HEAT TOLERANCE AND HEAT HARDENING IN CROP PLANTS MEASURED BY CHLOROPHYLL FLUORESCENCE

by

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Variable chlorophyll fluorescence (F_v), indicative of the capacity for electron flow through photosystem II, decreased as leaves became heat injured. In leaves heated at a rate of 1 °C per minute, F_v became undetectable above 43.3 °C in barley, 44.2 °C in pea, 45.2 °C in bean, 45.3 °C in tomato, 49.5 °C in maize and 51.0 °C in papaya. In heat hardened barley this temperature was increased by 6 to 9 °C compared with unhardened barley. Measurement of F_v in vivo provides a rapid method to monitor the onset of cellular heat injury, to rank plants in order of heat resistance and to follow heat hardening. It appears to be suitable for developing a mass screening test for heat tolerance.

1. INTRODUCTION

Plants inhabiting areas receiving high solar heat irradiation have evolved a remarkable variety of morphological and biochemical defences against the effects of high temperature. These

attributes are not shared by most plants used for production of food and fibre. The ever present need to extend agriculture into regions receiving high insolation, together with the increasing salinity in established growing areas creating as

it does pressures towards decreased transpirational rates with their attendant increases in leaf temperature, point to an urgent need for the selection and breeding of plants with increased tolerance to heat.

Non-destructive methods for comparing heat resistance and for following heat hardening are available and range from measurements of photosynthesis, F_0 fluorescence of chlorophyll, respiration, protein turnover and electrolyte leakage in intact tissue, to observations at the cellular level of vital staining, plasmolysis and protoplasmic streaming (2, 4, 14, 24, 29). Alternatively, assays of photochemical and enzymic activities in cell-free extracts can be used. None, however, is suitable as a method for screening large populations for heat resistance because of the complexity, time taken or subjective nature of the tests. We present here data to show that rate of rise in the variable component of chlorophyll fluorescence *in vivo* is suitable for routine comparisons of heat resistance and treatments for heat hardening in plant physiological and horticultural studies, and for the development of a screening procedure for heat tolerance in crop plants.

2. MATERIALS AND METHODS

2.1. Plant Material

Leaves that had just reached full expansion were used for heating experiments and were taken from 2 to 3 week old plants of barley (*Hordeum vulgare* L., cv. Svalöfs Bonus), bean (*Phaseolus vulgaris* L.), maize (*Zea mays* L.) and pea (*Pisum sativum* L.). The plants were grown in a growth chamber with a 16 hour day at 28 °C (6700 lux) and an 8 hour night at 24 °C. Leaves of *Carica papaya* L. were taken from 2 m high plants growing in a growth room at 20 °C under continuous light (1000 lux). Leaves of tomato (*Lycopersicon esculentum* Mill. var Supravite) were harvested from plants growing in a temperature controlled greenhouse (25 °C/20 °C).

Hordeum vulgare L. cv. Abyssinian was used for heat hardening experiments. Seeds were germinated for 2 days in running tap water and then transferred to a glass chamber surrounded by heated water and illuminated from above by white light (5000 lux, 12-hour day). Two heat

hardening regimes were employed. In one, plants were grown at 37 °C (day) 29 °C (night) for 10 days; in the other, plants were grown at 35 °C/28 °C for 4 days then at 40 °C/28 °C for 2 days. Control plants were grown at 25 °C/19 °C. The primary leaf was used for all experiments.

2.2. Chlorophyll fluorescence measurements during heating

Chlorophyll fluorescence induction kinetics on sections or discs of leaves were recorded with a fluorometer Model SF-10 (Richard Branker Research Ltd., Ottawa). The leaf material was placed on moist filter paper, abaxial surface down, on top of a fluted brass column standing in a water bath. The measuring unit of the fluorometer was positioned above the leaf tissue and separated from it by a layer of plastic film and an insulating 1-mm thick nylon annular disc. The heating rate of the water bath was controlled at 1 °C per min using either a Heto gradient heating bath type 02 PG 623 UL (Heto Birkerød, Denmark) or a Braun 1480 Termomix and Braun 1490 temperature programmer. Leaf temperature was monitored with a thermocouple (± 0.05 °C). Because of heat losses in the system, leaf temperature increased at slightly less than 1 °C per min. Plants were dark-adapted for one hour before the leaves were used, and the irradiance, time and frequency of the fluorescence measurements chosen to allow for dark recovery between readings.

3. RESULTS

3.1. Heat resistance

Figure 1 illustrates changes in chlorophyll fluorescence induction kinetics during heating at 1 °C per minute of leaves of two cereals, barley and maize, which prefer a cool and a warm growing season, respectively. Immediately upon irradiation a leaf, chlorophyll fluorescence yield rose (fluorescence of constant yield at physiological temperatures, F_0), then after a slight delay the fluorescence yield rose again (fluorescence of variable yield, F_v) to reach a peak after about one second, and then slowly declined. With heating, as the leaf cells became heat stressed, two prominent changes occurred in the fluorescence

Figure 1. In vivo chlorophyll fluorescence changes in barley and maize secondary leaves heated at 1 °C per min.

Leaf temperature at the start of each fluorescence measurement (irradiation time: 2.8 s; 11.2 $\mu\text{E m}^{-2} \text{s}^{-1}$) is shown. The measuring unit of the fluorometer was positioned on the leaf one-third of the distance from the tip, avoiding the central vein.

induction kinetics. Firstly, the rate of rise and the magnitude of F_v , i.e., the increment in fluorescence yield above F_0 , decreased to the point where it could no longer be measured and secondly, at somewhat higher temperatures, F_0 increased in size reaching a maximal value around 50 °C or higher. These changes took place at higher temperatures in maize than in barley.

F_0 also showed changes with temperature prior to the main increase commencing around 45 °C, the extent of the change varying with the leaves and species used. Studies with passionfruit leaves suggested that this change may be an indicator of early thermal damage (24).

The values for the maximal rate of the F_v rise, F_v , and F_0 are plotted against leaf temperature in Figure 2 for the two cereals and four dicots.

Figure 2. Changes in »constant« fluorescence (F_0), variable fluorescence (F_v) and the rate of the F_v rise (relative units s^{-1}) in leaves heated at $1\text{ }^{\circ}\text{C per min}$.

A fluorescence measurement (irradiation time: 2.8 s ; $11.2\text{ }\mu\text{E m}^{-2}\text{ s}^{-1}$) was taken with each degree rise in leaf temperature.

Again heating was at the rate of $1\text{ }^{\circ}\text{C per min}$. In the case of the pea leaf for example, both F_v and the rate of the F_v rise decreased above $38\text{ }^{\circ}\text{C}$ and could not be detected above $44.2\text{ }^{\circ}\text{C}$. F_0 increased slowly as the leaf temperature was raised, but above $43.9\text{ }^{\circ}\text{C}$ it increased more rapidly attaining a peak at $48\text{ }^{\circ}\text{C}$. The other plants tested showed similar trends with increasing leaf temperature; however, the quenching of F_v and the main increase in F_0 did not occur at the same temperatures for each plant and clear differences in heat sensitivity between the six species were discernible. If the temperature at which the curve for the rate of the F_v rise projected to zero was used as a basis of comparison of heat sensitivity, then barley and pea were the most sensitive, barley slightly more so than pea (Table I). Bean and tomato showed the same degree of heat sensitivity, but both were more resistant than either barley or pea. Maize was more resistant still and papaya (paw paw) the most resistant of all. The same ranking was obtained when the temperatures producing a 50% decrease in the rate of the F_v rise were compared (Table I).

The temperature at which F_0 starts to increase rapidly has been used previously to compare the heat resistances of different plant species (3, 4, 19, 22, 24, 27). In references (24) and (27), this value corresponds to the temperature value t_2 . This temperature value gave the same ranking as the one based on changes in F_v (Table I). The temperature at which F_0 reached a peak gave a similar ranking, although the temperature difference between the first 3 plants listed was only one degree.

3.2. Heat hardening

While growth temperature generally has little influence on heat tolerance, a specific hardening or change in heat tolerance can be elicited in many species by exposure to near lethal tempera-

Table I

Temperatures producing chlorophyll fluorescence changes in leaves heated at 1 °C per min.

Plant	50 % decrease in F_v rise	F_v rise = 0	Start of F_0 increase*	F_0 maximum
Barley	40.7 °C	43.2 °C	42.7 °C	48.6 °C
Pea	42.1 °C	44.2 °C	43.9 °C	48.0 °C
Bean	44.3 °C	45.2 °C	44.5 °C	49.0 °C
Tomato	44.9 °C	45.3 °C	44.0 °C	50.0 °C
Maize	47.3 °C	49.5 °C	46.2 °C	52.5 °C
Papaya	47.9 °C	51.0 °C	47.0 °C	53.5 °C
Temperature range	7.2 °C	7.9 °C	4.3 °C	5.5 °C

* temperature at which the main increase in F_0 begins, determined as described by SCHREIBER and BERRY (29). Figure 3. Measurement of heat hardening in barley leaf from the change in rate of the F_v rise during heating.

Plants were first heat hardened as described in Materials and Methods and the excised primary leaf heated at 1 °C per min. Fluorescence measurements (irradiation time: 1.2 s; 20 $\mu\text{E m}^{-2} \text{s}^{-1}$) were taken with every 1.5 degree rise in leaf temperature. Values for unhardened leaves are divided by 2.

Table II

Non-reversibility of the F_v rise and reversibility of F_o after heating.

Pea leaves were placed on a moist filter paper on an aluminium plate and covered with plastic film. Chlorophyll fluorescence induction kinetics were recorded for each leaf and the plate was then immersed in a water bath at the temperatures specified in the table. Heat treatment was terminated by immersing the plate in water at 21 °C for 4 min. Chlorophyll fluorescence was again recorded for each leaf and at various times thereafter during which time the temperature of the leaves was maintained at 21 °C. Each value is the average ($\pm$ standard error) for 8 leaves of estimations of the maximal rate of the F_v rise (chart units s^{-1}) and F_o (chart units).

Heat treatment	Fluorescence parameter	Before heating	After heating		
			6 min	36 min	22 hours
40 °C, 10 min	F_v rise, rate	43.4 $\pm$ 2.6	22.4 $\pm$ 2.4	20.8 $\pm$ 1.8	14.3 $\pm$ 2.6
41.5 °C, 10 min		42.1 $\pm$ 4.0	5.5 $\pm$ 0.4	6.5 $\pm$ 0.4	3.4 $\pm$ 0.8
47 °C, 5 min		39.5 $\pm$ 2.6	0	0	0
40 °C, 10 min	F_o	23.0 $\pm$ 1.6	20.1 $\pm$ 1.0	21.8 $\pm$ 1.1	18.2 $\pm$ 2.6
41.5 °C, 10 min		24.4 $\pm$ 2.5	20.6 $\pm$ 1.1	24.1 $\pm$ 1.8	24.4 $\pm$ 2.6
47 °C, 5 min		27.6 $\pm$ 2.6	27.8 $\pm$ 1.3	25.9 $\pm$ 2.6	29.3 $\pm$ 2.6

tures (2, 4, 19, 21, 22, 29). Figure 3 shows changes in the rate of rise of F_v in barley primary leaves heat hardened using two different temperature regimes for growth. In the unhardened leaves from plants grown at 25 °C/19 °C, F_v was undetectable above 43 °C–44 °C, as shown previously for secondary leaves (Figures 1 and 2). This temperature was increased by 6 °C to 9 °C in leaves from heat hardened plants (Figure 3). The increase in F_o also occurred at a higher temperature in hardened compared with unhardened plants and can also be used as an indicator of heat hardening in barley, as has been demonstrated previously for other plants (4).

3.3. Changes in F_v and F_o after heating

The experiments described above and the other studies cited indicate that if fluorescence measurements are made during heating, either the decrease in F_v or the increase in F_o can be used to compare heat resistances in leaves of different species. However, F_o may not be suitable to estimate heat damage using measurements made before and after a heat treatment (rather than during heating) as it has been reported (13) that the enhanced F_o yield in heated *Chlorella* cells decreased when the temperature was again lowered. In Table II it can be seen that

this was also the case for leaves; the heat induced increase in F_o (Figure 2) was reversible after heating whereas the decrease in F_v was not. In these experiments, three separate heat treatments were applied to different batches of pea leaves. These, in order of increasing temperature, produced a 48 %, 87 % and 100 % inhibition of F_v based on the decrease in the rate of the F_v rise determined from fluorescence measurements made before and after (6 min readings) the heat treatments. There was no recovery in leaves maintained for an additional 30 min at 21 °C after heating. Nor was there any long term recovery (22 hour readings) following the heat treatment. In contrast, values for F_o showed little change following all three heat treatments. While only a small increase in F_o would have taken place in the leaves heated at 40 °C and 41.5 °C, F_o would have attained the maximum value during heating at 47 °C for 5 min (Figure 2).

4. DISCUSSION

The Hill reaction in isolated chloroplasts is sensitive to heat (11), the heat labile site being located on the photooxidizing side of the photosystem II reaction centre (12, 28). Indeed, it appears to be one of the most heat sensitive

reactions in green plant cells and temperatures around 40 °C are often sufficient to cause thermal inactivation (1, 16, 17, 20, 26). The inactivation of photosynthesis by temperatures around 40–45 °C suggests that the oxygen evolving reaction is equally heat labile in intact tissue and essential cell functions such as respiration and maintenance of semipermeability of cell membranes are affected at considerably higher temperatures (4, 5, 6).

The fluorescence of chlorophyll *in vivo* provides a sensitive non-destructive probe for photosynthetic electron transfer activity in algae and green plant tissue (18). Visible irradiation on a leaf results in a red fluorescence emission from chlorophyll that first rises rapidly, usually within 1 ns, to the F_0 level. This is fluorescence of constant yield at normal physiological temperatures, but the yield increases in heat damaged leaves (13). However, F_0 decreases again when the temperature is lowered (ref. 13 and Table II), even though the photosynthetic apparatus may have suffered irreparable heat damage, and as such it is not feasible to use measurements of F_0 as the basis of a screening method in which large numbers of leaf samples are heated for a fixed time, rapidly cooled and the effect of the heat treatment determined. The heat-induced increase in F_0 is very useful though in comparing the heat resistance of a limited number of samples during continuous heating as demonstrated for desert species by ARMOND *et al.* (3), PEARCY *et al.* (19), SCHREIBER and BERRY (22) and HETHERINGTON and SMILLIE (10), and for alpine, temperate and tropical species by SMILLIE and NOTT (27).

After reaching F_0 , the fluorescence yield continues to rise more slowly reaching a peak after about 1 to 2 sec. This fluorescence is of variable yield (F_v) as it is responsive to changes in electron flow through photosystem II, being regulated by the redox state of photosystem II electron acceptors (7). Inhibition of a reaction on the photooxidizing side of photosystem II abolishes F_v ; conversely, inhibition on the photoreducing side enhances F_v . High temperatures preferentially inactivate on the photooxidizing side of photosystem II and reduce the F_v emission. Preferential inactivation on the photooxidizing side of photosystem II also occurs in plants grown at low light irradiances and then irradiated with intense visible light and in plants

from the lowland tropics after exposure to a temperature of 0 °C. Consequently, changes in rate of the rise in F_v *in vivo* can be used to assay for photoinhibition (8) and chilling injury (9, 25).

The results presented here demonstrate that changes in F_v can also be used to follow the progress of heat damage in leaves during heating (Figures 1 and 2), to rank plants for heat resistance (Figure 2) and to ascertain the extent of heat acclimation or hardening (Figure 3). In the plants tested relative heat resistances were as expected, with barley and pea being the most sensitive, followed by bean and tomato, maize, and papaya. Further, the ranking obtained using F_v agreed with that using F_0 , a fluorescence parameter whose suitability for measurements of relative heat resistance in plants has been documented (3, 10, 19, 22, 24, 27). The rate of rise in F_v has also been used to compare the heat resistances of non-hardened, heat hardened and dehardened spinach leaves (21).

The measurement of variable chlorophyll fluorescence *in vivo* would appear to be a suitable one on which to base a screening test for heat tolerance. Hill reaction activity and hence F_v is not immediately reversible following thermal inactivation (Table II), and thus heat damage can be assessed by determining F_v before and after a standardized heat treatment. The temperatures resulting in a decreased F_v are those sometimes experienced by plants growing in hot climates and are lower than the temperatures required to change F_0 . The discrimination of heat resistance among species is slightly better using F_v compared with F_0 (Table I). In any screening application, recording the rate of the maximal rise in F_v rather than the magnitude of F_v before and after heating may be preferable since it would not be necessary to wait until the fluorescence yield reaches a maximum and this would shorten the measurement time required for each sample. Using a portable fluorometer of the types described by SCHREIBER *et al.* (23) and MELCAREK *et al.* (15) the reading for each leaf sample could be completed within a few seconds.

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