Using Chlorophyll Fluorescence to Study Photosynthesis*

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* This article is not a review, but is a presentation explaining the basics and the applications of Chlorophyll fluorescence in Biology, and, is designed for beginning researchers and students.

1. Principles

Each quantum of light absorbed by a chlorophyll molecule rises an electron from the ground state to an excited state. Upon de-excitation from a chlorophyll a molecule from excited state 1 to ground state, a small proportion (3-5% in vivo) of the excitation energy is dissipated as red fluorescence. The indicator function of chlorophyll fluorescence arises from the fact that fluorescence emission is complementary to alternative pathways of de-excitation which are primarily photochemistry and heat dissipation. Generally, fluorescence yield is highest when photochemistry and heat dissipation are lowest. Therefore, changes in the fluorescence yield reflect changes in photochemical efficiency and heat dissipation.

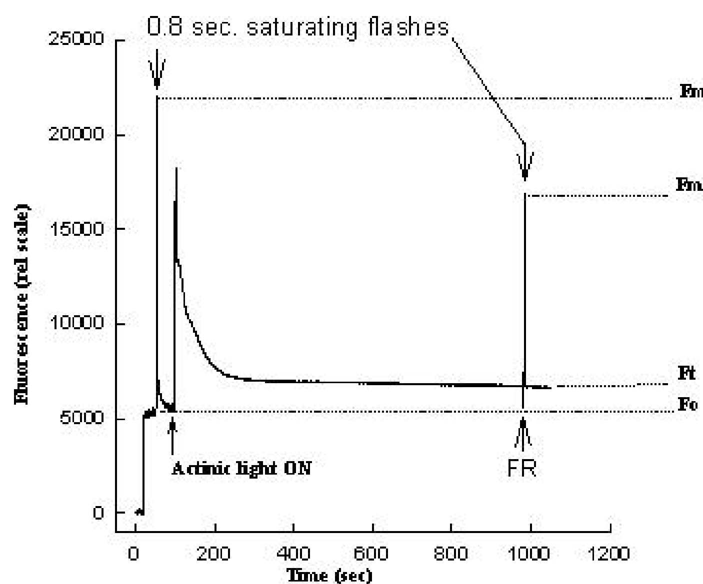

Figure 1: Measurement of chlorophyll fluorescence from a maize leaf by the saturation pulse method using a PAM-2000 instrument (Walz).

A typical measurement on an intact leaf by the saturation pulse method is shown in Fig. 1. The plant was dark adapted for 20 min prior to the measurement. Upon the application of a saturating flash (8000 µmol m^{-2} s^{-1} for 1 s), fluorescence raises from the ground state value (Fo) to its maximum value, Fm. In this condition, QA, the first electron acceptor of PSII, is fully reduced. This allows the determination of the maximum quantum efficiency of photosystem II (PSII) primary photochemistry, given by Fv/Fm = (Fm-Fo)/Fm. In healthy leaves, this value is always close to 0.8, independently of the plant species studied. A lower value indicates that a proportion of PSII reaction centers are damaged, a phenomenon called photoinhibition, often observed in plants under stress conditions. Upon subsequent application of constant illumination, a transient rise in fluorescence yield is observed. This is due to the lag phase before carbon fixation starts. Whilst electron transport starts within milliseconds upon illumination, some carbon fixing enzymes need to be light-activated. As a consequence, a substantial proportion of QA (the primary electron acceptor after PSII) is reduced during the first seconds of illumination and the fluorescence yield increases. Thereafter, upon the onset of photochemical and heat dissipation processes, the fluorescence yield is quenched and reaches a steady state value (Ft). Upon the application of a second saturation flash in the presence of actinic light, the maximum fluorescence obtained (Fm') is lower to that observed in the dark (Fm). Since at Fm', QA Is fully reduced again, the difference between Fm' and Ft reflects the photochemical part of fluorescence quenching, and the difference between Fm and Fm' reflects fluorescence quenching due to heat dissipation. From these data several parameters can be computed: The photochemical quenching [qP = (Fm'-Ft)/(Fm'-Fo)] reflects the redox state of the primary electron acceptor of PSII QA,. The non-photochemical quenching [qN = (Fm-Fm')/(Fm-Fo) or NPQ = (Fm-Fm')/Fm'] reflects energy dissipated as heat related to energization of the thylakoid membrane due to lumen acidification. The quantum yield of electron transfer at PSII (ΦPSII), calculated as (Fm'-Ft)/Fm', is a measure of the overall efficiency of PSII reaction centers in the light. Finally, Fv'/Fm' [(Fm'-Fo)/Fm'] reflects the efficiency of open reaction centers in light (see Chlorophyll *a* fluorescence measurements in plant biology for more details on the principles of chlorophyll fluorescence measurements). Additional information about the principles of chlorophyll fluorescence analysis can be found in numerous review articles (Maxwell and Johnson, 2000; see also Govindjee, 1995 for a historical overview).
Chlorophyll fluorescence allows us to study the different functional levels of photosynthesis indirectely (processes at the pigment level, primary light reactions, thylakoid electron transport reactions, dark-enzymatic stroma reactions and slow regulatory processes).
In the next sections, we show some examples from our own work to illustrate various possibilities of using chlorophyll fluorescence to study photosynthesis.

2. Possibilities to use chlorophyll fluorescence to elucidate the mode of action of substances interfering with photosynthesis

In this section, some examples of the effects of known substances are presented to illustrate the potential of chlorophyll fluorescence to study the mode of action of herbicides.

2.1. DCMU

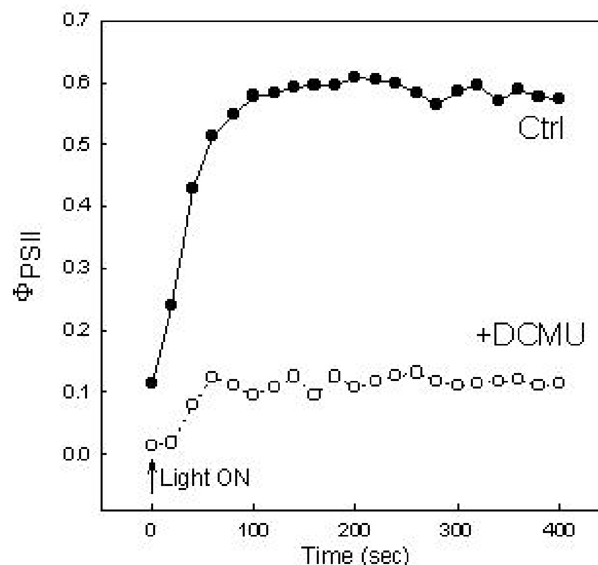

Figure 2: The effect of DCMU on electron transport in a maize leaf. Y. Fracheboud, unpublished result.

DCMU, a herbicide known as diuron, blocks electron transport on the acceptor side of PSII. In the example presented in Fig. 2, a maize leaf piece was poisoned by incubation for 30 min in the dark in a solution containing DCMU. Thereafter it was illuminated with a moderate actinic light (100 µmol m^{-2} s^{-1}) and saturation pulses were applied at 20 sec intervals. As seen from Fig 2, electron transport measured by ΦPSII is greatly reduced in the leaf treated with DCMU, due to a decrease of qP (not shown). From this it can be concluded that DCMU strongly inhibits electron transport shortly after PSII.

2.2. Methyl viologen

Known as the herbicide paraquat, methyl-violagen accepts electrons from PSI and generates superoxide. Fig 3. shows the fluorescence emitted by a maize leaf which was infiltrated with methyl viologen (MV) in solution for 30 min in the dark. After the application of a saturation pulse in the dark for the determination of the maximum PSII efficiency, the leaf was illuminated with a moderate actinic light (200 µmol m^{-2} s^{-1}), and saturation flashes were applied at intervals of 20 s. Upon illumination, the control leaf (treated with water) showed the typical fluorescence transient (called the Kautsky effect), which reached its steady state value after 12-15 min. In contrast, Ft in the MV treated leaf immediately reached steady state value upon illumination, as already found several years ago (Munday and Govindje, 1969). Since the Kautsky effect is due to the delayed start of carbon fixation compared to the light reactions, it is clear that MV is a very good electron acceptor. The high florescence yield obtained during the application of the saturation pulses in the MV treated leaf indicate a high rate of electron transport and little heat dissipation, whilst some non-photochemical fluorescence quenching is obvious in the control leaf, as indicated by the difference between Fm' and Fm. Therefore, in can be concluded that some limitation of electron flow by the carbon cycle is present in the control leaf, even under relatively low light. This illustrates the feed-back control of carbon fixation on electron transport.

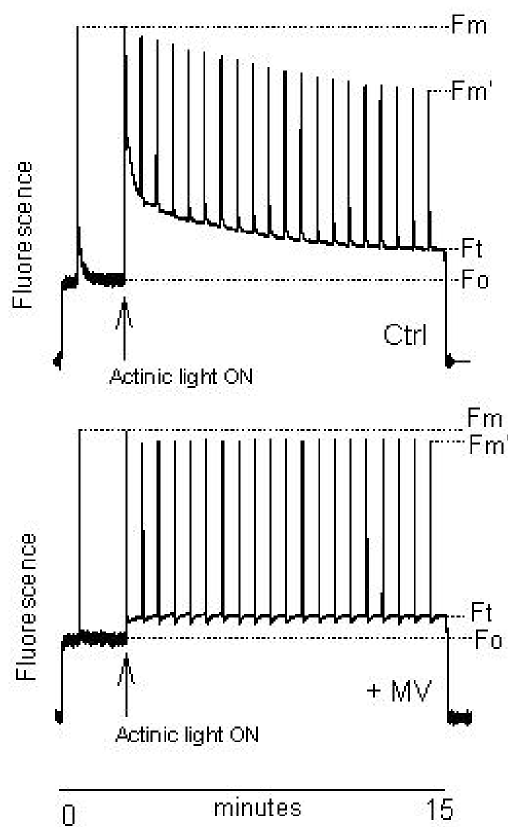

Figure 3: The effect of methyl viologen on the induction of chlorophyll fluorescence quenching in a maize leaf. Y. Fracheboud, unpublished result.

2.3. Nigericin

Nigericin is a protonophore which dissipates the proton gradient across the thylakoid membrane.

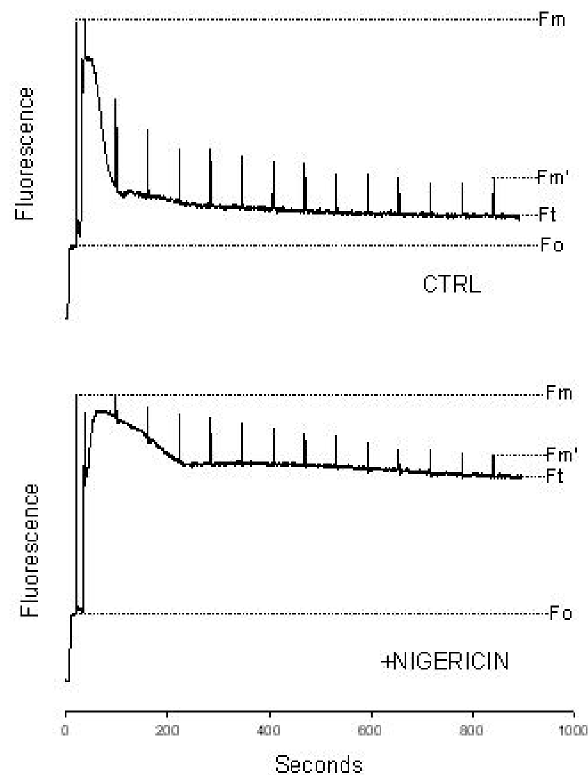

Figure 4: The effects of nigericin on the induction of chlorophyll fluorescence quenching in a maize leaf. Y. Fracheboud, unpublished result.

Fig. 4 shows the effects of nigericin application on the induction of chlorophyll fluorescence quenching in a maize leaf. A leaf segment was incubated for 30 min in a solution containing nigericin before beeing analysed with the saturation pulse method. After determination of Fv/Fm, the leaf was illuminated with a relatively strong actinic light (700 µmol m^{-2} s^{-1}) and saturation flashes were applied at 60 s intervals. Under such high light intensity, a large proportion of fluorescence quenching in the control leaf is due to heat dissipation, as indicated by the large difference between Fm' and Fm. In contrast, non-photochemical quenching is strongly inhibited in the leaf treated with nigericin, and results in a large increase of the steady state of fluorescence (Ft). This is because the proton gradient across the thylakoid membrane is necessary for the formation of the non-photochemical quenching.

3. The use of chlorophyll fluorescence to study the effects of environmental stresses on photosynthesis

Chlorophyll fluorescence is also very useful to study the effects of environmental stresses on plants since photosynthesis is often reduced in plants experiencing adverse conditions, such as water deficit, temperature, nutrient deficiency, polluting agents, attack by pathogens. In this section, we present some examples from our own work to illustrate the use of chlorophyll fluorescence to investigate temperature stress in maize.

3.1. The use of chlorophyll fluorescence to assess the allocation of electrons produced by photochemistry

The electrons produced by the photochemical process are not necessarily used for carbon fixation. In conditions where carbon fixation is limited (e.g. low temperature, shortage of CO2 due to stomatal closure, etc.) alternative sinks for electrons might be enhanced, namely photorespiration and reduction of molecular oxygen (part of Mehler reaction). Whilst the first can be considered as a waste of energy, the latter may lead to a dangerous oxidative stress. The allocation of electrons produced by the oxygen evolving complex can be studied by simultaneous measurement of the quantum yield of electron transfer at PSII (ΦPSII) measured by chlorophyll fluorescence and the quantum yield of CO2 fixation (ΦCO2) measured by gas exchange. If more electrons are used for photorespiration or for the Mehler reaction, the ratio ΦPSII/ΦCO2 will increase.

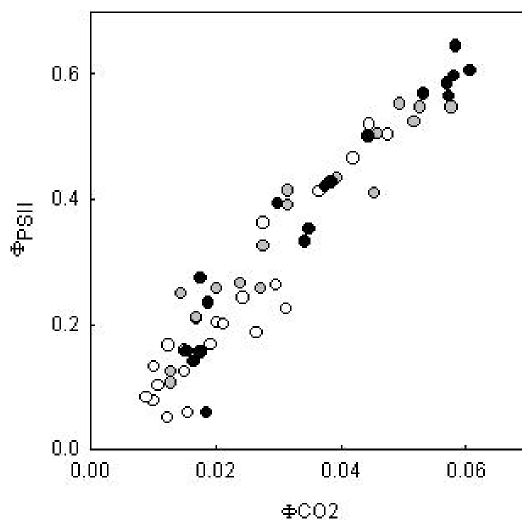

Figure 5: Relationship between ΦPSII and ΦCO2 in maize leaves grown in the field at different dates. Measurements were made between May 28 and 31 (white symbols), June 4 and 6 (grey symbols) and June 11and 13 (black symbols), 1996. All measurements were performed a 25°C. Adapted from Leipner *et al.*, 1999.

In the example presented in Fig. 5, segments from maize leaves grown in the field at different dates were sealed in a small chamber adapted for simultaneous measurements of chlorophyll fluorescence and carbon fixation by infra-red gas analysis. ΦCO2 was determined from CO2 fixation at different light intensities after correction for dark respiration and leaf absorptance. The result obtained indicates that the relation between ΦPSII and ΦCO2 is very simmilar in the different leaves. Therefore, it is unlikely that the symptoms of cold injury of leaves developed early in the season (low pigment content, low photosynthesis, not shown) which are typical for cold stress in maize (Haldimann *et al.*, 1995) are due to an oxidative stress through an excessive production of superoxide. Actually, these data suggest that ΦPSII can be used to estimate photosynthesis accurately in a wide range of environmental conditions and can be used as selection tool for cold-tolerance of photosynthesis (Fracheboud *et al.*, 1999)

3.2. Separating dark and light reactions

Upon exposure to low temperature, photosynthesis of maize leaves is strongly inhibited. Besides the obvious inhibition of the enzymes of the carbon cycle it is also possible that cold temperature inhibits the activity of the water splitting complex or electron transport because of a possible decrease of the thylakoid membrane fluidity. The two phenomena can be separated by substituting the carbon cycle with the electron acceptor methyl viologen (as in section 2.2). In the example shown in Fig. 6, maize leaves were infiltrated with methyl viologen for 30 min in the dark, before beeing exposed to low light (100 µmol m^{-2} s^{-1}) at given temperature. ΦPSII was measured after 20 min illumination by the application of a single saturation flash. The result (Fig 6) shows that maize leaves infiltrated with methyl viologen can perform water splitting and electron transport until PSI relatively well at low temperature. At 1.5°C, ΦPSII in MV treated leaves was only decreased by 24% compared to 25°C, whilst electron transport in control leaves was strongly inhibited at temperatures below 10°C.

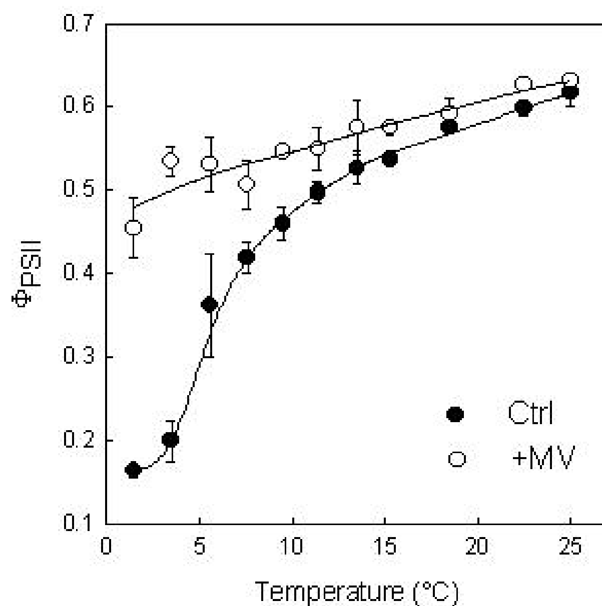

Figure 6: Response of ΦPSII to temperature in maize leaves infiltrated with methyl viologen. Y. Fracheboud, unpublished result.

3.3. Energy dissipation under field condition in relation to changes in pigment composition

Although it is convenient to study chlorophyll fluorescence in the laboratory under controlled temperature and light conditions, measurements under natural conditions are very relevant. Since about ten years, these have been made possible by the development of new compact instruments. With the help of special dark adaptation clips, it is now possible to monitor all the parameters presented in section 1 on attached leaves under field conditions.

With increasing illumination, more light energy is dissipated as heat in the pigment bed. This process requires energization of the thylakoid membrane and is associated with changes in the xanthophyll cycle pigments through a still debated mechanism. Under strong illumination, the xanthophyll cycle pigment violaxanthin (V) is de-epoxidised into antheraxanthin (A) which is further de-epoxidised into zeaxanthin (Z). Both A and Z seem to be involved in the energy dissipation mechanism (see Demmig-Adams and Adams III, 1996, for review) . Upon removal of excess light, A and Z are epoxidised back to V. Upon increased energy dissipation as heat, non-photochemical quenching of chlorophyll fluorescence is observed and the efficiency of open PSII centers decreases. In the example presented in Fig. 7, the energy dissipation mechanism in maize leaves was investigated in the field, by monitoring the changes in efficiency of open PSII centers (Fv'/Fm'). For this, a dark adaptation clip was fixed on the leaf, which was immediately subjected to the application of a saturation flash followed by 5 s illumination with far red. Far red light activates PSI,

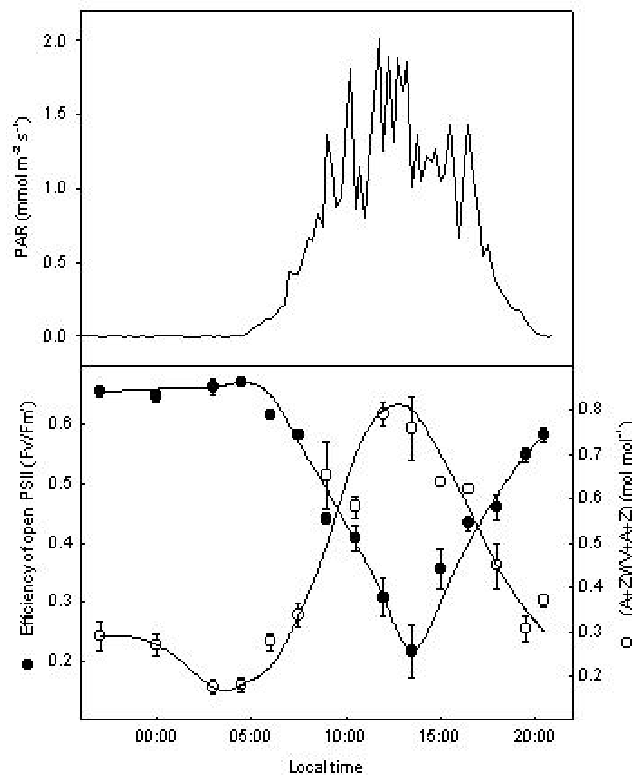

Figure 7: Changes in photosynthetically active radiation, efficiency of open PSII centers (filled symbols) and de-epoxidation state of xanthophyll cycle pigments (open symbols) of maize leaves in the field on June 7, 1995 at Eschikon (ZH). Y. Fracheboud, unpublished result.

and therefore sets all open PSII centers into a relaxed state. This allows re-determination of the ground state fluorescence Fo’, which is not necessarily identical to Fo measured after 10 or 20 min dark adaptation, especially in leaves under stress conditions. In this case, Fo’ is used instead of Fo for the calculations of the quenching parameters. The portion of leaf under the clip was then allowed to dark adapt for 15 min prior to the determination of the max. PSII efficiency by the application of a second saturation flash. Simultaneously, leaf samples were collected, and their pigment composition were determined by HPLC. The solar radiation was recorded with a weather station located at about 50 m of the experimental field. Fig. 7 shows that the efficiency of open PSII reaction centers (Fv’/Fm’) changed markedly during the course of the day, being the lowest when sun radiation was maximum. This decrease of Fv’/Fm’ was accompanied by an increase of de-epoxidation state of the xanthophyll cycle pigments (A+Z)/(V+A+Z). At noon, about 80% of the xanthophyll cycle pigments were in the form of zeaxanthin or antheraxanthin, whilst during the night, violaxanthin was the most abundant. When plotted against each other, Fv’/Fm’ and (A+Z)/(V+A+Z) show a remarkable inverse linear relation (Fig. 8) which implicates the involvement of the xanthophyll cycle in the down-regulation of PSII in natural conditions.

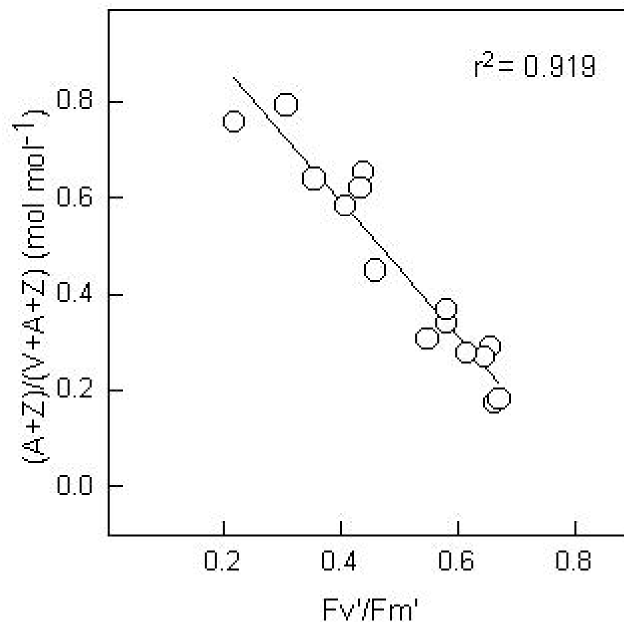

Figure 8: Relation between Fv’/Fm’ and the de-epoxidation state of the xanthophyll cycle pigments in maize leaves under field conditions. Y. Fracheboud, unpublished result.

4. Studies of specific components of the photosynthetic apparatus by chlorophyll fluorescence

In certain cases, chlorophyll fluorescence also allows to investigate specific components of the photosynthetic apparatus. In this section, we present examples where chlorophyll fluorescence was used to investigate the effects of temperature stress on water splitting , on the efficiency of PSII reaction centers and on the light harvesting complexes of maize leaves.

4.1. Inhibition of water splitting activity by heat

Upon sudden illumination of a dark-adapted leaf, PSII fluorescence yield increases following a triphasic kinetics (O-J, J-I and I-P, Strasser *et al.*, 1995; see also Fig. 9). The three phases have been interpreted as follows: the (O-J) phase corresponds to a complete reduction of the primary electron acceptor QA of PSII, the release of fluorescence quenching during the (J-I) phase is controlled by the PSII donor side (water splitting activity) and the third phase (I-P) corresponds to the release of fluorescence quenching by the oxidised plastoquinone pool (Neubauer and Schreiber, 1987; Schreiber and Neubauer, 1987). Thus, the (J-I) fluorescence phase is a useful indicator of water-splitting activity, although the exact mechanism involved remains to be established.

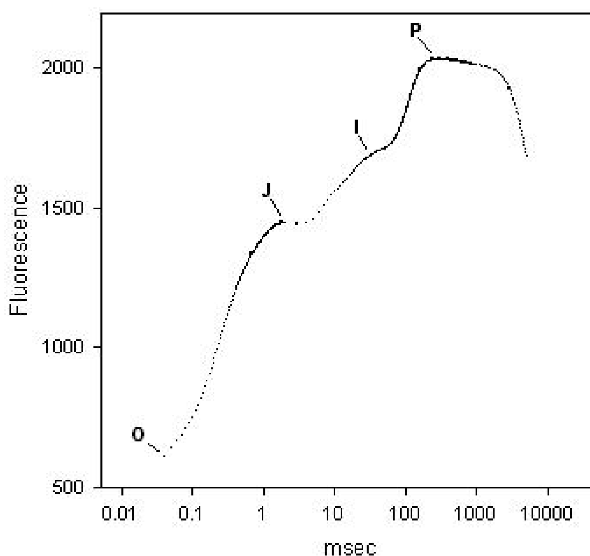

Figure 9: Induction of chlorophyll fluorescence in a dark-adapted maize leaf recorded with a PEA instrument (Hansatech). Adapted from Sinsawat (1999).

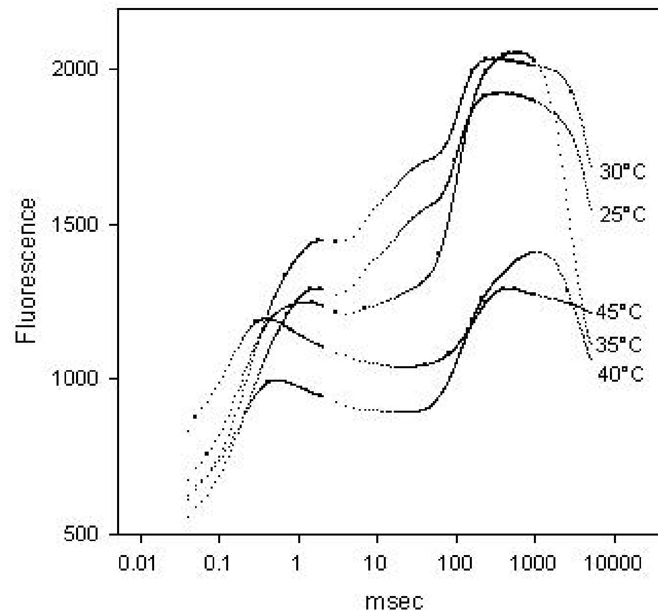

Figure 10: The effect of short heat stress in the dark on the OJIP transcient increase of chlorophyll fluorescence of maize leaves. Adapted from Sinsawat (1999).

Fig. 10 shows the effect of a 20 min heat treatment of maize leaves in the dark on the induction of chlorophhyll fluorescence. The result suggests that the water splitting activity becomes sensitive to heat stress in the dark around 35-40°C. The effects of heat on the OJIP transients shown in Fig. 10 are similar to those previously observed in peas (Srivastava *et al.*, 1997).

4.2. Damage to PSII reaction centers by high light and cold

Upon exposure to excess light, the D1 protein of PSII reaction center gets inactivated by phosphorylation and then degraded, leading to an inactive PSII center. The maximum PSII efficiency Fv/Fm can be used as an indicator of this process since there is a good correlation between degradation of D1 protein measured by radioactive labelling and Fv/Fm (Rintamaki *et al.*, 1995). Chilling sensitive plants are usually more susceptible to low temperature photoinhibition than chilling tolerant ones (Hetherington *et al.*, 1989). In the example shown in Fig. 11, segments of maize leaves were subjected to strong illumination for 8 hours at 6 or 25°C. to study the effect of the combination of low temperature and high light on photoinhibition. Thereafter the segments were placed in low light at 25°C, to investigate the recovery from the photoinhibitory treatment. Fv/Fm was measured by the application of a saturating pulse after 15 min of dark adaptation. The results indicate that photoinhibition was more pronounced at low temperature. Twenty hours after the end of the photoinhibitory treatment the leaves had recovered to a large extent, indicating that the damage was mostly reversible.

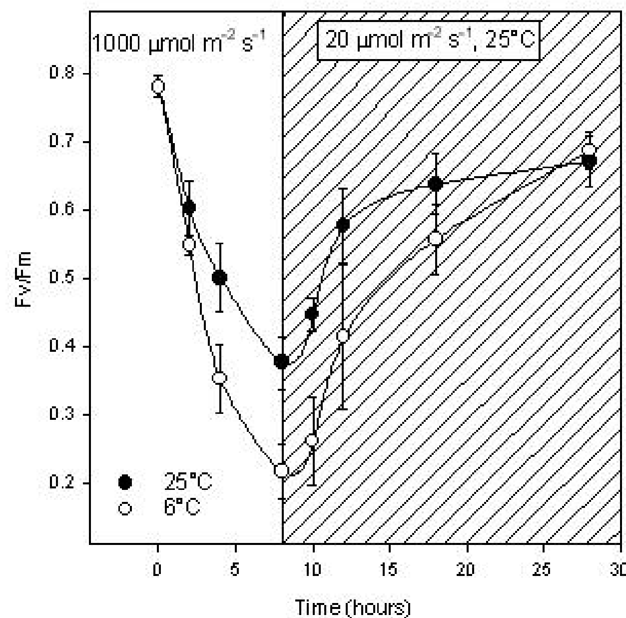

Figure 11: The effect of temperature on high light-induced photoinhibition in maize leaves. Adapted from Haldimann *et al.*, 1996.

4.3. Changes in the dark fluorescence yield (Fo) in relation to a possible dissociation of light harvesting pigment system of PSII from the PSII core

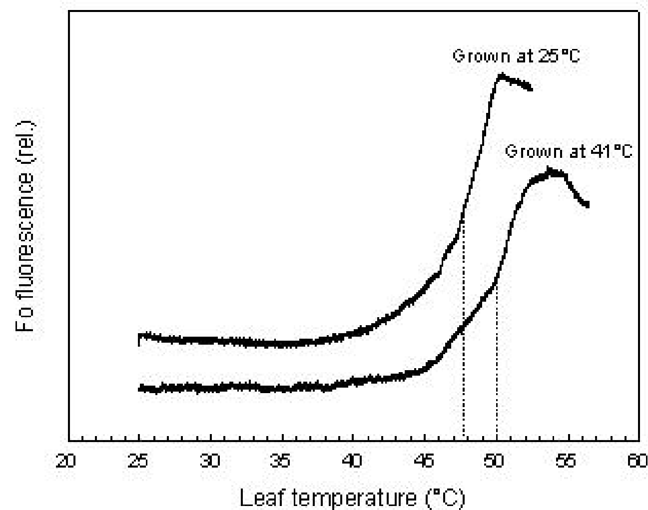

Figure 12: Change of Fo in maize leaves subjected to slow heating in the dark. The vertical lines indicate the temperatures coresponding to half increase of Fo. From Sinsawat, 1999.

Upon exposure to increasing temperature, the dark level of fluorescence Fo increases. From its theoretical expression, an increase of Fo can be interpreted as a reduction of the rate constant of energy trapping by PSII centers (Havaux, 1993) which could be the result of a physical dissociation of light harvesting complex from PSII core as it has been observed in several plant species as a result of heat damage. (e.g. Armond *et al.*, 1980). Furthermore, a close correlation has been found between change in Fo and necrosis test after heat stress in a large number of plant species (Bilger *et al.*, 1984). Fig. 12 shows the change of Fo in maize leaves heated at a rate of 1 °C min^{-1}. Clearly, a possible dissociation of light harvesting complexes from PSII core, occured only at very high temperature. Interestingly, the temperature corresponding to half increase of Fo was 2.3°C higher in the leaf developed at 41°C compared to the leaf developed at 25°C indicating that acclimation to heat stress can be induced by supra-optimal growth temperature.

5. Chlorophyll fluoresecence as a tool for breeding

The linear correlation between ΦPSII and ΦCO2 observed in maize (Fig. 5) suggests that ΦPSII can be used as a tool to select for plants with different photosynthetic capacity. This hypothesis was tested in a maize breeding population (with a high genetic variability) grown at low temperature. C.a. 600 seedlings were grown at 14-15°C and screened for ΦPSII (see Fracheboud *et al.*, 1999 for details about the method) . The eight individuals with the highest ΦPSII values and the eight individuals with the lowest ΦPSII values were transplanted in the field and allowed to set seeds by selfing. ΦPSII was then measured in the S1 offsprings and in the original population grown at low temperature.

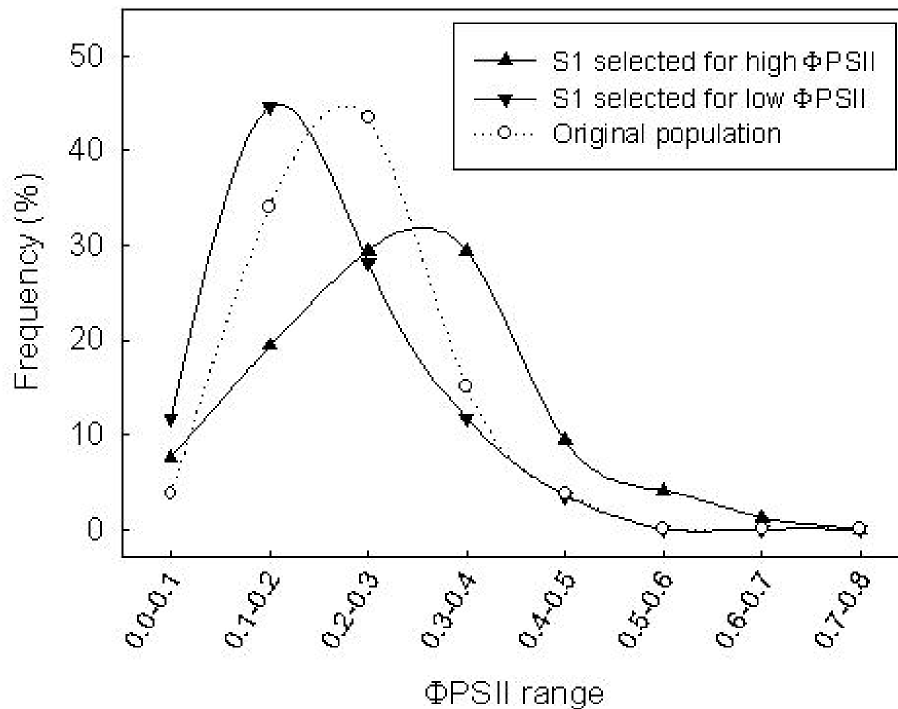

Figure 13: Distribution of ΦPSII in pooled S1 progenies of maize plants selected for high or low ΦPSII at low growth temperature (From Fracheboud *et al.*, 1998)

The result, shown in Fig. 13, indicates that ΦPSII can be used to breed both for cold-tolerance or cold-sensitivity of photosynthesis, since the distributions of the progenies are clearly shifted from the distribution of the original population. Furthermore, applying the selection for a second cycle yielded lines with different capacity of carbon fixation when

grown at low temperature (Fig .14). These results show that ΦPSII can be used for breeding.

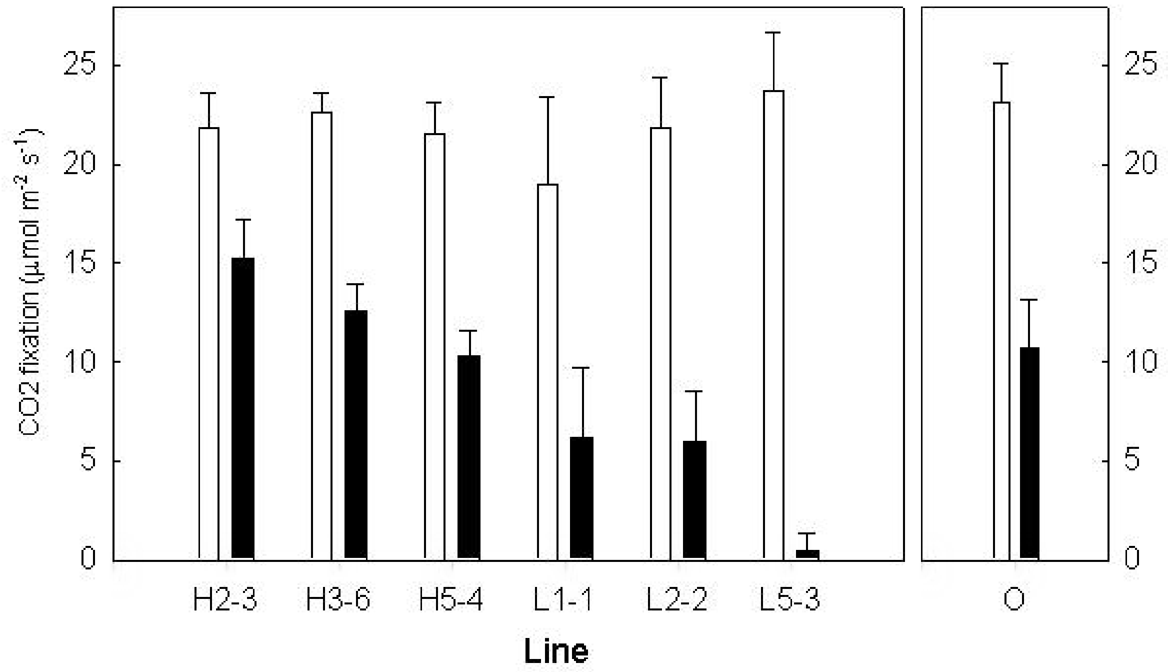

Figure 14: Carbon fixation in S2 maize lines selected for high ΦPSII (H2-3, H3-6 and H5-4), low ΦPSII (L1-1, L2-2, L5-3) and in the breeding population they are derived from (O) grown at 25°C (white bars) or 14°C (black bars). From Fracheboud, 1999.

Conclusions

The examples presented here illustrate how chlorophyll fluorescence can be used to study components of the photosynthetic apparatus and their reactions to changes in the environment as well as photosynthesis as a whole. This is interesting in the view that photosynthesis is a good indicator of adaptation of plants to their environment. Since the measurements are non-intrusive, fast and reliable, this makes chlorophyll fluorescence an attractive tool for environmental research and for breeding purposes.

References

Armond P.A., Bjorkman O. and Staehelin L.A. 1980. Dissociation of Supramolecular Complexes in Chloroplast Membranes - a Manifestation of Heat Damage to the Photosynthetic Apparatus. Biochim Biophys Acta 601: 433-442.

Bilger H.W., Schreiber U. and Lange O.L. 1984. Determination of Leaf Heat-Resistance - Comparative Investigation of Chlorophyll Fluorescence Changes and Tissue Necrosis Methods. Oecologia 63: 256-262.

Demmig-Adams B. and Adams III W., W. 1996. The role of xanthophyll cycle carotenoids in the protection of photosynthesis. Trends in Plant Science 1: 21-26.

Fracheboud Y. 1999. Cold adaptation of the photosynthetic apparatus of maize by growth at suboptimal temperature. In: M. Sanchez-Diaz, J. Irigoyen, J, J. Aguirreola, K. Pithan (eds), Crop development for the cool and wet climate of Europe. Office for the Official Publications of the European Communities, Brussels, pp. 88-98.

Fracheboud Y., Haldimann P., Leipner J. and Stamp P. 1999. Chlorophyll fluorescence as a selection tool for cold tolerance of photosynthesis in maize (*Zea mays* L.). J Exp Bot 50: 1533-1540.

Fracheboud Y., Leipner J. and Stamp P. 1998. Selection for cold tolerance in maize using chlorophyll fluorescence. In: P. Sowinski, B. Zagdanska, A. Aniol, K. Pithan (eds), Crop development for the cool and wet regions of Europe. Office for the Official Publications of the European Communities, Brussels, pp. 116-121.

Govindjee. 1995. 63 Years since Kautsky - Chlorophyll-a Fluorescence (Vol 22, Pg 131, 1995). Australian Journal of Plant Physiology 22: 711-711.

Haldimann P., Fracheboud Y. and Stamp P. 1995. Carotenoid composition in *Zea Mays* developed at sub-optimal temperature and different light intensities. Physiol Plant 95: 409-414.

Haldimann P., Fracheboud Y. and Stamp P. 1996. Photosynthetic performance and resistance to photoinhibition of *Zea mays* L. leaves grown at sub-optimal temperature. Plant Cell Environ 19: 85-92.

Havaux M. 1993. Characterisation of Thermal damage to the photosynthetic electron transport system in potato leaves. Plant Science 94: 19-33.

Hetherington S.E., He J. and Smillie R.M. 1989. Photoinhibition at Low-Temperature in Chilling-Sensitive and Chilling-Resistant Plants. Plant Physiol 90: 1609-1615.

Leipner J., Fracheboud Y. and Stamp P. 1999. Effect of growing season on the photosynthetic apparatus and leaf antioxidative defences in two maize genotypes of different chilling tolerance. Environ Exp Bot 42: 129-139.

Maxwell K. and Johnson G.N. 2000. Chlorophyll fluorescence - a practical guide. J Exp Bot 51: 659-668.

Munday J.C. and Govindje. 1969. Light-Induced Changes in Fluorescence Yield of Chlorophyll a in Vivo .3. Dip and Peak in Fluorescence Transient of Chlorella Pyrenoidosa. Biophys J 9: 1-&.

Neubauer C. and Schreiber U. 1987. The polyphasic rise of chlorophyll fluorescence upon onset of strong continuous illumination .1. Saturation characteristics and partial control by the photosystem-II acceptor side. Zeitschrift für Naturforschung 42: 1246-1254.

Rintamaki E., Salo R., Lehtonen E. and Aro E.M. 1995. Regulation of D1 Protein-Degradation During Photoinhibition of Photosystem-Ii in-Vivo - Phosphorylation of the D1 Protein in Various Plant Groups. Planta 195: 379-386.

Schreiber U. and Neubauer C. 1987. The polyphasic rise of chlorophyll fluorescence upon onset of strong continuous illumination .2. Partial control by the photosystem II donor side and possible ways of interpretation. Zeitschrift für Naturforschung 42: 1255-1264.

Sinsawat V. 1999. Acclimation of the photosynthetic apparatus of maize seedlings (*Zea mays* L.) to high temperature. PhD thesis ETH No. 13222

Srivastava A., Guissé B., Greppin H. and Strasser R.J. 1997. Regulation of antenna structure and electron transport in photosystem II of *Pisum sativum* under elevated temperature probed by the fast polyphasic chlorophyll *a* fluorescence transient: OKJIP. Biochim Biophys Acta 1320: 95-106.

Strasser R.J., Srivastava A. and Govindjee. 1995. Polyphasic chlorophyll *a* fluorescence transient in plants and cyanobacteria. Photochemistry and Photobiology 61: 32-42.