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An examination of factors contributing to non-photochemical quenching of chlorophyll fluorescence in barley leaves

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The 'dark' relaxation of the non-photochemical quench in leaves has been monitored by applying short saturating pulses of light, and measuring the yield of chlorophyll fluorescence. The non-photochemical quench relaxes with complex kinetics, revealing at least three components with half-times of about 1 min ('fast'), 5 min ('middle') and hours ('slow'). The 'fast' component only appears after illumination with high actinic light. This component also relaxes after switching to low actinic light, or if light pulses are applied in the 'dark'. It is selectively increased with tentoxin and decreased by nigericin, and is identified as high-energy state quenching (q_E). Very high light intensities are needed to saturate the fluorescence yield during light pulses when this component is present. The 'middle' component is saturated by relatively low actinic light intensities. The 'middle' component is affected by changing the light quality, when it correlates with a readjustment of Q_A oxidation, and it can be abolished by fluoride. It is associated with the redistribution of excitation energy (state transitions, q_T). The remaining 'slow' phase has a residual component at low light and increases markedly after illumination with very high light intensities. This component is attributed to photoinhibitory processes (q_I). It is discussed how q_E makes a very variable, and often small, contribution to non-photochemical quenching in leaves.

Introduction

Chlorophyll *a* fluorescence is increasingly being used as a non-intrusive probe in photosynthetic research. This has been made possible by an increased understanding of the parameters which affect the fluorescence yield [1-8], and the availability of commercial equipment allowing accurate measurement even under field conditions [6]. Since several factors can lead to a decreased yield of chlorophyll fluorescence, interpretation of this signal obviously depends on our ability to resolve the contribution made by each of these mechanisms. This resolution has largely depended on the work in aqueous photosynthetic systems (chloroplasts, protoplasts and algae). In these systems, specific inhibitors can be used to relate particular aspects of the fluorescence signal to a given mechanism [5,8,9]. Chlorophyll fluorescence can be quenched by using the light energy in photochemistry (q_Q), or by non-photochemical mechanisms [4,8]. Several factors can contribute to non-photochemical quenching. One major component involves high-energy quenching (q_E) [8], which is thought to reflect a mechanism for energy dissipation at PS II, triggered by a rising transthylakoid pH gradient or, more precisely, a rising proton concentration in the thylakoid lumen. However, state transitions [9], quenching by plastoquinone [10,11] and photoinhibition [12,13] have also been shown to contribute to the non-photochemical quench in various aqueous systems.

With intact leaves, alternative methods have to be employed. The photochemical quench can be resolved using the light saturation pulse technique [4,6], but the remaining non-photochemical quenching components

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Abbreviations: F_m, fluorescence yield with a saturating pulse in dark-adapted (12 h) material; F_0, ground level of fluorescence obtained using a weak non-actinic modulated beam in the dark; F_{vs}, fluorescence yield with a saturating pulse in any material which is not dark-adapted; LHCP, light-harvesting complex protein; PP_i, inorganic pyrophosphate; PS I, Photosystem I; PS II, Photosystem II; Q_A, the primary electron acceptor for Photosystem II; q_{NP}, total non-photochemical fluorescence quenching; q_E, high-energy state fluorescence quenching; q_I, fluorescence quenching due to photoinhibition; q_Q, photochemical fluorescence quenching; q_T, non-photochemical fluorescence quenching due to excitation energy redistribution (state transitions); q_f, 'fast' component of relaxation of fluorescence quenching in the presence of a weak, non-actinic, modulated light (see text for further details); q_m, 'middle' component of relaxation of fluorescence quenching (see text); q_s, 'slow' component of relaxation of fluorescence quenching (see text).

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have not been further resolved. As a result, many authors have tended to equate this remaining component (q_{NP}) with high-energy state quenching (q_E) as a measure for 'energisation' in leaves.

Recently, Demmig and Winter [14] have measured the dark relaxation of light-induced fluorescence quenching using the light-saturation technique [6]. To separate the various components of the non-photochemical quench, they measured Fv_s (the fluorescence yield during a pulse of light which fully reduces Q_A and, hence, abolishes photochemical quenching) after 2 min of darkness, and again after 12 h of darkness. The initial rapid relaxation of fluorescence quenching was assumed to be complete after 2 min and to result from relaxation of q_E. The remaining relaxation was measured as a single component between 2 min and 12 h. This showed a complex dependence on the intensity of the actinic light which had been used to illuminate the leaf, suggesting it was heterogenous. One part saturated at very low actinic light intensities, and was tentatively attributed to redistribution of excitation energy by state transitions (q_T). The other part, which only appeared at high light intensities, was ascribed to photoinhibition, and was shown to correlate with the formation of zeaxanthin [14]. However, there was no direct evidence for the identification of q_E, q_r and q_I, except for the expectation that q_E should collapse rapidly following the decay of thylakoid energisation in the dark [2,5,8,12], and that state transitions would be expected at low light intensities [9,16].

An alternative approach has been applied to barley protoplasts [9] where the kinetics of relaxation of fluorescence quenching in the dark was monitored using periodic light pulses. The kinetics could be separated into three components, using logarithmic plots, that corresponded closely to components identified using specific inhibitors [9]. A similar approach was used in the following experiments which were carried out to provide direct evidence that different components of the non-photochemical quench in leaves are associated with the development of the transthylakoid proton gradient (q_E) and with the redistribution of excitation energy, or state transition, (q_T). A third component, which reverses over a period of several hours, is also present (q_I). We will attempt to outline a rational approach to resolving these components using the light-saturation pulse technique. As will emerge, the contribution of q_E to the total non-photochemical quench varies greatly in leaves depending on the conditions.

Materials and Methods

Barley was grown as in Ref. 17. All leaf material was darkened for 12–18 h before use, unless stated otherwise. To supply inhibitors, barley leaves (10–12 cm long) were cut off about 3 cm from the base with a sharp razor, after immersing the plant under water. The detached leaves were transferred in groups of four into a microcuvette containing 700 μl of water plus 0.3 μg/ml tentoxin, 10 μM nigericin, or 20 mM NaF. For uptake of tentoxin or nigericin, the leaves were illuminated for 40 min (400 $\mu mol \cdot m^{-2} \cdot s^{-1}$) at 20 °C in moving air, and were then darkened for about 2 h in still air before starting the experiment. The leaves (0.3 g fresh weight) transpired about 300 μl of solution in this time. For uptake of fluoride, leaves were left in the dark at 20 °C for 12–15 h, over which time about 200 μl of solution was transpired.

O_2 evolution and chlorophyll fluorescence were measured using a PAM chlorophyll fluorescence measuring system (Walz, Effeltrich, F.R.G., Ref. 6) in a leaf disc O_2 electrode [7], modified as in Refs. 17 and 18. The saturating light pulses had a duration of 700 ms and an intensity of 3600 $\mu mol \cdot m^{-2} \cdot s^{-1}$. Photosynthesis was largely light-saturated at 300–400 $\mu mol \cdot m^{-2} \cdot s^{-1}$ in barley in the conditions used in these experiments. The non-actinic modulated measuring beam was used at 100 kHz in the light, and was switched to 1.6 kHz in the dark. The intensity of the measuring beam used in the dark was less than 0.1 $\mu mol \cdot m^{-2} \cdot s^{-1}$ and during actinic illumination was 1.5 $\mu mol \cdot m^{-2} \cdot s^{-1}$. Results were registered on a recorder with time compensation. Far-red light was supplied using a projector fitted with a cut-off filter (RG715). Actinic light was supplied from a Schott KL1500 light source fitted with a heat filter and with a heat reflector (Schott FL 103F).

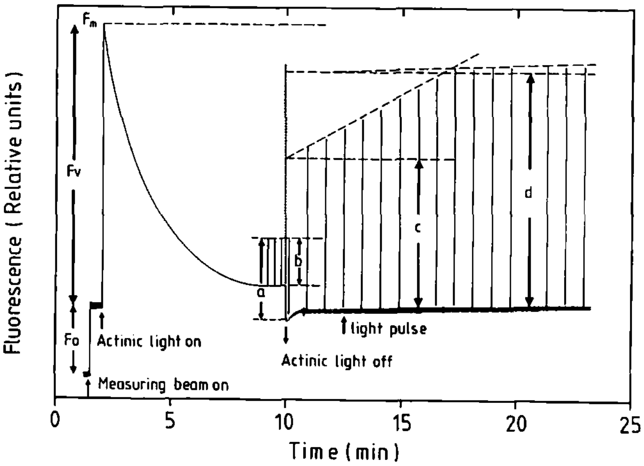

Fig. 1. Schematic representation of a typical chlorophyll fluorescence trace obtained from barley leaves. The trace shows the changes in fluorescence yield during and after actinic illumination and defines the parameters which are used to calculate the various quenching components (see text). Fv_s is the maximum fluorescence yield after a saturating light pulse minus the dark F_0 level at the time of the pulse. The amplitude of the fluorescence rise induced by a light pulse during actinic illumination is indicated by (b). The linear phases of the relaxation of fluorescence quenching for both the 'middle' and 'slow' components were extrapolated back to the time when the actinic light was turned off. The intercept values were used to measure the values (c) and (d).

Fluorescence parameters were calculated from values determined from experimental chart traces as shown schematically in Fig. 1: $q_Q = b/a$, $q_{NP} = 1 - a/F_v$, $q_f = 1 - a/c$, $q_m = 1 - c/d$ and $q_s = 1 - d/F_v$. This method of calculation takes into account changes that occur in the level of F_0 during the dark relaxation and that are associated with particular non-photochemical quenching components. When the kinetic components are calculated in this way the equation $1 - q_{NP} = (1 - q_f)(1 - q_m)(1 - q_s)$ holds true. Determination of the quenching components utilising the initial F_0 level obtained upon darkness causes an overestimate of q_m and q_s that can lead to large errors in the above equation.

Results and Discussion

Fluorescence quenching relaxation kinetics

Fig. 2 shows the rate of O_2 evolution and the yield of chlorophyll *a* fluorescence when barley leaf segments taken from dark adapted plants were illuminated for about 10 minutes and the redarkened. The actinic light (579 μmol $\cdot$ m^{-2} $\cdot$ s^{-1}) was saturating in these conditions (15 °C, saturating CO_2). There is a characteristic induction period [6,7], before attaining a steady-state rate of O_2 evolution and yield of chlorophyll *a* fluorescence. Pulses of high intensity light were given at 20-s intervals during actinic illumination, to reduce totally the primary electron acceptor of PS II (Q_A) and abolish photochemical quenching. After application of these pulses, the chlorophyll *a* fluorescence yield (Fv_s) is still appreciably quenched compared to the initial value obtained when dark adapted leaf material was given a saturating pulse (F_m). This implies that non-photochemical quenching mechanisms are also operating.

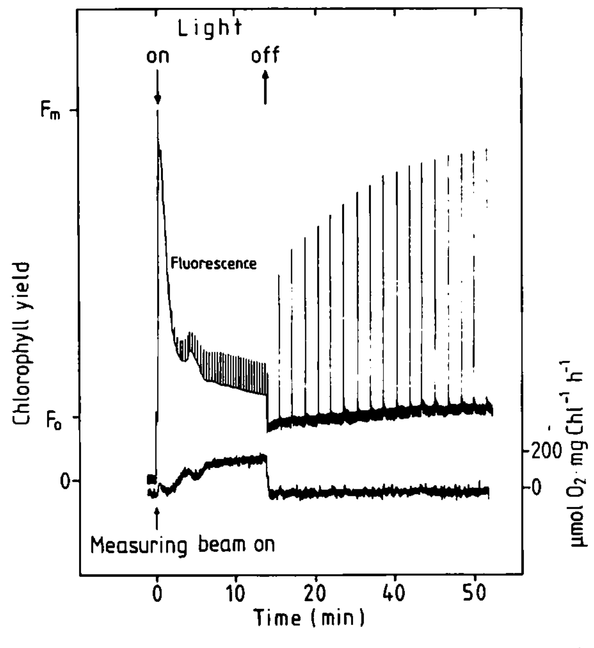

Fig. 2. 'Dark' relaxation kinetics of non-photochemical chlorophyll fluorescence quenching in barley. Leaf segments were taken from dark-adapted barley plants, and transfered to the O_2 leaf electrode. After 60 s, the weak fluorescence measuring beam was turned on, and F_0 was determined. The actinic light (579 μmol$\cdot$m$^{-2}\cdot$s^{-1}) was then turned on, and saturating pulses were applied.every 20 s. After about 12 min, the actinic light was turned off, a saturating pulse was given after 5 s darkness, and then every 100 s. The weak measuring beam was modulated at 100 kHz duirng actinic illumination, and 1.6 kHz in the 'dark'. The simultaneous recording of a differentiated signal for O_2-evolution is shown for comparison.

When the continuous actinic illumination is turned off, the fluorescence signal returns to a new depressed F_0 level; signal noise also increases at this point due to a reduction in the intensity of the measuring beam by reducing the modulation frequency. The rate at which the fluorescence level falls to the new F_0 level depends on the rate of reoxidation of Q_A. Under some conditions this can be quite slow, but can be accelerated by the application of a weak far-red light (data not shown). Generally, a slow relaxation of F_0 is associated with conditions where there is some limitation of photosynthesis, for example during photosynthetic induction, or the 'peaks' of fluorescence [7] during photosynthetic oscillations (data not shown), or after blocking ATP synthesis (see below). Once the F_0 signal has been established it starts to rise again, at first rapidly during the initial 1–2 min in the dark, and then much more slowly over the next 10–15 min.

By applying saturating light pulses at intervals in the dark the recovery of Fv_s can also be measured, revealing the 'dark' relaxation kinetics of the non-photochemical quench. In Fig. 2, a saturating light pulse was applied 5 s after removing the actinic illumination, and then every 100 s. The Fv_s value obtained after 5 s darkness was appreciably below the Fv_s value obtained by a pulse during steady-state photosynthesis. This is because the intensity of the light pulse used in this experiment was not saturating for fluorescence yield at this particular time point (see later). The subsequent pulses, which are saturating (see later), applied at 100 s intervals, reveal that Fv_s rises with increasing time in the dark, and eventually approaches the F_m value obtained at the start of the experiment. There is a rapid rise for the first 100 s in the dark, followed by a gradual linear rise for the next 12 min and then an even slower rise which continued for about 3 h (the full experiment is not shown in Fig. 2). We will refer to these provisionally as the 'fast' (q_f), 'middle' (q_m) and 'slow' (q_s) components, respectively, of the non-photochemical quench. As will be shown later, these components are similar to the components q_E, q_T and q_I, which are commonly refered to in the literature.

Experiments with different species of plants or individuals grown under different conditions reveal that the 'dark' relaxation kinetics are very variable (data not shown). This variability means that there may be no one

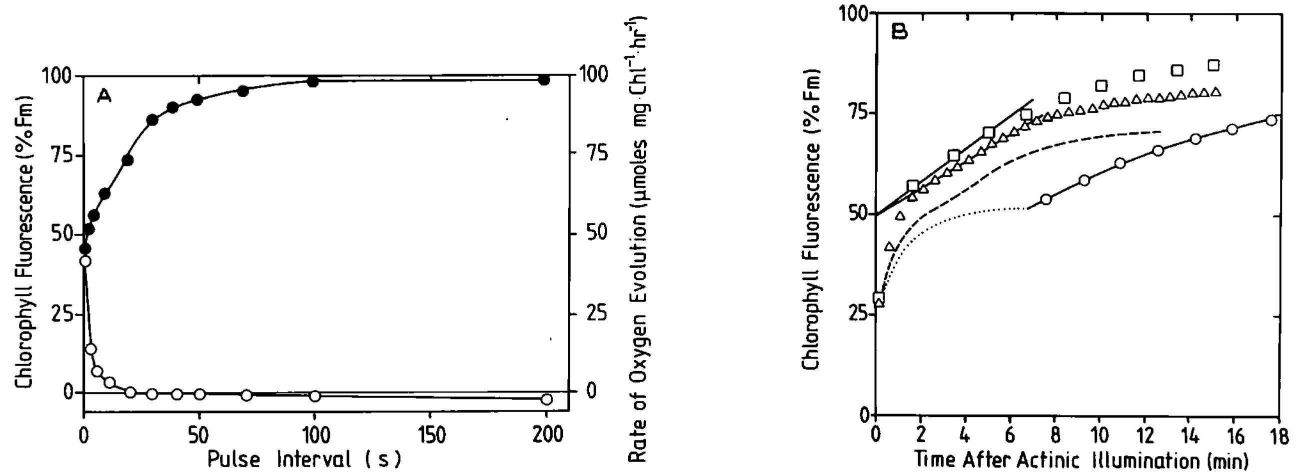

Fig. 3. Influence of pulse frequency on fluorescence yield from saturating light pulses in barley leaves. (A) Dependence of the steady-state value of Fv_s on the interval between saturating pulses given in the dark (●). For comparison the average rate of O_2 evolution is shown (○). (B) Effect of flash frequency on the 'dark' relaxation kinetics of Fv_s after removing actinic illumination. Barley leaves were illuminated for 12 min at 579 μmol·m^{-2}·s^{-1}, and were then darkened and saturating pulses applied 100 s (□), 30 s (△), every 20 s (———) or 7 s (······). For the latter, the flash frequency was then lowered from 7 s to 1100 s (○) after 6.5 min. The results are given as a percentage of the F_m, the fluorescence yield with the first flash given to dark-adapted leaves.

universal procedure for resolving the components of non-photochemical chlorophyll fluorescence quenching. Rather, the procedure may have to be defined for any one particular plant species and growth condition. In the remainder of this article, we will consider the resolution of the components in barley leaves.

Effect of pulse frequency and intensity on the relaxation kinetics

Our approach uses repetitive high intensity light pulses to measure the 'dark' relaxation kinetics of the non-photochemical quench [9]. This is only valid if the light pulses themselves have no significant effect on what we are trying to measure. We therefore investigated whether the fluorescence yield and kinetics are affected by the frequency or the intensity of the pulses.

Fig. 3A shows the effect of light pulse frequency on Fv_s in the absence of actinic illumination. Leaf segments from 12 h dark-adapted barley plants were illuminated with a weak (1.6 kHz) measuring beam, and were immediately given one pulse of saturating light (2100 μmol · m^{-2} · s^{-1}) to determine F_m. This value is set as 100% in Fig. 2A. Flashes were then applied at 200 s intervals until a new steady-state Fv_s value was obtained. The pulse frequency was then increased, and a new steady state Fv_s was determined. Fig. 3A shows that Fv_s decreased as the pulse frequency is increased. We also measured the rates of O_2 evolution. Clearly, Fv_s can be reduced by over 25% before net O_2 evolution begins, at a frequency of 1 pulse/20 s. The pulse frequency was then progressively decreased, and Fv_s rose again in a reversible manner (data not shown), although it took longer to attain each new steady state.

In Fig. 3B, we investigated whether the pulse frequency affected the 'dark' relaxation kinetics after removing the actinic illumination. Barley leaf segments were illuminated (570 μmol · m^{-2} · s^{-1}) until steady-state rates of photosynthesis were achieved. Actinic illumination was then stopped, and saturating light pulses were applied at various frequencies in the subsequent 'dark' period. Pulsing at intervals greater than 100 s had no measurable effect on the relaxation kinetics (data not shown as this would confuse the figure). However, at higher flash frequencies the relaxation kinetics became slower, and Fv_s levelled off at a lower fluorescence yield. Only the highest flash frequency used (every 7 s) led to an apparent small net evolution

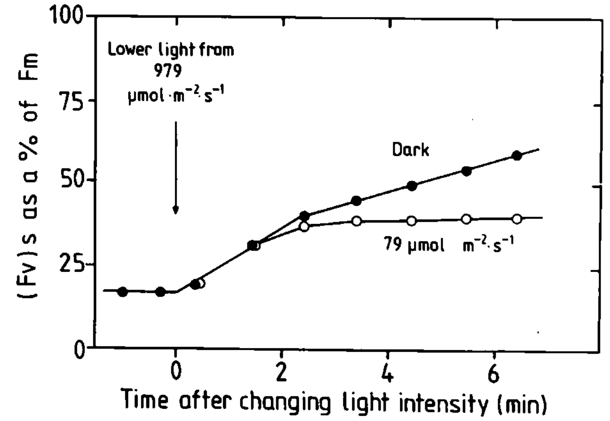

Fig. 4. Comparison of relaxation kinetics of Fv_s after decreasing the light intensity from saturating to limiting levels, or to darkness. Barley leaves were illuminated for 12 min at 979 μmol·m^{-2}·s^{-2}, and were then darkened (●) or the light was decreased to 79 μmol·m^{-2}·s^{-1} (○). Saturating pulses were applied every 60 s.

of O_2. At this frequency, Fv_s stabilised at a new steady-state value, equivalent to only 52% of F_m, after about 4 min 'darkness'. When the pulse frequency was reduced to 100 s, Fv_s now started to rise further but without any 'fast' phase. It should be noted that the stabilised Fv_s value achieved at a pulse frequency of 7 s is similar to the value obtained if we extrapolate the 'middle' phase obtained at a pulse frequency of 100 s or 30 s, and also resembles the point at which an inflexion appeared at a pulse frequency of 20 s.

We conclude that increasing pulse frequencies selectively prevents the reversal of the 'middle' component of the non-photochemical quench relaxation kinetics. Similar results were obtained for a variety of different temperatures, and after preillumination at a variety of different actinic light intensities. Reduction of the actinic light to a low intensity had a similar effect on these components (Fig. 4). Barley leaves were illuminated with saturating light and pulsed every 60 s. The relaxation of Fv_s was then compared after turning the actinic light off, or after decreasing it to 79 $\mu\text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$. The low actinic light did not interfere with relaxation of the 'fast' component, but it prevented relaxation of the 'middle' component. As will become evident later, the 'fast' and 'middle' components are also affected in a different manner by the intensity of the preceding actinic illumination.

The effect of the light intensity applied during the pulses is investigated in Fig. 5. Barley leaf pieces were allowed to attain steady-state rates of photosynthesis and then the actinic light was turned off. Light pulses, of a set intensity, were then applied after 5 s and then every 100 s. The experiment was repeated several times, using a different light intensity each time for the pulses.

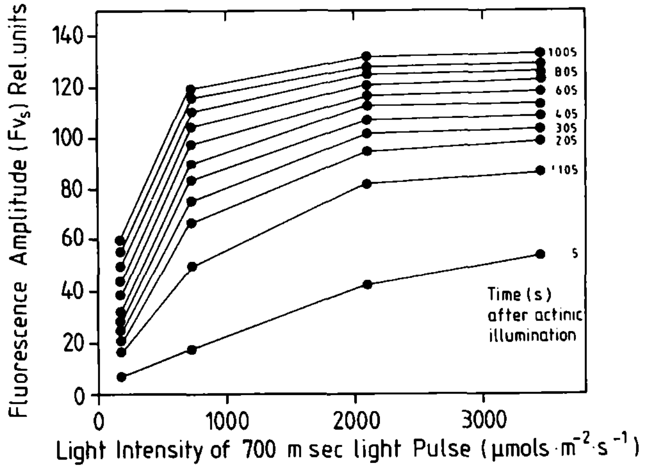

Fig. 5. The dependence of the amplitude of the fluorescence rise induced by a light pulse on the light intensity of the pulse. This was determined for light pulses applied at various times after turning off actinic illumination. Barley leaf pieces were allowed to attain steady-state rates of photosynthesis (580 $\mu\text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$) prior to removal of actinic illumination. Light pulses were then applied of varying intensities after determined times of darkness.

The measured amplitude of the fluorescence yield (Fv_s) is then plotted against the applied pulse light intensity. This reveals how the light saturation response of Fv_s is changing as the leaf adapts to the dark again. The pulse amplitude after 5 s darkness is the only light pulse that is not light saturated in our normal system (2100 $\mu\text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$) and this light pulse is not being used in the analysis of the fluorescence components anyway (see Fig. 1).

Nevertheless, Fig. 5 also reveals that the light intensity dependence ($I_{50\%}$) of the amplitude of the fluorescence rise is clearly reduced as the time after actinic illumination increases. The $I_{50\%}$ declines in a biphasic manner during the relaxation of fluorescence quenching in parallel to the 'fast' (q_f) and 'middle' (q_m) components (data not shown). This would be expected as the non-photochemical quenching mechanisms (q_e, q_t and q_I) all serve to reduce the amount of light energy available for photochemistry at PS II. Schreiber and Neubauer [19,20] have also previously shown that the fluorescence rise kinetics of a single light pulse have complex kinetics consisting of three phases (I_1, I_2 and P), each of which has a different light response curve. We will show elsewhere that the amplitude of the I_1 phase of the fluorescence rise shows only marginal changes during the 'middle' (q_m) and 'slow' (q_s) components of the relaxation of fluorescence quenching (Quick, Stitt and Horton, unpublished results).

Selective manipulation of the 'fast' component using inhibitors

If the 'fast' phase is due to q_E, it should be possible to manipulate this component selectively by feeding chemicals which alter the steady-state transthylakoid pH gradient. We therefore supplied a specific inhibitor of the chloroplast ATPase (tentoxin) [21] or an uncoupler (nigericin) to barley leaves. They were supplied via the transpiration stream for 40 min in the light, and the leaves were then dark adapted for 2 h. The detailed effect of these chemicals on metabolism has been described elsewhere [22].

Fig. 6A shows a typical fluorescence induction curve after illuminating barley leaf segments with low actinic light (79 $\mu\text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$). The experiment shown in Fig. 6 was carried out at low light intensities because the effect of tentoxin is clearest in these conditions. No significant 'fast' component is found when the actinic light is removed and light flashes are given every 100 s to monitor the relaxation of non-photochemical quenching. After feeding tentoxin, a large increase of non-photochemical quenching is seen, even at tentoxin concentrations which only marginally inhibit steady-state photosynthesis (Fig. 6B). The 'dark' relaxation kinetics demonstrate that this increase is totally accounted for by a selective increase of the 'fast' component. The 'middle' phase is present in both treatments, and is not

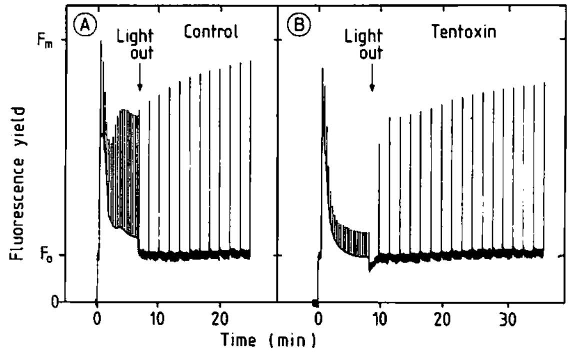

Fig. 6. Influence of tentoxin on the 'dark' relaxation kinetics of the non-photochemical quench. (A) Control. (B) 0.3 μg per ml tentoxin. The tentoxin was supplied for 40 min to detached leaves via the transpiration stream in the light, and the leaves were then darkened for 2 h before starting the experiment. The procedure was as in Fig. 1, except that the leaf segments were illuminated for 7 min at 79 $\mu mol \cdot m^{-2} \cdot s^{-1}$.

increased by tentoxin. At higher light intensities, non-photochemical quenching is already large in uninhibited leaves, and includes a substantial 'fast' phase (see below). There was an increase after feeding tentoxin, but the difference was less visually striking than at low light (data not shown).

Fig. 7 shows how marginal doses of nigericin affect the 'fast' component of these 'dark' fluorescence relaxation kinetics. High intensity actinic illumination (567 $\mu mol \cdot m^{-2} \cdot s^{-1}$) was used to generate a large 'fast' component (Fig. 7A). Preliminary experiments showed that many uncouplers lead to a large decrease of the F_m/F_0 ratio, and a large irreversible quenching of fluorescence relaxation kinetics. Moderate concentrations of nigericin, however, had little effect on the F_m/F_0 ratio (Fig. 7B). These concentrations lengthened the induction period, but had little effect on the steady-state rate of O_2 evolution. They markedly decreased the 'fast' component of the 'dark' relaxation (Fig. 7B). At higher nigericin concentrations the 'fast' phase was almost completely abolished, but other features of the fluorescence signal were also affected, including the F_m/F_0 ratio (data not shown). Similar results to nigericin were obtained by supplying 20–30 mM ethylamine or hexylamine as uncouplers (data not shown).

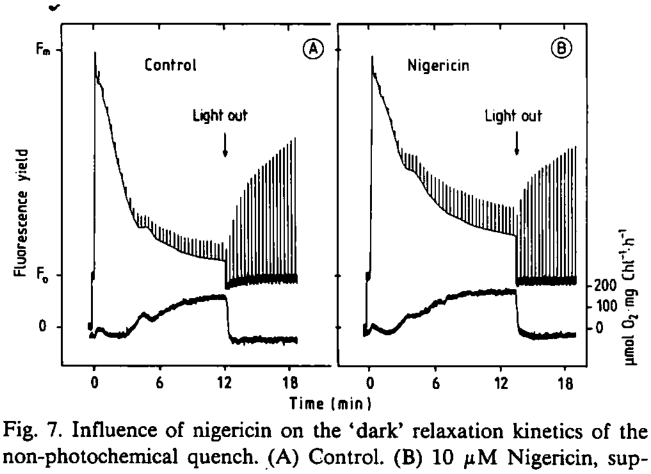

Fig. 7. Influence of nigericin on the 'dark' relaxation kinetics of the non-photochemical quench. (A) Control. (B) 10 μM Nigericin, supplied via the transpiration stream as in Fig. 6. The leaves were illuminated at an actinic light intensity of 579 $\mu mol \cdot m^{-2} \cdot s^{-1}$, and then darkened. Saturating light pulses were given every 20 s in the light and the 'dark'. The differentiated signal from the O_2 electrode is shown for comparison.

Changes of non-photochemical quencing are accompanied by changes of the F_0 signal. When leaves are illuminated using high light, the F_0 value obtained after darkening is 27% lower than the value in dark-adapted leaves (Fig. 7A). This decrease is abolished by nigericin (Fig. 7B). It is absent in low light, when no 'fast' phase is present (Fig. 6A), but appears when tentoxin is supplied to produce a large 'fast' phase (Fig. 6B). Further, the relaxation of Fv_s during the 'fast' phase is accompanied by a rapid rise of F_0 back towards its dark-adapted value (Fig. 7A and B).

Thus, chemicals which modify the magnitude of the transthylakoid proton gradient selectively alter the 'fast' component of the relaxation kinetics. We conclude that this component is analogous to the q_E mechanism described in aqueous systems. The accompanying quench of the F_0 signal is in agreement with observations that q_E quenches both F_0 and variable fluorescence in aqueous systems [8,9,12]. Obviously, q_E makes a considerable contribution to the non-photochemical quench in leaves in high light. However, its contribution in low light is negligible (see below).

Effect of light quality on fluorescence relaxation kinetics

We next carried out a series of experiments in which the quality of the actinic illumination was varied, to test whether the 'middle' component is related to state transitions (q_T) and, hence, to redistribution of excitation energy away from PS II. It is well established that light which is deficient in far-red intensities leads to phosphorylation of LHCP and a decrease of the PS II antennae size; conversely, light which is supplemented with far red light leads to dephosphorylation of LHCP and an increase of the PS II antenna cross-section [23–26]. In the following experiments, barley leaf segments were illuminated with a low intensity (52 $\mu mol \cdot m^{-2} \cdot s^{-1}$) white light (L2) or a white light (40 $\mu mol \cdot m^{-2} \cdot s^{-1}$) supplemented with far red light (greater than 715 nm, 20 $\mu mol \cdot m^{-2} \cdot s^{-1}$) (L1). These light intensities balanced to give approximately the same steady-state rate of O_2 evolution (25 μmol per mg Chl per h).

In the experiment of Fig. 8, leaf segments were illuminated alternately with L1 and L2. Between each light regime, the 'dark' relaxation of Fv_s was monitored using saturating pulses every 100 s. These light regimes were alternated several times, and Fig. 8 shows one typical cycle. There is a typical induction period after turning either light on (see Refs. 6 and 7), during which

Q_A becomes much more reduced and non-photochemical quenching is higher than at steady state. This transient high non-photochemical quench is due to q_E. Once these induction transients are completed and a steady rate of O_2 evolution is established, marked differences appear in the fluorescence quenching, depending on the light regime. Firstly, Q_A is far more oxidised in L1 than in L2. Secondly, the non-photochemical quench continues to decrease during illumination with L1, while it increases again in L2 light. Thirdly, after turning off L1 light there is only a small and slow increase of Fv_s over the next 7–8 min; in contrast, after turning off L2 there is a substantial increase of Fv_s for the next 7–8 min. It might be noted that there is a sudden rise of Fv_s within 5 s with both L1 and L2, which seems too fast to be associated with the 'fast' phase described in the previous section. These rapid changes in the amplitude of Fv_s also occur following changes in actinic light quality (Fig. 9); the presence of far red light causes a reduction in the amplitude of Fv_s and vice-versa. Fluorescence quenching by oxidised plastoquinone [10,11] could explain this if the plastoquinone pool was not completely reduced during high intensity light pulses.

In the experiment of Fig. 9A, dark-adapted leaves were illuminated with alternating L1 and L2 light, but this time without any intervening dark period. The changes of variable fluorescence in Fig. 9A are identical to those obtained in the classic demonstration of state transitions [24,27]. By combining them with saturating light pulses, more information is obtained about the reduction state of Q_A and, especially, about the accompanying changes of non-photochemical quenching. As expected, Q_a becomes more reduced immediately after switching from L1 to L2, and more oxidised immediately after switching from L2 to L1. These immediate changes are then gradually reversed. This takes about 7 min in light L2 ($t_{1/2} \approx 3$ min), but considerably longer in light L1 ($t_{1/2} \approx 9$ min). The adjustment to light L2 is accompanied by an increase of the non-photochemical quench while the adjustment to light L1 is accompanied by a decrease of the non-photochemical quench. The time-course of the fluorescence rise in L1 is comparable to that of the 'middle' phase of the dark relaxation kinetics (Fig. 2).

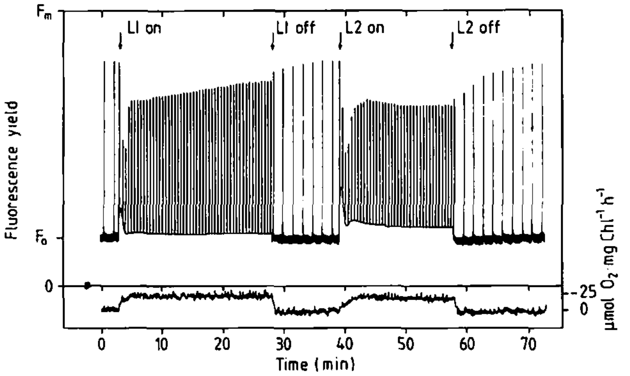

Fig. 8. Influence of light quality on the redox state of Q_A and non-photochemical quenching. Dark-adapted barley leaves were illuminated alternately with light L1 (40 $\mu mol \cdot m^{-2} \cdot s^{-1}$ white light plus 20 $\mu mol \cdot m^{-2} \cdot s^{-1}$ far-red light) or with light L2 (52 $\mu mol \cdot m^{-2}$ in the light. Between these light regimes, the leaf segments were darkened and saturating pulses were given after 5 s, and then every 100 s. The results are expressed as a percentage of the F_m signal obtained at the beginning of the experiment, and show one of three cycles. For comparison, O_2 evolution is also shown.

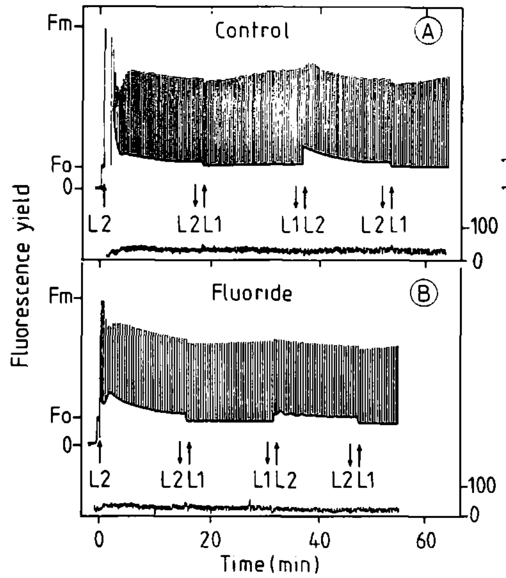

Fig. 9. Chlorophyll fluorescence quenching during transitions between white light, and white light enriched with far-red light. (A) Control. (B) Leaves supplied with 20 mM sodium fluoride for 12 h in the dark. In both experiments, leaves were illuminated alternately for 15–16 min with light L2 (white light) and light L1 (far red enriched, for details see Fig. 8 or text) without any intervening dark period. Saturating pulses were applied every 20 s. For comparison, the differentiated signal from the O_2 electrode is shown; O_2 evolution was about 25 and 20 $\mu mol \cdot m^{-2} \cdot s^{-1}$ above dark respiration in control and fluoride-treated leaves, respectively.

State transitions can also be manipulated by adding fluoride to inhibit the dephosphorylation of LHCP [9,23,24]. This should lead to an increased non-photochemical quench, because LHCP is 'trapped' in the phosphorylated form, which is not associated with PS II. We supplied 20 mM fluoride to leaves via the transpiration stream for 12 h in the dark (Fig. 9B). At these concentrations, fluoride has almost no effect on the light-limited rate of photosynthesis. The maximal rate is decreased (see below), mainly due to an inhibition of sucrose synthesis which results primarily from an accumulation of PP_i [28]. These leaves were subjected to an alternating regime of L1 and L2 light, exactly as in Fig. 9A. Fluoride effectively abolished the gradual adjustment of Q_A to the light quality. Fluoride also abolished the gradual changes of the non-photochemical quench which normally accompany this adjustment.

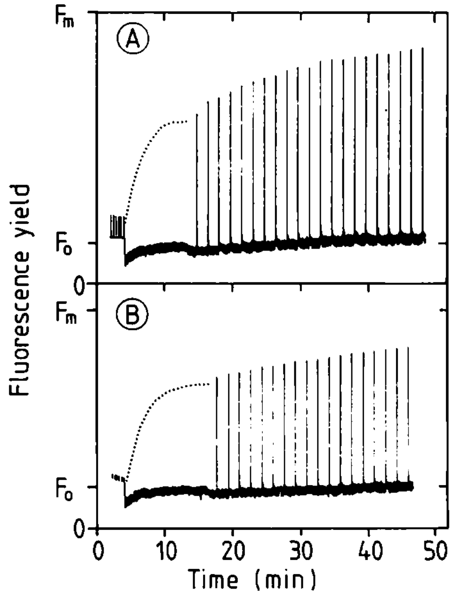

Fig. 10. Influence of fluoride on the 'dark' relaxation Kinetics of Fv_s in barley leaves. (A) Control. (B) 20 mM NaF supplied via the transpiration stream for 12 h. The leaf material was illuminated with saturating light (579 μmol·m^{-2}·s^{-1}) for 15 min, the actinic light was then removed, and saturating pulses were applied every 7 s until Fv_s has relaxed to new stable value, and then every 100 s. The fluoride treatment inhibited photosynthesis by 60% in these conditions.

Thus, suddenly changing the light quality to overexcite PS I leads to an immediate over-oxidation of Q_A, while sudden changes of the light intensity to overexcite PS II lead to an immediate overreduction of Q_A. This imbalance between the photosystems is then readjusted via a mechanism which is fluoride sensitive, and which is accompanied by changes of non-photochemical quenching over a time scale similar to that of the 'middle' phase of fluorescence quenching. We therefore conclude that the 'middle' phase is associated with the presence of state transitions (q_T) as has already been demonstrated in barley protoplasts [9].

Fluoride allowed us to ascertain the contribution of q_T to non-photochemical quenching at high light (Fig. 10). Leaves were supplied with 20 mM NaCl or 20 mM NaF via the transpiration stream overnight. They were then illuminated with saturating light (579 μmol · m^{-2} · s^{-1}) for 10 min, before removing the actinic light and pulsing every 7 s with saturating light for 12 min, and then every 100 s for another 35 min. The relaxation of the non-photochemical quench to a new plateau under the 7 s pulse regime provides an estimate of q_E (see Fig. 3), while the relaxation under the 100 s pulse regime reveals the contribution of the 'middle' (q_T) and later, of the 'slow' component (q_T). In these high light conditions, fluoride inhibited photosynthesis by about 60% (see above). Fluoride did not significantly alter q_E, and both sets of leaves also had a large 'slow' component (q_I). However the 'middle' phase, which was substantial in the controls, was effectively abolished in leaves which had been supplied with fluoride. We conclude that q_T may make a significant contribution to the total non-photochemical quench in leaves, even in conditions of saturating light. In this example, the contribution is almost half as large as that made by q_E. Similar results have already been obtained using protoplasts, although q_T was apparently not as large as in leaves [9].

Actinic light intensity effects on the quenching components

Utilising the dark relaxation technique, described above, the various components of fluorescence quenching were determined at steady-state rates of photosynthesis for a variety of actinic light intensities (Fig. 11). The data on the left-hand side of the panel are presented to allow the expansion of the low intensity region; at these low light levels the apparent quantum yield for photosynthesis was almost constant. Fig. 11A shows how the rate of oxygen evolution varies with light intensity; the rate is almost linear with respect to light intensity between 10 and 200 μmol · m^{-2} · s^{-1}; at light intensities below 10 μmol · m^{-2} · s^{-1} the measurement of the rate of photosynthesis is complicated by light-induced suppression of dark respiration. This is especially marked in well dark-adapted plant material (see Ref. 29) and leads to apparent quantum yields which are much higher than the theoretical maximum. At higher light intensities the linearity deviates as we approach light saturation. The reduction state of PS II (q_Q) (Fig. 11C) is almost linearly related to light intensity, becoming increasingly reduced as the light intensity is increased. The total non-photochemical fluorescence quenching (q_{NP}), shown in Fig. 11C, is present at the lowest light intensities measured and increases throughout the light intensity range.

The individual components of q_{NP} determined from the relaxation kinetics of fluorescence quenching show very different light response curves. The 'fast' component of dark fluorescence relaxation (q_f), which we attribute to high energy state quenching (q_E), shows a sigmoidal curve that rises sharply at the point where photosynthesis starts to deviate from linearity in the light response curve. As the rate starts to saturate so does q_e. This 'fast' relaxing component of fluorescence quenching is clearly not well suited to dissipate light energy at super-saturating intensities; q_e only increases from 0.5 to 0.65 where the light intensity has more than doubled (400 to 1000 μmol · m^{-2} · s^{-1}).

The 'middle' component of the dark relaxation kinetics (q_m) is clearly already present at the lowest light intensities used and is saturated at 200 μmol · m^{-2} · s^{-1} (Fig. 11D). This is consistent with q_m being associated with q_T and results from a mechanism that serves to balance excitation of the two photosystems at low light intensities. The values of q_m obtained here (up to 0.45) are high compared to other studies if this is indeed a true measure of q_T; a 25–30% decrease in F_m has been

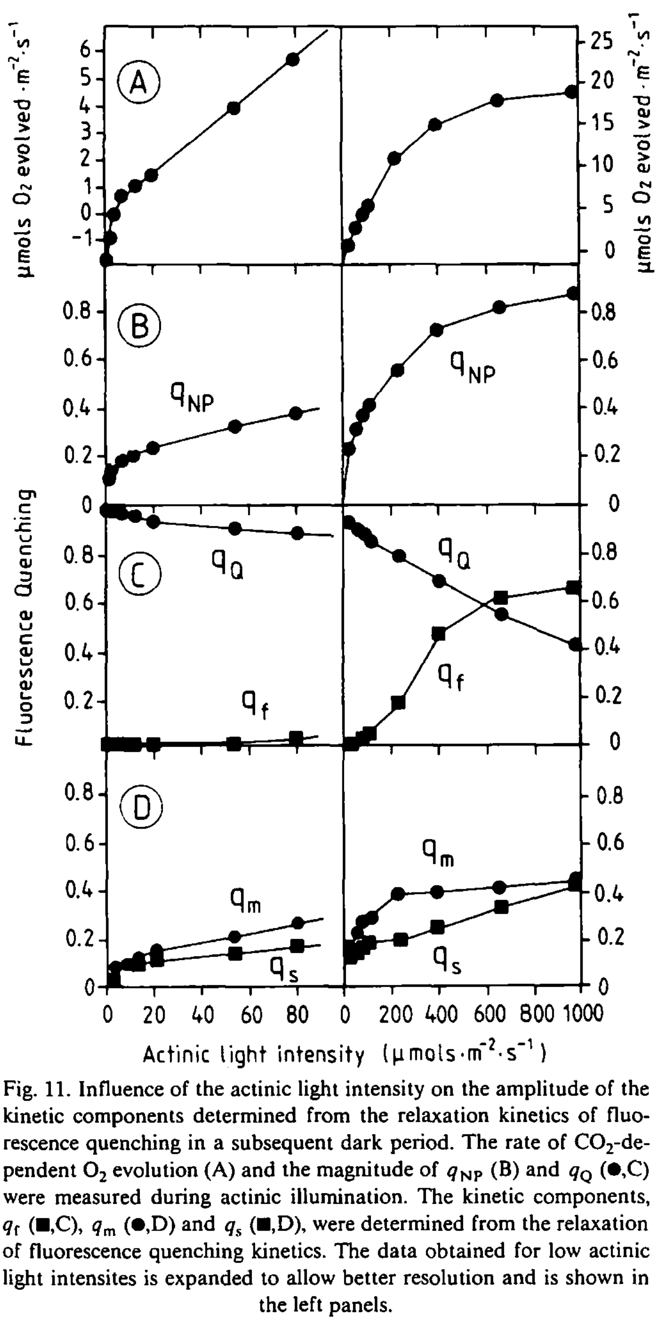

Fig. 11. Influence of the actinic light intensity on the amplitude of the kinetic components determined from the relaxation kinetics of fluorescence quenching in a subsequent dark period. The rate of CO_2-dependent O_2 evolution (A) and the magnitude of q_{NP} (B) and q_Q (●,C) were measured during actinic illumination. The kinetic components, q_f (■,C), q_m (●,D) and q_s (■,D), were determined from the relaxation of fluorescence quenching kinetics. The data obtained for low actinic light intensites is expanded to allow better resolution and is shown in the left panels.

reported in thylakoids associated with LHCP phosphorylation and light distribution to PS I [30] which would require a minimal q_T value of 0.25. In barley protoplasts values of q_T between 0.2 and 0.3 have been reported [5,9]. However, it is interesting to note that the F_m/F_0 ratios obtained in these aqueous systems (4.0–5.0) are significantly lower than those obtained in leaves (5.0–6.0).

Also shown in Fig. 11D is the 'slow' component of the fluorescence dark relaxation (q_s). This component shows a large increase in the super-saturating light, suggesting it may reflect photoinhibitory processes. This 'slow' relaxing component was shown to be sensitive to cycloheximide in barley protoplasts indicating the need for protein synthesis and 'repair'. However, there is also a residueal level of q_s at low light intensities. This may reflect an incomplete separation of the q_s and q_m components during our analysis or that this 'slow' relaxing component is heterogenous [9].

One factor might be complicating the quantitation of these different fluorescence quenching components. Schreiber and Neubauer [19,20] have indicated that the light pulse used to remove photochemical fluorescence quenching may not be light saturated under certain circumstances due to a PS II donor side limitation which can be restored by addition of DCMU. Direct comparisons of a saturated light pulse- and DCMU-induced fluoresencence rise, in barley protoplasts, also indicated that the light pulse underestimated the DCMU response by up to 20% except during early induction [5], again suggesting a possible donor side limitation. One possible explanation for this is the presence of a low efficiency population of PS II [20] with a reduced antenna size (PS II-beta centres) [32]. Reversion back to State 1 during the 'middle' component of the fluorescence quenching relaxation kinetics could reduce the population of PS II$_\beta$ centres and hence any PS II-donor side limitation. This would mean that q_{NP} would be overestimated as not all photochemical quenching would be removed by a light pulse during actinic illumination. The effect of a PS II donor side limitation on the 'fast' component (q_f) is more complex but could be predicted to cause an underestimation of the q_f and an overestimation of the 'middle' component (q_m). However, direct evidence of a PS II donor side limitation is required to resolve this potential problem.

Nevertheless, the data presented here demonstrate that there are non-photochemical quenching mechanisms in leaves that relax, in the absence of actinic illumination, too slowly to be associated with high-energy state quenching (q_e) and that they contribute significantly to the overall non-photochemical quench. Following the work of Weis and Berry [33], who show a direct correlation between q_E and the quantum yield of oxidised PS II centres, several reports have appeared in the literature showing a variety of different correlations [34–39]. Part of this discrepancy may well result from the variety of methods used to determine non-photochemical quenching and its components. For example, unless the components which we attribute to q_T and q_I are adequately resolved, there will be a serious overestimation of q_E, especially at low light. Most previous workers have used a single flash after an arbitrary time in the dark to determine q_E. Also, the time constants of the various quenching components are variable, not only between different plant species and environmental conditions (data not shown), but also for different experimental conditions [37]. Hence, we feel that the use of single light pulses, or of low light intensity pretreatments, are unlikely to allow the isolation of single components of the non-photochemical quench. We would suggest that monitoring the kinetics of relaxation of fluorescence quenching using repetitive light pulses provides a more accurate and reliable method for determining the contribution of the individual components. When this method is applied, it is apparent that there is a direct and almost stoichiometric relation between q_E and the apparent quantum yield [38,39].

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