Novel amplification of non-photochemical chlorophyll fluorescence quenching following viral infection in *Chlorella*

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Abstract In higher plants non-photochemical dissipation of excess light, trapped by the pigment pool of photosystem II, prevents photodamage to the photosynthetic apparatus. We report here that an algal virus infecting *Chlorella* strain Pbi induces non-photochemical quenching of photosystem II fluorescence, indicating enhanced loss of absorbed light energy from photosystem II. This phenomenon occurs soon after the establishment of the virus infection cycle and is observed at low irradiance (20 μmol quanta m^{-2} s^{-1}). At low light, infection associated non-photochemical quenching is not linked to extensive conversion of violaxanthin to antheraxanthin and zeaxanthin. However, such conversion occurs rapidly (2–10 min) in infected cells under conditions of high irradiance (100–300 μmol quanta m^{-2} s^{-1}). Under similar conditions uninfected *Chlorella* cells do not display significant changes in non-photochemical quenching.

Key words: *Chlorella*; Algal virus; Xanthophyll cycle; Violaxanthin de-epoxidase

1. Introduction

Aquatic green algae have a poorly developed mechanism for rapidly adjusting non-photochemical quenching (NPQ) to dissipate excess light energy and only display a gradual rise in NPQ at saturating irradiance [1,2]. Using *Chlorella* NC64A and the virus PBCV-1 [3], we have previously shown that photosynthesis is down regulated within the first 30 min of the viral infection cycle, without any associated development of non-photochemical quenching of chlorophyll *a* fluorescence [4]. Synchronous infection of a large number of cells is possible using saturating levels of virus under suitable conditions [5]. This allows the study of virus induced changes in a population of cells, all at the same stage of the virus infection cycle.

Using a related system, *Chlorella* Pbi cells [6] infected with the *Chlorella* virus CVM-1, we have observed a unique virus induced rise in NPQ associated with violaxanthin de-epoxidation at saturating light intensities. At low light intensities NPQ was observed shortly after infection but before a virus-induced decrease in the photochemical quenching coefficient (q_P). Evidence that suggests the NPQ response may be due to viral encoded factors is presented.

2. Materials and methods

Production and purification of *Chlorella* viruses and growth of host cells have been described previously [6,7]. *Chlorella* strain Pbi was grown on modified Bolds basal medium [7] supplemented with 2 g l^{-1} of Lab-Lemco powder (Oxoid Limited, London, UK). Cell growth conditions, harvesting of cells and methods used for photosynthetic measurement have been described previously [4]. A multiple of infection of 2 was used to infect cells with the virus CVM-1. This level of infection was saturating as determined by the methods of Rohozinski et al. [5]. Cell pigments were extracted and analysed using the methods described by Seaton et al [4].

3. Results and discussion

When *Chlorella* Pbi cells, maintained at an actinic light irradiance of 20 μmol quanta m^{-2} s^{-1}, were infected with the *Chlorella* virus CVM-1 a rise in NPQ was observed shortly after infection but before a virus-induced decrease in the photochemical quenching coefficient (q_P) occurred (Fig. 1A). As infection proceeded and photosynthesis was inhibited, the rise in NPQ stopped before dropping to a steady state value. The pattern of electron flow through the photosystems of infected cells can explain the dynamics of the NPQ response. A drop in q_P indicates that electron withdrawal from photosystem II has become limiting and the primary quinone acceptor (Q_A) pool becomes reduced. Consequently, proton release into the lumen was slowed, the proton gradient across the thylakoid membranes (ΔpH) is reduced, and NPQ fell. In infected cells the long term maintenance of NPQ above background levels, with reduced electron flow and a concomitant lower ΔpH (Fig. 1A), indicates that some permanent change has occurred in the photosystem II membrane–protein complex that is dependent on the virus replication cycle. However, the photosystems remain functional. When electron flow was maintained by the addition of the electron acceptor methylviologen (Fig. 1B) Q_A remains oxidised, the ΔpH was maintained, and NPQ increases significantly to a plateau value above that observed in infected cells under normal conditions (Fig. 1A).

The dependence of the virus associated NPQ on translation of viral transcripts is indicated by two lines of evidence. Firstly, it has previously been shown that host transcription is inhibited upon infection [3,8] and viral transcripts can be detected within 5 min of infection [8]. The time course of NPQ induction indicates that translation of de novo host transcripts is unlikely to be involved in the induction of the viral associated NPQ response. Secondly, by treating the cells with cycloheximide for 1 h before infection, translation of viral transcripts was limited. Under these conditions viral translation products are absent, no change in NPQ or q_P was observed, and the cells showed all the photosynthetic character-

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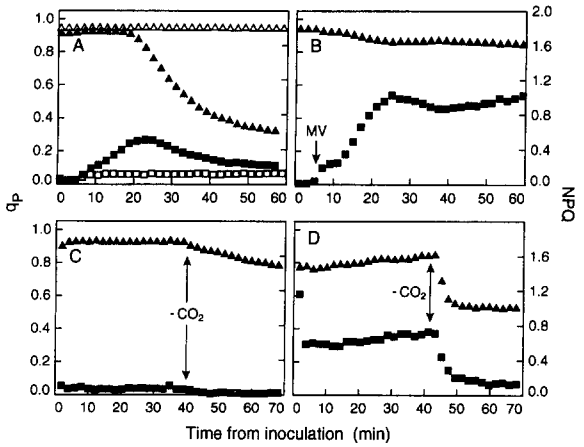

Fig. 1. Non-photochemical quenching associated with the infection cycle of *Chlorella* virus CVM-1. (A) The effect of virus infection on photochemical quenching (q_P, ▲) and non-photochemical quenching (NPQ, ■). The open symbols (△, □) show the response of *Chlorella* cells pre-treated with cycloheximide (10 μg ml^{-1}) for 1 h before infection. Cells were equilibrated without light for 5 min before exposure to an actinic light of 20 μmol quanta m^{-2} s^{-1} for 15 min prior to infection with a saturating amount of virus. (B) Methylviologen (75 μM) was added 5 min post-infection. (C) Healthy cells exposed to 1% O_2 in nitrogen 40 min after illumination with 20 μmol quanta m^{-2} s^{-1}. (D) Healthy cells exposed to 1% O_2 in nitrogen 40 min after illumination with 300 μmol quanta m^{-2} s^{-1}.

istics of healthy cells (Fig. 1A). The use of cycloheximide to prevent translation of early viral messages establishes that the expression of viral genes is essential for the rise in NPQ and drop in q_P.

The lack of a rapid and sustainable NPQ response in healthy *Chlorella* was demonstrated by reducing electron flow through the photosystems via a reduction in CO_2 availability. Healthy cells were treated with a mixture of nitrogen and 1% oxygen after 40 min of incubation, under normal conditions, in media equilibrated with air in a sealed chamber [4]. Under low irradiance (20 μmol quanta m^{-2} s^{-1}) *Chlorella* cells shown only minimal NPQ development upon illumination (Fig. 1C) and this situation was maintained for the 40 min before CO_2 removal. Limitation of CO_2 availability resulted in a slight decrease in both q_P and NPQ (Fig. 1C). The drop in q_P indicates that, in the absence of CO_2, the antennae are trapping excess light energy even at 20 μmol quanta m^{-2} s^{-1}. Restriction of electron flow through the photosystems results in reduction of the Q_A pool, a decrease ΔpH across the thylakoid membranes and subsequent loss of NPQ. This demonstrated that the maintenance of NPQ was closely coupled with electron flow in the cells.

With high irradiance (300 μmol quanta m^{-2} s^{-1}) healthy cells show a typical light saturation response with suppression of q_P (Fig. 1D) due to Q_A being reduced faster than reducing equivalents can be passed down the electron transfer chain of the photosystems. Under these conditions, excess energy trapped by the antennae of photosystem II was dissipated as heat via NPQ, thus q_P was lower and NPQ greater (Fig. 1D) than observed at low light (Fig. 1C). When CO_2 availability becomes limiting at high irradiance, electron flow through the photosystems decreases, resulting in the Q_A pool becoming even more reduced with a concomitant drop in q_P. This reduction in electron flow due to CO_2 limitation presumably results in a smaller ΔpH across the thylakoid membranes, and NPQ decreases in tandem with q_P. The fact that under high irradiance and CO_2 limiting conditions, NPQ drops (Fig. 1D) to a level approximately equal to that observed in low light treated cells (Fig. 1C), indicates that no gross change in the potential for NPQ has occurred.

The difference between the level of NPQ observed in cells, under low and high irradiance, in the presence of CO_2 (Fig. 1C,D) can be attributed to a larger ΔpH generated across the thylakoid membranes at high irradiance. This was presumable coupled with the presence of antheraxanthin and zeaxanthin in the thylakoid membranes and resulted in an elevated but relatively constant level of NPQ. The coupling of NPQ with ΔpH and violaxanthin de-epoxidation status has been documented by Gilmore and Yamamoto [9].

Our observations contrast markedly with those from terrestrial plants. In terrestrial plants exposure to saturating light irradiances induces the rapid and increasing development of NPQ that is closely associated with de-epoxidation of violaxanthin to antheraxanthin and zeaxanthin [10]. This process usually takes place over the first 5 min after exposure to saturating irradiances at which point NPQ reaches a plateau [10]. Difference in the behaviour of NPQ development in *Chlorella* cells and terrestrial plants is further demonstrated with limitation of CO_2 at saturating irradiance. *Chlorella* cells show a decrease in both q_P and NPQ when CO_2 under saturating irradiance (Fig. 1D). When leaves of terrestrial plants were deprived of CO_2 and exposed to moderate irradiance (100–800 μmol quanta m^{-2} s^{-1} depending on species) a rapid

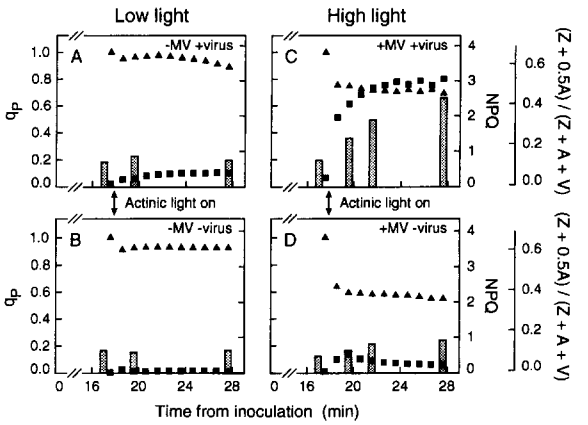

Fig. 2. The relationship between irradiance, chlorophyll fluorescence quencing and de-epoxidation status of the xanthophyll pool in infected healthy cells. (A) Infected cells exposed to 20 μmol quanta m^{-2} s^{-1} of light (q_P, ▲; NPQ, ■; de-epoxidation status, bars). (B) Healthy cells treated as in A. (C) Infected cells exposed to 300 μmol quanta m^{-2} s^{-1} in the presence of 75 μM methylviologen. (D) Healthy cells treated as in C. Cells were inoculated 17 min before illumination to avoid the induction phase of the infection cycle. Prior to switching on the actinic light a saturating flash was used to determine the maximum fluorescence yield. The de-epoxidation status was calculated as the ratio of de-epoxidated xanthophyll to total pool size, where A = antheraxanthin, V = violaxanthin and Z = zeaxanthin. The xanthophyll pool size did not change during the experiment and the xanthophyll–chlorophyll ratio remained constant. At the indicated time points (immediately before illumination then 2, 7 and 15 min post-illumination) 0.5 ml of cells (4×10^7 cells) was removed from the reaction vessel, centrifuged and the pigments were extracted.

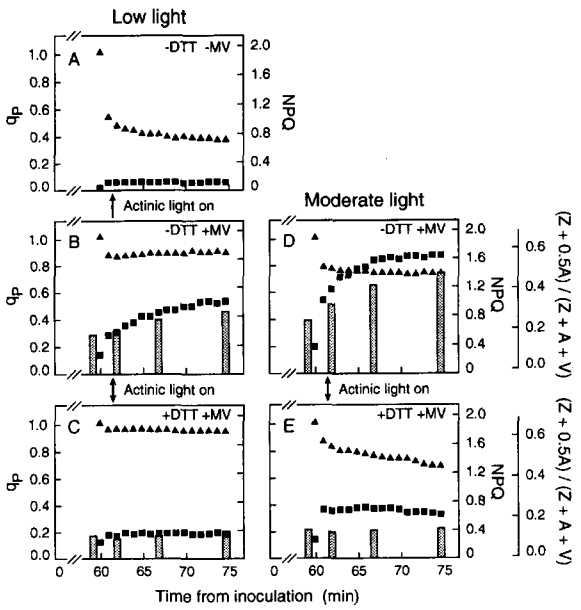

Fig. 3. Effect of dithiothreitol on virus associated de-epoxidation and related attenuation of NPQ. (A) q_P (▲) and NPQ (■) response of infected cells in the absence of methylviologen when illumination with 20 μmol quanta m^{-2} s^{-1}, 60 min post-infection. (B) q_P, NPQ and de-epoxidation status (bars) of infected cells exposed to 20 μmol quanta m^{-2} s^{-1} in the presence of 75 μM methylviologen. (C) As in B but with the addition of dithiothreitol (3 mM). (D) Response of infected cells exposed to 100 μmol quanta m^{-2} s^{-1}, 60 min post-infection in the presence of methylviologen. (E) Infected cells as in D, treated with both methylviologen and dithiothreitol. Where indicated cells were treated with methylviologen 5 min post-infection and dithiothreitol 20 min post-infection. Cells were kept in the dark for 60 min after infection to allow the infection cycle to become fully established before illumination and to prevent light induced de-epoxidation during this time.

rise in NPQ has been reported [11,12] and this response is closely coupled with rapid de-epoxidation of violaxanthin to antheraxanthin and zeaxanthin [12]. Thus, in terrestrial plants NPQ increases under conditions of CO_2 limitation whereas in *Chlorella* NPQ decreases. In both cases the ΔpH across the thylakoid membranes is important in the development of NPQ. In terrestrial plants removal of the terminal electron acceptor by treatment with an atmosphere of 2% O_2, 0% CO_2 promotes the buildup of a ΔpH across the thylakoid membrane in light [11] with subsequent induction of violaxanthin de-epoxidase activity and development of NPQ. However, in *Chlorella* it appears that ΔpH relaxes with reduced electron flow through the photosystems and NPQ decreases proportionally. In terrestrial plants electron flow is presumably maintained via Mehler-ascorbate peroxidase photorespiration [13] when CO_2 is limiting. Non-assimilatory electron transport and photon utilisation can thus sustain a ΔpH that promotes antheraxanthin and zeaxanthin synthesis [13]. Our observations using *Chlorella* cells indicate that uninfected cells may not possess a Mehler-ascorbate peroxidase photorespiration pathway and certainly lack a rapidly inducible NPQ response when subjected to saturating irradiance.

As the NPQ response in infected cells showed characteristics more akin to those of terrestrial plants, even at low light irradiance, we tested the possibility that NPQ development in infected cells is associated with induction of violaxanthin de-epoxidase activity. *Chlorella* viruses can replicate in the dark without temporal alteration of the life cycle [7]. We therefore maintained the cells without light during the first part of the infection cycle so that viral induced change and/or encoded products that are involved in the virus associated NPQ response accumulate in the dark. The advantage of doing this was that upon illumination the cells would rapidly develop NPQ without the complications associated with the induction phase of the replication cycle. Exposure of infected cells to low light, 17 min post-infection, induced a slight rise in NPQ, without significant de-epoxidation of violaxanthin to antheraxanthin and zeaxanthin (Fig. 2A). In similarly treated healthy cells, only a very low level of NPQ was evident and there was no de-epoxidation of violaxanthin after illumination (Fig. 2B). However, under high light conditions in the presence of methylviologen, there was a rapid rise in NPQ in infected cells that is coupled with the conversion of violaxanthin to antheraxanthin and zeaxanthin (Fig. 2C). In healthy cells exposed to high light and methylviologen, a low level of de-epoxidation does occur and continues over the period of illumination (Fig. 2D). Surprisingly, NPQ only increases marginally above the level observed at low light (Fig. 2B,D) and drops after reaching a peak at 2 min post illumination (Fig. 2D). These data indicate that healthy cells do not rapidly convert violaxanthin to antheraxanthin and zeaxanthin at saturating irradiances. However, infected cells display a unique light associated violaxanthin de-epoxidase activity resulting in the conversion of violaxanthin to antheraxanthin and zeaxanthin, and development of NPQ at saturating irradiance (Fig. 2C) that is well above background levels (Fig. 2D). Methylviologen was added to the high light treatments to maximise electron flow and hence proton translocation into the lumen of the thylakoid [14], which in turn enhanced zeaxanthin formation.

Feasible explanations for the behaviour of the infected cells are the possibility that virus infection alters electron flow through the thylakoid membranes, allowing the development of a lower pH in the lumen which in turn activates an endogenous de-epoxidase; alternatively lumen ascorbate concentration could be altered with subsequent induction of a Mehler-ascorbate peroxidase photorespiration system. However, the possibility that the virus may encode for a violaxanthin de-epoxidase cannot be overlooked. Our data demonstrates that expression of viral genes is essential for NPQ development in infected cells. As previously mentioned, transcription and translation cease almost immediately after infection whereas viral transcripts can be detected within 5 min of infection [3,8]. Furthermore, our previous studies using a related virus (PBCV-1) and host system (*Chlorella* NC-64a) showed no viral associated development of NPQ [4], suggesting that the virus CVM-1 codes for a unique function associated with NPQ development in the host cell upon infection. The observation of atypical violaxanthin de-epoxidase activity in the infected cells is thus consistent with the hypothesis of the virus encoding for this enzyme. The complexity of these viruses, their large genome (approximately 330 kb of DNA) and large number of genes they encode [3] is consistent with this hypothesis.

The link between virus-associated violaxanthin de-epoxidation and a rise in NPQ was confirmed with the use of dithiothreitol (DTT), a potent inhibitor of violaxanthin de-epoxidase [15–17]. As DTT is known to interfere with viral

attachment and penetration of the host cell wall, thus preventing the establishment of infection [3], the infection cycle was initiated 20 min before addition of DTT. Subsequently, the cycle was allowed to proceed for 60 min in the dark to avoid induction of de-epoxidase activity. Normal progress of the infection cycle over the first 60 min without illumination was confirmed by a control experiment. Infected cells illuminated with 20 μmol quanta m^{-2} s^{-1} 60 min post-infection (Fig. 3A) displayed a drop in q_P that, together with NPQ, rapidly attained levels normally observed at the equivalent stage of the infection cycle under continuous illumination (Fig. 1A). Addition of methylviologen to infected cells before illumination allowed the maintenance of electron flow through the photosystems upon illumination. This resulted in high q_P and the generation of a ΔpH across the thylakoid membranes (Fig. 3B). Under these conditions NPQ increased rapidly in the first 2 min of illumination followed by a gradual steady increase to a plateau over the next 14 min. No significant violaxanthin de-epoxidation occurred in the first 2 min of illumination. However, there was a subsequent steady increase in de-epoxidation (Fig. 3B) associated with a slow rise in NPQ. In the presence of DTT, the NPQ change was smaller (Fig. 3C) than that observed in its absence (Fig. 3B), reaching a plateau within 2 min of illumination. There was no significant de-epoxidation of violaxanthin (Fig. 3C) and no prolonged rise in NPQ. The difference in the initial NPQ response, in the presence and absence of DTT (Fig. 3B,C), suggests that a small amount of xanthophyll conversion can occur in the dark in infected cells. This leads to an increased NPQ once a ΔpH is established upon illumination. Under conditions of moderate light (100 μmol quanta m^{-2} s^{-1}), in the absence of DTT, there is a rapid increase in NPQ within 1 min and a plateau is reached 7 min later (Fig. 3D) with de-epoxidation occurring throughout the period of illumination. Addition of DTT eliminates violaxanthin de-epoxidation (Fig. 3E) so that NPQ reaches a plateau almost immediately after illumination. This NPQ value is quantitatively above that observed at low light (Fig. 3C) indicating that in DTT treated cells NPQ is determined by ΔpH alone.

Under high light de-epoxidation is triggered by lumen pH dropping below a threshold [9] that allows the low pH-dependent violaxanthin de-epoxidase to become activated [14,15]. By limiting CO_2 availability to healthy cells we have demonstrated that the development of NPQ in healthy *Chlorella* cells is almost solely dependent on ΔpH, acting on endogenous levels of antheraxanthin and zeaxanthin. This behaviour is functionally different to that observed in terrestrial plants that display a strong association between rapid violaxanthin de-epoxidation and NPQ [9,18,19]. In contrast, the development of NPQ in infected cells shares many characteristics with terrestrial plants.

Although we do not know the exact nature of the viral gene product responsible for the NPQ response in infected *Chlorella* cells we speculate that it may be a de-epoxidase. Once expressed, the viral de-epoxidase could migrate to the thylakoid membrane, become associated with the photosystem II light harvesting complex, and take part in the NPQ response. In higher plants the de-epoxidase has been demonstrated to have a pH-dependent mobility within the thylakoid lumen [15] that could be associated with part of the NPQ response that occurs before rapid violaxanthin de-epoxidation is initiated [17]. Violaxanthin de-epoxidation, being dependent on sufficient acidification of the thylakoid lumen, would be associated with any NPQ response greater in magnitude than that observed under conditions where the level of antheraxanthin and zeaxanthin are unchanged. Alternatively, viral gene products could become associated with the thylakoid membrane enabling the algal de-epoxidase in situ to catalyse violaxanthin de-epoxidation at rates not normally possible in unperturbed system. However, the lack of evidence for changes in the thylakoid membrane and associated physical disruption of the photosystems, together with no observed changes in chlorophyll content or fluorescence signal suggests that the former hypothesis is a more likely explanation.

The absence of xanthophyll related NPQ in green algae has been previously noted and constitutes a major difference between algae and land plants [1,20]. Of the green algae only *Ulva rotundata* has been intensively studied with regard to photoinhibition. On exposure of *Ulva rotundata* to high light there is only a very slow conversion of violaxanthin to zeaxanthin and antheraxanthin with conversion taking several hours. However, there is an increase in the size of the xanthophyll pigment pool in high light grown *Ulva* [20] indicating that some level of photoprotection is afforded by these pigments under high growth irradiances.

In higher plants non-photochemical quenching of excess energy prevents damage to the photosynthetic apparatus under conditions of high irradiance [19,21]. Rapid de-epoxidation of violaxanthin to antheraxanthin and zeaxanthin is strongly associated with the dissipation of excess light energy trapped by the pigment pool of photosystem II in terrestrial plants [18,22,23]. The lack of a rapid light induced violaxanthin de-epoxidation cycle in aquatic green algae suggests that the acquisition of such a cycle may have been a prerequisite for life in a terrestrial environment.

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