingly no correlation.

Keywords: *Arabidopsis*, NPQ, PsbS, photosynthesis, field experiment, metabolomics

Sammanfattning

Stressresponser i *Arabidopsis* med olika kapacitet för "icke-fotokemisk quenching"

Överskottsenergi kan vara skadligt för en växts membran och fotosynteskomplex. Vid överskott av solenergi blir fotosystemen mättade och växten behöver därför ett sätt för att göra sig av med all överskottsenergi, detta kallas för "icke-fotokemisk quenching" (NPQ). Fotokemisk quenching (fotosyntes), NPQ och fluoresens är tre alternativa vägar för exalterade klorofyller. PsbS är involverad i NPQ och associerar med fotosystem II.

De resultat som presenteras i denna avhandling kommer från studier av modellväxten *Arabidopsis thaliana* (Backtrav), i huvudsak gjorda på vildtypen i jämförelse med en mutant som saknar PsbS (*npq4*) och en transgen som överuttrycker PsbS (oePsbS). Vi har försökt att undersöka kopplingen mellan ljus- och herbivoristress och visar här att mängden PsbS påverkar både en specialist (*Plutella*) och en generalist (*Spodoptera*) insekt vid val av föda, samt *Plutella* även vid äggläggning. Växternas nivå av PsbS visade sig även påverka metabolomet och transkriptomet, och vi fann en uppreglering av gener i biosyntesen för jasmonat samt mer av själva hormonet jasmonat i *npq4* växter efter herbivori.

Eftersom vi har gjort många av experimenten ute i fält har vi även karakteriserat en typisk *Arabidopsis* växt i fält samt hur denna skiljer sig från den vanligt använda lab-växten. Dessutom har vi även undersökt naturlig variation av NPQ av *Arabidopsis* både i fält och på lab och resultaten visar, till vår förvåning, att det inte går att finna någon korrelation mellan dessa.

List of papers

I   Martin Frenkel, **Hanna Johansson Jänkänpää**, Jon Moen and Stefan Jansson
An illustrated gardener's guide to transgenic *Arabidopsis* field experiments.
New Phytologist, 2008, 180(2): 545-555

II   Martin Frenkel*, Carsten Külheim*, **Hanna Johansson Jänkänpää**, Oskar Skogström, Luca Dall Osto, Jon Ågren, Roberto Bassi, Thomas Moritz, Jon Moen and Stefan Jansson
Improper excess light energy dissipation in *Arabidopsis* results in a metabolic reprogramming.
BMC Plant Biology, 2009, 9(1): 12

III   **Hanna Johansson Jänkänpää**, Martin Frenkel, Ismayil Zulfugarov, Michael Reichelt, Jonathan Gerhenzon, Jon Moen, Choon-Hwan Lee and Stefan Jansson
Arabidopsis thaliana with reduced capacity for non-photochemical quenching show altered herbivore preference.
Manuscript

IV   **Hanna Johansson Jänkänpää***, Yogesh Misra* and Stefan Jansson
Metabolic profiling reveals metabolic shifts in *Arabidopsis* plants grown under different light conditions.
Manuscript

V   Yogesh Misra, **Hanna Johansson Jänkänpää**, Anett Z Kiss, Christiane Funk, Wolfgang P Schröder and Stefan Jansson
Arabidopsis plants grown in the field and climate chambers significantly differ in leaf morphology and photosystem components
Submitted

*These authors contributed equally to respective manuscript
The papers will be referred to by their roman numbers in the text.

Publications not included in thesis

Hanna Johansson Jänkänpää and Stefan Jansson
How to grow transgenic *Arabidopsis* in the field.
Book chapter in "Transgenic Plants: Methods and Protocols", Volume of
Methods in Molecular Biology published by Springer
Submitted

Raik Wagner, Harald Aigner, Adriana Pruzinská, **Hanna Johansson
Jänkänpää**, Stefan Jansson and Christiane Funk
Fitness analyses of *Arabidopsis thaliana* mutants depleted of FtsH
metalloproteases and characterization of three FtsH6 deletion mutants
exposed to high light stress, senescence and chilling.
New Phytologist, 2011, doi: 10.1111/j.1469-8137.2011.03684.x

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"Science is built of facts, as a house is built of stones: but an accumulation of facts is no more science than a heap of stone is a house"

-Henri Poincaré, 1905

Abbrevations

Arabidopsis	= *Arabidopsis thaliana*
Chl	= Chlorophyll
GS	= Glucosinolate
JA	= Jasmonic acid
LHC	= Light harvesting complexes
LHCII	= Light harvesting complex 2
NPQ	= Non-photochemical quenching
PQ	= Plastoquinon
Plutella	= *Plutella xylostella*
PS	= Photosystem
PSI	= Photosystem 1
PSII	= Photosystem 2
PsbS	= Photosystem B protein S
qE	= ΔpH dependent quenching
RC	= Reaction center
ROS	= Reactive oxygen species
$^1O_2$	= Singlet oxygen
$O_2^{\cdot-}$	= Superoxide
Spodoptera	= *Spodoptera littoralis*
VDE	= Violaxanthin de-epoxidase

Introduction

Plants and Evolution

The earth has been populated for at least 3.4 billion years by photosynthetic organisms, but for only a couple of million years by humans (Raven et al., 1999). That means photosynthesis as a way to gain energy is extremely purified and well developed. Through evolution, this system and process has become almost perfect and we believe that all the proteins and processes in photosynthesis have a specific role, since everything irrelevant should be long gone. Most plants live their whole life in the same spot, which demands special abilities for survival. Depending on the habitat they live in, plants will face different stresses such as, cold, drought, flooding, heat, excess light, pathogens and herbivory. Because of this, photosynthesis is not only a brilliant way to get energy (which humans nowadays try to mimic) and a well-developed system but also a highly flexible process and interesting to study.

Photosynthesis

Photosynthesis takes place in the thylakoid membrane inside the chloroplast of a plant cell. Thylakoids are both stacked on top of each other (grana) and have parts exposed to the stroma (stroma lamellae) (fig 1A). The photosynthetic apparatus consists of two main complexes; photosystem I (PSI) and II (PSII) where PSII appears first in the order of photosynthetic reactions. PSI is located in stroma-exposed positions whereas PSII occurs in the stacked parts (fig 1B). Since electron transport would have been easier if the photosystems had been positioned closer to one other, there must be a reason why they are not. The segregation of PS and formation of grana stacks has not been completely explained but there are many suggestions as to why they would spatially segregate. One suggestion for the lateral segregation is for access to the final electron acceptor $NADP^+$, which exists in the stroma (Allen and Forsberg 2001). Chow et al., 2005 proposes the reason to be prevention of spillover for excitation energy from PSII to PSI, especially under limiting light. Furthermore, had the photosystems not been laterally separated, PSII would probably have problems with energy capture, since PSI has faster kinetics for trapping excitation energy as compared to PSII (Trissl and Wilhelm 1993, Rojdestvenski et al., 2002).

One previous suggestion regarding grana stack formation has been that it is caused by a lipid-lipid interaction, but current opinion is that it is a consequence of protein-protein interaction either by PSII or LHC (Allen and Forsberg 2001).
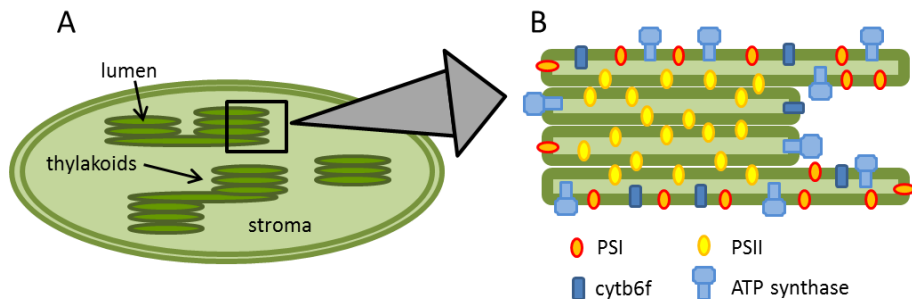
Figure 1. Chloroplast and thylakoid organization. A schematic picture of the organization of A) thylakoids in the chloroplast and B) photosynthetic membrane proteins in the thylakoid membrane. Model modified after Dekker and Boekema 2005.

Energy from sunlight is collect by chlorophyll (chl) and carotenoid "decorated" light harvesting complexes (LHC) that consist of 10 different major proteins, Lhca1-4 and Lhcb1-6, (Jansson et al., 1992). The abundance and composition of these harvesting proteins varies depending on light quality and intensity and other external and internal factors. The most common complex is a trimer called LHCII, this complex mostly consists of two Lhcb1 proteins and one Lhcb2 or Lhcb3 protein, but other combinations can also occur (Dekker and Boekema 2005).

Photosynthesis can be describes as a chain of reactions (Fig 2), where light energy is captured by excitation of chls in the LHC. The energy is passed on to the reaction center (RC) of PSII and close to PSII on the lumen side of the membrane a water molecule is oxidized at the manganese cluster. An electron is excited and a proton (H^+) is released. PSII transfers the excited electron through the plastoquinone (PQ) pool, cytochrome b_6f and plastocyanin to PSI where the chlorophylls in the LHCs of PSI capture new energy and the electron is excited again. The energy is passed to ferredoxin and NADPH is produced (Taiz and Zeiger 2002).

Plastoquinones (PQ) are small hydrophobic molecules that carry electrons from PSII and protons from the stroma. They function as the first electron carrier between PSII and PSI and since they deliver protons to the lumen

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they participate in proton pumping. The state (reduced or not) of the PQ pool is a clear signal of the state of the photosynthesis. Plastocyanin (PC) is a small lumen protein that transports electrons from the cytochrome b_6f to PSI.
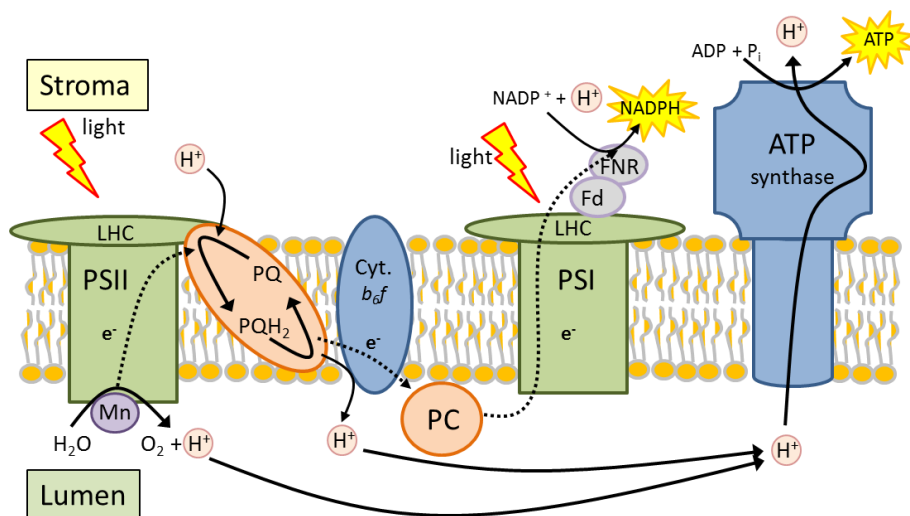
Figure 2. Reactions in photosynthesis. Photosynthesis is starting with excited chlorophylls in the light harvesting complex II (LHC). Energy is passed on to the reaction center of PSII. At the same time a water molecule close to PSII on the lumen side of the membrane is oxidized at the manganes cluster (Mn). An electron is excited and a proton (H$^+$) is released. PSII forwards the excited electron through the plastoquinone (PQ) pool, cytochrome b_6f and plastocyanin to PSI where the electron again is excited. Through ferredoxin (Fd) and ferredoxin NADP$^+$ reductase (FNR) the electron and a proton forms NADPH. This electron transport also results in a proton gradient, which drives the ATP synthase to form ATP. Modified after Taiz and Zeiger 2002.

The main electron flow in photosynthesis is linear, but there is also a cyclic electron flow. In cyclic electron flow the electrons are cycled around PSI either through the protein PGR5 pathway or through the NDH (NADH oxidase like complex) pathway (Johnson 2011). The cycling is generating a ΔpH gradient which also drives the synthesis of ATP (Johnson 2011). The role of electron cycling is not fully understood and hard to investigate since there are many processes in the stroma where ATP is needed. Thus the idea of a possible involvement of electron cycling in photoprotection (because of increased ΔpH) is interesting (Johnson 2011).

The photosystems themselves consist of many proteins both large and small, they are named Psa for PSI and Psb for PSII proteins and then letter wise (A-Z) in the order of identification. Two of the largest proteins of PSII are named PsbA and PsbD and they are the main reaction center. The protein I have been working with is small (22kDa) and associated to PSII (Dekker and Boekema 2005) and it is called PsbS. It was identified in 1984 by Ljungberg et al. (1984). Almost ten years later Wedel et al. (1992) connected PsbS to light harvesting because of its similarity to the light harvesting proteins. Later on it was suggested to be involved in non-photochemical quenching (NPQ) (Li et al., 2000b) as it still is, but the position and function of PsbS has since then been subject of debate (se page 9, "PsbS and qE").

Non-photochemical quenching (NPQ)

Can plants really utilise all the light energy they are exposed to? And if so, can they also convert all energy into carbohydrates? What happens to energy that does not go into photosynthesis and how do plants handle this? How are they prepared for variation in light energy such as a half-cloudy day or the seasonal changes in a boreal forest?

Up to a certain light intensity the correlation between light intensity and carbon assimilation is linear, however after that point the plant cannot make more carbohydrates despite more light energy because of the limitation of CO_2 uptake (Taiz and Zeiger 2002). This can happen during different conditions such as; relatively strong or fluctuating light (although what is considered as strong light depends on previous light conditions), dehydration, and low temperatures. Such conditions are very dangerous to plants but are nevertheless daily life for some plants and they have evolved ways to handle it. This chapter is about some of these adaptations.

After the point of equilibrium between light energy captured and carbon assimilation there will be an excess of light energy (Fig 3). More chlorophyll will be excited than the PSII reaction center (RC) can utilize, since the downstream PQ pool is saturated. Most chls are excited and want to pass their energy somewhere else, preferably to a carotenoid but in the worst case they react with oxygen and reactive oxygen species (ROS) are produced, usually singlet oxygen ($^1O_2$) or superoxide ($O_2^{.-}$) (Havaux and Niyogi 1999). Like the name reveals oxides are highly reactive and can easily cause a lot of damage to the photosystems and especially the membrane

lipids (Taiz and Zeiger 2002). A small abundance of oxides can be used in signaling but a larger amount is devastating to the plant (Triantaphylidès and Havaux 2009). With increasing light and electron saturation, the lumen becomes acidic due to the protons from the oxidized water and the PQ transport. Lumen acidification is an important first step in photoprotection and is sensed by the PsbS protein (Li et al., 2002b and Ruban et al., 2011). Plants have developed some ways to avoid the oxides and disarm the excited chlorophyll in a harmless way, through photoprotection and photoinhibition.
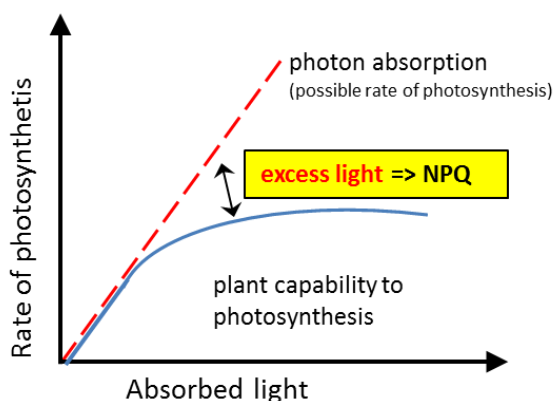
Figure 3. Why NPQ is needed. The rate of photosynthesis does only increase until usually carbon access becomes the limiting point. The excess light that is absorbed is harmful and must be dissipated. Modified after Li et al., 2009.

This process of NPQ is not only important to plants but also to some algae and cyanobacteria; maybe even more important to those, since they are mobile in the water and therefore exposed to a high variation of light intensity and quality. Light harvesting complexes (LHC) are antenna complexes where light energy is harvested and are highly conserved among the plant kingdom (Jansson 1999, 2006) but the evolution of PsbS seems to have been slightly different. In green algae a protein (also related to the light harvesting family) called LHCSR has been found to be responsible for qE (Peers et al., 2009). This protein has also an active form and together with PsbS has been found in the moss *Physcomitrella patens* (Alboresi 2010). Together these data suggest that LHCSR and PsbS have evolved in parallel whereas LHCSR seems to have been lost just after plants were entering land (Alboresi 2010). This thesis will only focus on NPQ in vascular plants.

Photoprotection and photoinhibition

Both photoprotection and photoinhibition are reversible processes and preferred by the plant as an alternative to uncontrolled damage to the PS and membrane. Photoinhibition has been considered a long term response and photoprotection a short term response (Long and Humphries 1994) but more recently these processes seem to be overlapping and therefore the difference between them maybe not so pronounced.

Photoinhibition is described as a slow but reversible retardation of photosynthesis and can, except as protection, be seen as a reduction in potential CO_2 uptake (Long and Humphries 1994). During photoinhibition the PsbA (D1) protein is damaged, targeted for degradation and replaced by a new functional D1 protein, a process called the D1 repair cycle. The other process, photoprotection, is the plant's most rapid strategy to protect PSII from excess light. Photoprotection is the process where excess energy is dissipated as heat and here follows a description of the most common processes of photoinhibition and photoprotection. I will present them starting with long term and ending with short term responses.

Leaf/chloroplast movement and reduction of antennae

Leaf movement and reduction of antennae size are two types of long term responses to excess light. Through leaf movement the plant re-localizes its leaves to a more beneficial angle where less absorption is possible and thereby excess light is reduced, this is light avoidance. The chloroplasts of the plant can also move and in case of excess light they can reorientate into a position where less chloroplast area is facing the light source (Murchie Murchie and Niyogi 2010). Another long term response is reducing the antenna size, consequently decreasing the amount of LHC and pigments in the thylakoid membrane. Leaf movement is a process that takes a long time, whereas chloroplast movement takes minutes to hours (Murchie and Niyogi 2010) and antenna reduction take days. Even though these are relatively slow processes they are very important for acclimation of the plant during changing light conditions, for example seasonal changes or if a nearby tree falls and there is a sudden gap in the canopy.

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State transition

Another strategy is state transition. This is done by phosphorylation and is the plant's strategy to switch between PSII and PSI excitation under increasing light or a change in spectral quality. During these conditions LHCII becomes increasingly phosphorylated (Tikkanen and Aro , in press) and moves from PSII and grana (state 1) to stroma exposed regions (state 2) where PSI complexes are located (Fig 1B). Under excess light the PQ pool is reduced which activates a kinase (STN7) that phosphorylates the LHCII complexes (Dietzel et al., 2008). Upon PQ oxidation the kinase is inactivated, LHCII de-phosphorylated and relocated to PSII (Dietzel et al., 2008). When LCHII change from state 1 to state 2 it limits the flow of electrons from PSII into PQ but also increases the flow of electrons from PQ to PSI, thereby the extra abundance of electrons situated in the membrane is cleaned up for a while (Allen and Forsberg 2001). It has been shown that it is only a small part of the total LHCII complexes that migrate during state transition in *Arabidopsis thaliana* (*Arabidopsis*) while most of the LHCII complexes relocate under state transition in *Chlamydomonas* (Allen 1992).

Although not directly connected with state transition, as mentioned previously, PSI can also cycle electrons either through the protein PGR5 pathway or through the NDH (NADH oxidase like complex) pathway (Johnson 2011). Independent of the pathway used, the cycling is generating a ΔpH gradient which also drives the synthesis of ATP (Johnson 2011). The purpose of electron cycling is not certain and hard to prove since there are many processes in the stroma where ATP is needed. Thus the idea of electron cycling possible involvment in photoprotection (because of increased ΔpH) is also interesting (Johnson 2011).

Carotenoids and the xanthophyll cycle

There are many different carotenoids in the world and in the xanthophyll cycle three main carotenoid pigments are involved: violaxanthin, antheraxanthin and zeaxanthin. For heat dissipation zeaxanthin is the most effective. The xanthophyll cycle is the conversion of violaxanthin to zeaxanthin by violaxanthin de-epoxidase (VDE) and with antheraxanthin as an intermediate. Lutein and neoxanthin are two other common carotenoids in plants and they both have fixed positions in the antenna proteins (Jahns and Holzwarth 2011). Lutein stabilizes the thylakoid membrane by quenching of triplet chl (Mozzo et al., 2008) and has thus also been suggested to contribute to photoprotection (Jahns and Holzwarth 2011).

The xanthophyll cycle was first discovered in 1957 by D.I. Sapozhnikov who observed variation in violaxanthin under light and dark conditions (Heyde and Jahns 1998). Much later, in 1987, the existence and involvement of the xanthophyll cycle in plant photoprotection was suggested by Demmig and co-workers (Demmig et al., 1987, Demmig-Adams 2005). This theory took 3 more years to prove, and it was Bilger and Björkman 1990 who depleted the VDE and consequently saw a decrease in the capacity of the plant's quenching. This was the final step to prove the involvement of the xanthophyll cycle in photoprotection through quenching.

In excess light energy there is an acidification of the lumen and this stimulates VDE to convert violaxanthin into zeaxanthin. Under normal or high lumen pH VDE is in a soluble form but in low pH the enzyme is membrane bound (Rockholm and Yamamoto 1996). When the excess of excited chlorophylls and electrons are reduced the lumen pH is raised again and the cycle is reversed; zeaxanthin is epoxidased through antheraxanthin back into violaxanthin (Demmig-Adams and Adams 1996). The de-epoxidation is a rather fast process (within minutes) and the ratio of violaxanthin to zeaxanthin in the xanthophyll pool varies over the day both depending on the conditions the plant experience and diurnal rhythm (Demmig-Adams and Adams 1996). Also epoxidation can occur within minutes but could take up to days depending of eventual additional stress (Demmig-Adams and Adams 1996).

Table 1. Processes of photoprotection and the time they take to accomplish.

Process	Protection method	Time intervall
Leaf/chloroplast movement	Light avoidance	Minutes-Hours-Days
Reduction of antennae size	Light avoidance	Hours-Days
State transition	Balancing	Minutes
Xanthophyll cycle	Heat dissipation	Minutes
PsbS quenching	Heat dissipation	Seconds

The capacity of xanthophylls to cycle between being more reactive (zeaxanthin) and less reactive (violaxanthin) enables them to have a protective as well as harvesting role in photosynthesis. In certain conditions the plant is favored by energy capture, while in other conditions there is

increased need for energy dissipation. The cycling of xanthophylls fulfills both these requirements and can change quickly.

PsbS and qE

The quickest plant photoprotection mechanism known today is heat dissipation through quenching; a process the PSII associated protein PsbS is mainly responsible for. The protein PsbS is a small (22kD) 4-helix membrane protein and together with the qE part of NPQ (qE corresponds to the heat dissipation dependent on ΔpH) has long been a subject of discussions such as; whether PsbS can bind pigments, whether it does bind pigments, where it is situated, and the nature of the process that protects PSII (Ruban et al., 2011).

The involvement of PsbS in photoprotection was discoverd through an *Arabidopsis* mutant that was very defective in qE (Li et al., 2000b). This mutant is called *npq4* and has a single mutation in the nuclear genome (Li et al., 2000b). It was also thought that PsbS binds chlorophyll and xanthophylls (Funk et al., 1994). It was the xanthophyll violaxanthin that had been isolated with PsbS and therefore the authors suggested PsbS to be essential for NPQ. One year later Funk et al. (1995) demonstrated that PsbS was also stable in the absence of pigments and later on Dominici (2002) more thoroughly investigated the pigment-binding property of PsbS. The experiments were conducted both *in vivo* and *in vitro* and they concluded that if PsbS binds pigments they are very loosely bound. Whether PsbS binds pigments or not is still debated but the majority of researchers believe it is not.

There are several old and new hypotheses about PsbS and especially qE. Over the last decade there have been four main hypotheses and they are presented in table 2. Two of them have been more questioned but the top-two have instead been developed. Here follows a history and brief description of the main hypotheses about qE.

In 2000, three different models of qE quenching were presented. One model from Li et al. (2000b) suggested PsbS itself was the quencher. Another model suggestion was from Bassi and Caffarri (2000), and they suggested that the minor antenna proteins Lhcb4 and Lhcb5 were the quenchers. The third model came from the lab of Peter Horton (Horton et al., 2000) and suggested that quenching happened in the LHCII antenna

after a conformational change, where zeaxanthin is the allosteric modulator. This third model is therefore called the allosteric model and describes two types of LHCII formations, one light harvesting form and one energy dissipating form. Regarding the PsbS quenching model, if PsbS were the quencher it must bind pigments, and at that time this question was not yet answerd. However, some years later Crouchman et al. (2006) investigated the NPQ capacity of PsbS in the absence of zeaxanthin and found that it does not rely on zeaxanthin; instead they suggested PsbS to be a proton receptor that induce an eventual conformational change in the antenna. Regarding quenching in the minor antenna, it has been shown that the Lhcb4 and Lhcb5 proteins do bind carotenoids (Bassi and Caffarri 2000) but also that qE is relatively unaffected in mutants of these proteins (Andersson et al., 2001). Therefore this model can only describe a fraction of the function behind qE. Furterh on, Holt et al. (2005) found the formation of a carotenoid radical cation to be connected with qE and by that suggested a chl-carotenoid heterodimer to be the site of quenching. In additions, Pascal et al. (2005) also raised a suggestion about the function and process of qE. It is similar to the aggregation model since it suggests that quenching occurs in the LHCII antenna, but according to this model the conformation is believed to be controlled by the xanthophyll cycle. The reason for conformation is thought to be the distance between a chl and a carotenoid, the distance is shorter when in a dissipative state. This is similar to the suggestion from Holt and co-workers.

More research of PsbS and qE have followed and PsbS has been proven to control the organization of PSII during membrane stacking (Kiss et al., 2007). It was also suggested that protonated PsbS is the driving force of a conformational change in LHCII and Kereïche et al. (2010) suggested PsbS to be a macro-organizer of the grana membrane. The authors examined 500 micrographs of the thylakoid membrane of each genotype (wt, *npq4* and oePsbS). For the overexpresser they found no individual with a crystalline formation while the wt had 5.0% and *npq4* had formed crystals in 8.6% of the micrographs. As a conclusion of these results a model was presented where PsbS is the key player to loosen the thylakoid membrane and thus improve the possibility for quenching (Kereïche et al., 2010). This model is a further explanation to the allosteric model. Further on, Ballottari et al. (2010), have investigated the quenching capacity of the different parts of PSII antenna and found both major and minor LHC to quench, but the latter one more efficiently.

Table 2. A summary of the most common qE hypotheses.

Model	Description	+/-	Results (supporting or not)
Allosteric model	Quenching in LHCII	+	(Protonation of PsbS is essential for its role in qE[a])
	PsbS induce conformational change of LHCII	+	LHCII can be involved in aggregation-dependent quenching[b]
		+	PsbS can enhance NPQ without zeaxanthin[c]
		+	*npq4* plants do form qE, but it is slower[d]
		+	PsbS gives a flexible membrane macro-organisation[e]
Direct quenching	Charge transfer quenching	+	Formation of a carotenoid radical cation is correlated with qE[f]
	Change of distance between chl and carotenoid	+	Minor Lhcb proteins are more efficient in quenching than LHCII[b]
	Monomeric Lhcb involved	-	(PsbS can enhance NPQ without zeaxanthin[c])
Minor antenna quenching	Quenching in minor antennae	+	Minor antenna does bind a lot of xanthophylls[g]
		-	Minor antennae mutants does not loose qE capacity[h]
PsbS quenching	Quenching at PsbS	+	PsbS is necessary for qE[i]
		+	Protonation of PsbS is essential for its role in qE[a]
		-	PsbS does not bind enough pigments[c]

References: [a] Li et al, 2002; [b] Ballottari, 2010; [c] Crouchman, 2006; [d] Johanson and Ruban, 2010; [e] Kereïche et al, 2010; [f] Holt et al, 2005; [g] Bassi and Caffarri, 2000; [h] Andersson et al, 2001; [i] Li et al, 2000

Consequently, PsbS has also been shown to be non-crucial for qE (Johnson and Ruban 2009) since *npq4* plants also showed qE, only it took much longer time to occur. Nowadays PsbS is thought to be a catalyst and induce NPQ on much smaller ΔpH than only zeaxanthin would do (Ruban et al., 2011). It is also possible that qE is not a homogenous process and can therefore not be described by one model alone.

Another outcome of this story is a model postulated by the group of Eva-Marie Aro in Finland whereby state transition and PsbS cooperate and cover each others' backs (Tikkanen et al., 2011). Under low light when NPQ is not functional, state transition (phosphorylation of LHCII) balances the energy between PSII and PSI to keep up a continous flow of electrons. The kinase (STN7) which induces LHCII phosphorylation is activated in low light. With increasing light intensity (and decreased lumen pH) PsbS is protonated and actively dissipates the excitation energy; under these conditions the kinase (STN7) is inactivated. When light is decreasing lumen pH is increasing, PsbS less protonated and the thermal dissipation is no longer active. The oxidation of stromal reductants quickly activate the kinase again (Tikkanen et al., 2011). This alternative model is thought to mainly be regulated by the different needs of the downstream electron acceptors.
As the name reveals photoinhibition (and photoprotection) is thought of as an inhibiting process that decrease the total yield of the plant. Nonetheless these inhibiting and protecting mechanisms might actually be helping the plant to produce higher yields in the long run since repairing PSII is a costly process.

How to measure NPQ

As described above NPQ is heat dissipation. Heat from a plant is not easy to measure especially since the dissipation does not lead to a large temperature change and there might be other factors than dissipation affecting the temperature. Therefore today an indirect method to indirectly measure heat dissipation, by use of chlorophyll fluorescence is commonly used.

When chlorophyll is excited the energy has three different destinies; photochemical quenching, non-photochemical quenching or fluorescence. In the presence of light the plant will maximize its photochemistry and any excess light will be fluoresced or dissipated as heat. By measuring the photochemistry and fluorescence we can measure the proportion of

photons (the light exposure is regulated) dissipated as heat (Maxwell and Johnson 2000).

Measurements are relative and two experiments cannot directly be compared without normalization owing to many factors may change e.g. plant day to day variation and the ageing of the lamp in the instrument (Maxwell and Johnson 2000). To measure the full capacity of PSII the plant is dark adapted prior to measurement. This relaxes the plant's photosynthetic apparatus and ensures all the PSII reaction centers are open (Maxwell and Johnson 2000). When measuring NPQ (or fluorescence to be more exact) the plant is regularly exposed to a pulse of a desired light intensity in combination with the presence or absence of actinic light. Figure 4 shows a normal fluorescence curve of a one hour dark adapted wildtype plant. The background fluorescence is seen in the first seconds of the baseline. Later, when saturating light pulses are given, all PSII complexes are saturated and closed and the maximum fluorescence capacity can be measured (Maxwell and Johnson 2000). The plant's increasing capacity of photosynthesis is visualized as decreasing fluorescence and measured from the F_t-line and the regularly appearing, decreasing peaks (F_m'). NPQ is simply measured as the first (and highest) peak minus the following peaks. But in a more complex description the NPQ measured is regarded to contain four different types of quenching: qT - quenching because of state transition; qI - quenching because of non-functional PSII; qZ – zeaxanthin dependent quenching; and the one we are mainly interested in, qE - quenching because of its dependence on delta pH (Jahns and Holzwarth 2011). To calculate qE a phase of dark relaxation (light is switched off) needs to be introduced, which can be seen in the end of the fluorescence curve in figure 4. When the light is switched off the plant PSII is relieved of electrons and through that is "relaxed" again. At the same time the capacity to fluoresce is increasing again. The qE part of NPQ is calculated as: **(F_m/F_m')-(F_m/F_{mr}')**

Krause and Weis (1991) for the definitions below.

F_0 = Emission by antennae chl a, mirrors the antenna size
F_m = Maximum total fluorescence, full reduction of PQ
F_m' = Maximum fluorescence at a specific time point under actinic light
F_{mr}' = Maximum fluorescence at a specific time point in dark
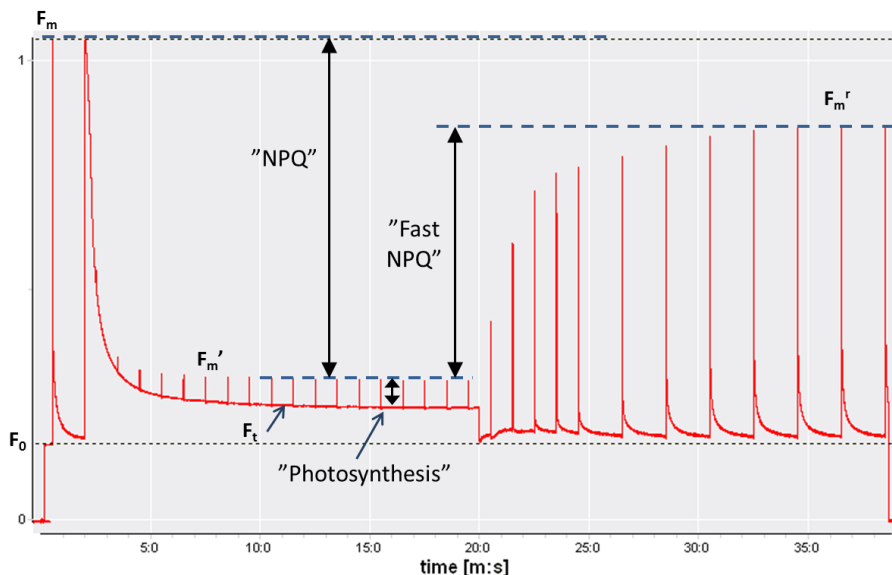$F_m - F_0 = F_v$ = Total variable fluorescence

Figure 4. Fluorescence curve of a wildtype plant with terms and what they describe in a physiological perspective. The arrows show what the fluorescence data mainly represents but these parameters need to be carefully calculated to be comparable with other fluorescence curves.

Model plant and genotypes

The model plant in these studies is *Arabidopsis thaliana*. In total, about 30 different ecotypes of *Arabidopsis* plants together with a mutant plant with a deletion in the PsbS gene (Li et al., 2000b) and a transgenic line that has an overexpression of the *psbs* gene (Li et al., 2002a) were used in these experiments. The ecotypes are from collections from around the world (Fig 5 and Table 3), some collected by colleagues (*tack* Pelle Ingvarsson!) but most of them ordered from NASC, the European Arabidopsis Stock Centre.

When studying the effect of NPQ capacity in relation to other biotic stresses the mutant (*npq4*) and the transgenic line (oePsbS) as well as wildtype Col-0 were used. In oePsbS the amount of PsbS protein is increased and as a result its capacity to rapidly quench excess energy is doubled (Li et al., 2002a). There is no visible phenotype for the three different genotypes (Fig 6).

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Figure 5. World map of the ecotypes used. Red dot shows where the experiments have been performed, Umeå in Sweden.

Table 3. Ecotypes of *Arabidopsis* and their place of origin.

Genotype	Full name	Location	Altitude
Aa-0	Aua	Aua/Rhön, field border, Germany	200-300
Br-0	Br	Brunn, Czech Republic	200-300
Can-0	Canary Islands	Las Palmas/Mirador, Canary Islands	1260
Col-0	Columbia	Landsberg, Germany	100
Cvi-0	Cape Verdi Islands	rocky wall with moss, Cape Verdi Islands	1200
Färjestad	Färjestad	Färjestad, Sweden	50
Kas-1	Kashmir	Kashmir, disturbed site, India	1580
Mr-0	Monte	Monte/Tosso, Italy	1000-1500
Mt-0	Martuba	Martuba/Cyrenaika, Libya	100-200
Old-2	Oldenburg	Oldenburg, bot.gardens, Germany	1-100
Ron-0	Ronda	Ronda, limestones, Spain	1000
Sf-2	San Feliu	San Feliu, Spain	1-100
Sthlm	Stockholm	Stockholm, Sweden	0-100
UK	United Kingdon	United Kingdom	
Van-0	Vancouver	University of British Columbia, field border, Canada	1-100
Ws2	Wassilewskija	Karma, Belarus	100-200

The field

Many of the experiments performed in this thesis have been done under semi-natural conditions. To detect true effects of a protein, especially if involved in photosynthesis, the plants should be exposed to more natural conditions than growth chamber with constant light, water and nutrients. By studying annual plants under semi-natural conditions, also survival and

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plant fitness can be investigated. Paper I describes the field conditions and handling of the plants in a detailed way.

Our field is conveniently situated within 100m of the lab and 300m of a weather station (www.tfe.umu.se/weather). Summer in Umeå is short but the lifetime of *Arabidopsis* is shorter. If *Arabidopsis* would grow naturally in Umeå (it has a wide distribution in Sweden except Umeå) it would probably overwinter as a leaf rosette because it is a winter annual. To study *Arabidopsis* under natural conditions we probably should have germinated the seeds in autumn and studied their true survival and behavior the following summer. However our permit does not allow it and keeping track of the plants under a snow cover is probably difficult. Our field experiments are described as semi-natural because of the growing method, the extra water we supply, and the fact that they are grown in separate pots to control competition.

Figure 6. Picture of *Arabidopsis* plants in the field, oePsbS, wildtype and *npq4*.

Plant to plant variation is usually larger when performing experiments outdoors, for which must be compensated with larger sample sizes. Over the years we have not only obtained different results from year to year but also experienced several unpredictable events. These things might frighten many plant scientists working in the lab but these are typically everyday situations faced by ecologists, and there are ways to handle the variation. After all, most of what we find in biology must have a relevant interpretation in nature. A minor difference of something may show there is a small change but what is the true impact of the data and the biological effect? Will or could this really have meant something for evolution?
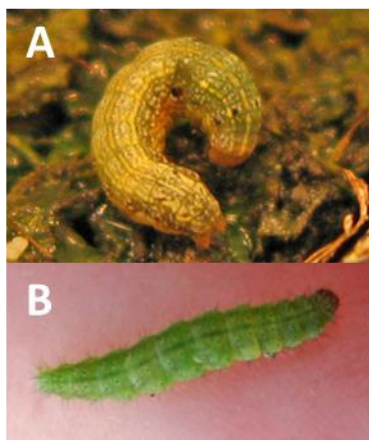
Herbivory

Plants in the field experience many different biotic stresses. Herbivory is one such stress and the one we have focused on. Herbivory is defined as consumption of plants by an insect or larger animal. Bacteria, fungi and other organisms can also consume or affect plant material but that is not considered as herbivory. Herbivores can be specialized on different parts of the plants (like only the nectar) or specialized on one or different plant species, those are called specialist herbivores. Herbivores that are unselective in what they are eating as long as it tastes good are called generalists. A generalist herbivore has the food preference whereby the more energy/tastiness/sugars the plant contains, but the less chemical defense it contains, the better. In contrast, specialist herbivores have learnt to recognize and overcome the specific defense compounds of their target plant (-s) and therefore can feed on these plants without the risk of getting poisoned.

In our experiments we used the generalist herbivore *Spodoptera littoralis* (Egyptian cotton worm) and the specialist herbivore *Plutella xylostella* (Diamond-back moth) (Fig 7). *Plutella* is a specialist on plants in the *Brassicaceae* (Sun et al., 2010) to which *Arabidopsis*, and also economically important plants like broccoli and cauliflower, belongs (cabbage-like plants). The most abundant chemical defenses in this plant family are the glucosinolates (mustard oils) (Fahey et al., 2001, Ratzka et al., 2002).

Plant defense

To defend themselves against herbivores plants have many different strategies. These include, for example, thorns, hairs, thicker leaves, the use of mimicry or chemical defenses. The chemical defense of a plant is usually based either on carbon or nitrogen and belongs to one of the three main groups; nitrogen compounds (N), terpenoids (C) or phenolics (C). All these compounds are secondary metabolites which have different effects on the herbivores and can either damage their intestines by inhibition or activation of enzymes, slow down digestion, or some other destructive action.

The chemical defense of a plant can be constitutive or induced (Kessler and Baldwin 2002). Since there is a nutritional cost for the plant to produce these compounds both types have their advantages. If constitutive, the plant is always protected but on the other hand if the herbivores (or pathogens) do not appear it is waste of energy. An induced chemical defense is more dynamic and uses energy for protection when needed, but will not be able to protect the plant until a short time after the start of the attack. Baldwin (1998) demonstrated this with the seed production of *Nicotiana attenuata* grown under different herbivore pressure with or without methyl jasmonate (MeJA). Induced plants in the control environment produced about 20% less seeds; but also shows the advantage in the presence of herbivory because only 1% of the induced plants compared to 33% of the control plants were grazed within the first week. The importance of JA for insect defense has also been shown in *Arabidopsis* (McConn et al., 1997), however MeJA is only an inducer and the chemical defense in *Arabidopsis* is by glucosinolates (Ratzka et al., 2002).

Figure 7. Larvae of the A) generalist (*Spodoptera*) and B) specialist (*Plutella*) herbivore.

Glucosinolates

Glucosinolates (GS) are sulfur-nitrogen, secondary metabolite products that upon contact with myrosinase — through hydrolysis — form toxic defense compounds (Halkier and Gershenzon 2006). These compounds are mainly nitrile, thiocyanate and isothiocyanate (Wittstock et al., 2003), the last one is the major hydrolysis product in Columbia wildtype plants (Mumm et al., 2008). Exactly how the toxic products act is not known but *in vitro* isothiocyanates can react with proteins and cleave disulfide bonds (Wittstock et al., 2003) which cause a problem in the insect gut. The GS occur as a naturally defense in the *Brassica* family. Glucosinolates can be divided in two subgroups; aliphatic and indolic GS, indolic GS are the ones mainly induced upon stress (Textor and Gershenzon 2008). The highest concentration of GS can be found in young leaves and seeds/siliques and the lowest in senescing leaves (Wittstock et al., 2003), which mirror the different tissues values to the plant. The distribution of GS within a leaf also

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varies. More GS can be found in the edges and at the veins, but less in the "middle" of the leaf (Shroff et al., 2008 et al. PNAS 2008).

Kliebenstein et al. (2001) investigated 39 different ecotypes of *Arabidopsis thaliana* and out of these they identified 34 different GS in the seeds, 22 of these types of GS were also present in the leaf. Since many of the GS only were found in one of the ecotypes and small peaks were not analyzed the authors believe that even more types of GS may be found in *Arabidopsis*. The many different hydrolysis products of GS in *Arabidopsis* also vary in type and abundance between different ecotypes (Wittstock et al., 2003). For the ecotype Columbia, three common GS (4MSOB, I3M, 8MSOO) have been reported to be the major compounds and together they respond to >75% of total GS (Shroff et al., 2008). To overcome this toxic product *Plutella* larvae have a GS sulfatase in their gut. Before the myrosinase has the chance to hydrolyze GS, the sulfatase does it and the GS derivates are no longer substrates to the myrosinase. The formation of toxic GS product is thus prevented (Wittstock et al., 2003, Ratzka et al., 2002).

Glucosinolates are a common defense strategy for many plants; however these compounds can also attract herbivores. In intact leaves indolic GS have been shown to work as oviposition "markers" for *Plutella* (Sun et al., 2010). Another experiment with a specialist herbivore (*Pieris rapae*) showed that the herbivore was attracted to oviposit on plants containing isothiocyanates (Mumm et al., 2008). These data show an evolutionary result of the specialist herbivore and host interaction, which of course is a drawback to the plant.

Stresses are often combined
In nature plants experience many different abiotic and biotic stresses at the same time. Sun, wind, herbivores, pathogens and competition from neighboring plants are some they continually have to face. Most experiments investigate the plant's reaction to one of those, some to two, but there are far too few plant experiments investigating the whole range of these stresses, which also is an important key to fully understand the plant and its functions.

Recently Schenke et al. (2011) investigated the crosstalk of pathogen and UV defense responses in *Arabidopsis*. The data they got indicate that the plants saved resources from flavonol production and used them for the

more urgent pathogen defense. In several other experiments — and with different species — drought has been shown to reduce the resistance to pathogens (Fujita et al., 2006). Regarding this result the authors also underline the fact that pathogens usually need humid conditions and therefore it makes sense to adjust between these two defense pathways. Further on it has been shown that many photosynthesis related genes are down-regulated during both abiotic and biotic stresses (Narusaka et al., 2004) and Muhlenbock et al., 2008 investigated the effect of excess excitation energy (EEE) and systemic acquired acclimation and found a crosstalk between these processes. The results of this crosstalk are very interesting and give us a broader understanding of the processes in a plant, and combined interactions.

In signaling crosstalk many hormones are usually involved but Abscisic acid (ABA) has been shown to have a particular role in crosstalk between abiotic and biotic stress responses (Fujita et al., 2006). This interaction of ABA is an indication that water stress is the most severe and threatening stress to the plant and has to be prioritized. In the work presented in this thesis we have been most interested in the hormone jasmonic acid (JA) since it is involved in the herbivore defense pathway but also made one experiment with salicylic acid (SA) that is involved in pathogen response (Kessler and Baldwin 2002). Both JA and SA are signaling molecules and an increase in one of those is usually the first response when a plant is exposed to herbivory or infections. JA has been shown to decrease fitness in *Arabidopsis* (Cipollini 2002, Baldwin 1998) but it has also been shown that upon herbivore attack the induction of a defense through JA can lead to increased fitness in for example wild radish *Raphanus sativus* (Agrawal 1998) and *Arabidopsis* (Baldwin 1998).

Proteins, metabolites and genes

Biochemical reactions and processes make you and me as well as *Arabidopsis* plants functional. It is the degree of action, response and flexibility in these processes that makes the plant acclimate and adapt to changes in environment and life conditions (plasticity). Acclimation can be seen as changes in e.g. the transcriptional, translational and metabolic level. Both primary and secondary responses can be studied by studying these different biochemical changes.

Changes in metabolite content are a very fast response towards changed conditions. Metabolites are very small compared to proteins and can be found anywhere in the cell. Metabolic responses can therefore be very quick and within an hour make a lot of changes in the plant metabolome (paper IV). Some metabolites involved in the Calvin-Benson cycle even turnover within a second (Stitt and Fernie 2003). Compared to metabolites, gene regulation can be considered slow. Micro arrays can be run to detect differences in the total transcriptome. It has been very helpful to study the mRNA changes but a changed level of mRNA must be interpreted with care. A decreased amount of specific mRNA might indicate that the plant does not need this protein as well as if the plant just overproduced this protein. The biological interpretation is very different in these two statements. Interpretation of metabolomics data is similar since it is all about relative values; a decreased amount of a metabolite does not say if the plant has little of this specific metabolite or very much of the one it is compared to.

For both metabolomics and transcriptomics the amount of data is huge and very complex, making univariate statistical methods inadequate, hence multivariate analysis of the data is advisable. In this thesis principal component analysis (PCA) and partial least square discriminant analysis (PLS-DA) has mainly been used. A PCA describes the anonymous data set and gives the best summary of the dataset's beaviour over time; the software modulates with all data points and finds the most descriptive components of the data. These components have a value between 0 and 1 where 1 is a perfect model. $R2$ describes the variation in the dataset and $Q2$ how well how well the model can predict new samples. When analyzing data with a PLS model every sample has to be given a group identity. The PLS model will identify which metabolites which are important to describe the separation between the groups.

Proteins are constantly synthesised but changes in protein levels are usually slow. When the conditions change the plant has to sense that and change, up-regulate the level of mRNA and translate it into proteins. For that, also sufficient amounts of nucleotides and amino acids must be accessible. Most of the proteins also need to be transported into their place and folded to be functional. Degradation of proteins are much faster; the protein is tagged by a degradation protein called ubiquitin and the tagged proteins are then found by proteases and degraded (Taiz and Zeiger 2002). Amino acids from degraded proteins are often recycled and used for new proteins. Unnecessary synthesis and degradation of proteins is costly for the plant

and therefore a change in protein content is not only a more slow and complex process but also tightly regulated (Buchanan et al., 2000).

Because of these changes the plant can — more or less — easily acclimate to new conditions, it is costly but possible and a question of survival. In this thesis mRNA and protein level as well as metabolites have been investigated in the search for an answer of how plants with varying levels of NPQ survive and acclimate under semi-natural conditions.

Background to aim

It was in the summer of 2000 that a former PhD student (Carsten Külheim) noticed a genotypic difference in seed outcome depending on herbivory. Plants in some, but not all, trays were quite heavily grazed by herbivores and if separating the data according to trays with and without herbivory a different result was observed. Under semi-natural conditions *npq4* plants had a lower amount of seeds and therefore seemed to be less fit than the wildtype. But under the pressure of herbivores they produced as many seeds as the wildtype (Paper II, Fig 6). Since PsbS obviously is beneficial for the plant (Kulheim et al., 2002) we have asked the question why they do not have more of this protein. This question, in combination with the data where plants lacking PsbS did better under herbivory, gave a hint of a possible answer and had to be investigated.

Aim

The general aim of this thesis was to understand how light and herbivore stress might be connected and more specificly the effect of different levels of PsbS and variation in NPQ. All work was done with the model plant *Arabidopsis thaliana* and many experiments were performed in the field, which means high and variable light as well as other abiotic and biotic influences. Two main questions were asked to investigate the light and herbivore interaction:

How are herbivores affected by the level of PsbS and what might be the reason for this?

What happens to plants with varying levels of PsbS during and after field acclimation?

Results and Discussion

Growth, fitness and natural variation in field

Many of the results generated in this thesis come from field studies. Paper I describes how to grow and handle transgenic *Arabidopsis* plants in the field. For growing transgenics we also have a permit where the Swedish board of Agriculture (Jordbruksverket) approves our experiments and the special protection measures we have made for that. In paper I the procedures and regulations are carefully described but in short we use a specially prepared area and cover the plants with a net cage to prevent spreading of eventual pollen with insects.

Another factor we need to take into account is that the light period is very different from the commonly used growth chamber light-dark cycle. During the most sunny days in our field in the northern part of Sweden (Umeå) the sun rise around 2:30 a.m. and set around 11 p.m. giving a 20.5 h light/3.5 h dark cycle. Even when the sun sets it is not pitch black as it would be in a growth chamber, but there is twilight, and we believe the plants do consider it as night. Since we mainly compare plants that are growing side by side in the field the light–dark cycle is usually not a complication. In some studies we do compare plants that are growing in short-day chambers to those in our field but for these studies the purpose is to highlight the differences between the "normal" *Arabidopsis* lab-plant and the more "normally growing" *Arabidopsis* plant out in the field.

Growth effects in field

Experimental design is always important but when preparing for field studies it might even be more important, since it will never be possible to repeat it exactly the same again. The size of the plants when they are transferred into the field will affect the outcome (Paper I, Fig 2), growth, and amount of seeds in this case. If the plant is bigger it will have higher photosynthetic capacity and thereby energy production. In general, bigger plants produce more seeds (Fig 8). Therefore it is essential to document the size of plants in the beginning of the experiment so eventual differences can be tracked and explained.
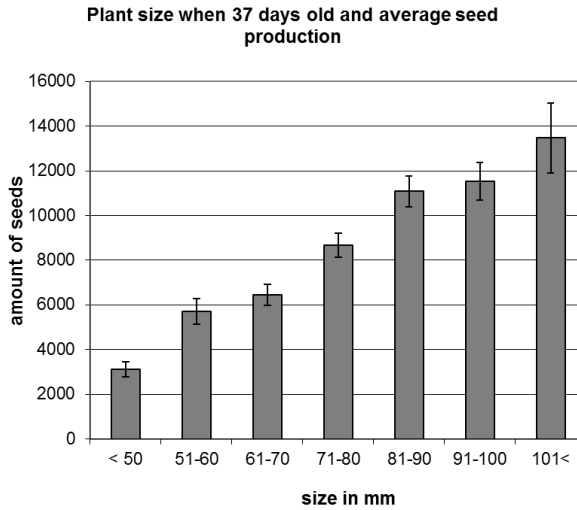
Plant size when 37 days old and average seed production

Figure 8. Plant size and seed production. Plants were 37 days old when size of rosette diameter was measured. Error bars are SE, n=12-86.

Since plants in the field, in contrast to the common lab plant (grown in big, single pots and addition of fertilizer) usually are limited by nutrients instead of light, plant-to-plant competition becomes more expressed and the arrangement of the plants should be very carefully done. If there are plants that have access to more nutrients it will definitely affect the results. Figure 9 shows a direct effect of the soil nutrient availability and plant size.

In paper V we describe the most prominent differences when comparing *Arabidopsis* plants that had been grown in a chamber under standard short day (SD) conditions to those that had been growing in our field. Again we focus on important factors for photosynthesis (growth/size, proteins, chlorophyll and photosynthetic measurements) and how in *Arabidopsis* wildtype they respond to different light intensities and under field conditions. Not surprisingly, plants in a chamber grew bigger before flowering than field plants did, but also leaf shape was different (Paper V, Fig 2 and 3). Field plants had more rounded leaves (Paper V, Fig 2) and the area of those was only a sixth of the area of chamber grown plants (Paper V, Fig 3). Also changes were seen on xanthophylls, chl a/b ratio, abundance of Lhca5 and early-light-induced proteins (ELIP). *Arabidopsis* field plants had more of the ELIPs (Paper V, Fig 7), more xanthophylls (Paper V, Table 1), a higher chl a/b ratio (Paper V, Fig 4) but lower amount of Lhca5 (Paper V, Fig 5) as compared to all the chamber plants (they were grown under 30, 300 and 600 µE).

Figure 9. Importance of plant arrangement. *Arabidopsis* plants at same age and under same conditions except for tray-distribution, where one tray holds 30 plants and the other only 10 plants.

These data are important for interpretation of results generated from chamber-grown *Arabidopsis* plants. Finding more of the protein ELIP and xanthophylls is not surprising since field plants usually experience light stress and that increases the amount of ELIP (Norén et al., 2003) and xanthophylls (Demmig-Adams and Adams 1992). Finding a decreased amount of Lhca5 however, is a bit surprising and may tell us some about the function of Lhca5; together with Lhca6, Lhca5 has recently been suggested to be involved in cyclic electron transport (Peng et al., 2009). The higher chl a/b ratio was also expected and correlates well with both light intensity (Paper V, Fig 4) and the amount of Lhcb1 and Lhcb2 proteins (Paper V, Fig 6) since they bind more chl b than Lhca proteins does.

Slugs, moth larvae and aphids usually find our field and the experimental plants at some time during the summer. They have different feeding techniques and feed on different parts of the plant. The most common herbivore in our field is the Diamond-back moth (*Plutella xylostella*), which we also have been rearing in the lab. For several years we have also been scoring the amount of herbivore damage in field but it is always difficult to measure what is not there (the missing leaf or parts of a leaf). The scoring data shows, however, that the herbivores tend to avoid eating on the *npq4* plants (Paper II, Fig 7), which also was the case in the cafeteria experiments (Paper III, Fig 1).

When measuring the growth of the three genotypes (oePsbS, wt and *npq4*) no difference in growth was found (Fig 10). However, Logan et al. (2008) and Krah and Logan (2010) have also measured the size of the same genotypes and they found *npq4* plants to be smaller because of retarded growth in the later developmental stages. The difference they found was pronounced after six weeks and experiments were performed in a greenhouse but with the influence of natural light. Since our growth measurements reaches over a longer period of time (7 weeks), the plants are bigger (probably because of longer days – 18 h light compared to about 15 h) and the plants were grown under semi-natural conditions, we believe there is no difference in growth between these three genotypes under our growth conditions.
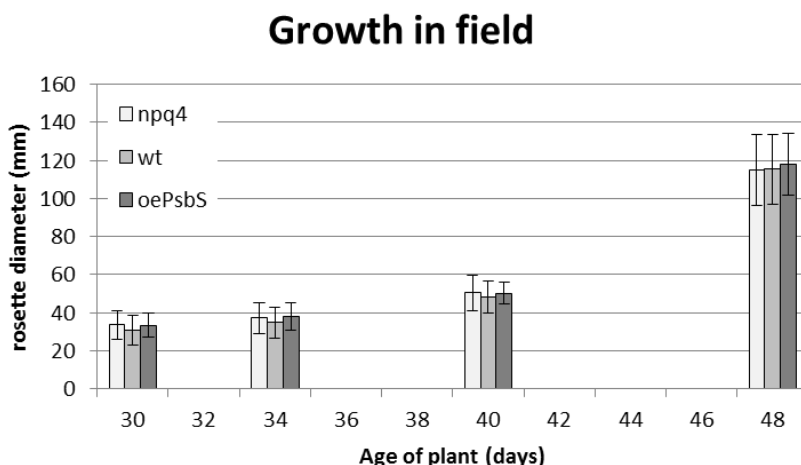
Growth in field

Figure 10. Growth in field. The growth of *Arabidopsis* plants (oePsbS, wt and *npq4*) when transferred to field.

Fitness studies

Fitness can relatively easily be measured in annual plants —like *Arabidopsis*— because they produce all their offspring at once. Total amount of seeds and their germination rate is a simple relative estimation of the Darwinian fitness of the plant. Other factors such as the growth rate of the offspring and their ability to reproduce will affect the fitness but quantification of this is very laborious so we only quantify seed production and germination rate. Our control experiments have shown that despite the relatively high variation between field samples, the seed averages are still reliable (Paper I, Fig 5). It is however also of importance to grow and compare to a wildtype of the same ecotype (Paper I, Fig 5).

We have been measuring seed fitness of plants grown under semi-natural conditions for many years and as seen in figure 11 the variation of production is enormous between and within years. In the "best" years the plants have produced several tens of thousands of seeds but in the "worst" years only hundreds. Some variation is due to heavy herbivory and the size of plants when they were transferred to the field but most variation is probably due to variation in the weather conditions, especially during the first week in field. It is amazing to see the huge year-to-year variation and also some individual performance differences (like a few centimeter high plant that still produces a few siliques), which really shows true survival and the purpose of life: reproduction. A balance between nutrients, light energy and competition obviously determines reproduction efficiency in the end.
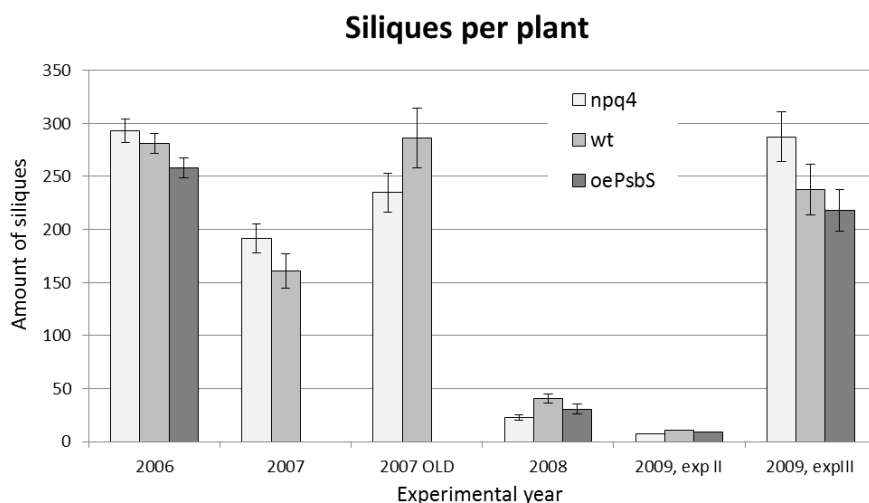
Siliques per plant

Figure 11. Year to year variation of silique production. Average amount of siliques produced by the three genotypes, during six different field experiments. Error bars are SE, n>15.

Natural variation, comparisons and weather

Arabidopsis is a natural inbreeder and the variation between two *Arabidopsis* plants of the same ecotype is very low. However, one summer the field studies showed a plant phenotype that seemed to be lab-specific. A few days after transfer of all plants to the field, the plants from another lab showed a more reddish color while our plants remained mainly green (Fig 12). This is not common and we do not have an explanation for this but it is an interesting observation. We have also compared our wt plants with wt from other labs without differences (see for example Paper I, Fig 5).

One factor that has, unsurprisingly been shown to be very important during field studies is the weather. Since weather is a name summarizing many different parameters itself, for example temperature, rain, humidity, wind and light intensity it is hard to make a clear correlation to it. Although for some experiments we have correlated the data with light intensity since most of the time light intensity reflects temperature and rain as well; and is also an important factor for plant growth.
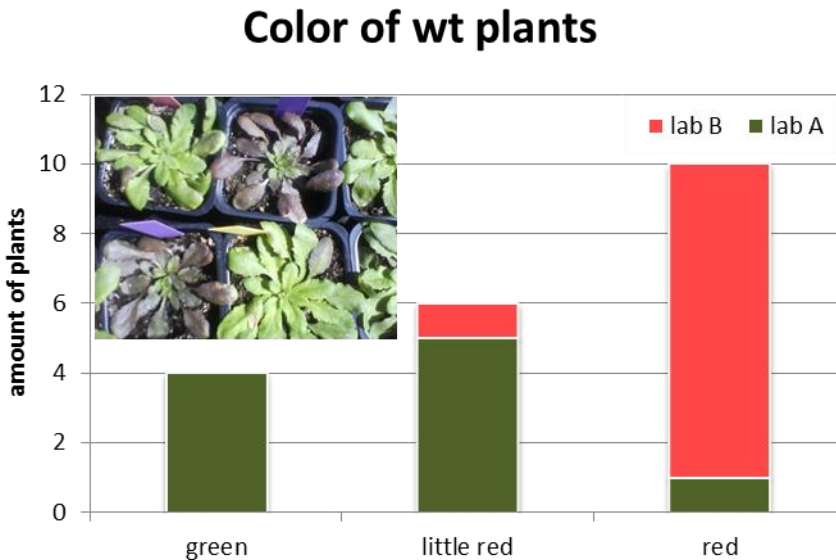

Figure 12. Occasional lab effect on plants. Color of *Arabidopsis* wt plant rosettes a few days after transfer to the field. Only difference is origin of seeds.

NPQ is dynamic

In 2009 Jung and Niyogi (2009) published a paper where they quantified NPQ in 62 different *Arabidopsis* ecotypes —grown under constant conditions— and the result showed a wide natural variation of NPQ capacity between the ecotypes. To compare plants with high and low NPQ we selected and ordered about ten ecotypes with the highest and lowest NPQ from the Nottingham Arabidopsis Stock Center (NASC). Together with some local wt-*Arabidopsis* collections and the *npq4* and oePsbS we repeated the experiment in both growth chambers and acclimated to our semi-natural conditions to further investigate the natural variation in NPQ capacity.

Maximum vs. "stand-by" NPQ

Arabidopsis has a very wide distribution and has been adapted for many different environments. The question is; how well prepared for light stress are plants that grow their whole life under constant and favorable conditions?

When we measure NPQ on plants from constant growth conditions we probably measure the "stand-by" capacity for NPQ. But plants in the field need to be active in photoprotection and values closer to their maximum capacity for NPQ can be measured. Data generated from the field in the summer of 2009 were compared to those of the growth chamber plants and major differences were found. As seen in Paper V, Figure 10B when the plants are grown in a chamber the measured values are more stable and similar between —and within— the ecotypes as compared to data from plants in the field (Paper V, Figure 10A). This might be an effect of the varying conditions outdoors, during the days of the measurements the light intensity, temperature and rain varied but most of the days were warm and sunny. Overall there seem to be no correlation between the NPQ-capacity of plants grown under constant light conditions and the NPQ capacity for the same ecotypes grown under semi-natural conditions (Fig 13), a surprising result.

Different ecotypes have different adaptations to NPQ

All ecotypes used in this study are listed in table 3 together with their country of origin and habitat. Most of the ecotypes look quite similar to each other but in Figure 14 four of the most extreme ecotypes can be seen. It is also known that they have different flowering time, probably to some extent because of the different latitudes and habitats they come from but mainly as a result of wintergreen or late summer flowering characteristics (Riihimäki et al., 2005). Here we investigate how the adaptation has affected NPQ capacity.

What can be seen in the scatter plot (Fig 13) of the fluorescence data is a wider span of NPQ capacity for field plants than chamber plants. All measurements are relative so if ecotype 1 is increased or ecotype 2 is decreased in NPQ capacity is not possible to say, only how they change relative to each other, and compared to the extremes (oePsbS and *npq4*).
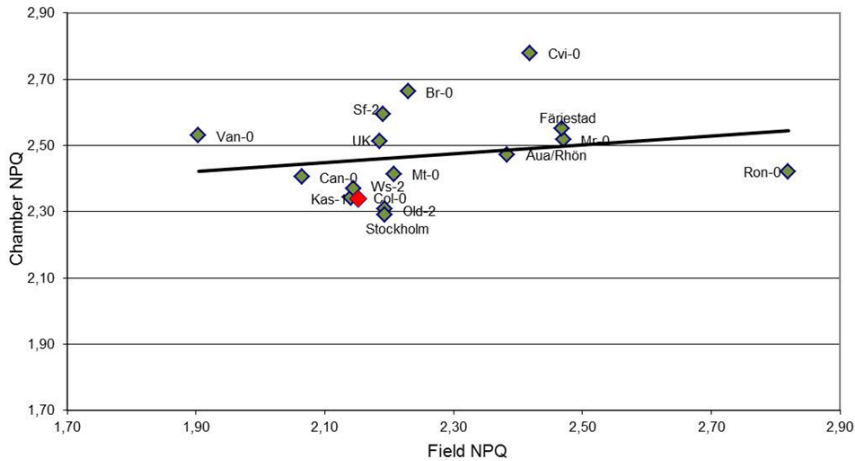
Figure 13. NPQ capacity of different ecotypes in the field and in the chamber. No correlation for the NPQ capacity under these two conditions could be found.
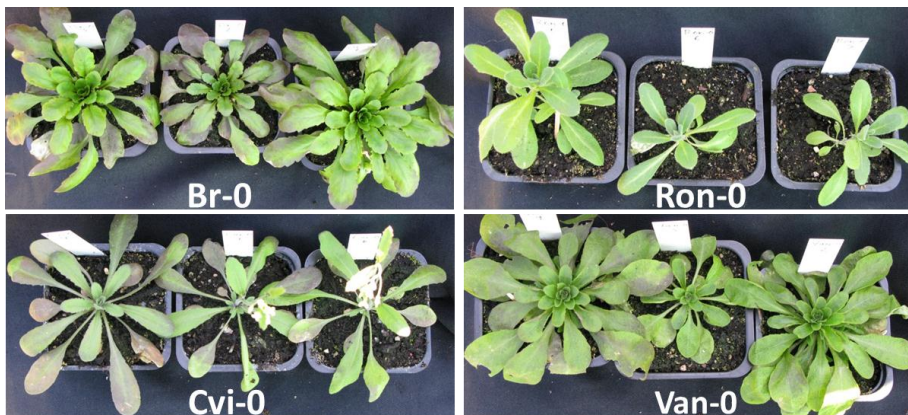
Figure 14. Four of the more extreme phenotypes among the ecotype plants. Pictures are taken one week after transfer to the field.

One of the most puzzling ecotypes is Ron-0. It was selected because it had the second lowest NPQ capacity in the Jung and Niyogi (2009) paper. In our field however, Ron-0 turns out to have a NPQ-capacity similar to oePsbS; and under our chamber conditions Ron-0 gives a little bit of a weird but relatively average response of NPQ capacity. Ron-0 could easily be separated from the other ecotypes because of its different appearance (Fig 14); it has small, thick, hairy leaves that do not grow flat to the ground but tilt upwards – one above the other. Ron-0 comes from a limestone habitat

in Spain and was collected at about 1000m above sea level. This is probably a very exposed and rather harsh environment to live in and could be the reason for the ecotype's different appearance and good NPQ capacity. But still it does not explain why Ron-0 had a very low NPQ capacity in the growth chamber.

Compared with the performance of the other ecotypes Br-0 has a relatively better NPQ "stand-by" than "maximum" capacity. One probably needs to know more about the background of Br-0 to understand its NPQ capacity and special appearance (Fig 14) but a guess would be that it comes from a shadowed part of a forest edge or field where it is not exposed to very high light intensities. Sf-2 and Van-0 are ecotypes that had a high NPQ capacity in chamber conditions but low in the field; the ecotype Van-0 was one of the lowest in field and had also minor changes in relative capacity between our chamber experiment and Jung et al. The Swedish ecotype Stockholm had an opposite performance with almost the lowest NPQ capacity in chamber but a relatively average capacity in field. Cvi-0 is collected at the Cape Verde Islands, has narrow leaves and is early flowering (Fig 14), the NPQ capacity both in our chamber and in field was high but when chosen for the experiment it was because it was rated as one of the lowest in the Jung-paper (Jung and Niyogi 2009).

The results from NPQ capacity under different conditions are suitable to answer the question of plant adaptation and acclimation to different environments. This also reflects that there is a difference in so called "stand-by" and maximum or activated NPQ.

Performance of extremes

The genotypes I have mainly been working with are extremes regarding qE due to mutation or transgenic modification. This is also the reason we wanted to investigate the natural variation for this capacity. The overexpressers (oePsbS) have much more of the protein PsbS (Li et al., 2002a) and almost two times (1.71) higher NPQ than the wt. The mutants *npq4* have no PsbS protein and 1/5 (0.21) of the NPQ capacity compared to the wt (Paper V, Fig 10). From the experiments with all the ecotypes the natural variation of NPQ capacity seems to be about 20 % between the highest capacity (Cvi-0/Ron-0) and the lowest capacity (Sthlm/Can-0).

As seen in Paper V, Figure 10, the difference in NPQ between all the wildtypes and oePsbS is bigger in the chamber than in the field. In one

experiment, half of the plants, after fluorescence measurement, were transferred to the field and then all plants were measured again 5 days later. The data from this experiment show oePsbS may even decrease the capacity of NPQ (and qE) after transfer to field (Fig 15), measured in the standard way. These are surprising results but after more analysis we suggest a possible reason for this; oePsbS in the field have a decreased F_m. A decreased F_m can mean the plants are a little bit more light stressed and therefore fluoresce less. If normalizing the data to F_0, the fluorescence curve of oePsbS outdoors was slightly lower than that of oePsbS chamber plants (fluorescence after 18 min: 1.04 +/- 0.023 in the field and 1.27 +/- 0.028 in the chamber), which indicates an increased capacity to NPQ. For wildtype the capacity of NPQ is increased after field transfer both before and after corrections for the lower F_m value. The increase is not large (about 20%, similar to the natural variation we found in the different ecotypes) but significant, interesting, and probably an acclimation response. We have not made shift experiments with *npq4* but if compared to the wt of chamber and field plants the relative value of NPQ follows the same pattern as wt. I believe the 20% natural variation in NPQ capacity is an adaptation to different environments, yet *npq4* plants actually grow well in field (Fig 10) and can induce qE in the longer perspective (Johnson and Ruban 2009).

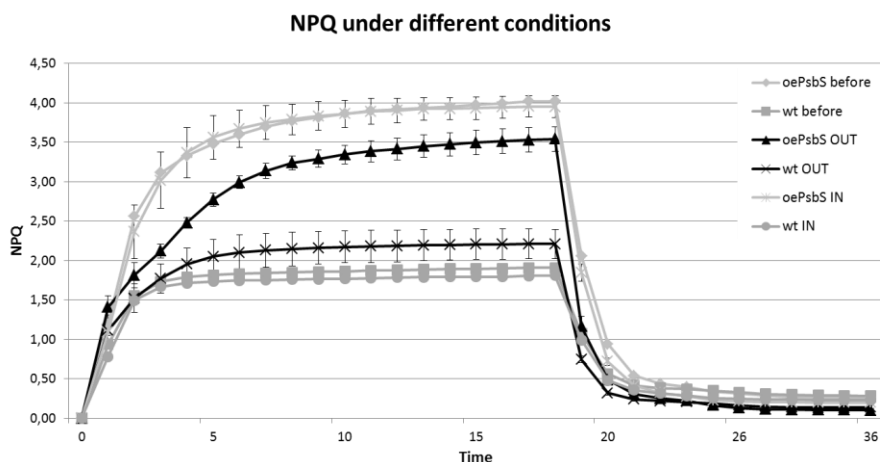**Figure 15.** Changes in NPQ capacity when transferred to the field. Plants were measured both before transfer and after five days in field (OUT) as well as in chamber (IN). Error bars are SD, n=5 for field plants and n=3 for chamber plants.

Field and NPQ effects of plant metabolites

Because I have been working a lot with field plants throughout my PhD studies we have also made efforts to characterize the differences between

"normal" lab plants and "naturally" growing field plants. This has been done mainly for wt but also partly for the three genotypes varying in amount of PsbS. In Paper V we describe some photosynthetically important factors (proteins, pigments and photosynthetic measurements) and how they in *Arabidopsis* wildtype respond to different light intensities and field conditions. Not surprisingly the results clearly indicate that lab and field environments demand different qualities from the plant. In addition to this we analyzed the metabolome of these plants and of a set of plants that were grown in a chamber under SD and then transferred to the field.

Taken together the data analyses of the wt metabolomics data show a huge variation over time when transferred to the field (Paper IV, Fig 4) and the metabolites in the chamber plants clearly correlate with light intensity (Paper VI, 1). Results from the principal component analysis (PCA) of the transferred plants indicate a quick and strong metabolic response towards the changed conditions and also reveal a slower phase — probably the start of acclimation— that occurs after the first field-day. Primarily the analysis of the plants shows an increasing abundance of amino acids and secondarily an increase in sugars.

Between two and four hours after transfer to the field the light intensity and temperature dropped (by ca 5°C and the light intensity decreased by a half) and 7 mm of rain fell. In the PCA plot (PaperIV, Fig 4) the 4h-group of samples takes a "step" back in the first component axis, towards the 1h-group of plants and most of the metabolites showed a reduction in abundance. This illustrates the profound importance weather could have on plant metabolism.

Sugar and amino acid increase under higher (and lower) light intensity

Sugars of plants grown under different light intensities in the chamber had a positive correlation with light intensity. After transfer to the field the total amount of identified sugars (fructose, glucose, glucose 1,6-anhydro beta and sucrose) in the plants also steadily increased (Paper IV, Table 2). These increases are probably a result of more light energy. In the field fructose had the highest increase and was over nine times more abundant on the fourth day in field. A circadian pattern in sugar fluctuations can also be seen, especially in fructose.

The amino acid glycine is the metabolite that gives the first and strongest response in plants transferred to the field; it increased fourteen-fold during the first hour and over thirty-fold during the second field day. This is probably due to its involvement in the photorespiratory pathway, which can be expected to increase as an effect of more light energy (Foyer et al., 2009). It is also possible that plants transferred from the growth chamber close their stomata. Closure of stomata will not release the oxygen produced in photosynthesis and the O_2/CO_2 ratio will increase, which also leads to more photorespiration (Raven et al., 1999).

Many other amino acids increased after transfer to the field (Paper IV, Fig 5 and Table 2) but also in low light (Paper IV, Fig 2 and Table 1). These results were initially surprising but there are many processes in a plant to take into account. The carbohydrate and amino acid increase seems to be the first metabolic response and we believe this is due to an increase in the carbon fixation in the presence of more light energy. But why should plants grown under low light have an increase in amino acids? We interpret our results that plants grown under lower light intensities (30µE in this case) but in nutrient rich soil, might have a different carbon—nitrogen balance. The rate of carbon fixation, and thus plant growth, is not high enough relative to the availability of nutrients. Probably this is a reason to store the valuable, fixed nitrogen as amino acids while waiting for more carbon.

An increase in both amino acids and carbohydrates is a positive signal for healthy plants that will grow well, however, under long days (LD) and field conditions plants are usually smaller. Is this just a faster energy and nutrient turnover? We know that *Arabidopsis* plants under high light develop faster (paper V) but why does faster development lead to decreased plant size? Is growing bigger, the normal lab plant's way to compensate for the lower energy income because of lower light and shorter days? In that case none of the lab plants live under very "natural" conditions when grown in too low (what is called normal) light and under too short light time periods.

Other metabolites and their behavior during field acclimation
From the metabolomics data set with plants transferred to field we studied 31 identified metabolites. Out of these 10 metabolites are amino acids, 4 are carbohydrates, 3 belong to the tricarboxylic acid (TCA) cycle, 8 are fatty acids and 6 metabolites we categorized as "others". None of the TCA cycle, fatty acids and "other" metabolites showed any specific or spectacular

results. Two out of the three identified metabolites in the TCA cycle were increased after only one hour in the field, probably as a response to increased light energy and therefore carbon fixation.

Beside all the known metabolites that we identified from this data set there are also five defined peaks that seem to be of interest. Two of the peaks are within a short distance of each other and vary in the same way; they will be called "the double-peak" (Fig 16). The metabolite behind the double-peak is clearly involved in acclimation and therefore may be involved in for example anthocyanin production. The other three unknown metabolites are present in low concentration from the start but 6 h after transfer to field they increase a lot – only with a varying amount. This increase also occurs at the termination of experiment and at 73 h the most extreme metabolite has increased over 20-fold. Apparently these metabolites are highly increased under semi-natural conditions and all of them have been found before (but unfortunately never identified). A metabolite that is highly present in field plants but not so much in chamber-grown plants is difficult to speculate about (since most data comes from plants grown in controlled conditions) and the pattern of continuous increase is not found in any of the known metabolites in our dataset.
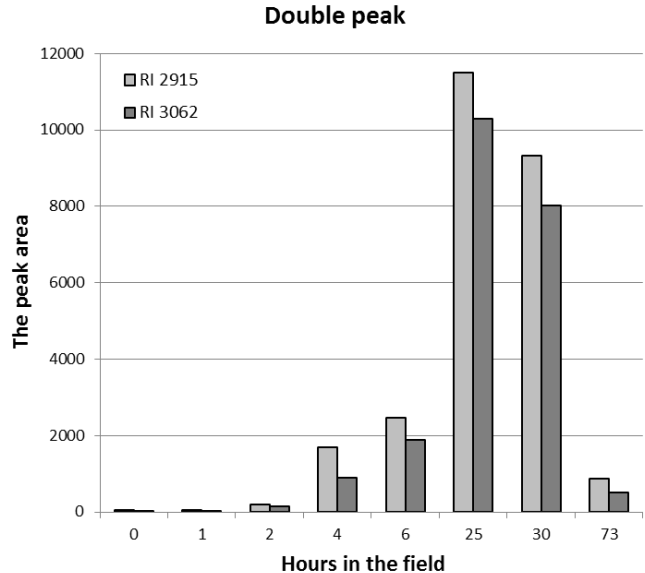

Figure 16. A metabolic double-peak. An unknown metabolite with a high response to field acclimation. Metabolic peaks were found at retention index (RI) 2915 and 3062.

Phenotypic differences of genotypes with a varying level of PsbS

With these field data parameters as a background the phenotype of the three genotypes with different NPQ capacity (oePsbS, wt and *npq4*) has been investigated in many different ways.

Metabolic changes due to NPQ capacity

We have investigated metabolic changes in the three genotypes wt, *npq4* and oePsbS. The metabolomic study of the genotypes in Paper II was conducted with plants in chambers and compared to plants of the same genotypes acclimated to the field for five days. During the field experiment the weather varied a lot, which is also instantly reflected in the metabolites. The most interesting outcome of this study is that the chamber plants do not separate on a genotype basis in the PLS while the field-acclimated plants do (Paper II, Fig 1). Huge differences in amino acids and carbohydrates can explain this separation, *npq4* plants are more enriched in most of the carbohydrates and oePsbS are generally more enriched in amino acids (Paper II, Table 1).

In the summer of 2008 and 2009 metabolomic studies of the selected genotypes were conducted with plants sampled at a series of time points after transfer to the field. Since the second study was more stable in weather conditions and sampling time points the 2009 dataset was selected to be thoroughly analyzed. For both studies the principal component analysis (PCA) of the data shows a good correlation of metabolic variation and time points (Paper III, Fig 5). There is a considerabe metabolic effect of the stress that occurs when the plants are transferred from constant conditions into the field (see more about this in Paper IV) and this outweights the genotypic effect of the plants. At specific time points however this reveals some differences between the genotypes. In Paper III, Table 1, all identified metabolites that at some time point show a significant difference between wt and *npq4* are listed. All metabolites that show a tendency to separate wt and *npq4* are less abundant in the latter both in the chamber and during fourth day in field (73h).

When comparing the experiments from Paper II and Paper III the results differ. In Paper II however, the plants were acclimated to the field for five days and then sampled at a specific day with good weather conditions. In the other experiment the plants were transferred to the field and then sampled regularly independently of weather conditions. Since we know how much the weather influences the results we believe this is the most

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likely explanation of the large differences in the results. It is also surprising that *npq4* and oePsbS partially separate from the beginning (i.e. in controlled conditions) in the time series experiment (Fig 17). In Paper III, Table 1 there are also 6 metabolites shown to be significantly different between wt and *npq4* in contrast to our previous data (Paper II, Fig 1 and data from field 2008) where no differences were observed. Therefore the conclusion from the metabolic studies is that acclimation and weather affect the plants in a pronounced way and relative to this, the genotypic differences are minor.
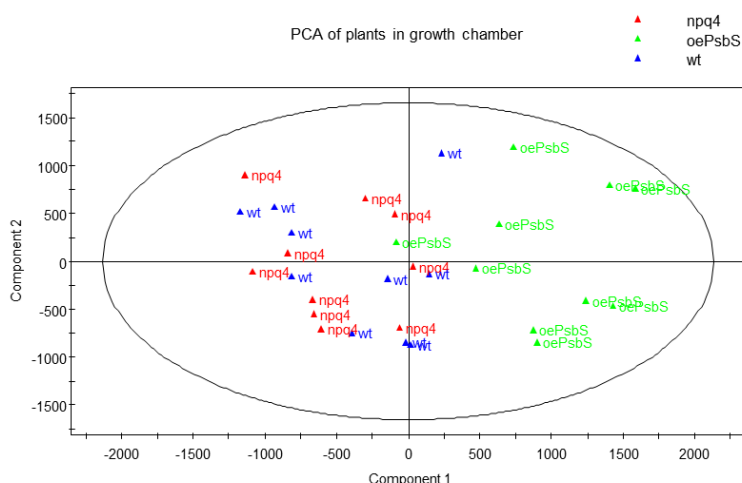

Figure 17. Principal component analysis of metabolites before transfer to the field. R2X[1]=0.281, R2X[2]=0.167.

Up regulation of JA pathway in npq4 plants

Microarray studies of the transcriptome of wt, *npq4* and oePsbS were performed on both chamber and field plants. Not surprisingly the results showed a higher expression of photosynthesis related genes in oePsbS, and ELIP was the only photosynthesis related gene that was induced in *npq4* plants; consistent with the knowledge that this protein is induced by light stress (Norén et al., 2003). We also found an increase in the jasmonic acid (JA) pathway in the *npq4* plants (Paper II, Table 2). To investigate this further we measured the protein profile of two proteins involved in the JA-pathway (LOX-C and AOX), the amount of JA in the leaves and two gene products related to the JA-pathway. The protein profile of LOX-C and AOS showed an increase in *npq4* plants, especially under HL (Paper II, Fig 4). A higher amount of JA was found in grazed plants of *npq4*, compared both with wt and non-grazed plants (Paper II, Fig 5). However, the real-time PCR

analysis of mRNA for genes (COI1 and VSP2) from the JA-pathway did not show any significant differences (data not shown) between the genotypes when light stressed. Taken together none of these results indicate that JA is increased in the light stressed *npq4* plants; nevertheless, both proteins early in the pathway and JA itself seem to increase in *npq4* plants under light or herbivore stress. Therefore we believe it is something upstream of JA, e.g. in the primary metabolism, that is responsible for the transcript-omic (Paper II), metabolomic (Paper II and III), protein (Paper II) and herbivore differences (Paper III) found between the genotypes.

Superoxides are produced at PSII in absence of PsbS

To further explore the metabolic status of the plants we investigated the amount of reactive oxygen species (ROS). In rice (*Oryza sativa L.*) superoxides have been shown to be produced in PsbS-deficient plants under light stress (Zulfugarov et al., submitted). It was also shown that the superoxides did evolve from PSII and not PSI, which is the site of production in wt plants (Scarpeci et al., 2008). Accumulation of superoxide was also found in *npq4* plants (Paper III, Fig 6) and we believe PsbS protects from superoxide production. Also hydrogen peroxide (H_2O_2) was measured and shows a similar pattern in both species but when investigated further the accumulation starts off 2-3 min after the accumulation of superoxides (Zulfugarov et al., submitted and Paper III, Fig 7) and therefore we believe the production of superoxide to be the initial response. Superoxide is easily dismutated into H_2O_2 through superoxide dismutase (SOD). Since the superoxide signals arise in the chloroplast and we also have found changes in the expression of nuclear genes (Paper II), the course of events is a retrograde signaling.

Herbivore preferences are connected to light stress

Plants with more PsbS are preferred by the herbivores

In the dual-choice experiment two herbivore species, *Plutella* and *Spodoptera* got the choice to feed on two plants of different genotypes. All these plants had been light-stressed in the field for some days before the experiment was performed in the lab. The data analysis revealed an overall preference for the plants with more PsbS (Paper III, Fig 1). Both the specialist and the generalist herbivore preferred to feed on these plants, which indicate that plants with more PsbS are tastier. Perhaps this is

because of sugar composition, other metabolites, less reactive oxygen species (ROS), less anthocyanins, or something else. It is also possible that a combination of these factors causes the herbivores to prefer the plants with more PsbS.

During the experiment many of the herbivores had been feeding on both *Arabidopsis* genotypes but finally consumed more of the genotype with more PsbS. This indicates that herbivores can identify something in the taste of the plants and determine between them. We can also speculate that there is induction in a defense pathway that is recognized and therefore rejected by the herbivore. In Paper II we concluded light-stressed *npq4* plants to be primed for other stresses. For example we have shown an up regulation in the jasmonic acid (JA) pathway in *npq4* plants (Paper II) and increased level of JA in *npq4* plants eaten by herbivores (Paper II, Fig 5). However, there is a discrepancy in this particular result; the question is why did the wildtypes that were eaten by herbivores not show an increase in JA?

Natural herbivory in the field was scored, and also resulted in a preference for more photoprotected plants (Paper II, Fig 7). Unfortunately, oePsbS was not included in the experiment. In the field we have a more diverse spectrum of herbivores but the most abundant is *Plutella*. Since both the specialist and generalist herbivores showed the same preference for more photoprotected plants in the dual-choice experiment, the diversity is probably not something we have to consider.

PsbS has no effect on larval growth and survival
To investigate the different genotypes as a qualitative food source, growth and survival of the generalist and specialist herbivore raised on plants of the three different genotypes (oePsbS, wt and *npq4*) was studied in the field. Neither the larval weight nor the survival rate was altered for either of the two herbivores on any of the three genotypes.

The *Spodoptera* experiment was performed three times during the same summer with very different effects on the plants; however no clear genotypic differences could be seen for any of the experiments. During the second experiment the weather was the warmest (most days above 20 degrees which is preferred by the larvae) and two —instead of one as in the other experiments— larvae were placed on each plant. The plants turned red and some plants were completely eaten by the end of the experiment

(Fig 18). The effect on the larvae however was not as pronounced but had high variation; in most cases one of the larvae and in some cases both larvae died during the experiment. Two larvae that survived on the same plant could vary a lot in weight and the bigger larva could weigh up to twice as much as the smaller one. Even though this could be a result of the competition between the larvae, or something else, I think it clearly shows that the variation between larvae is larger than the effect of food quality. A similar experiment was conducted for *Plutella* larvae with a similar result. In that experiment many larvae were competing for the same plants, which also may have affected the outcome of the experiment.
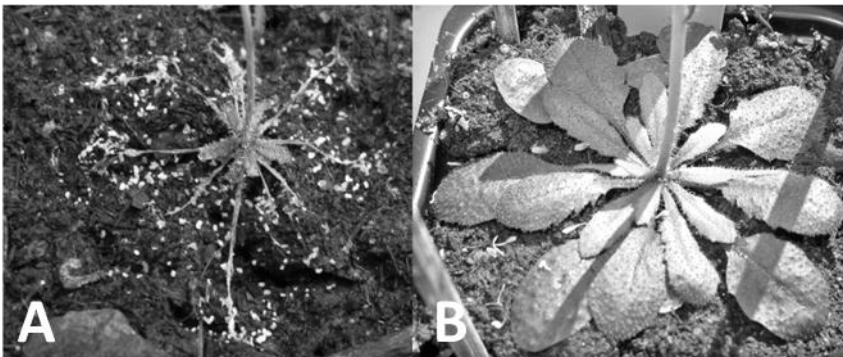

Figure 18. Plants from *Spodoptera* and glucosinolate experiment. A) Picture of a plant with larvae and B) control plant, when harvested after 3 weeks in field.

...but effects Plutella during ovipositoning

Specialist herbivores like *Plutella* usually recognize one or several of the plant's special defense compounds (Sun et al., 2010) which they use in order to track these plants. Since we believe the amount of NPQ to be connected with the defense against herbivory we also quantified the performance during ovipositioning of the specialist herbivore. We found a preference for oviposition on *npq4* plants (more light-stressed plants). On wt and oePsbS the percentage of eggs laid was below 30, but on *npq4* about 45%. This result indicates that *Plutella* probably could sense and differenciate between the genotypes, which could be connected to the plant's capacity of NPQ. The equal result of wt and oePsbS could be explained by an absence of change in the "signaling substrate" in these two, but an increase in *npq4* plants.

Specialist herbivores may oviposit on a plant they recognize through known compounds due to competition. The newly hatched larvae will probably

have less competition from other herbivores on a plant which has strong defenses. One possible drawback could be the quality of food. Indeed in an experiment by Li et al. (2000a), where larval growth was tested on plants with more or less defense compounds, the growth of the larvae was little decreased for *Plutella* but affected the generalist (*Spodoptera Eridania*) much more significantly. In our experiment (Paper III) we did not see any difference in larval growth as a measure of food quality.

Glucosinolates (GS) are not the key component of recognition
Previous data showed the specialist herbivore to be attracted by *npq4* plants during oviposition (Paper III, Fig 3) and the most common chemical defense in *Arabidopsis* is GS (Ratzka et al., 2002). Therefore we decided to measure the amount and composition of GS. Plant material from the growth and survival experiments with *Spodoptera* was sampled, as well as control plants without herbivores. At the termination of the experiment the plants looked very different and had a varying amount of leaf material remaining (Fig 18). One complication when measuring GS on plants after herbivory is to know which parts are left over; it could be that the remaining plant tissue contains more GS.

Total amount of GS did not differ between the genotypes but a small increase of GS was observed in the herbivore treatment compared to controls. A comparison of all GS (Paper III, Table 1) shows that some GS are increased more than others, especially some of the indolic GS.

In wt the amount of 4MOI3M (4-Methoxy-indol-3-yl-methyl) was induced both by time in the field (i.e. in control plants) and herbivory (Fig 19). The plants were covered by netting to keep the herbivores in place but it is not certain that pathogens could not get in and the results could therefore be influenced by pathogens, even though no visible damage was seen. Another theory is that surrounding plants with herbivores might have induced defense in control plants through volatile signaling (MeJA). Yet another factor to consider is that day 0 (when the first plants were sampled) is the fourth day in field. This means that some of the GS might already have increased, however we were mainly interested in comparing the effect of herbivory on the different genotypes and see if any of the genotypes had an increased defense in field conditions so this is not entirely relavant. Taken together, this analysis shows that GS are probably not the signaling substances increased in *npq4* plants and recognized by *Plutella*, which was contrary to our expectations since GS is the common defense from

Arabidopsis (Ratzka et al., 2002) and usually the marker for *Plutella* (Sun et al., 2010).
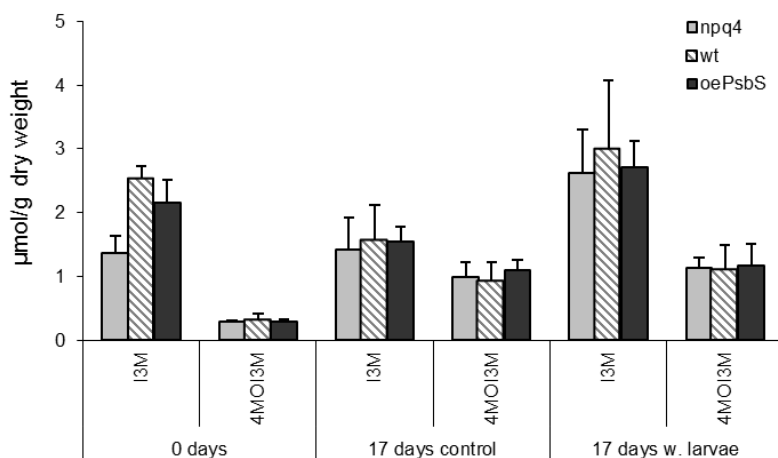

Figure 19. Measurement of two induced glucosinolates (GS). Concentration of GS was measured on field plants before (0 days) larvae and after 17 days with and without (control) larvae. Error bars are SD, n=4-10.

A model: How does the amount of PsbS influence herbivore responses?

PSII is considered the most vulnerable part of the photosynthesis apparatus (Maxwell and Johnson 2000) and, as mentioned previously, the plant has many ways of protecting it. It has long been known that PsbS is one of these protection mechanisms but as yet we do not know exactly how. Detection of superoxide production that probably arises from PSII gives us insight into the role of PsbS as a photoprotector and in Figure 20 we present a model describing how we think this affects the plant in a larger perspective.

When the plant is exposed to excess light, PsbS responds quickly to dissipate the light energy as heat. If there is an absence of PsbS, PSII is overloaded and excess capture of energy will form ROS (especially superoxides) which might damage the photosystem. We believe that this ROS production, through retrograde signaling, initiates a chain of events whereby the metabolic profile of both the chloroplast and the cytosol are altered. Through a pathway of question marks we believe the signal to reach the nucleus which results in a changed transcription profile. Something in these plants — and probably all of these processes taken
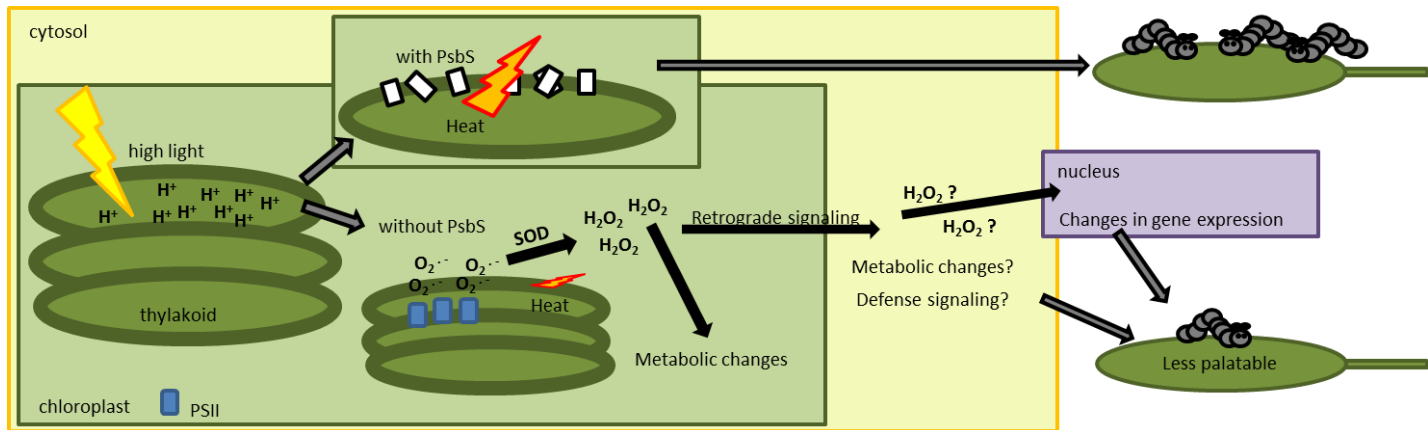
Figure 20. A model proposal. Reactions in a plant with PsbS (small chloroplast) and without PsbS (big chloroplast). PSII is overexcited and produces superoxide ($O_2^{\cdot-}$), which is dismutated into hydrogen peroxide (H_2O_2), and result in metabolic changes in the chloroplast. There is also a retrograde signal to the nucleus where transcript changes have been found. These reactions lead to a change where both a specialist (*Plutella*) and a generalist (*Spodoptera*) herbivore prefer to feed on plants with more PsbS.

together rather than one specific factor — affects the food preferences and ovipositioning of our test herbivores *Plutella* and *Spodoptera*.

I believe that the observed effects are not of huge impact in nature since the differences we have found are minor. However, such minor differences may cause changes in the longterm adaptation and this could be a reason for the variation in NPQ capacity between the ecotypes.

Conclusions and Future Perspective

We have been investigating the effect of variation in NPQ capacity in many different ways: gene regulation, the metabolome, fitness, growth rate, glucosinolates, herbivory and herbivore preferences, the amount of JA, and different ROS, and most of this has been investigated in semi-natural conditions.

How are herbivores affected by the level of PsbS and what might be the reason for this?

This thesis has shown that both a generalist (*Spodoptera*) and a specialist (*Plutella*) herbivore prefer to feed on plants with more PsbS. However, for oviposition *Plutella* preferred *npq4*, a plant without PsbS. Also in the field with naturally occurring herbivores, plants with PsbS were preferred for food. In an attempt to understand the reason behind the changed herbivore behavior relative to PsbS levels, we have analyzed transcriptomics, metabolomics, glucosinolates and reactive oxygen species. We did not find any differences in the amount or composition of glucosinolates, which are the obvious defense compounds of *Arabidopsis*, but we did find an up-regulation in the JA-pathway, minor metabolic changes and an increased production of superoxides in *npq4* plants. Unfortunately we have been unable to pinpoint the factor that is responsible for the alternation in herbivore behavior but perhaps *npq4* plants are primed by light stress and therefore more "paranoid" and more easily induce something that give some defense towards herbivory. Potentially, increased superoxide production could be responsible for this. Recently more research have been conducted on the correlation of abiotic and biotic stresses and this study adds to those that indicate such a correlation, and this calls for further studies. The level of PsbS is important but is not a question of survival, for the first generation, although it is likely that it will be under selection during evolution that leads to adaptation of

future generations. To fully understand the importance of NPQ capacity and especially qE it would be interesting to grow *Arabidopsis* plants with a varying level of PsbS in the field generation after generation. This could hopefully shine more light into the importance of qE for plant survival and fitness.

What happens to plants with a varying level of PsbS during and after field acclimation?

Arabidopsis plants transferred to the field show a strong and absolute response of increased amino acids and sugars. There is also a genotypic effect, but the acclimation effect is so strong that the amount of PsbS has only a minor influence on the result. In the longer term field plants showed increased chl a/b ratio, more xanthophylls and altered amounts of some proteins – such as increases in PsbS. Compared to chamber-grown plants, field plants are much smaller in size and have a rounder leaf shape, and when comparing growth of the genotypes oePsbS, wt and *npq4*, no differences were found. About 20 *Arabidopsis* ecotypes collected from around the world were grown both in chambers and acclimated to the field, before NPQ was measured. Some ecotypes had a high capacity in chambers but a low capacity in the field and vice versa. In general the span of NPQ capacity was bigger in the field than in chamber but otherwise no correlations could be observed for the different ecotypes.

These investigations of field acclimation and differences between field and lab plants have not only been described for the first time but more importantly also given a background for interpretation of both field and lab results. To continue these investigations it would be interesting to also measure the level of PsbS and maybe also xanthophylls in the ecotypes when grown under the different conditions. Since the day-length is different in the field it would also be interesting to do studies of the metabolomic circadian rhythm in field-plants and to compare to same day-length in chamber to really see what the metabolic differences in field are.

Personal reflections

When I started my PhD studies I did not really know what non-photochemical quenching (NPQ) was. According to the job-add I was not even supposed to work with NPQ in the extent I have. However, things change along the way and I think it is these changes and solving new challenges that is the general learning during a PhD education. During my first year I went to a NPQ workshop and got amazed by how many people could actually work on the same tiny protein, PsbS. This protein is strongly connected to one part of NPQ, and as presented, there are several hypotheses about this process. However, the process of how PsbS is involved in NPQ is of minor relevance for my work since I have mainly studied the effect of PsbS levels as well as natural variation of NPQ in *Arabisopsis thaliana*.

I have definitely learned a lot these past years and I hope you enjoyed reading the story of my work.

Hanna Johansson Jänkänpää
October 2011

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