Mitochondrial uncoupling protein is required for efficient photosynthesis

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Uncoupling proteins (UCPs) occur in the inner mitochondrial membrane and dissipate the proton gradient across this membrane that is normally used for ATP synthesis. Although the catalytic function and regulation of plant UCPs have been described, the physiological purpose of UCP in plants has not been established. Here, biochemical and physiological analyses of an insertional knockout of one of the *Arabidopsis* UCP genes (*AtUCP1*) are presented that resolve this issue. Absence of UCP1 results in localized oxidative stress but does not impair the ability of the plant to withstand a wide range of abiotic stresses. However, absence of UCP1 results in a photosynthetic phenotype. Specifically there is a restriction in photorespiration with a decrease in the rate of oxidation of photorespiratory glycine in the mitochondrion. This change leads to an associated reduced photosynthetic carbon assimilation rate. Collectively, these results suggest that the main physiological role of UCP1 in *Arabidopsis* leaves is related to maintaining the redox poise of the mitochondrial electron transport chain to facilitate photosynthetic metabolism.

mitochondria | *Arabidopsis* | electron transport | reactive oxygen species | photorespiration

Uncoupling proteins (UCPs) are integral to the inner mitochondrial membrane and catalyze a proton conductance across this membrane, dissipating the mitochondrial proton gradient (1). Unlike the F_1F_0 ATP synthase, which couples the energy inherent in this proton gradient to ATP synthesis, UCP dissipates the energy as heat (2). Indeed, the function of mammalian UCP1 that is present in brown adipose tissue is to support rapid uncoupled respiration for thermogenic purposes (3). Other mammalian UCPs can also catalyze proton conductance but their function as respiration uncouplers is less well established and their role is thought to be unrelated to heat production (4). UCPs are widely distributed in the eukaryotic kingdom and the presence of a plant UCP was first identified biochemically in 1995 (5). The presence of UCPs in plants gained widespread acceptance with the identification of the first plant UCP gene (6) and since then UCP genes have been identified in a wide range of plant species (7, 8). It is now apparent that UCPs are encoded by small gene families, although consensus on the precise composition of these families has not yet been reached (9, 10). Plant UCPs appear to be regulated in a similar fashion to their mammalian counterparts; they catalyze a fatty acid-dependent, nucleotide-inhibitable proton conductance (11–13) and are activated by superoxide and aldehyde products of lipid peroxidation (14, 15). Given that formation of superoxide and related reactive oxygen species (ROS) by the respiratory chain is increased nonlinearly at high mitochondrial membrane potential, activation of UCP by superoxide represents a feedback loop by which superoxide production can be regulated.

Although UCP activity will lead to heat generation, it is not believed that UCP is present in plants for the purpose of thermogenesis (8). Even in thermogenic *Arum* species, UCP is expressed in all tissues, not just in the thermogenic floral organs (16). Moreover, *in vivo* measurements of flux through the alternative and cytochrome respiratory pathways in sacred lotus flowers suggest that UCP activity is not increased during thermogenesis (17). In general, UCP genes appear to be ubiquitously expressed in plants (7) but the induction of *StUCP1* and *AtUCP1* in potato and *Arabidopsis* leaves by low temperature is suggestive of a specific role under this condition (6, 18). This expression pattern and the fact that UCP is activated by ROS have led to the suggestion that the physiological role for UCP might be to moderate ROS production during abiotic stress (8, 19).

Nevertheless, a firm physiological role for UCP remains to be established. It is not immediately obvious how UCP would be able to reduce ROS under conditions of abiotic stress, when physical factors such as altered membrane fluidity or inhibition of electron transport chain complexes are responsible for ROS production. In an attempt to clarify the role of UCP we investigated an insertional mutant of *Arabidopsis UCP1* (At3g54110). This article presents the results of our physiological and biochemical investigations of this mutant that establish a firm role for UCP1 in the photosynthetic metabolism of *Arabidopsis*.

Results

Characterization of the *ucp1* Mutant. Of the three UCP genes in the *Arabidopsis* genome, *AtUCP1* (At3g54110) predominates in terms of expression in a range of tissue types [supporting information (SI) Fig. 6]. To investigate the physiological role of UCP in plants, we exploited the presence of an insertional mutant of *AtUCP1* (*ucp1*) in the SAIL mutant collection (20). PCR was used to identify homozygous lines, and segregation analysis confirmed a single insertion. The insertion was in the first intron, and homozygous plants showed an almost complete absence of UCP protein in mitochondria from rosette leaves (Fig. 1*A*). The remaining UCP protein (≈5% of WT) is presumably encoded by other UCP genes. The presence of non-phosphorylating bypasses of the plant respiratory chain provides a possible route by which loss of UCP1 could be compensated for. There was no major change in NDA, the internal NADH dehydrogenase, but there was a modest increase (≈30%) in NDB, the external NAD(P)H dehydrogenase and a more substantial decrease in the alternative oxidase (AOX) protein (Fig. 1*A*). Although the increase in NDB could partially compensate for the loss of UCP, the increase in the NDB protein was rather slight and, on its own, only results in partial uncoupling of the

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Abbreviations: UCP, uncoupling protein; ROS, reactive oxygen species; HNE, 4-hydroxy-2-nonenal; AOX, alternative oxidase.

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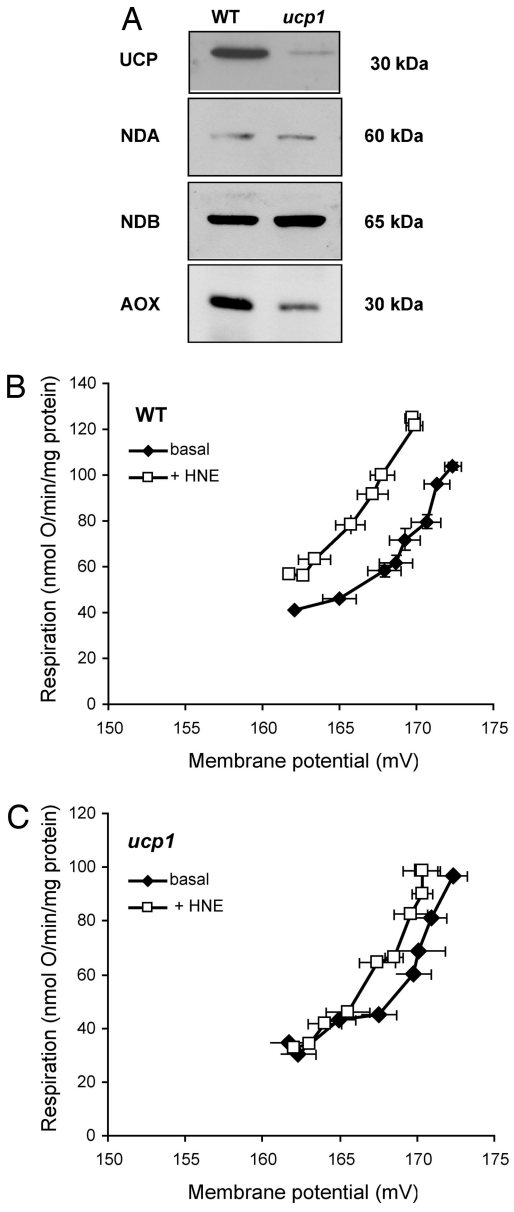

Fig. 1. Characterization of the *ucp1* mutant. (*A*) Western blots of mitochondrial protein probed with antibodies against UCP (51), mitochondrial NADH dehydrogenase A (NDA), Mitochondrial NADH dehydrogenase B (NBA) (52), and AOX (53) are shown. (*B*) UCP-catalyzed proton leak rate in mitochondria from WT is shown. (*C*) UCP-catalyzed proton leak rate in mitochondria from the *ucp1* mutant is shown. The basal proton leak rate (respiration in the absence of ATP synthase acitivity) and the UCP-catalyzed proton leak rate (respiration in the absence of ATP synthase activity and in the presence of HNE) are shown. Values are the means of three independent mitochondrial isolations ± SEM

electron transport chain (bypassing one of the three proton-translocating respiratory complexes). It is not immediately obvious why AOX protein should decrease but it is clearly not a compensatory change because one would expect an increase in this protein rather than a decrease. Loss of UCP protein was accompanied by a significant change in the proton-leak kinetics of isolated mitochondria (measured as the dependence of respiration rate in the absence of ATP synthase activity on membrane potential) (Fig. 1*B*). The basal proton-leak (respiration rate) kinetic was essentially the same in mitochondria from WT and *ucp1* plants. However, addition of 4-hydroxy-2-nonenal (HNE), a known activator of UCP (15), resulted in a significant increase in proton conductance (respiration) in the WT mitochondria (that was inhibitable by GTP; data not shown) that was abolished in the *ucp1* mitochondria. Thus, loss of UCP1 protein results in a loss of UCP-catalyzed proton leak in mitochondria. The *ucp1* mutant line therefore represents a useful resource for investigating the role of the AtUCP1 protein.

Oxidative Stress in the *ucp1* Mutant. At high mitochondrial membrane potential, the formation of ROS by mitochondria is increased. Thus, one consequence of dissipation of the proton gradient by UCP is a reduction in ROS formation (15, 21). We confirmed the relationship between UCP and ROS formation in the *ucp1* mutant. There was a general increase in protein oxidation in mitochondria from *ucp1* plants, and the activities of malic enzyme and aconitase, two mitochondrial enzymes known to be sensitive to oxidative inactivation (21, 22), were significantly decreased (SI Fig. 7 *A* and *B*). These observations are consistent with an increased rate of ROS production caused by the loss of UCP. However, other measures of oxidative stress suggest that the increase in ROS production rate is modest: the amounts and reduction state of the ascorbate and glutathione pools were unaltered (SI Fig. 7 *C* and *D*) and there was no increase in the accumulation of lipid peroxidation products in the *ucp1* mutant (SI Fig. 7*E*).

Given that the expression of *AtUCP1* is responsive to low temperature (18) and not to other stress conditions (23), we examined the growth of *ucp1* mutant plants under optimal and low temperatures (Fig. 2). Under optimal temperature conditions the mutant growth and development was similar to WT (Fig. 2*A*), but there was a small significant decrease in shoot mass (Fig. 2*B*). Root growth, however, was unaltered in the mutant (Fig. 2*C*), which was also the case at low temperature. Moreover, introduction of heavy metal (Cd^{2+}) or the respiratory inhibitor antimycin A [conditions that affect the root growth of antioxidant enzyme mutants of *Arabidopsis* (24)] also do not significantly impair root growth of the *ucp1* mutant in comparison with WT. Neither was cell damage after treatment with the oxidative stress-inducing herbicide, methyl viologen, significantly greater in the *ucp1* mutant (Fig. 2*D*). In fact, at some points during the observed time course, the degree of ion conductance was actually significantly less in the *ucp1* mutant. Overall, our observations are consistent with the assertion that loss of UCP1 causes only modest oxidative stress and suggest that UCP1 does not play a major role in tolerance of *Arabidopsis* to the tested stress conditions.

Metabolic Consequences of Loss of UCP1. The other physiological role that has been proposed for UCP in plants is that, by partially uncoupling electron transport from ATP synthesis, a higher flux through the tricarboxylic acid (TCA) cycle is facilitated (15). To establish whether UCP is important in this context in *Arabidopsis* plants, a broad metabolite abundance profile was determined in WT and *ucp1* leaves (SI Table 1). Overall, few significant changes in metabolite abundance between leaves of WT and *ucp1* plants were observed, suggesting that loss of UCP had no wholesale consequences for metabolism. Of particular note, the abundance of aspartate and glutamate, amino acids whose synthesis directly requires TCA cycle intermediates, was not affected. In fact, of the 15 amino acids measured, the only significant changes in the *ucp1* line were in serine and glycine levels, which were reduced by 29% and 52%, respectively.

Photosynthesis Is Decreased in the *ucp1* Mutant. The specific changes in serine and glycine levels point to a possible change in photosynthesis, and specifically in photorespiration (because glycine is oxidized to serine in the mitochondrion as part of the photorespiratory carbon recovery cycle). To investigate photo-

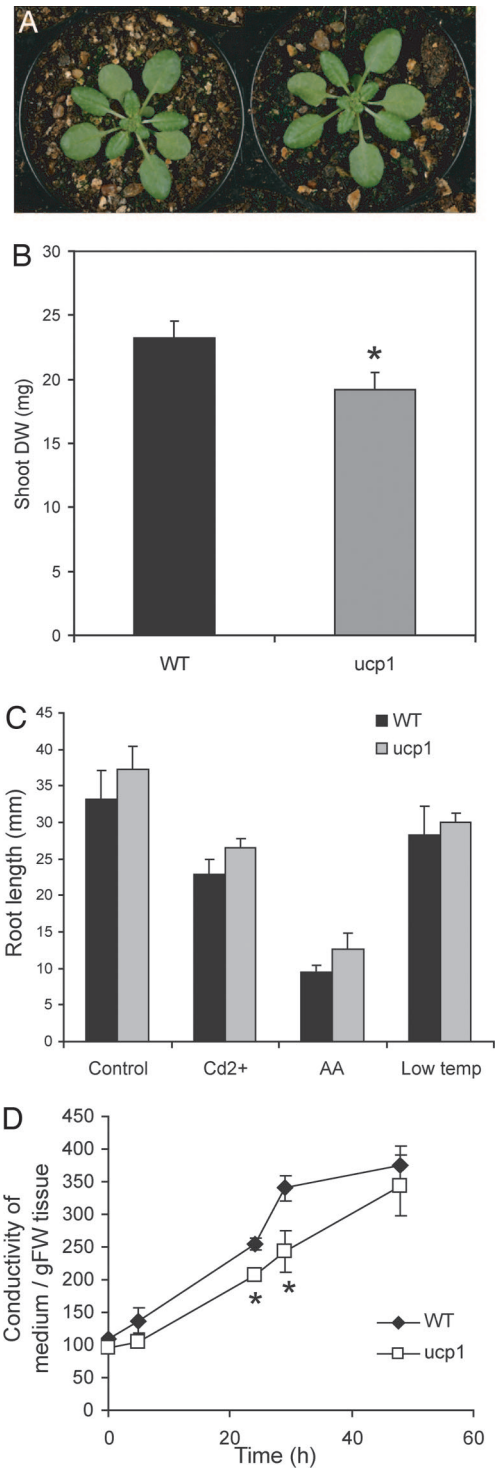

Fig. 2. Growth characteristics of the *ucp1* mutant. (*A*) *A. thaliana* WT (*Left*) and *ucp1* mutant (*Right*) were grown in compost for 3 weeks under the following conditions: 100 μmol of photons·m^{-2}·s^{-1}, 16:8 h light/dark cycle, 22°C. (*B*) Shoot mass (dry weight) after 3 weeks of growth. Values are mean of 15 plants ± SEM. * indicates significantly different to WT ($P > 0.05$). (*C*) WT and mutant seedlings were grown on vertical agar plates containing half-strength Murashige and Skoog salts for 15 days in the same growth conditions as stated above, except for the low-temperature treatment in which the temperature was 10°C in the light and 5°C in the dark. $CdCl_2$ and antimycin A were added at final concentrations of 25 and 10 μM, respectively. Values are means of 10 plants ± SEM. (*D*) Ion leakage rate in WT and ucp1 seedlings after treatment with 1 mM methyl viologen. All values are the mean ± SEM of three independent seedlings at each time point.

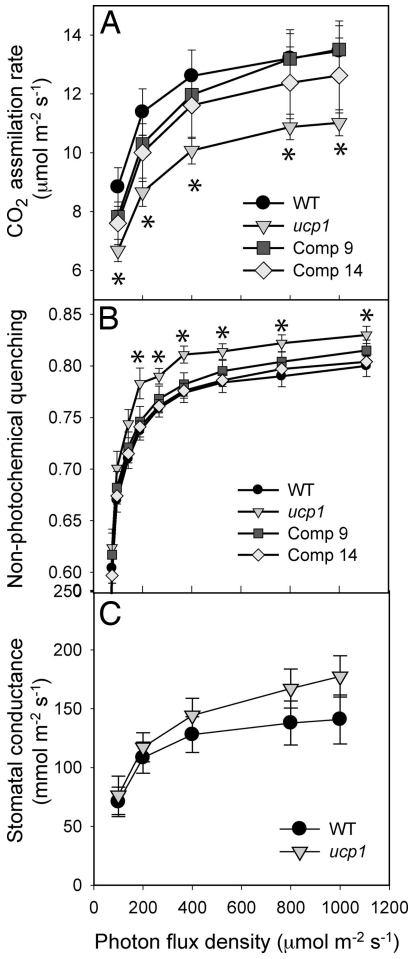

Fig. 3. Photosynthesis in the *ucp1* mutant. (*A*) CO_2 assimilation rate. Values are mean of five plants ± SEM. (*B*) Nonphotochemical quenching. (*C*) Stomatal conductance. Values are mean of six plants ± SEM. Comp 9 and comp 14 refer to two independent complemented lines. All data were statistically compared with WT using the *t* test; * indicates a significant difference ($P < 0.05$).

synthesis in the *ucp1* mutant, a range of photosynthetic gas-exchange and chlorophyll fluorescence parameters were measured. A significant decrease in the rate of CO_2 assimilation in the *ucp1* mutant was observed over a range of different light intensities. (Fig. 3*A*). Moreover, this decrease in photosynthesis could be rescued by complementing the mutant (Fig. 3*A*), demonstrating that the photosynthetic phenotype is specifically caused by the *ucp1* mutation. In addition, there was a significant increase in nonphotochemical quenching that was restored to WT values in the complemented lines (Fig. 3*B*). Gas exchange data further revealed that stomatal conductance was not significantly affected in the *ucp1* mutant (Fig. 3*C*), suggesting that a reduction in stomatal density or aperture size was not responsible for the decreased photosynthetic rate. Similarly, internal CO_2 concentration was unaltered between WT and *ucp1* mutant (data not shown). Furthermore, there was no decrease in either Rubisco protein amount or activity (or activation state) or in the activities of other Calvin cycle enzymes (Fig. 4), suggesting that a reduction in Calvin cycle capacity is not responsible for the decreased assimilation rate.

A possible link between mitochondrial coupling state and photosynthesis is the requirement for rapid oxidation of NADH produced in the mitochondrion during conversion of photorespiratory glycine to serine (25) (SI Fig. 8). Isotope-tracer experiments were used to test the impact of loss of UCP1 on

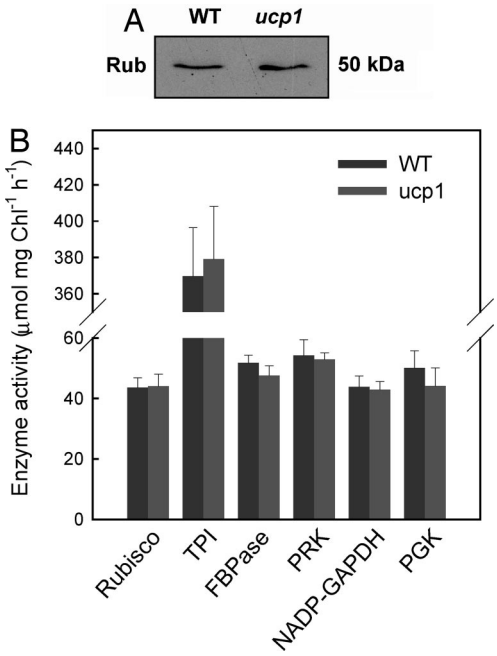

Fig. 4. Capacity of Calvin cycle enzymes. (*A*) Western blot showing Rubisco large subunit content. (*B*) Maximum catalytic activities of selected Calvin cycle enzymes. TPI, triose phosphate isomerase; pFBPase, plastidic fructose-1,6-bisphosphatase; PRK, phosphoribulokinase; NADP-GAPDH, NADP-dependent glyceraldehyde-3-phosphate dehydrogenase; PGK, phosphoglycerate kinase. All data are the mean of four independent leaf extracts ± SEM.

photorespiratory capacity. A dramatic decrease in the rate of conversion of glycine to serine was observed (Fig. 5), and the flux was restored to WT levels in a complemented line (Fig. 5). These data demonstrate that loss of UCP restricts photorespiratory flux. To test whether this inhibition of photorespiration is indeed responsible for the photosynthetic assimilation rate phenotype, we measured photosynthetic rate under high CO_2 conditions [concentration of CO_2, 5% (vol/vol)] in which the oxygenase activity of Rubisco is minimized and photorespiration is not active. Under these conditions we obtained the following photosynthetic rates (μmol·O_2·mg·chlorophyll^{-1}·h^{-1}): WT, 218 ± 13; *ucp1*, 205 ± 10. The photosynthetic rate in the *ucp1* mutant is not significantly different from WT under nonphotorespiratory conditions, strongly suggesting that the decrease in assimilation rate under ambient atmospheric conditions is linked to the photorespiratory restriction.

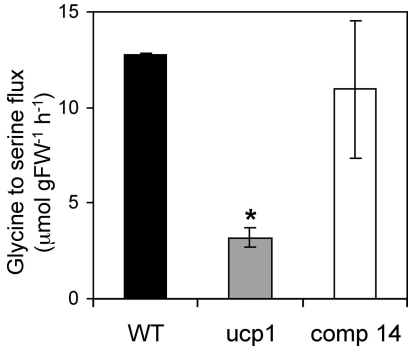

Fig. 5. Rate of photorespiratory glycine oxidation; *in vivo* glycine to serine flux. Values are the mean of four plants ± SEM. Comp 14 refers to an independent complemented line. All data were statistically compared with WT by using the *t* test; * indicates a significant difference ($P < 0.05$).

Discussion

UCP1 Is Required for Efficient Photosynthesis. Our analysis of the *ucp1* mutant strongly suggests that one of the circumstances in which UCP is required to adjust the bioenergetic poise of the respiratory chain is during photosynthesis. It is well known that mitochondria are important in leaves in the light (26). One of the less controversial roles that mitochondria are known to play is in the oxidation of glycine produced during the photorespiratory pathway. This process generates large quantities of NADH that must be oxidized to regenerate the NAD^+ required for the glycine decarboxylase reaction. A large part of this oxidation is thought to occur via malate dehydrogenase activity in the mitochondrial matrix, reducing oxaloacetate to malate (27). Export of the resultant reducing equivalents from the mitochondrion as malate can supply the NADH required for the peroxisomal reactions of photorespiration (see SI Fig. 8). Given that there is an exact stoichiometric balance between the amount of NADH generated by glycine oxidation in the mitochondrion and the amount required for hydroxypyruvate reduction in the peroxisome, it is tempting to assume that all NADH produced in the mitochondrion by photorespiration is oxidized by export of reducing equivalents. However, studies of mitochondrial respiratory mutants provide good evidence that some of the NADH is oxidized via the electron transport chain (25). Presumably, the capacity of the malate export pathway is not sufficient to oxidize all of the NADH produced. Photorespiration therefore leads to a high flux of electrons into the respiratory chain (see SI Fig. 8). Given that demand for mitochondrially generated ATP may be quite low during the light, this high electron flux would lead to the build-up of a high proton gradient across the inner mitochondrial membrane, which ultimately would restrict continued electron flux. In other words, unless there is a mechanism apart from the F_1F_0 ATP synthase to dissipate the proton gradient, then oxidation of NADH would be prevented and photorespiration would be inhibited because of an insufficiently rapid regeneration of NAD^+ required for glycine decarboxylase.

We propose that the initial restriction of electron transport in this scenario would lead to increase ROS production by the respiratory chain, which would lead to activation of UCP. In turn, UCP would allow a controlled dissipation of the proton gradient, relieving the thermodynamic constraint on electron flux and allowing continued photorespiration. Consistent with this hypothesis, there was a significant decrease in the photorespiratory flux of glycine to serine in the *ucp1* mutant. We also observed a decrease in photosynthetic assimilation rate in the *ucp1* mutant (which is most probably responsible for a slight decrease in the growth rate of shoot tissue), which was not present under nonphotorespiratory conditions. At first sight, it is not immediately obvious why a restriction in photorespiration would necessarily decrease carbon assimilation rate. However, a decrease in assimilation is also observed in photorespiratory mutants and it has been proposed that this is possibly caused by a limitation of ribulose-1,5-bisphosphate in the Calvin cycle because of reduced recycling of 2-phosphoglycolate into 3-phosphoglycerate via the photorespiratory pathway (28).

Given the restriction of glycine oxidation in the mutant, one would predict that glycine would accumulate. Indeed this accumulation is seen in transgenic plants with reduced glycine decarboxylase P-protein (29). However, in the *ucp1* mutant leaves in the light, the opposite occurred; there was a significant decrease in glycine content (SI Table 1), which suggests that there has been a regulatory response to decrease flux through upstream steps of photorespiration. The fact that glycine actually decreases suggests that the regulatory changes overcompensate slightly. In glycine decarboxylase mutants or antisense transgenics, the decrease in glycine decarboxylation is presumably too severe to be balanced by such a regulatory mechanism.

A similar uncoupling role during photorespiration has been proposed for the AOX (30) and the internal NADH dehydrogenase (31) proteins. Together, these two "nonphosphorylating" bypasses allow electron transport without proton translocation and could therefore, by a different mechanism, fulfill the same role as UCP. The question therefore arises as to why both nonphosphorylating bypasses and UCP are required to support photorespiratory glycine oxidation. The most likely answer to this apparent redundancy is that neither mechanism is completely effective. UCP activity will lead to a partial dissipation of the proton gradient, which will reduce, but not completely remove, the thermodynamic constraint on electron flux. Similarly, there is never a 100% flow of electrons down the alternative pathway (32) because AOX is always in competition with complex III for electrons from the ubiquinone pool. Thus, it is likely that both mechanisms operate together to facilitate the highest possible electron flux and photorespiration rate.

Conclusions

Overall, our analysis of the *ucp1* mutant has established a firm physiological role for UCP1 in higher plants. We have shown that uncoupling of the mitochondrial electron transport chain mediated by UCP is essential to permit the mitochondrial steps of the photorespiratory pathway. UCP probably works in conjunction with the nonphosphorylating bypasses of the respiratory chain, which also serve to partially uncouple mitochondria (30, 33). However, UCP and the nonphosphorylating bypasses are not functionally redundant because there are clear metabolic consequences in the *ucp1* mutant: photorespiration is inhibited and as a result photosynthesis is impaired. Although this role for *Arabidopsis* UCP1 is clearly specific to plants, our work nevertheless has implications for attempts to understand the role of UCP1 homologues in animals. Our results suggest that moderation of ROS production is perhaps not the main purpose of UCP1 homologues. Rather, activation of UCP by ROS is used as a mechanism to sense the demand on the mitochondrial respiratory chain and to facilitate increased electron transport rates when required. We therefore propose that the main purpose of UCP1 homologues may be to facilitate high electron transport flux for metabolic purposes. The exact nature of the metabolic pathways connected to UCP function will vary as a result of evolutionary specialization in different species and different cell types.

Methods

Plant Growth. *Arabidopsis thaliana* (ecotype Colombia 0) were grown on compost supplemented with Vermiculite at 22°C with a photoperiod of 16 h and a light intensity of 150–200 $\mu E \cdot m^{-2} \cdot s^{-1}$. For root-growth assays, plants were grown under the same conditions but in vertical Agar plates supplemented with 50% Murashige and Skoog salts.

Ion Leakage Assays. Two-week-old seedlings (grown on agar plates, conditions as above) were sprayed with a 1 mM solution of methyl viologen and incubated in the dark at 22°C. At time intervals up to 50 h, seedlings were removed from the plate and floated on 30 ml of double-deionized water for 3 h before the conductivity of the water was measured with a portable conductivity meter.

Insertional Mutant Isolation. An insertional line from the SAIL collection (536_G01.b.1a.Lb3Fa) was screened for homozygous insertions in AtUCP1 by PCR of genomic DNA by using the following primer pairs: GACGAAGATGTGAAGTAGACC/TAGCATCTGAATTTCATAACCAATCTCGATACAC and GACGAAGATGTGAAGTAGACC/TCAGTTTCTTTTGGACGCATCG. The amount of UCP1 protein was quantified by Western blot analysis using the antibody and conditions as detailed (14).

Complementation of the *ucp1* Mutant. The complemented lines were created by introducing into the *ucp1* mutant a 3.4-kb fragment of genomic DNA consisting of the AtUCP1 gene (At3g54110) and a 1-kb upstream promoter region. Mutant plants were transformed (using the floral dipping method) with the pKWG plasmid (34) containing the genomic DNA fragment. Kanamycin-resistant primary transformants were screened for AtUCP1 transcript abundance by RT-PCR using the following primers: ATGGTGGCGGCTGGTAAATC/TCAGTTTCTTTTGGACGCATCG. Two independent lines were selected that showed approximately WT levels of the AtUCP1 transcript.

Photosynthesis Measurements. CO_2 assimilation rate was measured with an infrared gas analyzer (35). Chlorophyll fluorescence parameters were measured by using a PAM-2000 fluorometer (36). Glycine-to-serine flux was determined by incubating leaf discs in the light (500 $\mu mol \cdot m^{-2} \cdot s^{-1}$) with 50 mM 1,2-^{13}C glycine (Cambridge Isotope Laboratories, Cambridge, MA) for 6 h exactly as described (25) and determining the fractional enrichment of serine in a leaf extract by using GC-MS according to ref. 37. Calvin cycle enzyme activities were assayed as described: Rubisco (38), glyceraldehyde-3-phosphate dehydrogenase and phosphoglycerate kinase (39), phosphoribulokinase (40), fructose-1,6- bisphosphatase (41), and triosephosphate isomerase (42). Rubisco antibody was a kind gift of Martin Parry (Rothamsted Research, Hertfordshire, U.K.).

Mitochondrial Isolation and Proton Leak Assays. Mitochondria were isolated from 50 g fresh weight of 10-day-old *Arabidopsis* seedlings (43), and proton leak was determined in mitochondria respiring succinate by using custom-built electrodes (15).

Indicators of Oxidative Stress. Protein carbonyls of mitochondrial proteins were visualized by Western blotting (44). Tricarboxylic acid cycle enzyme activities were measured in isolated mitochondria by using spectrophotometric assays (15). Reduced and oxidized ascorbate and glutathione were measured in perchloric acid extracts of leaf tissue (45). The lipid peroxidation productions, HNE and malondialdehyde, were measured according to ref. 46 with tissue prepared by grinding rosette leaves to fine powder under liquid N_2 and adding 1:2.97 (g fresh weight/ml) 20 mM Tris (HCL) (pH 7.4), and 30 μl of 500 mM butylated hydroxytoluene/g fresh weight.

Metabolite Assays. Metabolites were quantified in rosette leaves harvested at the midpoint of the photoperiod as described: amino acids (47), nucleotides (48), hexose phosphates and 3-phosphoglyceric acid (49), and organic acids, sugars, and other metabolites (50).

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