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Non Photochemical Quenching mechanism in higher plants and in the unicellular alga *Chlamydomonas reinhardtii*

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To Vincenzo, Maria Gemma and Giorgio…

My nephews and niece, the sunshine of my family

Abstract

Light is a necessary ecological factor for oxygenic photoautotrophic organisms. Plants and algae require light for life but it can sometimes become a limiting factor or even cause stress events. Among the different mechanisms evolved to cope with excessive light, the Non Photochemical Quenching (NPQ) seems to be the most efficient process in both plants and algae. The NPQ value can be attributed to three different components (qE, qT and qI). A central problem for eco – physiology is the quantification of the partitioning of the excitation energy into the different mechanisms.

To investigate the variability in the extent of the three components on external parameters, different experiment were performed. My research showed that utilizing inhibitors or mutants lines of *Arabidopsis* a variation in the components was inducted. The analysis of the fluorescence parameters were further developed and in this work a revised energy partition approach will be proposed.

Moreover, seen that it is very important avoid light stress situation this research proposed the NMR (Nuclear Magnetic Resonance) as a useful tool in the detection of stress situation in vegetal extracts.

In regard to *Chamydomonas reinhardtii* not so many information are collected on the NPQ. Interestingly, analyses of a suppressor of the *npq4* mutation seemed to partly restore the wild-type NPQ value. This suggests a role not only for protein LHCS3 , but also for LHCSR1.

Keywords: NPQ, energy partitioning, *Arabidopsis thaliana*, *Chlamydomonas reinhardtii,* photoprotection.

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List of abbreviations:

ROS: reactive oxygen species

NPQ: non photochemical quenching

qE: energy-dependent component of NPQ

qT: state-transition dependent component of NPQ

qI: photoinhibition related component of NPQ

HL: high light

LL: low light

Arabidopsis: *Arabidopsis thaliana L.*

Beta: *Beta vulgaris L.*

Spinacia: Spinacia oleracea L.

Chlamydomonas: Chlamydomonas reinhardtii

Vio: violaxanthin

Zea: zeaxanthin

Neo: neoxanthin

VDE: violaxanthin de-epoxidase

VE: violaxanthin epoxidase

LHC: Light Harvesting Complex

Lhc (s): proteins of the antenna complex

Chl *a*: chlorophyll *a*

Chl *b*: chlorophyll *b*

PSI: Photosystem I

PSII: Photosystem II

RC: reaction centre

SOD: super oxide dismutase

Φ_{PSII} : quantum yield of PSII

WT: wild type

Chapter 1: Introduction

Summary

During the last three years of PhD research, I investigated the mechanisms of heat dissipation and tried to find out how the different components of this process can vary after the occurrence of light stress in different higher plants species. Moreover, I examined different strains of *Chlamydomonas reinhardtii* with particular attention to mutants in the energy dissipation pathway. Non photochemical quenching is just one of the ways taken by plants and algae in order to avoid the photooxidative stress. In this chapter I shortly review other photoprotective mechanisms as well as examine the NPQ mechanisms I studied in detail.

Preface

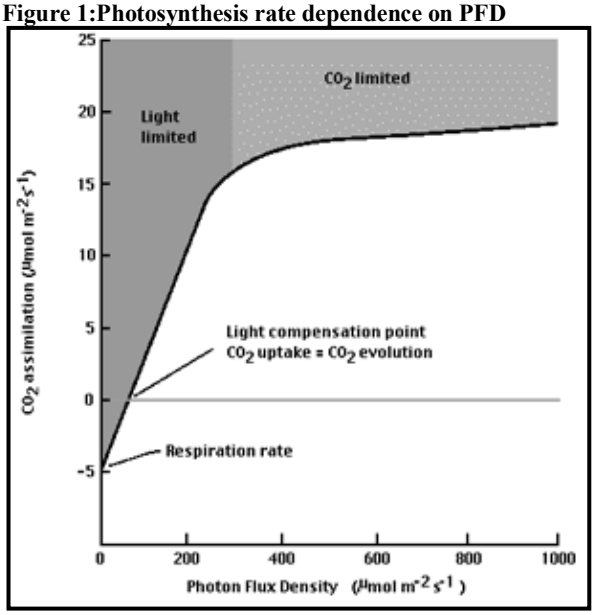
Some of the contents of the section 1.3 of this chapter will be part of a review from the title "qE in Plants versus Algae" by Thuy B. Truong and Krishna K. Niyogi (*unpublished*).

Contents of the section 1.7 of this chapter have been published as Guadagno, C.R., Virzo De Santo, A., and D'Ambrosio, N. (2010) A revised energy partitioning approach to assess the yields of non-photochemical quenching components. *Biochimica et Biophysica Acta – Bioenergetics*, 1797: 525 – 530.

1.1 "Too much of a good thing is a bad thing": high light damage and photoprotective mechanisms

Light is a necessary ecological factor for oxygenic photoautotrophic organisms. Plants and algae require light for life: it is source of energy through the photosynthetic mechanism and it is an important signal for their growth and development. However, light can sometimes become a limiting factor or even cause stress events. This love / hate relationship is clearly showed by the plot of photosynthesis rate versus photon flux density (PFD) which can be used to obtain useful information about photosynthetic properties of leaves (*Owens, 1994*) [Fig.1].

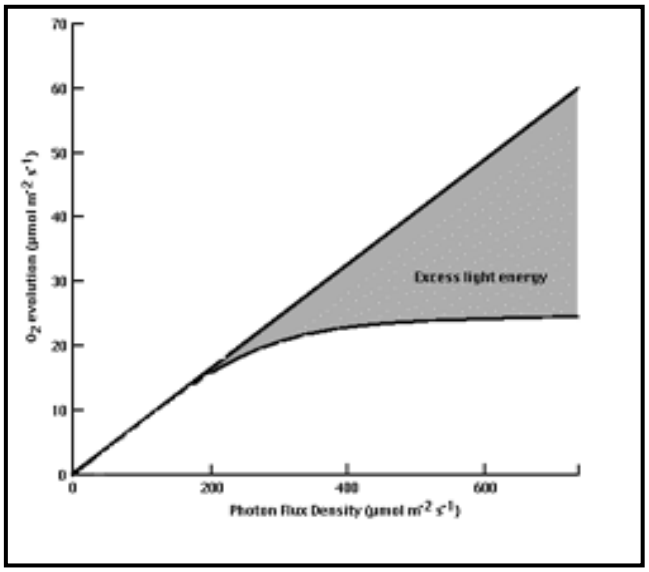
Figure 1:Photosynthesis rate dependence on PFD



Conventionally, the curve starts with negative values because in darkness a release of CO_2 is caused by respiration (*Vogelmann, 1998*). The point zero is called light compensation point and corresponds to the PFD where the rate of photosynthesis equals the rate of respiration. At this point, the uptake of CO_2 through photosynthetic pathways is exactly matched to the respiratory release of carbon dioxide, and the uptake of O_2 by respiration is exactly matched to the photosynthetic release of oxygen. At higher PFDs there is an increment in CO_2 absorption, until the absorbed CO_2 balances the CO_2 released through respiration and the curve reaches a plateau. At this point, further increments of PFD do not influence photosynthesis which is limited by the activity of carbon metabolism enzymes. This latter situation corresponds to the light saturation point, a useful measure of the leaf photosynthetic capacity. It corresponds to an exact PFD value that varies in relation to species and to diverse growth conditions. Consequently, photosynthetic organisms are not always able to utilize all the energy absorbed because the photochemical quenching via

photosynthesis can reach a saturation point. When the PFD exceeds the saturation [Fig.2], the photosynthetic apparatus can experience over excitation and over reduction, resulting in a generation of reactive species of oxygen (ROS) and consequent photooxidative damage.

Figure 2: Light saturation curve of photosynthesis



Additionally, algae and higher plants have to continuously cope with the natural variability of light radiation. Then, photosynthetic organisms have developed both short than longer term regulatory mechanisms in order to avoid energy overloading of photosynthetic apparatus (*Björkman, 1995*). The short-term responses can be considered a sort of light-shock response seen that they are the first fast lines of defense against the stress. On the contrary, the longer-term response leads to an acclimation of the organism to the light environment. In this chapter the avoidance mechanism and the light acclimation are briefly reviewed in the sections 1.1.1 and 1.1.2, while an introduction to the harmful reactive species and photoinhibition is given in sections 1.1.3 and 1.1.4. A concise outline of the Chl fluorescence as a probe of photosynthetic efficiency in vivo is reported in section 1.2. Finally, particular attention is given to the Non-Photochemical Quenching mechanisms (NPQ) as the subject of this project of research. This process is widely reviewed in section 1.3 and the different methods for the analysis of its components are reported in section 1.4 and 1.5.

1.1.1 Avoidance responses to light

In nature, the most direct way to avoid light stress consists in avoiding the absorption of excess light. This purpose can be reached through different short-term responses. Higher plants usually respond to the sun-tracking with related leaf movements activated by pulvinar actions. So, the leaves of some plants are able to change their orientation relative to the direction of solar radiation in order to minimize light absorption (*Björkman and Demming-Adams, 1994; Walters, 2005)*. These rapid and reversible movements of the leaves seem to be related to some carotenoids or flavoproteins (*Elheringer and Forseth, 1980; Björkman,1995*). Moreover, both plants and algae can adopt a very fast response within the cell: position and orientation of chloroplasts are strictly dependant on the incident PFD. In low light (LL) conditions, chloroplasts tend to be assembled perpendicular to the incident light, in order to increase the rate of the absorption. On the contrary, in high light conditions (HL) the chloroplasts line up to the edges of the cell until they are parallel to the incident light; as a consequence, an excessive light absorption can be avoided (*Björkman, 1995*). A blue light-receptor mediates chloroplast movements in plants, whereas the phytocrome, which absorbs in the bands of near and far-red, is the proteic pigment responsible for this response in green algae (*Wada et al., 1993*).

1.1.2 Acclimation to high light stress

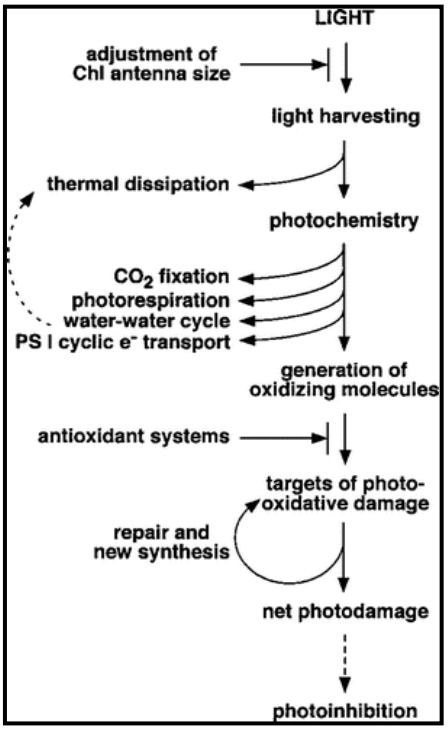
Plants and algae can exhibit long-term regulation too. This response acts on a time scale of hours or days and it leads to an acclimation to the HL environmental conditions. It is widely reported that sun and shade leaves show differences in leaf thickness, light transmission and stomatal distribution. Leaves of plants grown in full sunlight are usually thicker because the palisade cells are expanded and the stomata density is higher in respect to shade leaves (*Björkman, 1981*). Moreover, the capacity for photosynthetic electron transport and the rate of respiration are

higher in sun leaves than in shade leaves (*Björkman, 1981;Walters et al.1993)*. This increment is essentially due to the increases in the activity of the Rubisco enzyme and other features of the electron transport, as the cytochrome f. HL conditions usually cause a down-regulation of the nuclear genes for the Light-Harvesting Complex (LHC) proteins in the photosynthetic organisms that results in a smaller final amount of Lhcs proteins connected to both photosystems (*Maxwell et al., 1995; Teramoto et al., 2002; Ballottari et al., 2007*). Additionally, in case of HL stress the antenna size can be reduced through the proteolysis of the Lhc proteins (*Lindahl et al., 1995*). Lastly, seen that the chlorophyll *b* (Chl *b*) is mainly associated with peripheral Lhc proteins the chlorophyll a/*b* ratio increases in HL conditions. Although acclimation to HL conditions is still weakly understood, it surely consists in a complex network of different components. Actually, the perception of the excess light occurs in the chloroplast then, at least one signal transduction pathway is needed to transfer the information to the nucleus and to influence the gene expression.

1.1.3 Photoprotective pathway and highly reactive species formation

The organization of the photosynthetic apparatus is designed for absorbing several amounts of light energy and for transforming it into chemical energy. At a molecular level, the energy of a single photon causes an energetic perturbation that the photosynthetic apparatus can efficiently control in normal environmental conditions. On the other hand, if the light harvesting regulation or the energy dissipation mechanisms are not perfectly efficient, the production of toxic molecules may occur with consequent damages for the photosynthetic apparatus (*Horton et al., 1996*). Photoprotection is a complex multilevel process [Fig. 3].

Figure 3:Schematic diagram of photoprotective processes occurring within chloroplasts (*Niyogi, 1999*)



The fastest reply is the heat dissipation of the excessive absorbed energy. If this photoprotective strategy fails, toxic photoproducts, as Reactive Oxygenic Species (ROS), can be produced (*Blankeship, 1998*). These potentially harmful molecules generate at the three major sites of the photosynthetic apparatus: the LHC of the Photosystem II (PSII), the (RC) of PSII and the acceptor site of Photosystem I (PSI) (*Macpherson et al., 1993; Telfer et al., 1994; Niyogi,1999*). Firstly, a fundamental role is played by the chlorophyll (Chl) molecules which are excited by the absorbed light generating the state of singlet ($_1$Chl*). The excitation energy is rapidly transferred to other Chl molecules of the LHC but on the way to the RC some $_1$Chl* may generate triplets ($_3$Chl*), highly reactive species. The formation of $_3$Chl* is dependent on the lifetime of $_1$Chl* in the antenna. $_3$Chl* lifetime is longer than the one of $_1$Chl* and they can interact with O_2 molecules producing oxygen singlets ($_1O_2$*). These molecules are highly reactive and can be very harmful for the cellular components, especially lipids and membranes (*Ledford and Niyogi, 2005*). Other reactive species can be produced in the RC of the PSII where the harvesting of the excitation energy causes the charge separation for Chl dimer (P_{680}) and the Pheophitin molecule (Pheo). The produced radicals are reversible and the charge recombination may generate other triplets ($_3P_{680}$*) which can react with O_2 molecules generating reactive $_1O_2$*. Lastly, seen that the acceptor side of the PSI has a low redox potential, it is able to reduce O_2 molecules into the superoxide anion radical ($O_2\bullet^-$) that can be metabolized to hydrogen peroxide (H_2O_2) and this can cause the production of the oxidril radical ($OH\bullet^-$) (*Tjus et al, 2001; Asada, 2006*). All the three oxygenic molecules are highly toxic for the photosynthetic cells. All the reactions described above take place at every PFD value but they are accelerated in HL conditions

(*Niyogi, 2000*). A second line of defense in order to eliminate these toxic products is developed by carotenoids (Car). These molecules play a fundamental role in the photoprotective mechanism quenching the excited state of Chl. The excited Car molecule does not have enough energy for generating $_1O_2$* and passes to its basal state with contemporary heat dissipation (*Blankeship, 1998*). The quenching reaction is reported in Bassi and Caffarri, 2000:

$$^3Chl^* + {}^1Car \rightarrow {}^1Chl + {}^3Car^*$$
$$^3Car^* \rightarrow {}^1Car + heat$$

Another photoprotective strategy is the presence of the Ascorbate in the chloroplasts. This is a soluble antioxidant and it can directly neutralize $_1O_2$* and $OH\bullet^{-}$. Besides all these lines of defense, there are some scavengers enzymes, such as the superoxide dismutase (SOD), which are involved in ROS removal (*Sieffermann, 1987; Havaux and Niyogi, 1999*). However, if all these photoprotective mechanisms are not able to cope with the excess of light energy, the photoproducts can damage some molecules, as the D1 protein of the PSII (*Asada, 1996*). This is the only protein of the PSII which is completely replaced through the so called turnover of the D1 protein, and is not recycled (*Blankeship, 1998*).

1.1.4 Photoinhibition

When all the photoprotective responses fail, a series of molecular processes are triggered causing the inactivation of the RC of PSII with consequent inhibition of the photosynthetic process (*Aro, 1993; Melis, 1999; Takahashi, 2008*). The photoinhibition can be partial and totally reversible but it can also reach a drastic state in which the PSII is completely damaged (*Kok, 1956*). When the quantum yield of PSII (Φ_{PSII}) decreases but the maximum photosynthetic rate is stable, the photoinhibition is called dynamic. In this case, the excess of absorbed energy is moderate; the decrement in Φ_{PSII} is not permanent and the initial value is restored after a certain period of time.

On the contrary, when the maximum rate of photosynthesis and Φ_{PSII} decrease simultaneously, the photoinhibition is chronic (*Osmond, 1994*). In the first case the photoprotective mechanisms are efficient, but in the second case they are less efficient due to an overload of excitation energy (*Vogelmann, 1998*).

1.2 Chlorophyll fluorescence analysis

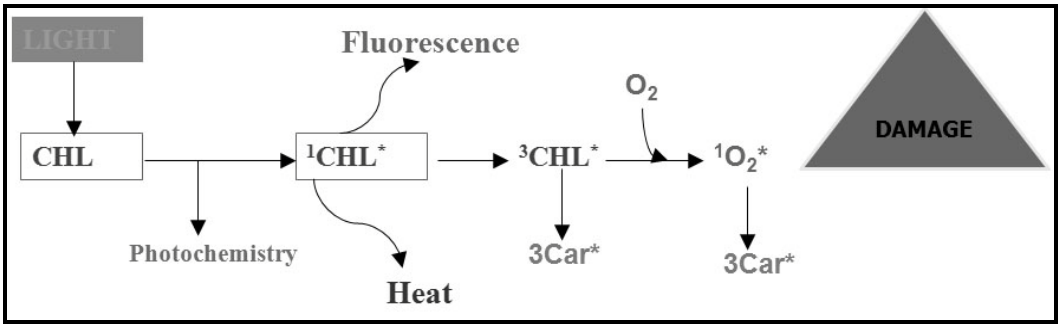
In all the photosynthetic organisms, Chl plays a central role in the harvesting and in photochemical transformation of the light energy. The excitation energy absorbed by Lhcs usually can undergo three fates [Fig.4]:

- it can be used to drive photosynthesis (photochemistry),

- it can be dissipated as heat or

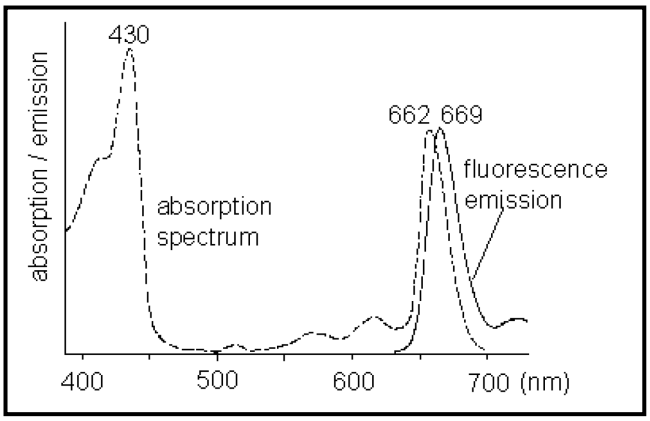
- it can be re-emitted as red fluorescence.

Figure 4: Possible fates for excited chlorophyll



These three processes occur in competition. Since the sum of rate constants is invariable, any increase in the efficiency of one process will result in a decrease in the yield of the other two. Therefore, determining the yield of chlorophyll fluorescence will give information about changes in the efficiency of photochemistry and heat dissipation (*Maxwell & Johnson, 2000*). At room temperature, Chl fluorescence is originated exclusively from the PSII (*Schreiber et al., 1995*). Although the percentage of the emitted fluorescence is very low (only 1-2%), this is easily assessable because the emission spectra of the fluorescence is slightly different from the one of the absorbed light, with a peak of emission in a wavelength region higher than the one of the absorption [Fig.5] (*Maxwell & Johnson, 2000*).

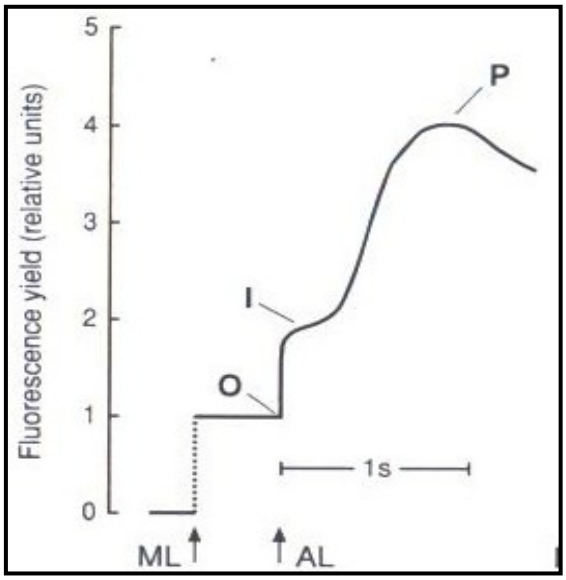
Figure 5:Difference in the absorption spectrum and fluorescence emission of Chl *a*



The intensity of the fluorescence signal is determined by different factors as the light intensity, the excitation energy funneled to the PSII and the heat dissipation efficiency (*Schreiber & Bilger, 1993*).

1.2.1 Kautsky effect

Figure 6: Kautsky effect (*Schreiber, 1993*)



For the first time, changes in the emission of the Chl fluorescence were observed by Kautsky and co-workers in 1930 *(Kautsky & Hirsch, 1931)*. They observed that when a leaf is irradiated with continuous light after a period of dark, the Chl fluorescence increases from a minimum value (O), through an intermedium value (I), to a maximum value (P) and then decreases again to a value similar to the original O [Fig.6]. This phenomenon is called the 'Kautsky effect'. The first value of fluorescence F_0 is recorded in darkness and it is caused by the Chl molecules of the LHC when all the RC and the plastoquinone (primary acceptor of electrons (Q_A)) are oxidized. When an actinic light is switched on, the first increase of the fluorescence value (O-I) reflects the reduction of Q_A which is in equilibrium with the second acceptor Q_B. The fluorescence level I reflects the equilibrium $Q_A^- Q_B \longleftrightarrow Q_A Q_B^-$ which is originated from the complete oxidation of the plastoquinone pool. The increment from the value I to P corresponds to the reduction of the

plastoquinone pool. The consequent decrease of the fluorescence value is due to the activation of the photochemical and non-photochemical processes *(Schriber & Bilger, 1993)*.

1.2.2 Saturation pulse method

Today, Chl fluorescence analysis has become an indispensable method for photosynthetic studies because it is a non intrusive tool and it can give useful information on the PSII very quickly (*Krause & Weis, 1991*). But, it has to be underlined, that this technique may sometimes lead to a difficult interpretation of the data (*Maxwell & Johnson,2000*). The instruments for chlorophyll fluorescence measurements are called fluorometers and they utilize the 'Saturation Pulse Method' which uses the application of saturating pulses of light in order to rapidly reduce the RCs of the PSII [Fig.7]. Consequently, the photochemical activity of PSII is temporarily inhibited and the pathway of the fluorescence is increased. In dark-adapted leaves, the saturating pulse causes the increase from the minimum value F_0 to the maximum value F_m: the difference between these two values is called F_v and represents the variable fluorescence. The ratio F_v/F_m is equal to the value of the maximum photochemical efficiency of the PSII. If an actinic light is switched on, the value of the variable fluorescence F is detectable and it is called F_s when it reaches the steady-state. At this point, the application of a saturating pulse causes the maximum emission of fluorescence in light conditions called F'_m (*Schreiber et al., 1995).* The recording of all these values allows for the calculation of the coefficients for the Photochemical and Non-Photochemical Quenching, PQ and NPQ respectively. These coefficients can be calculated according to the equations from Bilger and Björkmann, 1990. The value F_m reflects the amount of photochemical processes, while the difference between F_m and F'_m refers to the presence of non-photochemical processes.

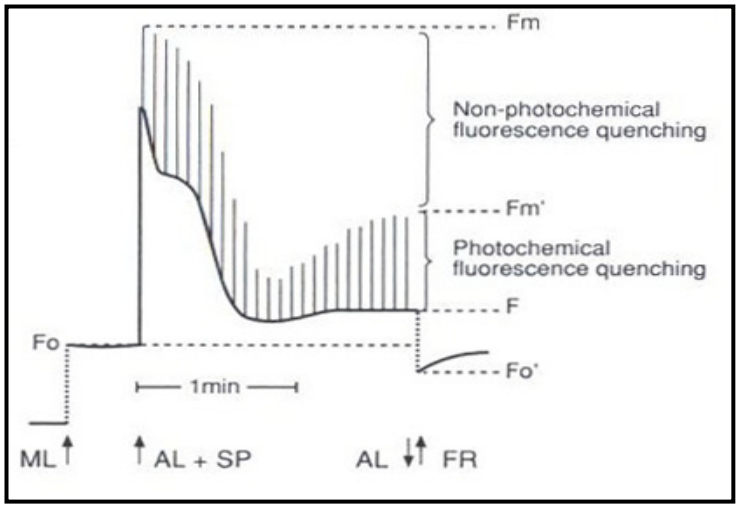
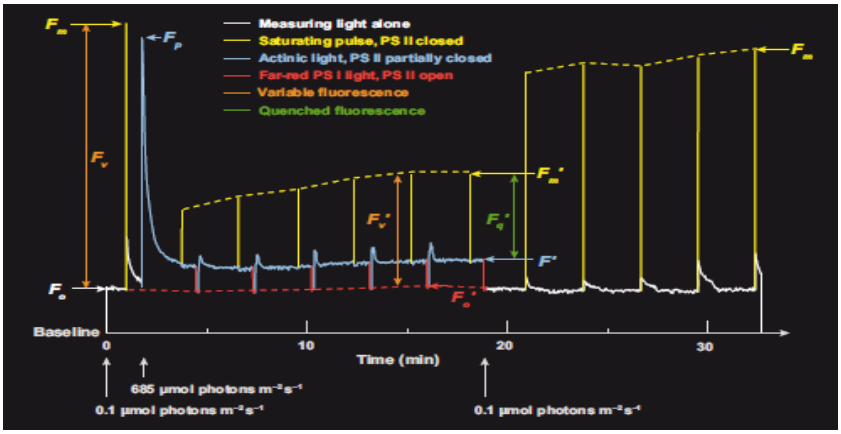


Figure 7: Classical protocol for chlorophyll fluorescence measurement (*Schreiber et al., 1995*)

Immediately after the actinic light is switched off, another minimum value for the fluorescence is measurable. This value is called F'_0 and is recorded through the application of a pulse of light with a wavelength in the far-red region (735nm), which is able to rapidly re-oxidize the RCs (*Maxwell & Johnson,2000*; *Schriber & Bilger,1993*). In stress conditions, often the value of F'_0 is lower than F_0 and it is used for the calculation of the coefficients. Later on, additional information has been added by different groups of researches and a complete review of all the fluorescence parameters was published by Neil Baker in 2008 [Fig.8]. The parameters denoted with a prime (') are from the leaf exposed to actinic light. The parameters without a prime are obtained from the leaf in the dark-adapted state. The different colors of the trace denote different light treatments. White: weak measuring light alone (0.1 µmol photons m^{-2} s^{-1}) that gives F_0. An important feature of this measuring beam is that its intensity must be low enough so it does not drive significant PSII photochemistry. Yellow: saturating light pulse (≤1 s duration, >6000 µmol photons m^{-2} s^{-1}) that gives F_m in darkness and F'_m in light.

Figure 8: Fluorescence analysis using modulated pulse method (*Baker, 2008*)

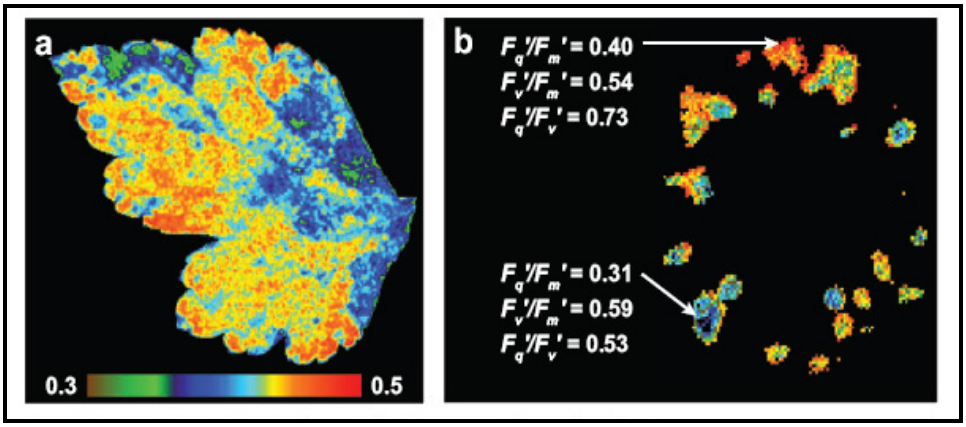


Blue: actinic light (685 µmol photons m^{-2} s^{-1}) that drives photosynthesis and gives F'_m. Red: far-red light (30 µmol photons m^{-2} s^{-1} at 720–730 nm for 4 s) that excites PSI preferentially, and thus oxidizes the plastoquinone and Q_A pools associated with PSII and gives F'_0. Orange: variable fluorescence calculated as $F_v = F_m - F_0$ from the dark-adapted leaf and $F'_v = F'_m - F'_0$ from the illuminated leaf. Green: fluorescence that is quenched from F'_m to F' by PSII photochemistry in the illuminated leaf, calculated as $F'_q = F_m - F'$ (*Baker, 2008*).

1.2.3 Imaging of chlorophyll fluorescence

In the last few years a great innovation has been revolutionizing the fluorescence method: the introduction of instruments capable of imaging fluorescence. These tools allow the resolution of the spatial heterogeneity of the photosynthetic performance (*Oxborough, 2004; Nedbal and Whitmarsh, 2004*).

Figure 9: Imaging the heterogeneity of photosynthetic activities of leaves and chloroplasts (*Baker,2008*)



Photosynthetic heterogeneity has been identified in many situations, e.g., during induction of photosynthesis and in response to stresses (*Meyer and Genty, 1999; Bro et al., 1996*). Non-imaging fluorescence measurements would often not detect such heterogeneity. Imaging of appropriate fluorescence parameters can provide information about the causes of the heterogeneity. Moreover, fluorescence imaging can be used in screening procedures to identify organisms with modified

photosynthetic performance, which has been done for algae (*Bennoun et Bèal, 1997; Niyogi et al., 1997*) and *Arabidopsis* mutants (*Niyogi et al., 1998*). Perturbations of metabolic processes not directly involved in photosynthetic metabolism often induce changes in fluorescence parameters (*Percival and Baker, 1991; Barbagallo et al., 2003*), which can be used to screen for such perturbations.

1.3 Non Photochemical Quenching

The topic of this project of research is the Non Photochemical Quenching (NPQ) of chlorophyll fluorescence as the most efficient photoprotective response in plants and algae. As soon as the absorbed energy by Lhcs exceeds the requirement for photochemical activity, this fast mechanism of heat dissipation is triggered, in order to prevent ROS production (*Denmig-Adams, 1992; Szabo, 2005; Eberhard, 2008*). NPQ is a composite of three different components, each one characterized by a peculiar kinetic behavior. The first component (qE), is the energy dependent quenching, the intermediate quenching (qT) is state transition related and the slow component (qI) is photoinhibition related (*Eberhard et al., 2008*). qE is very fast, its efficiency seems to be very high under the most part of circumstances and it is present in a wide range of organisms, from cyanobacteria to higher plants. Although this component is the most studied both in algae and plants, many are the unresolved doubts on this process.

For this project of research, both higher plants and the green unicellular alga *Chlamydomonas reinhardtii* have been utilized. Therefore, in the following section the NPQ mechanism, and especially the most studied qE component, will be reviewed in both organisms.

1.3.1 qE mechanism in higher plants

NPQ has been studied as a photoprotective mechanism, especially in higher plants. In particular, the model organism *Arabidopsis thaliana* has been playing a central role in the molecular

biology approach to NPQ for more than ten years. *Arabidopsis* is perfect for the aims of plant biologists because of its small dimensions, a relatively short life cycle and small size of the genome. Therefore, screens for mutants deficient in qE have been productive in analyzing the components of this mechanism. Conventionally, there are three essential factors for the activation of qE: the xanthophyll cycle, the pH gradient, and some proteins of Lhc superfamily. Nevertheless, it is still unclear how some components are involved in the mechanism.

1.3.1.1 The xanthophyll cycle in higher plants

Xanthophylls are oxygenated derivatives of carotenoids which play roles in light harvesting, photoprotection, and the structure of the photosynthetic antennas (*Young et al., 1997; Pogson et al., 1998*). The inter-conversion of these pigments is essential for qE mechanism. The cycle consists in the ΔpH dependent transformation of Violaxanthin (Vio) into Zeaxanthin (Zea), trough Anteraxanthin. This cycle is catalized by two enzymes: the violaxanthin-deepoxidase (VDE) and zeaxanthin-epoxidase (ZE) [Fig.10]. There are two schools of thought for the role of xanthophylls: a direct and/or indirect role. In the first case, Zea quenches chlorophyll a fluorescence directly, due to its lower S_1 energy state compared to that of Chl *a* (*Frank et al., 1994*). Instead, Vio has a higher S_1 state and it transfers light energy to Chl *a*. In the second case, xanthophylls have an allosteric role in qE. causing a conformational change in the light-harvesting antenna to mediate quenching. In this model, xanthophylls enhance the aggregation state of LHCII, and the energy state of the xanthophylls is less relevant than their structural and chemical properties (*Ruban et al., 1994; Pascal et al., 2005*). Zea, more hydrophilic and planar than Vio, induces qE and promotes the aggregate state. The addition of Vio delays LHCII aggregation, and this pigment acts as an "anti-quencher" (*Perez-Bueno and Horton, 2008*). Even if the real meaning of the xanthophylls cycle for the qE is unknown, these pigments are critical to qE and their importance is strongly established with *Arabidopsis* mutants.

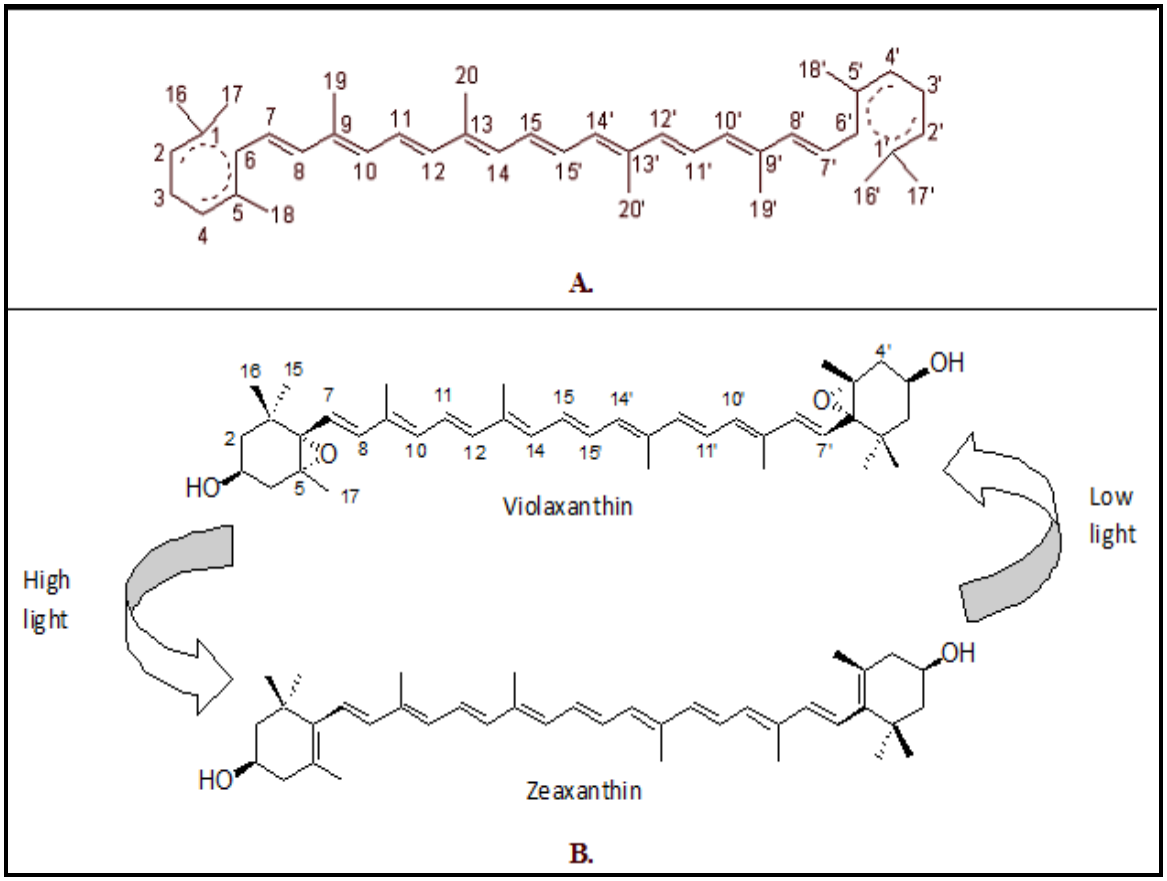


Figure 10: A) Carotene structure and numbering B) Xanthophyll cycle: violaxanthin and zeaxanthin structure and numbering

The *npq1* mutant, which cannot convert Vio into Zea due to the absence of the VDE gene, has reduced NPQ capacity compared to wild type (*Niyogi, 1999*). The *npq2* mutant that constitutively accumulates Zea, in turn, has a faster NPQ induction, but not higher NPQ than wild type (*Niyogi, 1999*).

1.3.1.2 pH gradient in higher plants

During photosynthesis the electron flow from PSII to PSI leads to the transport of protons (H^+) from the stroma to the luminal side of thylakoids with the consequent synthesis of ATP. This H^+ flux causes a pH gradient (ΔpH) which can become higher in presence of excessive absorbed light. This triggering through the ΔpH allows the quick induction and relaxation of qE [Fig.11].

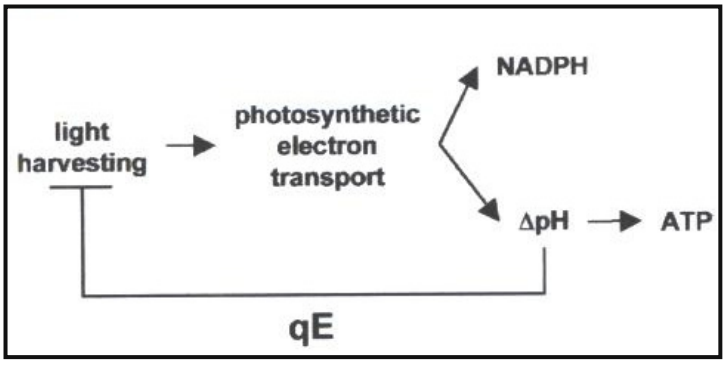
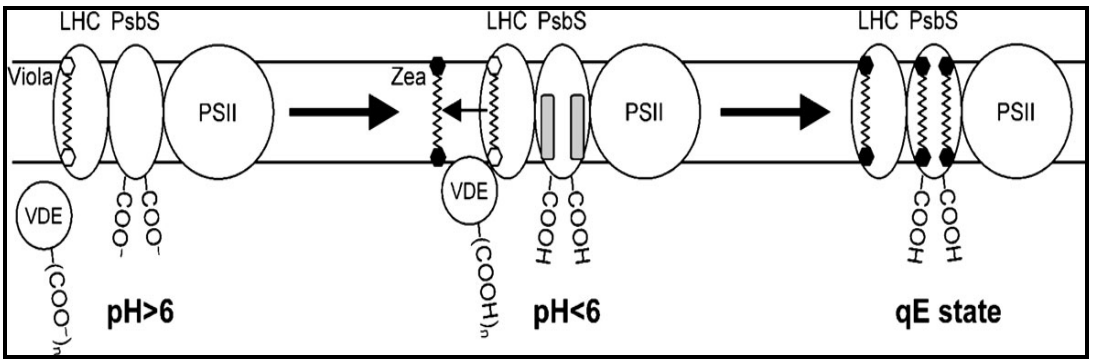


Figure 11: Feedback regulation of light harvesting via lumenal pH gradient (*Niyogi et al., 2004*)

It is established that the proton gradient is necessary for qE generation since qE cannot build up when the gradient is blocked via the addition of the uncoupler nigericin (*Quick et al., 1989; Jahns and Hyede, 1999; Muller et al., 2001*). In addition, *Arabidopsis* mutants unable to produce a proton gradient show lower NPQ than wild type (*Munekage, 2001; Okegawa, 2007*). *pgr1* mutant is defective in linear electron transport and it cannot generate the required threshold pH for qE activation under HL conditions. *pgr5* mutant has an impaired Cyclic Electron Flow (CEF) and its NPQ is also impaired. The ΔpH plays a double role in qE (*Niyogi et al., 2004*) [Fig.12]. Firstly, it activates the xanthophyll cycle. The VDE enzyme during normal light conditions floats about in the lumen and only when the pH reaches a level of about 5.5 in HL, the enzyme binds to the thylakoid membrane and is triggered to convert Vio into Zea (*Rockholm, 1996*). The second role of the gradient is the protonation of the acidic residues of Lhcs proteins. The significance of protonation of each of these protein is still unknown, but the protonation of PsbS is essential for qE induction.

Figure 12: Schematic model for the role of pH in qE mechanism (*Niyogi et al., 2004*)



1.3.1.3 Lhcs proteins in higher plants

The Light Harvesting Complex proteins (Lhcs) are divided into two categories: major and minor. The major antennas are LHCII trimers composed of Lhcb1, 2 and 3 and are connected to the PSII reaction centers through the minor antennas, made up of Lhcb4, 5, and 6. Both types bind to an assortment of chlorophylls and xanthophylls. Each major antenna protein binds to two luteins (Lut) at sites termed L1 and L2, one Vio/Zea at V1, and one neoxanthin (Neo) at N1. The minor antennas hold only one Lut at site L1, one Vio/Zea at site L2 and one Neo at N1 [Fig.13]. The only LHC related mutant that shows a substantial defect in NPQ capacity is the *Arabidopsis npq4* mutant. It lacks *PSBS* gene, coding for a member of the LHC family that contains four trans-membrane domains instead of the usual three (*Li et al., 2000*) [Fig.14]. It is demonstrated that two luminal glutamate residues are required for the function of this protein and they are responsible for sensing the lumen pH (*Li et al., 2000*). Mutation of each residue lowers the NPQ capacity to half of the wild type value.

Figure 13: Occupancy of the xanthophyll-binding sites in the major LHCII antenna complex

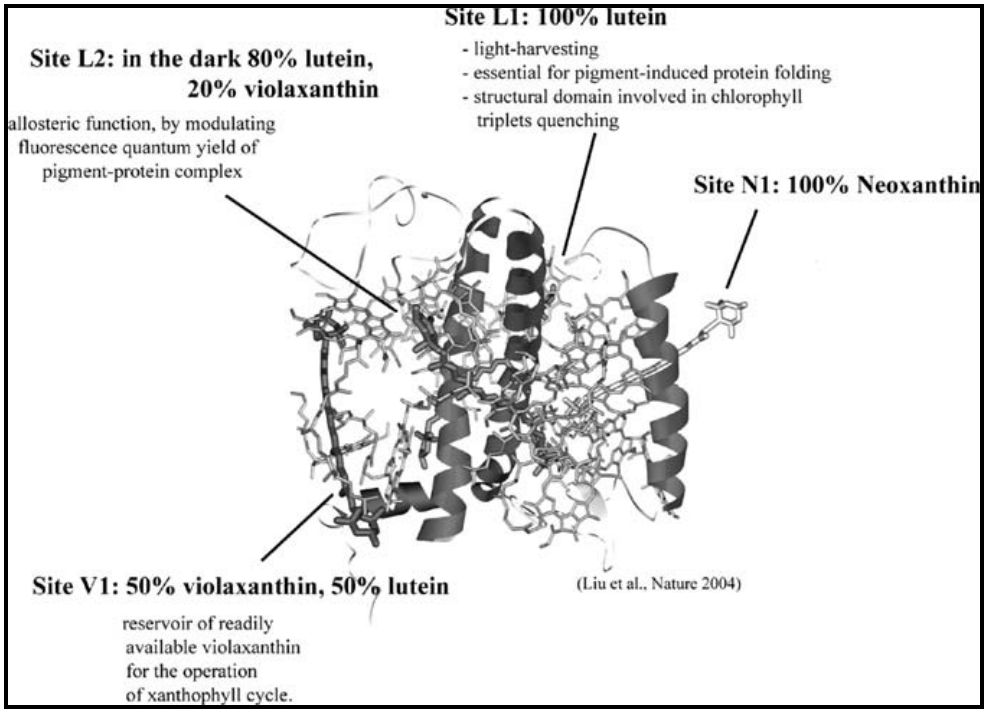
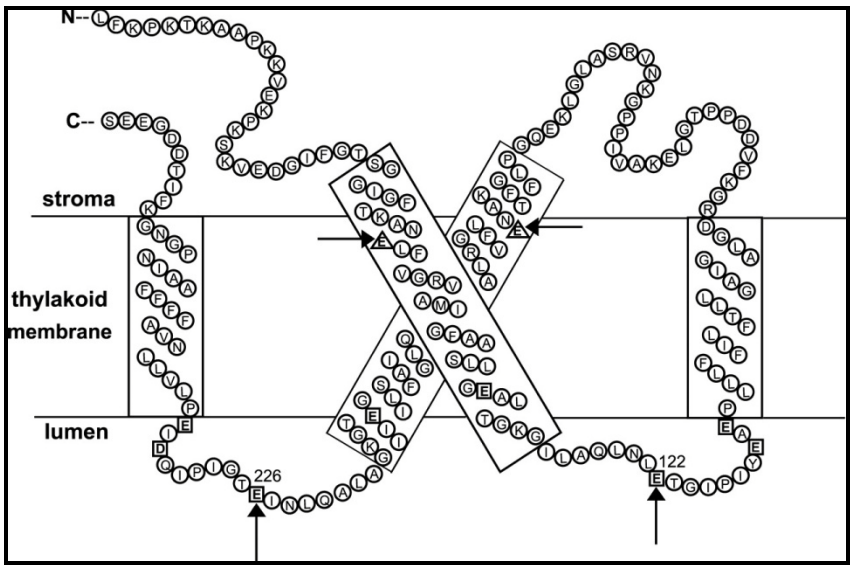


Figure 14: Topological model of *Arabidopsis* PsbS



Triangles and horizontal arrows denote positions of two highly conserved glutamates that serve as ligands to bound chlorophylls in LHCII. The two glutamates that are necessary for qE and DCCD binding are numbered and marked by vertical arrows (*Niyogi et al., 2004*).

1.3.1.4 Possible mechanisms of qE in higher plants

There are three current models for the qE mechanism in higher plants. They all agree on the aforementioned involved components but they differentiate from each other for the exact role of each component and the location of quenching they suggest.

The first model proposes that the HL condition causes a decrement in the pH value of thylakoid lumen and the protonation of Lhc proteins as PsbS with the contemporary production of Zea via the xanthophyll cycle (*Li et al., 2002; Niyogi et al., 1997*). The protonated PsbS induces a conformational change in antenna proteins and the formation of a quenching complex (*Li et al., 2002*). In this model, the de-excitation happens via charge separation and subsequent recombination in a Chl-Zea complex. Supporters of this model, through the transient absorption (TA) spectroscopy analyses, found a species at a 1000 nm that they ascribed to be a zeaxanthin radical cation (*Holt et*

al., 2005). This species was found only in the quenched state and was missing in the *npq4* mutant. TA spectroscopy of isolated minor antenna complexes showed the presence of the ZEA radical cation, whereas the trimeric LHCII showed no such species (*Avenson, 2009*). In HL, VIO is exchanged for ZEA that then can undergo charge transfer with a Chl dimer.

The aggregation of LHCII proteins plays a central role in the second model for qE. It was observed that oligomerization of LHCII trimers leads to quenching of chlorophyll fluorescence (*Pascal et al., 2005*). This quenching is accompanied by a twist in a Neo molecule, as shown by Raman spectroscopy (*Pascal et al., 2005*). This conformational change brings one or two chlorophylls together that can then transfer the excess energy to the S_1 state of a nearby Lut, which is located at site L1 in the major antennas (*Ruban, 2007*). This mechanism still requires the proton gradient as well as the PsbS protein and Zea, with Zea having an allosteric role.

A third possible mechanism is the Chl-Chl charge transfer (*Muller et al., 2010*). Using time-resolved fluorescence spectroscopy, they showed the presence of a 400 ps lifetime species that is associated with LHCII aggregates and is found in intact *Arabidopsis* in HL (*Miloslavina et al., 2008*). According to this model, the quenching mechanism arises from a Chl dimer that undergoes charge transfer and subsequent emission to the ground state, with no energy transfer to xanthophylls. Consequently, LHCII antennas are detached from the PSII cores in high light and most probably quenched by this Chl-Chl charge transfer mechanism as reported in Holzwarth et al., 2009. PsbS is required and believed to allow for the detachment of the LHCII from the supercomplex. However, the role of Zea in the qE mechanism is still unknown. It could either be involved in Chl-Car energy transfer or play a role in a charge transfer mechanism.

1.3.2 qE mechanism in *Chlamydomonas reinhardtii*

Figure 15: ***Chlamydomonas reinhardtii***

It is believed that photoprotection in the form of qE is crucial for the survival of the algae. The components required for qE in algae are similar to those in plants: the xanthophyll cycle, the proton gradient, and the antenna proteins. The model algal organism of choice in this study is *Chlamydomonas reinhardtii*, a unicellular alga of soil and freshwater [Fig.15]. This particular alga is very useful for plant biologists because its genome is totally known, its time of reproduction very fast and its adaptation to light conditions very peculiar. However, qE has also been studied in a number of other algae, particularly diatoms because of their prevalence in the ocean.

1.3.2.1 Xanthophyll cycle in Chlamydomonas

The xanthophyll cycle as above mentioned is only found in higher plants, ferns, mosses, and some groups of algae (*Latowski et al., 2004*). *Chlamydomonas* is an alga in which the xanthophyll cycle has been studied extensively with regards to qE. The *npq1* mutant in this alga, which lacks Zea, does not show the same qE defect as in the *Arabidopsis* mutant (*Niyogi et al., 1997*). Its induction phase is similar to wild type and total qE is only slightly lower than wild type. Although the *Chlamydomonas npq1* mutant is defective in the conversion of Vio into Zea, the basis of the mutation remains unknown. Moreover, there are no homologs of the plant VDE found in its genome, suggesting that either the enzyme responsible for the conversion is highly divergent from its plant counterpart or that it is an entirely new enzyme (*Anwaruzzaman et al., 2004*). Then, the *npq2* mutants are new alleles of *aba1*, the ZE gene. The high levels of Zea in *npq2* affected the kinetics of induction and relaxation but not the extent of NPQ (*Niyogi et al, 1998*) [Fig.16].

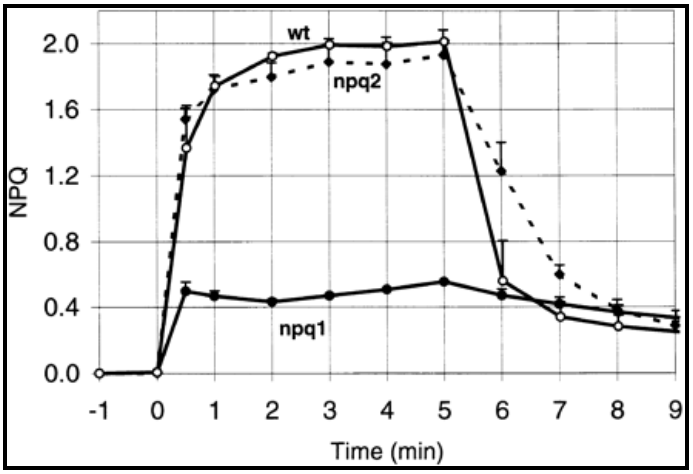


Figure 16: Time courses for induction and relaxation of NPQ in leaves of the wild type, *npq1*, and *npq2* of *Chlamydomonas* (*Niyogi et al., 1998*)

The double mutant *npq1lor1*, which lacks Zea and Lut, shows a more pronounced lack of qE; this suggests that Lut might play an important role in the qE of green algae too.

1.3.2.2 pH gradient in algal qE mechanism

The pH gradient importance in algal qE has been studied more in diatoms than in *Chlamydomonas*. However, in diatoms as in green algae and higher plants, the proton gradient is required to initiate qE. The role of the proton gradient goes beyond the activation of the xanthophyll cycle. The authors believe that the proton gradient is required in diatoms as it is in higher plants by activating LHC antenna components to be in the quenched protonated state. This would lead to a very similar scenario for the mechanism of quenching in both higher plants and algae.

1.3.2.3 LHCSRs proteins in Chlamydomonas reinhardtii

In *Chlamydomonas* the organization of the PSII supercomplex differs in terms of the number of trimers bound, due to the absence of the minor antenna protein CP24. Instead, there exists a number of novel antenna proteins found in green algae, including Light Harvesting Complex Stress Related (LHCSRs) proteins which are ancient light harvesting antenna proteins belonging to the Lhc superfamily with homologs in diatoms (*Peers et al., 2009*). The role of PsbS in algae is not

established as it has been for higher plants. Search for mutants lacking *PSBS* gene in *Chlamydomonas* has yielded no results. Biochemical studies showed that despite the fact that this alga contains two copies of the gene, neither protein is expressed in the chloroplast (*Bonente et al., 2008*). Insertional mutagenesis in *Chlamydomonas*has given some clues as to the nature of the sites of quenching in algae. The *npq5* mutant lacking *Lhcbm1,* a gene that encodes for one of the LHCII trimer proteins, exhibits low qE (*Elrad et al., 2002*). In turn, it contains fewer LHCII trimers compared to wild type. This correlation between LHCII trimers and qE deficiency implies that the quenching sites might just be in the trimers themselves, as suggested by Ruban et al. (*1999, 2005, 2007*). However, the same insertional mutagenesis produced another mutant, *npq4,* lacking LHCSRs isoform 2 and 3 (*Peers et al., 2009*). Lacking the two LHCSR3 isoforms, the NPQ phenotype of this mutant resembles that of the *Arabidopsis npq4* mutant. LHCSRs are good candidates for quenching sites in *Chlamydomonas*. Unlike other Lhc proteins, their expression is induced when the algae are exposed to high light.

1.3.1.4 qE mechanism hypothesis in Chlamydomonas reinhardtii

The molecular mechanism of qE in algae is not well studied as it has been in higher plants and new studies are just emerging. There is much interest in the LHCSR proteins and their roles in quenching. The same TA spectroscopy technique was applied to reconstituted protein- pigment complexes of LHCSRs and these exhibited the signal for a Lut radical cation (*Bonente et al., 2008*). This is unlike the TA data for *Arabidopsis*, in which Zea was the dominant signal and the signal for Lut only appeared when Zea was absent. However, the mechanism by which the excited chlorophylls are quenched apparently is the same via electron transfer from a xanthophyll species, and de-excitation by subsequent recombination.

1.4 qT: state transition related quenching

qT was supposed to be the result of a mechanism which balances the excitation pressure between PSII and PSI, through a reversible phosphorylation of Lhc proteins pool (*Haldrup et al.,2001*). State transition is signaled by the redox state of the plastoquinone pool and is independent of pH or xanthophyll concentration (*Bennett, 1991*). However, it was shown that qT might not be active under high light stress in plants, but rather during exposure to low or moderate light (*Walters and Horton, 1990*). Today, rather than the migration of LHCII from PSII to PSI, this intermediate quenching component is believed to be associated with the conversion of Vio into Zea and it is now designated as qZ (*Nilkens et al., 2010*). The induction and relaxation time of qZ coincide with the formation and re-epoxidation of Zea within the 10-15 minute time range that was attributed to qT before.

1.5 qI: photoinhibition related quenching

This quenching mechanism is more ambiguous than the other two. qI is commonly associated with the damage of the D1 protein that leads to photoinhibition and lower photosynthetic capacity (*Aro et al., 1993*), but also Zea seems to be involved in the triggering of this mechanism (*Jahns & Miehe, 1996; Thiele et al., 1996; Verhoeven et al., 1996*). The impaired PSII reaction centers are capable of quenching fluorescence directly (*Horton, 1996*), but the mechanism by which this is done is still unknown. qI may be a composite of many mechanisms and more studies are needed to uncover the processes involved.

1.6 Relaxation kinetics of NPQ components

NPQ value is calculated as: NPQ = (F_m – F'_m)/ F'_m (*Bilger & Bjorkmann, 1990*) utilizing the fluorescence value from the PAM (Pulse Amplitude Modulated fluorometers) as reported in section 1.1.2 of this chapter. In 1988, firstly Demming and Winter measured the relaxation in darkness of NPQ using the Saturation Pulse Mode. An alternative approach was proposed on barley leaves with a sequence of many pulses in a short period of ten minutes (*Quick and Stitt, 1988*). In 1990, Walters and Horton added a mathematical innovation to the method. Reporting the values of NPQ on a semi-logarithmic scale versus the time of recovering of NPQ in darkness, was possible resolving the three different components of NPQ [Fig.17].

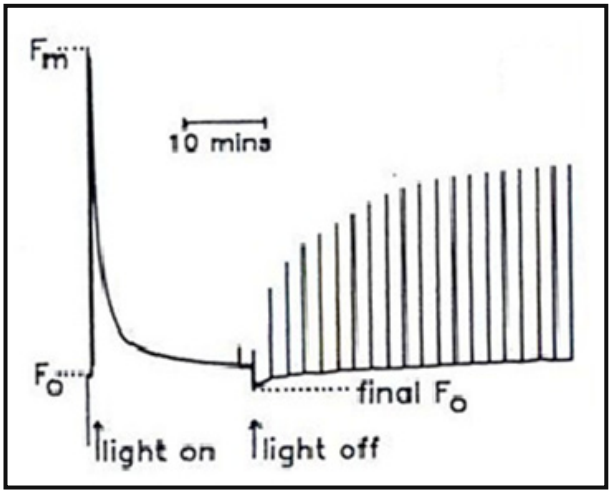


Figure 17: Curve of the fluorescence emission. A barley leaf with an actinic light on (950 µmol m-2 s-1) and saturating pulse (3000 µmol m-2 s-1) after a darkness period (*Quick and Stitt, 1988*)

They applied a linear regression to each significative variation of the slope finding intercept values (A, B and C) on the NPQ axis [Fig.18]. These values allowed to determinate the absolute values for each one of the three component according to the following formulas:

qI = A; qT = (B – A); qE = (C – B).

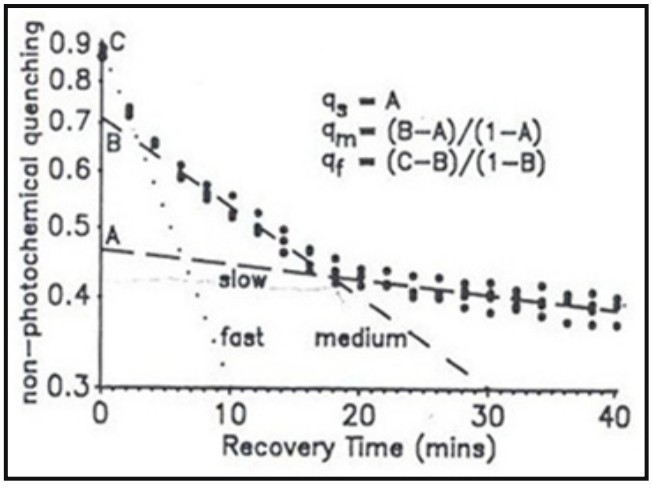


Figure 18: NPQ components relaxation kinetic on a semilogarithmic scale (*Walters & Horton, 1990*)

1.7 Energy partitioning approach for NPQ estimation

Quantifying the fate of excitation energy (energy partitioning) is important for a fully understanding of the response of photosynthetic apparatus to environmental factors as well as acclimation mechanisms. Besides the aforementioned dark relaxation kinetic method, a unified approach known as "energy partitioning in PSII complexes" was performed to assess directly the fraction of absorbed energy that is utilized via photochemistry or dissipated through different mechanisms (*Genty et al., 1989; Weis and Lechtenberg, 1989; Demming-Adams et al., 1996*). The main benefit of the quantum yield approach is that it takes into account the rate constants of every process involved in the quenching mechanisms of excited chlorophylls (*Hendrickson et al., 2004 and 2005*). For this reason, several methods of energy partitioning has been developed since Genty et al. proposed in 1989 their successful model (*Demming-Adams et al., 1996; Cailly et al., 1996; Kramer et al., 2004)*. However, an important step in this field was taken by Hendrickson et al. (2004) who introduced the quantum yield of NPQ (ΦNPQ), providing a more quantitative analysis of energy partitioning (*Kornyeyev and Hendrickson, 2007*). Recently, Ahn et al. (2009) have extended this approach, expressing the NPQ components as quantum yields (ΦqE, ΦqT + qI) and demonstrating the utility of this resolution (*Ahn et al., 2009*).

Because the experiments conducted for this project of research led to the elaboration of a revised method, an extended review of this approach is reported in chapter 4 of this thesis.

Chapter 2: Aim of the research

Summary

The project of research for my PhD has been based on the NPQ of the Chl *a* fluorescence. As reported in the previous chapter, this mechanism, even if it is widely studied, still has many unsolved interrogatives. This project has been developed pursuing several experiments, utilizing different approaches and techniques, with the ultimate goal of elucidating the role of the NPQ components in different environmental conditions. In this chapter, an outline of all the experiments performed and their reasoning are provided.

2.1 Goals of the experiments performed

NPQ is essential for the life of algae and higher plants. This complex mechanism is a composite of at least three different processes (qE, qT and qI). Most of the researches have been focused on the component qE (energy dependent) and some peculiar features for its activation have been found in higher plants (chapter 1). Only in the last few years, the researchers have been focused also on algae, but the mechanism in these organisms is still unclear. Moreover, it is still uncertain the variability of each component of NPQ when external parameters, such as incident light change. In this doubtful yet interesting background, different experiments have been performed with the aim of clarifying some of the characteristics of this powerful mechanism, such as NPQ.

This project started with an analysis of the effect of different light intensities on the resolution of the three components of NPQ in *Spinacia oleracea L.* (chapter 3). Previous experiments, conducted for my Bachelor degree, had revealed that the qE could not be the most important component of NPQ if leaves of *Arabidopsis thaliana* were exposed for 40 minutes at different light intensities (85 to 1200 μmol phot $m^{-2}s^{-1}$). Additionally, it had seemed that the *npq1* mutant, even lacking the VDE enzyme and consequently the qE component, could adjust the NPQ total value in response to PFDs changes (*D'Ambrosio et al., 2008*). Then, in order to confirm the suggested idea, the analysis of NPQ components was performed on *Spinacia* leaves at different light intensities with the additional infiltration nigericin. This uncoupler, inhibiting the qE component of NPQ, permitted the analysis of the variation for the other two components (qT and qI). For this experiment, the relaxation kinetic method from Walters and Horton (1990) was partly revisioned (chapter 3).

Later on, the article of Ahn *et al.* (2009) and the interest for the energy partitioning approach, led to the performance of an experiment on *Beta vulgaris*. This experiment, with 10

minutes of illumination at different light intensities and 40 minutes of dark relaxation, allowed the elaboration of a revised method for the assessment of the NPQ components (chapter 4). This approach merged together the relaxation kinetic of Walters and Horton (1990) and the energy partitioning method (*Guadagno et al., 2010*).

The publication of the aforesaid analysis, originated the collaboration with Dmytro Kornyeyev (School of Engineering, University of California, Merced, CA, 95343, USA ; Institute of Plant Physiology and Genetics, Vasylkivska St. 31/17, 03022, Kyiv, Ukraine). This cooperation has been funded on the common intent of reviewing the different approaches toward the definition of a unified method. The assessment of energy partitioning in PSII complexes was reached through a re-elaboration of data of chlorophyll fluorescence (chapter 5).

Then, thanks to a course on the Nuclear Magnetic Resonance (NMR) held by the University Federico II, I had the chance to learn the basis of this powerful technique. As a consequence, a collaboration with the Prof. Marina Della Greca (Department of Organic Chemistry, University of Naples Federico II) allowed the execution of NMR experiments on liquid extracts from different plant samples. The obtained spectra showed interesting results confirming the initial idea of the possibility of using the NMR for detecting light stress in plants (chapter 6).

All the aforementioned experiments have been performed at the Department of Structural and Functional Biology – University of Naples Federico II, in the laboratory of Photosynthesis with the Prof. Nicola D' Ambrosio as a supervisor.

Besides these, I have been taking advantage of an experience in a foreign laboratory since the second year of my PhD course. I have had the chance to spend total eight months in the laboratory of the Department of Plant and Molecular Biology – University of California, Berkeley, with the Prof. Krishna K. Niyogi as a supervisor.

During the first period in this laboratory abroad, an analysis of NPQ components repartition in different mutant lines of *Arabidopsis thaliana* was performed, in order to evaluate the reliability

of the revised energy partitioning method on different mutant lines in the NPQ mechanism (chapter 7).

During my second visit to the Berkeley laboratory, I have had the possibility to work for two different projects focused on the unicellular alga *Chlamydomonas reinhardtii.*

With the aim of clarifying the role of the different isoforms of LHCSR proteins in the alga, *npq4* mutant of *Chlamydomonas reinhardtii* has been transformed with UV mutagenesis and a suppressor screening has been performed. The phenotypical and biochemical characterization of the successful strain has been executed too (chapter 8).

Lastly, an HL transfer experiment of *Chlamydomonas reinhardtii* with chlorophyll fluorescence measurements and biochemical analyses has been completed, in order to find the time points of interest for NPQ induction and LHCSR proteins accumulation (chapter 9).

Chapter 3: Effect of different light intensities on the resolution of the three components of NPQ in *Spinacia oleracea*

Summary

In this chapter, the comparison between the relaxation kinetics of NPQ in control leaves of *Spinacia oleracea* and in leaves infiltrated with nigericin is proposed. The main aim of this experiment is to elucidate the contribute of qE to total NPQ. Furthermore, the influence of the ΔpH variation on the other two components (qT and qI) and on the total photosynthetic efficiency at different photon flux densities will be discussed.

3.1 Introduction

As reported in the first chapter, one of the strategies for plants and algae to avoid deleterious effects of excessive excitation energy is the thermal dissipation (NPQ). This mechanism is reported to be a composite of at least three mechanisms (qE, qT and qI). However, in literature it is widely reported that the ΔpH-dependent quenching (qE) represents the major and most important component, under the largest part of environmental conditions (*Kalituho et al. 2007*; *Crouchman et al. 2006*; *Niyogi et al. 2005*; *Ruban et al. 2002; Müller et al. 2001; Li et al. 2000*). Previous results, collected during the period of my Bachelor degree, showed that qE was not the main component of NPQ at certain experimental conditions. In particular, plants of *Arabidopsis*, line *Col-0* (wild type) and *npq4* (mutant lacking VDE) exposed to different light treatments for 40 minutes, did not show significative differences in the total NPQ value, even if the mutant line is unable to accumulate Zea in HL conditions. It seemed clear that the three mechanisms of quenching are able to adjust their extent in order to maximize the photoprotection and that qE is not always the major component NPQ (*D'Ambrosio et al., 2008*). In order to validate this evidence, the first experiment performed for this project of research evaluates the three components of NPQ, by their different relaxation kinetics in darkness after a light period, in intact leaves of *Spinacia oleracea*. Spinach leaves were infiltrated with nigericin. This antibiotic acts as an_ionophore and, inhibiting the trans-thylakoidal ΔpH, reduces or totally removes the qE component of NPQ (*Quick and Stitt, 1989; Ruban and Horton, 1995*). Furthermore, the influence of the qE changes on the other two components and on the PSII photochemical efficiency at different photon flux densities was investigated.

3.2 Materials and methods

Photosynthetic efficiency of fully developed *Spinacia* leaves was assessed by simultaneous measurements of chlorophyll fluorescence (MINI-PAM, Walz, Germany) at different light intensities: 85 (low light, LL), 450 (middle light, ML), 1200 (high light, HL) µmol photons m^{-2} s^{-1}. To allow the infiltration spinach leaves were cut off about 2 cm from the base and transferred in a microcuvette containing 50 µM nigericin solution (Sigma-Aldrich). Detached leaves were infiltrated with water as a control. The influence of detaching on photosynthetic performance was also assessed by measuring attached leaves.

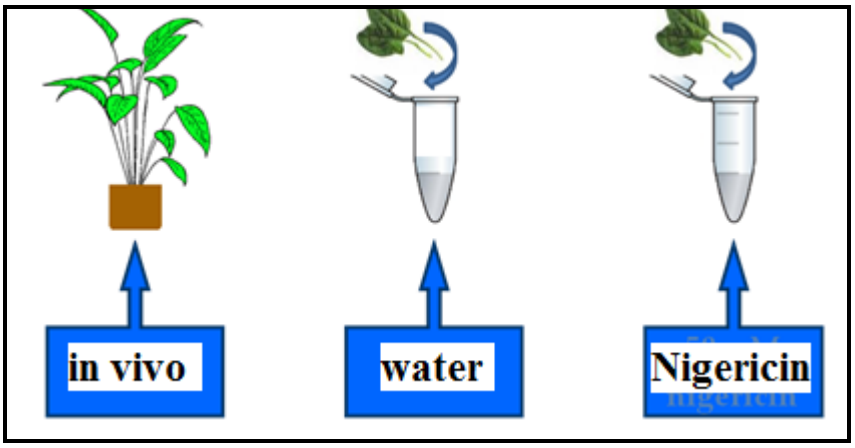


Figure 19: Different conditions for measurements of *Spinacia* leaves

All leaves were darkened for 20 minutes to measure the PSII photochemical efficiency (F_v/F_m) and then illuminated for 2 hours at different PFDs. In detached leaves two hours of illumination assured the uptake of nigericin which was valued by the measurement of transpiration (about 3.7 mmol H_2O m^{-2} s^{-1}). At the end of the light period, leaves were darkened to measure the relaxation kinetics of NPQ by applying saturating pulses at different time from the beginning of dark period (2, 5, 10, 20, 30 and 40 min). Resolution of three NPQ components (qE, qT and qI) was performed according to the modified Walters and Horton's procedure (*1990, 1991*). NPQ data were reported in a semi-logarithmic plot versus recovery time and the components of NPQ were

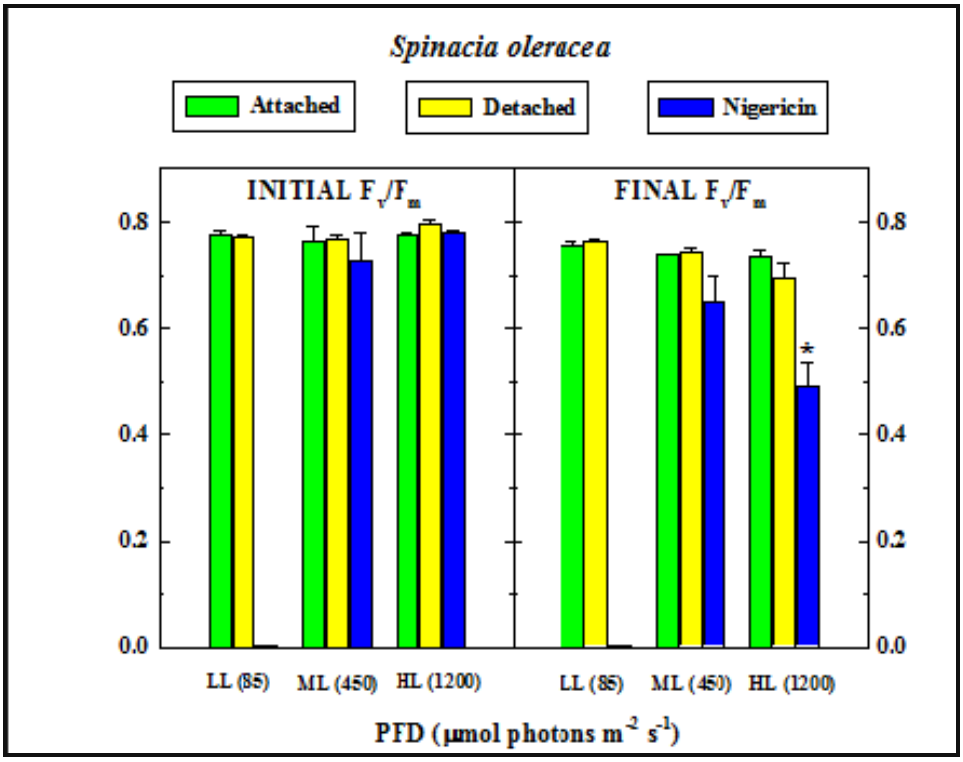
calculated by linear regression of three exponentially decaying components. The half-times for each components were referred as those of qE, qT and qI (*D'Ambrosio et al, 2008*).

All data reported are the average of measurements performed on three leaves. Data sets with a significance level of $P < 0.05$ according to Student's t test are marked with an asterisk.

3.3 Results and discussion

The value of F_v/F_m was checked in attached and detached leaves for assessing the possible decrease in the photosynthetic efficiency due to the detachment. Figure 20 clearly shows that this effect is null at all the PDFs considered. Only in the detached leaves infiltrated with nigericin the difference in the efficiency value is significant in respect to the attached leaves. This difference seems to be connected to the antibiotic infiltration and not to the previous detachment.

Figure 20: Fv/Fm values (initial and final) in spinach leaves at different light intensities



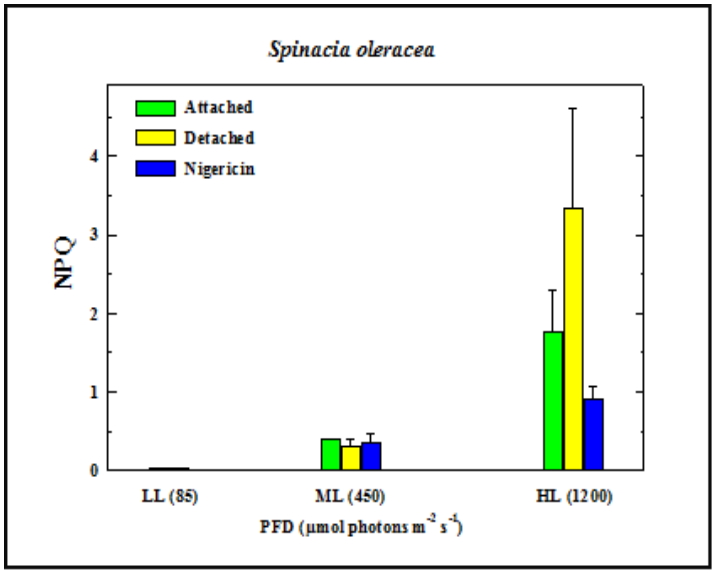
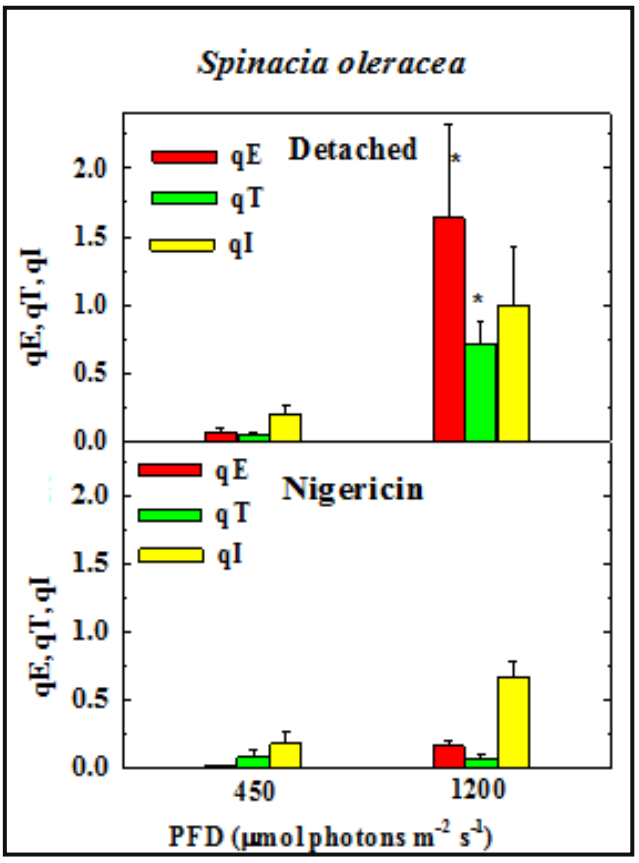


Figure 21: NPQ values in spinach leaves at different light intensities

At LL conditions, NPQ is absent in attached and detached leaves; consequently, no nigericin infiltration were done at this PFD (Fig.21). Additionally, at higher PFDs no significant decrement in the total NPQ value is observed in infiltrated leaves. On the other hand, nigericin infiltration induces a significant decrease of qE at ML (29 %) and HL (34 %) conditions, compared to detached leaves, and a change in the other two NPQ components (Fig. 22). It has to be highlighted that qE is not the major component of NPQ in our experimental conditions except for the highest PFD.

Figure 22:Absolute values for qE, qt and qI in spinach leaves at different light intensities

Interestingly, the results posits that the NPQ is a whole phenomenon which is able to funnel an invariable amount of energy at certain environmental conditions. At the same time NPQ is a composite of other mechanisms variable on the external conditions. It is suggested that the variation of one among qE, qT and qI causes the variation of the other two in order to maintain the same level of photoprotection. In fact, with the nigericin



infiltration, although the NPQ is unchanged, qE decrease influences significantly the other two components of NPQ. At ML condition a qT increase is observed, suggesting that the state transitions mechanisms completely compensate the qE decrease. On the other hand, at the highest PFD, qI strongly increases because a photoinibitory process occurrs. This is also outlined by a significant F_v/F_m decrement after illumination at the highest PFD in the infiltrated leaves (Fig. 20).

In conclusion, this experiment seems to confirm the idea that the value of the total NPQ is not affected by the variation in the qE extent during a long exposure to different light intensities. As for the *Arabidopsis* experiment (*D'Ambrosio et al., 2008*), even in this case the difference is showed in the consequent re-adjustment in the extent of the other two components. Consequently, the repartition of the light energy into the different mechanisms appears to be deeply dependant on the experimental conditions and sometimes qE is not the major component of total NPQ coefficient.

These results urge to elaborate a method which can allow the analysis of the energy partitioning considering the great variability of the NPQ mechanisms. This is the goal for the experiments reported in the next two chapters (chapter 4 and 5).

Chapter 4: A revised energy approach to assess the yields of NPQ components

Summary

Recently, the NPQ components have been resolved as quantum yields according to the energy partitioning approach that takes into account the rate constants of every process involved in the quenching mechanisms of excited chlorophylls. In this chapter, a fully extended quantum yield approach and the introduction of novel equations to assess the yields of each NPQ components are presented. Furthermore, a complete analysis of the yield of NPQ in *Beta vulgaris* exposed to different irradiances has been carried out. In agreement with experimental results here it is shown that the previous approach may amplify the yield of qE component and flatten the quantitative results of fluorescence analysis. Moreover, the significance of taking into account the physiological variability of NPQ for a correct assessment of energy partitioning is demonstrated.

Preface

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4.1 Introduction

As reviewed in the first chapter, the light energy absorbed by plants can be utilized via photochemistry or dissipated as heat or fluorescence. The above mechanisms are competitive and any variation in the quantum efficiency of one will elicit complementary changes in the yields of the others. Quantifying the fate of excitation energy (energy partitioning) is important for a fully understanding of the response of photosynthetic apparatus to environmental factors as well as acclimation mechanisms.

Thermal dissipation is ascribed to a composite of processes collectively termed NPQ (*Walters et al., 1996*). Initially, the resolution of these components was conducted by the analysis of dark relaxation kinetics of chlorophyll fluorescence quenching (*Quick and Stitt, 1989; Walters and Horton, 1993*). In the meantime, a unified approach known as "energy partitioning in PSII complexes" was performed to assess directly the fraction of absorbed energy that is utilized via photochemistry or dissipated through different mechanisms (*Genty, 1989;.Weis and Lechtenberg, 1989; Demming- Adams et al., 1996*).

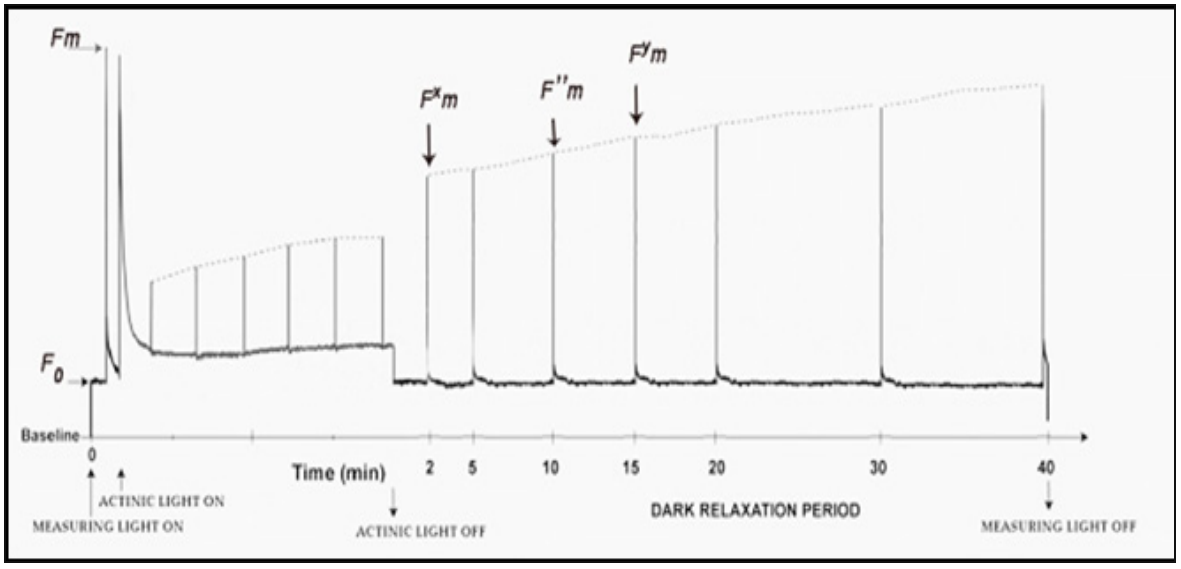
The main benefit of the quantum yield approach is that it takes into account the rate constants of every process involved in the quenching mechanisms of excited chlorophylls (*Hendrickson et al., 2004, 2005*). For this reason, several methods of energy partitioning has been developed since Genty et al. proposed in 1989 their successful model (*Demming- Adams et al., 1996; Cailly et al., 1996; Kramer et al., 2004)*. However, an important step in this field was taken by Hendrickson et al. (2004) who introduced the quantum yield of NPQ (Φ_{NPQ}), providing a more quantitative analysis of energy partitioning (*Kornyeyev and Hendrickson, 2007*). Recently, Ahn et al. (2009) have extended this approach, expressing the NPQ components as quantum yields (Φ_{qE}, $\Phi_{qT+\,qI}$) and demonstrating the utility of this resolution (*Ahn et al., 2009*). With the work proposed

in this chapter we further extend the quantum yield approach taking into account each component of NPQ (Φ_{qE}, Φ_{qT} and Φ_{qI}). Actually, we demonstrate that the method of Ahn et al. 2009 may amplify the yield of qE and flatten the quantitative results of fluorescence analysis because it does not separate Φ_{qT} and Φ_{qI} and considers a standard relaxation time for qE component. Subsequently, the previous method does not effectively take into account the physiological variability of NPQ. Thus, here we determine new equations that more accurately describe the quantum yields of three different NPQ components.

4.2 Materials and methods

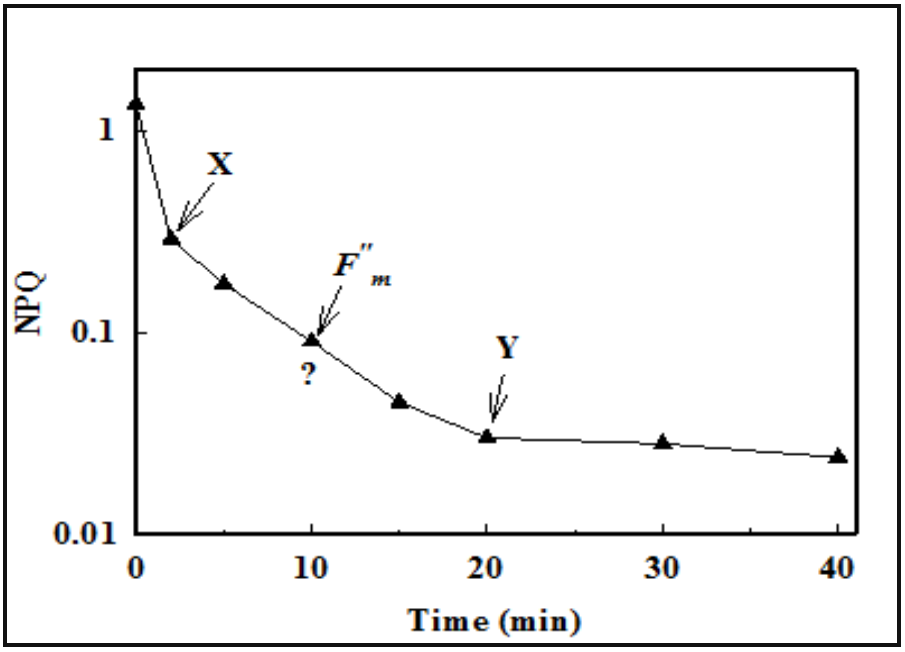
Garden beet (*Beta vulgaris* L.) plants were grown in pots filled with 1:1 peat:soil and watered when required. The plants were grown in a controlled growth chamber with 8/16 h day/night (D/N) photoperiod, 25/20 °C D/N temperature and 65/85 % D/N relative humidity (RH), with a growth irradiance of 150 μmol photons m^{-2} s^{-1}.Chlorophyll *a* fluorescence emissions were measured using a pulse amplitude modulated fluorimeter (PAM-2000, H.Walz, Effeltrich, Germany).

Figure 23: Example of fluorescence kinetic curve obtained from the experimental protocol in *Beta vulgaris* leaves. Fxm, Fym and F"m are fluorescence values utilized in revised equations and in Ahn et al. (2009)



After a dark period (30 min), the maximum fluorescence (F_m) was determined applying a saturating pulse (0.8 s) with an intensity higher than 5,000 µmol photons m^{-2} s^{-1}. Then, leaves of beet were exposed for 10 minutes to different photon flux densities (PFDs) (85, 180, 450, 750 and 1200 µmol photons m^{-2} s^{-1}) to obtain F'_m value. After the induction period the recovery of fluorescence signal in darkness for 40 minutes was monitored, applying seven saturating pulses at different time (2, 5, 10, 15, 20, 30, 40 min) from the beginning of dark period. The complete experimental protocol for the fluorescence measurements is reported in Fig. 23. NPQ data were expressed as NPQ = $(F_m-F'_m)/F'_m$ (*Bilger and Schriber, 1986*) and the resolution of the three NPQ components (qE, qT and qI) was performed according to the method of Walters and Horton (*Walters and Horton, 1990, 1991*) partially modified. NPQ data were reported in a semi-logarithmic plot versus recovery time and the components of NPQ were calculated by linear regression of three exponentially decaying components [Fig. 24].

Figure 24: Semi-logarithmic plot of NPQ values to relaxation time in darkness. The significant changes in the slope of the curve are termed x and y. No variation in the slope is detected for F"m at 10 minutes of relaxation



4.2.1 Fluorescence parameters and rate constants

In photobiology one of the main conceptions is that the quantum yield of any process, contributing to utilization or dissipation of absorbed light energy, is equal to the ratio of the rate constant for this process to the sum of all rate constants including the other utilization/dissipation processes (*Genty et al., 1989; Kramer et al., 2004; Kitajima and Butler, 1975; Rohacek, 2002*). So it is very important that useful parameters derived from fluorescence measurements can be defined in terms of ratios of rate constants. F_m and F'_m are maximum levels of fluorescence for a leaf sample in darkness or light conditions, respectively. F_s is the steady-state fluorescence level at light-adapted conditions. These quantities are defined as $F_m = G \frac{k_F}{k_C}$, $F'_m = G \frac{k_F}{k_C + k_{NPQ}}$, $F_s = G \frac{k_F}{k_C + k_{NPQ} + k_P}$, where k_P, k_{NPQ}, k_C are the rate constants for photochemistry, non-photochemical processes and photophysical decay, respectively, and G is the instrumental gain coefficient (*Hendrickson et al., 2004; Turro, 1991*). Recently Ahn et al. (2009) further dissected k_{NPQ} parameter as the sum of $k_{qE} + k_{qT} + k_{qI}$ introducing F''_m. This parameter is the maximum level of fluorescence measured at 10 minutes of darkness after the illumination period, defined as:

$$F''_m = G \frac{k_F}{k_C + k_{qT} + k_{qI}} \tag{4.1}$$

From above, the magnitude of NPQ, according to the Stern-Volmer coefficient, corresponds to the ratio of rate constants for the NPQ process relative to the intrinsic photophysical decay (k_C):

$$NPQ = \frac{F_m - F'_m}{F'_m} = \frac{k_{NPQ}}{k_C} \tag{4.2}$$

Similarly the ratio of the rate constants for photochemical pathways is equal to (*Ahn et al., 2009*):

$$PC = \frac{F'_m - F_s}{F'_m} x \frac{F_m}{F_s} = \frac{k_P}{k_C}$$

(4.3)

4.2.2 Analysis of energy partitioning

The main benefit of the energy partitioning approach is given from the following equation:

$$\Phi_{PSII} + \Phi_{NPQ} + \Phi_C = \frac{k_C + k_{NPQ} + k_P}{k_C + k_{NPQ} + k_P} = 1$$

(4.4)

that lets each yields to be solved so that the energy utilized through each pathway can be estimated (*Hendrickson et al., 2004; Kramer et al., 2004; Ahn et al, 2007*). Since the quantum efficiency of PSII photochemistry has been previously defined in terms of the ratio of rate constants as (*Hendrickson et al., 2004; Kramer et al., 2004; Genty et al., 1989)*:

$$\Phi_{PSII} = \frac{k_P}{k_C + k_{NPQ} + k_P} = \frac{F'_m - F_s}{F'_m}$$

(4.5)

the following ratios can be used to describe the quantum yields of NPQ and of chlorophyll photophysical decay of a light-adapted leaf sample (*Hendrickson et al, 2004; 2005*):

$$\Phi_{NPQ} = \frac{k_{NPQ}}{k_C + k_{NPQ} + k_P} = \frac{F_m - F'_m}{F_m} x \frac{F_s}{F'_m}$$

(4.6)

$$\Phi_C = \frac{k_C}{k_C + k_{NPQ} + k_P} = \frac{F_s}{F_m}$$

(4.7)

According to Ahn et al.(2009) it is also possible to separate the quantum yields for the different processes of NPQ. For example, Φ_{qE} is defined as:

$$\Phi_{qE} = \frac{k_{qE}}{k_C + k_{NPQ} + k_P} = \frac{F''_m - F'_m}{F''_m} x \frac{F_s}{F'_m} \tag{4.8}$$

while the sum of Φ_{qT} and Φ_{qI} is obtained from

$$\Phi_{qT+qI} = \frac{k_{qT} + k_{qI}}{k_C + k_{NPQ} + k_P} = \frac{F_m - F''_m}{F_m} x \frac{F_s}{F''_m} \tag{4.9}$$

Here we demonstrate how the equation (4.9) proposed by Ahn et al. (2009) does not take into account the physiological variability of NPQ relaxation kinetic and here we introduce two novel useful fluorescence parameters F^x_m and F^y_m. These can be defined as the levels of fluorescence corresponding at the time of the first and second variation in the slope of darkness relaxation kinetic of NPQ, respectively (*Walters and Horton, 1990; 1991; 1993*).

F^x_m can be termed as:

$$F^x_m = G \frac{k_F}{k_C + k_{qT} + k_{qI}} \tag{4.10}$$

and represents the maximum fluorescence obtained during the dark relaxation, after the qE component of NPQ has completely relaxed, and qT and qI remain as the only active components of NPQ (*Kornyeyev and Hendrickson, 2007; Kramer et al., 2004; Muller et al., 2001; Dall'Osto et al., 2005*). The maximum fluorescence during the dark period after the total relaxation of qT component is referred to as F^y_m and can be get out from:

$$F^{y}_{m} = G\ \frac{k_F}{k_C + k_{qI}}$$

(4.11)

where qI is the only NPQ component still active. Using the parameters proposed above, further equations expressing the quantum yield for each of the three NPQ components can be written. The efficiency of the fast component of NPQ (qE) can be defined as:

$$\Phi_{qE} = \frac{k_{qE}}{k_C + k_{NPQ} + k_P} = \frac{F^{x}_{m} - F^{'}_{m}}{F^{'}_{m}} x \frac{F_s}{F^{x}_{m}}$$

(4.12)

while the quantum yield for state transitions component can be calculate as:

$$\Phi_{qT} = \frac{k_{qT}}{k_C + k_{NPQ} + k_P} = \frac{F^{y}_{m} - F^{x}_{m}}{F^{x}_{m}} x \frac{F_s}{F^{y}_{m}}$$

(4.13)

Finally, the quantum yield for the slowest NPQ component results as:

$$\Phi_{qI} = \frac{k_{qI}}{k_C + k_{NPQ} + k_P} = \frac{F_m - F^{y}_{m}}{F^{y}_{m}} x \frac{F_s}{F_m}$$

(4.14)

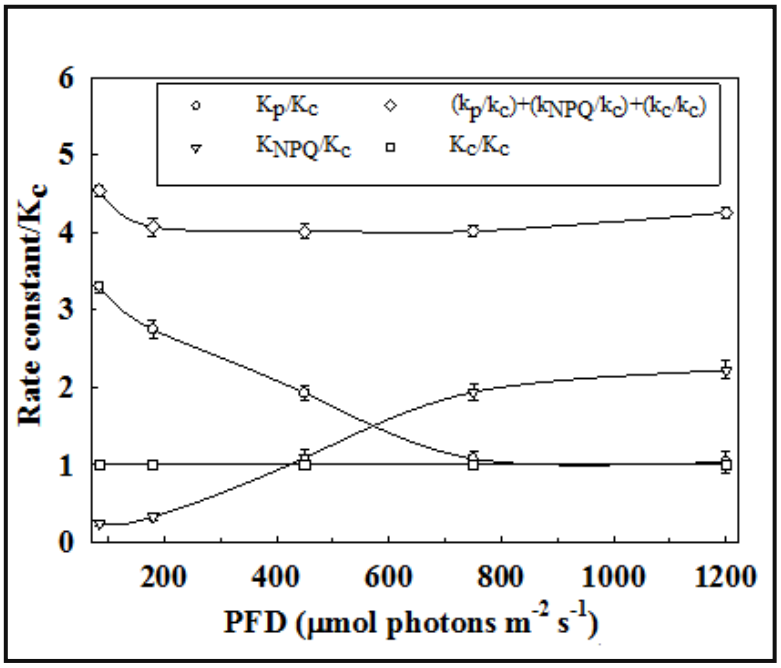
The accurate version of energy partitioning approach here proposed is useful to highlight information that could be hidden in the total Φ_{NPQ} parameter.

All data of chlorophyll *a* fluorescence reported in this chapter are the average of measurements performed on at least seventeen different plants. Differences between methods were analysed by the Student's t test based on a significance level of $P < 0.05$.

4.3 Results

In order to assess the light-dependent dynamics of the rate constants (Eqs. 4.2 - 4.3) we plotted the ratios of rate constants (k_{NPQ} , and k_P and k_C) at the steady-state conditions relative to k_C , as estimated in leaves of *Beta vulgaris* exposed to PFDs ranging from 85 to 1200 μmol photons m^{-2} s^{-1} [Fig. 25]. As expected, in accordance to previous works (*Ahn et al., 2009; Makino et al., 2002*), both k_P / k_C and k_{NPQ} / k_C demonstrate a dynamic trend in response to the variable PFDs.

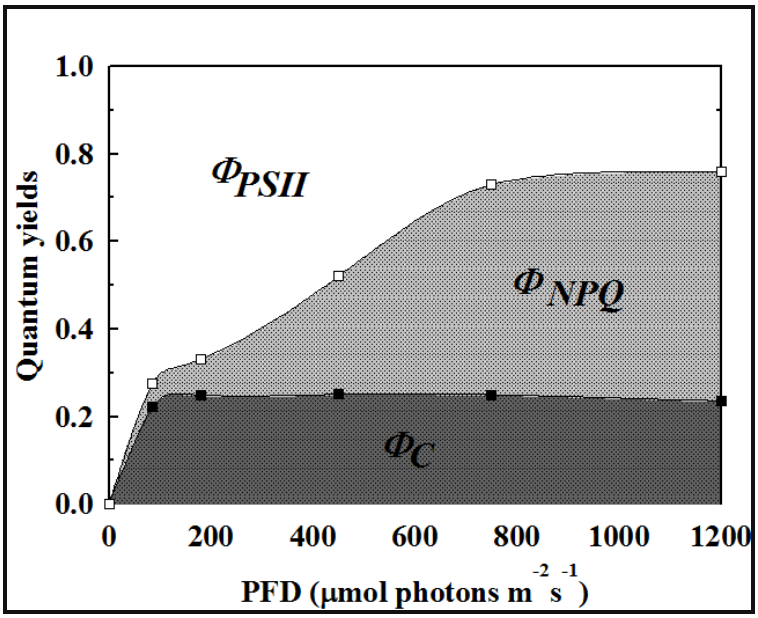
Figure 25:Plot of rate constants/kc versus PFD.



The k_P / k_C ratio decreases sharply from 85 to 750 μmol photons m^{-2} s^{-1} and gets to an almost constant value at the highest PFD. Differently, the k_{NPQ} / k_C first increases gradually from 85 to 180 μmol photons m^{-2} s^{-1}, then rises up to 750 μmol photons m^{-2} s^{-1} and reaches a value fairly constant at 1200 μmol photons m^{-2} s^{-1}. Although the k_C / k_C component is obviously equal to 1.0 over the entire range of PFDs, the sum of all the rate constants ($\sum_i k_i$, i = C, NPQ and P) is variable because k_P / k_C and k_{NPQ} / k_C have different light-dependent dynamics, especially at the lowest PFDs. Then, with the aim of reaching a comprehensive description of energy partitioning we analyzed our results using the quantum yield convention. Interestingly, this approach allows to estimate the fraction of absorbed irradiance consumed through various utilization and dissipation pathways by the application of Eqs. (4.5), (4.6) and (4.7), as underlined

from previous authors (*Kornyeyev and Hendrickson, 2007; Hendrickson et al., 2004; Hendrickson et al., 2005; Ahn et al., 2009*).

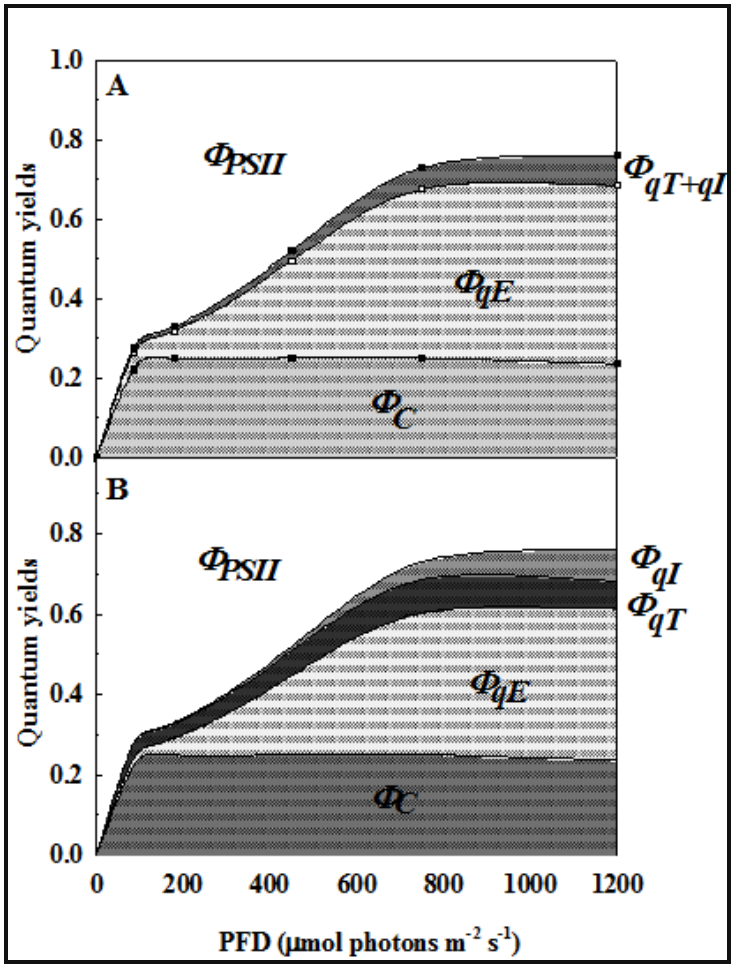
Figure 26:Estimated fraction of absorbed light energy consumed via various utilization and dissipation pathways in *Beta vulgaris* leaves after 10 min of illumination at different PFDs (85, 180, 450, 750 and 1200 µmol photons m-2 s-1). The measured parameters include photochemistry (Φ_{PSII}), non-photochemical processes (Φ_{NPQ}) and photophysical decay (Φ_C)



The fate of absorbed light energy according to Eq. (4.4) in beet leaves after 10 minutes exposure to irradiances between 85 and 1200 µmol photons m^{-2} s^{-1} is showed in Fig. 26. The area in dark grey corresponds to Φ_C and its value is quite constant at about 0.2 with little variations in the entire range of PFDs considered. As expected, these data demonstrate an increase of thermal dissipation, expressed as $\Phi_{NPQ,}$ strictly correlated to the light intensities. Furthermore, the residual upper section of this yield plot corresponds to Φ_{PSII} whose value decreases in a curvilinear manner with PFD. True to form, these results indicate that the major fraction of absorbed light energy is dissipated by non photochemical processes over the examined range of PFDs. Values of $\Phi_{PSII,}$ $\Phi_{C,}$ $\Phi_{qE,}$ and Φ_{qT+qI} for leaves exposed to different PFDs ranging from 85 to 1200 µmol photons m^{-2} s^{-1}, calculated according to the Eqs. (4.8) and (4.9) proposed by Ahn et al. (2009), are shown in Fig. 27A. The same data were also processed using our equations (4.12, 4.13 and 4.14) and the results

obtained are illustrated in Fig. 27B.

Figure 27:Quantum yields of photochemistry, qE, qT, qI and the photophysical decay (ΦPSII, ΦqE, ΦqT, ΦqI and ΦC, respectively) versus PFD in Beta vulgaris. Values are reported according to Ahn et al (2009) (A) and according to new equations proposed



Moreover, in both cases the total area occupied by the yields of NPQ components corresponds to the whole Φ_{NPQ} of Fig. 26. Indeed, the plot of Φ_{NPQ}, calculated according to the Eq. (4.6), versus the sum qE, qT and qI is linear with a slope of one whether they are calculated according to the Eq. (4.8) and (4.9) or whether in reference to the Eqs. (4.12), (4.13) and (4.14). The additivity underlined by Ahn et al. (2009) is still maintained as a key benefit of our revisioned approach [Fig.28].

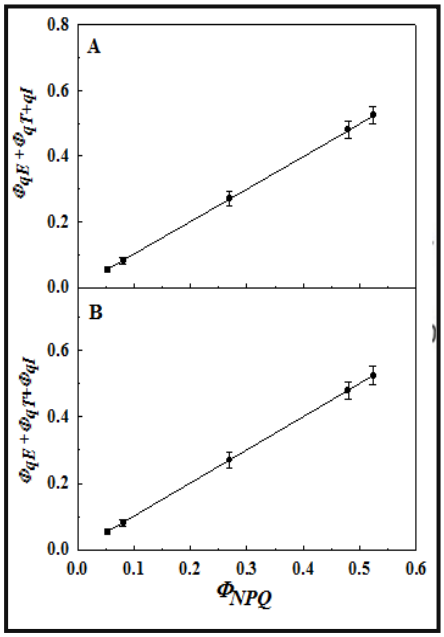
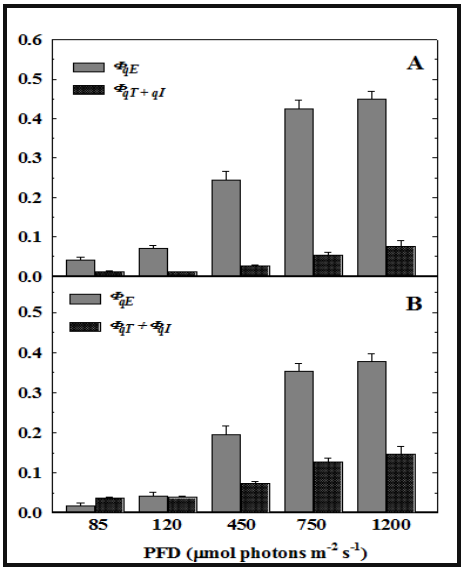


Figure 28:Plots of the sum of ΦqE + ΦqT+qI versus ΦNPQ according to Ahn et al. (2009) (A) and ΦqE + ΦqT + ΦqI versus ΦNPQ in our approach (B). Reported data demonstrate that the additivity is detected in both methods

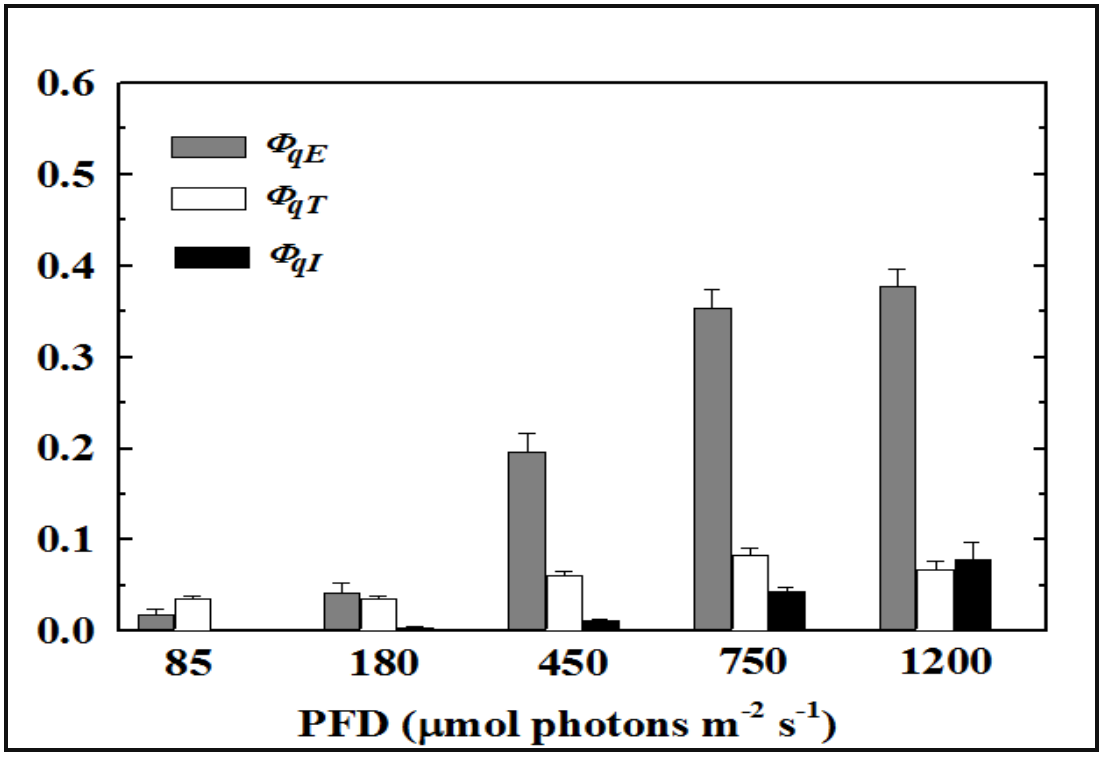
A comparison of the quantitative analysis of the Φ_{NPQ} components according to both procedures is presented in Fig. 29A-29B. In order to obtain a good quality corresponding with Φ_{qT+qI}, the value of Φ_{qT} and Φ_{qI} are represented as a sum [Fig. 29B]. Although the utilized equations are different, these data demonstrate that the major portion of Φ_{NPQ} over the PFD range is nearly always attributable to Φ_{qE}. Inevitably, the extent of Φ_{qE} is strongly light dependent and reaches its maximum at the highest PFDs. The quantitative contribution of each component in yield terms according to our revised equations is represented in Fig. 30.

Figure 29:The contributions of ΦqE and ΦqT+qI according to Ahn et al. (2009) (A) and the contributions of ΦqE and ΦqT + ΦqI in our revised approach (B) versus PFD



In particular, it has to be underlined that Φ_{qE} at the lowest irradiance of 85 μmol photons m^{-2} s^{-1} is not the major component of $\Phi_{NPQ,}$ then (at 180 μmol photons m^{-2} s^{-1}) it becomes higher than Φ_{qT} but without any significant difference.

Figure 30:The repartition into ΦqE, ΦqT and ΦqI in leaves of *Beta vulgaris* exposed to different PFDs ranging from 85 to 1200 μmol photons m^{-2} s^{-1}



Only with the increase of PFD (450 to 1200 μmol photons m^{-2} s^{-1}) Φ_{qE} value increases significantly and it becomes the main component of thermal dissipation as expected. So, according to a previous work (*Allen, 1992; Aro et al., 1996; Demming-Adams and Adams III, 2006*) the contribution of Φ_{qT}, depending on state transitions mechanisms, is the most important at the lowest PFDs, but interestingly it is still present even if in a moderate percentage at the higher PFDs. As usual in a short term quenching induction, Φ_{qI} is almost null up to 750 μmol photons m^{-2} s^{-1} and, although it reaches the highest value at 1200 μmol photons m^{-2} s^{-1} it is still significantly lower than Φ_{qE}. All these quantitative considerations about the contribution of each components to the total

NPQ yield have been clearly pointed out by using our revised approach of energy partitioning, otherwise by the previous method they were partly hidden.

4.4 Discussion

Our revised approach is based on the consideration that NPQ is a physiological mechanism characterized by a great variability. Sometimes changes which naturally occur in NPQ capacity, possibly on genetic basis, have been observed in different plant species (*Demming Adams, 1998; Demming-Adams and Adams, 1998*). Sun-acclimated plants have got up to four times as much NPQ capacity as low-light-acclimated plants of the same species (*Demmig-Adams, 1998; Demmig-Adams and Adams 1994; Osmondet al., 1993; Ruban, et al. 1993; Demmig-Adams et al., 1995; Brugnoli et al., 1994*).

The maximum extent of NPQ at saturation irradiance is also dependent on the plant growth conditions, primarily the quality of light, the air temperature and the plant species. This variability can influence the total NPQ extent but also the contribution of each single component to total thermal dissipation. Actually, qT and qI components may be larger than qE under certain physiological conditions and in different species as well as various mutants of the same species (*Ahn et al., 2009*). Therefore, we extended the energy partitioning approach taking into account the real relaxation time of each component of NPQ. Since the analysis of the kinetic curve of NPQ relaxation in darkness did not reveal any physiological variation in the NPQ components [Fig. 25], we reported NPQ values in a semi-logarithmic plot versus time appreciating the variations in the slope of the curve [Fig. 26]. As a result, the detection of two time points, termed as x and y, is achievable; these points correspond to clear changes in the slope and can be associated to the relaxation times of qE and qT component of NPQ. Consequently, the F^x_m and F^y_m, utilized in Eqs. (4.12), (4.13) an (4.14), are the fluorescence values at the time points x and y and are dependent on the environmental conditions and plant species. On the contrary, the point which corresponds to 10

minutes of dark relaxation, associated to F''_m is not characterized by any relevant variation in the NPQ value. So, we demonstrate that the application of a semi-logarithmic plot of NPQ values during time of dark relaxation is the basis for a careful choice of the relaxation time of NPQ components. In fact, the use of $F''_m = G \frac{k_F}{k_C + k_{qT} + k_{qI}}$ (*Ahn et al., 2009*) is not suitable for assessing a correct analysis of the yield of NPQ components. The F''_m is a too inflexible parameter because it is always taken after 10 minutes of relaxation in darkness of NPQ. At that time it is possible that qT component too is just relaxed and a consequent overestimation of Φ_{qE} value may occur. Moreover, by this method it is evidently not possible to specify if the increase in Φ_{qT+qI} originates with qT, qI or both components (*Ahn et al., 2009*). Using the new features, $F^x_m = G \frac{k_F}{k_C + k_{qT} + k_{qI}}$ and $F^y_m = G \frac{k_F}{k_C + k_{qI}}$, we were able to choose the more appropriate value. Our results clearly show that the value of Φ_{qE} calculated through the previous method is overestimated by 2 to 7 % with respect to the data calculated by our approach. The significant overestimation goes up with the increase of PFDs considered. To the same extent the value of Φ_{qT+qI} [Fig. 29A] is decreased significantly respect to $\Phi_{qT} + \Phi_{qI}$ [Fig. 29B] with a consequent flattening of the contribution of state transitions and photoinhibitory quenching to total NPQ. Therefore, our method unveils a significant overestimation for Φ_{qE} and a parallel as much significant flattening in the sum of the other two components ($\Phi_{qT} + \Phi_{qI}$) in the results analyzed according to Ahn et al. procedure (2009). Furthermore, our analysis comes up to the requirement of a complete separation for the contributions to total thermal dissipation of the three NPQ components as quantum yields [Fig. 30]. In fact, we were fully succeeded in the separation of Φ_{qT} and Φ_{qI} introducing the revised equations $\Phi_{qT} = \frac{F^y{}_m - F^x{}_m}{F^x{}_m} x \frac{F_s}{F^y{}_m}$ and $\Phi_{qI} = \frac{F_m - F^y{}_m}{F^y{}_m} x \frac{F_s}{F_m}$. Our revised energy partitioning approach allows easily the separation of Φ_{qE}, Φ_{qT} and Φ_{qI} but does not modify the extent of Φ_{PSII} and Φ_C (Fig.

27A-27B) considering that these latter parameters do not derived from the relaxation kinetic. However, the results of our reconsideration of the previously proposed equations for the calculation of the quantum yields for NPQ components do not change the additivity benefit [Fig.28] that still remains as a key gain of our approach. In summary, this original repartition leads to a complete analysis of energy partitioning giving the right importance to each NPQ component and demonstrates that is possible to fully analyze Φ_{NPQ} by using the new equations [Eqs. (4.12)-(4.14)] here introduced. We propose that this revised approach of energy partitioning will facilitate a better understanding of the natural photoprotection and of such a variable mechanism as NPQ.

Chapter 5: Assessment of energy partitioning in PSII complexes using chlorophyll fluorescence analysis: reviewing the different approaches toward the definition of a unified method

Summary

In most cases, analysis of NPQ components is based on the changes of either F_m or F_v values and calculations of non-photochemical quenching coefficients. Although it gives an idea about contribution of each component to light-induced NPQ, the approach does not provide values that can be directly compared to the efficiency of photochemical quenching (*Maxwell and Johnson, 2000*). The estimation of quantum yields of various NPQ components, seems to be an attractive alternative (chapter 4) because it provides researchers with a detailed picture of energy partitioning in PSII complexes. This makes it possible to obtain valuable information about how plants manage the light energy absorbed by PSII antennae. A number of different versions of the equations that calculate energy distribution in PSII have appeared, leading to a sense of confusion, but the arena for testing of ideas has been set. Several groups have recently proposed formulas that seem to have strong similarities and reflect basically the same approach despite differences in details. These studies used the representation of quantum yields as ratios of the rate constants (*Kitajima and Butler, 1975; Genty et al., 1989; Demming-Adams et al., 1996; Rohàček, 2002*) and described energy partitioning under illumination, when PSII reaction centers are partly closed. In this Chapter a review of the methods utilized for the energy partitioning approach is presented. The aim of this work, in collaboration with Dmytro Kornyeyev, was to demonstrate the similarities among different approaches and to pave the way for a unified complete method of the energy partitioning.

Preface

The dissertation presented in this chapter is part of the manuscript **"**Assessment of energy partitioning in PSII complexes using chlorophyll fluorescence analysis: reviewing the different approaches toward the definition of a unified method" from Kornyeyev, D., Guadagno, C.R.., and D'Ambrosio, N. (*submitted*).

5.1 Introduction

The chlorophyll fluorescence analysis has been applied to estimate the distribution of excitation energy in PSII complexes for a long time. The classical work by Butler and Kitajima (1975) described means to calculate the quantum yield of biochemistry, i.e., the probability for charge separation, introducing a parameter that estimates the efficiency of photochemistry in PSII complexes. The calculations were based on the comparison of the chlorophyll fluorescence levels under conditions when all reaction centers (RCs) of PSII were open (F_o) or closed (F_m). PSII units with open and closed RCs are often identified as those with the primary quinone acceptor (Q_A) in oxidized and reduced form, respectively. The quantum yield of PSII photochemistry was estimated as $\Phi_{PSII} = (F_m - F_o)/F_m = F_v/F_m$ (*Schreiber et al., 1995*).

However, as it was pointed out by Genty *et al.* (1989), only part of PSII RCs are opened under illumination. The comparison between the chlorophyll fluorescence levels obtained when PSII RCs were partially opened (F') or closed (F'_m) was proposed as an estimate of the quantum yield of linear electron transport ($\Phi_e = (F'_m - F)/F'_m = F'_q/F'_m$). This parameter is also known as operational efficiency of PSII (*Baker et al., 2007, Baker, 2008*). It has to be noted that the prime sign is used to distinguish the fluorescence parameters measured when samples (leaves) were light-acclimated. The parameter $1 - F'_q/F'_m$ has been shown to correlate with quantum yield of CO_2 fixation and, despite this correlation can be affected by alternative electron sinks (*Ort and Baker, 2002*), the parameter has tremendous practical value, proven by decades of extensive application (*Maxwell and Johnson, 2000*).

After the actinic light is switched off, all PSII RCs became open and the fluorescence reaches the level termed as F'_o. The ratio $(F'_m - F'_o)/F'_m = F'_v/F'_m$ estimates quantum yield of PSII biochemistry (Φ_{PSII}) in light-acclimated state. Unlike F'_q/F'_m (Φ_e), F'_v/F'_m (Φ_{PSII}) is called the

maximal quantum efficiency of PSII under the given light conditions (*Baker and Rosenqvist, 2004; Baker et al,. 2007*). Φ_e and Φ_{PSII} reflect real (under actinic illumination) and potential (light-acclimated samples with actinic light off) quantum yields of PSII photochemistry, respectively. The PSII operating efficiency is lower than the PSII maximum efficiency because a fraction of PSII centres are closed in the light-acclimated state (*Oxborough and Baker, 2000; Baker and Oxborough, 2005*).

The amplitude of F_v/F_m ratio measured for dark-acclimated leaves is higher than that of F'_v/F'_m for light-acclimated leaves due to the development of the structural and functional changes in the pool of PSII complexes leading to the increased probability for excitation energy in PSII to be dissipated as heat. Thermal dissipation of excitation energy, known also as the non-photochemical quenching (NPQ), is measured by analyzing the decrease (quenching) of the chlorophyll *a* fluorescence. Despite NPQ brings about a decrease in the quantum yield of PSII photochemistry, it is generally accepted that thermal dissipation helps plants to deal with excessive light energy absorbed by light harvesting complexes. In other words, NPQ plays an important role in photoprotection by regulating the distribution of excitation energy in PSII complexes (*Demmig-Adams and Adams, 1996; Müller et al., 2001; Ruban et al., 2007*).

The combined quantum yield of non-photochemical processes (thermal dissipation and fluorescence) can be estimated by subtracting quantum yield of PSII photochemistry from unity. Quantum yield of non-photochemical dissipation for illuminated sample (Φ_N) is equal to 1 - Φ_e (Φ_N = 1- F_q'/F_m' = F'/F_m'). In case of light-acclimated sample with all reaction centers open after the switching off of the actinic light, quantum yield of non-photochemical dissipation is equal. NPQ has several components that vary in the rate of relaxation in the darkness (*Quick and Stitt, 1989; Walters and Horton, 1993; Muller et al., 2001*). As reported in Chapter 1, three major components of NPQ have been identified: a fast-relaxing component that was associated with the ΔpH-

dependent high-energy-state quenching and termed as qE (*Niyogi et al., 2005; Pascal et al., 2005*), a component qT, which is associated with state transition and has medium rate of relaxation (*Quick and Stitt 1989*) and a slow-relaxing component qI related to photoinhibition of PSII (*Walters and Horton, 1993; Lee et al., 2001; Matsubara et al., 2004*).

5.2 Results and discussion

The origins of the approach mentioned above can be traced back to work by Cailly *et al.* (1996) who introduced the formula for the quantum yield of NPQ activated as a result of illumination ($\Phi_{NPQ} = \frac{F'}{F_m'} - \frac{F'}{F_m}$). It is an estimate for the sum of quantum yields of all light-induced components qE, qT, and qI for an illuminated sample ($\Phi_E + \Phi_T + \Phi_I$). The formula was brought back to light in Hendrickson *et al.* (2005). The manipulation with the time of dark relaxation allows one to distinguish between components that relax and do not relax during dark acclimation, i.e., $\Phi_E + \Phi_T$ and Φ_I, respectively (*Kornyeyev and Hendrickson, 2007*):

$$\Phi_E + \Phi_T = \frac{F'}{F_m'} - \frac{F'}{F_m''} \qquad (5.1)$$

$$\Phi_I = \frac{F'}{F_m''} - \frac{F'}{F_m} \qquad (5.2)$$

Here the double prime sign is used to mark the values of fluorescence measured during dark acclimation following a light treatment. F_m'' was determined after several hours of dark relaxation (*Ahn et al,. 2009*). It was applied a shorter time of relaxation assuming that 10 min period would allow for relaxation of qE but not qT, thereby separating Φ_E and $\Phi_T + \Phi_I$:

$$\Phi_E = \left(\frac{F_m'' - F_m'}{F_m'}\right)\frac{F'}{F_m'} = \frac{F'}{F_m'} - \frac{F'}{F_m''} \qquad (5.3)$$

$$\Phi_T + \Phi_I = \left(\frac{F_m - F_m''}{F_m} \right) \frac{F'}{F_m''} = \frac{F'}{F_m''} - \frac{F'}{F_m} \quad (5.4)$$

It is easy to notice that, after simplification, the right parts of the equations 5.3 and 5.4 look identical to the one of the equations 5.1 and 5.2, respectively. In other words, two approaches are equal mathematically. The difference is in the time of dark relaxation prior to measurements of F_m'' (10 min *versus* several hours) which allows to separate different groups of components (Φ_E and $\Phi_T + \Phi_I$ *versus* $\Phi_E + \Phi_T$ and Φ_I). It is important to note that a good correlation was observed between Φ_E and zeaxanthin cation formation (*Ahn et al,. 2009*).

It is possible to obtain an estimate for Φ_T by combining these two approaches.

$$\Phi_T = \Phi_{E+T} - \Phi_E = \frac{F'}{F_m''_{(10\,MIN)}} - \frac{F'}{F_m''_{(3\,HOURS)}} \quad (5.5)$$

$F_m''_{(10\ MIN)}$ is F_m''measured at 10 min of dark acclimation and $F_m''_{(3\ HOURS)}$ is F_m'' measured after long-term dark acclimation, for instance, 3 hours. However, as shown in Guadagno *et al.* (2010), 10 min does not always correspond to the dark period when qE is fully relaxed. Taking into account the natural variability of the relaxation kinetics of different NPQ components, our revised approach (*Guadagno et al., 2010*) has been successful in the separation of three components of NPQ by applying the energy partitioning approach to the well-known triple exponential decay method (*Walters and Horton 1991, 1993*). In Guadagno *et al*, 2010 it was proposed to detect two points (x and y) corresponding to the time of the first and second variation in the slope of darkness relaxation kinetic of NPQ measured by following the changes in F_m''. The values of maximal fluorescence at these points (F_{mx}'' and F_{my}'') were used to calculate the quantum yields of NPQ components as follows:

$$\Phi_E = \left(\frac{F_{mx}'' - F_m'}{F_m'} \right) \frac{F'}{F_{mx}''} = \frac{F'}{F_m'} - \frac{F'}{F_{mx}''} \quad (5.6)$$

$$\Phi_T = \left(\frac{F_{my}'' - F_{mx}''}{F_{mx}'} \right) \frac{F'}{F_{my}''} = \frac{F'}{F_{mx}''} - \frac{F'}{F_{my}''} \quad (5.7)$$

$$\Phi_I = \left(\frac{F_m - F_{my}''}{F_{my}''} \right) \frac{F'}{F_m} = \frac{F'}{F_{my}''} - \frac{F'}{F_m} \quad (5.8)$$

Here we assume that F_{mx}'' is an analog of $F_m''_{(10\ MIN)}$ and F_{my}'' is an analog of $F_m''_{(3HOURS)}$, it becomes clear that Eq 5.5 is related to Eq 5.7 (the other pairs are Eq. 5.2 and Eq. 5.8, Eq. 5.3 and Eq 5.8). The sum of The sum of $\Phi_E + \Phi_T + \Phi_I$ (Eq. 6-8) is equal to $\frac{F'}{F_m'} - \frac{F'}{F_m}$, which corresponds to Cailly *et al.* (1996) formula for quantum yield of light-induced NPQ. It shows that the approaches used in Cailly *et al.* (1996), Kornyeyev and Hendrickson (2007), Ahn *et al.* (2009) and Guadagno *et al.* (2010) are essentially the same from mathematical point of view. However, the recent development presented in the previous chapter seem to be more precise than all the approach previously described while the combination of two others might be more practical.

The calculations of Φ_E, Φ_T, and Φ_I discussed so far are based on the comparison of steady state and maximal levels of fluorescence at different time before, during, and after light treatment. Any movements of a sample or a fiber optic cable (including the movements of chloroplasts within the cells) during the entire experiment can produce an error in the calculations. It also means that, most likely, several fluorometers would be needed for measuring diurnal changes in the distribution of excitation energy in PSII in the field experiments.

Another approach, which is based on the comparison of Φ_{PSII} values have been introduced by Kornyeyev and Hendrickson (2007) (see more detailed description in *Kornyeyev and Holaday, 2008*). It was initially designed for splitting light-induced NPQ into reversible (qE + qT) and irreversible (qI) components. In order to adapt the equations from Kornyeyev and Holaday (2008) to more detailed partitioning like in Guadagno *et al.* (2010), we determined the values of F_v''/F_m''

corresponding to the time of the first $(F_v''/F_m'')_x$ and second $(F_v''/F_m'')_y$ variation in the slope of darkness relaxation kinetic of F_v''/F_m''. Those values represent quantum yields of PSII photochemistry in different situations. $(F_v''/F_m'')_y$ is higher than initial F_v'/F_m' for light-acclimated sample because qE and qT are relaxed at the time when $(F_v''/F_m'')_y$ is measured. This difference can be used to calculate to calculate $\Phi_E + \Phi_T$:

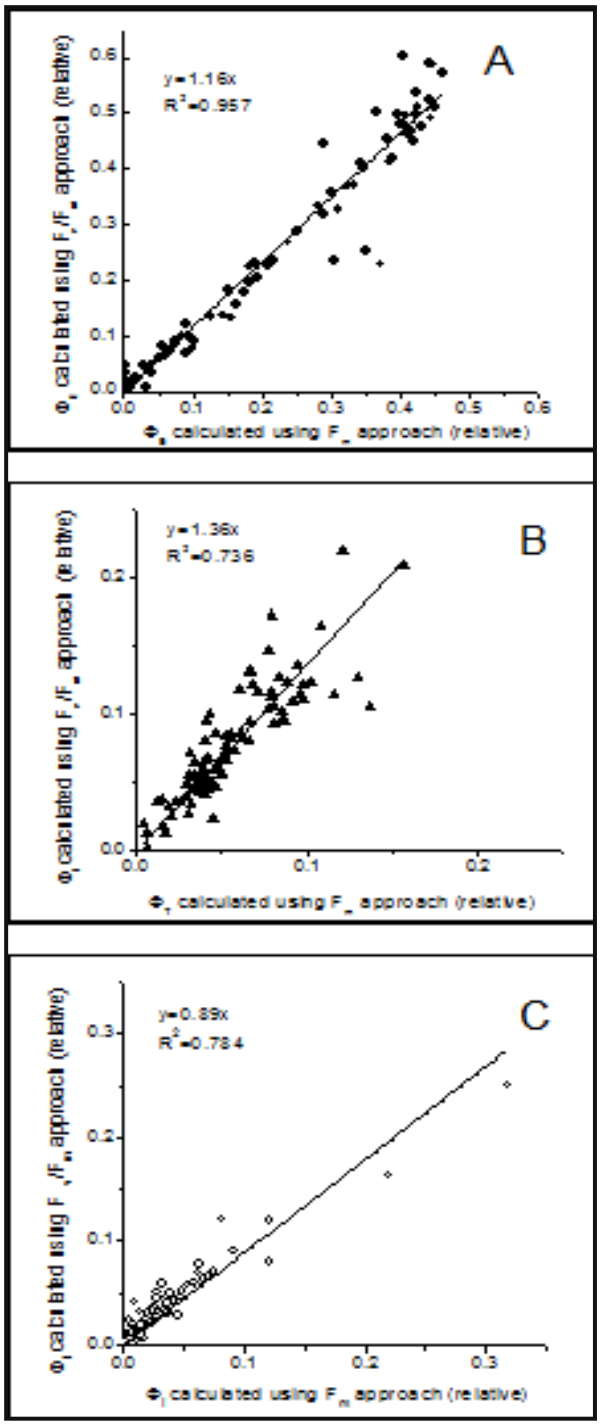
$$\Phi_E + \Phi_T = \left(1 - \frac{F_v'/F_m'}{(F_v''/F_m'')_y}\right)\frac{F'}{F_o'} \quad (5.9)$$

Note that first the quantum yields of PSII with open reaction centers are compared and then the coefficient F'/F_o' is used to account for closure of a fraction of PSII reaction centres, i.e., partial reduction of Q_A pool under illumination (this coefficient was introduced in Kramer *et al,*. 2004). Φ_E can be estimated in similar way by comparing F_v'/F_m' measured when the qE component is present and $(F_v''/F_m'')_x$ measured at the time when qE is assumed to be fully relaxed:

$$\Phi_E = \left(1 - \frac{F_v'/F_m'}{(F_v''/F_m'')_x}\right)\frac{F}{F_o'} \quad (5.10)$$

Then Φ_T is calculated by subtracting Φ_E (Eq. 5.10) from the sum $\Phi_E + \Phi_T$ (Eq. 5.9).

Figure 31:Relationship between values of quantum yields of three major components of non-photochemical quenching obtained by two approaches based on the analysis of Fm (Fm'') and Fv/Fm (Fv''/Fm'')



The composition of the equation for the estimation of the quantum yield of qI can begin with the comparison of quantum yields of photochemistry prior to light-treatment (F_v/F_m) and after the long-term dark-acclimation following the light-treatment, when both qE and qT are assumed to be relaxed $(F_v''/F_m'')_y$. Then $(F_v''/F_m'')_x/(F_v''/F_m'')_y$ can be added to account for occurrence of qE and qT in during acclimation to light. Finally, the coefficient F'/F_o' is used to account for closure of some PSII reaction centers under illumination. Thus, Φ_I for light-acclimated sample under illumination is estimated as following:

$$\Phi_I = \left(1 - \frac{(F_v''/F_m'')_x}{F_v/F_m}\right)\left(\frac{(F_v''/F_m'')_x}{(F_v''/F_m'')_y}\right)\frac{F'}{F_o'} \quad (5.11)$$

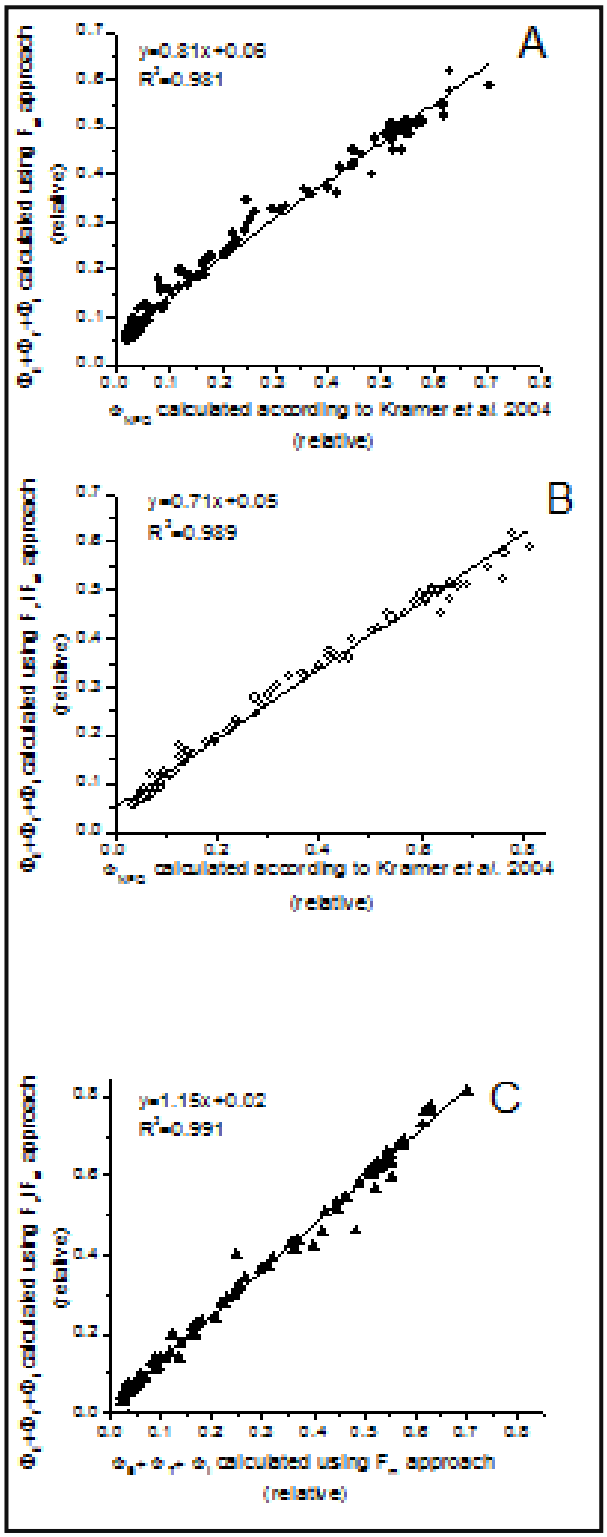
The values of three major NPQ components obtained by using the approaches based on measuring F_m (Eq. 5.6-5.8) and F_v/F_m (Eq. 5.10-5.11) are compared in Fig. 31. The strong correlation between the values of the parameters calculated in two different ways and the proximity of the slopes to unity suggests that these approaches yield similar results and can be used as alternatives.

The approach based on the changes in F_v/F_m gives more flexibility in designing experimental procedures because it allows for change of the optical path between the measurements

of F_v/F_m, F_v'/F_m' and F_v''/F_m'', i.e., moving the fiber optic cable from one sample to another in order to perform an experiment with a single device. This feature might be also very helpful for tracking diurnal and seasonal changes in the PSII energy partitioning. For instance, variations in chlorophyll content may greatly affect reliability of the results based on F_m measurements requiring determination of chlorophyll content for adjustment (*Porcar-Castell et al,. 2008*). This is not necessary in the case when the calculations are based on the comparison of F_v/F_m.

Another advantage of the F_v/F_m approach is that F_v/F_m values can be extrapolated to the maximal levels known for the species of interest, for instance 0.8. It can be done for the samples with unknown history (no pre-dawn F_v/F_m values measured) or for samples where the presence of sustained NPQ is suspected (the stressful conditions may not allow F_v/F_m to return to the maximal value due to the remaining residual qI). The maximal F_v/F_m values reported in the literature are in relatively narrow range (0.78-0.83) suggesting that such an extrapolation is realistic. In some cases, when the changes in F_m (or F_v/F_m) are close to the error of measurements, the obtained values of F_m'' (F_v''/F_m'') can be higher than those of F_m (F_v/F_m) leading to negative values of Φ_I calculated according to Eq. 5.8 (or Eq. 5.11). This can be corrected by assuming that $F_{my}''= F_m$ and $(F_v''/F_m'')_y = F_v/F_m$.

Figure 32:Relationship between values of quantum yield of light-induced NPQ (Φ NPQ) calculated according to Kramer at al. 2004 and the sum of the quantum yield of three major NPQ components (Φ qE + Φ qT + Φ qI) obtained using different approaches



In addition to light-induced NPQ, there is a thermal dissipation of excitation energy that is related to intrinsic properties of PSII (constitutional non-photochemical dissipation or non-light-induced quenching processes (*Baker 2008*)). It is responsible for the fact that maximal quantum yield of PSII photochemistry is always lower than unity (see *Kornyeyev et al., 2001; Kramer et al,. 2004*) and includes fluorescence along with thermal dissipation. The contribution of this NPQ component is estimated as a combined quantum yield of fluorescence and constitutional thermal dissipation ($\Phi_{C,\ F}$). Calculations are based on the following balance equation: $\Phi_{C,\ F} + \Phi_E + \Phi_T + \Phi_I + \Phi_e = 1$.

The calculations of the quantum yields for various processes discussed above are related to the entire pool of PSII complexes, i.e., they describe the bulk energy fluxes without separating the complexes in various states. Such model is justified by the complexity of the PSII heterogeneity. PSII can differ not only in the reduction state of Q_A but also in the ability to charge separation, phosphorylation of the proteins, connection to other PSII units and light-harvesting complexes, and

various combinations of all of the above. Indeed, it is unlikely that all PSII complexes would have all NPQ components present at the same time, so generalization seems to be reasonable in this case.

Other mathematical models that consider PSII pool as a combination of separate units and that take into account the connectivity between PSII units have been described (*Lavergne and Trissle, 1995; Lazar, 1999; Kramer et al., 2004*). However, up to now, no detailed separation of the NPQ components has been proposed on the base of those models.

Despite the model introduced in Kramer *et al.* (2004) does not account for various components of light-induced NPQ, we have performed calculations for quantum yield of combined light-induced NPQ (Φ_{NPQ}) according to Kramer *et al.* (2004) and compared it with the sum $\Phi_E + \Phi_T + \Phi_I$ obtained with two approaches described above.

A linear correlation between Φ_{NPQ} and $\Phi_E + \Phi_T + \Phi_I$ values is observed when the sum is calculated by following either changes in maximal fluorescence levels (Fig. 32A) or quantum yield of PSII photochemistry (Fig. 32B). As expected from data presented in Fig.31, the values of $\Phi_E + \Phi_T + \Phi_I$ calculated with two approaches show excellent correlation (Fig. 32C).

In conclusion, the analysis of the literature allowed us to sort out two major approaches for detailed characterization of energy partitioning in PSII complexes. They are based on the changes of either F_m or F_v/F_m during dark relaxation. The data presented in this chapter imply that those approaches can yield similar results along a wide range of light intensities. Moreover, these results seem to lead to the conclusion that a unified approach may be achieved in near future, in order to clarify and merge together all the important steps taken by different researchers up to now.

Chapter 6: Nuclear Magnetic Resonance for detecting light stress in plants

Summary

During my third year of PhD, I have had the chance of attending a course on the Nuclear Magnetic Resonance (NMR), at the University of Naples Federico II. NMR spectroscopy is a versatile technique used to analyze any molecule containing one or more atoms with a non-zero magnetic moment. The detection of the isotopes ^{1}H, ^{13}C, ^{14}N, ^{15}N, and ^{31}P causes the occurrence of at least one NMR signal in all the basic molecules for biological processes. So, NMR spectra show the identity of the molecules in a sample, and this technique can be used to identify and quantify metabolites in samples of biological origin (*Krishnan et al., 2005; Sobolev et al., 2005*). Additionally, NMR is a non-destructive technique, and spectra can be recorded from extracts or purified metabolites but also from more complex samples as whole plants (*Ratcliffe, 1994; Ratcliffe and Shachar-Hill, 2001; Ratcliffe et al. 2001; Ratcliffe and Shachar-Hill, 2005*).

Additionally, it is widely reported in literature that several moleculess are involved in the NPQ mechanism and their concentration vary in presence of light stress. Consequently, thanks to a collaboration with Professor Marina Della Greca, from the Organic Chemistry Department of the University of Naples Federico II, an experiment of stress detection on crude extracts from fresh leaves was performed.

In this chapter, the results obtained from the recording of ^{1}H NMR and the interesting prospective of using this modern technique as a probe for light stress detection, are presented.

Preface

The results presented in this Chapter are included in the manuscript "Nuclear Magnetic Resonance for detecting light stress in plants" by Guadagno, C.R.., Della Greca, M., Virzo De Santo, A., and D'Ambrosio, N. (*in preparation*).

6.1 Introduction

During the last century, the development of the global climate change has exacerbated the occurrence of light stress and plants may undergo major disadvantages. As a consequence, obtaining a fast and comprehensive method in order to sense and analyze the metabolic variations occurring in stressed is desirable.

Since 1975, when Schaefer *et al*. proposed their ^{13}C studies on carbohydrates and lipid metabolism (*Schaefer and Stejskal, 1975; Schaefer et al., 1980*), NMR has been applied to plants and many insights in plant physiology have been achieved thanks to this method. At the present time, high-field NMR methods permit to solve spectra of complex mixtures and to quantify the corresponding components without chemical separation and for this reason are routinely used in metabolomics and food chemistry (*Loughman, 1984; Pollesello et al., 1996; Putzbach et al., 2005; Tiziani et al., 2006; Valverde and This, 2008*). Compared to other analytical methods as ultraviolet–visible (UV-Vis) spectroscopy (*Lichtenthaler, 1987; Wellburn, 1994; Wrolstad, 2005; Kupper et al., 2007*), high-performance liquid chromatography (HPLC) (*Minguez-Mosquera, 1989; Khachick et al., 1992*) and thin-layer chromatography (TLC) (*Quach et al., 2004; Sherma et al., 2004; Valverde et al., 2007*), ^{1}H NMR spectroscopy is a fast analytical method for complex samples that provides huge quantities of complementary information without drawbacks as time losses and money squandering. Moreover, it gives a large amount of data without former chromatographic separation. As a result, NMR spectroscopy has been widely used to analyze crude extracts from plant or algae tissues (*Pollesello, 1993; Fan, 1996; Tiziani, 2006*) for the last 20 years. Consequently, NMR seems to be the best nominee for being a sensor of light stress and in this Chapter the use of NMR spectra as powerful tools for detecting harmful situations for higher plants is proposed.

The oxidative damage due to light stress in plants and the resulting photoprotective response are widely studied, as reported in the previous Chapters (*Barber and Andersson, 1992; Long et al., 1994; Niyogi, 1999; Sung and Niyogi, 2008*). Since the involvement of the lipidic fractions, especially pigments, in the plant response to light stress have been widely demonstrated during the last decades, NMR spectroscopy can be utilized to compare crude extract of leaves at different level of light stress, allowing an analysis of these compounds.

In this Chapter the analysis of the ^{1}H NMR (1D) spectra of two agronomic species (*Spinacia oleracea* and *Beta vulgaris*) exposed to different light intensities is shown. The main aim of the proposed experiment is the identification of possible relationships between light stress in plants and ^{1}H NMR signals variations. Particular attention is given to carotenoids and xanthophylls variations in order to correlate them to the NPQ variation (*Horton et al., 1996; Niyogi et al., 2005; Pascal et al., 2005*).

6.2 Materials and methods

6.2.1 Plant material

Garden beet (*Beta vulgaris* L.) and spinach (*Spinacia oleracea L.*) plants were grown in pots filled with 1:1 peat:soil and watered when required. The plants were grown in a controlled growth chamber with 8/16 h day/night (D/N) photoperiod, 25/20 °C D/N temperature and 65/85 % D/N relative humidity (RH), with a growth irradiance of about 150 μmol photons m^{-2} s^{-1}.

6.2.2 Leaf chlorophyll fluorescence measurements

Chlorophyll *a* fluorescence emissions were measured using a pulse amplitude modulated fluorometer (MINI-PAM, Walz, Effeltrich, Germany). After a dark period (30 min), the maximum fluorescence (F_m) and the maximum efficiency of PSII (F_v/F_m) were determined by applying a

saturating pulse (0.8 s) with an intensity higher than 5,000 μmol photons m^{-2} s^{-1}. Then, leaves were exposed for 60 minutes to a PFD of 900 μmol photons m^{-2} s^{-1} to obtain F'_m value. During the illumination period, the leaves temperature was in the range of 24-27 °C. The leaves were put on wet filter paper, in order to avoid eventual problems caused by high temperature. After the induction period, the recovery of the fluorescence signal in darkness for 40 minutes was monitored to assess the relaxation of NPQ, applying saturating pulses every 5 minutes from the beginning of dark period. NPQ is expressed as $(F_m-F'_m)/F'_m$ (*Bilger W. and Schreiber, U., 1986; van Kooten O. and Snel J.F.H., 1990*).

6.2.3 Sample preparation

Immediately after the fluorescence measurements *in vivo*, the whole leaves were sampled, frozen in liquid nitrogen and powdered in a ceramic mortar with a pestle. Grinded samples (about 0.4 g of dry weight) were homogenized with 100 % acetone in order to obtain a lipidic fraction extract. Extraction with solvents can be degradative for the photosynthetic part and especially lipids. Therefore, all extraction procedures were done rapidly and at low temperatures, 0-4 °C in a dim light using only glassware (*Christie W.W., 1993*). No attempt at purifying single components was made throughout the experiments.

6.2.4 NMR measurements

NMR spectra of the vegetal extracts were recorded at 297 K on a Varian Inova 500 spectrometer operating at the ^1H frequency of 499.709 MHz. ^1H spectra of acetone extracts were obtained using the following parameters: 256 transients, 64K data points, recycle delay of 2.5 s and

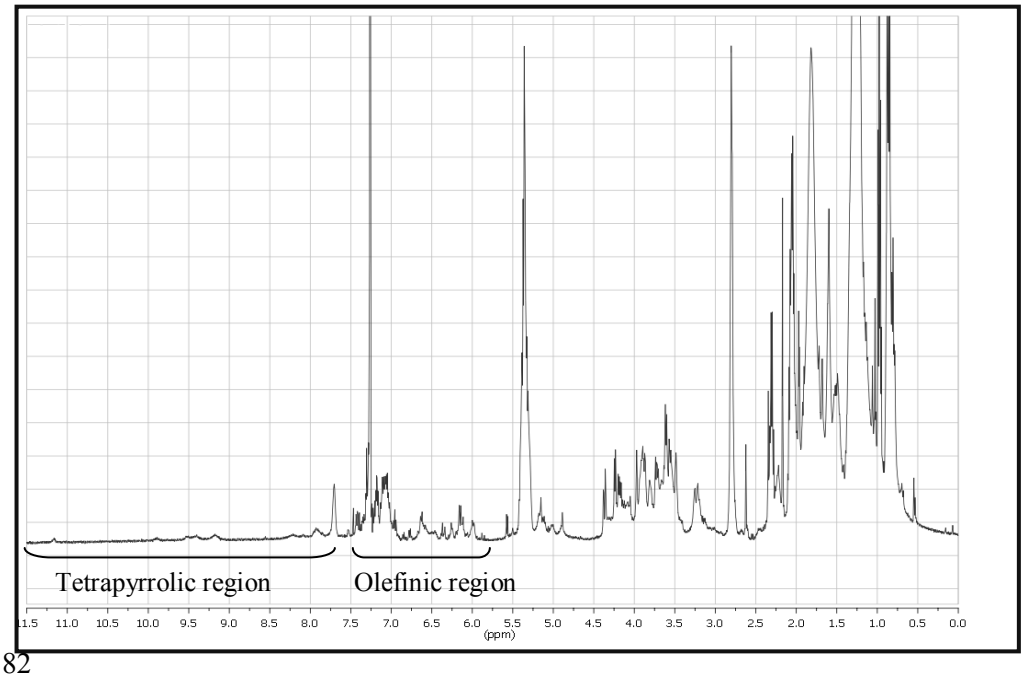
a 60° flip angle pulse of 4.8 μs. Molecules of interest were identified by comparison with previously published data (*Sobolev et al., 2005; Valverde and This, 2008*).

6.3 Results and discussion

6.3.1 Analysis of the ^{1}H NMR spectrum

Although the acetone extracts give rather complex ^{1}H-NMR spectra, similar for beet and spinach, a qualitative analysis of the overall spectrum for dark-adapted leaves conditions is possible [Fig. 33-35]. Peaks have been assigned after comparison with previously published data (*Englert, 1991; Khachick et al., 1992; Putzbach et al., 2005; Sobolev et al., 2005; Valverde et al., 2007*). In the downfield region, between 11.5 and 8.3 ppm, the signals from chlorophylls (Chls) and pheophytins (Pheos) are detected. Owing to their structural tendency to aggregate, the detection of the peaks for Chls is tricky respect to Pheos one. This is certainly caused by the Chls sensitivity to the solvent composition, as well as to concentration and temperature, as it is clearly reported in literature (*Hyvärinen et al., 1995; Sobolev et al., 2005; Valverde et al., 2007*).

Figure 33:^{1}H NMR spectrum in CDCl3 of a dark-adapted leaf of *Beta vulgaris*



In all spectra analyzed, the peak at 7.26 ppm is assigned to the solvent $CDCl_3$ and the region between 7.0 and 5.4 is called

olefinic for the presence of the signals from the long chain conjugated to double bonds of carotenoids (Cars).

Figure 34:[1]H NMR spectrum in CDCl3 of a dark-adapted leaf of *Spinacia oleracea*

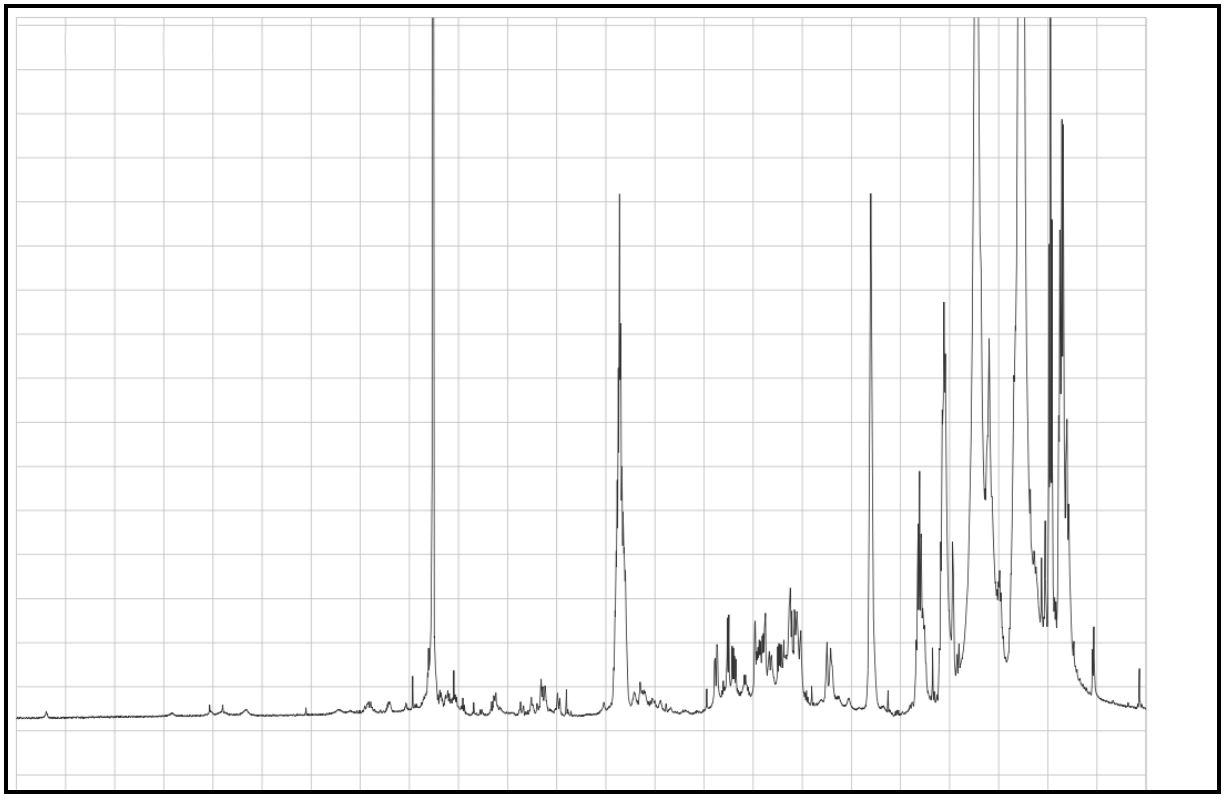
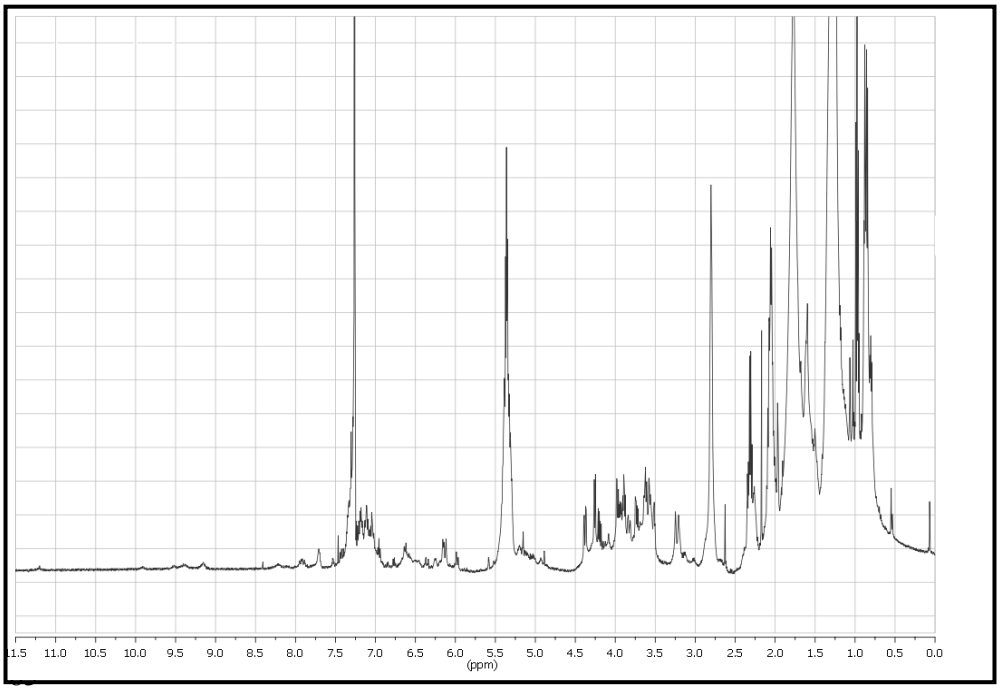


Figure 35:[1]H NMR spectrum in CDCl3 of *Beta vulgaris* exposed to 60 minutes of high light treatment (900 µmol photons m-2 s-1)



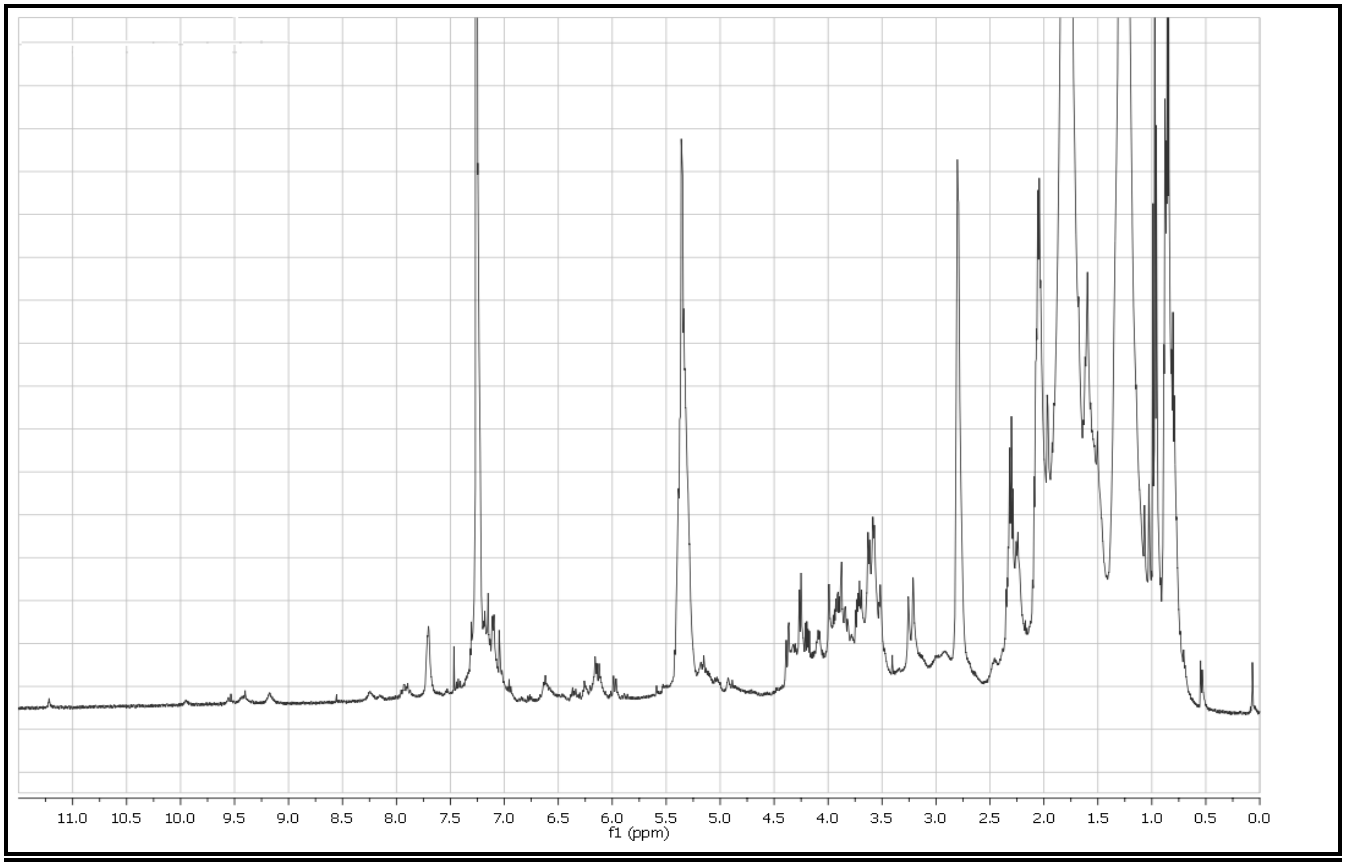
In fact, [1]H NMR spectra of Cars have the terpenic chain in common and they differ mainly by protons in the distal part of the molecules. Signals

from chains of Cars were assigned after previously published data (*Englert, 1991; Khachick et al., 1992; Putzbach et al., 2005; Sobolev et al., 2005; Valverde et al., 2007*).

The intense signal centered at 5.26 ppm is assigned to the double bound protons of fatty acids. Between 5.0 and 3.0 ppm, in the so called carbinolic

region, there is the presence of the signals from oxygenated molecules; in this range, all the glycerolipids, sterols and pheophytins are represented. Lastly, in the upfield region there is the presence of the aliphatic protons signals. Despite the fact that no important differences are present between the two species, there is evidence of remarkable differences for the same species before and after the light exposure [Fig. 33-36].

Figure 36: ^{1}H NMR spectrum in CDCl3 of *Spinacia oleracea* exposed to 60 minutes of high light treatment (900 □mol photons m-2 s-1)



6.3.2 Light stress effect on the NMR spectrum

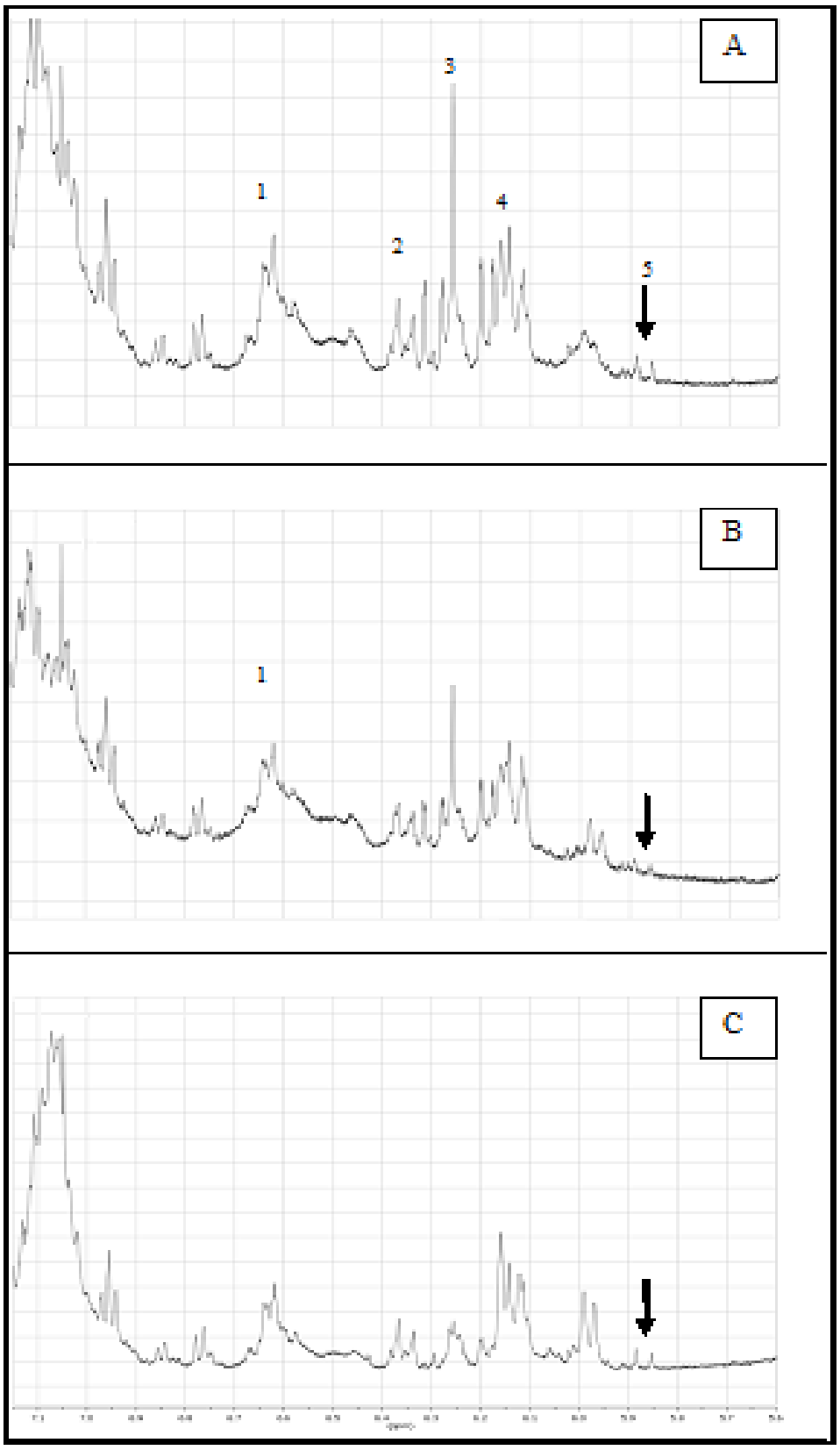
In this experiment ^{1}H NMR spectroscopy is used in order to test the possible correlation between light stress and NMR signals in beet and spinach leaves. Any visible change in the recorded spectra could be related to mechanisms involved in the response to light stress and then activated during the light treatments [Fig. 33-36]. In both species, the light stressed samples show visible differences respect to the dark-adapted ones. These variations are spread all over the 0-11.5 ppm range but they seem to become significant for the signals from oxygenated compounds (5.0-3.0) and in the olefinic range (5.6-7.15). In this latter region, Cars signals are concentrated and, as expected, signals from this part of the spectra seem to have a strong connection with light exposure [Fig. 37-A and 37-B].

The important role of carotenoids in the photoprotective process is well established (*Demming-Adams and Adams, 1996; Horton et al., 1996; Niyogi, 1999; Bonente et al., 2008*). Carotenoids ensure the protection of photosynthetic apparatus through a series of different mechanisms: chlorophyll triplet quenching, reactive oxygen species scavenging and activation of heat dissipation *via* non-photochemical quenching mechanisms. The results of peak assignments for pigments are given in Table 1.

Table 1: Chemical shifts of carotenoids in aceton extract.

Peak number	Assignment	δ_H (ppm)
1	Cars (CH-11/11') Cars (CH-15/15')	6.69 – 6.59
2	Cars (CH-12/12')	6.39 – 6.31
3	Cars (CH-14/14')	6.26 – 6.22
4	Cars (CH-7/7') Cars (CH-8/8') Cars (CH-10/10')	6.16 – 6.10
5	Vio (CH-7/7')	5.86

Figure 37: Details of the 5.6-7.01 region of the 1H NMR spectrum in CDCl3 of A) dark adapted leaf, B) light adapted leaf and C) dark adapted leaf relaxed leaf of *Beta vulgaris* after high light treatment



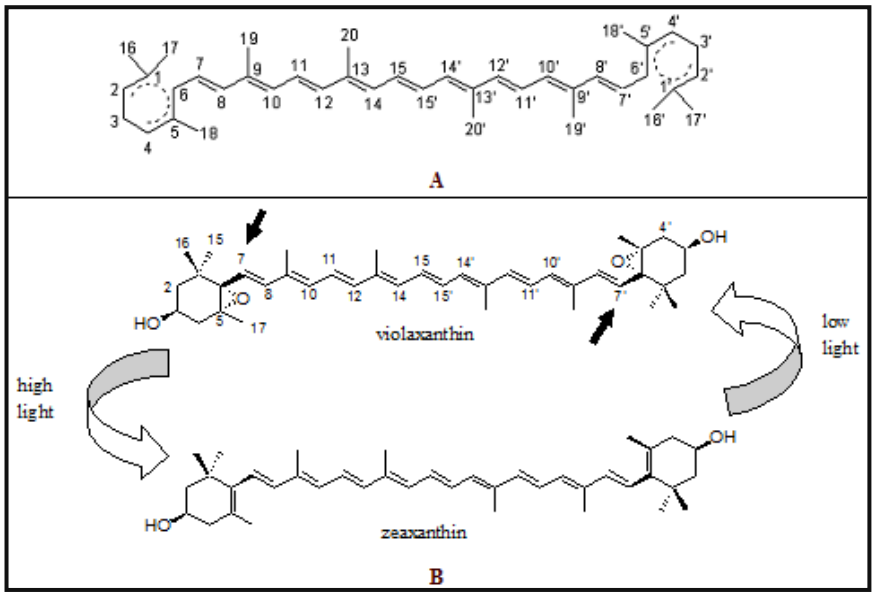
Specifically, the peaks in group 1 were attributed to H-11, H-15, H-11′, and H-15′ of the terpenic chain that Cars have in common. The peaks of groups 2, 3, and 4 were assigned to the terpenic chain of Cars too, but in this case there was an overlapping with vinyl group peaks of Chls and Pheos. The peak of group 2 contains signals from H-12 and H-12′, whereas the one of group 3 from H-14 and H-14′. Lastly, the peak of group 4 contains the signals from H-7, H-8, H-10, H-7′, H-8′, and H-10′, all from the terpenic chain of Cars. The signals of pigments were not fully resolved because they were overlapped with other compounds (mainly lipids and fatty acids).

6.3.3 Violaxanthin signal detection and NPQ correlation

The main aim of the experiment reported in this Chapter, is to assess the potential of ^{1}H NMR as a useful tool for detecting the light stress in plants. It is possible by comparing two spectra, one from a dark adapted leaf and another one from a light stressed sample, to find out an exclusive signal, which has a specific behavior and gives an immediate response about the occurrence of a light stress.

Among all the Cars signals we point our attention on a characteristic signal at 5.86 ppm. This doublet is reported in literature (*Valverde, J. and This, H., 2008*) as a typical signal of VIO the oxygenated carotenoid essential for the triggering of NPQ (Chapter 1) [Fig.38A]. When the absorbed light exceeds the photochemical demand, the luminal pH decreases and VIO is de-epoxidated into ZEA, through the so called xanthophylls cycle [Fig. 38B]. The decrease in the concentration of this pigment and in presence of the protein PsbS the triggering of NPQ mechanism occurs (*Müller P., Li X.-P. and Niyogi K.K., 2001; Niyogi K.K., 1999*). From the detailed analysis of the spectra, [Fig. 37] it is immediately noticeable the decrease of the VIO signals in the stressed sample [Fig. 37B] compared to the dark-adapted ones [Fig. 37A]. This decrement is present in both species utilized [no detailed spectra for *S. oleracea* are shown].

Figure 38:A) Carotene structure and numbering B) Xanthophyll cycle: violaxanthin and zeaxanthin structure and numbering. Black arrows indicate H-7 and H-7' causing the doublet at 5.86 ppm of 1HNMR spectra



In dark conditions Fv/Fm values were monitored as a reference of the physiological state of leaves. Then, we continuously monitored the value of chlorophyll fluorescence signal throughout the light treatment in order to appreciate the NPQ induction and lastly to detect the relaxation of the quenching during the subsequent dark period [Table 2]. NPQ passed from a null value to 1.068 for *Spinacia* and 1.102 for *Beta*, after 60 minutes of light treatment. As expected, when the actinic light was turned off the NPQ value showed a strong decrement in both species [Fig. 39]. These values show a perfect correlation with variations of VIO signal in the NMR spectra.

Table 2: NPQ values for spinach and beet leaves. All values are the average of three measurements.

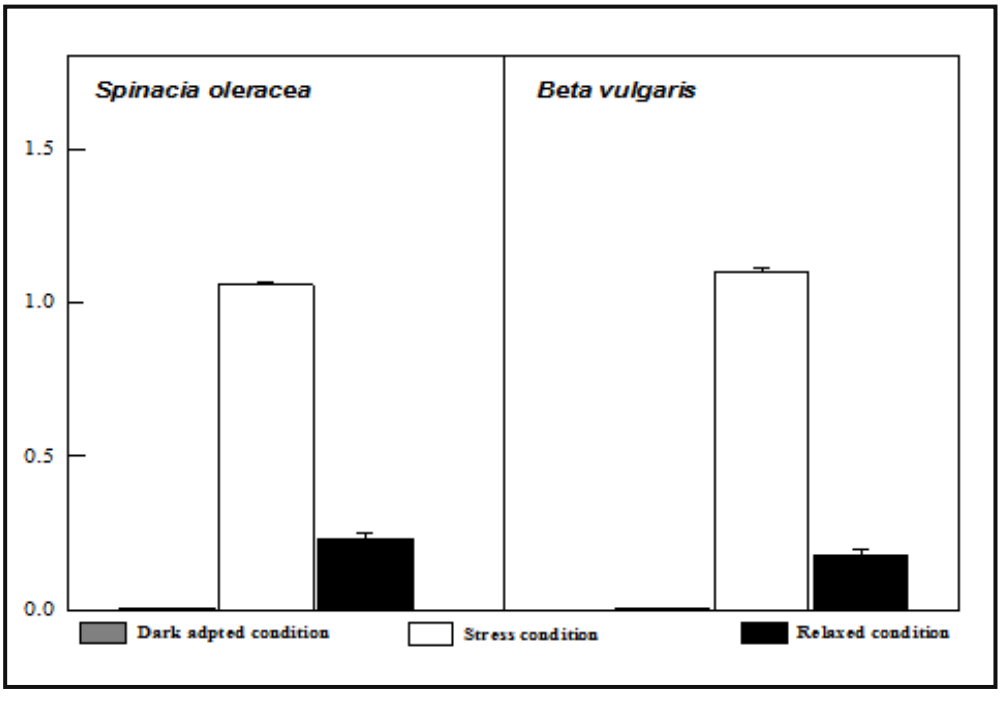
Species	Dark adapted leaves (Fv/Fm)	Light adapted leaves (NPQ)	Relaxed leaves after light treatment (NPQ and Fv/Fm)
Spinacia oleracea	0.754	1.058	0.234 - 0.699
Beta vulgaris	0.772	1.102	0.180 – 0.710

In order to validate the hypothesis of a detection of light stress induction from the NMR spectrum we also recorded spectra from relaxed leaves after the end of the light treatment when the NPQ is supposed to be relaxed [Fig. 37C]. In this case, the VIO signal is re-established in accordance with the low value of NPQ after 40 minutes of dark relaxation [Fig. 39]. Our results demonstrate the potential of ^{1}H NMR technique as a stress detector in *Spinacia* and *Beta* leaves.

Above all, it is possible by this method to monitor the VIO concentration variations, gaining insights on important issues on the NPQ induction/relaxation. Alternatively, this kind of information can be obtained only by long and expensive HPLC analysis otherwise the same information is undetectable by using other techniques, as UV-spectrometry.

Here the utilization of the ^{1}H NMR technique as a faithful mirror of the physiological state of plants is proposed. In particular, a correlation between VIO signal and NPQ value is demonstrated and it is shown that is possible discriminate between light stressed and dark-adapted leaves by recording NMR the spectra.

Figure 39:NPQ values in spinach (left) and beet (right) leaves. NPQ values are reported for dark adapted leaves (grey), leaves under high light treatment (white) and relaxed after an high light treatment (black)



In addition to this evaluation of VIO de-epoxidation state, the spectra also give a huge amount of information which makes it possible to analyze the other compounds present in the leaf extract. In

conclusion, ^{1}H NMR technique is a perfect tool for stress detection and good results can be very efficiently obtained, because the sample preparation is fast and easy. In the future, further work has to be addressed to get a quantitative analysis of the compounds.

Chapter 7: Analysis of NPQ components in *Arabidopsis thaliana* mutants

Summary

During my PhD experience, I had the chance of joining the laboratory of Professor K.K. Niyogi twice. The first time, I spent my period in Berkeley working on *Arabidopsis thaliana* as a model organism for the NPQ mechanism. Seen that my host laboratory is a leader in the NPQ research, I had the opportunity of working with a great availability of *Arabidopsis* mutants. With the main aim of testing the revised energy partitioning method (chapter 4) on mutants with different NPQ phenotypes, the analysis of the different components on NPQ was conducted. In this chapter, the results for the NPQ as absolute values and in terms of quantum yields for the different Arabidopsis lines, are presented.

Preface

The contents presented in this Chapter are the result of a collaboration with Matthew Brooks and K.K. Niyogi, Plant and Molecular Biology Department, University of California, Berkeley (*unpublished data*).

Some of the contents of this Chapter are included in the paper Is qE always the major component of non-photochemical quenching? In : *Photosynthesis. Energy from the Sun: 14th International Congress on Photosynthesis,* 1007–1010 (J. Allen, E. Gantt, J. Golbeck, and B. Osmond eds.) Springer, by N. D'Ambrosio, C. R. Guadagno, and A. Virzo De Santo (2008).

7.1 Introduction

Plants have developed different strategies to avoid deleterious effects of light energy absorbed in excess. One of these strategies is the thermal dissipation of absorbed light energy refereed as NPQ of chlorophyll fluorescence (chapter 1). During last decades, the intensive research activities on NPQ contributed to define three basic mechanisms of thermal dissipation. Moreover, several mutants of *Arabidopsis* that affect thermal dissipation have been isolated by video imaging of Chl fluorescence quenching (*Niyogi, 1999*).

Although in literature it is reported that the ΔpH-dependent or energy component (qE) represents the major component of NPQ (*Crouchman et al. 2006; Kalituho et al. 2007; Li et al. 2000; Müller et al. 2001; Niyogi et al. 2005; Ruban et al. 2002*), the results from the former experiments show the great variability of the NPQ components at different environmental conditions (chapters 3 and 4).

The purpose of the experiment reported in this Chapter was to assess the different extent of the NPQ components for different mutants of *Arabidopsis*, grown in different light conditions. Moreover, in order to evaluate the applicability of the revised energy partitioning approach, presented in chapter 4 of this thesis, the modified method was also applied to wild type and mutants.

The mutants chosen for this experiment were all defective in at least one of the three NPQ components for obtaining as many as possible information on the NPQ components and their variability. Besides the *Col-0* (wild type line), this is the list of the *Arabidopsis* mutants analyzed for this experiment: *npq4.1*, *npq1.2*, *lut2*, *npq1lut2* and *Stn7*. In the *npq4-1* mutant of *Arabidopsis*, the nuclear gene encoding PsbS has been completely deleted, resulting in the absence of PsbS protein and a severe defect in qE (*Li et al., 2000*). The *npq1.2* lacks a functional VDE, this line shows less NPQ than wild type but more than *npq4.1* (*Li et al., 2000*). The third mutant, *lut2*, lacks Lut but is capable of

accumulating Zea in high light and shows somewhat less qE than the WT (*Pogson et al., 1998*). The *npq1lut2* mutant completely lacks both Zea and Lut and thus has no qE (*Niyogi et al., 2001*). Lastly, *Arabidopsis stn7* knockout plants display much less LHCII phosphorylation than wild-type plants and are not able to perform state transitions (*Bellafiore et al., 2005*; *Bonardi et al., 2005*).

In this work the three components of NPQ, by their different relaxation kinetics in darkness, in intact leaves from wild-type and mutants plants of *Arabidopsis,* grown at different light intensities, were assed.

Moreover, using the same fluorescence protocol, the NPQ kinetic of two suppressors (named *6.22* and *0.26*) of the *npq4* mutation were analyzed.

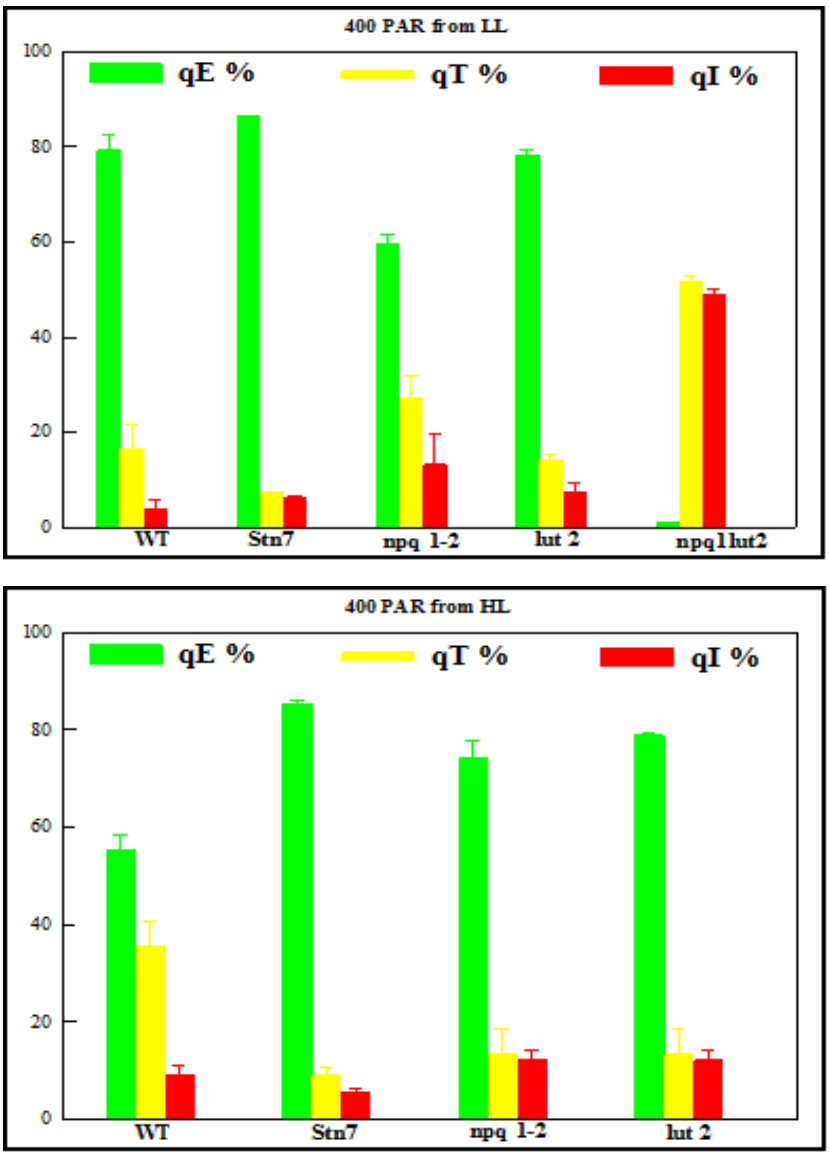
7.2 Materials and methods

Seeds of wild-type (ecotype *Columbia-0*) and *npq4.1*, *npq1.2*, *lut2*, *npq1lut2* and *Stn7* mutants of *A. thaliana* were available in the laboratory of K.K. Niyogi in Berkeley. Seeds of the suppressor *6.22* and *0.26*, were obtained through an EMS (Ethyl Methane Sulfonate) mutagenesis. Plants were grown in soil and located in a growth chamber under two different light conditions: the following conditions: 100 and 300 µphotons m^{-2} s^{-1}. All the other conditions were the same for both sets of plants: temperature 25°C/22°C (D/N), photoperiod of 14 h light/10 h dark, 65%/80%,H (D/N). Chlorophyll fluorescence was measured at room temperature by a Fluorescence Monitoring System fluorometer (FMS, Hansatech). Minimal (Fo) and maximum fluorescence (Fm) were measured to determine the maximum quantum efficiency of PSII [Fv/Fm = (Fm– Fo)/Fm]. Then leaves were exposed for 10 min at 400 µphotons m^{-2} s^{-1} to induce NPQ. At the end, leaves were darkened to measure the relaxation kinetics of NPQ by applying saturating pulses at different time from the beginning of dark period (2, 5, 10, 20, 30 and 40 min). Resolution of three NPQ components (qE, qT and qI) was performed according to the modified procedure reported in

D'Ambrosio et al., 2008. The energy partitioning analyses in the PSII were obtained applying the revised approach reported in Chapter 4 of this thesis (*Guadagno et al., 2010*).

7.3 Results and discussion

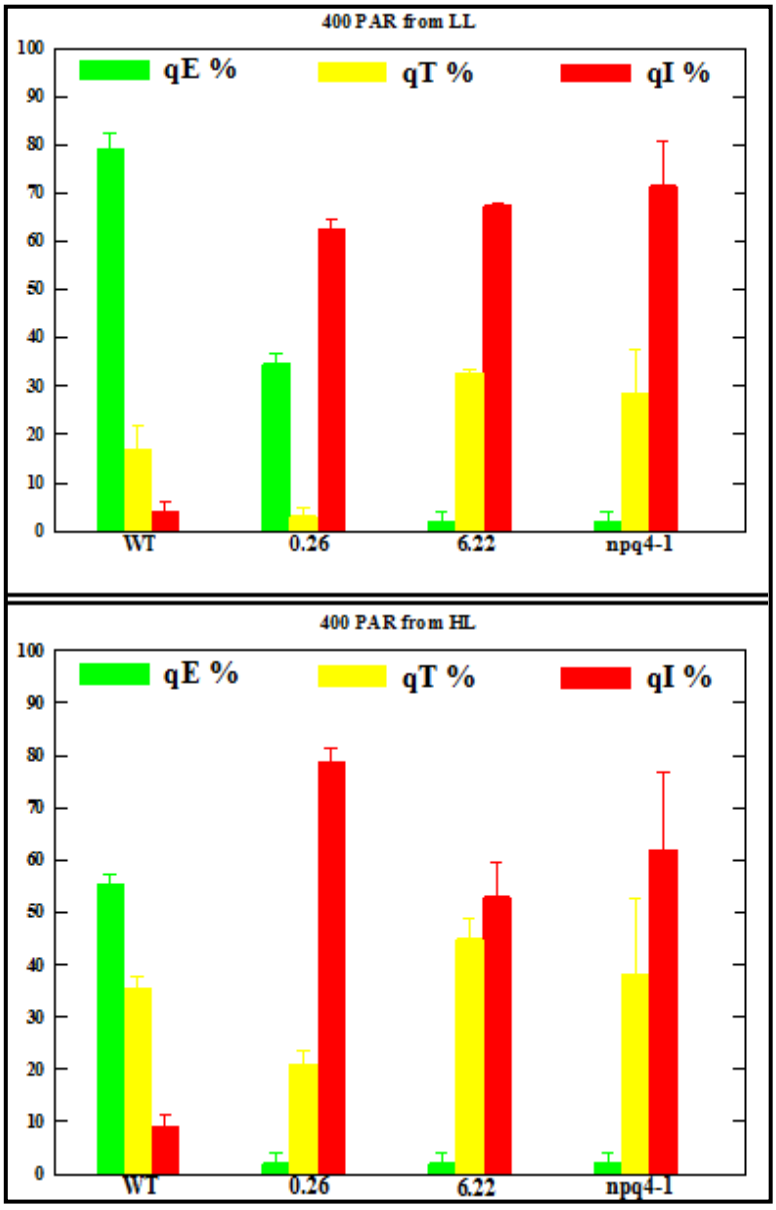
Figure 40: Repartition of NPQ in components qE, qT and qI in *A.thaliana* grown in LL and HL in WT and mutants lines



The repartition in the three components qE, qT and qI of the total NPQ for *Arabidopsis* plants, grown in LL or HL conditions, is represented in Figure 40. In both conditions, the the qE component is the most important for all the mutants lines considered and for the wild type strain. The mutant line *npq1lut2* seems to be an exception. This particular line completely lacks both Zea and Lut and it has no qE (*Niyogi et al., 2001*). In LL conditions, the results of our experiment point out this characteristic: *npq1lut2* has no qE component but 50% in qT and 50% in qI component. However, the absence of the qE component seems to affect the fitness of this mutant that is unable to grow in our HL experimental conditions [Fig.40]. It is interesting that the lack of the two pigments Zea and Lut causes an increment in the qI component

in LL conditions: this results seem to suggest that this component do not have a strong connection with these two pigments in these experimental conditions.From the above mentioned results, qE is demonstrated to be the major component of NPQ for all the mutant lines considered except to *npq1lut2*.

Figure 41: NPQ components in percentage in *A.thaliana* leaves in LL and HL



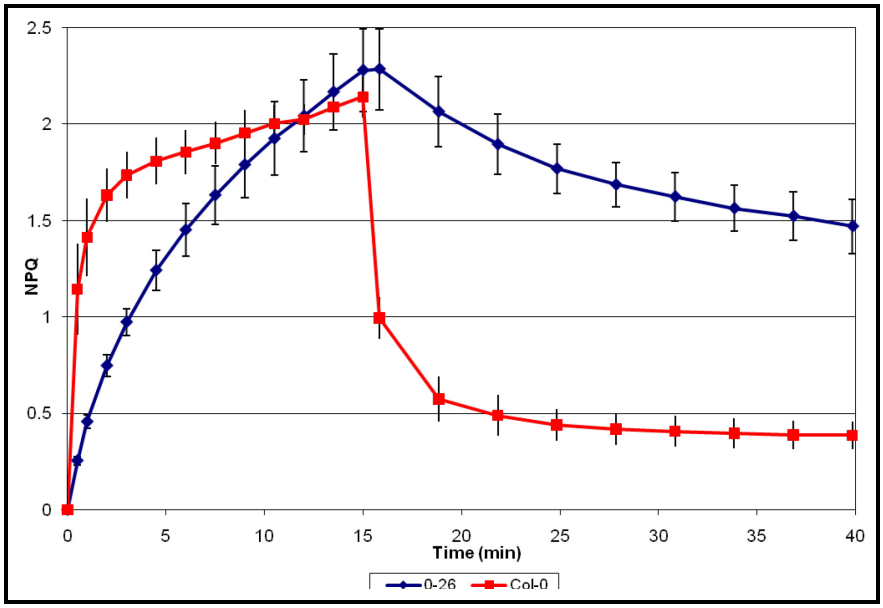
On the other hand, analyzing the second set of mutants, qT and qI components are showed to be very important in both growing conditions [Fig.41]. In particular, the qI component plays an important role for plants acclimated in LL in order to avoid the short stress caused by the actinic light of the NPQ induction period.

These results represent the first phenotypic characterization for the strain *0.26* and *6.22*. These two mutant lines are the result of an EMS mutagenesis in the *npq4.1* background. They were selected through a screening with imaging chlorophyll fluorescence. They both restore the wild type NPQ value and for this reason are indicated as suppressors of the *npq4.1* mutation. Interestingly, using the revised deconvolution method (*D'Ambrosio et al., 2008*), it is possible to observe that both suppressors restore the NPQ value as the wild type line, but the repartition in the three components is very dissimilar from the *Col-0*. As a matter of fact, qE is the

major component for the wild type while the same role is played by qI in the two suppressors [Fig.41]. In Figure 42, the difference between Col-0 and 0.26 is better highlighted by the graph representing the kinetic of induction and deconvolution of the NPQ value. It is clear that for the suppressor the relaxation of NPQ is very slow in respect to wild type one, indicating an higher presence of the qI component.

These results confirm the aforementioned (Chapter 3) idea that when one of the components of NPQ is decreased, the other two can adjust their extent in order to avoid decrement in the fitness of the plant. The genetic characterization of the two lines is still ongoing at the Berkeley laboratory: further results will tell us if *0.26* and *6.22* are real suppressor of the *npq4.1* and where the point mutation is located in the *Arabidopsis* genome.

Figure 42: NPQ kinetic in *A. thaliana* WT and 0.26 mutant



In regards of the energy partitioning approach, a parallel between the wild type line and the *Stn7* mutant is proposed at the different growth conditions [Fig.43-44]. Surprisingly, the extent of in the mutant does not vary between LL and HL whereas they increases significatevely for the wild type if grown in HL condition. This result seems to propose that the efficiency for this mutant is reduced in HL conditions due to the lack of state-stansition.

Figure 43: Energy partitioning representation for the mutant Stn7 in LL and HL

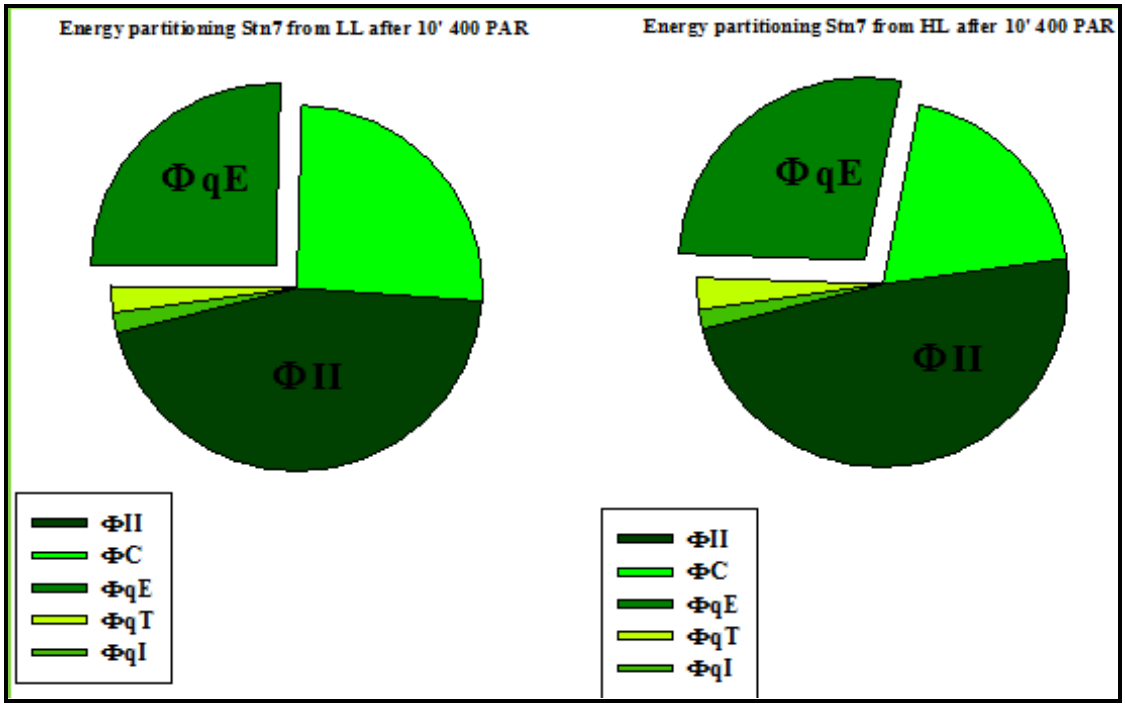
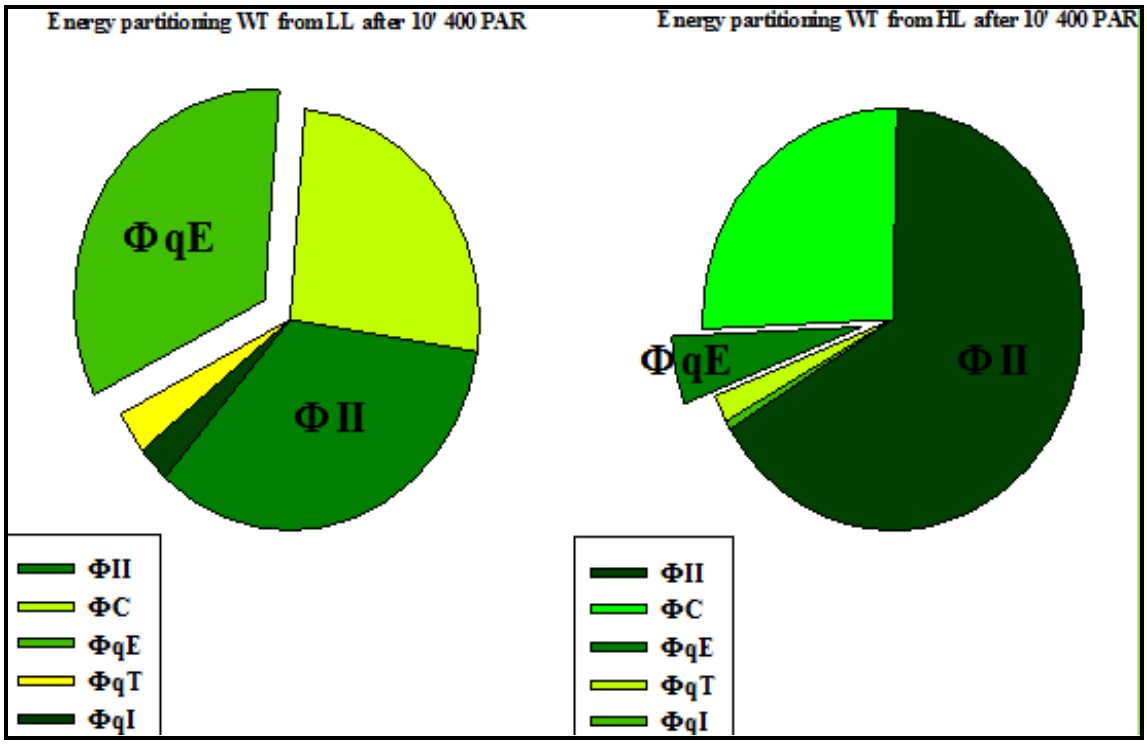


Figure 44: Energy partitioning representation for the mutant WT in LL and HL



In conclusion, the experiment reported in this Chapter allows to think that both suppressors of *npq4.1* do not restore the qE component of NPQ but only qI, further work will particularly characterize the mutation. Moreover, this is another proof of the variability of the NPQ components: again, qT and qI can supply the qE deficiency. Besides the analysis of the suppressor, this experiment demonstrate the reliability of the revised method, presented in the previous chapters; it seems to be a good approach for easy and fast confirmation of mutants phenotypes.

Chapter 8: *Chlamydomonas reinhardtii* experiments at Berkeley laboratory

Summary

During my second visit to the Berkeley laboratory, in accordance with Professor K.K. Niyogi, I focused my work on the microalga *Chlamydomonas reinhardtii*. NPQ mechanism in this organism is totally unknown but protein LHCSRs seem to play an important role in this process (Chapter 1). The results of three different experiments are reported in this chapter. Firstly, the characterization of suppressors of the *npq4* mutation obtained *via* UV mutagenesis is presented. Then, results of an experiment with the overexpressor mutant for the LHCSR3 protein are summarized. Lastly, an high light transfer experiment with wildtype strain of *Chlamydomonas* was carried out, in order to find out the time points of interest for the NPQ induction.

These three experiments were all performed with the aim of correlating the NPQ value to the LHCSRs proteins accumulation.

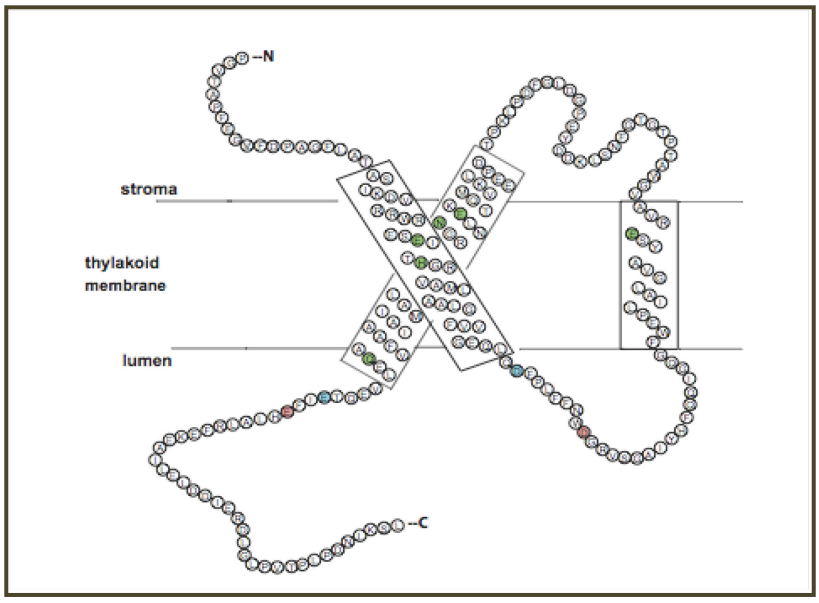
Preface

The contents presented in this Chapter are the result of a collaboration with Thuy B. Truong (PhD student) and K.K. Niyogi, Plant and Molecular Biology Department, University of California, Berkeley (*unpublished data*).

8.1 Introduction

Insertional mutagenesis in *Chlamydomonas* has given some clues as to the nature of the sites of quenching in algae. There exists a number of novel antenna proteins found in green algae, as the LHCSRs (Light Harvesting Complex Stress Related proteins) [Fig.45]. These ancient light harvesting antenna proteins seem to be strictly related to NPQ mechanism and their expression is induced when the algae are exposed to high light (*Peers et al., 2009*). LHCSRs proteins are present in *Chamydomonas* in three different isoforms LHCSR1-LHCSR2-LHCSR3 [Fig.46]. Two of these isoforms and their correlation with the NPQ are the main subject of the experiments reported in this Chapter. In particular, different strain of *Chlamydomonas*, were utilized in order to clarify their role in the triggering of the NPQ mechanism. The wild type strain ($4A^{+}$), has the expression of all the isoforms of LHCSRs proteins. The overexpressor utilized in these experiment, instead, shows an overexpression only in the isoform LHCSR3 but this seems to be enough for causing an important increase in the NPQ value (*Peers and at., 2009*). Genetic analyses showed that a single nuclear mutation is responsible for the low NPQ phenotype of the *npq4* mutant. Thus this strain is not affected in either of two linked PSBS genes, previously shown to be critical for qE in plants, which are located on linkage group I. The flanking genomic DNA fragment sequenced in the npq4 mutant is 2 kilobases upstream of two genes (LHCSR3.1 and LHCSR3.2) that encode identical LHCSR proteins

Figure 45: LHCSR protein structure



(LHCSR3).

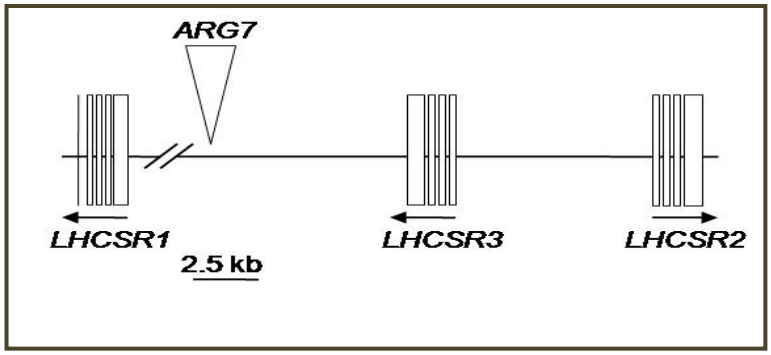
Figure 46: Identity between LHCSR1 and LHCSR3

SeqA	Name	Len(aa)	SeqB	Name	Len(aa)	Score
1	LHCSR1	253	2	LHCSR3.1	259	82
1	LHCSR1	253	3	LHCSR3.2	259	82
2	LHCSR3.1	259	3	LHCSR3.2	259	100

```
LHCSR3.1          MLANVVSRKASGLRQTPARATVAVKSVSGRRTTAAEPQTAAPVAAEDVFAYTKNLPGVTA 60
LHCSR3.2          MLANVVSRKASGLRQTPARATVAVKSVSGRRTTAAEPQTAAPVAAEDVFAYTKNLPGVTA 60
LHCSR1            --MAMMMRKAAAVPAS-SRRSVAVNSVSGKRTVSGK--AGAPVP-EDVLAYAKTLPGVTA 54
                      :: ***:.:  : :* :***:****:**.:.:  :.***. ***:**:*.******

LHCSR3.1          PFEGVFDPAGFLATASIKDVRRWRESEITHGRVAMLAALGFVVGEQLQDFPLFFNWDGRV 120
LHCSR3.2          PFEGVFDPAGFLATASIKDVRRWRESEITHGRVAMLAALGFVVGEQLQDFPLFFNWDGRV 120
LHCSR1            PFDNVFDPAGFLATASVKDVRRWRESEITHGRVAMLAALGFIVGEQLQDFPLFFNFDGRV 114
                  **:.************:*************************:*************:****

LHCSR3.1          SGPAIYHFQQIGQGFWEPLLIAIGVAESYRVAVGWATPTGTGFNSLKDDYEPGDLGFDPL 180
LHCSR3.2          SGPAIYHFQQIGQGFWEPLLIAIGVAESYRVAVGWATPTGTGFNSLKDDYEPGDLGFDPL 180
LHCSR1            SGPAIYHFQQIGQGFWEPLLIAIGVAESYRVAVGWATPTGTGFNSLKDDYEPGDLGFDPL 174
                  ************************************************************

LHCSR3.1          GLKPTDPEELKVMQTKELNNGRLAMIAIAAFVAQELVEQTEIFEHLALRFEKEAILELDD 240
LHCSR3.2          GLKPTDPEELKVMQTKELNNGRLAMIAIAAFVAQELVEQTEIFEHLALRFEKEAILELDD 240
LHCSR1            GLKPTDPEELKTLQTKELNNGRLAMIAIAAFVAQELVEQTEIFEHLVLRFEKEVILELED 234
                  **********.:*********************************.******.****:*

LHCSR3.1          IERDLGLPVTPLPDNLKSL 259
LHCSR3.2          IERDLGLPVTPLPDNLKSL 259
LHCSR1            VERDLGLPLTPLPDNLKAI 253
                  :********:*********::
```

Integration of plasmid DNA into the *Chlamydomonas* genome is often accompanied by a deletion and/or rearrangement of adjacent DNA, and genomic polymerase chain reaction (PCR) analysis showed that neither LHCSR3.1 nor LHCSR3.2 is intact [Fig. 47].

Figure 47: LHCSRs genes in *npq4* mutant of *Chlamydomonas*



Correspondingly, LHCSR3.1 and LHCSR3.2 messenger RNAs are undetectable in *npq4*. A third LHCSR gene (LHCSR1), which encodes a protein that is 82% identical to LHCSR3 [Fig.46], is located on the same linkage group and is 240 kilobases upstream of the insert. This gene is intact and is transcribed in the *npq4* mutant (*Peers et al., 2009*).

In the first experiment, strain of *np4* were mutagenized in order to find suppressors of the mutation capable of NPQ restoration. The UV mutagenesis induces point mutation and through this method different results can be expected. It would be possible identify: regulatory factors that would express LHCSR1 in *npq4*; mutations that increase the pH gradient or mutations that allow for higher NPQ independent of LHCSR.

In the second experiment performed, the NPQ induction in the overexpressor of LHCSR3 in LL conditions was assessed. The purpose was to look for necessary factor (s) for the qE induction in regards to LHCSRs.

Lastly, an high light transfer experiment with the $4A^{+}$ strain was performed to look at the timeline of qE induction and LHCSRs expression and see the eventual correlation.

8.2 *np4* suppressors: mutagenesis and characterization

8.2.1 Materials and Methods

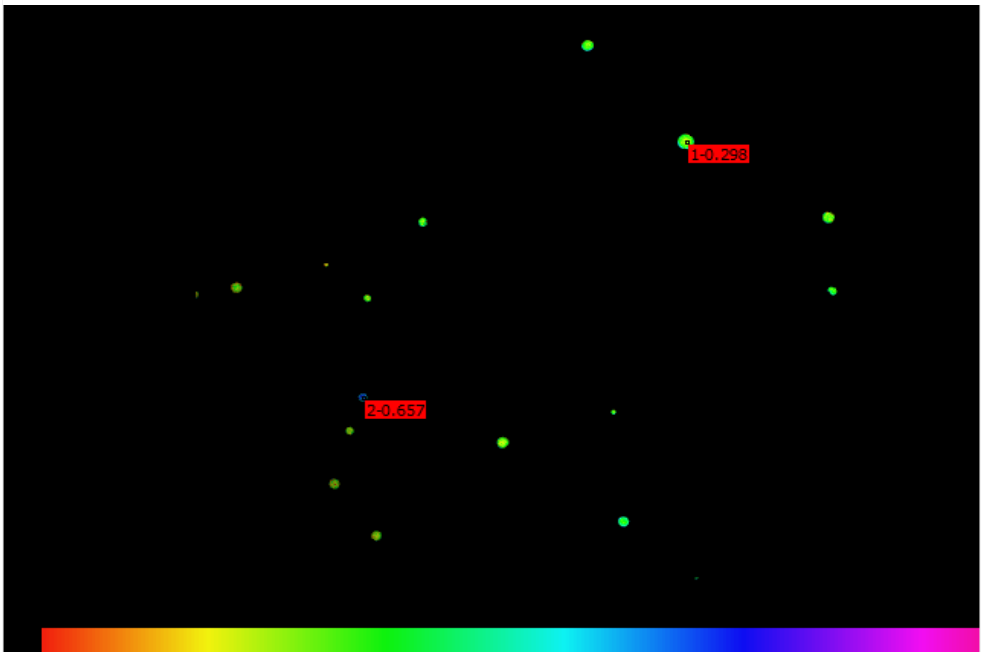
The *npq4* mutant was generated from the arginine-requiring CC-425 background as described previously (*Niyogi et al., 1997*). Liquid coltures were grown in LL conditions (40 µmol photons $m^{-2}s^{-1}$) up to a cell concentration of 4 millions cells/ml. Then, under a safety hood, using the Stratalinker UV crosslinker the mutagenesis was inducted with an energy level 70,000 µjoules.

Petri dishes with mutagenized colture and control dishes at different dilution were grown photoautotrophically for 10 days. At the end of this period, a screening via imaging fluorescence (Imagin PAM, Walz) was completed. The colonies with higher NPQ value respect to the control (*npq4*) were re-patched on new plates and further analyzed. Chlorophyll fluorescence measurements of *Chlamydomonas cells* were performed with an Hansatech FMS2 system. Cells were dark-acclimated for 30 min before measurement, then gently filtered onto a glass-fibre filter and placed on the instrument's leaf clip. The maximum efficiency of PSII, (Fv/Fm), was measured after a far-red pulse to ensure transition into state I. Fo is the fluorescence resulting from the measuring light

alone. Fm is the maximum fluorescence measured during a brief, saturating flash of light. Fv is the variable fluorescence. Cells were exposed to actinic light of 500 µmol photons $m^{-2}s^{-1}$ to induce NPQ. Total NPQ was calculated as (Fm-Fm')/Fm', where Fm' is the maximum fluorescence measured in the light-adapted state (during or after actinic light illumination). Chlorophyll content per cell and Western Blot analyses were executed on samples from cultures grown in LL (about 40 µmol photons $m^{-2}s^{-1}$) and HL (400 µmol photons $m^{-2}s^{-1}$) conditions. HPLC analyses on cell cultures (15.000.000 cell concentration) were performed too.

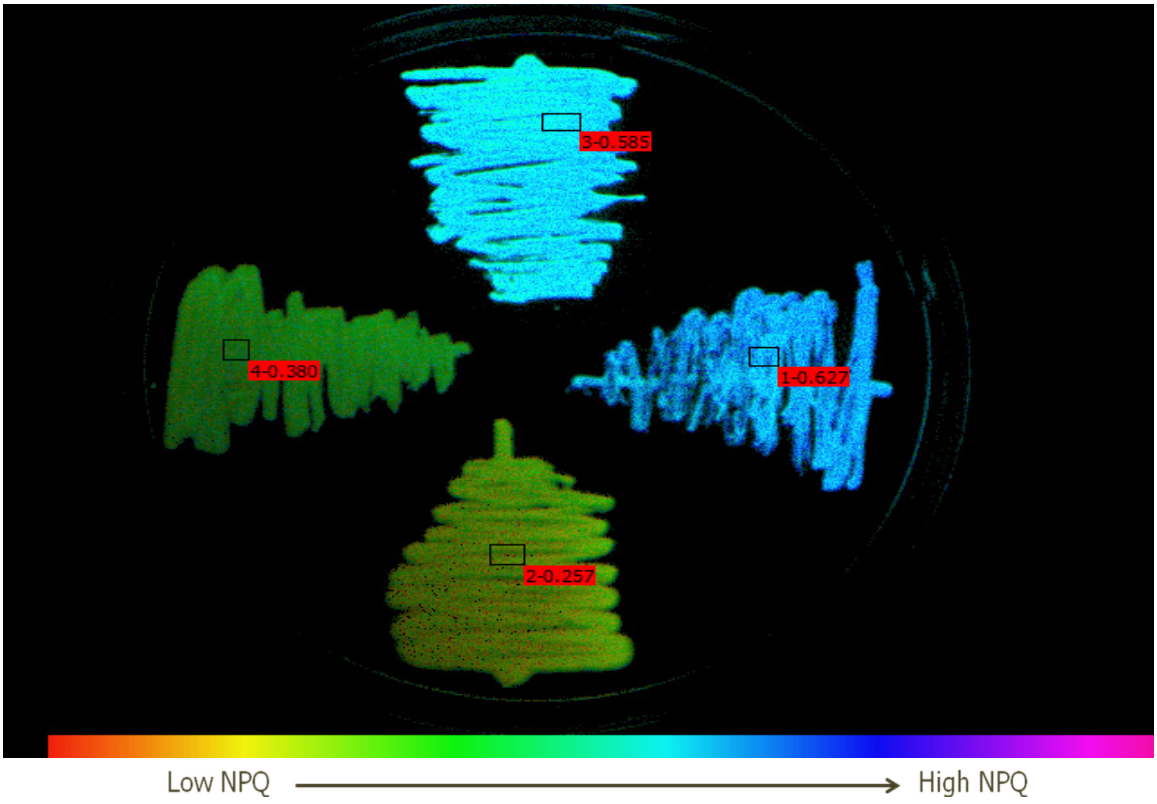
8.2.2 Results and discussion

Figure 48: Image of chlorophyll fluorescence of a mutagenized plate of *Chlamydomonas npq4* mutant



After ten days in HL in photoautotrophic conditions, the UV mutagenized and the control plates of *Chlamydomonas npq4* were screened via Imaging PAM. Occasionally, on mutagenized plates some colonies showed an higher value for NPQ respect to the average value of the colonies on the same plate [Fig. 48]. In the reported figure, the average of the NPQ value is 0.298, value perfectly fitting with the average value of *npq4* mutant strain grown in HL conditions. In this case, one colony showed an higher value for NPQ (0.657) and was re-patched on a new plate in order to grow in HL condition a bigger amount of biomass and compare it with *npq4* and $4A^{+}$ colonies. This higher values of NPQ were detected on several plates but once re-plated the phenotype often disappeared, as a consequence of a stressed condition [Fig.49- left strain].

Figure 49: Re-patched plate of *Chlamydomonas* strains. Top: 4A+; Right: SUPP1; Bottom:*npq4* and Left: supposed suppressor. In red NPQ values



Two strains were found having a strong phenotype for the NPQ restoration and were named SUPP1 and SUPP2. From these two colonies, liquid cultures were started in order to assess the NPQ dynamics using the chlorophyll fluorescence pulse modulated method. In LL conditions, both SUPP1 and SUPP2 showed an higher value in respect to the *npq4* for NPQ after the induction period, even without any strong differences in the dynamic behavior [Fig.50]. Interestingly, when the cultures were grown in HL conditions, the phenotypes seemed to be much more stronger. It is clear from the graph that SUPP1 was able to partly restore an NPQ value similar to the $4A^{+}$ strain than to *npq4* [Fig. 51]. On the other hand, from the chlorophyll fluorescence measurements, SUPP2 seemed not dissimilar from the *npq4* strain. As a conclusion, the fluorescence reply induced to think that SUPP1 could be a real suppressor of the *npq4* mutation and for this reason capable of NPQ restoration. However, the induced NPQ was quickly relaxed within the first minutes in darkness for the WT, *npq4* and SUPP2 but not in SUPP1, where the relaxation happened after about 13 minute of darkness. This result seemed to suggest a restoration of other components of NPQ besides qE, but further analysis are due to validate this hypothesis.

Figure 50: NPQ induction and relaxation behaviour for different strains of *Chlamydomonas reinhardtii* in LL conditions

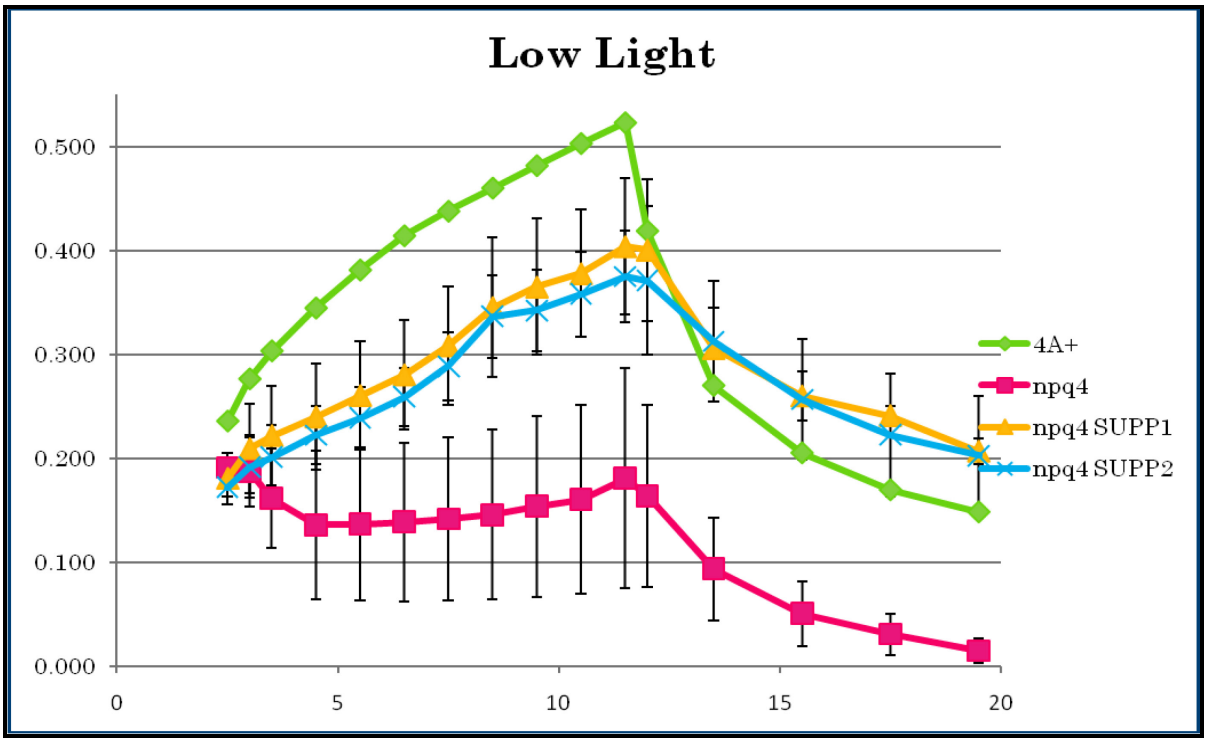
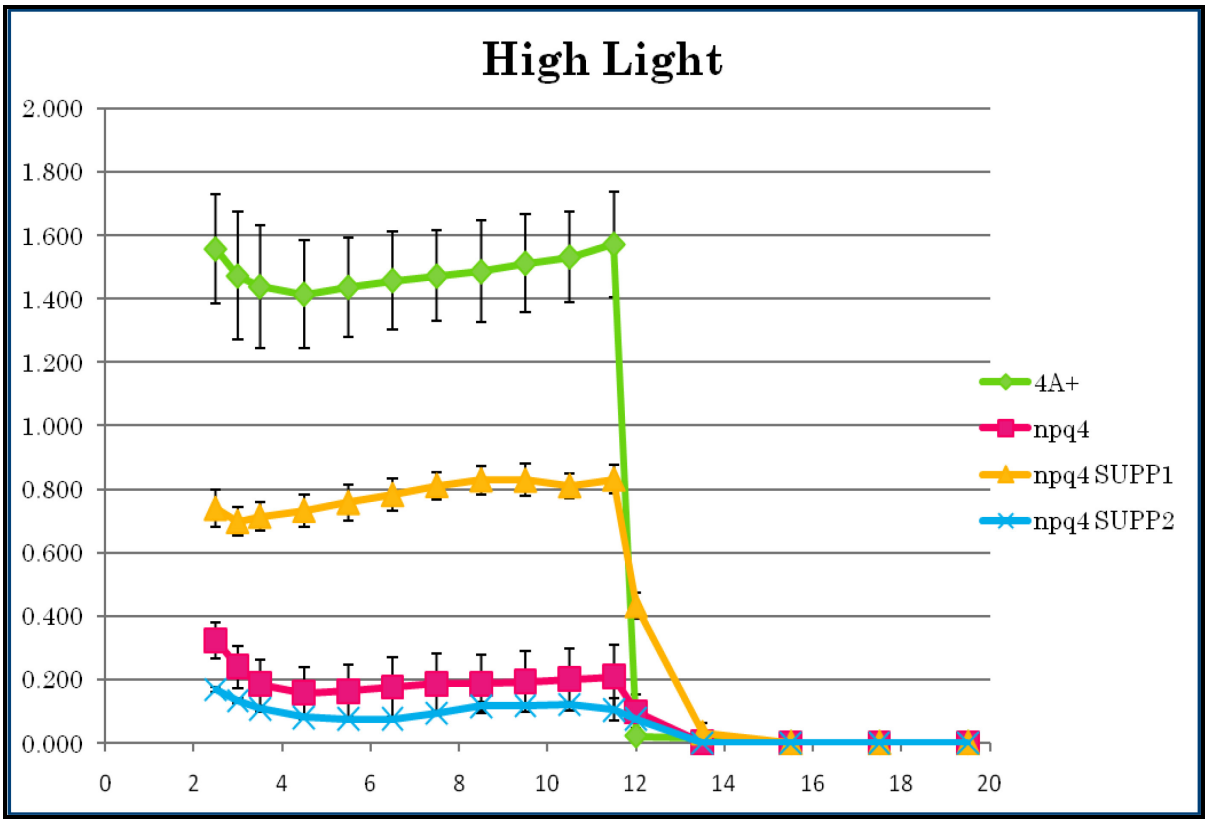
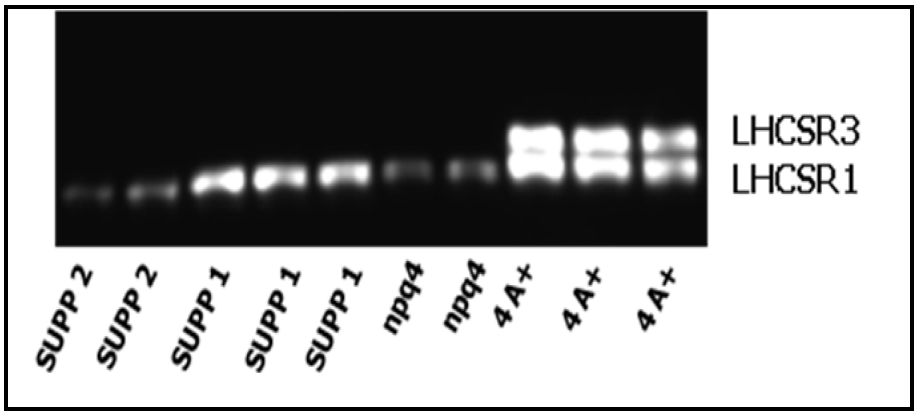


Figure 51:NPQ induction and relaxation behaviour for different strains of Chlamydomonas reinhardtii in HL conditions



Contemporary to the fluorescence measurements, analyses of immunoblotting were performed on samples taken from the different cultures in HL conditions. The loading were done on chlorophyll content basis and a primary antibody against all the LHCSRs (isoform 1 and 3) were utilized [Fig.52].

Figure 52: Western Blot analyses for different strains of *Chlamydomonas* grown in HL conditions



In particular, the $4A^+$ strain showed an high concentration of both isoforms of LHCSRs and, as expected, the *npq4* presented only a light band for the isoform1. In regard to the two hyphotized suppressors, SUPP1 interestingly showed an higher amount of LHCSR1 protein, suggesting an unexpected relation between this isoform and the qE or total NPQ restoration. On the contrary, SUPP2 showed a protein LHCSRs profile totally similar to the *npq4*, supporting the idea that this is not a real suppressor of the mutation. Additionally, it is worthy of note that analyses through HPLC showed these values for Zea concentration: 4 A^+ = 15.9 mmol/mol and SUPP1 = 61.7 mmol/mol. These results indicate that LHCSR1 may link Zea more than the WT or an overexpression of the same protein may cause an alteration in the production of the pigment during the xanthophylls cycle.

In conclusion, this experiment was successful in the induction and primary characterization of SUPP1, a suppressor of the *npq4* mutation. Further genetic analyses are needed to a complete collocation of the mutation. Before my departure from the Berkeley laboratory, I sequenced the LHCSR1 gene, designed different primers and executed a series of crossing of the new strain back to WT and *npq4,* in order to start the genetic classification of the mutation.

8.3 Suppressor of LHCSR3 overexpressor

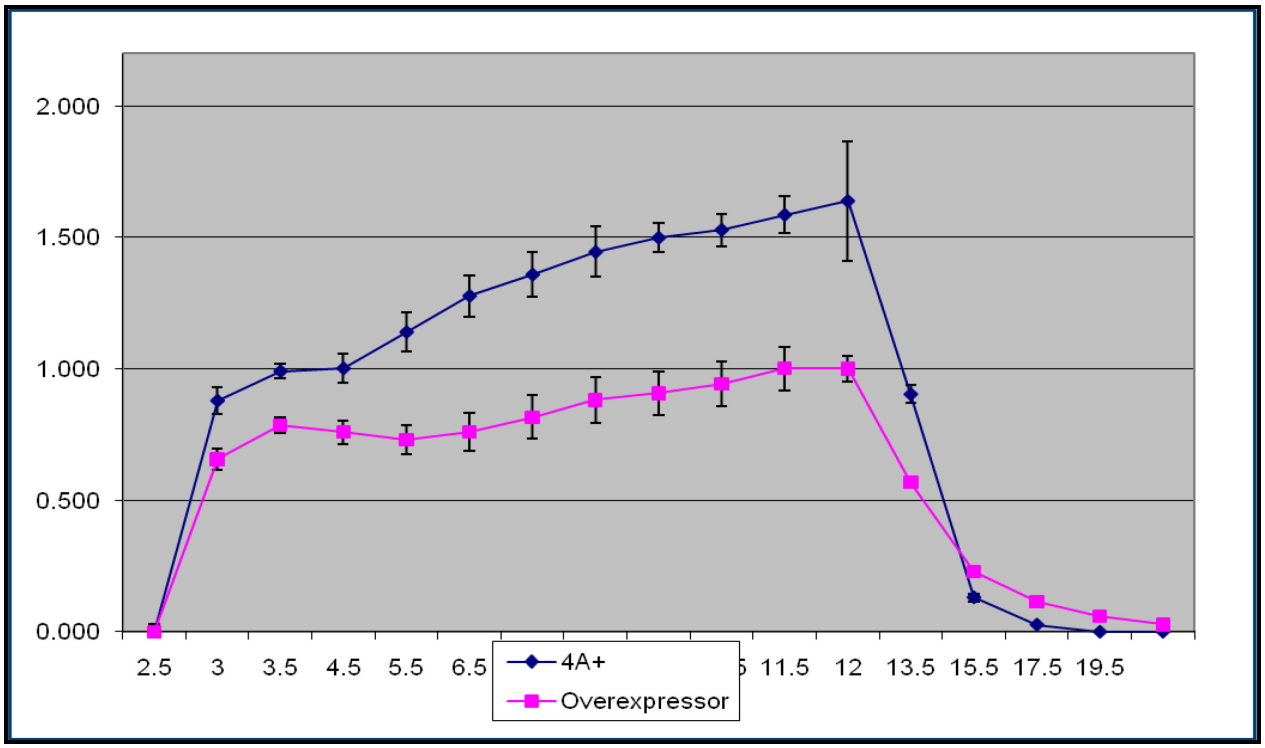
8.3.1 Materials and Methods

Liquid photoautotrophic cultures of overexpressor of the LHCSR3 and WT as a control were grown in LL conditions (about 40 µmol photons $m^{-2}s^{-1}$). Chlorophyll fluorescence measurements of *Chlamydomonas* cells were performed with an Hansatech FMS2 system. Cells were dark-acclimated for 30 min before measurement, then gently filtered onto a glass-fibre filter and placed on the leaf clip of the instrument. The maximum efficiency of PSII, (Fv/Fm), was measured after a far-red pulse to ensure transition into state I. Fo is the fluorescence resulting from the measuring light alone. Fm is the maximum fluorescence measured during a brief, saturating flash of light. Fv is the variable fluorescence. Cells were exposed to actinic light of 500 µmol photons $m^{-2}s^{-1}$ to induce NPQ. Total NPQ was calculated as (Fm-Fm')/Fm', where Fm' is the maximum fluorescence measured in the light-adapted state (during or after actinic light illumination). Chlorophyll content per cell and Western Blot analyses were executed on samples from cultures.

8.3.2 Results and discussion

For this experiment, the induction and relaxation of NPQ for the overexpressor in LL conditions was performed. Moreover, immunoblot analyses of LHCSRs protein levels was executed too. There were two possible expected results. In the first case, if NPQ would be not induced and LHCSR3 overexpressed, other factors could be related to NPQ induction. In the second case, with NPQ not induced and LHCSR3 not overexpressed. As showed in figure 53, the overexpressor grown in LL did not present a higher NPQ value in respect to the WT strain.

Figure 53:NPQ behavior versus time (minutes) in the WT and LHCSR3 overexpressor of *Chlamydomonas*



From the immunoblot results, LHCSR3 proteins were not overexpressed. In the showed gel, there is a bright band for the $4A^{+}$ correspondent to one of the two bands typical for this strain of LHCSR3 protein. For the overexpressor, two different cultures, from two different tetrads after crossing, showed a very light band in correspondence of LHCSR3. Unfortunately, seen that neither NPQ nor LHCSR3 overexpression are induced in LL conditions, it is not possible concluding anything about whether this protein is sufficient for the NPQ induction.

Figure 54: Western Blot analyses of LHCSR3 overexpressor in LL conditions

8.4 High light transfer experiment of wild type strain of *Chlamydomonas reinhardtii*

8.4.1 Materials and method

Liquid photoautotrophic cultures of WT $4A^+$ were grown in LL conditions (about 40 µmol photons $m^{-2}s^{-1}$). After five days, different cultures were merged in a big glass bowl of 650 ml in volume. On a stirrer, the bowl was transferred in the HL chamber (400 µmol photons $m^{-2}s^{-1}$). Samples were collected every hour in HL and a first one was collected from the cultures as soon as taken from LL condition. Chlorophyll fluorescence measurements of *Chlamydomonas* cells were performed with an Hansatech FMS2 system. No dark adaptation before measurement was executed, then gently filtered onto a glass-fibre filter and placed on the leaf clip of the instrument. The maximum efficiency of PSII, (Fv/Fm), was measured after a far-red pulse to ensure transition into state I. Fo is the fluorescence resulting from the measuring light alone. Fm is the maximum fluorescence measured during a brief, saturating flash of light. Fv is the variable fluorescence. Total NPQ was calculated as (Fm-Fm')/Fm', where Fm' is the maximum fluorescence measured in the light-adapted state (after actinic light illumination).For this particular experiment, initial Fm value from LL induction was used for NPQ calculation. Chlorophyll content per cell and Western Blot analyses were executed on samples from cultures.

8.4.2 Results and discussion

From the time point zero, correspondent to the measurement from LL condition, the NPQ value increases almost constantly through the first five hours in the HL conditions. This point seems to be an important time point where the curve of NPQ versus time reaches a plateau [Fig.55].

After twenty-four hours, the NPQ value is stable if the standard error bars are taken into account. It is to be underlined that the reported value utilize the first recorded Fm value. This adjustment is necessary due to the great variability of Fm value during the time [Fig.56].

Figure 55: NPQ value versus time during HL transfer of 4A$^+$

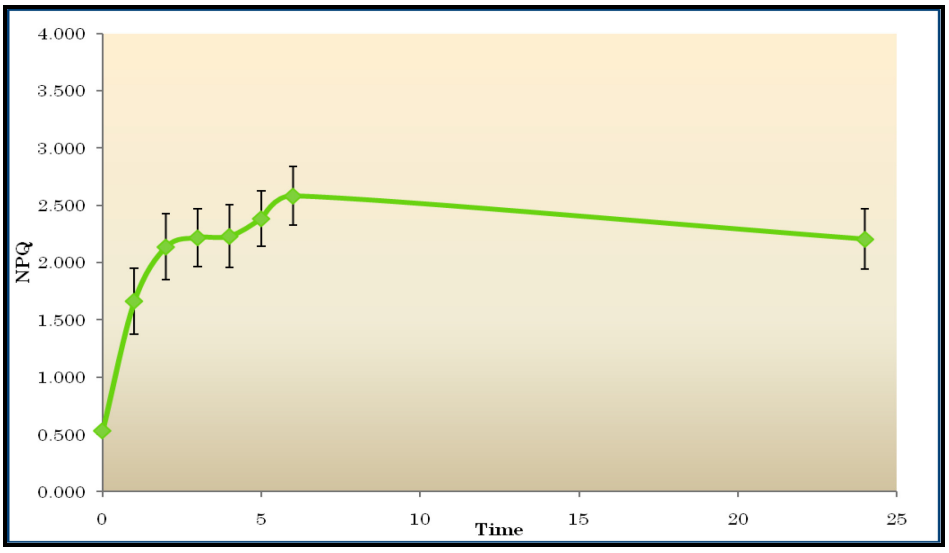


Figure 566:Fm and F0 values versus time during HL transfer of 4A$^+$

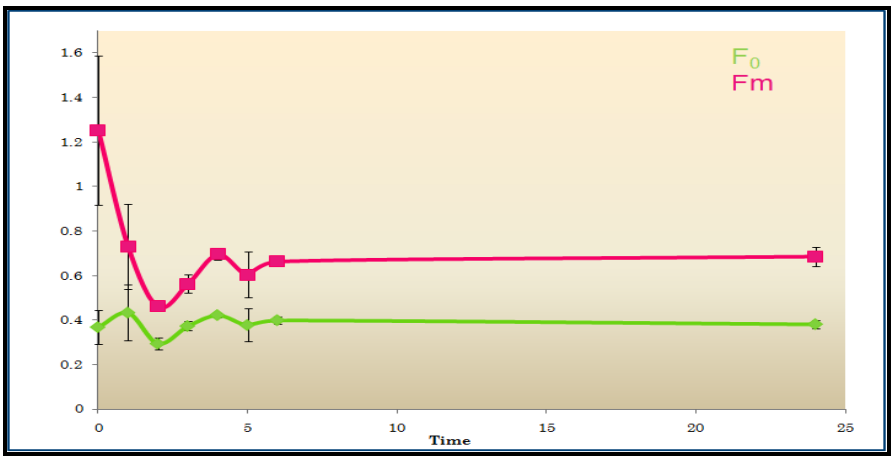
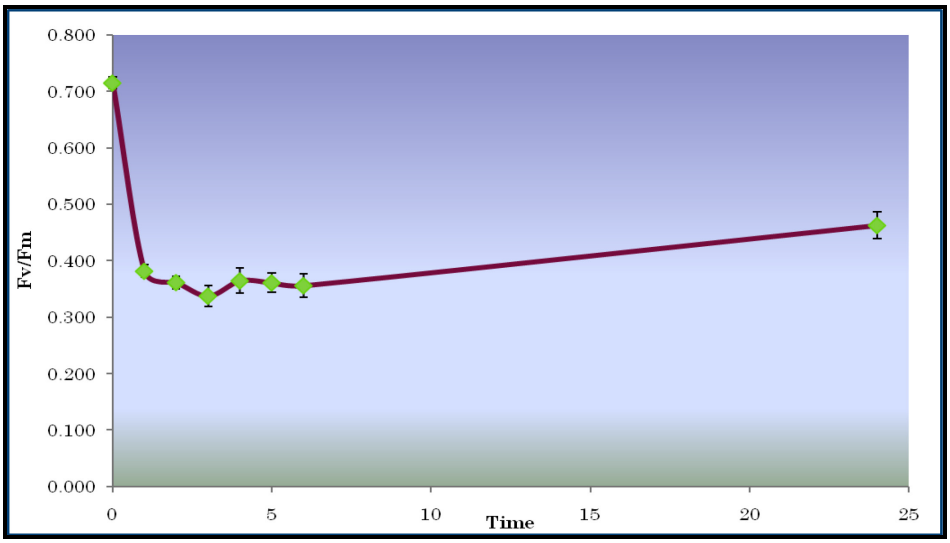
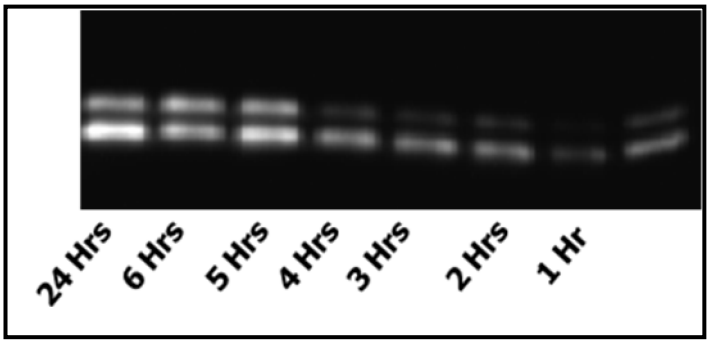


Figure 577:Fv/Fm value versus time during HL transfer of 4A$^+$



As a reference of the fitness for the culture during the HL transfer, the value Fv/Fm is reported against the time [Fig.57]. Every hour a sample for the protein analyses was collected too.

Figure 58: Immunoblot analyses of LHCSRs during HL transfer of 4A^{+}



It is very clear from the gel [Fig. 58] that the LCHSR3 proteins are accumulated during the hours passing. Very strong is the difference between four and five hours. This accumulation seems to be related with the second smaller increment in the NPQ value at the same time point. These results suggest that NPQ values and LHCSR3 are related during the HL transfer in these experimental conditions. It is important that in this experiment no dark adaptation was performed on the cultures.

In conclusion, the important time point for the LHCSR3 accumulation seems to be collocated between four and five hours from the transfer in HL. Further analyses will be performed at the Berkeley laboratory in order to validate this result with RNA seq to see which other genes are co-expressed in order to determine the other factors necessary for qE.

9.1 Conclusions

Thanks to the development of this PhD thesis I had the opportunity of studying an important and still unclear mechanism as the Non Photochemical Quenching of chlorophyll fluorescence. This process is a composite of different features and many of those are still under debate. Additionally, I had the great opportunity of spending part of my PhD at the University of California-Berkeley in the laboratory of Prof. K.K. Niyogi. This experience gave me the opportunity of comparing and contrast NPQ attributes between plants and algae.

In this background, the results presented in this thesis allow to drawn some important conclusions:

- An original revised method for the energy partitioning in PSII has been developed (chapter 4) and a first step towards an unified method for evaluating the NPQ components has been taken (chapter 5). This methods allow to take into account the great variability of the NPQ mechanism and clarify the importance of each component (qE, qT and qI) under different experimental conditions (chapter 7). In this project the method has been experimented on different species of higher plants (*Spinacia oleracea*, *Beta vulgaris* and *Arabidopsis thaliana*) and it seems to perfectly fit different protocols and variabilities.
- An innovative prospective has been outlined in chapter 6, with the NMR experiment. Here the utilization of the ^{1}H NMR technique as a faithful mirror of the physiological state of plants has been proposed. In particular, a correlation between Vio signal and NPQ value has been demonstrated and it has been shown that is possible discriminate between light stressed and dark-adapted leaves by recording NMR the spectra.
- In regard to *Chlamydomonas reinhardtii* it has been demonstrated that during an HL transfer the LHCSRs are accumulated in a linear manner with the time. This result underlines the importance of these proteins not only for short-term HL stress but in longer HL transfer too.

- Lastly, an important result has been achieved with the characterization of SUPP1, suppressor of the *npq4* mutation of *Chlamydomonas reinhardtii*. This strain has an higher value in the concentration of LHCSR1 related to higher NPQ value. This suggests a crucial role for this protein in the NPQ mechanism besides the LHCSR3.

The conclusions here summarized seem to be of large interest for the NPQ research.

As matter of fact, the revised energy partitioning method can be a useful tool for the achievement of the NPQ repartition into the three different components. Further work is needed to merge this result into the NPQ background and to hopefully achieve an unified method with the collaboration of other research groups interested in the NPQ mechanism.

Moreover, the description of SUPP1 of *Chlamydomonas reinhardtii* will help the characterization of the LHCSR1 clarifying the role of this protein in the NPQ mechanism of this model organism.

Acknowledgment

My last remaining task is to acknowledge all those people that have contributed to the work described in this thesis.

First of all, I wish to thank my supervisor Prof. Nicola D'Ambrosio for introducing me to the world of plant biology. The joy and enthusiasm he has for his research was contagious and motivational for me, even during tough times in the PhD pursuit.

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My thanks go out to all the past and present PhD students, PostDocs and Researchers of the Ecology group at the University of Naples and of the Niyogi lab at the California University – Berkeley, they made the laboratory a convivial place to work.

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