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Phenotyping of dark and light adapted barley plants by the fast chlorophyll *a* fluorescence rise OJIP

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Chlorophyll *a* fluorescence of dark adapted leaves of barley plants (*Hordeum vulgare* L.) upon exposure to actinic light was measured. We compared the photosynthetic behaviour of ten cultivars of barley plants in the dark and light adapted states. A significant relationship between the light adaptation (S_1 to S_2 transition) of the photosynthetic Performance Index (^{l}PI/^{d}PI) and the normalised Area (^{l}Sm/^{d}Sm) evaluated by the JIP-test was observed. The two parameters might provide a basis to rank the plants according to their tolerance to light stress conditions, i.e. the studied cultivars can be split into three groups with a different response to high light stress: tolerant, intermediate and sensitive.

Abbreviations: ABS = absorption, Chl *a* = chlorophyll *a*, ET_o = energy flux for electron transport, F_o ($F_{50\mu s}$) and F_M = initial and maximum Chl *a* fluorescence, F_{2ms} = Chl *a* fluorescence measured at 2ms, F_V = maximum variable Chl fluorescence, F_s = steady state fluorescence, k_P and k_N = photochemical and non-photochemical rate constants, L, K, J, I, H, G = intermediate steps in the Chl *a* fluorescence transient between F_o and F_M, P = fluorescence level with the highest intensity, here equals F_M, PI = photosynthetic performance index, PS II = photosystem II, φ_{Po} = maximum quantum yield of primary photochemistry, Ψ_o = efficiency with which a trapped exciton can move an electron into the electron transport chain, Q_A = primary bound plastiquinone, γ = fraction of reaction centre chlorophylls relatively to the total chlorophyll, RC = reaction centre, Sm = normalised area above the fluorescence rise between F_o and F_M, tF_M = time needed to reach the maximum fluorescence intensity, TR_o = energy flux for trapping, V_J = relative variable chl *a* fluorescence at the J-step, V_t = relative variable fluorescence transients, (^{d}) and (^{l}) = dark adapted and light adapted states.

Introduction

Presently, the most common approach to reduce the effect of environmental stresses is to modify the environment by means of irrigation, the use of chemical products, etc. These methods are, however, expensive, disturb the ecosystem and are effective only for a short time. Selection of new varieties of cereal plants, tolerant to several environmental stresses and at the same time with a high crop yield is one of the biggest challenges to plant breeders (Vivekanandan and Saralabai 1997). To achieve an efficient selection, fast phenotyping and quantification of stress tolerance of plants is necessary. Several methods have been used for monitoring the physiological effects of environmental stresses on plants. Chlorophyll *a* fluorescence has been applied extensively as a fast and non-invasive tool for elucidating various aspects of the photosynthetic apparatus in higher plants (Force *et al*. 2003). It has been used as a measure of chloroplast function and, indirectly, as a measure of whole plant and plant organ physiological status (Strasser *et al.* 2000). Photosynthesis is central to plant productivity and monitoring of changes in photosynthesis is a very effective way to detect early changes in the physiological state. The state of the photosynthetic apparatus in the light adapted, compared to dark adapted state, has been previously studied and the photosynthetic parameters were used to estimate the deviation of the sample in one state relative to a reference state (Strasser *et al*. 1999, Flexas *et al*. 2002). The JIP-test using the OJIP chlorophyll fluorescence transient as described by Strasser *et al*. (2000) can be used to compare the physiological states of the samples, e.g. dark adapted and light adapted states, and estimate the rate of specific (per RC) or apparent (per leaf surface) photosynthetic electron transport and quantum yield for trapping ($\varphi_{Po} = TR_o / ABS$) and electron transport ($\varphi_{Eo} = ET_o / ABS$). In the dark adapted state, the Ferredoxin-$NADP^+$-reductase and the Calvin-Benson cycle are inactivated (Roháček and Barták 1999). In the light adapted state photosynthesis is in steady state, which means that there is a balance between the electrons added to the electron transport chain and the electrons used by metabolic processes (Strasser *et al*. 2000). If a strong saturating light pulse is given to a sample already in the steady

state, the initial fluorescence provided by the light pulse becomes a measure for the steady state fluorescence F_s. Each saturating pulse creates a fast closure of all reaction centres, as reflected by a fast fluorescence rise.

The goal of this work was to compare the light responses of ten cultivars of barley plants (*Hordeum vulgare* L.) using a fast method based on the analysis of the polyphasic fluorescence rise OJIP and to establish a more detailed approach to distinguishing the L, K, H and G steps in the Chl *a* fluorescence transient. The different steps of the polyphasic fluorescence transient are labelled in alphabetical order from the slower to the faster part of the transient. The most marked step at 2ms is called the J step. Up to the J step we get information about single turnover events of the primary reactions of photochemistry, mainly Q_A reduction. During the time interval from 2ms to ~200ms multiple charge separation occurs and the redox components of the electron transport chain become reduced. The different kinetic phases of this process show up in the fluorescence rise as the intermediate steps J, I, H, G and P. The step with the highest fluorescence intensity is called P (peak). The steps I, H and G can often only be distinguished by calculation of derivatives or calculation of differences between fluorescence transients. In the single turnover range F_o (measured at 50µs) to F_J (measured at 2ms), the step K (at about 250µs to 350µs) and the step L (at about 100µs to 200µs) can be visualised. An increase of the OJ-amplitude, according to the JIP test, corresponds to a slow-down of the dark reactions beyond Q_A ($\Psi_o = ET_o / TR_o$) (Strasser *et al*. 2000). An increase of the K step is a measure of a partial inactivation of the oxygen evolving complex (Strasser 1997). A more prominent L step indicates the transformation of a sigmoidal fluorescence rise toward an exponential rise. This indicates a decrease of energetic connectivity (grouping) between Photosystem II (PS II) units (Strasser and Stirbet 1998). We compared the photosynthetic behaviour of the 10 varieties of barley plants in two physiological states: the dark adapted state (first fast fluorescence rise) and the light adapted state (fifth fast fluorescence rise), during the adaptation period with actinic light. The goal is 1) to rank the tested 10 cultivars according to their light tolerance and 2) to get some insight into the mechanisms and the difference in behaviour of the three groups of plants: light sensitive, light intermediate and light tolerant.

Materials and Methods

Plant material

Ten cultivars of barley plants (*Hordeum vulgare* L.) provided by INIA (Instituto Nacional de Investigación y Tecnologia Agraria y Alimentaria) of Spain were used in our study: BG77–45 (A), BG79–35 (B), BG74–75 (C), Zaida (D), BG87–42 (E), BG91–87 (F) BG78–39 (G), BG79–72 (H), BG74–66 (I) and BG76–16 (J). After germination in petri dishes for 48h, seedlings were placed in pots (4 litres). They were grown in regularly watered soil substrate (1/3 sand, 1/3 peat and 1/3 compost) under long day conditions (illumination 16h day^{-1}, HQI 400 W) in a greenhouse. The experiments were performed on two week old barley leaves.

Chlorophyll a fluorescence

Chlorophyll *a* fluorescence transients were measured at room temperature with a portable fluorimeter (Plant Efficiency Analyser, built by Hansatech Instruments, King's Lynn Norfolk, UK) with high time resolution (10µs). After 60min dark adaptation, the leaves were exposed to strong 1s light pulses (600W m^{-2}), which were provided by an array of six light-emitting diodes (peak 650nm) focused on the sample surface (4mm^2). The light stress was applied by pulses which were given at a rate of one pulse per min for 5min with actinic light (18W m^{-2}) between the strong light pulses. The Chl *a* fluorescence emission induced by the strong light pulses was measured and digitised between 10µs to 1s by the instrument. Parameters describing the light adapted state can be compared with the corresponding parameters referring to the dark adapted state. The dark adapted state serves as a reference to the light adapted state. In the following we will refer to the dark adapted state as (^{d}) and to the light adapted as (^{l}) (Strasser *et al*. 1999).

JIP-test

Based on the measurement of the OJIP fluorescence transient, the JIP-test uses the model of the energy fluxes in biomembranes to calculate several phenomenological and biophysical expressions for a given physiological state (Strasser 1986). It was shown to be a very useful tool for the *in vivo* investigation of the adaptive behaviour of the photosynthetic apparatus and, especially, of PS II to a wide variety and combination of stressors, as it translates shape changes of the OJIP transient to quantitative changes of a set of parameters (Srivastava and Strasser 1996, 1997, Krüger *et al*. 1997, Strasser *et al*. 1998, 2000, Nussbaum *et al*. 2001). All photosynthetic parameters were calculated according to the equations of the JIP-test, by using the program Biolyzer (Maldonado-Rodriguez 1999–2002). Under our conditions, ^lF_o is taken as equal to ^dF_o. The expressions for the photosynthetic Performance Index relative to an equal chlorophyll absorption (PI_{abs} = PI) and the normalised Area (Sm) are:

$$Sm = Area / (F_M - F_o) \text{ where } Area = \int_{F_o}^{F_M} (F_M - F_t)dt$$

This is the area above the fluorescence rise between F_o and F_M.

$$PI = [\gamma_o / (1-\gamma_o)] \cdot [\varphi_{Po} / (1-\varphi_{Po})] \cdot [\Psi_o / (1-\Psi_o)]$$

where Sm is a function of the number of electrons transported by PSII in the time range from 0 to tF_M, the time to reach the maximum fluorescence intensity. The symbol γ_o represents the ratio of reaction centre chlorophylls and the total chlorophyll of PSII in the time range of the fluorescence rise (50µs to 1s). φ_{Po} is the fraction of excitons trapped per photons absorbed. It corresponds to the maximum quantum yield of primary photochemistry. Ψ_o is the fraction of electrons transported beyond Q_A- per excitons trapped by the RC of PSII. It is the probability that the energy of a trapped exciton is used for electron transport beyond Q_A. F_t is the fluorescence intensity at time t and F_o and F_M are the minimum and maximum fluorescence intensities of the fluorescence

rise with saturating light.

The Performance Index (PI) can be calculated with the measured fluorescence signals as follows:

$\gamma = chl_{RC} / chl_{total} = chl_{RC} / (chl_{antenne} + chl_{RC})$ and therefore

$\gamma / (1 - \gamma) = chl_{RC} / chl_{antenne}$ this corresponds with the terminology of the JIP-test where:

$RC / ABS = (RC / TR_o) \cdot (TR_o / ABS) = V_J / (dVdt_o) \cdot \varphi_{Po}$

V_J is the relative variable fluorescence at the J-step calculated as

$V_J = (F_{2ms} - F_{50\mu s}) / (F_M - F_{50\mu s})$,

φ_{Po} is the maximum quantum yield of primary photochemistry TR_o / ABS where φ_{Po} is calculated as

$\varphi_{Po} = 1 - (F_{50\mu s} / F_M) = F_V / F_M$ therefore

$\varphi_{Po} / (1 - \varphi_{Po}) = F_V / F_o = k_P / k_N = (F_M - F_{50\mu s}) / F_{50\mu s}$

$\Psi_o = ET_o / TR_o = 1 - V_J$ therefore

$\Psi_o / (1 - \Psi_o) = (1 - V_J) / V_J = (F_M - F_{2ms}) / (F_{2ms} - F_{50\mu s})$

Statistical Analysis

Table 1 shows the averages and standard deviations for the three main expressions (F_v / F_M, Sm and PI) of a group for 30 dark adapted samples. All data were analysed using the SPSS 9.0 for windows statistical package. The Student-test was used to compare means for the two parameters ($^{l}Sm / {}^{d}Sm$ and $^{l}PI / {}^{d}PI$) at $P < 0.05$.

Results and Discussion

Dark and light adapted states

The fast fluorescence rise of plants which were dark adapted for 1h defines the dark adapted state. After 5min of actinic illumination, the light adapted state was reached. It was characterised by the fast fluorescence rise provided by the fifth strong light pulse of 1s. Upon illumination of the sample after dark adaptation, a rise of the Chl *a* fluorescence is induced from an initial minimum value F_o (point O) to a maximum level F_M (labelled as point P) as shown in Figure 1. The measured fluorescence transients show that the PSII fluorescence yield increases following triphasic kinetics (O–J), (J–I) and (I–P) (Strasser *et al*. 1999). The three phases have been interpreted in the following way: (O–J) is the photochemical phase, leading to the single turnover reduction of Q_A to Q_A-. The intermediate level I is suggested to be related to a heterogeneity of components in the filling up of the plastoquinone pool. The P level is reached when all the accessible electron carriers between Q_A and ferredoxin are reduced to PQH_2 (Strasser *et al*. 1995). Upon a more detailed analysis, the O to J phase can be split into the O, L, K, J bands and the J to P phase into the J, I, H, G…P bands.

Performance Index (PI) and normalised Area (Sm)

In Figure 2, the different cultivars were ranked on the basis of the ratio light adapted to dark adapted state of the Performance Index and the normalised Area separately. In this way we tried to identify genotypes that respond in the same way to light stress. For further investigation three classes of barley plants were formed. The first class contains two varieties: BG77–45 (A) and BG79–35 (B), the second class contains four varieties: BG74–75 (C), Zaida (D), BG87–42 (E) and BG91–87 (F) and the third class contains four varieties: BG78–39 (G), BG79–72 (H), BG74–66 (I) and BG76-16 (J).

A high correlation ($r^2 = 0.8$) was found between the rank of the photosynthetic Performance Index ($^{l}PI / {}^{d}PI$) and the corresponding normalised Area ($^{l}Sm / {}^{d}Sm$) (Figure 3). The photosynthetic Performance Index (PI) is a parameter which can be used like a biological indicator of vitality (Strasser *et al*. 1999). According to the equation mentioned in the Material and Methods, PI is the product of the expressions containing the fraction of RC per chlorophyll, the yield of the capturing of energy (φ_{Po}) and the yield of transported electrons per excitons trapped (Ψ_o). The normalised Area (Sm) is a measure of the energy needed to close all reaction centres. Thus, these two parameters indicate the vitality of the plants subjected to the given environmental stress. The variations of these two parameters indicate that the plants respond differently to light stress and thus their light stress tolerance varies. These results are the basis for our study. Because it agrees with the idea that the ratio of these two parameters in light adapted relative to dark adapted states can con-

Table 1: Average and standard deviation for the three main expressions (F_v / F_M, Sm and PI) of a group for 30 dark adapted samples. Different letters indicate significant differences between means at $P < 0.05$

	Average ± SD (n = 30)			± SD in %			Average (P = 0.05)	
Cultivars	$^{d}F_v / {}^{d}F_M$	^{d}Sm	^{d}PI	$^{d}F_v / {}^{d}F_M$	^{d}Sm	^{d}PI	$^{l}Sm / {}^{d}Sm$	$^{l}PI / {}^{d}PI$
BG74–66	0.77±0.01	15.3±1.88	11.00±2.43	1.30	12.3	22.1	0.14^{bcd}	0.14^{bc}
BG74–75	0.75±0.03	19.6±3.08	8.14±2.33	4.08	15.7	28.7	0.27^{bc}	0.17^{b}
BG76–16	0.76±0.02	19.9±3.41	9.96±2.48	2.18	17.1	24.9	0.10^{d}	0.10^{bc}
BG77–45	0.76±0.01	17.9±2.90	9.71±1.23	1.31	16.2	12.7	0.36^{a}	0.21^{ab}
BG78–39	0.76±0.02	18.1±2.45	9.61±3.01	2.45	13.5	31.3	0.21^{bcd}	0.15^{bc}
BG79–35	0.77±0.02	18.1±2.84	11.56±2.25	2.10	15.7	19.5	0.34^{ab}	0.29^{a}
BG79–72	0.76±0.02	16.2±2.26	8.48±1.93	2.37	13.9	22.7	0.20^{bcd}	0.13^{bc}
BG87–42	0.78±0.01	16.4±2.69	10.11±2.61	1.20	16.5	25.8	0.23^{bc}	0.19^{ab}
BG91–87	0.77±0.01	18.6±2.59	11.32±2.70	1.84	13.9	23.8	0.23^{bc}	0.20^{ab}
Zaida	0.77±0.02	19.9±4.00	10.70±3.88	1.99	20.2	36.2	0.23^{bc}	0.16^{b}
Sensitive group	0.76±0.02	17.5±2.80	9.60±2.60	2.63	16.0	27.1	0.16^{c}	0.13^{c}
Intermediate group	0.76±0.02	18.5±3.60	10.20±3.30	2.63	19.5	32.4	0.24^{b}	0.18^{b}
Tolerant group	0.76±0.02	18.3±3.00	10.20±2.40	2.63	16.4	23.4	0.35^{a}	0.25^{a}

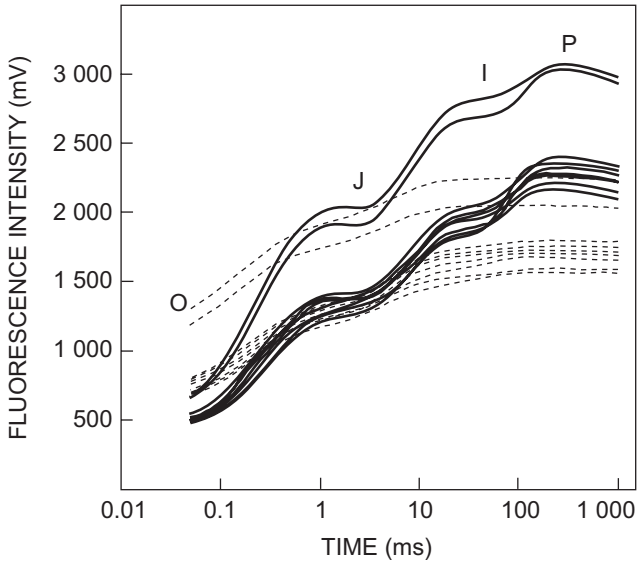

Figure 1: Chlorophyll *a* fluorescence transients of different varieties of barley plants in dark adapted (solid curves) and light adapted (dashed curves) states. The marked visible steps of the transient are called OJIP. A more detailed analysis (see Figures 5 and 6) reveals the bands O, L, K, J, I, H, G and P

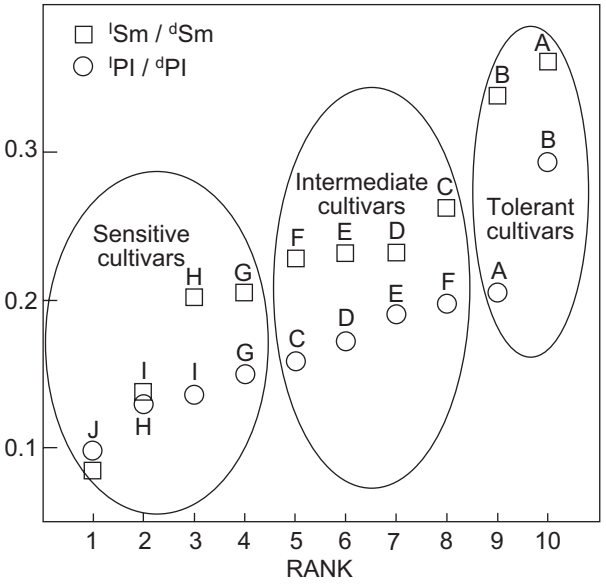

Figure 2: Ranking of the 10 different varieties of barley plant according to the Performance Index (^{l}PI / ^{d}PI) and the normalised Area (^{l}Sm / ^{d}Sm) respectively. The letters A to J correspond to the 10 different cultivars

tribute to the understanding of the physiological response and to identify a ranking among the varieties of barley plants. The ratio light adapted to dark adapted state has also been used to describe the effect of other environmental stresses. Flexas *et al*. (2002) used the ratio F_s / F_o for the early detection of water stress, where F_s is the steady state fluorescence level in the light adapted state and F_o the initial fluorescence level of the dark adapted state.

As shown in Figures 2 and 3, three possible plant groups were defined as: tolerant, intermediate and sensitive which had respectively a high, medium and low ratio of the expressions (^{l}PI / ^{d}PI) or (^{l}Sm / ^{d}Sm). The variability observed between different groups in their response to high light stress is probably due to the efficiency of utilisation of absorbed light by chlorophyll used in photosynthesis. The sensitive group has a low Performance Index, the same result was found in sensitive genotypes under cold stress (Fracheboud *et al*. 1999).

In Figure 4, the Chl *a* fluorescence transients of 60min dark adapted and light adapted barley plants for each stress class (sensitive, intermediate and tolerant), are plotted on a logarithmic time scale. The transients of the groups of Figures 2 and 3 were averaged and plotted in Figure 4. These curves show two intermediate steps J and I between the minimum level O and the maximum level P. The J and I steps are more prominent in the dark adapted than in light adapted states. The initial Chl *a* fluorescence yield, the O level (50µs), reflects the minimal fluorescence yield when all Q_A are in the oxidised state. The maximal measured fluorescence intensity, P, here equals F_M since the excitation intensity was high enough to ensure the closure of all photosystem II RCs. In the inset of Figure 4, the relative variable fluorescence transients (V_t) are shown, [$V_t = (F_t - F_o) / (F_M - F_o)$]. The plants of each group are represented by specific fluorescence curves.

In Figure 5, fluorescence transients of light and dark adapted leaves were normalised between 50µs and 250µs and the difference light minus dark curves were plotted. An additional peak around 100–120µs can be observed. The shape of the induction curve in this (L) region is influenced by the excitation energy transfer between PS II units, commonly denoted as connectivity or grouping (step L) (Strasser and Stirbet 1998). The difference between the curves of the dark and light adapted states in the same group shows that the variation between dark and light adapted states in the grouping probability G decreases (ungrouping) from the sensitive group to the tolerant group.

Figure 6a shows that the succession of the variable fluorescence transient curves between J and P of different groups is identical in dark and light adapted states. The J level of the fluorescence transient is higher in the sensitive group; the J level is strongly determined by the redox state of the electron carriers at the PS II acceptor side (Strasser *et al*. 2000). In Figure 6b, the difference (ΔV) between light adapted states of two groups (S-In, T-In or In-In) present three curves which have a peak labelled as I–H step, ($V_S - V_{In}$) has a positive peak contrary to ($V_T - V_{In}$) which has a negative peak. (^{l}V – ^{d}V) in the Figure 6c, shows two peaks between I–H and between the zones H–G, the succession of the three fluorescence transients is the same as in the Figure 6a. The $\Delta V = V_s - V_{In}$ or $\Delta V = V_T - V_{In}$ of the dark adapted states show also two peaks labelled as H and G steps (Figure 6d).

Previous studies indicated that in the range between the L and J a K step becomes dominant after heat treatment (Guissé *et al*. 1995). In our room temperature study this step

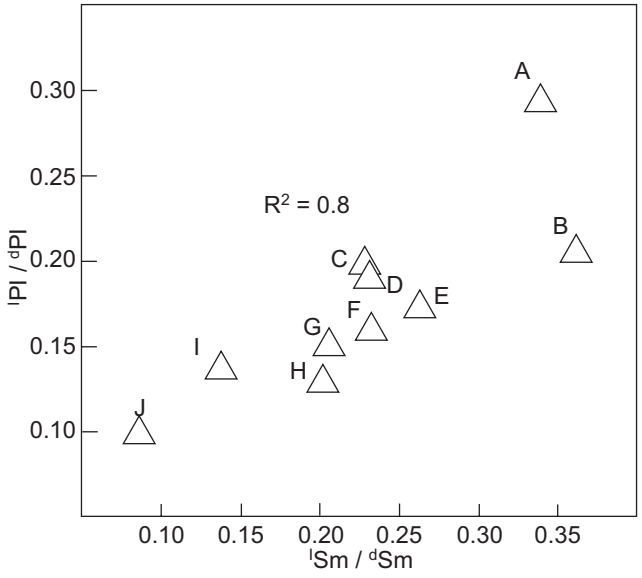

Figure 3: Correlation between Performance Index (ᴵPI / ᵈPI) and the normalised Area (ᴵSm / ᵈSm) for the different cultivars A to J

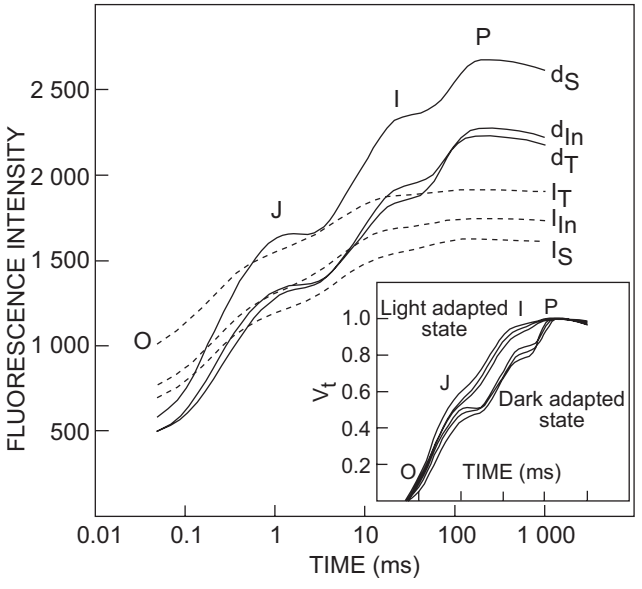

Figure 4: Polyphasic fluorescence curves of the dark and light adapted states for the three groups of barley plants (S: sensitive, In: intermediate and T: tolerant). Chl *a* fluorescence transient of barley plants in dark adapted state is marked as (ᵈ) and in light adapted state as (ᴵ). Inset shows Chl *a* fluorescence transients normalised between $^{d}F_o$ and $^{d}F_M$ or $^{I}F_S$ and $^{I}F_M$

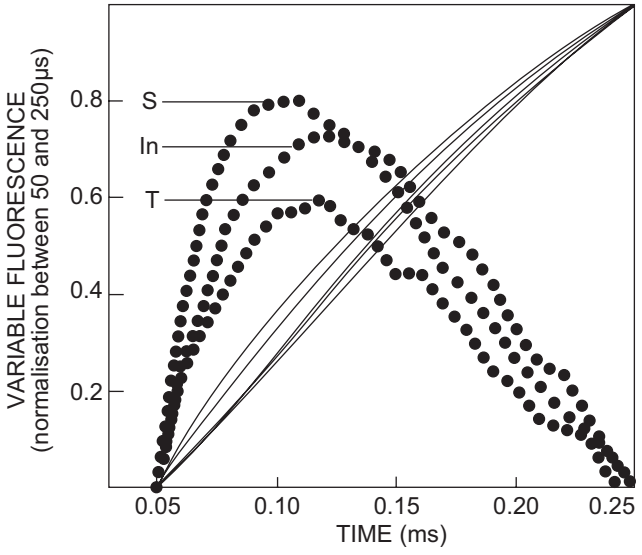

Figure 5: The L-band of the fluorescence transient. Chl *a* fluorescence normalised between F (50µs) and F (250µs) (solid curves). The dotted curves correspond to the difference between the curves in the light adapted and in the dark adapted states (S: sensitive cultivar, In: intermediate cultivar and T: tolerant cultivar)

does not show up.

The experimental results show the existence of an adaptive mechanism of plants to adapt optimally to light. In fact, the environmental conditions never cease to manifest alterations and the system is perpetually undergoing stress — stress adaptation processes, searching for harmony with its environment (Tsimilli-Michael and Strasser 2002). The efficiency of the utilisation of absorbed light energy by the photosynthetic apparatus is controlled by regulatory mechanisms that determine how much excitation energy is used and how much is dissipated as heat or in another form of energy. The thermodynamics for optimality, sub-optimality creates a driving force, under which the system undergoes state changes towards a new constellation of conformational parameters (Strasser 1986). It is evident that changes in the light environment have a profound influence on the structure and function of the photosynthetic apparatus (White and Critchley 1999). The way a specific cultivar adapts to the environmental light conditions seems to correlate to its sensitivity or tolerance to light and others stress. A close analysis of the different time zones of the fluorescence transient OLKJIHGP as shown in Figures 5 and 6 allow us to define expressions for the ranking and distinction of different cultivars into classes of stress sensitivity. Cultivars that lose the energetic connectivity between PS II antennae and have a lower electron transport rate, are more light sensitive (Figure 5). This is seen already in the dark adapted state and it remains in the light adapted state (Figures 6a, 6b, 6d). In terms of relative variable fluorescence, the light sensitive cultivars show a well pronounced higher I band (10ms) and a less pronounced H band (30ms) than the intermediate cultivars (Figure 6c).

In conclusion, the analysis of the measured fluorescence transients (dark and light adapted states) can be used as a source to predict the vitality of a photosynthetic sample and the tolerance of the plant in a stress environment. The results presented, which have been obtained by applying the proposed method to barley plants, justify the development of a procedure for phenotyping and for quantification of the health status of plants in precision farming and crop agriculture research. Ongoing additional studies will improve these

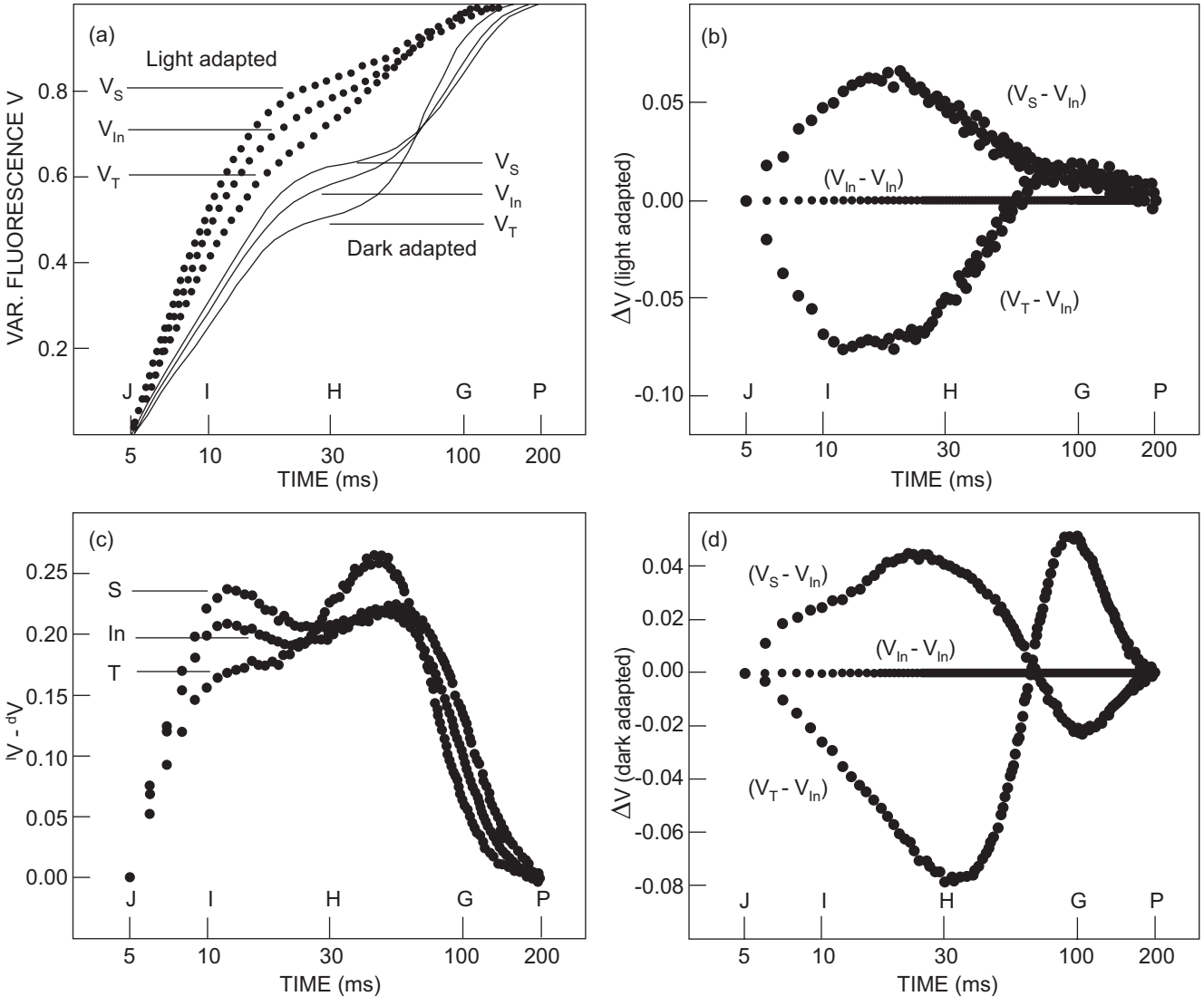

Figure 6: Transients of the relative variable fluorescence V of the three groups (S: sensitive, In: intermediate and T: tolerant) of barley plants normalised between the J (2ms) and P (500ms) steps (a). The difference of the fluorescence transients in the light adapted state show a band between J and P steps (b), this band is observed in the difference of fluorescence transients in dark adapted state (d) with another band G. In (c), the difference of the curves in light and dark adapted states of sensitive, intermediate and tolerant groups show the distinguishable bands between J and P called I, H and G. V stands for the relative variable fluorescence normalised between the J and P steps: $V = (F_t - F_{2ms}) / (F_M - F_{2ms})$

techniques. Direct fluorescence measurement with high time resolution combined with the JIP-test allow monitoring of the dynamics of the sample over five orders of magnitude in time (for HandyPea, a newer version of the PEA instrument used in this study from 20µs to 1s) with an experimental time of only one second.

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