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Biochimica et Biophysica Acta 1187 (1994) 145–151

BBA Biochimica et Biophysica Acta

Control of oxidative phosphorylation in plant mitochondria: the role of non-phosphorylating pathways

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Received 3 March 1994

Key words: Mitochondrion; Respiration; Alternative oxidase; Oxidative phosphorylation; Ubiquinone; (Plant)

1. Introduction

A knowledge of the factors that regulate respiratory activity and oxidative phosphorylation in plant cells in vivo is of particular importance since, for some plant species, a negative correlation exists between yield and the rate of leaf respiration [1]. For instance slow-respiring populations, in some plant species, show increased yield when compared with fast-respiring populations [2]. Such an observation may be a reflection of more efficient use of carbohydrate, increased efficiency of oxidative phosphorylation or a decrease in energy-utilising reactions [3]. Thus, a low plant respiratory rate may be the result of lowered respiratory costs for energy-requiring processes such as maintenance respiration, growth or ion uptake. Whatever the outcome of such studies it is obvious that a detailed understanding of the regulation of plant respiration is paramount for a fuller picture of the mechanisms that control plant growth and metabolism.

Although the precise mechanism that controls respiratory activity and oxidative phosphorylation in plant cells in vivo, as in other systems, is uncertain, it probably involves the carbohydrate status (i.e., the flux of carbon), the levels of the adenine nucleotides, the activity of the adenine nucleotide translocator and ATP synthase, the magnitude of the protonmotive force and perhaps more importantly, with respect to plant tissues, the extent to which non-phosphorylating pathways of the respiratory chain are engaged [4,5].

In addition to complexes I–IV, it is well-established that plant mitochondria possess routes of substrate oxidation and a terminal oxidase that by-passes the proton-pumping activity of complexes I, III and IV (Fig. 1). These include an NAD(P)H dehydrogenase located on the outer surface of the inner mitochondrial membrane, an internal rotenone-insensitive route that by-passes complex I and a cyanide- and antimycin-resistant alternative oxidase Substrates can therefore be oxidised protonmotively with subsequent ATP produc-

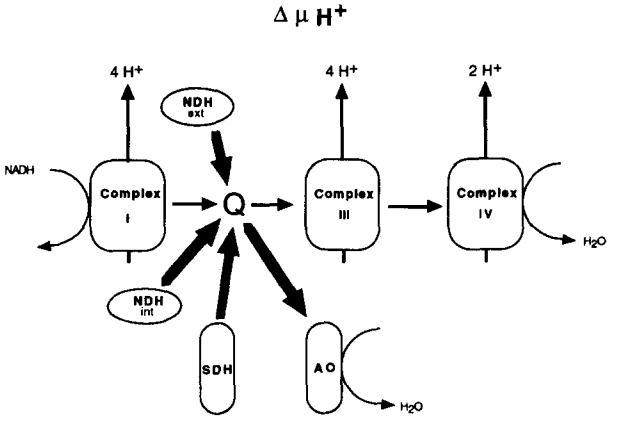

Fig. 1. A diagrammatic representation of the plaznt mitochondrial respiratory chain. The bold arrows represent non-phosphorylating routes of electron transfer to oxygen. NDH ext, the external NADH dehyrogenase; NDH int, the internal rotenone-insensitive pathway; SDH, Complex II; AO, the alternative oxidase; Q, the ubiquinone pool; $\Delta\mu H^+$, the proton electrochemical gradient.

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SSDI 0005-2728(94)00088-M

tion only when the oxidation pathway includes Complex I and/or Complexes III and IV.

2. Characteristics of the alternative oxidase

The alternative oxidase branches from the main mitochondrial respiratory chain at the level of the ubiquinone pool, it is non-protonmotive and an integral membrane protein with its active site located on the matrix side of the inner membrane [6]. Activity of the alternative oxidase strongly correlates with the appearance of three proteins with masses of 35, 36 and 37 kDa in thermogenic tissues and 35 kDa in non-thermogenic tissues [7]. Monoclonal antibodies cross-react not only with proteins from aroid (thermogenic) species but also with proteins from widely divergent plant species such as tobacco, pea and potato in addition to fungi and trypanosomes suggesting that not only is cyanide-resistance widespread amongst the plant kingdom but also highly conserved [8]. The cDNA encoding the precursor of the alternative oxidase protein has been isolated and characterised from a number of sources including *Sauromatum* [9], *Arabidopsis* [10], soybean [11] and the yeast *Hansenula anomala* [12]. Expression of the *Arabidopsis* alternative oxidase cDNA in *E. coli* (haem A deficient) is sufficient to support growth in the presence of cyanide [10]. Amino-acid sequence comparison reveals a high degree of homology amongst all species particularly within the two putative membrane-spanning helical regions and the C-terminal region. Among the plant sequences, only two of the three Cys residues are conserved and comparison with the yeast sequence reveals that only one of these residues is likely to be important for the functioning of the oxidase [13]. Analysis of the sequence also reveals that there are three possible metal-binding motifs located in the highly conserved C-terminal region that faces the matrix. Recent evidence suggests that the conserved Cys residue (Cys-172) may be involved in disulphide linkage between adjacent monomers of the alternative oxidase protein [13]. The monomeric reduced form of the protein is considered to be the active form of the enzyme and it has been proposed that the reversible nature of the intermolecular disulphide linkage may provide an additional mechanism for regulating enzyme activity in vivo [13]. Although the mechanism of conversion between the oxidised and reduced states of the alternative oxidase protein is unknown, one distinct possibility that has been suggested [13] is via thioredoxin, which has been demonstrated to be present within the plant mitochondrial matrix [14]. Despite an understanding of the relationship of the alternative oxidase to the main respiratory chain, the identification of both a gene and its associated protein, the role of the alternative oxidase in plant metabolism, apart from thermogenic tissues is far from clear. Although the pathway has been suggested to provide an energy overflow mechanism to enable the TCA cycle to operate under high cytosolic ATP levels [15] which may exist in the light, the evidence in favour of this hypothesis is contradictory (see [16,17]). An understanding of both the structure and control of the alternative oxidase is therefore necessary to not only clarify its function but also determine the extent to which it is engaged in vivo.

3. Regulation via the redox poise of the Q-pool

As indicated in the previous section a knowledge of the extent to which the alternative pathway is engaged in vivo is of particular importance since operation of the pathway results in by-pass of energy-conservation at sites 2 and 3 (which is potentially wasteful) and hence may be important in determining overall carbon balance of the plant. Extensive kinetic investigations [6,18–22], resulting from the development of a "Q-electrode" to measure the redox poise of the mitochondrial Q-pool [18], have revealed that engagement of the alternative pathway in non-thermogenic tissues is governed largely by the redox state of the Q-pool such that electron flow through the pathway only occurs when the reduction state of the pool exceeds a certain threshold value. Beyond this threshold engagement increases as a non-linear function of the Q-pool reduction state. In thermogenic tissues the relationship between the respiratory rate and the level of Q-pool reduction is linear. A two-step reduction model, based upon Q-pool assumptions, has been developed [4,6,21,22] the steady-state rate equation of which has been used to simulate data describing the relationship between Q-pool redox poise and alternative oxidase activity. The simulations appear to accurately describe such relationships and furthermore indicate that variations in the observed relation between Q-pool reduction state and alternative pathway activity among different plant mitochondria is mainly associated with differences in the rates of reaction between the reduced oxidase and oxidised ubiquinone. Additionally the simulations suggest that the reduction of oxygen by the alternative oxidase proceeds via the initial formation of a four-electron reduced enzyme [22].

4. Regulation in terms of metabolic control analysis

Although the metabolic control theory [23] has been used successfully to examine the regulation of respiratory activity in mammalian mitochondria [see 24 & 25] until recently plant respiratory systems [26–29] have not been studied in detail. The application of control

theory to both mammalian and plant mitochondrial systems has led to the idea that control of respiratory flux does not reside in a single step but is distributed over a number of different steps and the contribution of these steps to the overall control of the respiratory rate varies markedly with the metabolic condition of the mitochondria which in turn depends principally on factors such as the availability of ADP and respiratory substrates. Earlier investigations [26] revealed that under state 3 conditions control is distributed (in order of importance) between cytochrome oxidase, the bc_1 complex and the ATP-synthase with the adenine nucleotide translocator having no control over flux under these conditions. It should be noted, however, that the control coefficients determined by Padovan et al. [26] do not add up to 1 suggesting that there are other controlling processes. Under state 4 conditions (ADP limiting) neither cytochrome oxidase nor the bc_1 complex exerted major control suggesting that control is located in other steps such as the dehydrogenases, the proton leak and the substrate carriers. More recently, the top-down approach of metabolic control analysis has been applied to characterise the control of respiration in potato tuber mitochondria [27,29]. Control of the respiratory rate was found to be exerted mainly by the respiratory chain itself except at rates close to state 4, where the proton leak assumed an equal or larger degree of control. Control over the phosphorylation rate was also found to be predominantly by the respiratory chain except at low respiratory rates when control by the phosphorylation system became important. Control over the proton leak rate was shared between the respiratory chain and the proton leak with the phosphorylation system exerting negative control on the proton leak. Relatively little control was found to be exerted on the magnitude of the protonmotive force, which probably reflects the equal and opposite effects that the respiratory chain and phosphorylation system have in maintaining the protonmotive force at a near constant level. Under such conditions the proton leak exerted little control on the protonmotive force except close to state 4. Kesseler et al. [27] also attempted to define the control exerted by the alternative oxidase over the respiratory chain, proton leak, phosphorylating system or Δp and concluded that none of these parameters were controlled by the alternative oxidase. Interestingly, in contrast to previous kinetic investigations [6,19,21,22], Kesseler et al. [27] concluded that respiratory flux through the alternative oxidase remains unchanged as respiration is varied between state 4 and state 3. It is more conceivable that, since mitochondria from fresh potato tubers were used (which possess negligible alternative oxidase), the respiratory rate observed in the presence of cyanide was not due to alternative oxidase activity but more likely to a residual rate of non-mitochondrial origin. In a recent examination of the distribution of control of succinate oxidation in cucumber cotyledon mitochondria Hill et al. [28] concluded that, in contrast to Padovan et al. [26] and Kesseler et al. [27], that the adenine nucleotide translocator and dicarboxylate carrier exerted the most control with relatively little at the level of the respiratory chain. These results [28], however, probably do not reflect the situation in vivo, since succinate is generated intramitochondrially and hence the dicarboxylate carrier is unlikely to exert significant control over respiration under these conditions.

In an attempt to more clearly define the extent of control by the alternative oxidase, we have examined the distribution of control during succinate oxidation by potato callus mitochondria which have previously been demonstrated to possess considerable alternative oxidase activity. The system is defined as the respiratory chain only, comprising of succinate dehydrogenase (activated by ATP), the cytochrome pathway and the alternative oxidase linked by the quinone pool as the common intermediary metabolite. All other factors affecting respiration (such as the succinate/fumarate and oxygen/water couples, the energy state of the mitochondria) are considered to be constant (sources and sinks) in the steady-state under consideration. Consequently, control by the phosphorylation system or proton leak is not considered here.

Experimental data that has been used to analyse control was obtained, for each of the three pathways, from malonate titrations of succinate oxidase activity using potato callus mitochondria. Oxygen consumption and steady-state redox poise of the quinone pool were measured simultaneously during these titrations, as previously described for soybean cotyledon mitochondria [19], thus allowing the steady-state values of oxygen uptake (v_{ss}) and the proportion of quinone in the reduced state (Q_r/Q_t) to be determined (the point where the sum of rates of quinone reduction by the three pathways is zero). To determine the control coefficients, the elasticity coefficients of the three pathways with respect to Q_r/Q_t were calculated from the slopes at steady-state of the three v vs. Q_r/Q_t curves. Slopes are determined from hyperbolic fits to the curves (Krab and Van den Bergen, unpublished results; Ref. [30]). From the same fits, the rates through the individual pathways at steady-state can be calculated. Using the matrix method [31], the control of the three pathways on the overall rate (v_{ss}), on Q_r/Q_t and on the ratio of rates through the cytochrome and alternative pathways can be calculated.

An experimental problem encountered in such an approach is that the v vs. Q_r/Q_t curve for succinate dehydrogenase both under state 3 and 4 conditions is very steep in the majority of plant mitochondria we have studied to date i.e., the Q_r/Q_t ratio hardly changes when v is varied by inhibitor titration of the

Table 1
Control coefficients of mitochondria respiring on succinate

	Control by	$C(V_{ss})$	$C(Q_r/Q_t)$	$C(r_{ca})$
A				
State 3	succinate dehydrogenase	0.739	0.835	−0.342
	cytochrome pathway	0.259	−0.827	1.339
	alternative oxidase	0.003	−0.008	−0.997
State 4	succinate dehydrogenase	0.548	0.420	−1.020
	cytochrome pathway	0.357	−0.332	1.805
	alternative oxidase	0.095	−0.088	−0.785
B				
State 3	succinate dehydrogenase	0.003	0.003	−0.001
	cytochrome pathway	0.987	−0.003	1.001
	alternative oxidase	0.010	−0.000	−1.000
State 4	succinate dehydrogenase	0.001	0.001	−0.002
	cytochrome pathway	0.789	−0.001	1.002
	alternative oxidase	0.210	−0.000	−1.000

A. Calculated from steady-state data obtained with isolated potato callus (Van den Bergen et al., unpublished). B. Calculated after multiplying the elasticity coefficients of succinate dehydrogenase with respect to Q_r/Q_t by 1000. V_{ss}, steady-state rate of oxygen uptake; Q_r/Q_t, steady-state Q reduction; r_{ca}, steady-state value of rate through cyt. pathway divided by rate through alt.ox. C, control coefficient exerted by enzyme or pathway specified by (parameter).

quinol-oxidising pathways (see Fig. 1 in Ref. [32]). Consequently, it is not possible to determine the elasticity of succinate dehydrogenase with respect to Q_r/Q_t (it has a very large negative value). An exception to this case are potato callus mitochondria, where the v vs. Q_r/Q_t curves for succinate dehydrogenase are less steep (Van den Bergen et al., unpublished observations). Table 1 summarises the values of the control coefficients obtained by the above approach using potato callus mitochondria under state 3 and 4 conditions. From Table 1A it is apparent that within the boundaries of the system considered, in potato callus mitochondria, succinate dehydrogenase has the greatest degree of control both on the overall rate and on the redox poise of the Q-pool both in state 3 and 4. As anticipated transition from state 3 to state 4 results in an increased control exerted by the alternative oxidase but interestingly also by the cytochrome pathway at the expense of the dehydrogenase.

From Table 1, it is possible to estimate what would happen when the succinate dehydrogenase elasticity coefficient is decreased to the large negative values observed with other plant mitochondria (Table 1B). All control on Q_r/Q_t and by succinate dehydrogenase drop to essentially zero. In fact, virtually all control on v_{ss} under state 3 conditions is by the cytochrome pathway and predominantly so under state 4. Decreasing the elasticity coefficient of succinate dehydrogenase does increase the control exerted by the alternative pathway under state 4 but substantially less than that of the cytochrome pathway. Under both state 3 and 4, the control coefficients of the cytochrome and alternative pathway for r_{ca} (steady-state value of the rate through the cytochrome pathway divided by the rate through the alternative oxidase) approach 1 and −1, respectively.

5. Control of oxidative phosphorylation in vivo

The preceding sections clearly demonstrate that there is no simple answer as to what does control respiration and oxidative phosphorylation even in vitro. Obviously there are a great many factors which regulate these two processes and the degree to which each exerts control will vary dependent upon the metabolic condition of the cell. Perhaps one of the most dominant of the factors that influence overall respiratory activity in vivo will be the concentration of cytosolic adenylates. Whether control is mediated by the phosphorylation potential, the absolute concentration of ADP or any other function of the cytosolic energy state is not of prime importance since alteration to any or all of these parameters will result in changes in the efficient operation of the respiratory chain.

A knowledge of the extent to which respiration and oxidative phosphorylation are controlled in vivo is of particular importance during a transition of the plant cell from dark to light and under conditions where photorespiration may occur [4,5]. In mature photosynthetic plant cells evidence in the literature suggests there is a considerable increase in the cytosolic levels of ATP upon illumination due to indirect transfer of ATP from the chloroplast stroma (see Refs. [17,33]). Such increases would impose severe restraints upon coupled respiratory activity as a result of an increase in Δp, which in turn will influence the redox status of the components of the electron transport chain. As apparent from the preceding section, changes in the redox poise of components, such as Q_r, will dictate the degree of engagement of the non-phosphorylating pathways. Thus, although increases in cytosolic ATP (and decreases in cytosolic ADP), in the light, may result in a decrease in mitochondrial ATP synthesis activity, it may well not necessarily result in complete inhibition of respiratory activity due to the engagement of these pathways. Indeed it is well documented that considerable increases in respiratory activity can be achieved when additional substrates are added to isolated plant mitochondria even under conditions of ADP limitation (see Refs. [4,5]). This is considered a result of increased engagement of the alternative oxidase (when present) and the internal rotenone-insensitive bypass, and/or an increase in the proton leak.

Such considerations are particularly relevant in a discussion as to the extent to which respiratory activity is controlled under photorespiratory conditions. During photorespiration glycine is converted to serine via

the dual activities of glycine decarboxylase and serine hydroxymethyl transferase [34]. Glycine decarboxylase is a multienzyme complex which catalyses the conversion of glycine with the concomitant reduction of NAD^+ and release of CO_2 and ammonia. This reaction, which is restricted to leaf tissue, adds further to mitochondrial function and activity since the continued operation of the decarboxylase requires the continual re oxidation of the NADH either by the operation of increased respiratory activity and/or as a result of substrate(s) shuttle functioning (see Refs. [5,17,35]). Although the exact extent to which either of these processes are involved in the re-oxidation of NADH is uncertain [35], results, using inhibitors of either the ATP synthase or glycine decarboxylase, suggest that mitochondrial oxidative phosphorylation does contribute ATP to the cytosol even under illuminating conditions (see Refs. [5,17,35]). Such results might initially appear contrary to the idea of adenylate control upon mitochondrial respiratory activity in the light. However, as noted earlier, increases in cytosolic ATP (and decreases in cytosolic ADP), which occur upon illumination, will result in an increased protonmotive force and, as a consequence of non-ohmicity of the proton leak, a lowered yield of ATP in the light. In this context, it is important to note that as the respiratory rate decreases the efficiency of ATP production (i.e., the ADP/O ratio) actually increases, in a protonmotive independent manner, towards the theoretical maximum [32,36]. In other words, oxidative phosphorylation becomes more efficient at low respiratory rates even though the actual yield of ATP may decrease. For instance Table 2 shows that, in fresh potato tuber mitochondria, when the rate of NADH oxidation is faster than that of succinate it is generally accompanied by a lower ADP/O ratio. When the succinate-dependent state 3 respiratory rate is reduced from 118 to 30 nmol O_2 per min per mg by the addition of malonate (at a constant protonmotive force), the subsequent addition of NADH increases the respiratory rate considerably (from 30 to 116 nmol O_2 per min per mg) but results in a decrease in the ADP/O ratio from 1.41 to 1.10. Such changes in coupling efficiency can also be elicited with NAD^+-linked substrates operating via the internal rotenone-insensitive pathway. Thus, the observed ADP/O ratio appears to dependent upon respiratory flux, with increases in flux resulting in a decrease in the ratio consistent with that observed with yeast mitochondria [36].

Table 2
The effect of substrate on coupling efficiency in potato mitochondria

Substrate	State 3 (nmol/min per mg)	State 4 (nmol/min per mg)	P/O
Succ	118	38	1.37
NADH	200	90	1.14
Mal/Glu	70	14	2.31
Succ + malon	30	16	1.41
with NADH	116	40	1.10
Mal/Glu + rot	60	16	1.39
with Succ	114	36	1.15

Thus, although mitochondria may contribute to the cytosolic ATP pool in the light it may well be at a reduced ADP/O ratio compared to that observed in the dark. This is particularly the case if non-phosphorylating pathways are engaged. Although there are considerable uncertainties regarding the exact rate of respiration in vivo, the available evidence suggests that the rate is regulated by the supply of ADP and, perhaps more importantly, that mitochondrial respiration in the light is *not* maximal, since it can be increased in vivo by addition of uncoupling agents.

6. Mitochondrial dysfunctions in energy-coupling

Similar to mammalian tissues, it is increasingly apparent that defects in mitochondrial function, particularly at the level of oxidative phosphorylation, are present in plant tissues, for example non-chromosomal stripe mutation (NCS) [37] and cytoplasmic male sterility (CMS) [38,39]. Both classes of defect appear to be due to maternally inherited mutations and result from specific rearrangements and deletions in mtDNA. Such mutations appear to severely disrupt the efficiency of oxidative phosphorylation and as such affect plant growth and development.

Several different NCS mutations have been characterised including two in the cytochrome *c* oxidase subunit 2 gene (NCS5 and NCS6), one in the NADH dehydrogenase (Complex I) subunit 4 gene (NCS2) and two in the co-transcribed S3/L16 ribosomal protein genes (NCS3 and NCS4) [37]. All mutations appear to be lethal during kernel development and limit leaf growth at other stages. The cytochrome *c* oxidase and NADH dehydrogenase mutations appear to reduce mitochondrial phosphorylation efficiency and interestingly chloroplast structure and function, specifically in the efficiency of carbon fixation and photosynthetic activity. Whether the effect upon chloroplast development is a consequence of impaired efficiency of mitochondrial oxidative phosphorylation leading to decreased cytosolic ATP levels (which would affect organelle biogenesis and development) is yet to be established.

Cytoplasmic male sterile genotypes are known to exist in many monocotyledon and dicotyledon plant species and results in impaired pollen development. In one example CMS results in the synthesis (by the

mitochondrial genome) of a novel 25 kDa protein [38]. Characterisation of mitochondria from CMS tissue suggests that although electron transport and oxidative phosphorylation per se, are not affected, it does appear to result in an increased engagement of the alternative oxidase in some species. Operation of this pathway would decrease the efficiency of oxidative phosphorylation thereby reducing cytosolic ATP, the levels of which are critical for normal development.

Another example of impaired efficiency in mitochondrial energy transduction is seen in the Texas male-sterile cytoplasm (*cms-T*) of maize [39]. CMS in *cms-T* maize is due to a mtDNA rearrangement again resulting in the presence of a novel 13 kDa mitochondrial protein [39]. The 13 kDa protein is located in the inner mitochondrial membrane and associated non-specifically with a number of the respiratory chain complexes (see Ref. [40]). In the presence of a family of pathotoxins (T-toxins), produced by certain fungi or the insecticide methomyl, a pore across the inner mitochondrial membrane is formed by URF13-pathotoxin [40]. This pore induces a dramatic increase in the inner membrane's ion permeability properties, resulting in the leakage of Ca^{2+} and NAD^+, organelle swelling and perhaps most importantly an uncoupling of oxidative phosphorylation [see 39].

Whether altered efficiency in mitochondrial energy transduction is at the root of CMS in all species, or whether defects in numerous mitochondrial activities can produce sterility, will only be revealed by further analysis of the respiratory and phosphorylative properties of mitochondria from CMS lines of other species.

7. Conclusion

Fine-tuning of the efficiency of mitochondrial energy conservation (or the P/O ratio) may play a crucial role in the determination of the cytoplasmic adenine nucleotide levels. That this can have drastic effects on the physiology of both plant cells and the whole plant is illustrated particularly by the NCS and CMS mutants. An understanding of these effects consequently requires identification of the key points of control followed by quantitative description using tools such as kinetic modelling and metabolic control theory. In this approach sufficient attention should be given to verification that the boundary of the system studied is well-defined (i.e., to ensure that sources and sinks are indeed constant) so that within this boundary control theory can be applied.

Control analysis that has been performed to date indicates that in or close to state 4 the rate of respiration is mainly controlled by the proton leak (the main protonmotive force-dissipating process under these conditions). In mitochondria that do possess alternative oxidase activity, energy dissipation may proceed directly by means of electron transfer by this pathway. Obviously the alternative pathway will contribute to control of the respiratory rate under conditions where it is most active, which for the majority of plant mitochondria is state 4. In the present study, we have explored the distribution of control within the electron-transferring network of potato callus mitochondria. We found that during succinate-dependent state 4 respiration, the alternative oxidase contributes 10% or more to control of the respiratory rate. Our intention is to extend these studies from the electron-transferring network to include the complete mitochondrial energy-conserving system in an attempt to understand the interplay between electron transfer and energy conservation of other controlling factors such as the protonmotive force, Q-pool reduction and substrate supply. This will be done using mitochondria isolated from developing tissues in order to correlate changes in control properties with changes in physiology.

Acknowledgements

This work was supported in part by the Foundation for Chemical Research (SON) with financial aid from the Netherlands Organisation for the Advancement of Research (NWO), and travel funds from the British Council (UK-Dutch Joint Scientific Research Project - JRP071). G.L. gratefully acknowledges an SERC studentship.

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