Doctoral thesis

CHANGES IN HIGHER PLANTS INDUCED BY A DEFINED HEATING REGIME AND HEAT STRESS-RELATED PLANT RECOVERY

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Contents

I hereby declare that this thesis has been written by myself by using the resources given in the list of references.

In Olomouc, ...

Jiří Frolec

List of papers

The presented thesis is based on the following publications listed in chronological order [I-II]. The articles are referred to by their roman numerals in the text and are enclosed in the Supplement.

[I] Frolec J, Ilík P, Krchňák P, Sušila P, Nauš J (2008) Irreversible changes in barley leaf chlorophyll fluorescence detected by the fluorescence temperature curve in a linear heating/cooling regime. Photosynthetica 46: 537–546

[II] Frolec J, Řebíček J, Lazár D, Nauš J (2010) Impact of two different types of heat stress on chloroplast movement and fluorescence signal of tobacco leaves. Plant Cell Rep 29: 705-714

Declaration

I declare that my role in the preparation of the publications was as following:

[I] Chief author – working on all measurements and experimental procedures, preparation of the manuscript

[II] Chief author – working on part of measurements and experimental procedures, preparation of the manuscript

Jiří Frolec .....

On behalf of the co-authors, the above-mentioned declaration was confirmed by:

Prof. RNDr. Jan Nauš, CSc. .....

Doc. RNDr. Petr Ilík, Ph.D. .....

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Education

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Position:

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2006-2010	"Variability of components and interactions in plant pathosystem and effect of factors on their manifestation"; Ministry of Education, Youth and Sports of the Czech Republic, No. MSM 6198959215
2008-2010	"Integration of doctoral studies in biochemistry, plant physiology and biophysics"; Czech Science Foundation, No. 522/08/H003

Research fellowship

01/2010 – 03/2010	Institute of Plant Biology, Biological Research Center, Hungarian Academy of Science, Szeged, Hungary. Supervisor: Dr. Győző Garab

List of publications

Frolec J, Ilík P, Krchňák P, Sušila P, Nauš J (2008) Irreversible changes in barley leaf chlorophyll fluorescence detected by the fluorescence temperature curve in a linear heating/cooling regime. Photosynthetica 46: 537–546

Frolec J, Řebíček J, Lazár D, Nauš J (2010) Impact of two different types of heat stress on chloroplast movement and fluorescence signal of tobacco leaves. Plant Cell Rep 29: 705-714

Conference presentations

Frolec J, Ilík P, Krchňák P, Sušila P, Nauš J (2008) „Irreversible changes of leaf chlorophyll fluorescence detected during a linear heating/cooling regime". The COINS, Vilnius, Lithuania

Frolec J, Řebíček J, Lazár D, Nauš J (2009) "Impacts of two different types of heat stress on tobacco leaves". The Chemical Biophysics Symposium, Toronto, Canada

Frolec J, Řebíček J, Lazár D, Nauš J (2010) "Impact of two different types of heat stress on chloroplast movement and fluorescence signal of tobacco leaves". Visegrad Meeting, Tata, Hungary

Citations

Frolec J, Řebíček J, Lazár D, Nauš J (2010) Impact of two different types of heat stress on chloroplast movement and fluorescence signal of tobacco leaves. Plant Cell Rep 29: 705-714 (cited paper [I])

Lípova L, Krchňák P, Komenda J, Ilík P (2010) Heat-induced disassembly and degradation of chlorophyll-containing protein complexes in vivo. Author(s): Lipova L, Krchnak P, Komenda J, et al. Source: Biochim Biophys Acta 1797: 63-70 (cited paper [I])

Shu Z, Shao L, Huang HY, Zeng XQ, Lin ZF, Chen GY, Peng CL (2009) Comparison of thermostability of PSII between the chromatic and green leaf cultivars of Amaranthus tricolor L. Photosynthetica 47: 548-558 (cited paper [I])

Abbreviations

μ	coefficient of irreversibility defined as ratio of F intensity at 35 ºC after cooling and during heating of the sample
ABA	abscisic acid
ATP	adenosine-5'-triphosphate
Chl	chlorophyll
CP43 and CP47	the antenna chlorophyll binding proteins of PSII
CT	time-dependent collimated leaf transmittance
Cyt	cytochrome
D1 and D2	PSII reaction center proteins
DCMU	3-(3´,4´-dichlorophenyl)-1,1-dimethylurea
DGDG	digalactosyldiacylglycerols
DSC	differential scanning calorimetry
E_a	activation energy
F_0	minimal chlorophyll *a* fluorescence intensity
FLR	chlorophyll *a* fluorescence rise
F_M	maximal chlorophyll *a* fluorescence intensity, corresponding to all Q_A reduced
F_P	fluorescence intensity at the P step in FLR
FTC	fluorescence temperature curve
F_V	variable fluorescence defined as ($F_M - F_0$)
F_V/F_M	maximum quantum efficiency of primary photochemistry
H_1 (H_2)	normalized amplitude of increasing (decreasing) part of the CT curve
HS	heat stress
HSP(s)	heat-shock protein(s)
LHCI	chlorophyll-containing light harvesting complex of PSI
LHCII	chlorophyll-containing light harvesting complex of PSII
M_1 (M_2)	first (second) maximum of FTC during heating
MGDG	monogalactosyl-diacylglycerols
$NADP^+$	nicotinamide adenine dinucleotide phosphate
NPQ	non-photochemical Chl *a* fluorescence quenching
OEC	oxygen-evolving complex
P680	primary electron donor in PSII
P700	primary electron donor in PSI
PAR	photosynthetically active radiation
PG	phosphatidylglycerol
P_N	net CO_2 assimilation rate
PQ	plastoquinone
PSI	photosystem I
PSII	photosystem II
Q_A	first quinone electron acceptor in PSII
Q_B	second quinone electron acceptor in PSII
RC	reaction centre
Rubisco	Ribulose-1,5-bisphosphate carboxylase/oxygenase
S_1 (S_2)	normalized tangent of increasing (decreasing) part of CT curve
sHSPs	small heat-shock protein(s)
S_M	normalized area above the FLR curve

SQDG	sulphoquinovosyldiacylglycerels
T	temperature
T_C	critical temperature for the heat-induced fluorescence rise
T_{C1} (T_{C2})	first (second) critical temperature interval of irreversible fluorescence changes
T-jump	temperature jump
T_m	maximal T of linear heating or temperature of sample incubation
V_I	relative amplitude of the J step in FLR
V_J	relative amplitude of the J step in FLR
Y_D	tyrosine 160 of D2 polypeptide / tyrosine-D accessory donor
Y_Z	the secondary electron donor in Photosystem II (tyrosine 161)

Abstract

In the case of plants, high temperatures are one of the commonest environmental stress factors. Their impact however depends on the intensity and duration of the heat stress (HS). Although plants have developed various strategies for avoiding/tolerating high temperatures, serious HS results in irreversible heat damage. Recovery from less serious HS, nonetheless may be more important than the HS itself under time-varying field conditions.

In the first chapter of the thesis, the most significant structural and functional changes caused by elevated temperatures are summarized for both light and dark phases of the photosynthetic process. Considerable attention is also given to the reversibility of these changes as these provide clues to the phenomenon of heat tolerance.

The experimental part, based on two publications, focuses on study of heat stress effects and the reversibility of the resultant changes. The use of a variety of heating regimes (T-jump and linear heating with various heating rates) and comparison of their impact, formed the major part of the work.

The first experimental part deals with the reversibility of the fluorescence temperature curve (FTC) measured using four different heating/cooling rates (0.5, 1, 2 or 3 °C min^{-1}). The degree of fluorescence irreversibility after the heating/cooling cycle, a set of tangents of selected linear parts of the FTC and a denaturation model of transforming a photosystem II (PSII) from being fully functional into to an adversely changed one, were used for detailed evaluation of the measured data. A fully reversible response of PSII function as reflected in the reversibility of chlorophyll fluorescence, was found for maximal temperatures (T_m) of linear heating up to 42 °C. A partially reversible response occurred up to temperatures between 52 and 59 °C depending on the heating/cooling rate (from 0.5 to 3 °C min^{-1}). We applied the model to calculate activation energies (E_a) of this initial increase in the fluorescence irreversibility separately for each heating rate. Four different approaches led to values of from 30 to 50 kJ mol^{-1}, and these decreased slightly with the increasing heating rate. The assumptions used for the E_a evaluation suggest that the unrecoverable part of fluorescence changes is caused by irreversible closure of certain PSII reaction centers (i.e. the accumulation of Q_A^-).

The second part of the thesis deals with the impact of linear and temperature-jump heat stress on light-induced chloroplast movement. The leaf segments were either

linearly (2 °C min^{-1}) heated up to T_m or incubated for 5 min in a water-bath at the same temperature. The changes in light-induced chloroplast movement caused by the HS pretreatment were detected after the particular heating regime at 25 °C using a method of time-dependent collimated transmittance (CT). For each sample, the chlorophyll *a* fluorescence rise (FLR) was also measured to determine changes in PSII function caused by the HS and for comparison of sensitivity of the two methods. To evaluate the effect of the HS regime on the samples more accurately, we calculated 6 fluorescence parameters from the JIP-test and 4 transmittance parameters (amplitudes and rates of chloroplast translocation for both accumulation as well as avoidance response). The HS began to inhibit the chloroplast movement at lower stress temperatures (40 - 42 °C) than PSII function, as reflected in the FLR curves (42 - 45 °C). This difference in sensitivity of CT and FLR was higher for the T-jump than for the linear HS, indicating the importance of the applied heating regime. For the highest T_m (45 °C), the motility of chloroplasts was almost completely inhibited. We tentatively propose that the inhibition of chloroplast movement at higher temperatures may enhance the shielding function of the exposed upper layer of chloroplasts and help protect other chloroplasts located deeper inside the leaf from photoinhibitory damage. Moreover, measurement of collimated transmittance changes, caused by light-induced chloroplast movement, proved to be a sensitive method for use too, in the early detection of the adverse effects of elevated temperatures.

1. INTRODUCTION

Plant photosynthetic reactions take place in chloroplasts. These organelles contain an internal system of interconnected membranes – thylakoids, the site of photosynthesis. The main pigment-protein complexes are embedded within thylakoid membranes separating an inner aqueous phase, lumen, from outer stroma (Fig 1). High temperatures can alter the composition of the main components of the thylakoid membrane, lipids and proteins, as well as the spatial reorganization of the pigment-protein complexes.

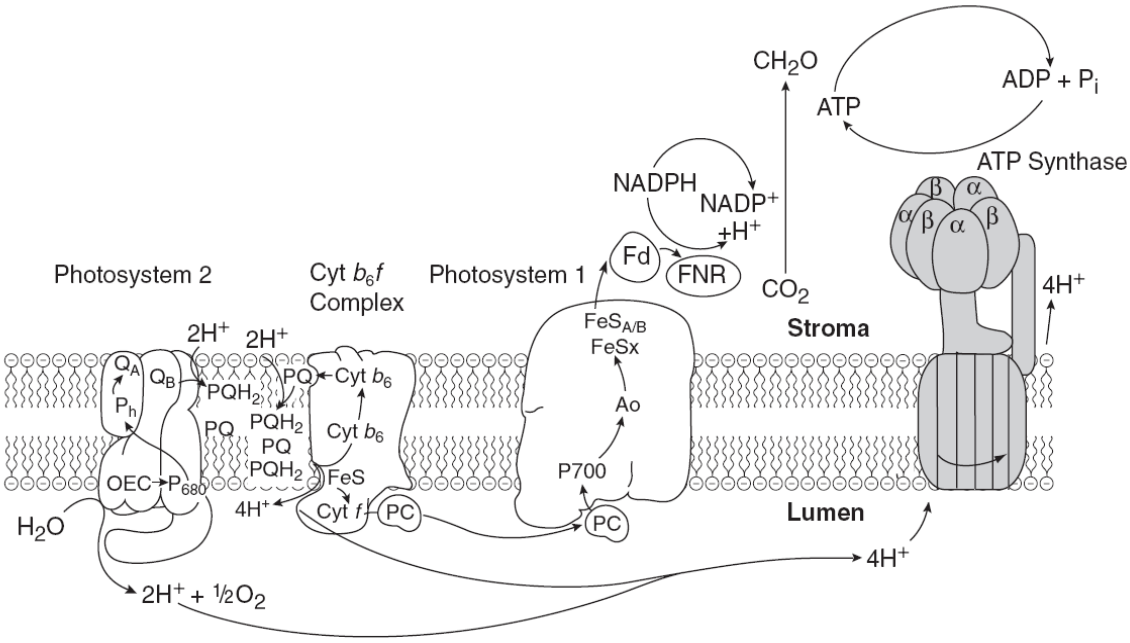

Figure 1. *Pigment-protein complexes and photosynthetic linear electron transport in thylakoid membrane (reprinted from Blankenship 2002 with permission[1]). Each complex is composed of several subunits and a large number of cofactors. The linear electron flow begins at the oxygen-evolving complex (OEC) by extracting electrons from water and continues via photosystem II, cytochrome b_6f complex and photosystem I to Ferredoxin (Fd). Ferredoxin carries the electron to the enzyme, ferredoxin NADP$^+$ oxidoreductase which reduces NADP$^+$ to NADPH. The electrochemical gradient of protons (H^+) generated within this process is used for the synthesis of ATP and, molecular oxygen (O_2) is released into the atmosphere.*

[1] With the kind permission of Wiley-Blackwell publishers, reprinted from Blankenship RE (2002) Molecular mechanisms of photosynthesis. Blackwell, London. Page 9, Figure 1.4.

These structural changes can impair the normal photosynthetic functions. For a more comprehensive understanding of plant reactions, this chapter summarizes the features of heat stress (HS) impact on the plant photosynthetic apparatus in more detail than those determined by papers listed in the Experimental part. Additional information can be found in related reviews by Berry and Björkman (1980), Weis and Berry (1988); Bukhov and Mohanty (1999); Carpentier (1999), Georgieva (1999), Wahid et al. (2007) and Allakhverdiev et al. (2008).

1.1. HEAT-INDUCED STRUCTURAL CHANGES IN CHLOROPLASTS

1.1.1. Thylakoid membrane composition

Plant membranes comprise a lipid bilayer and are formed mainly by monogalactosyl-diacylglycerols (MGDG) digalactosyldiacylglycerols (DGDG), sulphoquinovosyldiacylglycerels (SQDG) and the phospolipids, in particular phosphatidylglycerol – PG. The relative content of these lipids is about 45-50% (MGDG), 25-30% (DGDG), approximately 10% (SQDG) and around 13% (PG) in the case of PG (Harwood et al. 1994). Several membrane lipids, mainly DGDG, SQDG and phospolipids form the lipid bilayer. In contrast, MGDG belongs to the family of non-bilayer-forming lipids, i.e. after isolation from higher plants it forms non-bilayer structures - usually inverted cylindrical micelles (Quinn and Williams 1985; Williams 1988).

1.1.1.1. Changes in thylakoid membrane lipid composition

The fluidity of the thylakoid membrane is vital for maintaining the proper function of the lipid bilayer. It can be described as the relative diffusional motion of molecules within the membrane (Vigh et al. 1998) and its overall fluid character is due to its fatty acid saturation and the ratio of MGDG/DGDG. A heat-induced increase in molecular motion of membrane lipids causes increase in membrane fluidity (see Los and Murata 2004 for review) and this can lead to disintegration of the membrane. The subsequent plant acclimation reactions are a decrease in the MGDG/DGDG ratio (Süss and Yordanov 1986; Dörmann 2005; Chen et al. 2006) and increase in the incorporation of saturated fatty acids into the thylakoid membrane (Süss and Yordanov 1986; Larkindale and Huang 2004). These changes result in decreased membrane fluidity, i.e. in preservation of membrane properties. Higher growth temperature has also been shown to increase saturation in leaf lipids (Pearcy 1977) and reduce the MGDG/DGDG ratio (Wang and Lin 2006). However, the former findings contrast with the cyanobacterium-related study of Gombos et al. (1994) who showed that the desaturation of membrane lipids can to some extent stabilize photosynthesis under HS conditions.

It has been observed that some carotenoids, apart from their light collecting and quenching functions, can increase the thermostability of the photosynthetic apparatus against heat stress, due to decrease in membrane fluidity (see Havaux 1998 for review).

This role is attributed mainly to zeaxanthin accumulation and its interaction with the lipid phase of the thylakoid membranes (Havaux and Gruszecki 1993; Havaux et al. 1996; Tardy and Havaux 1997). A similar effect to increasing the thermostability of the thylakoid membrane is attributed to isoprene (2-methyl, 1,3,-butadiene), a compound emitted from the photosynthesising leaves of many plant species (Sharkey et al. 2001b).

1.1.1.2. Changes in protein composition

1.1.1.2.1. Denaturation of protein complexes in thylakoid membrane

More severe heat stress can result in the denaturation of existing proteins. For identification of the thermal denaturation of all main thylakoid protein complexes, a variety of approaches have been used but mainly differential scanning calorimetry (DSC) and electrophoretic methods.

Endothermic transition during DSC measurement occurring at about 42 °C has been attributed to OEC disruption (Cramer et al. 1981). Thompson et al. (1986; 1989) observed this transition in spinach photosystem II (PSII) membranes at 48 °C.

It is widely accepted that the D1 protein in PSII is very vulnerable to light stress. However, it seems to be very sensitive to high temperatures as well. Heat treatment of spinach thylakoids (40 °C for 30 min) resulted in cleavage of the D1 protein and production of 23 kDa N-terminal fragments whereas the D2 protein, a subunit of cytochromes b559, CP43, CP47, and LHCII did not cleave under the same HS (Yoshioka et al. 2006). It is assumed that a filamentation of temperature-sensitive protease is involved in the primary cleavage of the D1 protein and the well-known degradation process of this protein under light stress is similar to that induced by HS (Yoshioka et al. 2006).

Smith and Low (1989) observed that during calorimetric scans (DSC) of chloroplast membranes, denaturation of the whole PSII reaction centre complex occurred at around 60 °C. Shi et al. (1998) detected denaturation of the PSII core complex using the Fourier transform-infrared spectroscopy at similar temperatures (from 55 °C to 65 °C after 3 min incubation). Further analysis of DSC scans revealed that at the same temperature of around 60 °C, the core complex antennae of PS II, CP43 and CP47 was also denaturated (Smith and Low 1989). Interestingly, circular dichroism spectroscopy measurement showed that CP47 is more thermally stable than CP43

(Wang et al. 1999). These authors detected heat-induced denaturation of CP43 and CP47 after 5 min heat treatment at 50 °C and 63 °C, respectively.

The denaturation of the LHCII in spinach chloroplast membranes, determined by DSC at standard linear heating rate 1 °C min^{-1}, took place at 76 °C (Smith et al. 1989). This is in line with the complete degradation of LHCII observed in barley leaves between 70 and 80 °C (Lípová et al. 2010). The corresponding temperatures for irreversible denaturation (around 72 °C) have also been reported in the case of LHCII macroaggregates (Krumova et al. 2005). The temperature shifted from 69 °C to 74 °C with increase in scanning rate from 0.125°C min^{-1} to 2 °C min^{-1} showing the importance of absorbed heat.

Recently was investigated degradation of PSI. The PSI cores partially dissociated from light-harvesting complexes of PSI (LHCI) around 60 °C and formed aggregates (Lípová et al. 2010). Denaturation of PSI core polypeptides started at around 70 °C (Hu et al. 2004; Lípová et al. 2010) and they were completely degraded around 90 °C (Lípová et al. 2010). LHCI subunits were found to be more stable than PSI (Hu et al. 2004). Although the content of LHCI proteins began to decrease at a similar temperature, they were still present in the samples even at 100 °C. Further, a transition centred at around 65 °C has been shown to originate from denaturation of the CF_1 subunit of thylakoid ATPase (Smith et al. 1986; Nolan et al. 1992).

It has been shown that DSC transitions in pea thylakoids are reversible (Nolan and Vickers 1989; Nolan et al. 1992). Nolan and Vickers (1989) examined thylakoid membranes of cucumbers and peas during both heating and subsequent cooling scans in order to observe reversibility. Whereas cucumber thylakoids exhibited almost no reversibility, thylakoids of peas had reversible transitions at 50 °C and 68 °C. The results indicate that the calorimetric profiles of thylakoids from different plants can vary. Interestingly, at high temperatures, the granal thylakoids of pea denature irreversibly, whereas reversible transitions reflect changes in components associated with stromal thylakoids (Nolan et al. 1992).

1.1.1.2.2. Heat shock proteins

Plants respond to elevated temperatures by inducing the synthesis of a group of proteins called heat-shock proteins (HSPs). Expression of HSPs is increased when plants are exposed to high temperatures (see Wang et al. 2004; Efeoğlu 2009 for recent

reviews) but can also be triggered by exposure of plant tissue to other environmental stress factors, for example starvation (Zarsky et al. 1995), virus infection (Aranda et al. 1996; 1999), light stress (Stapel et al. 1993), metal toxicity (Orzech and Burke 1988) and even low temperatures (Sabehat et al. 1998). The increase in HSP synthesis is induced primarily by the so-called heat shock factor (see review by Nover et al. 1996). There are five main conserved classes of plant HSPs (named according to their molecular weights): HSP100, HSP90, HSP70, HSP60 and small (17 to 30 kDa) HSPs (Waters et al. 1996). HSPs are usually synthesized in the cytosol and subsequently transported into chloroplasts (Vierling et al. 1986; Carpentier 1999).

1.1.1.2.2.1. Synthesis of heat shock proteins

HSP expression correlates with cellular resistance to high temperatures and the accumulation of HSPs leads to increased thermotolerance (Lin et al. 1984; Lindquist and Kim 1996; Maestri et al. 2002; Jinn et al. 2004; Charng et al. 2006). A particularly important HSP for induced thermotolerance seems to be HSP104 (Lindquist and Kim 1996).

HSPs allow plants to reduce the impact of HS in several ways that are, however, still the subject of research. One of the main features of HSPs is their chaperone function. As molecular chaperones, the HSPs play a critical role in protein folding and coping with proteins denatured by heat or other stresses. This function is attributed mainly to the HSPs of a family of 60, 70 and 90 kDa (Carpentier 1999). Moreover, HSPs prevent aggregation of already denatured proteins or induce a refolding of stress-denatured ones (Lee et al. 1995; Wang et al. 2004). Other HSPs can assist if the protein aggregation has already taken place. In particular, HSP104, in coaction with additional specific chaperones, promotes the refolding of aggregated proteins and in this way assists the plant with inactive proteins (Glover and Lindquist 1998).

Apart from their chaperone functions, some HSPs may be associated with the thylakoid membranes (Süss and Yordanov 1986; Glaczinsky and Klopstech 1988; Carpentier 1999; Tsvetkova et al. 2002). For the pea, the binding temperature begins between 36 °C and 40 °C and incubation at 42 °C for 15 min is sufficient to induce the binding (Glaczinsky and Klopstech 1988). The association can regulate membrane fluidity and preserve membrane integrity during thermal stress (Tsvetkova et al. 2002). Süss and Yordanov (1986) suggested that interactions of HSPs with the outer

chloroplast envelope membrane might enhance formation of the DGDG species leading to increased thermotolerance due to decrease in membrane fluidity at elevated temperatures (see Section 1.1.1.1.).

It has been observed that small chloroplast HSPs (20-30 kDa) directly protect the thermolabile photosystem II under HS (Schuster et al. 1988; Stapel et al. 1993; Heckathorn et al. 1998). As a consequence, the electron transport chain from the donor side of PSII to the acceptor side of PSI was found to be preserved during HS treatment at 47 °C for 2 min (Heckathorn et al. 1998). The protective function of HSPs seems to be particularly important under the combined effects of light and heat stress (Schuster et al. 1988; Stapel et al. 1993). Further, an important role in photoprotection and reparation of PSII under photoinhibitory conditions is attributed to HSP70 (Schroda et al. 1999; 2001).

Although plant HSPs in most studies are detected within several hours, the formation of HSPs in cells may be more flexible, e.g. 10-15 min - treatment at temperatures of about 40 °C is sufficient for triggering (Nover and Scharf 1984) or even accumulation (Moisyadi and Harrington 1989) of HSPs in cells. Heat shock response depends not only on maximal stress temperature but also on applied temperature regimes. A gradual temperature increase (0.5 °C min^{-1} to 50 °C) permits far greater amounts of protein synthesis (including HSPs) than sudden heat shock (Howarth 1991). A similar positive effect on amount of synthesized HSPs was observed in the case of sample pre-incubation at moderately elevated temperatures (36 °C) before exposure to high temperatures (Ginzburg and Salomon 1986). The authors suggested that this effect could be due to an induction process, most probably the synthesis of heat shock mRNA, which is more sensitive to HS than the protein synthesis itself. Thus, if this induction is allowed, HSPs synthesis can follow at a more elevated temperature, resulting in the development of heat tolerance.

1.1.1.2.2.2. The role of HSP in recovery from HS

Some HSPs directly prevent irreversible heat-inactivation of other proteins under supraoptimal temperatures (e. g. Lee and Vierling 2000). The reversibility of HSP levels is characterized by decrease in their expression after restoration of the system to physiological temperature. The persistence of HSPs has been determined for example in pea leaves by Chen et al. (1990) and DeRocher et al. (1991). They investigated the

expression of small HSPs after a combined heating regime. HSP18.1 had a half-time of 37±8 h (DeRocher et al. 1991), which is quite similar to the 52±12 h half-life estimated for the chloroplast-localized HSP 21 (Chen et al. 1990). In addition, HSP 21 fully recovered to its initial level after about 7 days. Since the half-life of these proteins is long, they may play an important role during recovery (Chen et al. 1990; DeRocher et al. 1991). Interestingly, the levels of the HSP22 proteins from maize disappeared after heat stress more rapidly, with a half-life time about 4h and were undetectable after 21 h of recovery (Lund et al. 1998). For some HSPs, the duration of this period can be dependent on light intensity, as has been shown in the case of HSP 23 (Debel et al. 1994).

Synthesized HSPs play an important role in plants throughout these periods because the cells exposed to another heat shock are better protected from the effects of high temperatures. These HSPs also improve the recoverability under non-stress conditions. Forreiter et al. (1997) used firefly luciferase as a reporter of chaperone activities *in vivo*. HSP90 accelerated the luciferase renaturation during recovery. HSP70 had anologous effects if coexpressed with HSP17.6, showing that HSPs can assist in normalization of cellular functions during recovery from stress (Forreiter et al. 1997). Similar experiments have shown that sHSPs also improve the recovery process (Lee and Vierling 2000). Based on extensive genetic studies in yeast, a crucial role for plant recovery after different stress treatments is attributed to HSP104 (see Nover and Scharf 1997 for review).

Heat induced recompartmentalization of the chloroplast matrix that ensures effective transport of ATP from thylakoid membranes towards those sites where HSPs are being formed, is proposed as a metabolic strategy of plant recovery from heat stress (Süss and Yordanov 1986).

HS induces the expression of HSPs as well as decrease in normal protein expression including production of both photosystems and photosynthetic electron transport chain components (Süss and Yordanov 1986). High temperatures reversibly and irreversibly inhibited the synthesis of standard chloroplast protein in heat adapted and non-adapted bean plants, respectively (Süss and Yordanov 1986). These authors suggested that the inability to accumulate significant amounts of HSPs was due to rapid inactivation of the photosynthetic apparatus as a reason for their results. The experiments carried out by Nover and Scharf (1984) focused on the reversibility of proteins synthesized under standard conditions at room temperatures. They showed that

recovery at 25 °C was slow after 2 h at 39 °C, whereas an immediate recovery was observed in the preinduced culture (15 min at 40 °C followed by a 3-h incubation at 25 °C) after the second heat shock (1 h at 41 °C followed by 25 °C for 1 h). The response to repeated HS with respect to HSPs has been detected in millet seedlings as well (Howarth 1991). Full recovery occurred after 4 days under a regime of daily high T treatment of 2h at 50 °C followed by incubation for 22h at 35 °C.

The dark phase of photosynthesis is considered particularly sensitive to HS (see Section 1.2.2.1). Demirevska-Kepova et al. (2005) have reported that the high stability of Rubisco under HS in light may be related to the elevated levels of Rubisco binding protein, which is required for the assembly of the Rubisco holoenzyme. Since an increase in Rubisco activase and Rubisco binding protein was observed under 24-h HS and subsequent recovery in light, the results support the function of Rubisco binding protein as an HSP. The combination of high temperature and darkness had a severe effect on the investigated proteins and the damage caused by HS was unrecoverable. Taken together, although the role of many HSPs has not been fully elucidated, many can significantly improve the HS-related recoverability of plants.

1.1.2. Structural organization of thylakoid membrane

Pigment-protein complexes inside chloroplasts are arranged into stacked and unstacked regions called granal and stromal thylakoids, respectively. These regions are differentially enriched in photosystem I and II complexes (Fig. 2). Photosystem II is found almost exclusively in the appressed grana regions, whereas PSI and ATP synthase are found primarily in the non-appressed stromal regions (see Allen and Forsberg 2001 for review).

1.1.2.1. Reorganization of thylakoid membrane under HS

Heat stress alters the normal distribution of pigment-protein complexes. Temperatures above 35 °C caused detachment of PSII cores from LHCII and their lateral migration from the grana regions out into the stromal region, leaving behind the LHCII in appressed zone (Weis 1984; Sundby and Andersson 1985; Sundby et al. 1986). An analogous process is known to take place under strong light conditions, when some of the LHCII associated with the PSII core (i.e. in so-called State 1) are

phosphorylated, detached from PSII reaction center and connected to PSI (State 2), which results in increasing the PSI absorption cross-section (Allen and Forsberg 2001). As elevated temperatures usually accompany high light intensities, Sundby et al. (Sundby and Andersson 1985; Sundby et al. 1986) suggested that the temperature-dependent segregation of PSII from LHCII could be a regulatory mechanism to prevent overexcitation and subsequent damage of PSII due to high light intensities

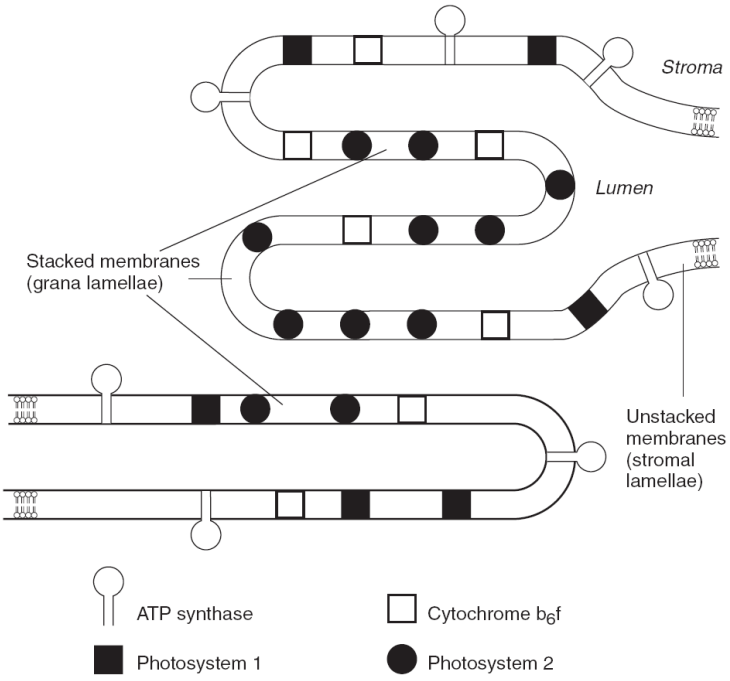

Figure 2. *Spatial distribution of main pigment-protein complexes in chloroplast (reprinted from Blankenship 2002 with permission*[2]*). Thylakoid membranes inside chloroplasts are formed into stacks called grana, which are connected by nonstacked membranes called stroma. Photosystem II is located in the stacked membranes while photosystem I in unstacked regions. For more details see text.*

The normal morphology of grana stacks in thylakoid membranes is usually distorted under HS (Armond et al. 1980; Gounaris et al. 1983; 1984). Measurements using freeze-fracture electron microscopy showed that incubation of bean chloroplasts for 5 min at 35 - 45 °C causes complete destacking of grana (Gounaris et al. 1983; 1984). Loosening of grana was also observed after linear heating (4 °C min^{-1}) up to 55 °C and was accompanied by swelling of the thylakoids (Ilík et al. 1995b).

[2] With the kind permission of Wiley-Blackwell publisher reprinted from Blankenship RE (2002) Molecular mechanisms of photosynthesis. Blackwell, London. Page 135, Figure 7.8.

The normal lipid bilayer (Fig.1) can exist in different states denoted as liquid crystal phase or gel lamellar phase (Williams 1988). Enhanced temperature is one of the factors causing the phase transitions from these phases to the inverted hexagonal phase. Treatment of bean chloroplasts above 45 °C caused phase-separation of non-bilayer lipids from membranes into aggregates of cylindrical inverted micelles (Gounaris et al. 1983; 1984).

1.1.2.2. Reversibility of structural changes in membranes

The above-mentioned detachment of PSII cores from LHCII was, up to 5 min incubation at 40 °C, almost fully reversible upon lowering the temperature (Sane et al. 1984; Sundby and Andersson 1985; Sundby et al. 1986). Both the heat-induced destacking as well as the phase-separation after incubations at temperatures higher than 45 °C were irreversible (Gounaris et al. 1983).

Heat has also been reported to induce aggregation of LHCII (Gounaris et al. 1984; Tang et al. 2007). Tang et al. (2007) observed *in vivo* and *in vitro* aggregation of LHCII caused by HS 10 min incubation at 35 °C or higher. An observed linear relationship between the formation of LHCII aggregates and NPQ represents LHCII aggregation as a protective mechanism to dissipate excess excitation energy. This heat-induced aggregation was reversible and caused probably by specific association between hydrophobic domains of different LHCII.

There is wide agreement that the reversible changes in the membrane structure precede the irreversible effects. These HS-induced modifications in different membrane properties reflecting injury, acclimation and recovery processes, are closely linked together and can occur simultaneously (Santarius and Weis 1988).

1.2. IMPACTS OF HIGH TEMPERATURE ON PHOTOSYNTHETIC FUNCTIONS

1.2.1. Effects of heat stress on components within thylakoid membrane

1.2.1.1. Oxygen-evolving complex

The oxygen-evolving complex (OEC) is located approximately at the membrane–lumen interface and is functionally connected with the PSII reaction centre. The OEC consists of a cluster of four manganese atoms, which catalyse the oxygen evolution, Ca^{2+} and Cl^- cofactors and three extrinsic proteins of 17, 23, 33 kDa (PsbQ, PsbP, PsbO, respectively) which are associated with the lumenal surface of the PSII reaction center and form the OEC (Fig. 3). This complex uses light energy trapped by PSII to extract electrons from water for the photosynthetic electron transfer chain and to produce molecular oxygen which is released into the atmosphere (see e.g. Goussias et. al 2002; Barber 2004; Renger and Kühn 2007).

1.2.1.1.1. Inactivation of oxygen evolution

OEC is generally considered one of the most sensitive components of the photosynthetic electron transport chain to HS conditions. The loss of two of the four manganese atoms results in the complete loss of oxygen-evolving activity (Nash et al. 1985). Heat treatment also causes release of the 33, 23 and 17 kDa proteins from PSII (Nash et al. 1985; Enami et al. 1994; Yamane et al. 1998; Barra et al. 2005). The release of the 17 kDa protein is most probably responsible for the heat-induced loss of the essential Ca^{2+} ion from the manganese complex (Barra et al. 2005). Ca^{2+} ion release has been also suggested as a first step in the heat-jump response of OEC (Pospíšil et al. 2003). The binding of the 17 kDa and 23 kDa protein has been shown to enhance the binding kinetics of the Ca^{2+} cofactor (Ghanotakis et al. 1984). According to Nash et al. (1985) the removal of the 23 and 17 kDa proteins from PSII particles causes only slight decrease in the heat stability of OEC.

In contrast, the 33 kDa protein seems to be essential for oxygen evolution. In particular, the 33 kDa protein is known to stabilize the manganese cluster (Nash et al. 1985; Rivas and Heredia 1999; Kuwabara et al. 2003). It has been shown that heat-inactivation of oxygen evolution is directly related to release of the 33 kDa protein (Enami et al. 1994). Its separation with high concentration of $CaCl_2$ or urea also always

results in a large decrease in the OEC activity (Miyao and Murat 1983; Ono and Inoue 1984). Release of this protein is easier in the absence of LHCII as has been shown in the case of *chlorina*-f2 mutant (Havaux and Tardy 1997).

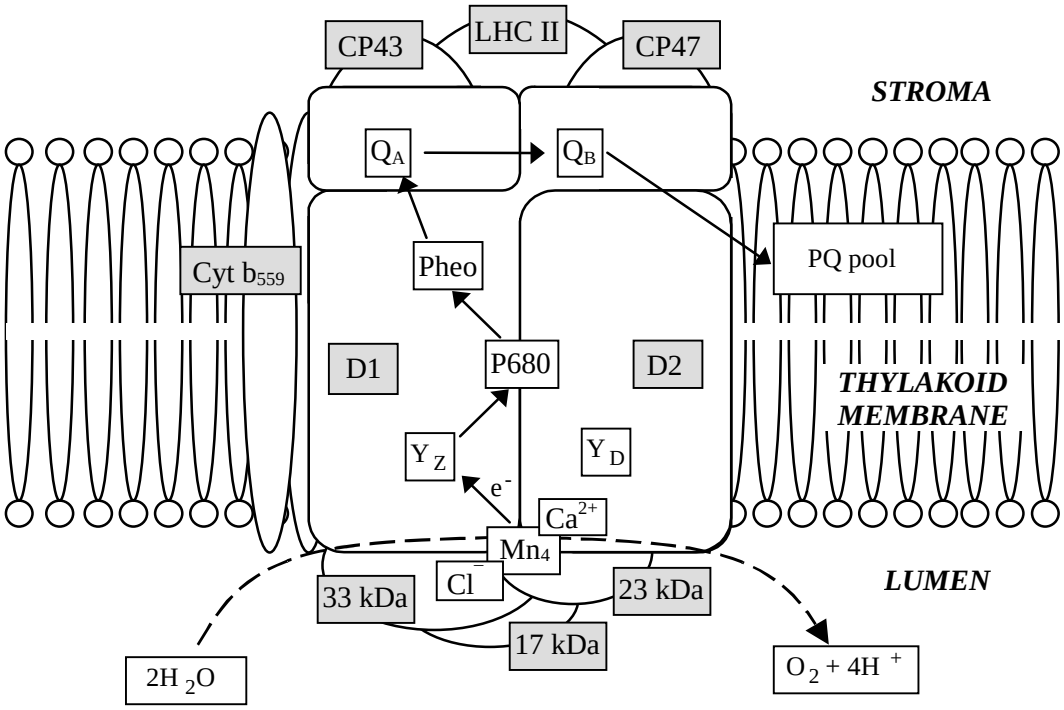

Figure 3. *Schematic structure of photosystem II (PSII) and its electron transport chain within the thylakoid membrane. Only major subunits of PSII (indicated in some cases by molecular masses, in other cases by name) are shown. Electron extracted from water reduces redox-active tyrosine residue (Y_Z) that then reduces the paired-chlorophyll and species P680, the primary electron donor in the photosystem II reaction center. The electron continues via pheophytin (Pheo) to the first (Q_A) and the second (Q_B) quinone electron acceptor in PSII and further, as indicated in Fig. 1.*

The effects of HS on OEC have also been investigated in many studies using chlorophyll *a* fluorescence rise (FLR). This curve is characterized by increase in fluorescence intensity from minimum to maximal yield via several peaks known as O, J, I and P. It mainly reflects gradual accumulation of reduced first (Q_A) and second (Q_B) quinone electron acceptors or following electron carriers in thylakoid membrane (for reviews see Lazár 1999; 2006; Lazár and Schansker 2009). Under HS conditions the shape of FLR is markedly changed and a new rapid K-peak usually appears (Fig. 4). The appearance of the K-step in the FLR the curve is attributed mainly to inhibition of the oxygen evolving complex (Guissé et al. 1995; Lazár et al. 1997; Srivastava et al.

1997; Strasser 1997). It has been shown that a decrease of rate constant of the J-P increase and suppression of amplitude of the J-P phase reflects the inhibition of OEC activity as well (Pospíšil and Dau 2000) because of the incomplete reduction of the electron transport chain.

The thermostability of OEC can be improved by abscisic acid (ABA) treatment (Li et al. 2003), glycinebetaine or bicarbonate (Klimov et al. 2003). Digalactosyldiacylglycerol (DGDG) has also been shown to stabilize OEC through the better binding of extrinsic proteins (Sakurai et al. 2007).

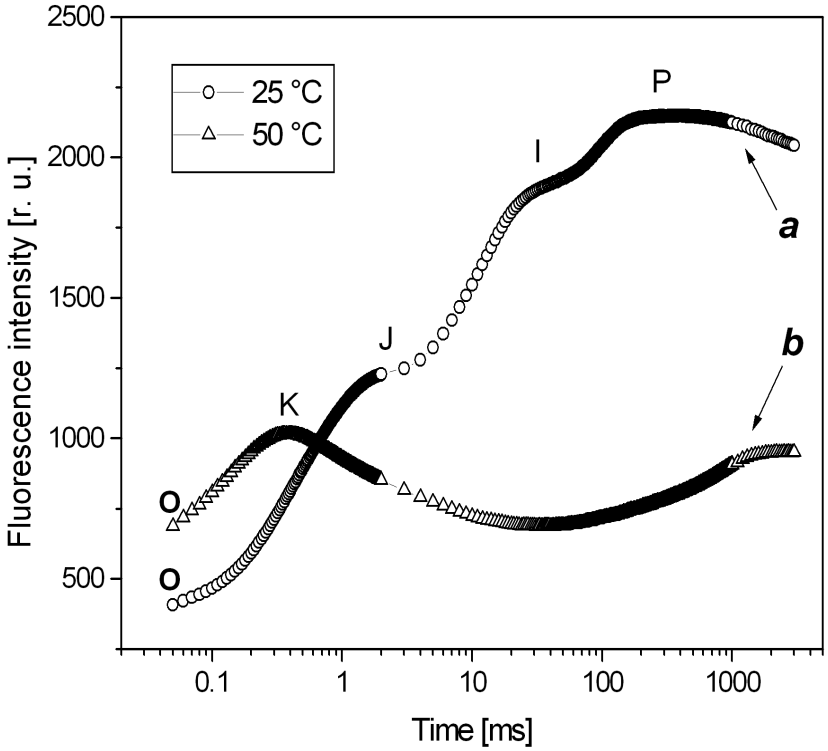

Figure 4. *Chlorophyll fluorescence induction curves of tobacco leaf segments measured at 25 °C (curve **a**) and at 50 °C (curve **b**) after linear heating at the rate of 2 °C min^{-1}. Exciting light 3000 μmol (photons) m^{-2}s^{-1} of red light (650 nm). The x-axis of the graph is logarithmic.*

1.2.1.1.2. Recovery of oxygen-evolving complex

The post-HS recovery ability of the water-splitting complex has been documented in several studies. The flash induced oxygen evolution of intact spinach chloroplasts was decreased at high temperatures (5 min treatment), but it showed partial recovery when the samples were cooled down and incubated at 25 °C in the dark (Yamane et al. 1998). The yield of oxygen evolution was fully reversible (relative to 25

°C) only up to about 38 °C. Čajánek et al. (1998) obtained similar results with barley leaves when partial decrease in oxygen evolution after 10 min incubation at 37,5 °C was found to be mostly reversible within 10 min at 30 °C, and at 42,5 °C irreversible. The quantum yield of oxygen evolution during 2h recovery from heat shock (36.5 °C or 40.5 °C for 15 min) was irreversible with reference to control in intact leaves (Havaux 1993a).

A higher degree of recovery of oxygen-evolving activity can also occur in samples stressed at even higher temperatures (up to 50 °C) but much longer periods of incubation at room temperature are usually necessary. If barley leaves were subjected to heat pulses at 50 °C for 40 s, the oxygen evolving activity was almost fully reversible after 48 h recovery in the light at growing temperature (Tóth et al. 2005). In darkness, recovery did not occur. The results contrast with measurements carried out by Havaux et al. (1987), who observed that the oxygen evolution of tobacco leaves can be completely restored from 3-min incubation at 48 °C after 24 h incubation at room temperature in the dark.

One of the main features of OEC reversibility resides in the action of 33 kDa protein. This manganese-cluster stabilizing protein, which is usually released during heat treatment, can rebind again to its functional site when the samples are cooled down to room temperature after heat stress *in vitro* (Yamane et al. 1998). The 33 kDa protein itself seems to relatively stable under high temperatures because its secondary structure which was completely lost during severe HS, returned to a state similar to that of an unheated one after lowering the temperature (Lydakis-Simantiris et al. 1999). Even in this case, the 33 kDa protein was capable of rebinding to PSII and the activity of OEC was restored. Also the initial content of OEC extrinsic proteins in the sample could be recovered. It took about 3 days in maize leaves (in the case of 23 kDa protein) and 4 days (for 17 and 33 kDa proteins) after exposure of plants to two successive 20h periods at 45 °C (Heckathorn et al. 1997). However, only partial recovery of water oxidation capacity was found in wheat leaves (Mohanty et al. 1987). After 10 min at 45 °C and 47 °C and for the following 3 days at 25 °C, the samples reached only about 20% and 60% of control values.

These recovery experiments confirm the proclaimed fragility of OEC: a few minutes incubation at temperatures higher than about 45 °C leads to longer recovery times and more sophisticated repair mechanisms generally need to be involved.

1.2.1.2. Reaction center of PSII

The photosystem II core complex acts as an electron transfer domain, which facilitates the light driven charge separation leading to water oxidation in OEC and to the reduction of plastoquinone pool. It is a large multi-subunit protein complex containing more than 20 polypeptide subunits. Five major transmembrane subunits are D1, D2, cyt b559, core antennae CP43 and CP47 (Fig. 3). The D1 and D2 proteins bind the primary electron donor of PSII (P680), pheophytin, tyroines Z (Y_Z) and D (Y_D) and quinones Q_A, Q_B (see reviews by Goussias et al. 2002; Vassiliev and Bruce 2008).

1.2.1.2.1. Heat-induced inhibition of the PSII core function

The acceptor side of PSII is generally considered more stable under HS than OEC but it is still one of the most sensitive parts of the photosynthetic machinery (Berry and Björkman 1980; Havaux 1993a, Carpentier 1999; Allakhverdiev et al. 2008).

More severe HS leads to denaturation of PSII components, especially the fragile D1 protein (see Section 1.1.1.2.1.). However, even mild HS is sufficient to trigger the inhibition of electron transport (ET), the most important functional change in PSII. It has been shown that HS induces inhibition of ET at the acceptor side of PSII, concretely, the electron transfer from primary (Q_A) to secondary (Q_B) quinone electron acceptor of PSII (Bukhov et al. 1990; Cao and Govindjee 1990). Cao and Govindjee (1990) have suggested that this functional inhibition may be connected with a structural change in D1 and D2 proteins. This is in line with the Fourier transform infrared spectroscopy measurement carried out by De Las Rivas and Barber (1997). Their experiments showed main conformational transition of PSII core proteins at around 42 °C, which may correlate with the well-known sensitivity of the reaction center of PSII to HS as well as to photoinhibitory conditions (De Las Rivas and Barber 1997). The impairment in electron transfer from Q_A to Q_B could be related to a shift in the redox potential of Q_A (Ducruet and Lemoine 1985, Bukhov et al. 1990). Later experiments carried out on linearly heated tobacco thylakoids confirmed this view (Pospíšil and Tyystjärvi 1999). The heat-induced inhibition of electron transport is related to increase in the midpoint potential of the Q_A/Q_A- couple from the standard value of -80 mV at 25 °C to +40 mV at 50 °C. HS-induced structural changes in D1 and D2 proteins of PSII could be a reason for the conversion of primary quinone electron acceptor QA from low to high potential form (Pospíšil and Tyystjärvi 1999).

There are a number of factors that can improve the thermostability of PSII. For instance, high concentration of osmolyte (sucrose or glycinebetaine) allows the stabilization of the PSII core complex together with the OEC (Allakhverdiev et al. 1996; 2008). If HS has already inactivated the OEC, ascorbate might protect the PSII core due to donation of electrons instead of OEC (Tóth et al. 2009). Treatment by ABA as well as NaCl also enhances the thermostability of PSII (Ivanov et al. 1992; Chen et al. 2004). Analogously, protection by bicarbonate against thermoinactivation of the acceptor side of PS II has been reported at temperatures of 42–50 °C as well (Pospíšil and Tyystjärvi 1999).

1.2.1.2.2. Chl fluorescence and PSII core under HS measured by its means

The light absorbed by the photosynthetic apparatus, drives the electron transport within the thylakoid membrane. However, a minor fraction of captured light is emitted as fluorescence (F), which presents a competing process as deactivation of excited pigments. Its measurement provides a useful diagnostic way for studying the functional state of the photosynthetic apparatus (see Krause and Weis 1991; Lazár 1999; Sayed 2003 for review). This experimental method is rapid, relatively sensitive, non-destructive and it has been extensively used to detect and understand HS-induced changes in the plant photosynthetic apparatus.

In contrast to the low-temperature chlorophyll (Chl) fluorescence signal, where a substantial part of the emission comes from PSI (Krause and Weis 1991), the fluorescence measured at room (and supraoptimal) temperature originates presumably from chlorophyll *a* molecules in PSII (Stahl et al. 1989; Krause and Weis 1991). The part of the fluorescence emission ascribed to PSI can be significant, especially in the case of wavelengths of approximately 700 nm and higher (Pfündel 1998; Agati et al. 2000). Despite the fact that this should be taken into account, it is generally considered that chlorophyll fluorescence reflects mainly the emission from PSII (Lazár 1999).

There are two extreme states of the PSII reaction center. The minimal chlorophyll fluorescence intensity, designated as F_0, is measured if the reaction centers of PSIIs are „open“ (i.e. Q_A is oxidized). If the fluorescence level is maximal (F_M) the reaction centers are „closed“ (i.e. Q_A is reduced). Thermal inhibition of PSII is reflected by a decrease in variable portion of Chl fluorescence ($F_V = F_M-F_0$) and the potential quantum yield of photochemical reaction of PSII (F_V/F_M) associated with the

photochemical activity of the photosystem (e.g. Krause and Weis 1991). The decline in F_V might arise from F_0 increase and simultaneous F_M decrease (Briantais et al. 1996; Pospíšil et al. 1998; Wen et al. 2005). The F_V/F_M ratio in particular has become an important parameter which under optimal physiological conditions was found to reach the value of about 0.83 (Björkman and Demmig 1987).

Heat injury of the photosynthetic apparatus is associated with a pronounced rise in the intensity of Chl fluorescence under low exciting light (Schreiber & Berry 1977). The basal fluorescence (F_0) is physiological parameters that have been shown to correlate with heat tolerance (Havaux 1993b; Yamada et al. 1996).

The principle of the F_0 rise consists of several events and it has been shown that the main causes differ from species to species (Yamane et al. 2000). The main reason for heat-induced rise in F_0 is generally considered to be accumulation of reduced Q_A resulting from inhibition of electron transport from Q_A to Q_B and associated blocking of the reaction center of PS II (Schreiber and Armond 1978; Cao and Govindjee 1990; Pospíšil and Nauš 1998; Kouřil et al. 2004). The shift in redox potential of Q_A mentioned above (see Section 1.2.1.2.1.) is probably the cause of the inhibition and the consequence of this effect is also known as conversion of Q_B-reducing PS II centres to Q_B-non-reducing PSII centres (Guenther and Melis 1990; Klinkovský and Nauš 1994). In DCMU-treated leaves, where Q_B is displaced from the Q_B pocket of D1 protein in PS II, the accumulation of Q_A^- is probably due to thermal inhibition of the S2 state of OEC and Q_A^- (Kouřil et al. 2004).

An alternative explanation for the F_0 increase after exposure of samples to high temperatures revolves around the separation of LHCII from the PSII core (Schreiber and Armond 1978; Yamane et al. 1995; 1997). However, this interpretation was later disputed in relation to the decreased absorption cross-section of the fluorescing PSII core which is detached from its antenna (Pospíšil and Nauš 1998; Kouřil et al. 2004).

The higher back electron transfer from Q_B^- (Kouřil et al. 2004) or from PQ pool (Bukhov et al. 1990; Sazanov et al. 1998; Yamane et al. 2000) to Q_A may also contribute to the HS-induced F_0 increase. A later effect, reduction of Q_A through the PQ pool in the dark, has been found to be the main cause in species such as potato, tobacco and others (Yamane et al. 2000).

The overwhelming majority of authors have used abrupt temperature change for investigation of heat stress effects. However, the dependence of fluorescence intensity on linearly increasing temperature, usually designated as the fluorescence temperature

curve (FTC), has been used for monitoring PSII as well (e.g. Schreiber and Berry 1977; Pospíšil and Nauš 1998; Kuropatwa et al. 1992; Nauš et al. 1992a; 1992b; Nauš and Ilík 1997; Ilík et al. 1995a; 1995b; 2000; 2003; Kouřil et al. 2004; Lípová et al. 2010).

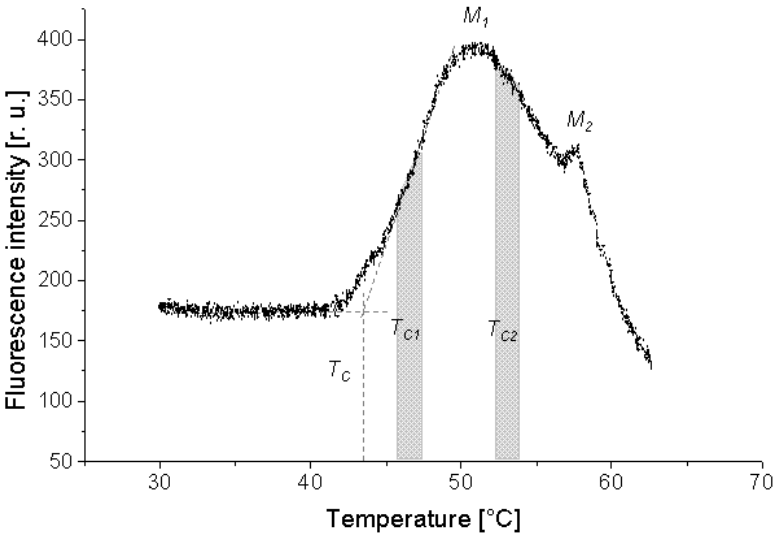

Figure 5. *Typical course of fluorescence temperature curve (FTC) of barley leaf with its first (M_1) and second (M_2) maximum reached during linear heating (0,5 °C min^{-1}). Approximate positions of the critical temperature of the main fluorescence increase (T_C) and intervals of critical temperatures of irreversible fluorescence changes (T_{C1} and T_{C2}) are marked according to Nauš and Ilík (1997).*

Two characteristic maxima of FTC can usually be distinguished (Fig. 5). The heat-induced enhancement of fluorescence intensity to the first one (M_1) is caused mainly by the above-discussed blockage of electron transport from Q_A to Q_B. The second maximum (M_2) probably originates in highly fluorescing Chl *a* molecules, which are released from the chlorophyll-containing protein complexes denaturing at 55–60 °C (Ilík et al. 2003).

The temperature of the fluorescence increase to M_1, also designated as the critical temperature (T_C), has been used as an indicator of the thermal stability of the thylakoid membrane (e.g. Havaux et al. 1988; Taub et al. 2000; Ducruet et al. 2007). Following the high temperature hardening of plants, the T_C shifts to higher temperatures (Smilie and Nott 1979, Havaux 1993b, Lazár and Ilík 1997). In addition, good correlations were found between T_C and the temperature of the K peak in the FLR curve (Fig. 4 curve b) on progressive HS incubation (Lazár and Ilík 1997) and between T_C and the leaf temperature at which the capacity for photosynthetic CO_2 fixation begins to decline (Seemann et al. 1984).

1.2.1.2.3. Recovery of the acceptor side of PSII

1.2.1.2.3.1. Recovery following abrupt temperature changes

As mentioned above, Chl fluorescence is a valuable probe of the photosynthetic apparatus. There are a number of studies, in which fluorescence parameters were used (mostly F_V/F_M or variables characterizing the fluorescence quenching) for monitoring the recovery of PSII (Havaux 1993a; Briantais et al. 1996; Yamane et al. 1997; 1998; Tsonev et al. 1999; Tóth et al. 2005; Nishiyama et al. 2006). The HS-treatment of PSII results in an increase in F_0, decrease in F_M and concomitant decrease in variable fluorescence (F_V) accompanied by inhibition of PSII activity. The quenching of F_M has been reported to be irreversible, while the increase in F_0 was partially reversible (Briantais et al. 1996). Two overlapping phenomena probably contribute to the F_0 increase. A moderate and reversible increase in F_0, beginning at 30 °C, which is probably caused by a partially reversible decrease in the quantum yield of PSII photochemistry, and a major F_0 increase starting at around 40 °C and probably originating from an irreversible decrease in connection of small chlorophyll-protein complex to the rest of the PSII (Briantais et al. 1996).

The reversibility of PSII function detected by fluorescence techniques has also been monitored together with CO_2 assimilation parameters (Crafts-Brandner and Law 2000; Sharkey et al. 2001a; Haldimann and Feller 2004; Sinsawat et al. 2004; Kim and Portis 2005; Zhang and Sharkey 2009). The fluorescence parameters mostly recovered within a relatively short period (up to one hour) following incubation for 10 – 30 min at temperatures of around 40 °C or slightly higher.

In some cases, usually after application of more severe HS, a much longer period (one day or more) was required for full recovery (Karim et al. 1999; Tóth et al. 2005; Kreslavski 2008; 2009). A model for such recovery of PSII disrupted by HS has been suggested (Tóth et al. 2005). The process involves degradation of damaged PSII units and *de novo* synthesis of the PSII core. It takes 48 h and is possible only in the light, not in the dark (Tóth et al. 2005). The exposure of heat-stressed samples to moderate light during HS is known to be advantageous for the photosynthetic apparatus, especially PSII (Havaux et al. 1991; Kreslavski 2009) while strong light exposure increases the adverse impacts of HS (e.g. Al-Khatib and Paulsen 1989, Kreslavski et al. 2008).

It seems that the low light is also required for recovery in the case that *de novo* synthesis to replace damaged proteins does not take place. Nishiyama et al. (2006) have

observed inactivation of PSII by moderately high temperatures (41 °C for 20 min), which was reversed upon transfer of soybean cells back to 25 °C. The reversible heat-induced inactivation might involve perturbation of the manganese cluster and the recovery required light, but not the synthesis of proteins (mainly D1) *de novo* (Nishiyama et al. 2006). This was designated as the first (reversible) step while the second step, induced by incubation at a temperature of 45 °C or higher, was irreversible. It remains to be determined whether irreversible inactivation by high temperatures is due to critical damage to the PSII complex itself or to the repair mechanism (Nishiyama et al. 2006). However, the samples were monitored only within 100 min and thus the recovery process described by Tóth et al. (2005) probably did not take place. However, the sequence proposed by Nishiyama et al. (2006), i.e. the first reversible event caused by changes at the donor side of PSII followed by an irreversible step(s), was suggested earlier by Yamane et al. (1998).

Recently, Kreslavski et al (Kreslavski et al. 2009) explained the absence of significant recovery of photosynthetic activity in wheat seedlings after 20-min incubation at 44 °C due to presumably greater formation of reactive oxygen species with respect to lower stress temperatures, at which the recovery of samples took place. For this reason, along with the importance of light, a prooxidant-antioxidant balance might play a crucial role in the complete recovery of the photosynthetic apparatus from heat-induced inactivation of PSII (Kreslavski et al. 2009).

1.2.1.2.3.2. Recovery following gradual heating

The enhanced dark Q_A reduction reflected by increased fluorescence during linear heating of darkened spinach leaves to 40 °C was found to be largely reversible after the leaves were returned to 20 °C (Bukhov et al. 1999). The reversibility of FTC was also investigated during linear heating followed by spontaneous cooling (Nauš et al. 1986) or under a combined regime of linear heating and incubation at a given temperature (Nauš et al. 1992a). The critical temperatures of the fluorescence temperature curve, T_{C1} and T_{C2} (Fig. 5), were postulated earlier in the temperature regions 45-48 °C and 53-55 °C, respectively (Nauš et al. 1992a). Lowering the temperature of the sample after reaching a temperature below T_{C1} or T_{C2} regions leads to a partially reversible transition of F to the preceding level (at 30 °C or M_1 respectively). Irreversible structural changes of the photosynthetic apparatus

corresponding to individual parts of the FTC have been recently identified (see Section 1.1.1.2.1 and Lipová et al. (2010)).

1.2.1.3. Photosystem I

Linear electron transport in the thylakoid membrane is enabled through the consecutive action of both photosystems: PSII and PSI (Fig. 1). Photosystem I is a multisubunit protein complex located in the thylakoid membranes which receives electrons from plastocyanin and reduces $NADP^+$ to NADPH through a system of electron carriers. Excitation energy is transferred to P700, the primary electron donor and the subsequent charge separation and electron transport lead to the reduction of ferredoxin. For further information on PSI structure and function see detailed reviews by Chitnis (2001), Hihara and Sonoike (2001) and Jensen et al. (2007).

1.2.1.3.1. PSI function at elevated temperatures

It has been known for some time that PSI function is more thermostable than that of PSII (Berry and Björkman 1980; Havaux 1993a). In line with this view, several other studies have shown that denaturation of PSI components generally takes place at higher temperatures than those of PSII (see Section 1.1.1.2.1.).

The photosynthetic performance of PSII is reduced at supraoptimal temperatures (see Section 1.2.1.2.1.). Interestingly however, the level of PSI photochemical activity was reported to increase (at temperatures around 35°C or higher) as measured in terms of O_2 uptake (Armond et al. 1978; Gounaris et al. 1983; Thomas et al. 1986; Mohanty et al. 1987). These experiments were mostly based on monitoring PSI-mediated ET from artificial electron donor (2,6-dichlorophenolindophenol) to acceptor (methylviologen). It has been proposed that the stimulation by HS is due to a conformational change at the level of the cyt b_6f complex resulting in creation of new reducing sides or greater affinity for the artificial electron donors (Boucher et al. 1990; Thomas et al 1986). However, the inactivation of PSI-mediated O_2 uptake was later ascribed to decline in the activity of membrane-bound superoxide dismutase or its release from the membrane (Lajkó et al. 1991; Boucher and Carpentier 1993). Apart from oxygen uptake measurements, a higher rate of light-induced P700 oxidation at temperatures up to 50 °C suggested higher activity of PSI (Ivanov and Velitchkova 1990). The increase was

ascribed to an increase in the PSI absorption cross-section due to heat-induced structural reorganization of membrane complexes (see Section 1.1.2.1.). Moreover, no stimulation of PSI activity was observed in the case of thylakoid membranes incubated at 48 °C with $NADP^+$ as terminal electron acceptor (Lajkó et al. 1991). The presupposed absence of real increase in ET activity was confirmed by measurement of gradual decline in energy storage in PSI at temperatures higher than 40 °C (Velitchkova and Carpentier 1994).

1.2.1.3.2. Cyclic ET around PSI and its role in recovery

Under certain stress conditions, cyclic electron transport around PSI can take place. In this process, the electrons are returned from NADPH (or ferredoxin) back to the donor side of PSI via plastoquionone, cyt b_6f and plastocyanin (see Joliot and Joliot 2006 for a review). This might prevent photoinhibition of PSII and provide extra ATP (Fork and Herbert 1993; Joliot and Joliot 2006).

An increase in this electron flow is a frequently reported effect of moderate HS on photosynthetic reactions (Havaux 1996; Bukhov et al. 1999; 2000). The regulation of cyclic ET around PSI may help plants to tolerate high temperatures by maintaining thylakoid proton gradient and it can assist with non-radiative dissipation of excess photon energy under the combined effects of high light and HS (Jin et al. 2009). In addition, the transmembrane proton gradient generated by cyclic ET, protects the thylakoid membrane itself from heat-induced damage (Zhang and Sharkey 2009). The cyclic ET also indirectly facilitates the reparation of heat-induced damage, because it supplies enough energy for synthesis of cytoplasmic, chloroplastic as well as heat shock proteins through the production of ATP molecules (Bukhov et al. 2000). This was confirmed by observation of a higher rate of cyclic ET during recovery from HS (Kreslavski et al. 2008).

1.2.2. Photosynthetic CO_2 assimilation

In the first stage of photosynthesis, light-dependent reactions collect the light energy and generate the energy-storage molecules ATP and NADPH (Fig. 1). These products are used to capture and reduce carbon dioxide which is converted into organic compounds during the second photosynthetic stage. This dark phase of photosynthesis

(Calvin cycle) takes place in chloroplast stroma. Ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) is a key enzyme used in the Calvin cycle to catalyse the first major step in carbon fixation (e.g. Blankenship 2002).

1.2.2.1. Effects of high temperature on CO_2 assimilation

It has been considered for many years that PSII, especially its donor side, is the primary target of high temperature effects (see part 1.2.1.1.). However, several studies have suggested that net CO_2 assimilation rate (P_N) is the most sensitive photosynthetic process in plants (for review see Salvucci and Crafts-Brandner 2004a). The PSII function, as measured by some basic chlorophyll fluorescence parameters (F_0, F_M or maximal quantum yield of PSII photochemistry F_V/F_M), appears to be more stable towards moderately elevated temperatures than CO_2 assimilation (Feller et al. 1998; Crafts-Brandner and Law 2000; Crafts-Brandner and Salvucci 2002; Tang et al. 2007). Although the CO_2 assimilation rate can be limited by the electron transport rate in PSII (Wise et al. 2004) or by the cellular content of extrinsic proteins in OEC (Heckathorn et al. 1997), the inhibition of Rubisco activity is usually considered as a basis for the lower P_N at moderately elevated temperatures (Weis 1981a; Law and Crafts-Brandner 1999; Kim and Portis 2005). It is not the activity of Rubisco *per se* but the light-dependent Rubisco activation that is particularly sensitive to HS (Weis 1981b; Feller et al. 1998).

In general, the activation state of Rubisco is regulated by another stromal enzyme called Rubisco activase (see Portis 2003 for review). Thus, the reason for Rubisco inhibition can be presumed to be due directly to loss of Rubisco activase activity. This premise has been proven in several studies (Feller et al. 1998; Law and Crafts-Brandner 1999; Crafts-Brandner and Law 2000; Crafts-Brandner and Salvucci 2002; 2004; Salvucci and Crafts-Brandner 2004b). For instance, the activation state of Rubisco decreased at temperatures exceeding 32.5 °C, with nearly complete inactivation at 45 °C (Crafts-Brandner and Salvucci 2002). The sensitivity of activase to HS is much greater than that of Rubisco (Salvucci et. al 2001) and varies in different plant species (Law and Crafts-Brandner 1999). The inhibition of activase can be attributed to changes in its structural properties, i.e. to formation of high-molecular-weight aggregates (Feller et al. 1998), or to disruption of activase subunit interactions with each other or with Rubisco (Crafts-Brandner and Law 2000). Reversible association of a specific protein

(chaperonin-60β) with Rubisco activase might protect it from thermal denaturation and thus contribute to plant tolerance to HS (Salvucci 2008).

In addition to these findings, it has been also suggested that decline in CO_2 assimilation rate is caused by limitation of Ribulose-1,5-bisphosphate regeneration, rather than Rubisco activase inhibition (Cen and Sage 2005; Kubien and Sage 2008; Sage et al. 2008). Thus, the process of photosynthesis limitation at elevated temperatures is still a subject of discussion. Moreover, photorespiration, an alternative Rubisco reaction , is increased at temperatures exceeding the optimum for assimilation due to relatively higher concentration of oxygen in relation to carbon dioxide at higher temperatures (Blankenship 2002) and this also leads to concomitant decrease in the efficiency of CO_2 assimilation.

The rate of photosynthesis (P_N) at higher temperatures depends on ambient atmospheric concentration of CO_2 (Taub et al. 2000; Wang et al. 2008). Taub et al. (2000) presented evidence that most tested species grown under elevated atmospheric CO_2 (550-1000 μmol mol^{-1}) showed increased tolerance of photosynthesis to HS. The thermotolerance of P_N under elevated CO_2 is increased in C3 plants, but decreased in C4 plants (Wang et al. 2008). These results, along with some other studies, indicate that the reaction of P_N under HS conditions depends especially on the plant species.

1.2.2.2. Recovery of CO_2 assimilation

In spite of its susceptibility to high temperatures, the CO_2 assimilation process is quite capable of recovery from HS-induced changes (Weis 1981a; Heckathorn et al. 1997; Feller et al. 1998; Crafts-Brandner and Law 2000; Sharkey et al. 2001a; Haldimann and Feller 2004; Kim and Portis 2005). A threshold condition for recovery of the activation state of Rubisco is generally considered the maintainance of leaf temperature around 40 °C for several minutes or up to half an hour (Feller et al. 1998; Crafts-Brandner and Law 2000; Haldimann and Feller 2004; Kim and Portis 2005). However, it is very difficult to compare the results from these studies because of the use various plant species (oak, tobacco, cotton and wheat) and different stress regimes (T-jump - e.g. Sharkey et al. 2001a; gradual temperature increase - Kim and Portis 2005; combined – Haldimann and Feller 2004). The reversible decarbamylation of Rubisco at moderately high temperatures might be a protective mechanism by which the plant avoids more serious damage to Rubisco and the rest of the photosynthetic apparatus

(Sharkey et al. 2001a). Kim and Portis (2005) concluded that Rubisco activase is critical for recovery after inhibition of photosynthesis by exposure to high temperatures. Finally, physical denaturation of activase has been observed at temperatures above 40 °C that cause irreversible inactivation of Rubisco (Feller et al. 1998). Interestingly, full post-heat stress (45 °C) recovery of P_N in maize coincided with recovery of levels of OEC proteins (Heckathorn et al. 1997) indicating that carbon fixation recovery can be limited by HS-induced decrease in these proteins. The recoverability of P_N can be improved by isoprene (Sharkey et al. 2001b). The photosynthesis of bean leaves fed fosmidomycin (which eliminates isoprene emission) recovered less, following HS treatment (2 min at 46 °C) than in the case of control leaves or fosmidomycin-fed leaves in air supplemented by isoprene (Sharkey et al. 2001b).

2. GOALS OF THE THESIS

The main goal of this thesis was to increase our understanding of some aspects of thermal damage in plants in relation to various heat stress regimes. A secondary aim was study of the recoverability of plants to these HS-induced changes. Two separate projects were undertaken:

1) The aim of the first project was to characterize the HS-related changes in the chlorophyll fluorescence signal and its reversibility in barley leaves exposed to a linearly increasing and decreasing temperature with different heating/cooling rates

2) The aim of the second project was to uncover the response of light-induced chloroplast movement in tobacco leaves to linear and temperature-jump heat stress

3. EXPERIMENTAL PART

3.1. IRREVERSIBILITY OF HEAT STRESS-INDUCED CHANGES DETECTED BY THE CHLOROPHYLL FLUORESCENCE IN LINEARLY-HEATED BARLEY LEAVES

The relation of the chlorophyll fluorescence intensity to linearly increasing temperature is usually depicted as the fluorescence temperature curve (FTC). The curve presents a record of the continual response of the plant photosynthetic apparatus to gradual heating and it reflects many structural as well as functional changes.

Recoverability from heat injury is very important for plants. The reversibility of the FTC was investigated during linear heating followed by spontaneous cooling (Nauš et al. 1986) or under a combined regime of linear heating and incubation at given temperatures (Nauš et al. 1992a). Other experimental methods have also helped to characterize some irreversible changes caused by linear heating (Lipová et al. 2010).

Here, we revealed the basic features of the HS-related recovery of barley leaves using four linear heating/cooling regimes with different heating rates. In order to carry out a detailed analysis of measured FTCs, five slopes of linear parts of each FTC, degree of fluorescence irreversibility at 35 °C and activation energies of the initial fluorescence irreversible increase were evaluated. The results are described and discussed in more detail in the attached paper [I].

3.1.1. Material and methods

Barley plants (*Hordeum vulgare* L., cv. Akcent) were cultivated in a homemade growth chamber for 9 d in a 16 h / 8 h light/dark regime (85 ± 20 μmol photons m^{-2} s^{-1} of white fluorescent light, relative humidity 50 ± 10 %) in artificial soil composed of perlit and Knop solution at 22 ± 3 °C. The plants used for measurements were in growth stage 12 according to Zadox (Zadox et al. 1974).

Chlorophyll fluorescence intensity was measured with a fluorimeter PAM 2000 (Walz, Effeltrich, Germany) excited by weak red light (655 nm, 0.3 μmol photons m^{-2} s^{-1}) and detected at wavelengths higher than 700 nm.

Heating device and linear heating

A laboratory-made heating device was used for the sample heating. The segments placed on a holder were immersed in a stirred distilled water-bath. The heating/cooling of the water bath used Peltier cells. The temperature of the bath was determined by a thermocouple and the regime was controlled by an application written in LabView 3.1 (National Instruments, Austin, Texas). The leaf segments were immersed in distilled water and heated at 0.5, 1, 2 or 3 °C min^{-1} from room temperature up to the maximal temperature T_m and then cooled at the same rate to 35 °C.

The FTC regression lines and E_a calculation

For detailed evaluation of the FTC, we established five tangents (S1 - S5) of some linear parts of the up/down linear FTC (Fig. 6). The temperature range for these lines was established so as to reach a minimal deviation between fluorescence intensity and the corresponding regression line. The first (designated S1) and second (S2) tangent is defined in the temperature range from 46 °C to 48 °C and from 51 °C to T_m, respectively. The S3 line was discernible only for T_m above 54 °C. The temperature range for the S3, S4 and S5 tangents was established at 54–48 °C, 48–42 °C and 42–35 °C, respectively. The slopes of tangents of the linear FTCs parts were estimated for these temperature ranges by a linear regression procedure using Microsoft Excel 2000.

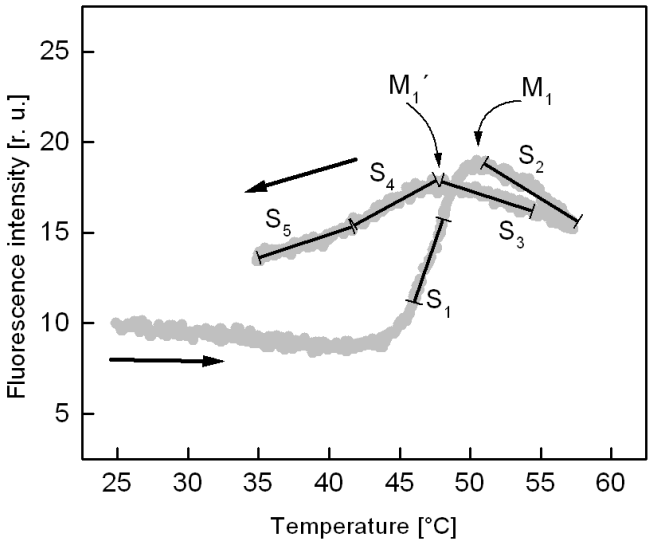

Figure 6. *A schematic illustration of tangents (S1 - S5) of the linear parts of the up/down linear FTC. The temperature ranges of the tangents are 46–48 °C (S1), 51 °C–T_m (S2) during heating and 54–48 °C (S3), 48–42 °C (S4) and 42–35 °C (S5) during subsequent cooling. The arrows indicate heating (to the right) and cooling (to the left) time-course. The position of the first FTC maximum reached during heating (M_1) and cooling (M_1´) are marked.*

3.1.2. Results and discussion

Coefficient of irreversibility and critical temperatures

In order to investigate the recovery of FTC, the barley segments were heated in a water-bath from room temperature up to maximal temperature T_m (chosen range from 42 °C to 65 °) and then linearly cooled to 35 °C at the same rate. Typical curves for linear heating (right-hand arrow)/cooling (left-hand arrow) of the samples for all measured rates (0.5, 1, 2 and 3 °C min^{-1}) and for T_m = 60 °C are shown in Fig. 7. This illustrates that the FTC course depends on the heating rate and maximal temperature of heating (T_m).

The recoverability of chlorophyll fluorescence after the heating/cooling cycle was determined using the coefficient μ, representing the degree of fluorescence irreversibility at 35 °C (i.e. ratio of fluorescence intensity at 35 °C after cooling and the fluorescence intensity at the same temperature during heating of the sample). The μ versus T_m dependences (Fig. 8) showed two distinct phases, an increasing and decreasing one, indicate the two main processes in the irreversible changes. The decrease in μ values during the decreasing phases (Fig. 8) to its initial values thus does not mean a fully reversible reaction but gradual disruption to fully irreversible state (Fig. 7a).

The reversibility was higher, as can be expected, for higher heating/cooling rates and lower T_m. The initial phase with values of μ greater than 1 increases and reaches a maximum at temperatures between 54 °C and 61 °C in relation to heating rate (Fig. 8). This increase in μ in all probability reflects an increase in the number of irreversibly closed (blocked) PSII centers. Interestingly, this first phase does not reflect the original FTC (Fig. 7). The dependence of μ on T_m increases smoothly with no maximum corresponding to the M_1 maximum (around 50 °C). The reverse part of FTC can also reach a maximum (designated as M_1´, Fig. 7) that partly copies the original curve.

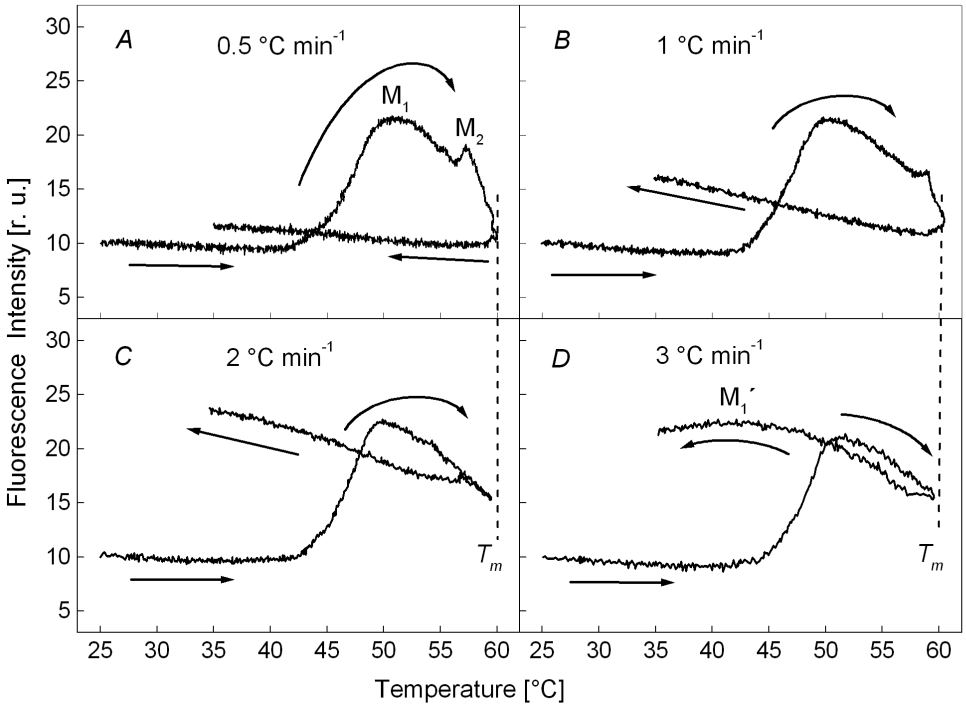

Figure 7. *Typical courses of the up-down linear FTC representing the changes in the measured F_0 level of a spring barley leaf segment for all measured heating/cooling rates. The maximal temperature of heating (T_m) was 60 °C. The curves are normalized to the fluorescence intensity at 25 °C. The arrows indicate heating (to the right) and cooling (to the left) temperature-course. The positions of the first and second FTC maxima reached during heating (M_1 and M_2) and the maximum reached during cooling (M_1´) are marked.*

A fully reversible response of barley leaves Chl fluorescence (μ = 1) was observed for T_m = 42–43 °C. The critical temperatures of the fluorescence temperature curve, T_{C1} and T_{C2}, were postulated earlier in the temperature regions 45-48 °C and 53-55 °C, respectively (Nauš et al. 1992a). Lowering the temperature of the sample after reaching a temperature below T_{C1} or T_{C2} regions led to a partially reversible transition of F to the preceding level (see Section 1.2.1.2.2.). A starting point of the steeper increase in μ (see Fig. 8, arrows) and the region of μ (Fig. 8) maximum could present a similar triggering region to T_{C1} and T_{C2}, respectively. The particular critical temperature occurred within temperature regions 43–48 °C (T_{C1}) and 54–60 °C (T_{C2}) depending on the heating rate.

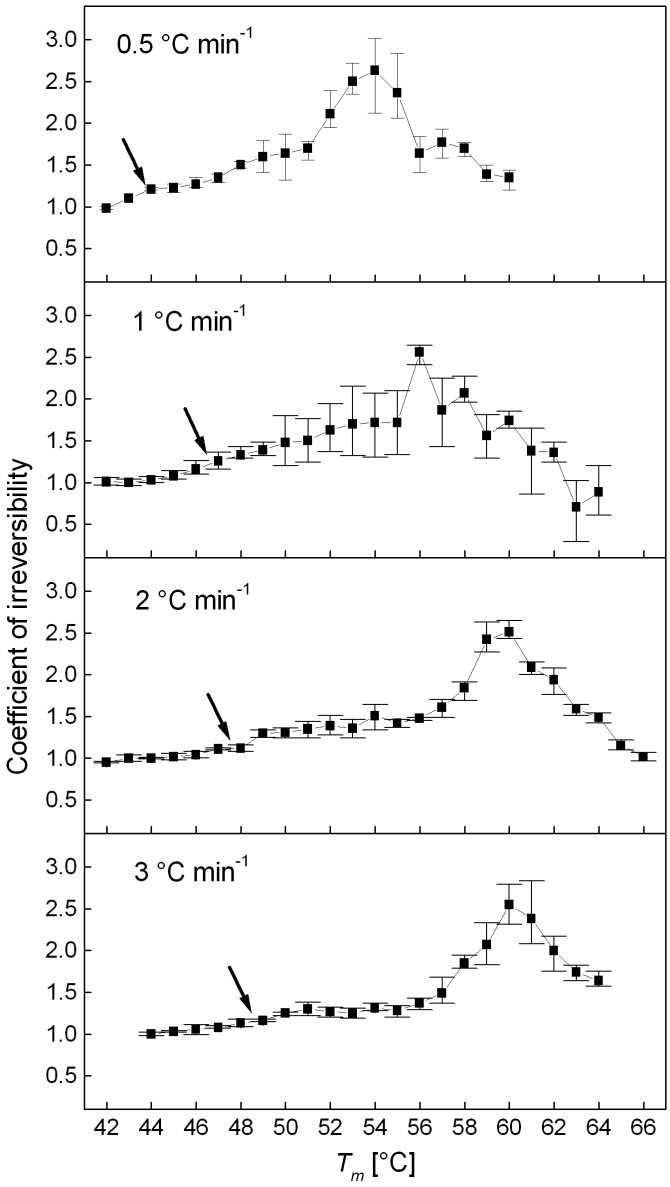

Figure 8. *The dependence of the fluorescence coefficient of irreversibility at 35 °C (μ) on the maximal temperature of heating (T_m) for different heating/cooling rates. The arrows indicate an approximate starting point of a steeper increase (i. e. 10% or more). The means of 3 experiments and the variation interval are shown.*

Tangents

A detailed characterization of the course of the up/down linear FTC can be made by a set of 5 linear parts (Fig. 6) and the slopes of their corresponding tangents (Fig. 9). The slopes of the S1 and S2 tangents, describing the heating part (to the M_1 maximum), were only changed slightly in our measurements (not shown). The S3, S4 and S5 tangents describe the fluorescence course during the linear cooling from T_m to 35 °C (Fig. 6). The temperature T_m that triggered a maximal fluorescence increase during the

first phase of cooling is represented by the minimum of the S3 tangent slope. An increase in the heating rate from 0.5 to 3 °C min^{-1} led to a shift of this minimum from T_m = 55 °C to 60 °C higher temperatures (Fig. 9). Similarly, the minima of S4 occurred at temperatures (T_m = 54 °C, 56 °C, 60 °C and 61 °C for the heating rate of 0.5, 1, 2 and 3 °C min^{-1}, respectively) reaching negative values. The dependencies of S5, reflecting the last phase of FTC cooling, had lower amplitudes but similar courses as in the case of S4.

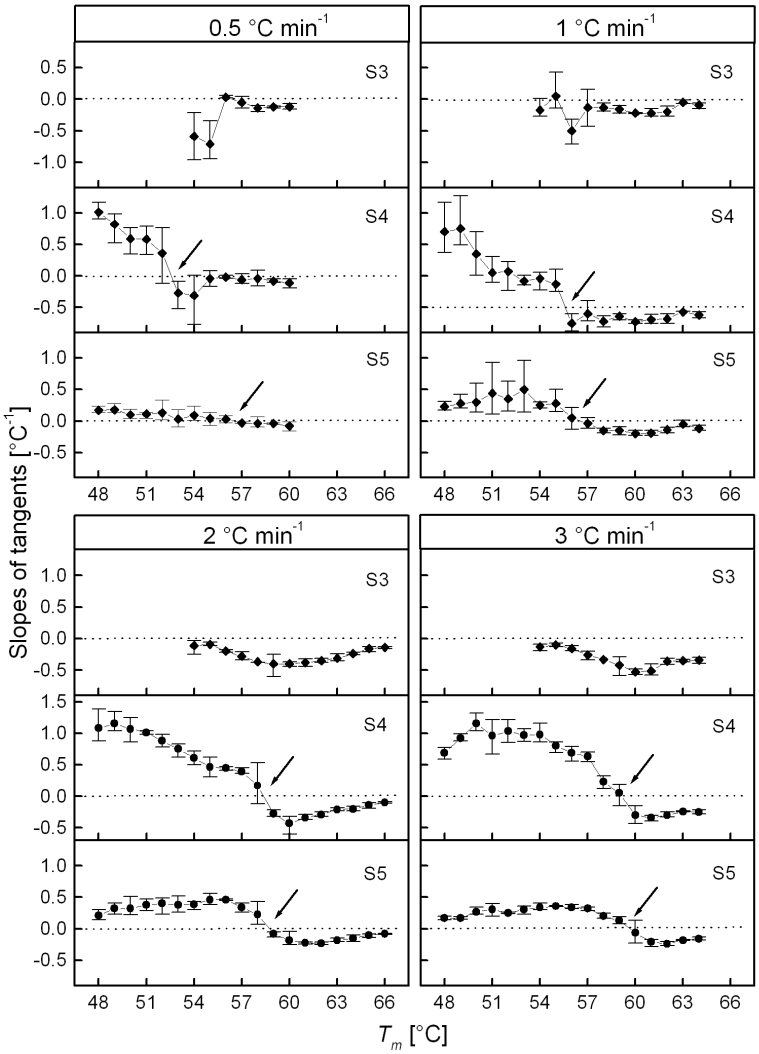

Figure 9. *The dependence of slopes of tangents (S3, S4, S5) on the maximal temperature of heating (T_m) for each measured heating/cooling rate. The means of 3 experiments and the variation interval are shown. The arrows indicate a change of sign of a given slope from positive to negative. Evaluated from the up/down linear FTCs of spring barley leaf segment.*

The slopes of the S4 and S5 tangents usually change their sign with increasing T_m (see Fig. 9, arrows). The maximum in the reverse part of the curve (M_1´) can be detected in the region of T_m where the slope S3 is negative and S4 positive. This can be

found between 54 and 59 °C but only for higher rates of heating/cooling (2 and 3 °C min^{-1}, Fig. 9).

A very relevant indication for the recovery threshold of PSII reflected by the FTC curve seems to be the change in sign from positive to negative of the slope of the S4 tangent (Fig. 9, arrows). These points occurred between 52 and 59 °C depending on the heating/cooling rate. For T_m below these temperatures, the changes in the sample are partly reversible. After this point, the signs of the S3 and S4 slopes are the same and the cooling part of FTC shows an increasing trend only with no maximum.

Activation energies

In order to better characterize the irreversible processes during our heating regimes, we attempted to calculate the activation energies (E_a) necessary for the fluorescence irreversibility increase. The initial increasing part of the $\mu(T_m)$ curve (up to T_m = 52, 54, 58 and 58 °C for the heating rate 0.5, 1, 2 and 3 °C min^{-1}, respectively) was used for such an evaluation. Since each of the possible reasons for increase in μ may be in principle characterized as a denaturation of some PSII transforming them from the fully functional state to an adversely changed one, we applied a simple protein-denaturation model to calculate the E_a. In the model, the proteins are irreversibly transformed from a native to a denatured state with a temperature-dependent rate constant (see e.g. Bischof and He 2005), which changes with temperature according to the Arrhenius or Eyring equation. In this model, a quantity designated as fraction of denatured proteins (F_d) needs to be estimated. We predicted that the fraction of denaturated centers was either proportional to measured excess of μ (F_{d1}) or to fluorescence irreversibility derived from the F_V/F_M parameter (F_{d2}). Thus, we obtained four options for the evaluation of E_a; the quantities F_{d1} and F_{d2} were calculated using both possible (Arrhenius as well as Eyring) temperature dependences of the rate constant.

For each of these options were numerically evaluated the activation energies separately for each heating rate (Tab. 1). Four different approaches led to values ranging from 30 to 50 kJ mol^{-1} (Tab. 1). These values decreased slightly with the increasing heating rate and they are similar to those of enzymatic membrane processes and, indicate rather some minor structural changes in PSII. For denaturation, a higher activation energy is needed (Bischof and He 2005).

Table 1. *Calculated activation energies of the fluorescence irreversible increase. For each combination of the model and heating rate, the obtained activation energy (kJ mol^{-1}) (left columns) and corresponding sum of least squares (right columns) are shown.*

Method	Heating rate (°C min^{-1})							
	0.5		1.0		2.0		3.0	
Arrhenius dependence (F_{d1})	49.8	0.032	48.7	0.008	48.2	0.044	47.7	0.075
Eyring dependence (F_{d1})	47.0	0.032	45.9	0.007	45.4	0.044	44.9	0.075
Arrhenius dependence (F_{d2})	31.2	0.005	30.2	0.001	29.5	0.006	29.1	0.011
Eyring dependence (F_{d2})	41.1	0.005	40.2	0.001	39.6	0.007	39.1	0.011

The best fit was obtained with an assumption of closing PSII at the acceptor side characterized by the tendency of fluorescence to increase up to the F_M level (F_{d2} option, Tab. 1). Therefore the most probable mechanism of the initial irreversible changes is the closure of certain PSII reaction centers (i.e. the accumulation of Q_A^-), which is probably connected with disintegration of OEC as the most susceptible part of PSII.

3.2. IMPACT OF TWO DIFFERENT TYPES OF HEAT STRESS ON CHLOROPLAST MOVEMENT IN TOBACCO LEAVES

The spatial arrangement of chloroplasts in the plant leaf can change as a response to light intensity. The translocation towards weakly illuminated leaf parts (the accumulation response or *diastrophe*) and away from strongly illuminated regions (the avoidance response *parastrophe*) may optimise the photosynthetic performance in a given plant protecting the chloroplasts from excess light (Kasahara et al. 2002).

Despite the fact that high temperatures are a common environmental factor, little is known about the chloroplast movement under HS conditions. Thus, the goal of this study was to characterize precisely how HS affects the light-induced motility of chloroplasts as detected by measurements of light-induced courses of collimated transmittance (CT). Using distinct heating regimes (linear and T-jump) allowed us to examine their impact. In addition, the fast chlorophyll fluorescence rise (FLR) was measured on the stressed leaves to monitor the function of PSII. Since chlorophyll fluorescence has been shown to be dependent on chloroplast movement (Brugnoli and Björkman 1992), the reactions of chlorophyll fluorescence and leaf transmittance measurements to elevated temperatures were compared as well. The results are described and discussed in more detail in the attached paper [II].

3.2.1. Material and methods

Plant material

Tobacco plants (*Nicotiana tabacum* L. cv. Samsun) were cultivated in regularly watered soil substrate in pots in a growth chamber (SGC.170.PFX.J, Weiss-Gallenkamp, Loughborough, England) for 2.5 - 3.5 months in a regime consisting of 8 h dark (temperature 21 °C, relative humidity 55 %), 15 h light (24 °C, 60 %, 100 μmol (photons) $m^{-2}s^{-1}$ of photosynthetically active radiation (PAR)) with 30 min of linear light-rise (dawn) and 30 min light-decrease (sunset).

Sample handling

The third leaves counted from the apex taken from ten different plants were used for five independent measurements under the T-jump regime and five under linear HS. Seven segments (for seven different T_m) of dimensions 0.9 x 2.0 cm (in the case of T-

jump regime) or 0.9 x 1.5 cm (linear regime) were cut off from each leaf and used for the measurements. Before HS treatment each leaf segment was put into a mask with central opening made from filter paper moistened with water (to reduce the water loss of the segment) and illuminated for 15 min [at 22 °C, RH: 24%, 20 μmol (photons) $m^{-2}s^{-1}$] to insure the *parastrophe* chloroplast position, then packed into aluminum foil. The foil insured the heat transport and prevented infiltration of the segment by the water from the bath. The samples (packed leaf segments) underwent one of two different regimes of HS before the FLR and time-course of light induced changes in CT were measured:

1) Linear regime: a laboratory-made heating device described in Frolec et al. (2008) was used for this HS regime. The samples were immersed in distilled water and heated at 2 °C min^{-1} from room temperature (25 °C) up to the maximal temperature T_m (25, 30, 35, 38, 40, 42 or 45 °C) and then cooled at the same rate to the final temperature (25 °C).
2) T-jump regime: 5 min treatment of sample in distilled water at 25 °C was followed by 5 min incubation at T_m (25, 30, 35, 38, 40, 42 or 45 °C) in thermostat U10 (Prüfgeräte-Werk Medingen, Dresden, Germany) and 5 min at 25 °C in thermostat (Transsonic T460/H, Elma, Singen, Germany). The transfer of a sample to the bath took about 1 s (the “jump”).

Experimental methods and parameters calculations

Fluorescence induction curves were measured from the adaxial leaf side by fluorometers PEA (Hansatech Instruments, King's Lynn, England) or FluorPen FP100 (PSI, Brno, Czech Republic). The irradiance was about 3000 μmol (photons) $m^{-2}s^{-1}$ of red light (peak wavelength 650 nm) for PEA and 3000 μmol (photons) $m^{-2}s^{-1}$ of blue light (455 nm) for FluorPen (a test showed that the FLR curves from a control leaf and measured by both fluorometers were the same within experimental error; data not shown). The signal was detected at wavelengths higher than 700 nm. The fluorescence parameters used here were calculated from the following equations (based on the JIP-test; see Strasser et al. 2004): F_0 = fluorescence intensity at 50 μs, $F_V/F_P = (F_P - F_0) / F_P$, $M_0 = 4(F_{300\,\mu s} - F_0) / (F_P - F_0)$, $V_J = (F_J - F_0) / (F_P - F_0)$, $V_I = (F_I - F_0) / (F_P - F_0)$, S_M = area (between fluorescence curve and F_P) / $(F_P - F_0)$ in the case of linear time scale.

Light-induced chloroplast movement was detected using leaf transmittance measurement, similar to that described in Nauš et al. (2008). To induce the chloroplast

movement, the samples were illuminated from the adaxial leaf side by a blue light (using Schott BG 12 filter) with two different intensities. The leaf segment was illuminated for 25 min with strong blue light [380 μmol (photons) m^{-2} s^{-1}] to cause the chloroplast movement in the direction to the anticlinal cell walls and for the next 35 min by a weak blue light [5 μmol (photons) m^{-2} s^{-1}] which caused a back movement characterized by a decrease in leaf transmittance. The source of cold white light was Schott KL 2500 (Schott Glas, Mainz, Germany). The transmitted light was conducted by a light-guide to the spectroradiometer LI-1800 (LI-COR, Lincoln, Nebraska). The transmittance signal was measured at 436 nm every 30 s. To obtain a particular value, the signal obtained with the leaf was divided by the signal detected in the same arrangement without the leaf.

From the light-induced time courses of the collimated transmittance were evaluated the normalized amplitudes (H_1, H_2) and the slopes (S_1, S_2) of the CT curves.

Statistical analysis

Data sets with $P < 0.05$ were regarded significantly different. Statistical software SigmaStat (Systat, Chicago, USA) version 3.0 was used for the testing. Each measurement (fluorescence inductions, transmittance measurements) was performed five times (n = 5). The statistically significant difference for the given parameter was evaluated for particular T_m with respect to the preceding T_m.

3.2.2. Results and discussion

Linear HS

Most measured FLR curves showed the characteristic shape with O, J, I and P steps. The changes in fluorescence signal detected after the linear regime started at about T_m = 42 °C (Fig. 10a). The character of all measured FLR curves and their responses to HS (i.e. gradual decrease of FLR intensity at J, I, and P steps reflecting gradual loss of photochemistry) correspond with other results (e. g. Guissé et al. 1995; Lazár et al. 1997; 1999; Tóth et al. 2005; 2007). Similarly, decline in the time-course of CT curves of the linearly heated samples was observed at the highest used T_m = 42 °C and 45 °C (Fig. 10b).

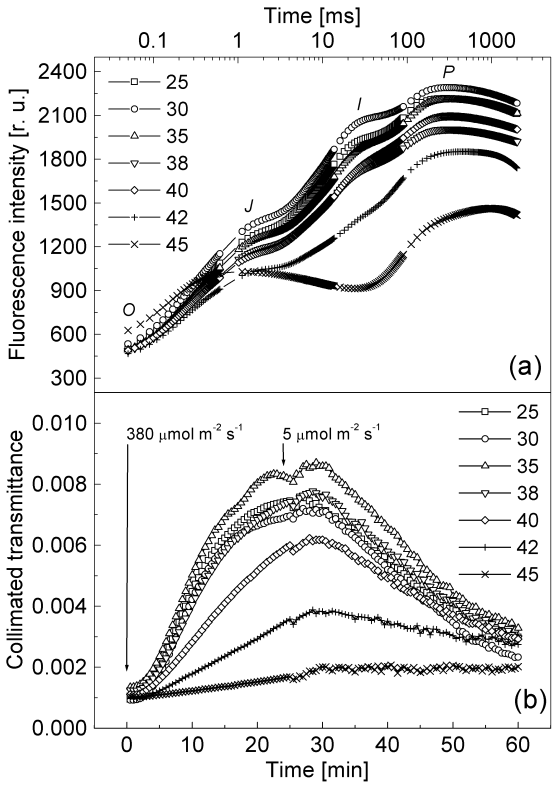

Figure 10. *Average (n = 5) FLR (**a**) and time-course of CT (**b**) curves of tobacco leaf segments measured at 25 °C after linear HS regime (2 °C min^{-1} up to given maximal temperature T_m and cooled at the same rate to 25 °C). The illumination during each CT measurement (**b**) was changed after 25 min from 380 to 5 μmol (photons) m^{-2} s^{-1}*

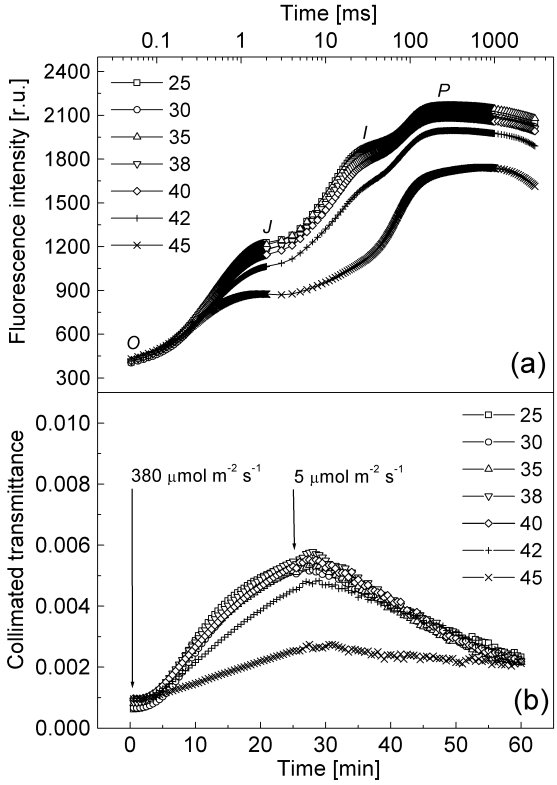

Figure 11. *Average (n = 5) FLR (**a**) and time-course of CT (**b**) curves of tobacco leaf segments measured at 25 °C after T-jump HS regime (5 min incubation at given T_m followed by 5 min incubation at 25 °C). The illumination during each CT measurement (**b**) was changed after 25 min from 380 to 5 μmol (photons) m^{-2} s^{-1}*

In order to estimate the effect of HS regime on the samples more accurately, we evaluated and compared several fluorescence and transmittance parameters. From standard JIP-test (for review see Strasser et al. 2004) we chose 6 "basic" parameters derived from the FLR curves (Fig. 10a and 11a): the minimal fluorescence at the beginning of FLR (F_0), maximal quantum yield of PSII photochemistry (F_V/F_P), approximation of the slope of the initial part of fluorescence rise (M_0), relative variable fluorescence at 2 ms (V_J), at 30 ms (V_I) and the normalized area (S_M) above the FLR curve. From the measured CT curves (Fig. 10b and 11b) we introduced the normalized parameters of the maximal collimated transmittance (H_1) and slopes of the linear section of the time-course (S_1) for the increasing part and analogically H_2 and S_2 for the decreasing part of CT curves.

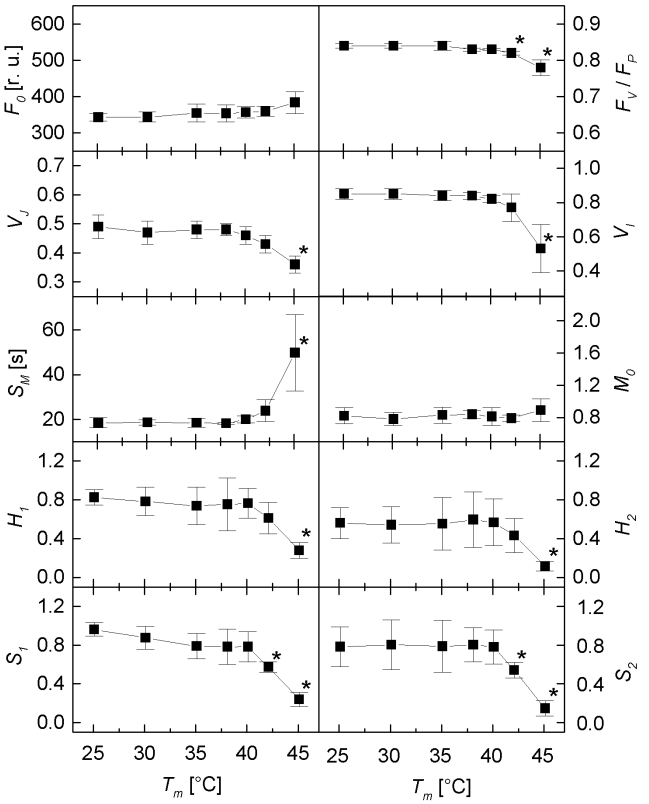

Figure 12. *Chl fluorescence and leaf CT parameters calculated from the FLR and CT curves measured at 25 °C after linear HS regime (2 °C min^{-1} up to given T_m and cooled at the same rate to 25 °C). The values are mean ± SD of five independent experiments. Statistically significant differences between neighboring means (P < 0.05) are presented by asterisks*

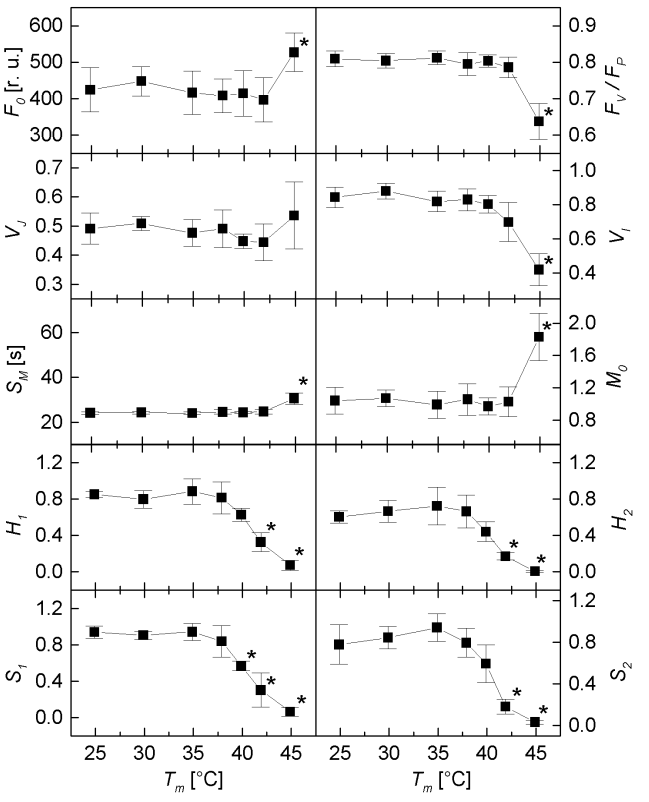

Figure 13. *Chl fluorescence and leaf CT parameters calculated from the FLR and CT curves measured at 25 °C after T-jump regime (5 min incubation at given T_m followed by 5 min incubation at 25 °C). The values are mean ± SD of five independent experiments. Statistically significant differences between neighboring means (P < 0.05) are presented by asterisks*

Parameters defined in this way reflect the amplitude (H_1 and H_2) and rate (S_1 and S_2) of chloroplast translocation. Statistically significant changes in parameters measured using linear regime mostly occurred for T_m = 45 °C (Fig. 12).

T-jump HS

The first pronounced changes in the shape and fluorescence intensity of the FLR curve measured after T-jump regime were detected at T_m = 42 °C (Fig. 11a). The decline in the extent of CT signal started at about T_m = 40 °C and the changes in CT curves during the illumination were almost fully inhibited at T_m = 45 °C (Fig. 11b).

Fluorescence parameters of curves measured after T-jump HS changed mostly at 45 °C while transmittance changes occurred in the interval T_m = 40 – 45 °C (Fig. 13).

Although the statistically significant differences in transmittance parameters were largely observed for T_m higher than 40 °C, there was a tendency to reach maxima of the transmittance parameters after incubation for T_m = 35 °C (Fig. 13) corresponding with the original CT curves (Fig. 11b). This maximum indicates that a T-jump incubation for 5 min at T_m = 35 °C was optimal for chloroplast movement.

Comparison of FLR and CT under both regimes

Our measurement of CT and FLR curves revealed that the chloroplast movement is more sensitive to higher temperatures than PSII photochemistry for our regimes (see all statistically significant changes in calculated parameters in Figs. 12 and 13). Interestingly, these differences in sensitivity were only slightly greater after linear HS (compare the reaction of S_1 and S_2 with FLR parameters at T_m = 42 and 45 °C in Fig. 12) in contrast to much greater in the case of T-jump regime (compare the reaction of CT parameters with FLR parameters for T_m = 40, 42 and 45 °C in Fig. 13).

The question, however, was whether the regimes and stress temperatures used, would occur under field conditions. Additional pilot experiments under field conditions showed that tobacco leaves can reach temperatures around 45 °C in the sunshine and that the temperature rise after exposure of the leaf is approximately linear and more rapid than slow leaf cooling induced by transfer of the sample back into the shadow.

Impaired chloroplast movement and its possible physiological role

The shape of CT curves in Fig. 10b and 11b reflects the chloroplast translocation. The increase in CT signal after onset of strong blue light is caused by the avoidance response resulting in increased transmittance through the cells. *Vice versa,* dim light induced accumulation of chloroplasts close to illuminated cell walls resulting in a decrease in light transmittance. All CT time-course parameters, the amplitude (H_1 and H_2) and rates (S_1 and S_2) of chloroplast translocation, began to decline at about T_m = 40 °C (Figs. 12 and 13). The decrease reflects gradual inhibition of chloroplast translocation for both avoidance (H_1, S_1) as well as accumulation response (H_2, S_2).

As the plant cytoskeleton plays a crucial role in chloroplast movement (for review see Takagi 2003), we tentatively propose that cytoskeleton changes are responsible for the inhibition of chloroplast movement at higher T_m. This is in accordance with observation of disruption of microtubules and actin filaments exposed to similar HS conditions (Müller et al. 2007). Other reasons too, for the inhibition of

chloroplast movement are possible, e.g. inhibition of signal transduction mediated by phototropins or impaired linkage between chloroplasts and actin filaments (Oikawa et al. 2008; Kadota et al. 2009).

One of the most accepted physiological functions of chloroplast movement is to avoid excess light (Park et al. 1996; Kasahara et al. 2002). Strong light under natural conditions is often accompanied by elevated temperatures that can result in decreased light-induced chloroplast movement. The HS-induced inhibition of chloroplast translocation in strongly illuminated regions of the first layer of the mesophyll cells prevents the chloroplast movement in the direction of the sidewalls of the cell under strong light. We tentatively propose that such HS-induced interception of greater amounts of light at the illuminated surface cells better protects the remaining chloroplasts inside the leaf from excess light.

4. CONCLUSION

The focus of this thesis was the effects of HS on the photosynthetic apparatus of plants and study of the reversibility of the resultant HS induced changes. The use of a variety of heating regimes and comparison of their impact was given careful attention. The response of the photosynthetic apparatus was monitored using chlorophyll fluorescence techniques in particular.

In the first paper, we focused on characterization of the effects of linear heating/cooling regimes, an unexplored approach in the field of heat stress research. Considering the initial signal, a fully reversible response of PSII function as reflected by the reversibility of chlorophyll fluorescence, was observed for a maximal temperature (T_m) of linear heating up to 42 °C. The evaluation of tangents revealed that the reaction of the samples was leastwise partly reversible up to maximal temperatures ranging from 52 (in the case of heating rate 0.5 °C min^{-1}) to 59 °C (3 °C min^{-1}). Further evaluation of initial irreversible changes in fluorescence led to an estimation of the activation energy needed for this process (on average 41 kJ mol^{-1}) and reflecting some minor structural changes in PSII. The assumptions used for the E_a evaluation suggested that the irreversible changes are caused by closure of certain PSII reaction centers (i.e. the accumulation of Q_A^-), which is considered the main reason for fluorescence increase in heated samples (see Section 1.2.1.2.).

The second paper deals with the impact of distinct heating regimes, linear and T-jump, on the light-induced movement of tobacco chloroplasts. While a 5-min incubation at 35 °C presented optimal conditions for the translocation, the inhibition of chloroplast movement began after incubation at about 40 °C and was almost complete at 45 °C. The fluorescence parameters from the O-J-I-P curves, measured in the same samples, responded generally at higher T_m. This difference in sensitivity was higher for the abrupt than for the gradual HS. Although chloroplast translocation can affect the fluorescence signal under normal conditions, their responses to HS seem to be different, according to our results. The observed inhibition of light-induced chloroplast movement is probably due to cytoskeleton disruption and it is suggested that this impairment might contribute to protection of the majority of chloroplasts inside the leaf from photoinhibitory damage. The measurement of changes in collimated transmittance showed that the light-induced chloroplast movement is affected under relatively mild

HS. For this reason, we suggest this may be a sensitive indicator of the adverse effects of elevated temperatures.

This thesis shows the importance of various heating regimes as the plant response to heat shock is strongly influenced by the kind and severity of the HS. It is not surprising that slow temperature increase causes greater irreversibility of some physiological parameters than faster heating to the same stress temperature (Fig. 7) as the slower temperature increase presents a longer exposure to higher temperatures and thus greater risk of plant damage. On the other hand, some of the results, namely the slight decrease in E_a with the increasing rate of heating as well as the lower adverse impact of linear heating with respect to T-jump, support the view that gradual temperature increase is more tolerable to plants than rapid temperature increase.

This very rapid acclimation process is activated in a relatively short period of time and thus it takes place during the action of the HS itself. This might consist in the protective effect of some heat shock proteins, whose synthesis is greater under gradual temperature increase (Ginzburg and Salomon 1986, Howarth 1991). Similar results were obtained in the case of net CO_2 assimilation rate measurement (Law and Crafts-Brandner 1999; Crafts-Brandner and Salvucci 2002) showing that this feature may be common to various plant levels.

Leaf temperatures in the field often change temporarily and very rapidly and this could be simulated by T-jump regime under more controlled laboratory conditions. However, the main temperature changes, which take place during the diurnal cycle or within sun-shade alternations, are rather gradual and thus progressive heating regimes would be more appropriate for reflecting the natural conditions.

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7. SUPPLEMENT

Separate papers are attached