Chapter 4
Fluorescence as a Tool to Understand Changes in Photosynthetic Electron Flow Regulation

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1 Introduction

The physiological state of a chloroplast is strongly influenced by both biotic and abiotic conditions. Unfavourable growth conditions lead to photosynthetic stress. Chlorophyll *a* fluorescence is a widely used probe of photosynthetic activity (specifically PSII), and therefore stress which specifically targets the electron transport pathway and associated alternative electron cycling pathways. By manipulating the processes that control photosynthesis, affecting the chlorophyll *a* fluorescence, yields detailed insight into the biochemical pathways. Light that is captured by a chlorophyll molecule can be utilised in three competing processes; electron transport, energy dissipation (via heat) and chlorophyll *a* fluorescence emission. Electrons produced by water-splitting are not always used in carbon fixation; if the incident irradiance generates more electrons than the dark reactions can use in carbon fixation, damage will occur to the photosynthetic apparatus. If carbon fixation is inhibited by temperature or reduced inorganic carbon (Ci), ATP or NADPH availability, then the photosystem dynamically adjusts and uses alternate sinks for electrons, such as molecular oxygen (water-water cycle or Mehler ascorbate peroxidase reaction). The process of stress acclimation leads to a number of photoprotective pathways and we describe how inhibitors can be used to identify these particular processes. In this chapter, we describe the processes controlling electron transport as influenced by light-induced stress.

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2 Electron Usage in Photosynthesis

Photosynthesis drives the light reactions which ultimately lead to carbon fixation; however predicting photosynthetic rates from fluorescence is a complex issue. As outlined in other chapters of this book (Chapter 3 and Chapter 6) different fluorescence tools are available to measure the electron flow through Photosystem II (PSII). The quantum yield of PSII can be multiplied by the amount of absorbed quanta which can be obtained from the incident light and either the PSII absorption cross section or the spectral overlap between the light spectrum and the *in-situ* absorption spectra. From these data, the electron transport rate per chlorophyll molecule over time can be assessed for an entire day to determine the daily primary production (Wagner et al. 2005). However, growth and photosynthesis are rarely equivalent. Electrons transported by PSII can follow several competing pathways: the majority of the electrons are normally used to reduce CO_2 to carbohydrates, allowing the synthesis of other cellular macromolecules like proteins, lipids or nucleotides, but some of them might be lost by alternate

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cellular processes (see alternate electron cycling) or dissipated (non-photochemical quenching). Therefore, the ratio of electrons per carbon incorporated into the biomass may vary tremendously, either by losses or by the synthesis of highly reduced biomolecules like proteins or lipids.

2.1 Alternative Electron Cycling (AEC)

In principle, PSII electron transport rates should match the gross rates of oxygen evolution. Studies have tried to verify this assumption with divergent results (Falkowski et al. 1986; Kolber et al. 1998; Suggett et al. 2001; Jakob et al. 2005). It was shown that linearity between PSII electron transport and oxygen evolution can be found, but non-linear behaviour was also observed, especially under conditions when photosynthesis was over-saturated (excess irradiance). These experiments indicated that PSII electron transport might over-estimate the primary production under some conditions, because oxygen evolution rates were found to be lower than PSII electron flow (Gilbert et al. 2000). Several explanations for this disparity have been suggested:

(a) Cyclic electron flow around PSII (Prasil et al. 1996; Lavaud et al. 2002)
(b) Water-water cycle (Asada 1999) where oxygen uptake on the acceptor side of PSI leads to superoxide which is then dismutated to H_2O_2 and then detoxified to water and
(c) Cyclic electron flow around PSI (Bendall and Manesse 1996)

These processes can be summarised as alternative electron cycling (AEC) which are not energetic losses (such as non-photochemical quenching: NPQ), because at least the water-water cycle and the cyclic flow around PSI generate a proton gradient which can be used for additional ATP synthesis. Therefore, it is suggested that alternative electron cycling is a normal stress response and might be of less importance under balanced growth conditions. Recently, Wagner et al. (2005) described an experimental setup to estimate the alternative electron cycling activity by comparing the electron flow through PSII with oxygen evolution relative to the amount of absorbed quanta. The result is shown in Fig. 1.

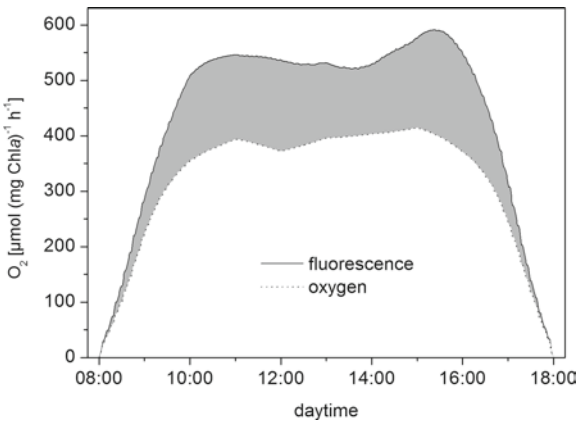

Fig. 1 Modelling of fluorescence and oxygen-based photosynthesis rates in *Phaeodactylum tricornutum* grown in a turbidostat under sine light conditions (10 h light period). Photosynthesis-irradiance curves were measured hourly and fitted using the dynamic model of Eilers and Peters (1988). With the derived fitting parameters, oxygen and fluorescence-based electron transport rates can be calculated for any given light intensity during the daily course of the light climate. The difference between fluorescence-based electron transport rates and oxygen-based photosynthesis rates (grey area between the curves) is linked to the proportion of alternative electron cycling

Obviously, at low light intensities in the morning and in the late afternoon, the fluorescence-based electron transport rates closely match the oxygen evolution rates as measured by a Clark-type electrode, whereas at high light intensities the "alternative electron cycling" can account for up to 40% of the fluorescence-based electron transport. This mismatch is not due to inappropriate measuring techniques, but to the physiological variability between linear and alternative electron pathways across the photosynthetic membrane. Interestingly, the ratio of linear to alternative electron cycling is not only light-dependent, but can be linked to species-specific physiological regulation, as shown in Fig. 2.

When light pulse frequency is manipulated, the relationship between electron transport and oxygen evolution is further altered. In the sine light (SL) climate which simulates a sunny day, the ratio PAM/oxygen is always higher than in the exponentially fluctuating light (FL) climate (Fig. 2), where the light intensity oscillates with a frequency of half an hour between the maximum value and zero. The green

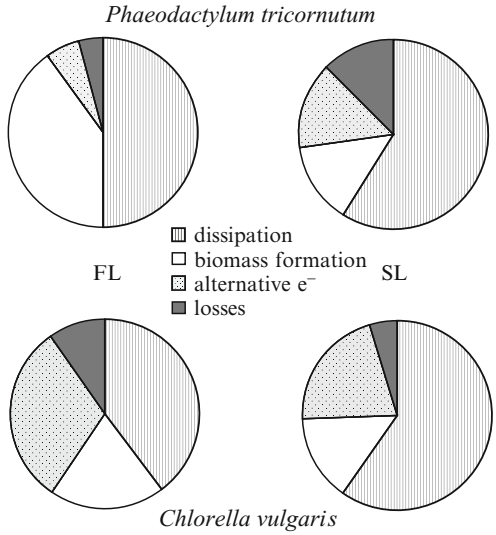

Fig. 2 The fate of absorbed photons in the comparison of *Phaeodactylum tricornutum* and *Chlorella vulgaris* grown in a turbidostat under dynamic light conditions (fluctuating light, FL; sine light, SL). Data are given as percentage of absorbed quanta (Q_{phar}). Energy losses by dissipation include the conversion of absorbed light into heat and fluorescence and were derived from [(1-q_P) Q_{phar}], where q_P is the photosynthetic quantum efficiency at Photosystem II measured by PAM fluorescence. The amount of quanta lost by alternative electron sinks was calculated from the difference of fluorescence and oxygen-based photosynthesis rates and the assumption of a quantum efficiency of 0.125 [mol O_2 (mol quanta)$^{-1}$]. The amount of quanta used for biomass formation was derived from Φ_μ according to Jakob et al. (2007). Energy losses which are not directly quantifiable as absorbed quanta, like mitochondrial respiration, have been depicted as 'losses'

alga, *Chlorella vulgaris,* performs much more alternative electron transport than the diatom, *Phaeodactylum tricornutum,* under both light conditions (FL and SL). In the sine light where the cells are exposed to a photon flux which exceeds the capacity of the Calvin cycle, the alternative electron cycling is highest indicating, that it can act as a photoprotective mechanism which compliments other photoprotective processes. Given that alternative electron cycling in the green alga was higher than in the diatom, this corresponds with the observation that in diatoms the energy dissipation capacity of the diadinoxanthin/diatoxanthin xanthophyll cycle is more active than in green algae or higher plants (Ruban et al. 2004; Goss et al. 2006) and will be discussed later in this chapter. It should be noted that green algae and higher plants have a different suite of xanthophyll pigments to diatoms and dinoflagellates.

2.2 Electron Usage to Produce New Biomass

Under continuous light, the oxygen production or the uptake of inorganic carbon shows a clear linear relationship with biomass production (Toepel et al. 2004) indicating that 55–60 μmol oxygen released is equivalent to 1 mg dry weight. However, the ratio of oxygen released to carbon incorporated is highly variable, for several reasons. Firstly, the reduction in biomass strongly depends on the species and the environmental conditions (Kroon and Thoms 2006). For example, under N or P limitation, the relative proportion of carbon incorporated into carbohydrates is strongly increased and therefore the reduction in biomass is relatively low. The energetic cost of converting the products of the Calvin cycle into lipid or proteins are incorporated by higher rates of mitochondrial respiration. Therefore, it can not be expected that the ratio of oxygen production in the light, per oxygen molecule consumed in the dark to be constant. This ratio is modulated, not only by the availability of nutrients and the reduction in biomass, but also by the turn-over rates of proteins and lipids. It is well documented that cells growing under high-light have significantly higher mitochondrial respiration rates, as well as under nutrient replete conditions and optimal temperature (Wilhelm and Wild 1984). Table 1 shows that the ratio photosynthesis/respiration varies not only in response to the light climate (sine versus fluctuating light) but also with the C/N ratio. Therefore, the ratio of photosynthetic electrons to carbon incorporated into the newly formed cells has to be variable. However, such parameters have not been measured under an adequate range of conditions or with sufficient species to make broad speculation.

The conversion of electron transport rates into actual new biomass requires accurate estimates for the ratio of electrons per carbon in the macromolecules of the new biomass. In future, the FTIR spectroscopy (Stehfest et al. 2005) might become a tool to measure this parameter, and is also possible with single cells.

Table 1 Comparison of *Phaeodactylum tricornutum* and *Chlorella vulgaris* with respect to the activity of alternative electron transport (expressed as the ratio of fluorescence-based to oxygen-based photosynthesis rates; P_F/P_O), to C/N ratios (given as mol mol^{-1}), and the activity of mitochondrial respiration (expressed as the ratio of respiration rate to net photosynthesis rate; R/P_{net}). Algal cultures were grown in a turbidostat under dynamic light conditions (10 h and 12 h light periods) which have been applied either as a non-fluctuating sine light climate or as oscillating light (osc. Light). In addition, *P. tricornutum* was exposed to nitrate-limited conditions (N-limited) (Data are adapted from Wagner et al. 2006 and Jakob et al. 2007)

Species	Growth condition	P_F/P_O	C/N	R/P_{net}
P. tricornutum	Replete – sine light (10 h)	1.4	7.7	0.8
P. tricornutum	Replete – osc. light (10 h)	1.3	7.9	1.0
P. tricornutum	Replete – sine light (12 h)	1.6	6.6	0.4
P. tricornutum	Replete – osc. light (12 h)	1.1	6.7	1.4
P .tricornutum	N-limited – sine light (10 h)	1.6	14.5	0.7
P. tricornutum	N-limited – osc. light (10 h)	1.2	10.8	1.5
C. vulgaris	Replete – sine light (12 h)	2.1	6.8	0.4
C. vulgaris	Replete – osc. light (12 h)	2.0	6.8	0.9

This opens the perspective to improve the robustness of estimates for primary production by using advanced fluorescence techniques.

3 Effect of Light Stress on Fluorescence Signatures and their Interpretation

When captured light energy cannot be completely utilized for metabolic processes, the excess energy accumulates within the photosynthetic apparatus (Nixon and Mullineaux 2001). This typically occurs when the light intensity is too high (over minimum saturating irradiance: E_k). However, this also occurs when the cells are suddenly switched from a dark/low light environment or to a higher irradiance (not necessarily over E_k) depending on the physiological state of the cells and their response to other environmental cues. Accumulation of excess energy within the photosynthetic apparatus can be harmful for photosynthesis, and especially for the activity of PSII, because the over-reduction of the primary electron acceptor (Q_A) generates free radicals which leads to oxidative stress (Ledford and Niyogi 2005); stress which will ultimately cause a decrease in the photosynthetic rate (i.e. photoinhibition). Photosynthetic organisms have developed a number of fast photoprotective (or photoacclimative) processes to minimize the level of oxidative stress, especially linked to the dissipation of the excess absorbed energy (Niyogi 2000). Non-photochemical quenching (NPQ) is believed to be one of the most important of these mechanisms for the fast regulation of photosynthesis in higher plants as well as in algae (Szabo et al. 2005; Demmig-Adams and Adams 2006; Lavaud 2007). It should be noted that NPQ is not a form of AEC, but rather it is especially efficient for organisms growing in a fluctuating light environment where it helps to balance the absorption of light energy with its use, and ultimately plays a role in the maintenance of their fitness (Külheim et al. 2002; Ralph et al. 2002; Demmig-Adams and Adams 2006; Lavaud 2007).

Non-photochemical quenching (NPQ) originates in the light-harvesting antenna (LHC) of PSII. When the available excitation energy exceeds the photochemical capacity, it can then be dissipated as heat (or reallocated) before it reaches the PSII reaction center. This process arise from reactions not directly related to photochemistry, which have been defined as 'non-photochemical quenching' to be distinguished from the processes dealing with the 'photochemical quenching' (qP) which is directly related to photochemistry and the linear transport of electrons (Fig. 3a) (Maxwell and Johnson 2000; Baker 2008). In that framework, the redox state of quinones (Q_A and Q_B) and plastoquinones can strongly influence the emission of fluorescence in parallel to NPQ under high light conditions (Perkins et al. 2006). NPQ reduces the lifetime of excited chlorophylls (^{1}Chl*) and thereby the quantum yield of Chl *a* fluorescence, which is seen by a decrease of F_m to F'_m level (see Fig. 3a). For that reason, it is calculated as $(F_m–F'_m)/F'_m$ (or $F_o–F'_o/F'_o$; Lavaud et al. 2002). In higher plants, green algae and dinoflagellates, where the NPQ mechanism has been investigated, it consists of three components (Fig. 3a) (Stroch et al. 2004;

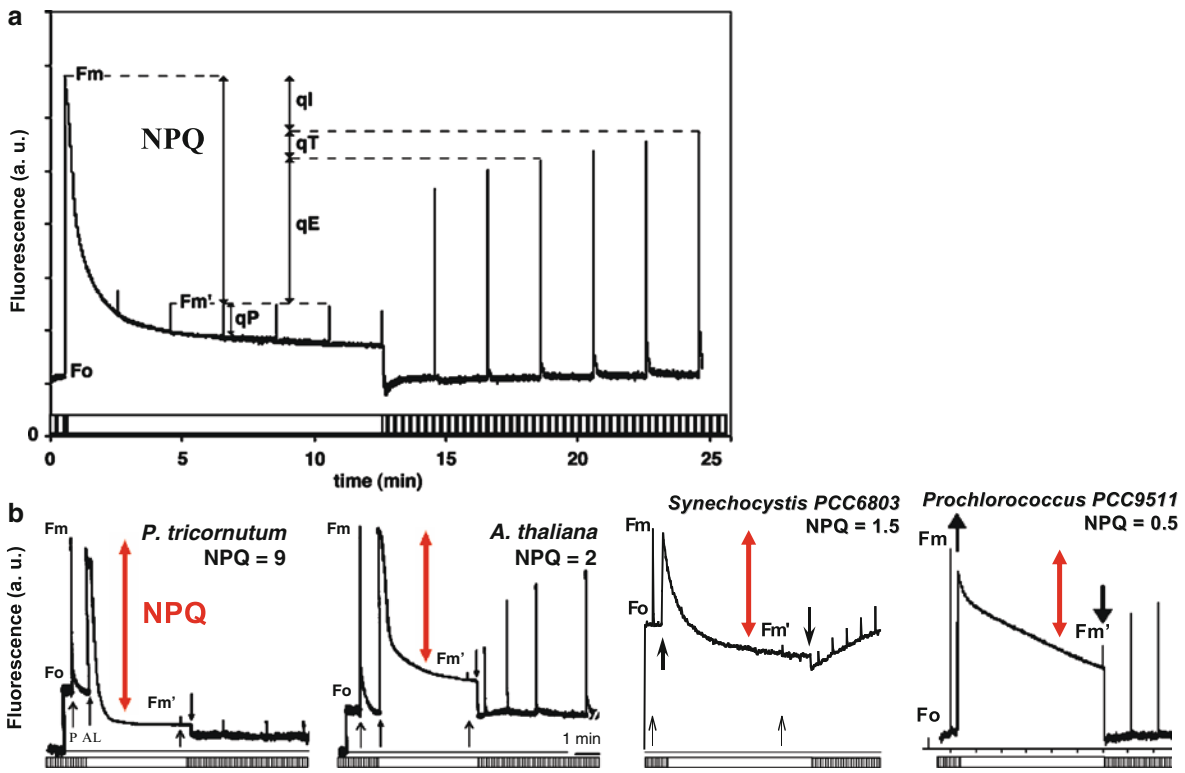

Fig. 3 (**a**) Chlorophyll *a* (Chl *a*) fluorescence signal as measured with a PAM fluorometer on an *Arabidopsis thalania* leaf. After dark-adaptation, in the presence of the detector beam (dashed bottom bar), the minimal fluorescence level (F_o) is measured. When a saturating light pulse (P) is given, the photosynthetic light reactions become saturated and fluorescence reaches a maximum level (F_m). Upon continuous actinic light (AL On, white bottom bar) with moderately excess light (750 µmol photons m^{-2} s^{-1}; growth light was 130 µmol photons m^{-2} s^{-1}), a combination of qP and NPQ lowers the fluorescence yield. NPQ (qE + qT + qI) is the difference between F_m and the measured maximal fluorescence after a saturating light pulse during illumination (F'_m): NPQ = $(F_m\text{-}F'_m)/F'_m$. After switching off the actinic light (AL Off), the quenching on the F_o level can be observed (F'_o). Also, the recovery of F'_m within a few minutes reflects relaxation of the qE component of NPQ. qT takes a longer time to relax while qI is a sustained quenching. Adapted from Müller et al. (2001). (**b**) Characteristic Chl *a* fluorescence signals as measured with a PAM fluorometer in cells of the diatom *Phaeodactylum tricornutum*, leaf of the higher plant *A. thalania*, cells of the cyanobacterium *Synechocystis* PCC6803, and cells of the Prochlorophyte *Prochlorococcus* PCC9511, illumination was: 5 min-2000 µmol photons m^{-2} s^{-1}. The time scale is given on the *A. thaliana* trace. Adapted from Ruban et al. (2004), Cadoret et al. (2004), Bailey et al. (2005). For (**a**) and (**b**): F_o, minimum fluorescence level in the dark; F'_o, minimum fluorescence level after light exposure (detector beam only for both); F_m, maximum fluorescence level in the dark; F'_m, maximum fluorescence level at light; AL, actinic continuous light (bold arrow up/down: AL on/off); P, over-saturating pulses (600–800 ms duration, thin arrows: pulse fire). Bars: dashed, detector beam only; white; detector beam+AL on

Hill et al. 2005; Szabo et al. 2005): the energy-dependent quenching (qE) which is regulated by the built-up of a trans-thylakoid proton gradient (ΔpH) and the operation of the xanthophyll cycle (XC); state-transition quenching (qT); which relies on the redistribution of excitation energy between photosystems by physical modulation of the cross-section of light-harvesting antennas (Ruban and Johnson 2009) and the sustained quenching which is heterogeneous (Demmig-Adams and Adams 2006) which partially depends on xanthophylls (Garcia-Mendoza and Colombo-Pallotta 2007) as well as on photoinactivation/photoinhibition (qI) of PSII (Stroch et al. 2004). Quantification of these three components is either based on their relaxation kinetics in the dark (Müller et al. 2001) or requires photosynthetic inhibitors (Horton and Hague 1988). The characteristics of their relaxation kinetics can vary according to environmental stresses and between

groups of organisms. Such that, qE relaxes very rapidly (within tens of seconds after the offset of light), qT takes several minutes (shorter for cyanobacteria and rhodophytes), while qI is sustained and can last for hours even days under certain extreme environmental conditions (Demmig-Adams and Adams 2006; Garcia-Mendoza and Colombo-Pallotta 2007). Furthermore, in diatoms qE usually relaxes very slowly in comparison to higher plants (compare the two organisms in Fig. 3b) so that it could be confounded with qI due to overlaps with time. In general, with non-stressed leaves, qE is the major component under moderate to saturating irradiance. qI can become prominent under over-saturating irradiances and possibly in combination with other stresses (nutrient/water deficiency, temperature and salinity) (Demmig-Adams and Adams 2006). In this context, qT is not as relevant since it generally only makes a small contribution to overall relaxation of fluorescence (see Fig. 3a) (Nixon and Mullineaux 2001). qT is usually significant only under low light levels (Mullineaux and Emlyn-Jones 2005) while some dinoflagellates increase qT under thermal and light stress (Hill et al. 2005). The amplitude and kinetics of the whole NPQ process and the importance of each component (Fig. 3b) can be extremely divergent between taxa, especially among microalgal groups (Casper-Lindley and Bjorkman 1998; Hill et al. 2005; Juneau and Harrison 2005), and even between species within a taxonomic group (Lavaud et al. 2004; Lavaud et al. 2007). For example, qE shows high amplitude and fast onset in diatoms and brown macroalgae, while being of minor importance in most of the green microalgae (Finazzi et al. 2006) and cyanobacteria (Kirilovsky 2007). Nevertheless, within the diatoms (see Chapter 7) as well as higher plants (Johnson et al. 1993) there are clear differences in qE amplitude that have been highlighted. Whereas, qT is currently unknown in diatoms (Owens 1986) and brown macroalgae (Fork et al. 1991), and of only moderate importance in higher plants and dinoflagellates (Hill et al. 2005), yet highly developed in some green microalgae and cyanobacteria (Finazzi 2005; Mullineaux and Emlyn-Jones 2005).

Amongst the three components of NPQ, qE has a major influence on the Chl *a* fluorescence signal under normal growth conditions (Logan et al. 2007; see also Chapter 7). The interpretation of qE is possibly the most complex of the NPQ components, as it is linked to faster regulation of photosynthesis than qT and qI with most organisms, especially under naturally fluctuating environment (Lavaud 2007). Frenkel et al. (2007) demonstrated that qE is critical for maintaining the fitness of plants under natural temperate-light conditions, rather than qT. Also, in cyanobacteria and green microalgae, qT has no significant physiological importance in photoprotection towards high-light stress, yet is more relevant in low light conditions (Mullineaux and Emlyn-Jones 2005; Ruban and Johnson 2009) and for acclimation to different light quality (Pfannschmidt 2005). The qT mechanism has been documented, as well as its impact on the fluorescence signal, especially in cyanobacteria and green microalgae (see Campbell et al. 1998; Nixon and Mullineaux 2001; Finazzi 2005; Mullineaux and Emlyn-Jones 2005). Even though qI has been well documented in some species of higher plants growing in extreme environments (Demmig-Adams and Adams 2006), its occurrence and control mechanism remains unknown in some of the algal groups. Also, the part of qI which depends on xanthophylls is also linked to the qE process (Demmig-Adams and Adams 2006), although clear mechanistic differences have only been recently demonstrated (Dall'Osto et al. 2005).

The qE mechanism has been described in a molecular context for higher plants and green microalgae (Standfuss et al. 2005; Cogdell 2006; Ruban et al. 2007). The machinery triggering and controlling qE amplitude and kinetics is now quite well known for groups of algae like the diatoms and brown macroalgae (Goss et al. 2006; Lavaud 2007), as well as in the cyanobacteria and prochlorophytes (Bailey et al. 2005; Kirilovsky 2007). The NPQ process is based on a feed-back reaction from the linear electron transport through the build-up of a transthylakoid ΔpH and subsequent acidification of the thylakoid lumen (see Nixon and Mullineaux 2001). Consequently, the activity of the ATP synthase (Dal Bosso et al. 2004) the cytochrome b_6f (Munekage et al. 2001), or the cyclic electron flow around PSI (Miyake et al. 2005) can indirectly influence qE. Hence, in a simple direct relationship, the higher the irradiance, the higher the electron transport rate, the higher the accumulation of protons in the lumen, the higher qE. In some organisms such as diatoms, it appears there is a relative independence of the PSII redox-state from the proton-motive electron transfer

and subsequent NPQ (Ruban et al. 2004; Lavaud et al. 2007). To summarize NPQ responses, the lumen acidification triggers two events (Fig. 4): (1) the protonation of specific sites of the LHC antenna, and (2) the activation of an enzyme, a de-epoxidase, which drives the conversion of epoxized xanthophyll to a de-epoxidized form. This conversion is reversible as the backward reaction is driven by an epoxidase which also depends on the trans-thylakoid ΔpH. The accumulation of de-epoxidized xanthophylls thus depends on the balance between the activity of both enzymes within the xanthophyll cycle (XC) (see Lavaud 2007 for a detailed description). In higher plants, green microalgae and brown macroalgae, the XC involves the conversion of violaxanthin to zeaxanthin (ZX) via antheraxanthin (AX) (Fig. 4) while the diatoms and dinoflagellates use diadinoxanthin (DD) which is converted to diatoxanthin (DT) under elevated light (see MacIntyre et al. – Chapter 7). Both protonated LHC protein(s) and the presence of DT or ZX/AX in the LHC antenna of PSII are thought to act together as the trigger of the qE. The whole LHC antenna switches into a dissipative mode when excess excitation energy should be converted into heat while Chl *a* fluorescence is quenched (Fig. 4) (Stroch et al. 2004; Szabo et al. 2005). More precisely, protonation would promote and transduce conformational changes ('aggregations') which bring pigments closer together and especially chlorophyll/xanthophyll molecules. In higher plants the 'special' polypeptide which undergoes protonation is PsbS (Niyogi et al. 2005). The function of PsbS is essentially to sense the lumen pH, that is linked to several H^+-binding amino acid residues present on the luminal loops of this protein (Dominici et al. 2002; Li et al. 2002). In green microalgae (Peers, G and Niyogi, KK, personal communication, 2008) and diatoms (Zhu and Green 2008), the Li-818 proteins which are up-regulated under high light, could play a similar role as PsbS in qE. Simultaneously, with PsbS protonation, de-epoxidized xanthophylls would also act as 'allosteric regulators' by amplifying the conformational changes within the whole LHC antenna. The physical process by which excitation energy is effectively converted into heat has only recently been understood (Holt et al. 2005; Pascal et al. 2005; Ruban et al. 2007). The qE mechanism is rather similar in other organisms like the diatoms and the brown macroalgae given some peculiarities (see Chapter 7). In other groups like the red algae, cyanobacteria and prochlorophytes, the process is quite different even though it involves xanthophylls and special proteins of the antenna system (Lavaud 2007). Therefore, qE in these taxa is not as controlled as in higher plants or diatoms since these organisms do not display a finely regulated xanthophyll cycle, also cyanobacteria and prochlorophytes show no involvement of a trans-thylakoid ΔpH (Kirilovsky 2007).

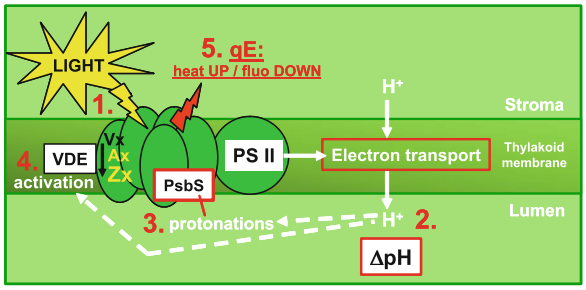

Fig. 4 Simplified model of the qE mechanism in higher plants (see the text for a full description). The numbering refers to the sequence of the qE process steps. AX, antheraxanthin; H^+, protons; PS II, photosystem II; VDE, violaxanthin de-epoxidase; VX, violaxanthin; ZX, zeaxanthin; ΔpH, transthylakoid proton gradient. (Adapted from Lavaud 2007)

Quenching based in the PSII reaction center (as opposed to LHC antenna) has also been observed in higher plants (Bukhov et al. 2001; Stroch et al. 2004) and green microalgae (Finazzi et al. 2004), and possibly in diatoms (Eisenstadt et al. 2008). Appearance of reaction centre quenching depends on the balance between light and carbon fixation fluxes (Finazzi et al. 2004) along with a clear temperature influence (Kornyeyev et al. 2004). This quenching appears to drive both qE and qI components of NPQ with both fast and slow relaxation kinetics, respectively. In contrast to the antenna-based quenching, it cannot cause changes in the F_o level (Maxwell and Johnson 2000). Nevertheless, as well as the antenna-based quenching it requires thylakoid acidification, but it does not require de-epoxidized xanthophylls (Bukhov et al. 2001; Finazzi et al. 2004). The qI part of this reaction center based quenching is associated with a reversible inactivation of a sub-population of the PSII (Finazzi et al. 2004) as well as with PSII photodamage (Kornyeyev et al. 2004).

4 Use of Chemicals for the Differentiation of Photosynthetic Processes

Photosynthesis is a complex interaction of complementary processes such as alternate electron cycling (AEC) and non-photochemical quenching (NPQ). A common method of isolating specific processes is using biochemical inhibitors such as herbicides. Electron transport inhibitors, uncouplers, artificial electron acceptors and donors have all proved to be essential tools in elucidating the function of various components of the photosynthetic electron transport chain, metabolic pathways and photosynthetic regulatory processes. Using herbicides to understand the regulation of photosynthesis and related biochemical pathways requires the basic understanding of how these herbicides interact with the photosynthetic apparatus.

Determining the appropriate concentration of herbicide is very important and often problematic, because depending on the organism, cells can have different cell wall composition, membrane transporters and a variation in the number of reaction centres per cell, thus requiring different concentrations of herbicide (Durnford et al. 1998). Therefore, any concentrations specified herein are only an indication of what has been used based the range of concentrations found in the literature. The most effective and correct way to determine the concentration at which a cellular response occurs is by titration of the herbicide against a known cell density or chlorophyll *a* concentration while measuring the physiological impact (oxygen evolution or chl *a* fluorescence).

4.1 Inhibitors of Linear Electron Transport

DCMU (3′-(3,4-dichlorophenyl)-1′,1′-dimethylurea), also known as Diuron, is the most extensively used inhibitor of photosynthetic electron transport. DCMU inhibits electron transport between PSII and PSI, impacting on the acceptor side of PSII by supplanting a bound plastoquinone from the Q_B^- binding site of PSII (Fig. 5). Binding of this herbicide to the Q_B^- site of PSII, results in the effective blocking of electron flow and leads to the subsequent inhibition of photosynthesis. Blocking of electron flow is a consequence of the herbicide being incapable of receiving electrons, and therefore electrons remain trapped in Q_A, the first quinone acceptor (Kleczkowski 1994). This trapping of electrons prevents the reduction of plastoquinone, by holding the electrons in the D1 dimer, thus affecting the redox state of the PQ pool, which becomes completely oxidised (Durnford et al. 1998).

DCMU causes a rapid increase to maximum fluorescence (F_m), where all PSII reaction centres are closed and the plastoquinone pool fully oxidised (Trebst 2007). DCMU has no impact on the membrane potential of the thylakoid in darkness, yet completely inhibits the light-induced membrane pH gradient. The amount of DCMU required for the inhibition of 50% of PSII reaction centres will vary depending on cell concentration and species. Published concentrations range from 1–20 μM (Falkowski and Raven 2007). However, incremental increases in the amount of DCMU added to cells will result in changes in variable fluorescence and the rate of Q_A re-oxidation (Durnford et al. 1998), which will invariably allow for the determination of the appropriate concentration of DCMU needed to elicit the desired effect. In addition, the light acclimation state of the cells needs to be taken into account, as cells grown at low photon flux densities will have a plastiquinone (PQ) pool that is predominantly oxidized and therefore the addition of DCMU will have very little effect on the redox state of the PQ pool (Durnford et al. 1998) similarly, chlororespiration can alter the PQ redox state, allowing fluorescence yield to occur with saturating DCMU concentrations (Wilhelm and Duval 1990). In the presence of saturating DCMU concentrations, fluorescence yield becomes maximum ($F_o \Rightarrow F_m$ so $F_v/F_m \Rightarrow 0$), as Q_A can no longer pass electrons to PQ, so electron transport stops and the maximum amount of captured energy is dissipated as fluorescence.

Like DCMU, DBMIB (2,5-dibromo-3-methyl-6-isopropyl-p-benzoquinone) is also an inhibitor of electron transport, however it blocks further along the electron transport chain near the Cytochrome b_6f complex (Trebst 2007). DBMIB is thought to interfere at the Reiske iron-sulfur centre (Trebst 2007), thus blocking photosynthetic electron flow through the Cytochrome b_6f complex. DBMIB binds close to the Qo pocket (Cramer et al. 2006), the plastoquinol binding site of the Cytochrome b_6f complex (Fig. 5), inhibiting the reoxidation of PQH_2 thus keeping the PQ pool completely reduced (Trebst 1980).

Some precaution should be taken when using DBMIB, because site of action is concentration dependent, as well as redox sensitive, where DBMIB becomes reduced under light and oxidised in the dark (Bukhov et al. 2003). At low concentrations, DBMIB inhibits electron transport on the reducing side of the PQ, but at

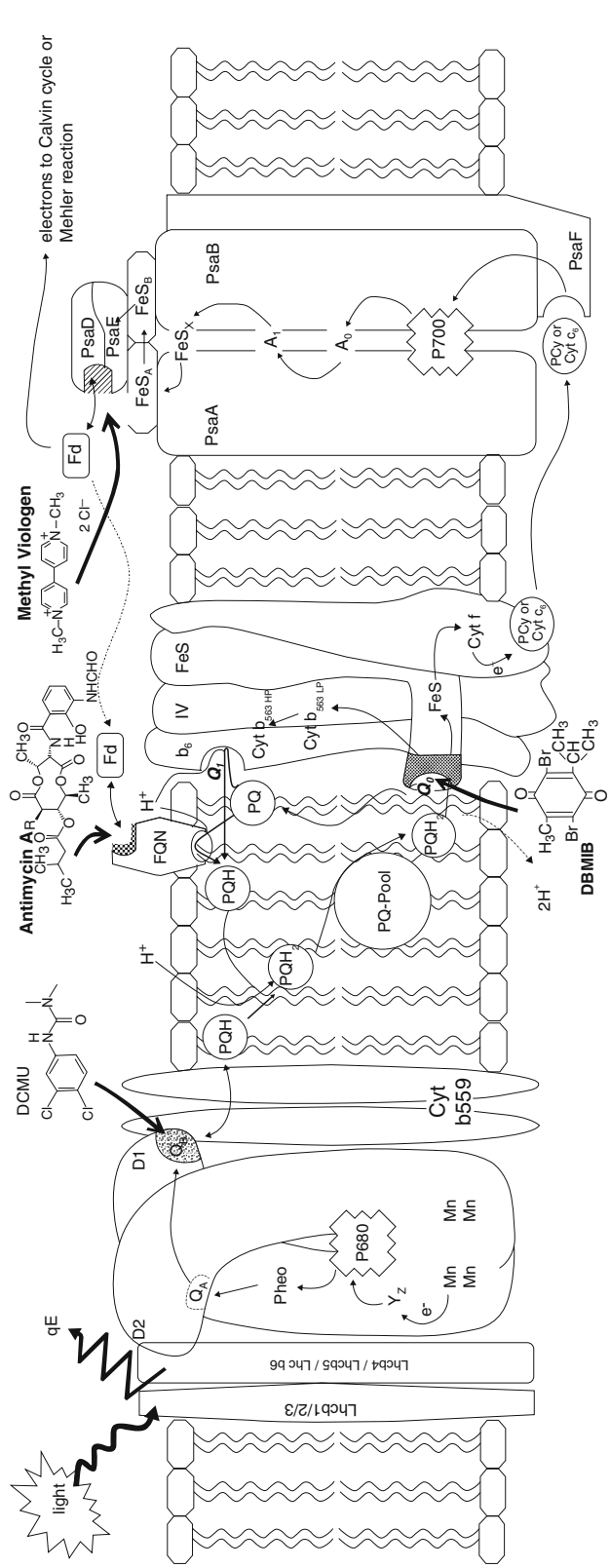

Fig. 5 Electron transport flow through PSII, cytochrome b_6f complex and PSI. The D1, D2 and Cyt b559 proteins are shown. The thin arrows indicate the electron flow pathway through PSII, while the thick arrow indicates the site of inhibition by DCMU. The figure shows the DCMU molecule binding to the Q_B site of PSII, thereby effectively blocking the continuation of electron flow from PSII to the plastoquinone, cytochrome b_6f complex and onto PSI. After Q_B the thin arrows indicate electron flow pathways and proton (H^+) pathways. The thick arrow in the lower middle of the diagram indicates the site of impact by the inhibitors DBMIB and antimycin A. One DBMIB molecule competes with the PQH_2 resulting in the blocking of the release of electrons from the plastoquinone to PSI at the Fe-S complex. Antimycin A (upper middle) inhibits the reduction of ferrodoxin in PSI, intercepting electron transport at the ferrodoxin-plastoquinone reductase, resulting in the blocking of cyclic electron transport. Electrons move through PSI indicated by thin arrows, while the thick arrow (upper right) indicates the impact site of methyl viologen. Methyl viologen interacts at the binding site of ferredoxin, accepting the terminal electron, thus preventing the reduction of ferredoxin and the continued pathway of the electron to carbon fixation or cyclic electron transport

higher concentrations excess DBMIB will inhibit the Q_B site of PSII, located on the oxidising side of the PQ (Moreland 1980; Rich et al. 1991). To prevent fluorescence quenching from oxidised DBMIB, it can be used in conjunction with an excess of sodium ascorbate (Kufryk and Vermaas 2006). For every Cytochrome b_6f complex, one molecule of $DBMIB_{red}$ is needed for complete inhibition of electron transfer through the Cytochrome b_6f complex (Rich et al. 1991). DBMIB can also inhibit mitochondrial electron transport (Durnford et al. 1998). This is a good example of co-inhibition, where some inhibitors have more than one impact site, and therefore interpretation of results must carefully consider the possibility of an alternate component of the cell machinery being affected by the inhibitor. DBMIB reduces minimum fluorescence (F_o) as well as the rise of variable fluorescence (F_v). While $DBMIB_{red}$ quenches chlorophyll *a* fluorescence, it does so less efficiently than the oxidised form ($DBMIB_{ox}$) and both forms alter NPQ estimates (Tyystjarvi et al. 1999).

4.2 Inhibitors of Cyclic Electron Transport

The antibiotic, antimycin A, is an effective inhibitor of one of the alternate electron cycles (AEC), cyclic electron transport around PSI (Tagawa et al. 1963). It has been proposed that inhibition of photosynthetic electron transport by antimycin A is associated with the ferredoxin-plastoquinone reductase (FQR) activity in cyclic electron transport (Simonis and Urbach 1973; Moss and Bendall 1984; Cleland and Bendall 1992). In addition to inhibiting cyclic electron transport, antimycin A is also known to inhibit excess light energy dissipation measured through NPQ (Oxborough and Horton 1987). The decline in qE (energy-dependent quenching) formation in the presence of this antibiotic is due to a change in the redox state of the electron transport chain. However, since antimycin A has no direct impact on linear electron transport rate, the redox change is most likely the result of a change in the redox state of a component located in the cytochrome complex (Oxborough and Horton 1987). Before inhibiting cyclic electron transport, it is important to understand that there are two potential transport pathways that cycle around PSI (Joët et al. 2001; Munekage et al. 2004). The first, cycles electrons from ferrodoxin to the PQ pool and is sensitive to antimycin A, while the second, involves the NDH complex which is insensitive to the antibiotic (Joët et al. 2001). In the case of the NDH cycle, it is not yet fully understood and no known inhibitor has been identified. Published concentrations of antimycin A range from 0.1–50 μM (Falkowski and Raven 2007).

4.3 Inhibitors of Alternative Electron Cycling (AEC)

Distinguishing between different electron pathways is important to describe the discrepancies often seen between oxygen evolution and chlorophyll *a* fluorescence under stressful conditions. Molecular oxygen can be reduced downstream of PSII at various sites, using different forms of AEC. In the case of the Mehler reaction, oxygen is reduced at the acceptor side of PSI (Mehler 1951) where it competes for electrons with both linear and cyclic electron transport pathways (Heber 2002). The Mehler reaction itself cannot be inhibited; however, the addition of potassium cyanide (KCN) can be used to inhibit the formation of H_2O and monodehydroascorbate (MDA) during the ascorbate peroxidase reaction, which is part of the Mehler cycle (Neubauer and Yamamoto 1992). The inhibition of H_2O formation as a result of altered peroxide turnover, impacts on the zeaxanthin-dependent light energy dissipation, by suppressing zeaxanthing formation and consequently NPQ (Neubauer and Yamamoto 1992). In fluorescence, the addition of KCN results in a decline in NPQ as well as a decrease in linear electron flow, shown as a suppression of q_P (Neubauer and Yamamoto 1992). Published KCN concentrations vary from 0.1 mM to 3 mM (Neubauer and Yamamoto 1992, Hormann et al. 1994, Singh et al. 1996).

Another more recently discovered pseudo-cyclic electron transport pathway which cycles around PSII via the plastoquinol terminal oxidase (PTOX), reduces molecular oxygen by utilising electrons from the PQ pool to generate H_2O (Cournac et al. 2000; Peltier and Cournac 2002; Josse et al. 2003). This alternative electron flow around PSII (upstream of PSI and Cytochrome b_6f) is believed to be advantageous in both a high-light environment and under iron limitation (Bailey et al. 2008), as it alleviates PSII excitation pressure by transporting electrons directly to oxygen while simultaneously ensuring that the electrons bypass the iron-demanding cytochrome b_6f and PSI complexes

(Mackey et al. 2008). Propyl-gallate (PGal) is an oxidase inhibitor specific to PTOX (Cournac et al. 2000; Bailey et al. 2008). PGal helps determine the role PTOX plays in alternative electron flow, and establish whether or not electrons are being used to reduce oxygen through PTOX activity (Mackey et al. 2008). The addition of 1 mM PGal results in a decrease in electron flow through PSII (Bailey et al. 2008; Mackey et al. 2008), highlighting the role the oxidase plays in keeping the PSII reaction centres oxidised in cells where Cytochrome b_6f and PSI activity are limiting. As in the case of DBMIB, PGal has more than one impact site in eukaryotes, as it can also lead to the inhibition of mitochondrial electron transport (Bailey et al. 2008).

4.4 Inhibitors of CO_2 Fixation

Iodoacetamide has been used as an inhibitor of carbon fixation (Miller et al. 1988; Miller and Canvin 1989), however when added during steady state photosynthesis, it inhibits CO_2 very slowly and may induce O_2 uptake in the light (Miller and Canvin 1989). An alternative inhibitor of CO_2 fixation is D, L-glyceraldehyde (Stokes and Walker 1972), which at very high concentrations (>25 mM) completely inhibits CO_2 fixation (Shelp and Canvin 1989) and blocks the conversion of triose-P to ribulose-1,5-bisphosphate (Stokes and Walker 1972). However, more recently, glycolaldehyde (GA) has become the preferred inhibitor of CO_2 fixation, as it uses concentrations an order of magnitude lower than those of D, L-glyceraldehyde, while rapidly and effectively inhibiting CO_2 fixation (Sicher 1984) without inhibiting CO_2 or HCO_3^- transport (Miller and Canvin 1989; Rotatore et al. 1992). The addition of GA to cells eliminates the chlorophyll *a* fluorescence quenching that is seen with the addition of inorganic carbon; however, oxygen evolution is greatly impacted by the presence of GA (Miller and Canvin 1989).

4.5 Electron Transport Uncouplers

Uncouplers function by dissociating electron transport from ATP synthesis during photosynthetic phosphorylation (Moreland 1980; McCauley et al. 1987). This is accomplished by dissipating the energised state (H^+) of the membrane (ΔpH) before the energy can be utilised in ADP phosphorylation (Moreland 1980) and thus prevent the formation of the trans-thylakoid ΔpH gradient. In addition to this major effect on the energy budget of the cell, the electron flow continues but the collapsed proton gradient no longer regulates electron transport rate. This type of inhibitor can be useful when examining processes triggered by ΔpH, such as NPQ and in particular qE. Common uncouplers of photophosphorylation include ammonia chloride ((NH_4Cl), carbonyl cyanide 4-trifluoromethyloxyphenylhydazone (FCCP) and nigericin.

Ammonium chloride (NH_4Cl) is a potent uncoupler of electron transport. As described above, it works in the classical way by relaxing the pH gradient across the thylakoid membrane, inhibiting ATP synthesis. The addition of NH_4Cl before the application of saturating light will prevent all quenching of F'_m. In contrast, if the uncoupler is added after fluorescence quenching has already formed (following a series of saturating pulses), it will result in a complete reversal of all F'_m quenching (Delphin et al. 1998).

Carbonyl cyanide p-trifluoromethoxy phenylhydrazone (FCCP) is a powerful uncoupler of photophosphorylation. It acts as an ionophore completely dissipating the pH gradient, while leaving the electron transport system uninhibited (Canaani and Havaux 1990). FCCP prevents the long-term fluorescence induction, meaning that the inhibition of the induction is likely the result of an increase in the dark decay processes (Canaani and Havaux 1990). Typical concentrations of FCCP are 1–10 μM (Shyam et al. 1993; Sigalat et al. 1993; Singh et al. 1996). At low concentrations FCCP quenches PSII fluorescence, indicative of the reoxidation of Q_A^- (McCauley et al. 1987), while it requires much higher concentrations to perform in its function as an uncoupler of oxidative phosphorylation (Canaani and Havaux 1990). When incubated with cells under photoinhibitory light, FCCP accelerates photoinhibition and rapidly quenches fluorescence yield (McCauley et al. 1987; Shyam et al. 1993; Singh et al. 1996).

Another type of uncoupler is the protonophore, such as nigericin which dissipates the proton gradient across the thylakoid membrane. Nigericin relaxes the ΔpH gradient by antiporting H^+ at the expense of K^+ across membranes, resulting in the collapse qE (Pressman et al. 1967). As a result of a breakdown in the pH gradient, the addition of nigericin to illuminated samples,

results in an increase in F_m' and strong inhibition of NPQ with a concomitant large increase in steady state fluorescence F_t. The typical concentration range for nigericin is 1–5 mM (Falkowski and Raven 2007).

4.6 Electron Acceptors

Electron acceptors are compounds with very strong reducing capacity, such as methyl viologen (N,N′-Dimethyl-4,4′-bipyridinium dichloride; MV^{2+}) also known as Paraquat. Methyl viologen is an artificial electron acceptor, intercepting electron flow between PSI and the Calvin cycle by competing with ferrodoxin for the binding site at PSI (Fig. 5) (Dan Hess 2000). MV^{2+} is an extremely powerful electron acceptor, due to the nature of the bipyridinium salts, which temporarily become a stable radical with the addition of an electron, neutralising the positive charge of the cation (Moreland 1980; Peon et al. 2001). MV^{2+} oxidizes the primary acceptor (ferrodoxin) of linear electron transport, allowing a ΔpH to become established. However, this temporary neutral radical rapidly reverts back to its ion form, a process that results in the production of superoxide radicals (Hormann et al. 1993; Dan Hess 2000). MV^{2+} can be used to demonstrate damage to the electron transport chain beyond PSI (typically Calvin cycle), where incubation with MV^{2+} will oxidise the electron transport chain and increase Φ_{PSII} by supplementing the slow carbon fixation rate. In the presence of MV^{2+}, non-photochemical quenching (NPQ) is reduced, because the excess electrons that are usually held up by the Calvin cycle, are being accepted by the MV^{2+} allowing for continual rapid electron transport and a reduced need for excess light energy dissipation in the form of NPQ. Published concentrations of MV^{2+} range from 0.05 to 1 mM (Falkowski and Raven 2007).

In conclusion, we have illustrated how photosynthetic electron transport is strongly influenced by a range of internal feedback processes (AEC and NPQ) to ensure maximum efficiency, whilst preventing potential damage from excess excitation energy. Light stimulated processes such as NPQ are closely linked with pigments, however the control mechanisms are species-specific and show wide variability. Chemical inhibitors can be used to isolate specific components of the electron transport chain allowing a mechanistic understanding of the control of these photosynthetic pathways.

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