COMPARISON OF TWO MEASURES OF PSI ELECTRON TRANSPORT IN WINTER RYE

by

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A Thesis

submitted to the Department of Biological Sciences

in partial fulfilment of the requirements

for the degree of

Master of Science

1995

Brock University

St. Catharines, Ontario

Abstract

The number of P700 (the reaction centre of Photosystem I) converted to $P700^{+}$, in winter rye, was determined by measuring the absorbance change at 820nm . It was found, with a single turnover flash, that thylakoids isolated from cold grown plants have a 50% greater number of P700 oxidized than thylakoids isolated from warm grown plants. Incubation of thylakoids in the dark at 35 C did not change the number of P700 oxidized. The conversion of P700 to P700+ with a single flash can be compared to a steady state rate of electron transport using a Clark electrode. The results for P700 oxidation using the absorbance change at 820 nm measure effects within the PSI complex whereas the results obtained from a Clark electrode measures steady state electron transport between the cytochrome b/f complex and the PSI complex. In contrast to the results for P700 oxidation it was shown, using a Clark electrode, that both thylakoids from cold grown plants and thylakoids incubated at in the dark 35 C exhibited 50% higher rates of electron transport than thylakoids from warm grown plants. The correlation between the higher rate of steady state PSI electron transport observed in thylakoids isolated from cold grown winter rye and number of active PSI reaction centres localizes the site of the increase to the PSI reaction centre. In contrast the lack of correlation after incubation at 35 C indicates the increase in the rate of light saturated electron transport in thylakoids isolated from cold grown plants and thylakoids incubated in the dark at 35 C occur by different mechanisms.

Acknowledgements.

I would first like to thank Dr. Doug Bruce and Dr. Norman Huner who showed more patience with me than perhaps I deserved over the last years. May your future graduate students cause you less grief.

Dr Ralph Chapman and Phillis Chapman who helped edit this work and saved my supervisors and myself many hours of editing. In addition, thanks to Jean King and Dr William Hopkins who introduced me scientific research. Also thanks to Dr Helga Baten who also provided help with this work.

To Chris, Moira, Christina, Dr Guy, and Cynthia for making the lab a less lonely place.

To one who would not wish their name recorded for keeping me in some semblance of sanity in the fall of '93.

And to Bethany Grace little for listening to me after that when even I found my conversation boring.

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List of Abbreviations

CF_oCF_1	The Proton driven ATP synthetase complex of plants
DAD	diaminodurene
DCMU	Dichlorolmethylurea
DCPIP	Dichlorrophenolindophenol
DHQ	dihydroduroquinone
LHC II	Light harvesting complex of photosystem II
MV	Methyl Viologen
P680	The core chlorophyll of photosystem II
P700	The core chlorophyll of photosystem I
*psa*A-E,I-L	Genes coding for PSI polypeptides
PsaA-E,I-L	PSI polypeptides coded by corresponding gene
PSI	Photosystem I
PSII	Photosystem II
SDS	Sodium dodecysulfate
SOD	Superoxide dismutase
TMPD	tetramethylphenylenediamine

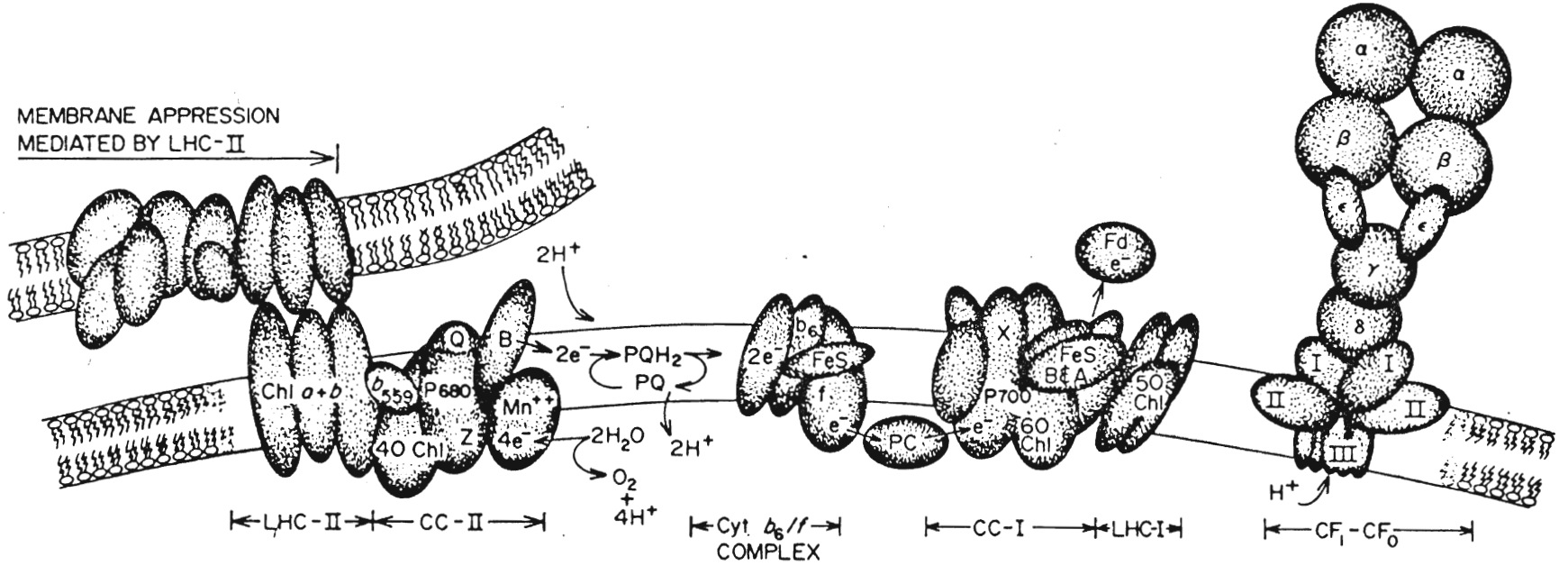
INTRODUCTION

Structure and Function of Thylakoid Membranes, Electron Transport, and the light reactions of photosynthesis

As higher plants are used as the subject of this thesis the discussion of photosynthesis will be limited to photosynthesis in C3 plants in which CO_2 is fixed by ribulose bisphosphate carboxylase-oxygenase in the stroma of mesophyll chloroplasts (Coombs, 1976). Leaf mesophyll cells contain membrane-bound organelles called chloroplasts. These chloroplasts are bounded by two membranes and contain a third set of membranes called the thylakoid membranes. Photosynthetic electron transport occurs on the thylakoid membrane. The reactions that comprise photosynthesis can be separated into the light dependent reactions and light independent reactions. In this work we are only concerned with the light dependent reactions.

The light dependent reactions involve the transfer of electrons from water to provide chemical reducing power in the form of NADPH. Concurrently with the production of NADPH, a proton gradient is generated which is harnessed to produce chemical energy in the form of ATP. These processes involve the electron transport chain which consists of two photosystems, photosystem I (PSI) and photosystem II (PSII), their antenna complexes and intersystem electron carriers. All these components are associated with the thylakoid membrane (Figure 1).

Figure 1 The complexes of the electron transport chain and their location in the membrane. Electrons flow from water through PSII, plastoquinone, the cytochrome b6/f complex, plastocyanin, PSI and are accepted by ferredoxin. PSII and LHCII are found primarily in the appressed region. PSI and the CF_oCF_1 complex are found primarily in the non-appressed region. DCMU blocks electron transport at Q_B, reduced DCPIP will donate electrons to the cytochrome b6/f complex, plastocyanin, and PSI and methyl viologen will accept electrons from the reducing side of PSI.

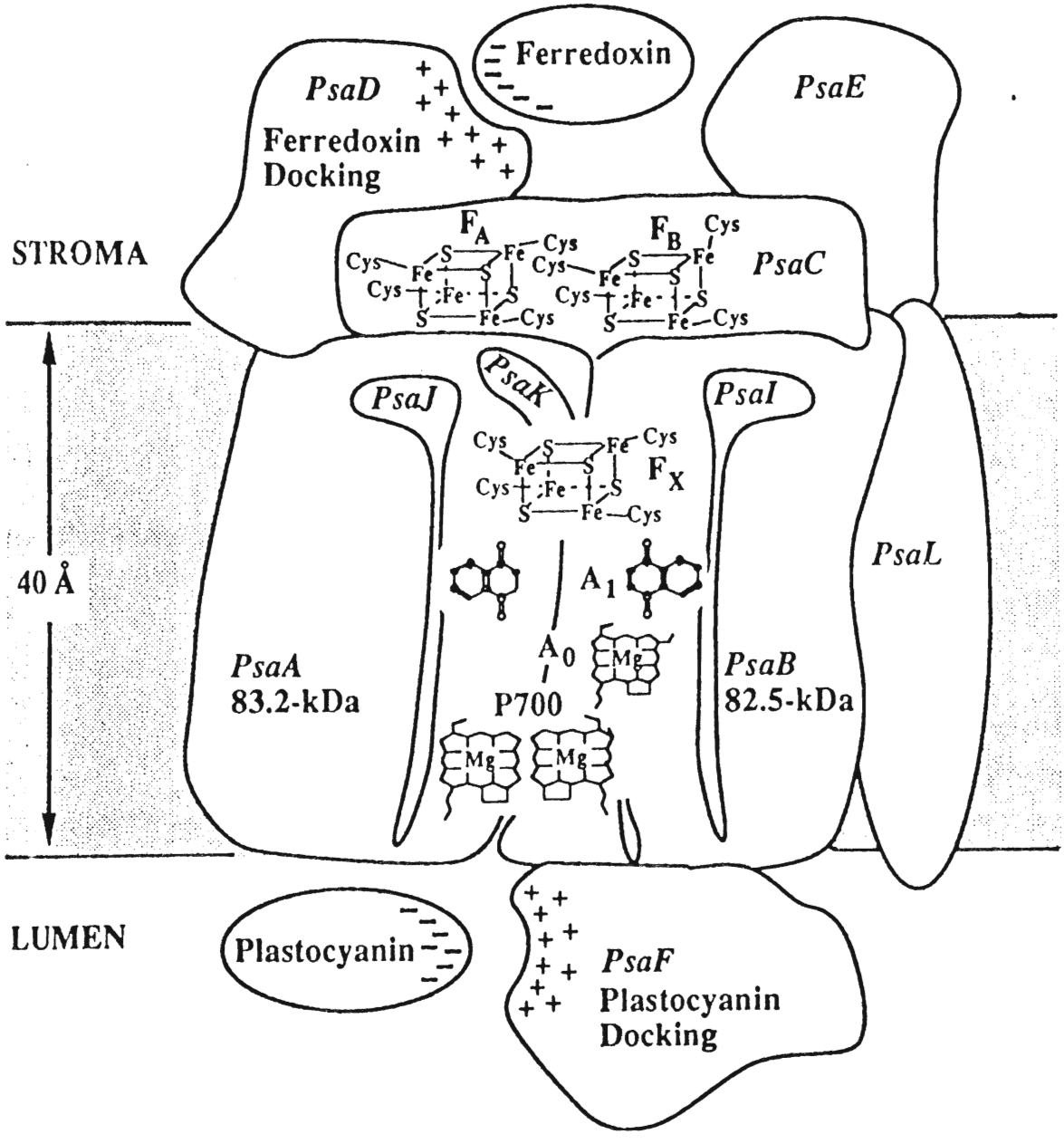

MEMBRANE APPRESSION
MEDIATED BY LHC-II
2H+
Chl a+b
b559
P680
Q
B
2e-
PQH2
PQ
2H+
Mn++
4e-
2H2O
O2
+
4H+
40 Chl
Z
2e-
b6
FeS
f
e-
PC
X
P700
e-
60 Chl
FeS
B¢A
50 Chl
Fd
e-
α
α
β
β
ε
ε
γ
δ
I
I
II
II
III
H+
LHC-II
CC-II
Cyt. b6/f
COMPLEX
CC-I
LHC-I
CF1-CF0

Energy from the photons absorbed by the chlorophyll and accessory pigments of the Light Harvesting Complex of Photosystem II (LHCII) is transferred to the core antenna and finally to the reaction centre of photosystem II. The PSII reaction centre consists of 5 integral proteins. Two of these proteins, D1 and D2, provide the binding environment for the reaction centre P680, pheophytin, and the primary and secondary quinone electron acceptors (Q_A and Q_B). (Vermaas, 1988). In the reaction centre, the energy from light is used to raise the energy level of one electron on the chlorophyll dimer, P680. The presence and position of other internal electron carriers causes the electron to leave the chlorophyll, resulting in the oxidation of P680 to $P680^+$. The rapid transfer of the electron through pheophytin to the primary and secondary quinone acceptors stabilizes the charge separation. The electron then leaves the photosystem II complex as it is passed on to plastoquinone, cytochrome b6/f complex and plastocyanin which ultimately donates electrons to photosystem I.

$P680^+$ must be reduced to P680 before another charge separation can occur. The electrons for this reduction come from water. This is facilitated by a group of polypeptides with a manganese prosthetic group attached to photosystem II, the water splitting complex.

The light harvesting complex of photosystem I also absorbs photons, funnelling the energy to the reaction centre of photosystem I (Figure 2). The energy is transferred to P700, the reaction centre chlorophyll of PSI. Charge

Figure 2 Proposed architecture for the PSI reaction centre. Excitation energy is pass to the chlorophyll dimer P700. P700 loses an electron which is then transferred to the chlorophyll A_o, then to a quinone A_1, then to three iron-sulphur groups F_x, F_A and F_B, before leaving PSI bound to ferredoxin. Data from Golbeck (1992).

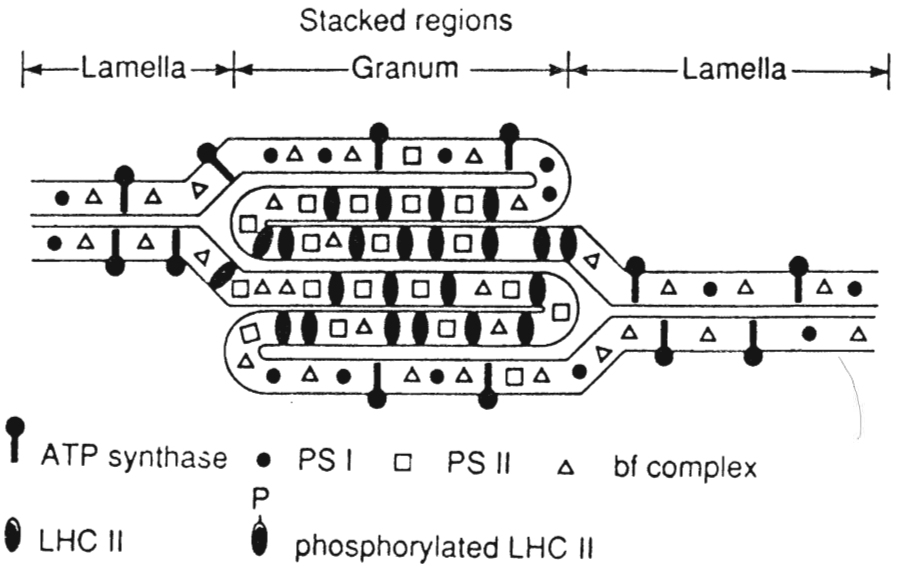

Ferredoxin
PsaD
Ferredoxin Docking
PsaE
STROMA
F_A
F_B
PsaC
PsaJ
PsaK
PsaI
F_X
40 Å
A_1
A_0
PsaL
PsaA
83.2-kDa
P700
PsaB
82.5-kDa
Mg
LUMEN
Plastocyanin
PsaF
Plastocyanin Docking

separation within PSI occurs in a manner similar to that in photosystem II. The result is the oxidation of P700 to $P700^{+}$. The electron lost by P700 is passed through a chlorophyll acceptor, A1, to phylloquinone, to a group of iron-sulphur proteins and on to bound ferredoxin. Cyclic electron transport results from the photosystem I reduction of the cytochrome b_6/f complex. The non-cyclic electron transport pathway converts $NADP^{+}$ to NADPH. The electron lost by the photooxidation of the photosystem I reaction centre is replaced by the electrons from reduced plastocyanin which has received electrons from the photosystem II reaction centre.

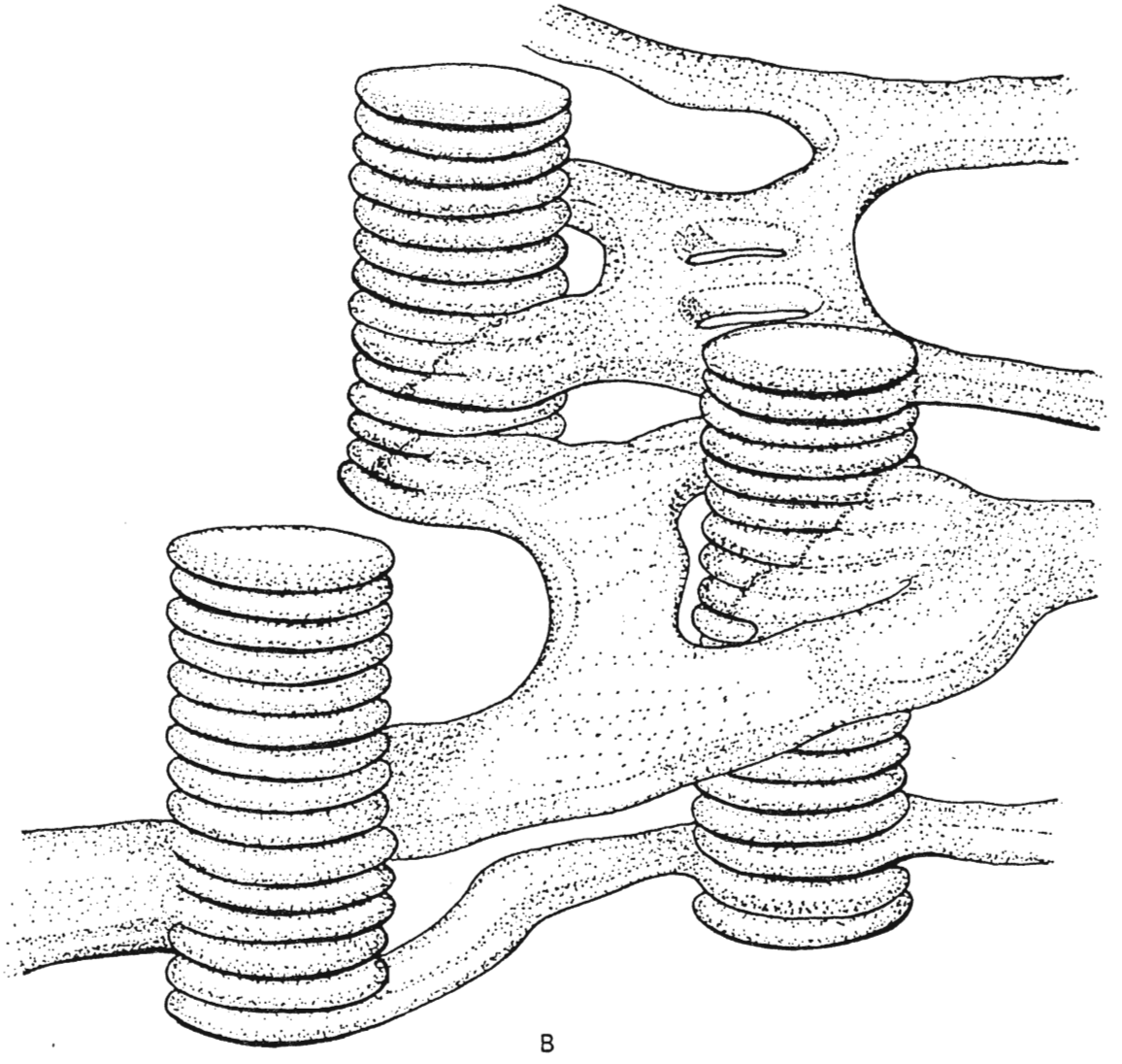
The photosystem I reaction centre core complex is composed of 10 polypeptides (Figure 2). A heterodimer (PsaA and PsaB) binds P700 and the primary acceptor chlorophyll (A1). The secondary acceptor, phylloquinone, and the iron-sulphur centre, Fx, also bind to these polypeptides. To this is bound a polypeptide, PsaC, which binds the iron-sulphur centres, Fa and Fb, and the Ferredoxin docking polypeptide, PsaD. The heterodimer also binds a number of additional smaller polypeptides of unknown function, PsaE, Psa I, PsaJ, Psa K, Psa L (Golbeck, 1992).

The thylakoid membrane exhibits distinct regions of organization or latural heterogeneity. It is divided into (i) appressed regions which form the granal stacks and (ii) non-appressed regions which constitute the stromal lamellae (figure 3). As thylakoids have a net negative charge at neutral pH (Barber 1982) the electrostatic repulsive forces on the surfaces of the membrane must be screened by cations for

Figure 3 The structure of thylakoid membranes. Thylakoid membranes are divided into stacked and unstacked regions. The appressed regions are found between the granal stacks and the rest of the membrane forms the non-appressed region. Modified from (top) Nicholls and Ferguson 1992 and (bottom) Bidwell 1974.


Stacked regions
Lamella
Granum
Lamella
State I
ATP synthase
PS I
PS II
bf complex
LHC II
P
phosphorylated LHC II

A



B

the membranes of the granal stacks to become appressed (Staehelin and DeWit 1984). The grana unstack if isolated in media with low cation concentrations (Barber and Chow, 1979). The growth conditions of the plant also alter the degree of stacking in vivo. Plants grown under low light conditions have a higher degree of stacking and wider granal stacks than plants grown under high light conditions (Lichtenthaler, 1981). This is because thylakoid stacking is also dependent on the presence of the light harvesting complexes of photosystem II (LHC II). The N-terminal region of LHC II has 33-34 positively charged amino acids which regulate the appression of the thylakoid membranes (Anderson, 1986). The presence of appressed and non-appressed regions results in lateral heterogeneity in eukaryotic thylakoid membranes.

LHC II and photosystem II are found primarily in the appressed regions while photosystem I and the CF_oCF_1 are found in the non-appressed regions (Prezelin and Nelson 1990). The cytochrome b6/f complex is distributed between the two. Plastocyanin and plastoquinone shuttle electrons between the cytochrome b6/f complex and photosystem I and between photosystem II and the cytochrome b6/f respectively.

The proton gradient is established as a result of the following events. First, the photolysis of water leads to release of protons in the lumen of the thylakoids. Second, The oxidation-reduction of plastoquinone by the cytochrome b6/f complex is coupled to proton transport from the stroma to the thylakoid lumen (Prezelin and Nelson 1990). Last, the conversion of $NADP^+$ to NADPH results in the loss of free

protons on the stromal side of the thylakoid membrane. The proton gradient is used by the CF_o-CF_1 ATP synthetase complex to synthesize ATP (Dilley et al. 1987). Thus, the end products of the light reactions are ATP and NADPH.

These molecules are used in the Calvin-Benson pathway to fix CO_2 yielding a variety of phosphorylated intermediates. Among the most important are triose phosphates which are phosphorylated 3 carbon molecules by which energy is exported from the chloroplast to the cell (Stitt 1990).

Theoretically, under saturating CO_2 concentrations, the stoichiometry of the reaction is such that each CO_2 fixed will release one O_2. Three ATP and two NADPH molecules are required for the reduction of one CO_2. Theoretically this has a quantum requirement of nine photons/ CO_2 or, the reciprocal, a quantum efficiency of 0.111 mole CO_2/ mole photons (Walker, 1988). In reality not all of the ATP and NAPDH from photosynthesis is used in carbon fixation. Nitrogen and sulphur may also be reduced and the synthesis of lipids and amino acids require chemical energy. Cell requirements for different ratios of ATP and NADPH can result in changes in electron transport that result in electrons following a cyclic path which generates a proton gradient and thus produces ATP without generating NADPH. Environmental constraints, such as a CO_2 limitation, may have the same result (Prezelin and Nelson 1990). Additionally, light absorbed by the light harvesting apparatus may be lost as fluorescence or heat. All of these factors lower the apparent quantum efficiency of photosynthesis.

In the dark reactions, ribulose bisphosphate carboxylase-

oxygenase of the Calvin-Benson cycle can use O_2 with the loss of CO_2 through the process of photorespiration. This results in an increase in the photon requirement for CO_2 fixation.

The rate of O_2 evolution increases with increasing light flux until light saturation occurs. Under light saturating conditions, available light no longer limits photosynthesis, the rate of photosynthesis is then limited by the rates of the enzymes in the carbon fixing Calvin cycle.

Even if light is unlimited, the rate of O_2 evolution can still change. Growth temperature affects oxygen evolution and other aspects of plant metabolism as well as plant development.

Cold hardening in plants.

Most plants grow optimally at temperatures of 10 to 35°C. Fluctuations above and below this range may limit plant growth and development. Plant responses to low temperature vary but can be grouped into two different categories. Chilling sensitive plants will suffer damage and death from short exposure to low nonfreezing temperatures. Cold tolerant plants are generally defined as plants that can grow at temperatures between 10° and 0°C. The tolerance of plants to low temperature varies with growth conditions and different species show different levels of cold tolerance. Plants which have the ability to acclimate to cold temperatures by growth and development at low temperatures are termed cold-hardy. Prolonged exposure to low non-freezing temperatures over a period of weeks or months results in a cold acclimated state

and the plants in this state are termed cold-hardened. Plants must attain this cold acclimated state in order to survive subsequent exposure to freezing temperatures.

Plants grown at temperatures above 10°C generally do not survive temperatures below -6°C. Growth at low temperatures can increase the tolerance of a plant to sub zero temperatures. Rye grown at 20°C will survive temperatures of -4°C, whereas rye grown at 4°C will survive temperatures down to -30°C (Huner et al 1981).

Interest in freezing tolerance dates back to 1741 (Levitt 1956) with descriptions of the survivability of various tissue samples frozen to various temperatures. Since the turn of the century, research has focused on the site and mechanism of cellular freezing damage. This includes examination of freezing points of lipids and proteins of plant tissues. (Siminovicht 1968; Prestegard 1978; Dowgert 1984; Steponkus 1984; Yoshida 1984a; Johnson-Flanagan and Singh, 1986; Guy 1990).

The nature of the cold-hardening process and the changes during cold-hardening have also been investigated. Changes in protein and lipid compositions have been examined (Siminovitch et al., 1968; Guy and Carter, 1984; Yoshida, 1984 b; Yoshida and Uemura, 1984; Lynch and Steponkus, 1987). Work has also been done on gene expression and protein synthesis during cold hardening (Guy, et al., 1985; Sarhan and Chevier, 1985; Guy, et al., 1987; Guy and Haskel, 1987; Guy 1990). Plants transferred to a low temperature environment have been shown to synthesise new proteins.

Unlike heat shock, which produces similar proteins in many organisms, the new proteins synthesised as a response to low temperature vary among species. Because of this variation the functions of many of these new proteins are not known. Several of these proteins appear to be slightly altered forms of enzymes that appear in major metabolic pathways (Guy, 1990). It is hypothesised that these proteins compensate for cold lability of some proteins and the change in kinetics of others at low temperatures (Guy, 1990). Other proteins may help the cell survive freezing. Winter rye, for example, exports proteins to the intracellular space. These proteins promote ice crystal formation outside the cell and control the size and shape of ice crystals formed. Thus, ice crystal formation is confined to the outside of the cell and the damage that would be done by formation of large crystals is avoided (Griffith, 1992).

The age and metabolism of tissues is important for cold-hardening. Light and CO_2 are required for cold hardening (Dexter, 1933; Tysdal, 1933). Sucrose, an end product of photosynthetic carbon metabolism, has been correlated with cold-hardening (Steponkus and Lanphear, 1967) and field grown winter wheat seedlings have to be of a certain age for proper cold hardening to occur (Andrews et al. 1960; Andrews and Pomeroy, 1974; Fowler, 1982).

Thus, energy from photosynthesis is an absolute necessity for a plant to cold-harden under natural conditions. Since photosynthesis is necessary for cold-hardening and since cold-hardening occurs at temperatures below 10 C, photosynthesis

must occur at these temperatures. It is not surprising then that cold-hardening results in photosynthetic adjustment (Öquist, 1983,).

Changes in winter rye as a result of cold hardening.

Low temperature growth of plants results in distinct morphological, anatomical, and biochemical changes in the photosynthetic apparatus compared to plants grown under higher temperatures. In winter rye, growth at lower temperatures is associated with: a 21 day lag phase before maximum vegetative growth, a 3-4 fold decrease in total leaf area/plant and stretched plant height, a 4 fold increase in leaf dry weight, and increased pigment content (Krol et al, 1984). Also there is a decrease in stomatal frequency (Huner et al, 1981), a change in the distribution of stomata, a decreased epidermal cells size (Griffith et al, 1985), and a 1.5 times increase leaf thickness due to a increased mesophyll cell size (Huner et al, 1981).

Growth at low temperature alters chloroplast morphology and ultrastructure. Fewer thylakoids per granum are found in the chloroplasts of plants grown at low temperatures (Huner et al 1985b). Freeze fracture analysis showed that LHCII-photosystem II units in the thylakoid membrane of rye grown at 20°C exist as particles of about 16.0 nm while LHCII-photosystem II units of plants grown at 5°C exist as particles of 13.6 nm (Huner et al, 1984).

In vitro separation of chlorophyll:protein complexes of thylakoids from cold and warm grown rye indicated that the

monomeric form of LHCII predominates in the thylakoids from cold grown plants while thylakoids from warm grown plants primarily exhibit the oligomeric form of LHCII (Huner et al 1987). This change is associated with a 72% decrease in the trans 16:1 content of thylakoid phosphatidyldiacylglycerol. Analysis of lipids and fatty acids from purified LHCII revealed that phosphatidyldiacylglycerol was specifically associated with LHCII in both warm and cold grown plants. The difference being that there was 54% less trans 16:1 acid associated with phosphatidyldiacylglycerol of LHCII from cold grown plants. Enzymatic removal of phosphatidyldiacylglycerol in LHCII samples resulted in equal ratio of LHCII oligomer/monomer. Only phosphatidyldiacylglycerol containing trans-16:1 could reconstitute oligomeric LHCII from monomeric LHCII (Krupa et al 1992). It was concluded that the trans 16:1 content of phosphatidyldiacylglycerol specifically changes the organization of rye LHCII.

Additional evidence for organizational changes in LHCII is found in the fact that low temperature growth changes the fluorescence emission spectra of rye thylakoids at 680 and 695 nm (Reynolds 1988) and alters the denaturation transition of LHCII as measured in situ by differential scanning calorimetry (Huner et al 1987).

While low temperature growth caused reorganization in LHCII it had no effect on the chlorophyll a:b ratio, the thylakoid polypeptide complement or photosystem II or photosystem I photosynthetic unit size (Griffith et al, 1984).

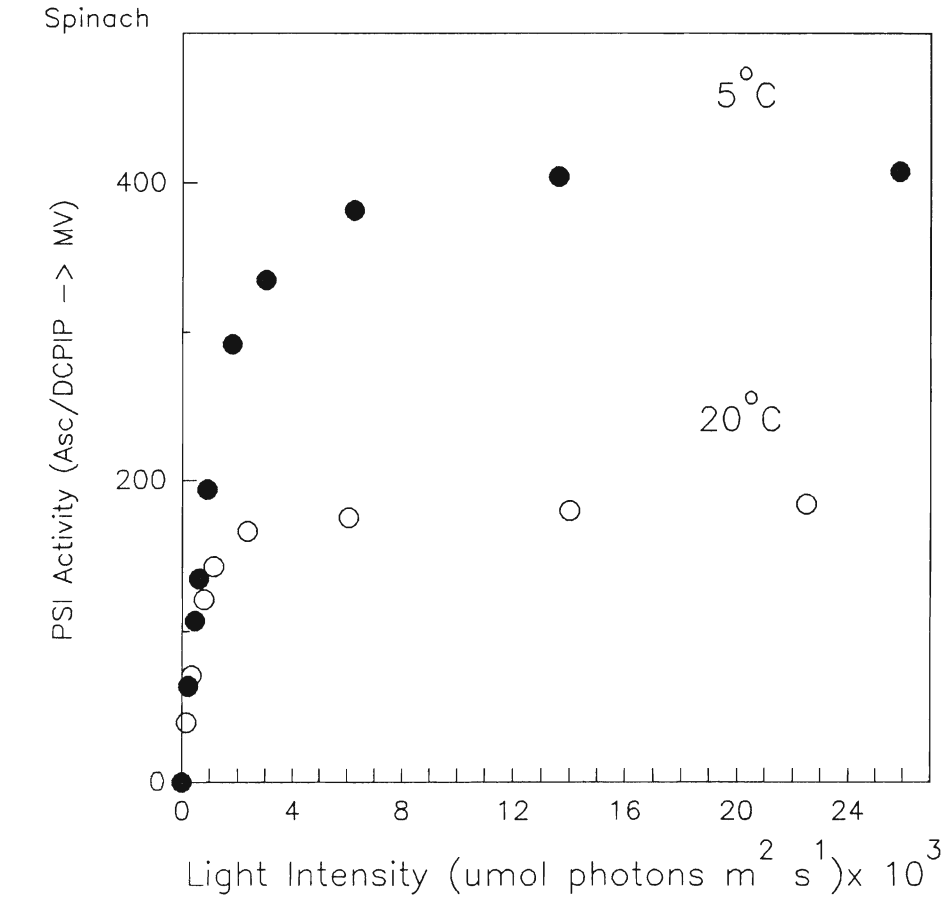
Low temperature growth does not appear to affect the size

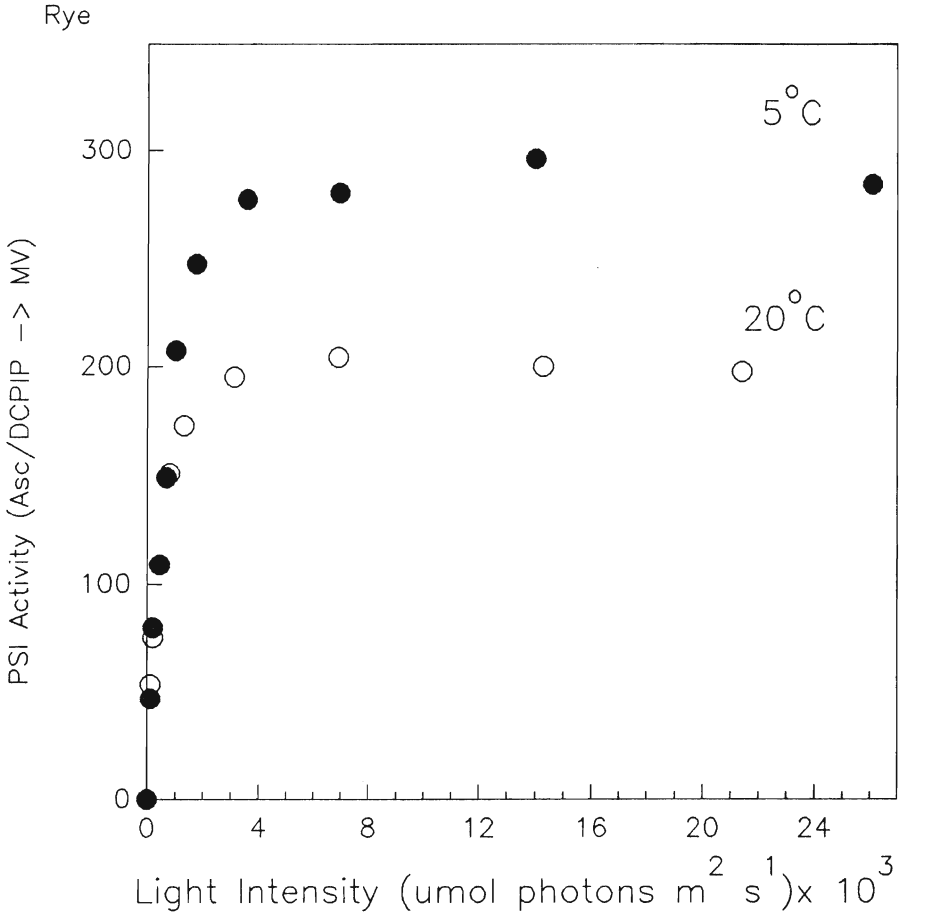
or proportion of the two photosystems but it has a marked effect on electron transport rates. Cold grown plants exhibit a light saturated whole chain electron transport rate 1.5 fold greater than warm grown plants. This is perhaps due to a 1.6 fold increase in the light saturated rates of photosystem I associated electron transport (DCPIP/Asc --> MV)(Huner et al, 1985a) (figure 4). It is not due to a change in the number of P700 on a per chlorophyll basis in cold grown plants as measured by light minus dark electron spin resonance spectra of P700 (Huner et al, 1984).

Curiously, isolation of thylakoids from cold grown plants in buffers which do not contain added cations (Mg^{2+},Na^{+}) resulted in light saturated rates of photosystem I electron transport which were not significantly greater than the rates of thylakoids isolated from warm grown plants. It should be remembered that the initial rate of light saturated photosystem I electron transport in thylakoids isolated from warm grown plants is not dependent of the presence of cation in the isolation buffer.

This increase in activity has also been seen in many other cold tolerant species of plants such as wheat, peas, spinach, broadbean, *Brassica napus*, and periwinkle but the mechanism is still unknown (Huner 1990). It has been postulated that the increase in photosystem I activity is either an organizational change in the photosystem I core complex, or a change in the interaction between photosystem I and the intersystem electron transport carriers, plastocyanin and cytochrome b6/f. It is possible that there is an increase

0**Figure 4** Light saturation curves of PSI activity in thylakoids isolated from 5 C and 20 C grown Muskateer rye and spinach. Thylakoids were isolated in the presence of NaCl and $MgCl^2$. PSI activity was measured as Asc/DCPIP -> MV and calculated O_2 consumed $(mg\ Chl)^{-1}\ h^{-1}$. From Huner and Reynolds (1989).


Spinach
5°C
20°C
PSI Activity (Asc/DCPIP -> MV)
0
200
400
0
4
8
12
16
20
24
Light Intensity (umol photons m 2 s 1)x 10 3


Rye
5°C
20°C
PSI Activity (Asc/DCPIP -> MV)
0
100
200
300
0
4
8
12
16
20
24
Light Intensity (umol photons m 2 s 1)x 10 3

in the PSI unit:Chlorophyll ratio but as mentioned previously and as will be shown in this work there is evidence against this hypothesis.

Cold hardening also leads to an increase in the resistance to photoinhibition in spinach (Somersalo and Krause 1989, 1990 Boese and Huner 1990) and in cereals. The increase in the resistance to photoinhibition was observed both in intact leaves of cereals (Oquist and Huner 1991, Hurry and Huner 1992) and isolated mesophyll cells of cereals (Lapointe and Huner 1992) but not in isolated thylakoids (Lapointe et al. 1991). A unique feature of this resistance is that growth at low irradiance (250 μmole photons m^{-2} s^{-1}) decreases susceptibility to high irradiance (1500 μmole photons m^{-2} s^{-1}). Resistance to photoinhibition requires that the leaves grow and develop at low temperatures. Oquist and Huner (1992) showed that the change in resistance reflects a change in the capacity to keep Q_A oxidized under high light and low temperature conditions. Equalization of the redox state of Q_A removes any difference in the susceptibility to low temperature photoinhibition. It has been suggested that the increased capacity for photosynthesis in cold grown plants may, impart, explain the increased capacity to keep Q_A oxidized under condition of high light.

Effects of mild heat treatment on the rate of electron transport in winter rye.

Huner and others have suggested that the increase in the rate of photosystem I electron transport with upon low

temperature growth is due to an organizational change in photosystem I and not a change in composition as the polypeptide and pigment compositions of cold grown and warm grown rye thylakoids were not significantly different (Huner et al 1984, Elfman et al 1984). There are several reasons for this: (1) examination of the polypeptide complement of purified photosystem I showed no difference between warm and cold grown plants; (2) photosystem I reaction centre polypeptide content on a per chlorophyll basis does not change; (3) Chlorophyll-protein complexes indicate that the proportion of PSI to total Chlorophyll does not change; (4) PSI unit size in Chl/P700+, as measured by electron spin resonance, does not change; (5) in freeze fracture, particle density on the PFS fracture face does not change; (6) If thylakoids from cold grown plants are isolated in the absence of cations, the light response curve is identical to that of thylakoids from warm grown plants; (7) mild heating (at 30-60°C) of thylakoids from warm grown plants produced an increase of 50-100% in the rate of photosystem I electron transport, similar to the increase of 50-100% after growth under cold hardening conditions (Santarius, 1975, Reynolds and Huner 1990).

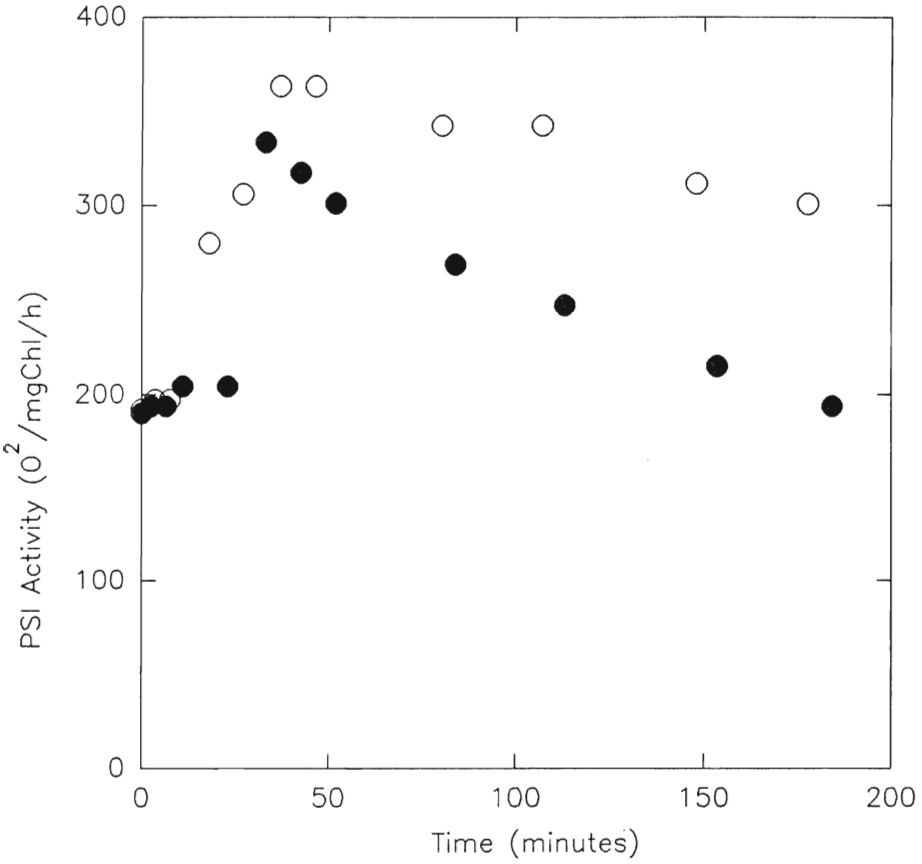
It has been shown in spinach that heating to 30°C inhibits cyclic and noncyclic photophosphorylation, coupled and uncoupled ferricyanide reduction, photosystem II activity, establishment of a light-dependent proton gradient and several stromal enzymes (Santarius, 1975). Mild heating also inhibits most photosynthetic processes in Euglena (Kataoh and San

Peitro, 1967) and liverwort thalli (Weis, Wamper and Santarius, 1986). The exception to this inhibition is photosystem I activity.

Other researchers found an increase in photosystem I activity during temperature treatment but were unable to explain the basis of this apparent activation (Williams, Sen and Fork, 1986; Stidham, Uribe and Williams, 1983). Increased photosystem I activity has also been observed in thylakoids isolated from primary wheat leaves when thylakoids were exposed to various elevated temperatures (Figure 5) (Mohanty et al, 1987). In pea, increased activity was observed over the temperature range 40 to 50°C. It was proposed that an increased accessibility of the electron donor, DCPIP, following heat treatment was responsible for this increase. Evidence for this increased accessibility can be found in the fact that the stimulation by the heat treatment was shown to involve the CN- sensitive pathway of plastocyanin (Thomas et al, 1984).

Both functional and physical dissociation of LHC II from photosystem II is induced in vivo by heating. Evidence for this can be found in freeze fracture electron microscopy as seen by a selective loss of particles greater than 90 angstroms in diameter, presumably LHCII, on the exoplasmic fracture face as a result of heat treatment (Armond, Bjorkman, and Staehlin, 1980). In vitro investigation showed reorganization of the thylakoid membrane through a heat induced dissociation of LHCII from photosystem II. The photosystem II and LHCII migrate to the non-appressed regions.

Figure 5 Rate of light saturated PSI (DCPIP/Asc -> MV) activity in thylakoids isolated from 20 C grown Muskateer rye after transfer to a 37 C incubation bath. Thylakoids were isolated in the presence open circles or absence closed circles of added cations. Both treatments how an increase in rate of PSI electron transport within 10 minutes of transfer. From Chapman (1991)


400
300
200
100
0
PSI Activity (O^2/mgChl/h)
0
50
100
150
200
Time (minutes)

This was correlated with a stimulation of photosystem I activity. It may be that the redistribution of excitation energy in favour of photosystem I prevents over-excitation and photodamage of photosystem II at high light intensities. Under natural conditions high temperatures are usually associated with high irradiance (Sundby et al ,1986)

The redistribution, by a plant, of excess excitation energy between two photosystems is termed a state transition. When plants are exposed to light which is primarily absorbed by photosystem II (light II) there is a redistribution of the energy in favour of photosystem I. When plants are subjected to light primarily absorbed by photosystem I (light I) this process is reversed. This redistribution was first noticed while studying slow fluorescence changes. Over time, a plant subjected to light to would deliver more energy to photosystem I at the expense of photosystem II (Bonvertura and Myers, 1969). The condition induced by light I and light II is termed State 1 and State 2 respectively.

There are essentially two different mechanisms for altering the distribution of light energy between photosystem II and photosystem I. The first mechanism, termed spillover, involves an increase in the direct transfer of energy between photosystem II and photosystem I. This necessitates a close physical association between photosystem I and photosystem II. This mechanism does not invoke gross movement of LHCII complexes. A second mechanism which changes the absorptive cross-sectional area results from a change in the fraction of light energy absorbed by one photosystem (Fork and Satoh,

1986). This increase in absorptive cross-section occurs because of the physical dissociation of LHCII from photosystem II and its migration to photosystem I.

Temperature affects energy distribution between the two photosystems. In *Chlorella cells*, *Canna* Leaves, and spinach thylakoids, mild heat treatments (45-48°C for two minutes) induces a shift in energy distribution from State I to State II (Sane et al, 1984). Asne et al (1984) have proposed that the heat induced transition of State II is a mechanism to prevent photooxidative damage to photosystem II by transferring the excess excitation energy to photosystem I which is more resistant to photooxidative damage. The heat treatment of isolated thylakoids appears to result in many organizational and functional alterations which mimic light-induced state transitions. It has been suggested that these changes may represent an in vivo mechanism to prevent heat damage to the photosynthetic apparatus (Havaux and Lannoye, 1987).

The measurement of electron transport.

There are several methods of studying electron transport. Some of these are discussed below.

O_2 electrode

Electron transport can be measured through the use of an oxygen electrode. A chamber which contains an aqueous solution is divided into two chambers by a semi-permeable membrane. This membrane is permeable to oxygen. One chamber of the cell

contains the thylakoid suspension. The other has a cathode and an anode bridged by a potassium chloride solution. The anode reacts with molecular oxygen to convert it to hydrogen peroxide and then to water. At the same time chloride ions react with the silver cathode to complete the circuit. This produces a current proportional to the oxygen concentration. Thus, an increase in the O_2 concentration in one chamber will equilibrate across the oxygen permeable membrane separating the two chambers and change the signal measured from the electrode in the other chamber.

The rate of whole chain photosynthetic electron transport can be measured as the rate of oxygen production. Alternatively, artificial electron donors and acceptors, and specific inhibitors can be used to monitor various parts of the electron transport chain. For instance, DCMU can be used to block electron transport between photosystem II and the cytochrome b6/f complex. DCPIP and ascorbate can be used to donate electrons to the cytochrome b6/f complex, plastocyanin, and photosystem I. methyl viologen will accept electrons from the reducing side of photosystem I and subsequently reduce oxygen to form hydrogen peroxide (Figure 1). If DCMU, DCPIP, ascorbate and methyl viologen are present in a buffer with thylakoids which are exposed to light electron transport will occur, but this electron transport will be limited to cytochrome b6/f complex, plastocyanin and photosystem I and will be measurable with the oxygen electrode as oxygen consumption.

Using a Clark type oxygen electrode to measure

photosystem I electron transport, Huner and co-workers showed that cold hardening temperatures caused a 1.5 times increase in the rate of light saturated PSI electron transport over that of non hardened plants (Huner 1985). This increase was seen in whole chain electron transport (H_2O -> MV) and using DCPIP as an electron donor. No increase was seen with DAD, TMPD, or DHQ (Huner and Reynolds, 1989). The increase required the presence of added cations in the thylakoid isolation buffers. If thylakoid membranes are isolated in the absence of added cations, the rate of light saturated electron transport was not significantly different from the rate of light saturated electron transport of thylakoids isolated from non-hardened plants in the presence of added cations (Huner and Reynolds, 1989).

Similarly, thylakoids from non-hardened plants heated in the dark exhibited a stable increase in the rate of light saturated PSI electron transport to 1.5 times the initial rate after 10 minutes of dark incubation. If the same treatment is used on thylakoids isolated from non hardened plants in the absence of added cations an identical increase in rate is observed initially, but this increase is followed by a rapid decay in PSI electron transport.

If the incubation temperature is varied from 0° to 60°C, there is no change in the extent of increase in photosystem I activity only in the rate of increase and subsequent decay. An increase in temperature decreases the time required for the maximal stimulation of PSI activity as well as the rate of decay of PSI activity. Likewise, a decrease in temperature

increases the time required. At 5°C, the maximal stimulation of PSI takes 280 minutes and the decrease takes place between 520 and 600 minutes after incubation in the dark. Thus, the extent of photosystem I stimulation occurs independent of the incubation temperature.

Since the extent of the increase in PSI activity takes place at all temperatures, it does not appear to be a response to temperature per se but may reflect in vitro destabilization of thylakoid membranes due to isolation. It is interesting to note that this increase in PSI activity is not observed for thylakoids isolated from cold hardened rye in the presence of cations. Thylakoids from cold grown rye can be stimulated only when isolated in the absence of cations. Furthermore, this increase in photosystem I activity occurs in the presence of saturating levels of SOD which dismutates $O_2^-\cdot$, a response to the isolation procedure the rate of which is temperature dependent.

Furthermore, it has been shown that any divalent cation at 5 mM concentration or any monovalent cation at 100 mM concentration will protect against decay. The decay in PSI activity in the absence of cations is not prevented by the presence of proteolytic inhibitors. (Chapman and Huner, 1993)

Measurement of absorbance changes in P700

As useful as the oxygen electrode is, it cannot examine parts of the electron transport chain smaller than 2 or 3 components without first isolating those components. This

isolation might then change the properties of the components. The oxygen electrode also measures a continuous rate over several minutes. To examine smaller portions of the electron transport chain it is necessary to use other techniques.

Photosystem I has an absorption spectrum which reflects the interaction of various wavelengths of electromagnetic radiation with the outer electrons of the molecules of which the photosystem is composed. When excited, the reaction centre of photosystem I, P700 may lose an electron which will change its absorption spectrum slightly. There is a bleaching around 700 nm and an increase in absrobance at 820 nm. With sensitive detectors the extent of this absorbance change can be measured. Since the extent of this absorbance change represents the number of reaction centres that are oxidized, it is a measure of electron transport. In addition to measuring the extent of electron transport under light saturating conditions, it is possible to measure the extent of P700 oxidation with various intensities of light and obtain the effective size of the photosystem I complex as an optical cross section.

Optical Cross Sections.

Since light is required for electron transport, the effect of increasing light intensity on the number of electrons moved is analogous to the effect of increasing substrate concentration on a typical enzyme. However, the only way to increase an object's ability of absorb light is to

increase the effective surface area of the object. Since photosystem I is very efficient at transferring excitation energy to the reaction centre, its effective size is close to the actual size (Nobel 1983). As with enzyme kinetics the rate of electron transport is plotted against the log of the intensity of the exciting illumination. Exciting illumination is expressed as the number of photons per square meter per second.

It has been shown that the rate of electron transport will vary with light intensity following the Poisson single hit probability distribution (Eqn 1).

(Eqn 1.) $A_i=A(1-e^{(-ic)})$

In equation 1 "A_i" is the amplitude of the signal with a single turnover flash of intensity "i", "A" is the amplitude at saturating single turnover flash intensities, "i" is the flash intensity and "c" is the optical cross section. This models the proportion of a fixed population of photosystems which are hit by at least one of a varying number of photons. Similarly if we know the density of the photons and the effective size of the photosystem it is possible to determine the curve that represents those conditions. Since a known concentration of photons is used and a curve is generated from this, curve fitting can be used to determine the curve of best fit, which will reveal the effective size of the photosystem. This is independent of the concentration of the photosystem because the same density of photons will trigger half the

photosystems regardless of the number in a given area. The affect of a greater concentration of photosystems would be an increased signal under saturating concentrations of photons. Only if the effective size of the photosystems gets larger will the system saturate at a lower concentration of photons (Figure 6).

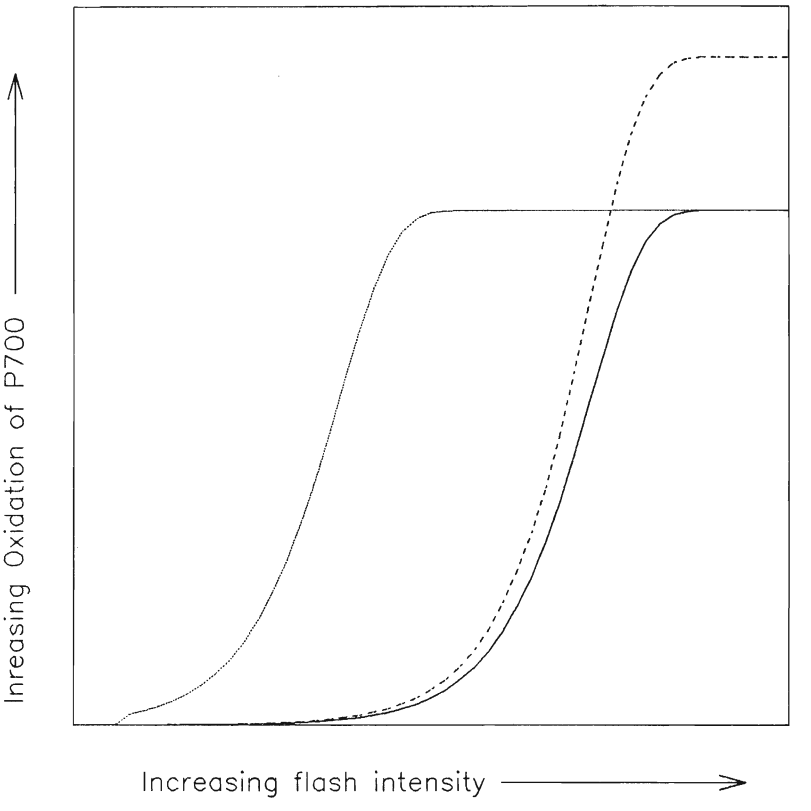
Equation 1 assumes that both the photons and the photosystems are distributed randomly and that the sizes of all the photosystems are uniform.

The experimental curve will follow a single component equation only if there is a single homogeneous population of photosystems. If there are 2 or more populations of photosystems the experimental curve will be best modeled by the sum of 2 or more curves as represented by the sum of two Poisson distributions. If the two component curve fits better than the single component curve, then heterogeneity in photosystem size is indicated.

Objectives

It had been indicated perviously that both the growth of plants at low temperatures and the incubation, in the dark, at elevated temperatures, of isolated thylakoids from plants grown at warm temperatures leads to a 1.5 to 2 fold increase in the rate of light saturated electron transport as measured by a Clark electrode. It may be that these two increases in rate occur by the same mechanism. Since a better understanding of this phenomenon might lead to a better understanding of the

Figure 6 The two possible ways an optical cross section can change. If the solid line represents an optical cross section from a hypothetical sample. The long dashed line represents an optical cross section from a sample with a greater concentration of photosystems of the same size. The short dashed line represents an optical cross section from a sample with the same number of photosystems of an effectively larger size.


Inreasing Oxidation of P700
Increasing flash intensity

effects of climate stress on photosynthesis, further research into this subject could be useful.

For the purposes of study the following hypothesis was formed: the increased electron transport activity in PSI observed in the thylakoids of winter rye as a result of growth of plants at low temperature occurs by the same mechanism as the increased electron transport activity in PSI resulting from heating of isolated thylakoids from warm grown rye, in the dark

This hypothesis was tested by localizing the site of rate increase caused by low temperature growth of winter rye and that caused by heating of isolated thylakoids. Electron transport as the proportion of P700 oxidized by a single turnover flash was measured. P700 oxidation can be detected as an increase in absorption of light at a wavelength of 820 nm. At this wavelength, the change in absorbance can be measured without the interference of fluorescence or of the single turnover flash. It was thus possible to determine if the change in rate of electron transport was due to a change in P700 or a change in electron carriers other than PSI.

In this study I show an increase in the proportion of P700 oxidized in isolated thylakoids from cold hardened plants. The extent of this increase is similar in magnitude to that reported previously (B.P Chapman and N.P.A. Huner 1993). However, no increase in the proportion of P700 oxidized was seen in heated thylakoids from non hardened plants. This indicates that the increase in PSI activity due to low temperature growth and that due to the heating of thylakoids

from warm grown rye, while similar in magnitude, occur by different mechanisms.

METHODS AND MATERIALS

Plant Material

Winter rye was grown in coarse vermiculite in 4 cm plastic pots. Water and Hoagland's nutrient solution were supplied as required. Warm-grown plants were grown for 2-3 weeks at an irradiance of 200 micromoles m^{-2} s^{-1} and a 16 hour photoperiod at 20°C. Cold-hardened plants were grown initially under controlled conditions with a day/night temperature regimen of 20°C/16°C day/night at light intensities of 250-200 micromoles m^{-2} s^{-1} and a 16 hour photoperiod. After 7 days, cold-hardened plants were transferred to a temperature regime of 5°C/5°C day/night with all other conditions held constant. This ensured that fully expanded leaves of warm and cold crown rye were of comparable physiological age (Krol et al 1984).

Isolation of thylakoid membranes.

Two or three leaves were harvested and cut into approximately 2 cm square sections. They were then ground for 30 seconds with a cold mortar and pestle in a solution of 50 mM Tricine, 5 mM MgCl2 and 10 mM NaCl at pH 6.8. All subsequent steps in the isolation were carried out at 4°C. The extract was centrifuged at 12000xg for 1 second. The thylakoid-containing supernatant was centrifuged again at the same speed for 10 minutes. The resulting thylakoid pellet was resuspended in a solution of 0.1 M sorbitol, 40 mM Tricine 5, mM MgCl2 and 10 mM NaCl pH 6.8.

77K Fluorescence Emission

Isolated thylakoids were removed from the suspention before and immediately after the determination of the proportion of P700 oxidized and immediately frozen in liquid nitrogen. 77K fluorescence emission spectra were collected with a laboratory built spectrofluorimeter based on a Jarrel Ash 1/4m spectrograph and EG&G diode array detector (1420) controlled by an EG&G detector interface (1461) accessed by an Intel 80386 compatible computer. Excitation light was supplied by a 100 W tungsten halogen lamp dispersed by a Jobin Yvon H 20 spectrometer. The fluorimeter was calibrated using a standard Neon lamp (Instrumentation Laboratory Inc.). (Salehian 1991)

Light saturated PSI electron transport

PSI electron transport was measured using a Clark-type electrode and calculated as μmol O_2 consumed (mg Chl)$^{-1}$ h^{-1}. The reaction mixture consisted of 50 mM sodium phosphate buffer (pH 6.8) containing, 200 μM methyl viologen, 1 mM sodium azide, 20 μM DCMU 30, μM DCPIP, 5 mM ammonium chloride. (Huner 1985). All reaction mixtures were prepared within an hour of the experiment. All activity measures were made at 25 C under saturating light conditions.

Determination of P700 flash saturation curves

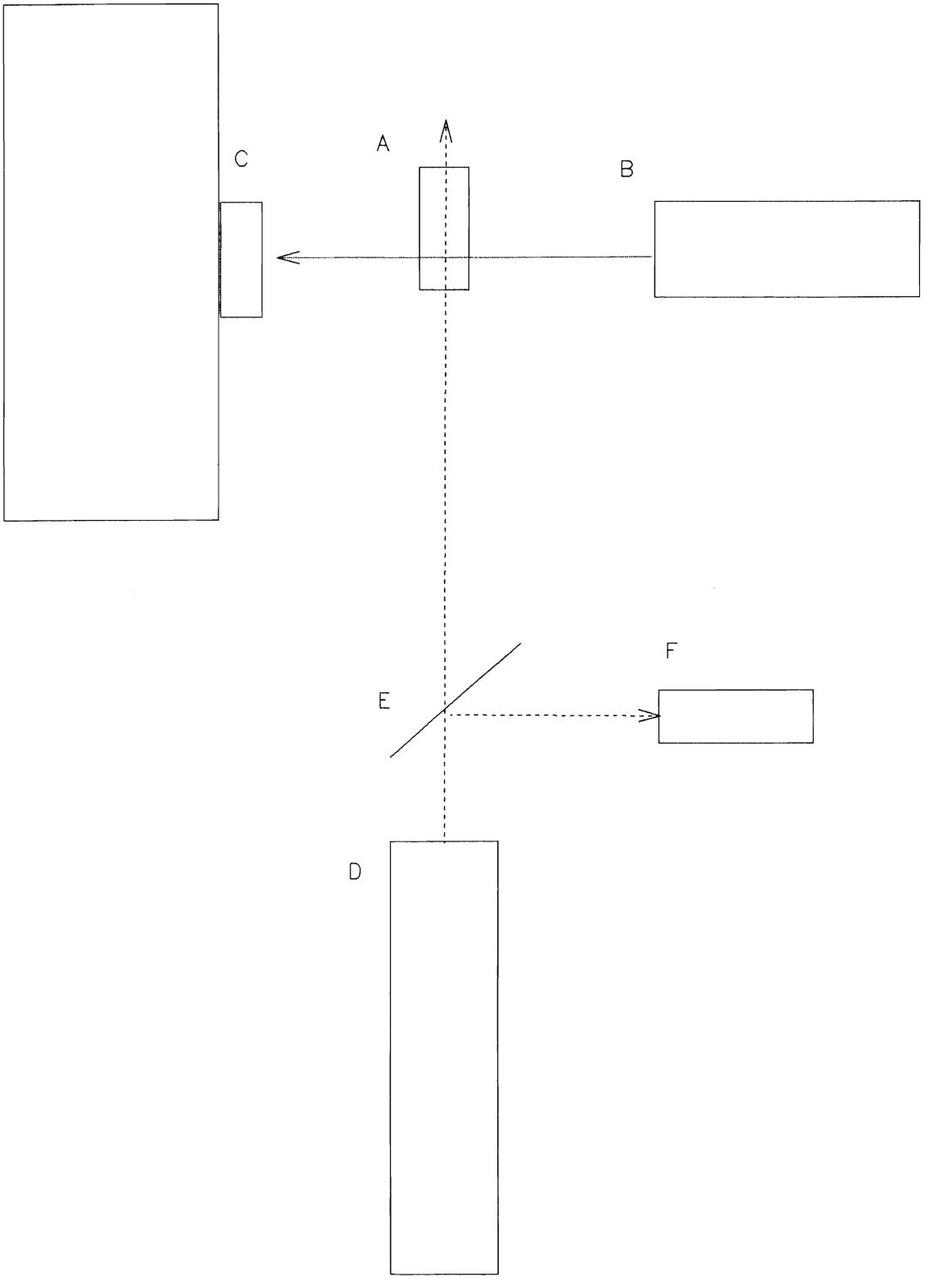
A small amount of thylakoid suspension was diluted in 50 mM sodium phosphate buffer (pH 6.8) containing, 200 μM methyl viologen, 1 mM sodium azide, 20 μM DCMU 30 μM DCPIP, 5 mM

ammonium chloride. (Huner 1985)

Photo-oxidation of PSI was assayed by the flash-induced increase in absorbance at 820 nm (ΔA820) using a laboratory-built single beam spectrometer. An 820 nm laser diode (Spindler and Hoyer, Milford) supplied the measuring beam which was detected by a pin 10D photodiode (Eg&G, Princeton) protected with an IR transmitting glass colour filter (Corion). A variable beam-splitter directed a fraction of the measuring beam towards a matched reference photodiode whose output was subtracted from the measuring photodiode to compensate for laser intensity fluctuations. The reference photodiode was also used to subtract any stray fluorescence induced by the actinic laser pulse. A multistage low noise amplifier (Brock Electronic shop) transferred the A820 signal to a Tektronics TD 540 digital storage oscilloscope. The oscilloscope was under the control of an 80286 personal computer (Computan, St. Catharines) and software developed by the Brock Electronics Shop. The actinic laser pulse (250 ns duration) was generated by a tunable Phase-R DL-32 flash-lamp pumped dye laser. DCM (Exciton) was used as the laser dye, the excitation wavelength was 661 nm. The way these components were set up is shown in figure 7.

Implicit in a technique used to measure absolute absorption cross section is the critical requirement for homogenous illumination over the measurement area (Doug Bruce, personal communication). To ensure homogeneity, the excitation flash was supplied to the bottom of a standard 1 cm by 1 cm sample cuvette via a randomized fibre optic bundle (Oriel)

Figure 7 A simplified diagram of the equipment used to measure the extent of oxidation of $p700^{+}$. The two lines represent the 820 nm measuring beam (dotted line) and the single turnover excitation pulse (dashed line). The other components of the system are a cuvette (A); an 820 nm diode laser (B); an 820 nm detector (C) linked to an oscilloscope (not shown); a dye laser (D); a beam splitter (E); and a pyrometer (F).


A
B
C
D
E
F

held beneath the cuvette. The 820 nm measuring beam passed through the cuvette 1 cm above the bottom perpendicular to the excitation flash. The 1 mm by 4 mm measuring beam profile was oriented horizontally with the long axis parallel to the bottom of the cuvette. In this way the pathlength of the actinic flash through the measuring region was minimized to 1 mm. The combination of a randomized fibre optic and an optically thin measurement region ensured homogenous illumination of all cells contributing to the A820 signal.

A small fraction of the actinic pulse was directed toward a light pulse energy meter (Molectron, J3-05) so that the energy of each laser pulse could be averaged simultaneously at a flash repetition rate of 0.25 Hz.

Slab gel electrophoresis of thylakoid polypeptides

Thylakoids from both warm and cold grown plants were isolated at 4 C in 50 mM Tricine (pH 7.8) containing 0.4 M sorbitol and 10 mM NaCl with two 5 s bursts of a Waring Blender. Membranes were washed once in double distilled water, once in 1 mM EDTA (pH 8.0) and twice in 50 mM Tricine (pH 8.0). The membrane pellets were resuspended in 0.3 M Tris (ph 8.8) containing 13% (v/v) glycerol and 1% (w/v) SDS polyacrylamide slab gels according to Waldron and Anderson (1979). Room temperature scans of pigmented complexes and their characteristic absorption spectra were obtained using Shimadzu UV-250 spectrophotometer.

Membrane polypeptide analysis.

Thylakoid membranes were isolated in 50 mM Tricine pH(7.6) containing 17% (w/v) sucrose, 2 mM $MgCl_2$ and 1 mM EDTA and then centrifuged at 1000xg for 15 min. The pellet was solubilized with SDS (SDS:chlorophyll = 20:1) in the dark at room temperature according to the method of Chua (1980). SDS polyacrylamide slab gel electrophoresis (SDS-PAGE) was performed in a 12% (w/v) polyacrylamide gel according to Chua (1980). the gel were stained with 0.1% (w/v) Coomassie brilliant blue R-250 and destained in methanol: acetic acid: water (3:1:10).

Curve Fitting

Sigma Plot's curve fitting routine was used to fit the 'cumulative one-hit' Poisson distribution (equation 1) to the data. The cumulative one-hit Poisson distribution has been shown to represent the change in proportion of reaction canters oxidized in response to the change in the number of photons in a single turn-over flash (Mauzerall and Greenbaum 1989). In the equation A_i represents the proportion of P700 oxidized, i the light intensity and A and c were variables. A represents the amplitude of the oxidation at saturation and c the cross sectional area. Both a one and two component curve was fit to the data. Sigma Plot uses the Leverberg-Marquardt method of nonlinear least squares to determind goodness of fit.

As a test of the robustness of the curve fit, curves were created in which the amplitude of the components were

progressively further from the best fit and the sum of square residuals for each of these curves was calculated. The curve fit was judged to be very precise if the least squares increased rapidly as the theoretical curve was moved away from its best fit.

RESULTS

Effects of Growth Temperature on 77K Fluorecence Emission

The results shown in Figure 8 confirm that the isolated thylakoid samples from winter rye grown at 5 C were cold-hardened and those grown at 20 C were non-hardened rye. Figure 8 illustrates the fluorescence emission spectra for isolated thylakoids from cold and warm grown rye. Thylakoids from warm-grown rye showed a greater proportion of 680 nm fluorescence at 77 K than thylakoids from cold-grown rye. These results are consistent with published results for thylakoids from warm and cold grown rye (Huner et al 1989) This indicates that thylakoid samples employed in this study were comparable to those used in previous studies.

Electrophoretic Analysis of Polypeptide and Chlorophyll-protein complexes

Figure 9 shows electrophoretic separation of the polypeptides from isolated thylakoids of cold and warm grown winter rye. No major differences were observed in the polypeptide complements of thylakoids from the two treatments.

When the chlorophyll-protein complexes of the samples were separated electrophoretically the results indicated no change in proportion of PSI to PSII. Densimetric scans of the gels were taken (Figure 10) of the various protein-chlorophyll complexes. The identity of these bands was confirmed by a spectra of each band (Figure 11).

The densimetric scans show the change in proportion of

Figure 8 77K fluorescence emission spectra. The 77K fluorescence emission spectra of thylakoids from warm (dotted line) and cold (solid line) grown rye.

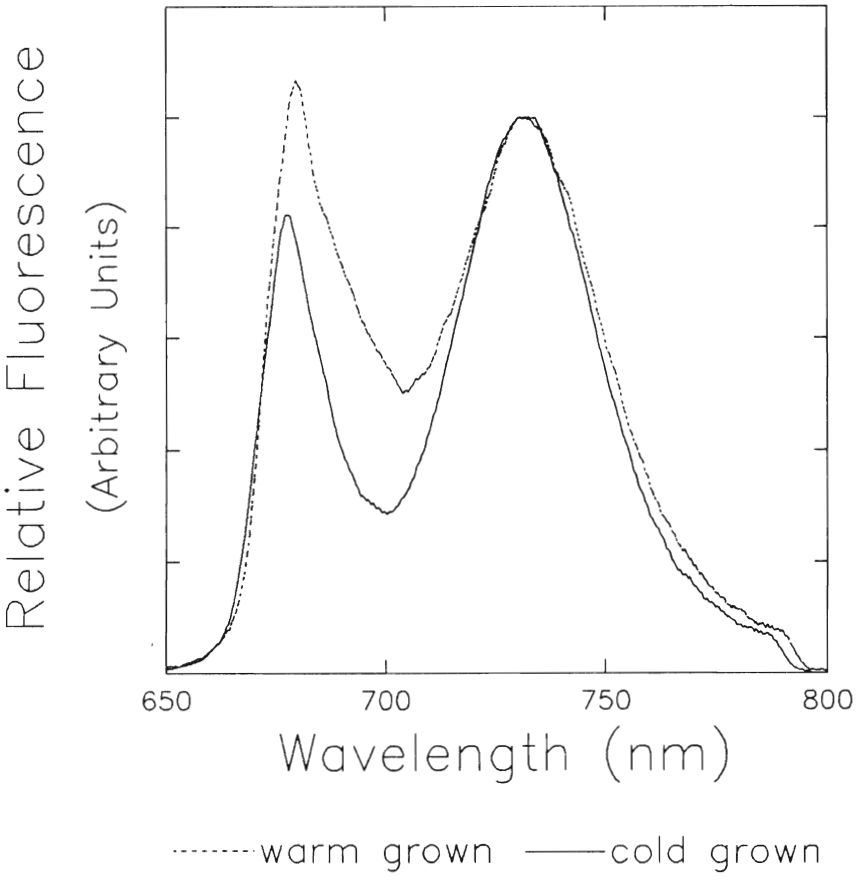

Relative Fluorescence
(Arbitrary Units)
650
700
750
800
Wavelength (nm)
warm grown
cold grown

Figure 9 Polyacrylamide discontinuous gel (12%) of isolated thylakoid membranes solublized in SDS. Lanes from left to right contain: Molecular weight standards; Thylakoids from winter rye grown at 20 C; Thylakoids from winter rye grown at 5 C. Thylakoid samples were normalized to 2 mg Chl per mL and 20 ul were put in each lane.

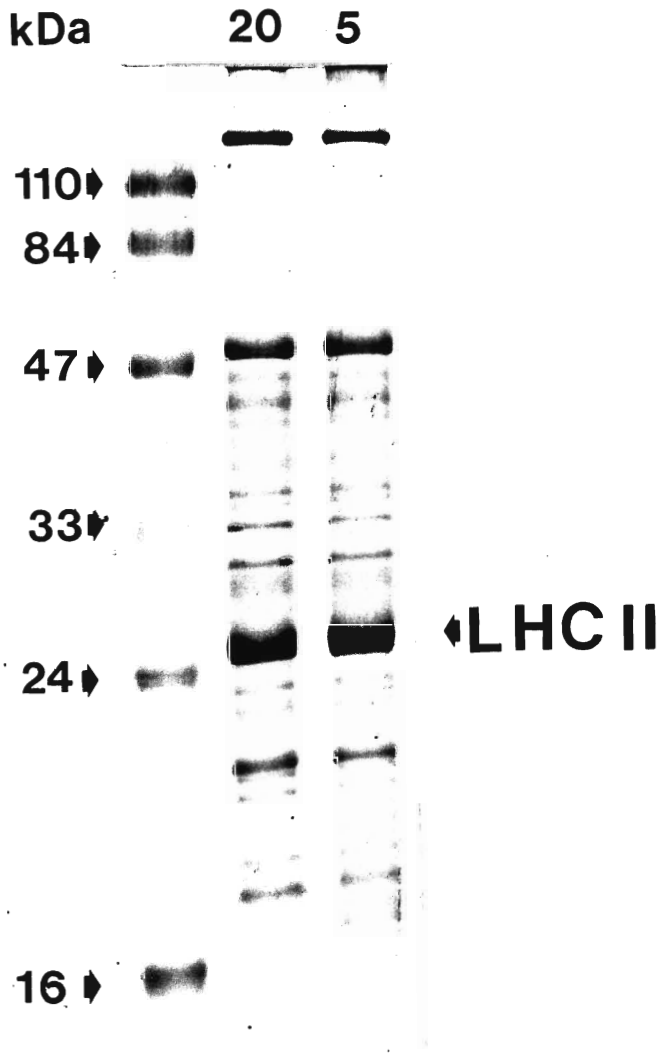

kDa
20
5
110
84
47
33
24
16
LHC II

Figure 10 Spectrophotometric scans of Chl-protein complexes in a nondenaturing green gel. Thylakoid membranes isolated from rye leaves of cold grown (A) and warm grown (B) plants and separated by electrophoresis are shown. Peak 1, PSI; Peak 2 oligomeric LHCII; Peak 3, PSII; Peak 4, monomeric LHCII. The scanning wavelenght was 671 nm and the scale bar represents .5 AU.

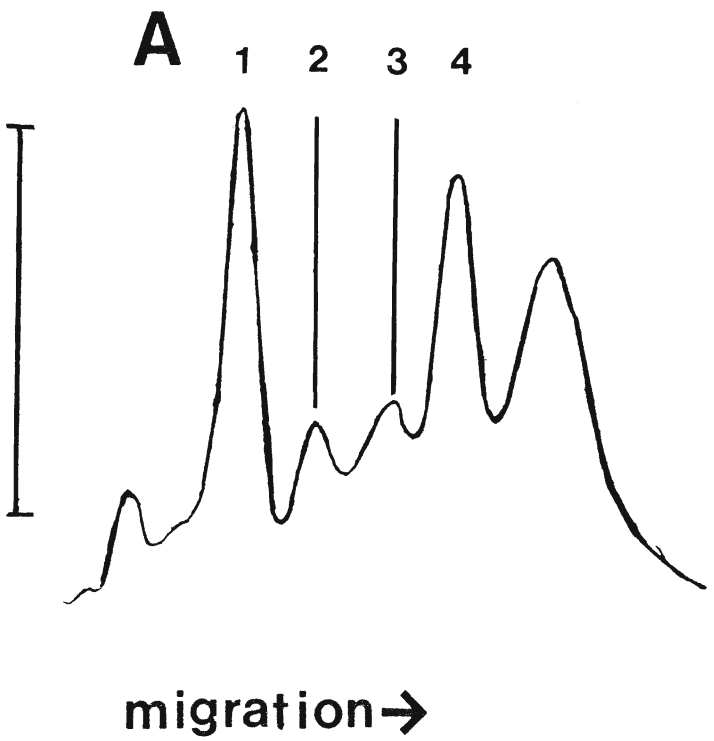
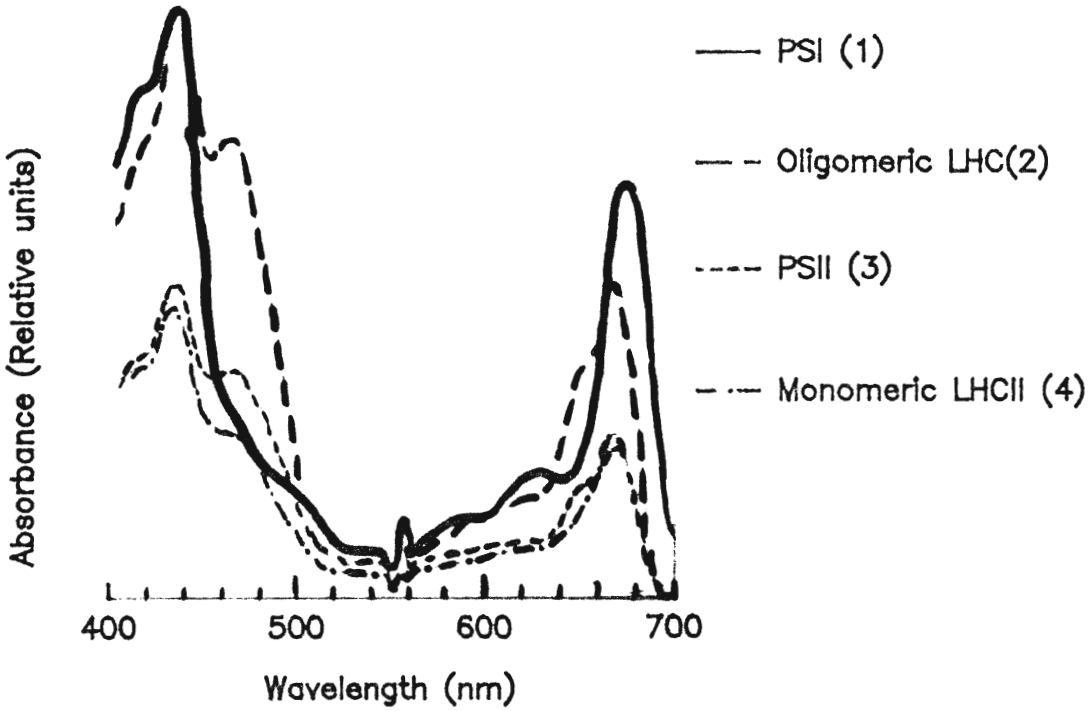

A
1
2
3
4
migration→
B
1
2
3
4
migration→

Figure 11 Absorbance spectra of peaks 1-4 in the warm grown plants in Figure 10 A.


PSI (1)
Oligomeric LHC(2)
PSII (3)
Monomeric LHCII (4)
Absorbance (Relative units)
400
500
600
700
Wavelength (nm)

oligmeric and monomeric LHCII which have been previously reported (Huner et al 1987, Huner et al 1989). It has also been shown that there is no difference between cold grown rye and warm grown rye in the proportion of PSI to PSII normalized to the amount of chlorophyll (Huner et al 1987, Huner et al 1989).

P700 Oxidation

Previous work with the Clark electrode examined the continuous rate of electron transport through the cytochrome b6/f complex, plastocyanin, and PSI. In order to measure electron transport instantaneously and narrow the scope to PSI electron transport was measured as the oxidation of P700. The ratio of the absolute absorbance at 820nm with the concentration of chlorophyll was determined for thylakoid suspensions of both cold-grown and warm-grown rye. This ratio did not vary significantly between cold-grown and warm-grown rye.

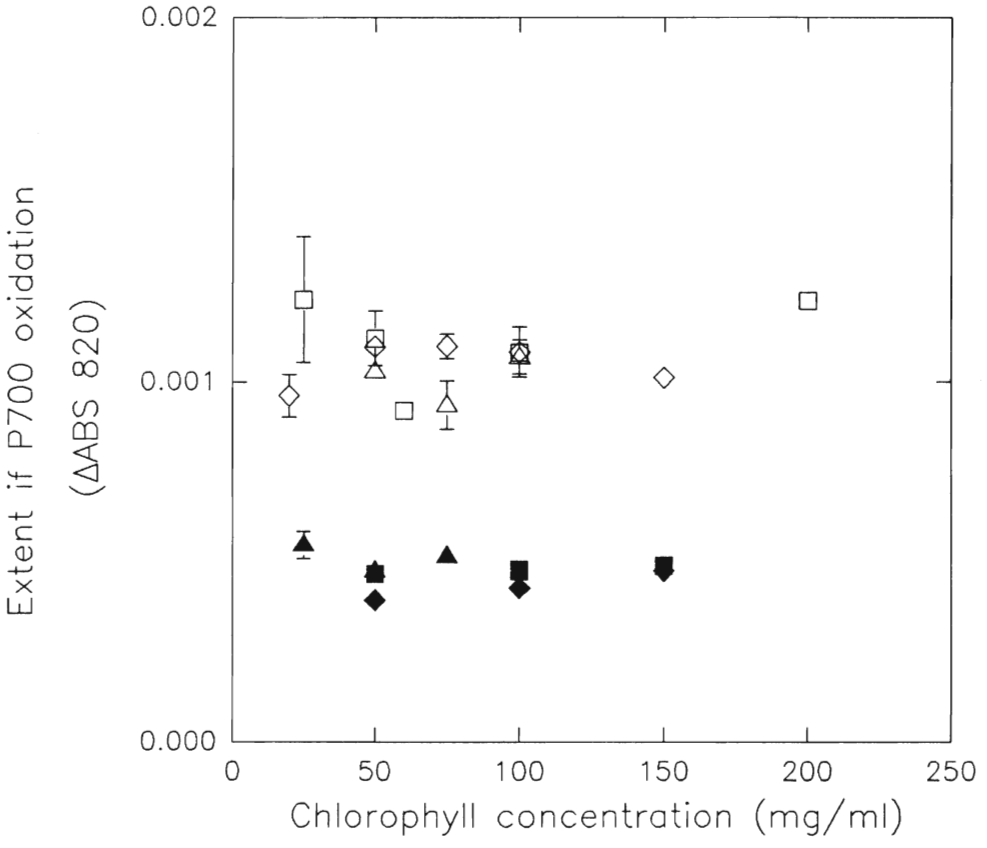
The proportion of P700 converted to P700+ in isolated rye thylakoid membranes was measured directly while the sample was subjected to saturating monochromatic light pulses at 661 nm. Thylakoids were isolated from both cold grown and warm grown plants and P700 oxidation was measured at a range of Chl concentrations. Regardless of chl concentration, the proportion of P700 converted to P700+ on a per chlorophyll basis under saturating light pulses in thylakoids isolated from cold-grown plants was 70% greater than thylakoids isolated from the warm-grown rye (Figure 12 and Table I)

The proportion of P700 converted to P700+ in thylakoids

Table I. The extent of photooxidation of P700 measured as a proportional increase in absorbance at 820nm.
Warm grown, cold grown and warm grown plants that were incubated in the dark at 37°C for 20 minutes (heated) were measured to determine the proportional increase in absorbance at 820nm. This table shows the mean and standard deviation for the increase in all three treatments. At least 5 repeats of each treatment were taken.

Sample	ΔABS 820nm
Warm grown	$5.62 \times 10^{-4} \pm 0.66 \times 10^{-4}$
Heated	$5.83 \times 10^{-4} \pm 0.62 \times 10^{-4}$
Cold grown	$9.74 \times 10^{-4} \pm 0.79 \times 10^{-4}$

Figure 12 The extent of photo oxidation of P700 by a saturating excitation pulse in cold grown and warm grown rye. The extent of photo oxidation of multiple samples (represented by the different symbols) of cold grown (open symbols) and warm grown (closed symbols) was determined under saturating single turnover flashes for a variety of chlorophyll concentrations.


0.002
0.001
0.000
0
50
100
150
200
250
Extent if P700 oxidation
(ΔABS 820)
Chlorophyll concentration (mg/ml)

from cold and warm grown rye was measured as a function of exciting flash intensity (Figure 13). Increasing flash intensity converted a greater proportion of P700 to P700+ (Δ ABS 820 nm) in thylakoids isolated from both cold and warm grown rye. There was no apparent large scale change in optical cross section which be seen as a change in the light intensity needed to oxidize all the P700 (Figure 14 and 15). This experimental data from warm (Figure 14) and cold grown (Figure 15) were fit to a one component single hit Poisson distribution and a two component distribution. The sum of square residuals was lower for the two component model than for the one component model. This gives the suggestion of two components in the fit, however, the noise precludes any firm conclusions.

For the two component fits the ratio of the smaller amplitude to the larger amplitude and the ratio of the smaller cross section to the larger cross section were determined to see if growth at low temperature changed the distribution of energy between these two populations or caused any changes in the optical cross sections of these two populations. These results are summarized in Table II. While there is a great deal of variation within each group, there is not a significant difference between thylakoids from cold and warm grown rye. It appears that ΔP700 in thylakoids of cold and warm grown rye PSI have similar optical cross-sections, but different concentrations of P700+ under saturating conditions.

Figure 13 The extent of P700 photooxidation as a function of the intensity of a single turnover flash (671 nm). Measurements from cold grown (hollow circles) and warm grown (closed circles) have bee plotted to show (A) the absolute increase and (B) the increase as a percent of the increase at saturation. Each point represents an average of 16 measurements

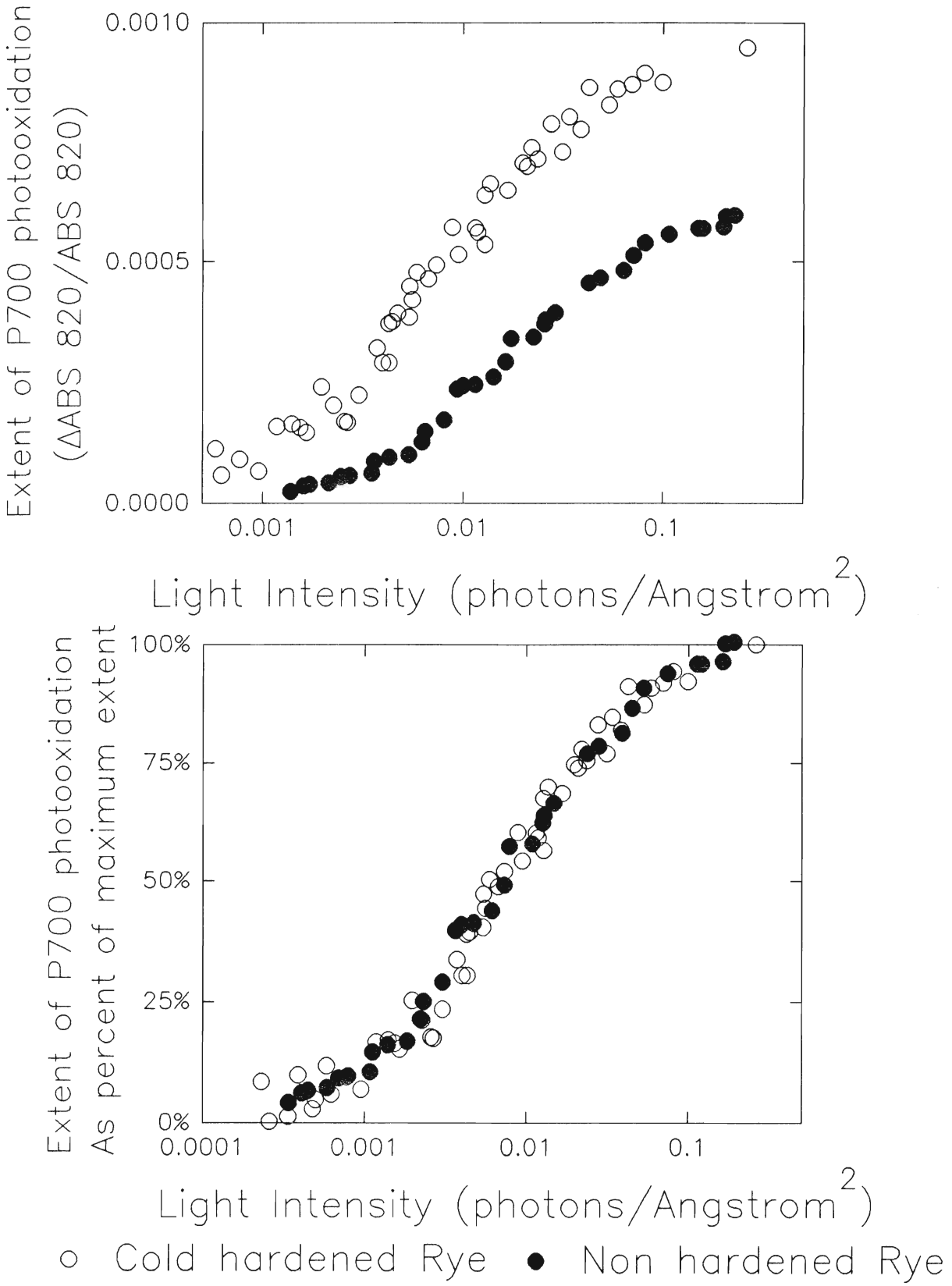

Extent of P700 photooxidation
(ΔABS 820/ABS 820)
0.0010
0.0005
0.0000
0.001
0.01
0.1
Light Intensity (photons/Angstrom^2)
Extent of P700 photooxidation
As percent of maximum extent
100%
75%
50%
25%
0%
0.0001
0.001
0.01
0.1
Light Intensity (photons/Angstrom^2)
○ Cold hardened Rye
● Non hardened Rye

Figure 14 The extent of warm grown P700 photoxidation as a function of the intensity of a single turnover flash (671 nm) and curve fits. Each point represents an average of 16 measurements. A single (solid line) and double (dotted line) component single hit poisson distribution were fit to the experimental results (A). For the single component fit the optical cross section is 107 photons per angstrom^2 and the amplitude at saturation is 5.45 x 10^{-4}. For the two component fit the optical cross sections and amplitudes at saturation are 250 photons per angstrom^2 and 2.88 x 10^{-4} and 31.8 photons per angstrom^2 and 2.97 x 10^{-4}. The residuals from the curve fit have also been plotted (B).

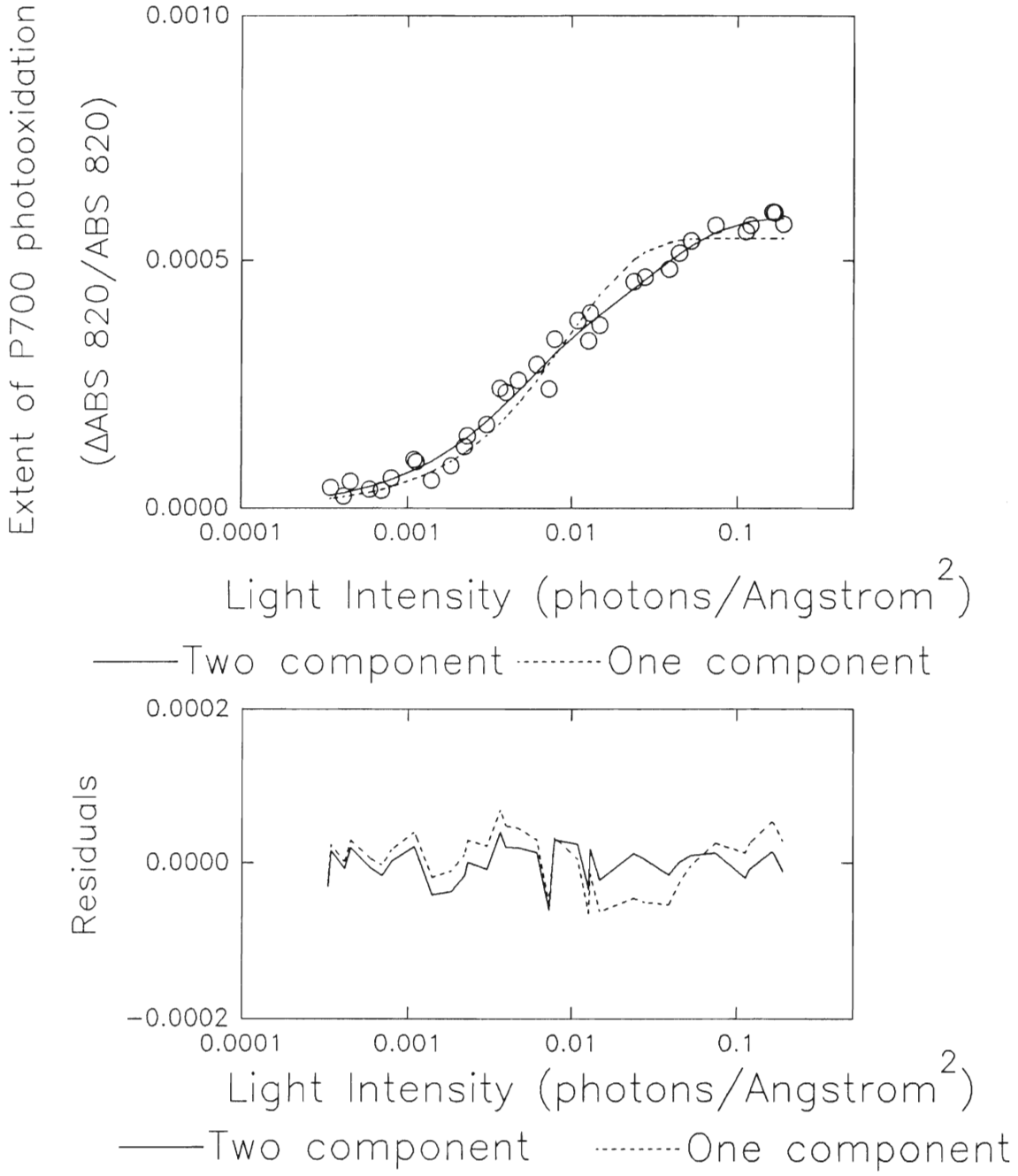

Extent of P700 photooxidation
(ΔABS 820/ABS 820)
0.0010
0.0005
0.0000
0.0001
0.001
0.01
0.1
Light Intensity (photons/Angstrom^2)
Two component
One component
Residuals
0.0002
0.0000
-0.0002
0.0001
0.001
0.01
0.1
Light Intensity (photons/Angstrom^2)
Two component
One component

Figure 15 The extent of cold grown P700 photoxidation as a function of the intensity of a single turnover flash (671 nm) and curve fits. Each point represents an average of 16 measurements. A single (solid line) and double (dotted line) component single hit poisson distribution were fit to the experimental results (A). For the single componet fit the optical cross section is 110 photons per angstrom^2 and the amplitude at saturation is 8.38 x 10^{-4}. For the two componet fit the optical cross sections and amplitudes are 163 photons per angstrom^2 and 6.27 x 10^{-4} and 14.43 photons per angstrom^2 and 3.88 x 10^{-4}. The residuals from the curve fit have also been plotted (B).

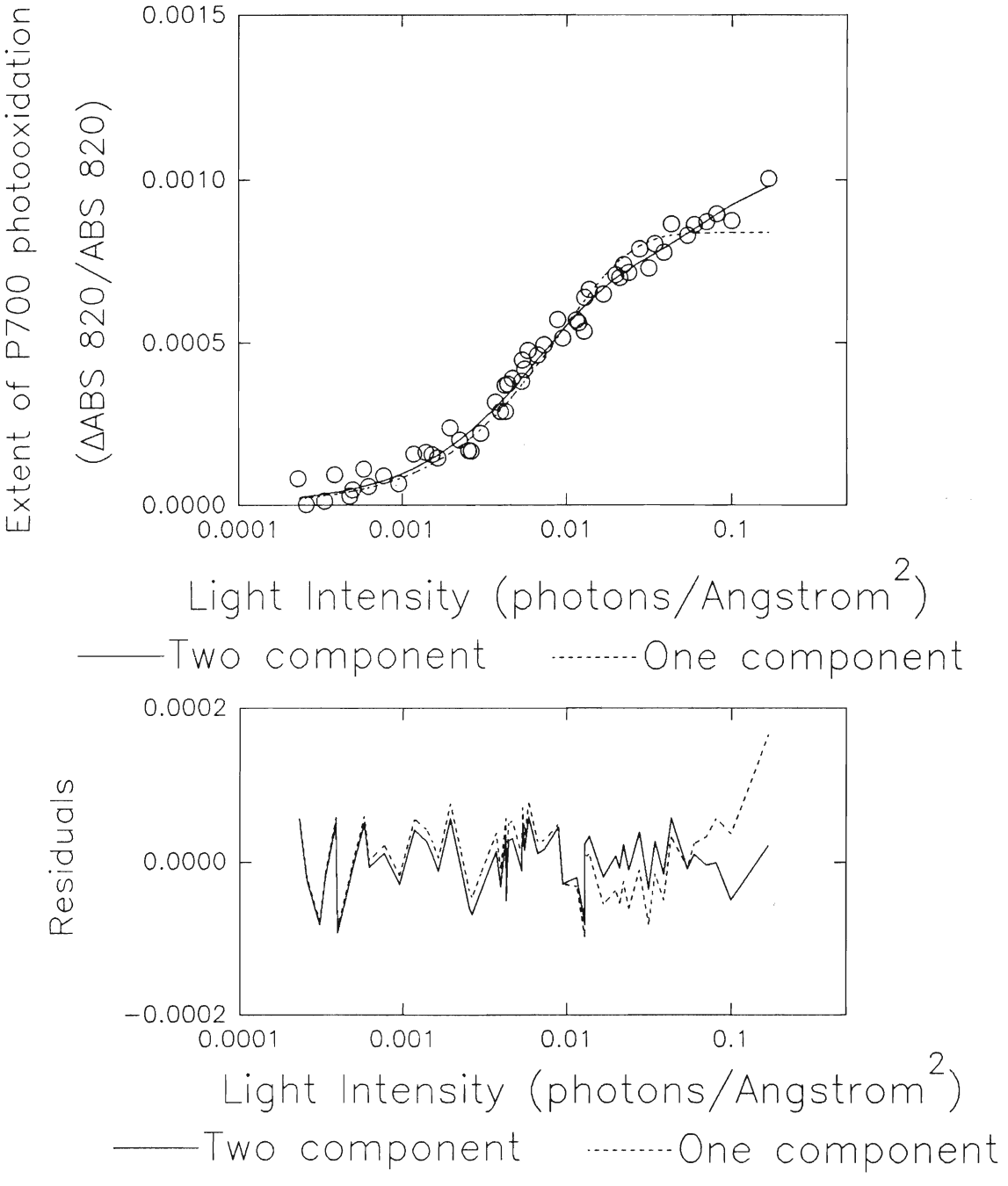

Extent of P700 photooxidation
(ΔABS 820/ABS 820)
0.0015
0.0010
0.0005
0.0000
0.0001
0.001
0.01
0.1
Light Intensity (photons/Angstrom^2)
Two component
One component
Residuals
0.0002
0.0000
−0.0002
0.0001
0.001
0.01
0.1
Light Intensity (photons/Angstrom^2)
Two component
One component

Table II. Optical cross sections of warm and cold grown winter rye.
Mean cross section and standard deviation of at least 5 experiments

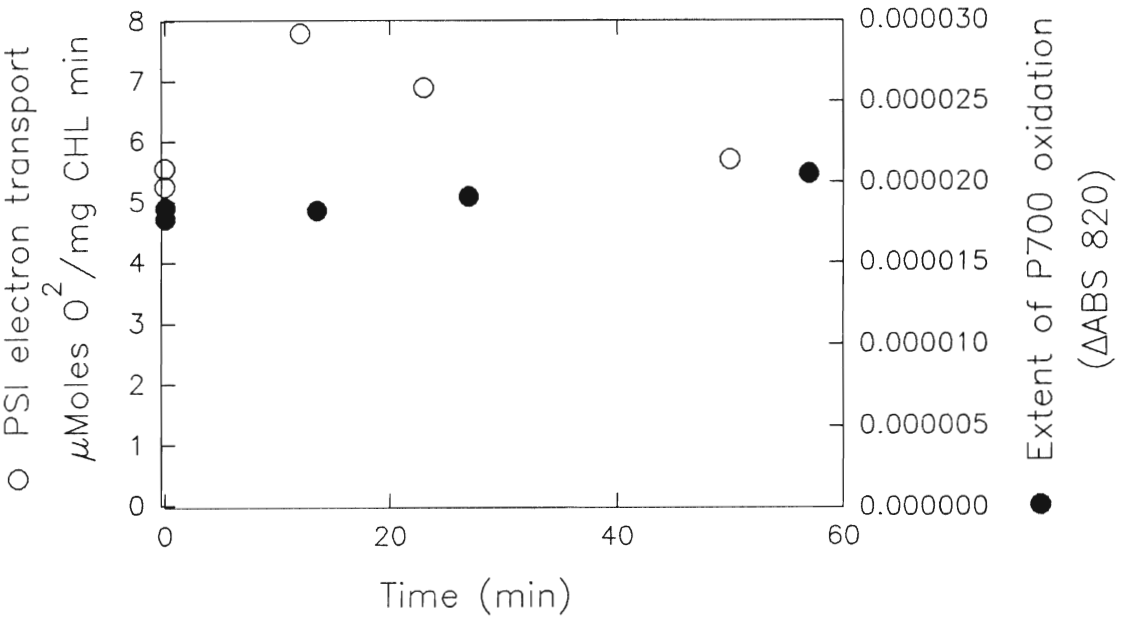
Sample	Cross section (angstroms^2)
Warm grown	136 ± 47
Cold grown	99 ± 41

Effect of temperature on PSI electron transport

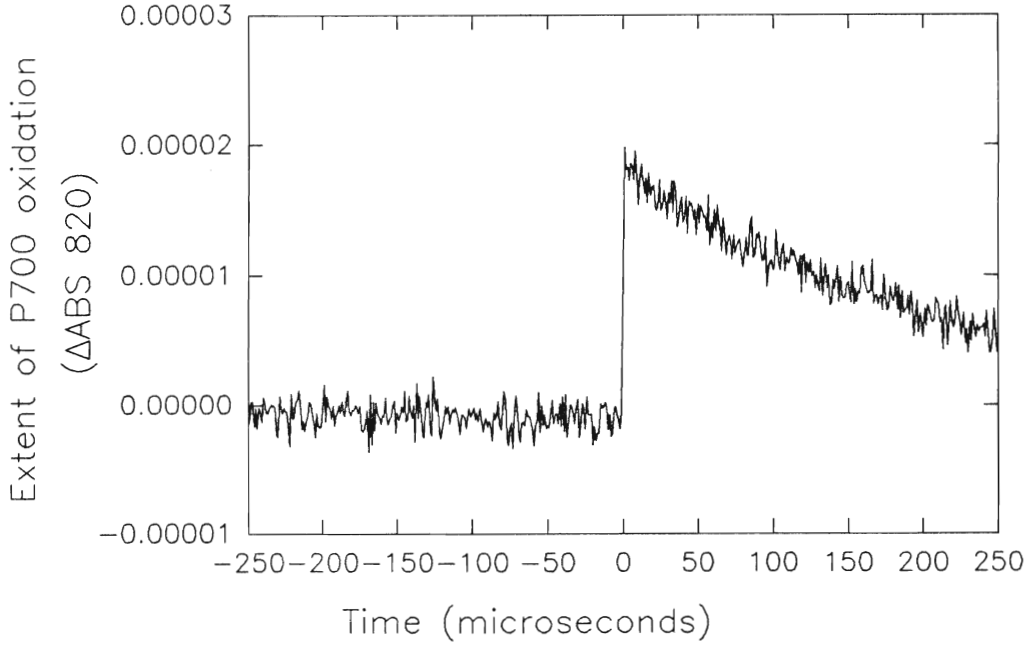
It has previously been shown (Chapman and Huner 1993) that thylakoids isolated from warm grown plants incubated at 35 C in the dark exhibited an increase in the rate of light saturated PSI electron transport of 50-100% within the first 15 minutes. If the thylakoids were isolated in the absence of added cations this increase was followed by a decay in rate to 75-125% of the original rate (Figure 5). In order to determine if this increase and decay were due to changes in the PSI complex or elsewhere in the chain, we examined thylakoids isolated from warm grown plants in the absence of added cations. As seen in Figure 16-A, the rate of light saturated PSI electron transport as measured from DCPIP/ascorbate to methyl viologen had the characteristic kinetics. A 45% increase in rate was seen in the first twelve minutes of dark incubation at 35 C. This was followed by a decrease in rate between 12 and 50 minutes to 105 % of the original rate. Measurements of the photoxidation of P700 taken from the same sample at the same time (Figure 16-A) showed no significant change in the extent of P700 photoxidation. A slow increase in the change in absorbance was seen but this may be due to changes in the organization of the membrane leading to changes in the light scattering properties of the thylakoid membranes during heating. Alternatively, the slow loss of water splitting on the donor side of PSII might lead to a component of the absorbance at 820 being due to the production of P680+. In either case this increase in not believed to be a result of a change in PSI.

Figure 16 The rate of electron transport and the extent of photoxidation of P700 of thylakoids from warm grown rye. Thylakoids from warm grown rye were isolated and then incubated in the dark at 35 C. The start of this incubation was assigned time 0 and measurements of the rate of light saturated electron transport (open symbols) and the extent of photoxidation (closed symbols) were made from one sample at various times during this incubation (A). A single trace showing the change in absorbance after the single turnover flash (time 0) indicating oxidation of P700 and its subsequent rereduction (B).

A


O PSI electron transport
μMoles O2/mg CHL min
0
1
2
3
4
5
6
7
8
0
20
40
60
Time (min)
0.000000
0.000005
0.000010
0.000015
0.000020
0.000025
0.000030
● Extent of P700 oxidation
(ΔABS 820)

B


Extent of P700 oxidation
(ΔABS 820)
0.00003
0.00002
0.00001
0.00000
−0.00001
−250 −200 −150 −100 −50 0 50 100 150 200 250
Time (microseconds)

Rereduction of $P700^{+}$

During the previous experiment, measurements of the rate of re-reduction of the P700+ were also taken from the same sample, at the same time (Figure 16-B). The rate at which absorption decreased after the initial increase was the same for samples taken at 0, 14, 27, 57 and 130 minutes. There is no change in rate of re-reduction during heating that could explain a fluctuation in PSI electron transport by 45% (Table III). This indicates that the change in rate of light saturated PSI electron transport is due to a change in the electron transport chain between PSI and methyl viologen.

Table IIII The times for rereduction of thylakoid membranes incubated at 35°C in the dark. Data from one experiment.

Time of Incubation	Time for 1/2 rereduction
0 min	166^{+}_{-} 22 ms
14 min	163^{+}_{-} 10 ms
27 min	173^{+}_{-} 8 ms
57 min	186^{+}_{-} 14 ms

DISCUSSION

This work demonstrates that the extent of photooxidation of PSI (Δ ABS 820 nm) in thylakoid membranes from cold hardened winter rye plants subjected to single turnover flashes, is 50% to 100% higher than that seen in thylakoid membranes from warm grown rye. An increase of 50% to 100% is also seen in the PSI electron transport rate (DCPIP/Asc -> MV) measured in thylakoids isolated from cold hardened winter rye over that measured in thylakoids from warm grown rye.

No increase was seen in the extent of photooxidation of PSI (Δ ABS 820 nm) from warm grown ryes subjected to single turnover flashes and incubated at elevated temperatures in the dark with or without the presence of added cations. However, an increase of 50% to 100% in the PSI electron transport rate (DCPIP/Asc -> MV) was seen in these thylakoids. Electron transport measured with DCPIP as a donor and methyl viologen as an acceptor determines the overall rate from DCPIP -> the cytochrome b6/f complex -> plastocyanin -> the PSI complex -> methyl viologen. A change in the rate of transport in any one of these steps might lead to a change in the overall rate. Photo-oxidation relies on the conversion of P700 -> P700+ and is only affected by changes within the PSI complex.

This demonstrates that the increase in rate of PSI electron transport detected by the Clark electrode in isolated thylakoids of cold grown winter rye and the increase in rate of PSI electron transport detected in thylakoids isolated from winter rye and incubated at elevated temperature occur by different mechanisms.

The only caviat with this conclusion is the fact that we have not proven the increase detected in cold grown rye, by the two methods are the result of the same change. It is possible, for example, that the growth of rye plants at low temperatures could lead to a change in the spectrum of P700+, in which case the absorbance at 820 nm of cold grown rye could differ from that of warm grown rye such that each P700+ in cold grown rye would absorb 1.5 times the light in warm grown rye at that wavelength. Thus, the increase in photooxidation could be unrelated to the increase of electron transport in cold grown rye. If this were the case, the increase in the rate of electron transport seen in thylakoids of cold grown rye and that seen in thylakoids from warm grown rye incubated at elevated temperatures could occur by the same mechanism. Hence, the hypothesis postulated in the objective would be true.

However; since it would require an alteration in direction and degree of absorbance in cold grown rye that just happens to precisely mimic the change in electron transport the chances of these effects being different are limited.

A subtraction spectrum of oxidized minus reduced P700 at 820nm would be required from both warm and cold grown rye to disprove a suggestion that cold hardening alters the spectrum of P700+. However, we believe such a coincidence is unlikely.

It appears that the increase in rate of PSI electron transport due to low temperature growth occurs by a change in the PSI particle itself. This is demonstrated by the increased concentration of P700+. Likewise, a lack of elevated

concentrations of P700+ seen in the heating of isolated thylakoids indicates that the increase in PSI electron transport rate occurs at some location other than PSI.

We will now examine our experimental results more closely to provide possible explanations for them.

The effects of heating isolated thylakoids.

It has already been shown that the increase in PSI electron transport observed when isolated thylakoids are incubated at elevated temperatures does not result from a change in the PSI complex. We will now look at other possible mechanisms. One possibility is the inactivation of superoxide dismutase. It has been shown (Boucher and Carpentier 1993) that heat will disrupt membrane bound superoxide dismutase, an enzyme which catalyses the conversion of superoxide anions back to oxygen. Methyl viologen accepts electrons and then converts oxygen to superoxide anion which is converted to hydrogen peroxide which, in turn, is eventually converted to water and oxygen. Superoxide anion is converted by superoxide dismutase. If superoxide dismutase is inactivated the superoxide anion is not converted back to oxygen. The oxygen concentration falls at an increased rate. As it is the rate at which the oxygen concentration falls that is being used as an indicator of the rate of electron transport, the rate of electron transport inferred is higher than the actual rate of electron transport.

Two facts refute the idea that the increase in rate of light saturated PSI electron transport of isolated thylakoids incubated at elevated temperatures in the dark is caused by

the inactivation of superoxide dismutase. First, the addition of saturating concentrations of soluble superoxide dismutase to the reaction medium did not affect the rate of PSI electron transport in thylakoids from cold grown rye relative to thylakoids from warm grown rye (Huner 1985). Second, more superoxide dismutase is inactivated with increasing incubation temperature (Boucher and Carpentier 1993). If the increase in rate were dependent on inactivation of superoxide dismutase it would be expected that higher incubation temperatures would cause greater increases in the rate of light saturated PSI electron transport. Since PSI activity increases to the same extent regardless of the temperature of incubation (20-60 C) (Reynolds and Huner, 1990) it is unlikely that this increase is caused by the inactivation of superoxide dismutase.

Alternatively, heating may affect the interaction of plastocyanin with PSI to increase the rate of PSI electron transport. There is indirect evidence of a change in the interaction of plastocyanin and PSI. The stability of the increase in rate of PSI electron transport is dependent on the presence of divalent cations in the isolation medium (Chapman and Huner 1993). Divalent cations have been shown (Olsem and Cox 1982) to effect the binding of plastocyanin to PSI. The levels of divalent cations which induce maximum binding of plastocyanin to PSI are also the levels of cations needed to stabilize the increase. This explanation would predict a change in the rate of rereduction of PSI which was not observed.

If this change is due neither to a change to PSI nor modification of accessibility to the electron donor site of we are left with the electron acceptor site of PSI as a possible site of modification. This would mean that heating is affecting the ability of methyl viologen to accept electrons from PSI. Whatever the explanation, it is clear that the increase in rate is not due to a change in PSI per se.

To clear up this question, experiments could be done using alternate electron acceptors, such as ferredoxin, to see if the increase in electron transport is dependent on the electron acceptor. In addition more experiments involving the rereduction of P700 would be useful in understanding these effects. One experiment indicated that there is no change in the rate of rereduction of P700 as the rate of electron transport changes. Further study of the rereduction of P700 I would isolate further the site of the change in rate with heating.

Recent experiments (Huner Unpublished) have show that thylakoids isolated from warm grown plants exposed to either high temperatures in the dark or to low temperatures in the light exhibit a stimulation of light saturated PSI activity (DCPIP -> methyl viologen) to a similar extent. However, no change in the extent of P700 oxidation as measured by 820 was observed in vivo.

Thus, the phenomenon of increased light saturated PSI activity in vitro may simply reflect an artifact of thylakoid membrane isolation and concomitant destabilization of the thylakoid membrane with time in vitro. Stressed plants with

either high or low temperatures prior to thylakoid isolation may simply enhance this instability of thylakoid membranes upon isolation.

The effects of low growth temperature on winter rye.

Any model of the effects of low growth temperature on light saturated electron transport in winter rye must deal with seemingly contradictory experiments. Measurements from the Clark electrode show an increase in the rate of light saturated PSI electron transport with cold hardening (Figure 4). Electron spin resonance experiments show no increase in the steady state P700+ signal on a per chlorophyll basis with cold hardening (Huner, Elfman, Krol and McIntosh 1984). Single turnover flash induced production of P700+ measured by a change in absorbance at 820 nm indicated that more P700 was oxidized in cold grown plants on a per chlorophyll (Figure 12).

These 3 seemingly contradictory facts can be explained if cold growth effects the rate of rereduction. An increased rate of rereduction with cold growth would assist a higher rate of electron transport through PSI seen in the Clark electrode. A higher rate of rereduction would mean that less of oxidized P700 would appear in a steady state reaction, such as the electron spin resonance experiment but a time resolved reaction such as the experiments measuring the change in absorbance at 820nm would still show more P700+.

To test this hypothesis it would be necessary to measure and compare the rereduction rates of cold grown and warm grown rye.

While changes in the rereduction rate will explain why different measures of the oxidation of P700 give different results, they cannot be responsible for the increased oxidation of P700 measured with the single turnover flashes. To explain the increase in electron flow through PSI we must find another explanation.

As described above the increase in the rate of PSI electron transport is clearly due to changes within PSI. The simplest explanation for this is that there is an enrichment of PSI relative to total chlorophyll in the cold grown plants. However, it has been shown that, even though there is approximately a 1.5 fold increase in the amount of P700 oxidized per chlorophyll, the ratio of chl a:b does not differ between warm grown and cold grown plants (Huner et al. 1984). The ratio of the non-chlorophyll pigments, B-carotene, lutein, violaxanthin and neoxanthin also remains unchanged on a per milligram chlorophyll basis between cold and warm grown rye (Huner et al. 1984). We have shown (Table I) that the relative proportions of PSI to PSII are also unchanged by cold growth. Thus this simple explanation is not sufficient.

If there is no change in P700 concentration we must find an alternate explanation for the increased proportion of P700 oxidized.

While this explanation would be sufficient other possibilities should still be investigated. It is possible, as

has been mentioned previously, that a change within PSI occurs with low temperature growth which alters the absorbance of P700. Such a change in absorbance would not explain why there is an increase of the same magnitude in PSI electron transport, measured in a Clark electrode.

Another hypothesis is that a fraction of total PSI in the warm grown rye is inactive. The normally inactive fraction would be activated by cold growth.

Optical cross sections of thylakoids indicate that there is no change in the effective size of the PSI particle but there are more PSI particles transporting electrons in cold grown rye. Since all experimental samples were normalized for the amount of chlorophyll in the sample this indicates that there is more P700 oxidized on a per chlorophyll basis in thylakoids from cold grown rye than thylakoids from warm grown rye. In electrophoretic gel studies on protein-chlorophyll complexes of warm and cold grown rye, we have shown (Table I) that there is no difference in the proportions of chlorophyll associated with each of the photosystems and no difference in the electrophoretic mobility of the complexes. This would indicate that there is no change in the size or proportion of PSI with cold-growth. While some questions have been raised as to the accuracy of this methodology as a measure of the proportion of PSI (Tom Owens personal communication) it seems unlikely that a two fold increase in cold grown plants could have been missed. In addition other researchers (Shubin et al 1986) have found evidence for inactive PSI.

As both warm and cold grown rye have the same absorbance spectra but, according to this hypothesis, only part of the PSI of the warm grown rye is transporting electrons, this hypothesis predicts that warm-grown rye would have to release more energy in a form other the electron transport than cold grown rye. One possible avenue of release would be as fluorecence but previous studies have shown that the fluorecence from cold grown rye is less than that of warm grown rye (Huner et al 1992). The next most likely possibility would be that the energy was released as heat. Photoacoustic experiments could be done to determine if warm grown plants generated more heat than cold grown plants with a non saturating single turnover flash. If there is more heat from warm grown plants, this would be a strong indicator of inactive PSI in warm grown plants.

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