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Superoxide Stimulates a Proton Leak in Potato Mitochondria That Is Related to the Activity of Uncoupling Protein*

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The ability of plant mitochondrial uncoupling proteins to catalyze a significant proton conductance *in situ* is controversial. We have re-examined conditions that lead to uncoupling of mitochondria isolated from the tubers of potato (*Solanum tuberosum*). Specifically, we have investigated the effect of superoxide. In the absence of superoxide, linoleic acid stimulated a proton leak in mitochondria respiring NADH that was insensitive to GTP. However, when exogenous superoxide was generated by the addition of xanthine and xanthine oxidase, there was an additional linoleic acid-stimulated proton leak that was specifically inhibited by GTP. Under these conditions of assay (NADH as a respiratory substrate, in the presence of linoleic acid and xanthine/xanthine oxidase) there was a higher rate of proton conductance in mitochondria from transgenic potato tubers overexpressing the *StUCP* gene than those from wild type. The increased proton leak in the transgenic mitochondria was completely abolished by the addition of GTP. This suggests that superoxide and linoleic acid stimulate a proton leak in potato mitochondria that is related to the activity of uncoupling protein. Furthermore, it demonstrates that changes in the amount of StUCP can alter the rate of proton conductance of potato mitochondria.

The flux through the tricarboxylic acid cycle and the flow of electrons through the mitochondrial respiratory chain are coupled to the synthesis of ATP via the proton gradient across the inner mitochondrial membrane. The activity of the F_1F_0 ATP synthase complex returns protons to the matrix allowing continued electron transport (with concomitant proton translocation into the intermembrane space) and recycling of NAD as a substrate for the tricarboxylic acid cycle. This strict coupling of tricarboxylic acid cycle, electron transport, and ATP synthesis matches respiratory activity to ATP demand. However, there are circumstances in which maintaining a coupled state is not desirable. Plants are autotrophic organisms and the tricarboxylic acid cycle is a source of carbon skeletons for a number of biosynthetic pathways (1, 2). Under these conditions, a higher tricarboxylic acid cycle flux is required than is the case for ATP synthesis alone, and the tricarboxylic acid cycle must be partially uncoupled from electron transport. Uncoupling may also be beneficial in reducing the production of reactive oxygen species by the respiratory chain and thereby avoiding oxidative stress (3). The respiratory chain of plant mitochondria therefore contains a number of non-phosphorylating bypasses such as the alternative oxidase and NAD(P)H deydrogenases (4, 5). These electron carriers do not translocate protons and are not dependent upon the dissipation of the proton gradient by ATP synthase.

An alternative means of attaining a regulated uncoupling of plant mitochondria is via the activity of the mitochondrial uncoupling protein (UCP).[1] UCP was first discovered in the brown adipose tissue of mammals where it functions to catalyze an uncoupled respiration of fatty acids to generate heat for thermogenesis (6). Consistent with this role, UCP1 is activated by anionic fatty acids, which are thought to directly participate in its catalytic function (7–9). Since then, a number of UCP1 homologues have been discovered in mammals (UCP 2–5), which were initially also assumed to catalyze mitochondrial proton leak (although not necessarily for the generation of heat energy). However, there is still considerable controversy as to whether the UCP1 homologues actually catalyze a proton leak *in vivo* and the function of these proteins remains to be established (10).

In plants, an activity reminiscent of UCP1 has also been identified (11). This activity was characterized by a reduction in membrane potential of isolated potato mitochondria that was stimulated by anionic fatty acids and inhibited by nucleotides, characteristics that distinguish uncoupling protein from other anion carrier protein-mediated proton leak. Two years later the first plant UCP gene was cloned (*StUCP* from potato) (12), and since then UCP genes have been identified from *Arabidopsis* (13, 14), skunk cabbage (15), wheat (16), and rice (17). UCP-like activity has been observed in isolated mitochondria from a number of plant species and the potato UCP activity has been purified and its proton transport properties recovered by reconsitution into liposomes (18). Furthermore, reconstitution of the *AtUCP1* gene product into liposomes has provided the first link between a plant UCP gene and proton transport activity (19). However, a recent study has shown that

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[1] The abbreviations used are: UCP, uncoupling protein; Tes, 2-{[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]amino}ethanesulfonic acid; BSA, bovine serum albumin; AOX, alternative oxidase; WT, wild type; TPMP, triphenylmethylphosphonium.

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when care is taken to avoid artifacts caused by extraneous effects of nucleotides on other electron transport chain components, the fatty acid-stimulated proton leak of potato mitochondria is not inhibited by nucleotides (20). This casts considerable doubt as to whether UCP contributes in any significant way to proton leak in plant mitochondria.

In this paper we sought to undertake a careful re-examination of the conditions that lead to proton leak in potato mitochondria and to establish under which conditions, if any, UCP contributes to this proton leak. We confirm previous observations that fatty acids stimulate a proton leak in potato mitochondria respiring NADH and that this leak is not inhibited by nucleotides. However, when exogenous superoxide is generated (by the addition of xanthine and xanthine oxidase) an additional proton leak is observed that *is* sensitive to GTP. This suggests that, as is the case in animals, plant UCP requires the presence of superoxide for full activity (21, 22). We provide further evidence that this fatty acid-dependent, superoxide-stimulated, and nucleotide-sensitive proton leak is related to UCP activity by studying proton leak in mitochondria isolated from transgenic potato plants overexpressing the potato *StUCP* gene.

EXPERIMENTAL PROCEDURES

Chemicals—Unless otherwise indicated all chemicals were from Sigma, Poole, UK).

Growth of Potato Plants—Potato (*Solanum tuberosum* L c.v. Desiree) were grown by planting sprouted tubers in 150-mm diameter pots containing general purpose compost. The plants were maintained in a glasshouse at 16–25 °C with a 16-h photoperiod of natural daylight supplemented to give a minimum irradiance of 150 $\mu E\ m^{-2}\ s^{-1}$. Tubers were harvested after ~10 weeks and stored at 4 °C for at least 1 week prior to use.

Production of Transgenic Plants Overexpressing the StUCP cDNA—The full-length *StUCP* cDNA (12) was cloned into the binary plant expression vector pBinAR (23) between the 35S cauliflower mosaic virus promotor (24) and the polyadenylation signal of the T-DNA octopine synthase gene (25) using standard techniques. The resulting construct was introduced into *Agrobacterium tumefaciens* and used to transform *Solanum tuberosum* L c.v. Desiree as described previously (26).

Northern and Western Blot Analyses—Total RNA was extracted from leaves and analyzed by Northern blot using radiolabeled StUCP as a probe as described previously (12). UCP protein content in isolated mitochondrial samples was assessed by Western blot analysis using an antibody raised against soybean UCP as described in Ref. 27.

Isolation of Potato Tuber Mitochondria—All procedures were done at 4 °C. Approximately 100 g of tuber material was homogenized into 100 ml of extraction medium (0.3 M mannitol, 50 mM Tes-NaOH (pH 7.5), 0.5% (w/v) BSA, 0.5% (w/v) polyvinylpyrrolidone-40, 2 mM EGTA, and 20 mM cysteine) using an electric juice extractor. The resulting homogenate was filtered through Miracloth (CN Biosciences, Nottingham, UK) and centrifuged at 1,500 × *g* for 5 min. The supernatant was then centrifuged at 18,000 × *g* for 10 min to recover an organelle pellet. This pellet was resuspended in wash buffer (0.3 M mannitol, 20 mM Tes-KOH (pH 7.5)) and layered onto a stepped gradient of Percoll (Amersham Biosciences Ltd., Little Chalfont, UK) consisting of steps of 50, 28, and 20% (v/v) Percoll with 0.3 M mannitol as an osmoticum. After centrifugation at 43,000 × *g* for 30 min, mitochondria were recovered from the 28%/50% Percoll interface. Mitochondria were further purified on a second self-forming Percoll gradient consisting of 28% Percoll with 0.3 m sucrose as an osmoticum.

Measurement of Proton Conductance—Proton conductance was determined by simultaneous measurement of oxygen consumption and mitochondrial membrane potential using electrodes sensitive to oxygen and the potential-dependent probe, $TPMP^+$ as described previously (28). A reaction chamber of capacity 2 ml was constructed such that the mitochondrial suspension was in contact with both electrodes. Mitochondria (400 μg) were resuspended in 2 ml of assay medium (0.3 M mannitol, 1 mM $MgCl_2$, 100 mM KCl, 10 mM KH_2PO_4 (pH 7.0) 0.1% (w/v) BSA (fraction V, fatty acid free, Roche Diagnostics Ltd., Lewes, UK)) containing 50 μM xanthine, 1 μM oligomycin, and 0.1 μM nigericin (to collapse the difference in pH across the inner membrane). The electrode was calibrated with sequential additions of $TPMP^+$ to a final concentration of 5 μm. Then NADH was added to a concentration of 1 mM to start the reaction. Membrane potential was progressively inhibited by the addition of KCN to a final concentration of between 0.6 and 70 μM. At the end of each run, 2 μM carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone was added to dissipate the membrane potential completely, releasing all the $TPMP^+$ back into the medium and allowing correction for any small electrode drift. Linoleic acid (final concentration, 300 μM), GTP (final concentration, 2 mM), xanthine oxidase (0.015 unit; Roche Diagnostics Ltd.) and superoxide dismutase (24 units, CN Biosciences (UK) Ltd., Nottingham, UK) were added as indicated.

RESULTS

Effect of Superoxide on Fatty Acid-stimulated Proton Leak in Potato Mitochondria—Plant UCPs are activated by fatty acids and inhibited by nucleotides. To establish whether UCP can contribute to proton conductance *in situ*, we investigated linoleic acid-induced proton conductance by measuring the kinetics of proton conductance of isolated potato mitochondria as membrane potential was titrated with KCN (28). Oligomycin was added to inhibit ATP synthase activity such that the rate of oxygen consumption was proportional to the rate of proton leak. Oxygen consumption and membrane potential were measured simultaneously using an oxygen electrode and a $TPMP^+$-selective electrode (28), respectively. External NADH rather than succinate was used as a respiratory substrate and GTP rather than ATP was used as an inhibitory nucleotide to avoid complications arising from activation of the succinate dehydrogenase complex (20). To ensure that the observed oxygen consumption was proportional to proton conductance it is necessary that alternative oxidase (AOX) activity is absent or inhibited. The addition of the AOX inhibitors, *n*-propyl gallate or octyl gallate, did not significantly decrease the rate of oxygen consumption indicating that AOX activity was negligable in the mitochondria used in these studies (data not shown). This is consistent with previous observations that AOX activity is very low in potato tubers (1). We did not routinely include these inhibitors in the assay as they had a slight uncoupling effect (data not shown). Xanthine (50 μm) was included in all assays to allow the generation of superoxide upon addition of xanthine oxidase. Xanthine alone had no effect on the basal rate of proton conductance (data not shown).

Addition of linoleic acid to a concentration of 300 μM (giving a molar ratio of fatty acid to albumin of 20) resulted in an increased rate of proton conductance (Fig. 1*a*). However, as was previously reported (20), this proton conductance was not inhibited by the addition of GTP (Fig. 1*a*). Since mammalian UCPs 1 and 2 have been shown to require the presence of superoxide for full activation (21, 22), we investigated the effect of superoxide on the linoleic acid-induced proton conductance (Fig. 1*b*). We found that in the presence of linoleic acid, superoxide induced an additional proton conductance (compare Fig. 1*b* with 1*a*). Furthermore, the addition of GTP reduced this rate of proton conductance back to a level similar to that observed in the presence of linoleic acid alone (Fig. 1, *a* and *b*). The superoxide effect was not seen in the absence of linoleic acid (data not shown).

Proton Conductance in Mitochondria from Transgenic Potato Tubers Containing Increased UCP Content—Transgenic potato plants were generated that expressed the *StUCP* cDNA (12) under the control of a constitutive promotor (see "Experimental Procedures" for more details of the constructs used to generate transgenic plants). On the basis of an initial screen of *StUCP* expression in 100 transgenic lines, two independent lines were selected that consistently showed increased abundance of the *StUCP* mRNA (data not shown). Northern analysis of *StUCP* mRNA content confirmed the increased expression of *StUCP* in these two lines (Fig. 2*a*). These lines contained increased levels of UCP protein (as a proportion of total mitochondrial protein) as determined by immunodetection of the UCP protein with an

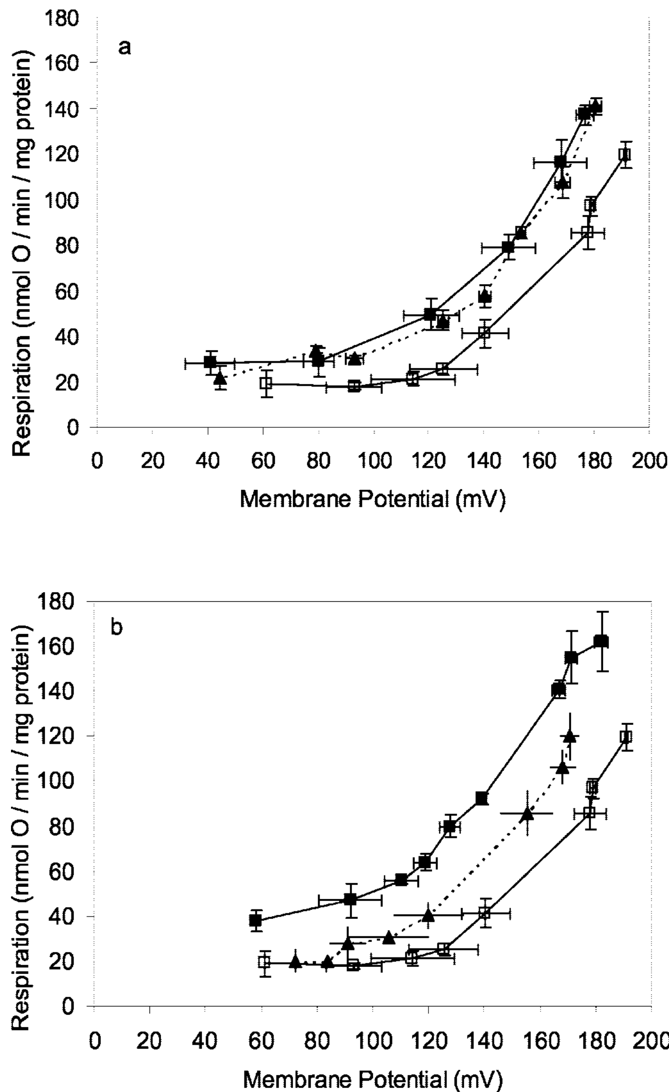

FIG. 1. **The effect of superoxide on proton leak kinetics of potato tuber mitochondria.** For further details see "Experimental Procedures." Potato tuber mitochondria were incubated with 1 mM NADH as a substrate and titrated with KCN in a medium containing 0.1% (w/v) BSA and 50 μm xanthine (□). Additions were 300 μM linoleic acid (■) or 300 μM linoleic acid plus 2 mM GTP (▲). These additions were made in the absence (*a*) or presence (*b*) of exogenous superoxide generated by the addition of 0.015 unit of xanthine oxidase. Values are means ± S.E. of three independent experiments.

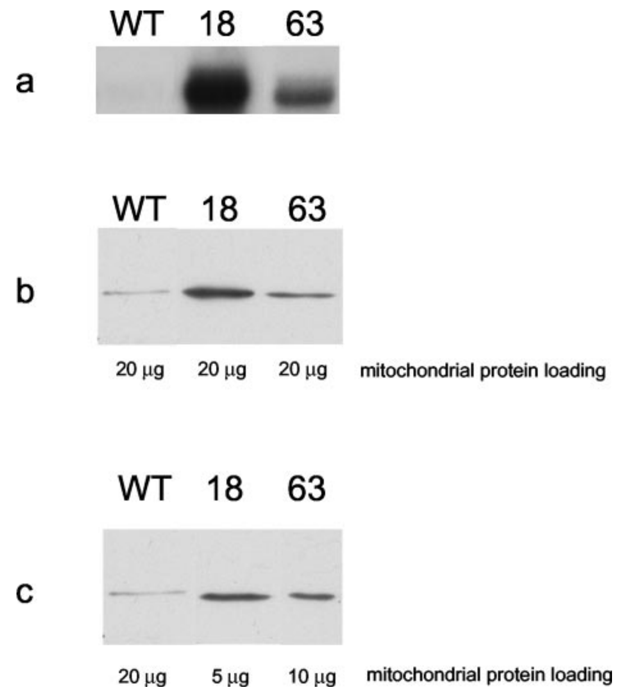

FIG. 2. **StUCP mRNA and UCP protein abundance in tubers of transgenic potato lines overexpressing the StUCP cDNA.** *a*, Northern blot analysis of *StUCP* expression. 25 μg of total RNA from leaf tissue was separated on agarose gels, transferred to a nylon membrane, and probed with radiolabeled *StUCP* under stringent conditions. *b* and *c*, Western blot analysis of UCP protein abundance. 5–20 μg of tuber mitochondrial protein was separated on acrylamide gels and transferred to a nitrocellulose membrane. Blots were briefly stained with Ponceau Red to confirm even transfer and protein loading (not shown). Blots were probed with anti-UCP antiserum (27). *b* shows equal protein loading, and *c* shows protein loaded at the amounts used for quantification of the Western blot.

antibody raised against soybean UCP (27) (Fig. 2*b*). To quantitate the increase in UCP protein, we established the linearity of response of the UCP antibody to increasing amounts of mitochondrial protein (data not shown) and loaded appropriate amounts of protein such that the signal for each line was within the linear range (Fig. 2*c*). Band intensity was determined using the Multianalyst software package (Bio-Rad Laboratories Ltd., Hemel Hempstead, UK). Band intensity was expressed per milligram of total mitochondrial protein to give a measure of UCP content. We estimated that line 18 contains 13 times as much UCP as WT and line 63, twice as much. The mitochondrial samples used for this analysis were derived from a pool of three independent mitochondrial isolations of each line and were the same mitochondrial samples used for proton leak assays.

We investigated the effect of this increased UCP content on proton conductance (Fig. 3). In the presence of linoleic acid alone the proton leak of mitochondria from the two transgenic lines was indistinguishable from wild type (Fig. 3, *a* and *d*). However, when xanthine/xanthine oxidase was added (to generate superoxide) in addition to linoleic acid there was an increased rate of proton conductance in mitochondria from the transgenic lines (Fig. 3, *b* and *e*). This increased rate of proton conductance was specifically dependent on the presence of superoxide as the addition of superoxide dismutase returned proton leak to wild type levels (Fig. 3*b*, *inset*). Furthermore, the superoxide-stimulated proton conductance was completely abolished by the addition of GTP (Fig. 3, *c* and *f*). We calculated the rate of proton conductance from the curves shown in Fig. 3 by assuming an H^+/O ratio of 6 for oxidation of external NADH. The proton conductance was calculated at a membrane potential of 130 mV. In comparison to wild type there was a statistically significant increase in proton conductance in the transgenic lines (*t* test; $p < 0.05$) of 3.0-fold in line 18 and 2.3-fold in line 63 (Table I).

DISCUSSION

Plant genomes contain homologues of mammalian UCPs and these gene products demonstrate UCP-like activity when reconstituted into liposomes or overexpressed in heterologous organisms such as yeast (18–20). This activity is characterized as a fatty acid-stimulated proton conductance that is inhibited by nucleotides such as ATP and GTP. However, despite a number of papers that report a similar activity in isolated plant mitochondria (11, 29–31), a recent and more rigorous study of isolated potato mitochondria failed to find evidence of a fatty acid-stimulated proton leak that was inhibitable by nucleotides (20). This latter work casts some doubt as to whether UCP catalyzes a proton leak in plant mitochondria, although there are several different interpretations of this data. One possibility is that UCP does catalyze a proton leak, but that there is

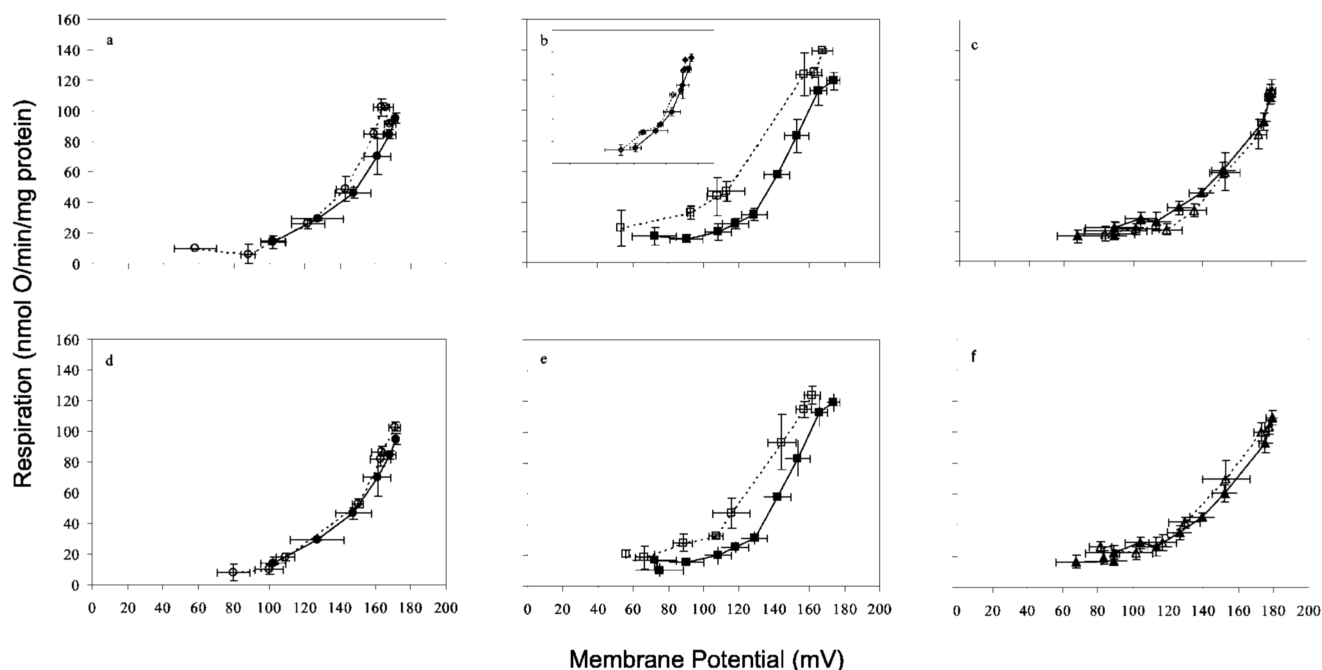

FIG. 3. **Proton leak kinetics of mitochondria isolated from wild type tubers and from transgenic tubers with increased StUCP protein content.** For further details see "Experimental Procedures." Wild type (●, ■, ▲) and transgenic (○, □, △) mitochondria were incubated with 1 mM NADH as a substrate and titrated with KCN in a medium containing 0.1% (w/v) BSA, 300 μM linoleic acid, 50 μM xanthine (●, ○), plus 0.015 unit of xanthine oxidase (■, □) or plus 0.015 unit of xanthine oxidase and 2 mM GTP (▲, △). Two independent transgenic lines were tested: line 18 (*a–c*) and line 63 (*c–e*). The *inset* in *b* shows the effect of addition of 24 units of superoxide dismutase prior to addition of xanthine oxidase to wild type (◆) and transgenic tubers (line 18; ◇). Values in all graphs are means ± S.E. of three independent experiments.

TABLE I
Effect of increased UCP content on proton conductance in transgenic potato tuber mitochondria

Proton conductance values were calculated from the proton leak kinetics shown in Fig. 3, assuming an H^+/O ratio of 6 for oxidation of external NADH. Values are shown for wild type mitochondria and those from two independent transgenic lines (line 18 and line 63) containing increased content of UCP. Proton conductance was calculated at a membrane potential of 130 mV under the conditions indicated (see Fig. 3 for more details). Values are the means ± S.E. of three independent experiments.

Condition	Proton conductance at 130 mV (nmol of H^+/min/mg of mitochondrial protein/mV)		
	WT	Line 18	Line 63
Linoleic acid + superoxide	1.09 ± 0.36	3.24 ± 0.21[a]	2.50 ± 0.49[a]
Linoleic acid + superoxide + GTP	1.36 ± 0.14	1.18 ± 0.46	1.51 ± 0.40

[a] Indicates significantly different from wild type (*t* test; $p < 0.05$).

insufficient UCP protein in potato to detect this leak. On the basis of estimates of the specific activity of potato UCP when the *StUCP* gene is overexpressed in yeast and estimations of UCP content in potato mitochondria, this is the possibility favored by Hourton-Cabassa *et al.* (20). However, since isolated mitochondria do not experience the same bioenergetic conditions as mitochondria *in vivo*, an alternative possibility is that the conditions of assay were not sufficient to fully activate UCP.

We have shown that in the presence of exogenous superoxide, linoleic acid stimulates a proton leak in isolated potato mitochondria that is inhibited by the nucleotide, GTP (Figs. 1 and 3). This is characteristic of UCP activity and suggests that superoxide may be required for full activity of potato UCP, as is the case for mammalian UCPs (21, 22). A reactive oxygen species-dependent uncoupling of wheat mitochondria in the presence of fatty acid has previously been observed, but the nucleotide sensitivity of this effect was not tested (32). To investigate whether superoxide-dependent, fatty acid-stimulated proton conductance is indeed related to the activity of UCP, we examined the effect of increased mitochondrial UCP content in transgenic plants overexpressing the *StUCP* gene. Mitochondria from two independent transgenic lines contained 13-fold (line 18) and 2-fold (line 63) more UCP protein than WT (Fig. 2). This confirms that overexpression of *StUCP* results in a measurable increase of UCP protein and that this protein is correctly targeted to the mitochondrion. The rate of proton conductance in mitochondria isolated from these two lines was signficantly higher than WT when assayed in the presence of superoxide and linoleic acid together but unaltered in the presence of linoleic acid alone (Fig. 3). This confirms that superoxide-dependent, linoleic acid-stimulated uncoupling is catalyzed by StUCP. The fact that this UCP-related uncoupling is completely inhibited by GTP (Fig. 3) provides a specifc assay for UCP that can be utilized in future studies. The increase in rate of proton conductance was proportional to the increase in UCP protein content in one of the lines (line 63; proton conductance rate, 2.3-fold WT and UCP protein content, 2-fold WT), providing further evidence that the change in proton conductance is directly linked to UCP. However, in a second line the increase in proton conductance was much less than the increase in UCP protein content (line 18; proton conductance rate, 3.0-fold WT and UCP protein content, 13-fold WT). It is not clear why the relationship is not directly proportional in this line, although it may be related to the greater increase in UCP protein content. Previously, it has been observed that the uncoupling effect of UCP when overexpressed to very high levels is artifactual, presumably due to a misfolding of the UCP protein in the mitochondrial membrane (33). Under such circumstances it is conceivable that the direct relationship between UCP protein content and proton conductance rate may break down. How-

ever, the fact that the increased proton conductance in line 18 is completely inhibited by GTP (Fig. 3*b*) leads us to believe that the additional UCP in line 18 is correctly folded and inserted into the membrane (since if it were not, the resulting artifactual uncoupling would be unregulated). Alternative explanations for the lower than expected increase in proton conductance rate in this line are that some unknown endogenous factor is limiting the proton leak rate or that above 3-fold expression only some of the overexpressed UCP is inserted correctly into the membrane, while the rest has no effect on proton leak rate in these mitochondria.

This work, for the first time, shows that changes in UCP content can affect the rate of proton leak in plant mitochondria providing firm evidence that plant homologues of mammalian UCPs do function as uncoupling proteins *in planta*. Furthermore, we have demonstrated that xanthine/xanthine oxidase stimulates this proton leak, which suggests that superoxide is required for full activity of potato UCPs. The abolishment of the xanthine/xanthine oxidase effect by superoxide dismutase confirms that is the specific presence of the superoxide anion that is responsible for the activation of UCP. Using the conditions we have described (isolated mitochondria respiring NADH in the presence of nigericin and oligomycin) it is possible to specifically assay UCP as the superoxide-dependent, fatty acid-stimulated proton leak that is inhibited by GTP. Previously, specific assay of UCP *in situ* in plant mitochondria has been complicated by the possibility that other carrier proteins can also catalyze a fatty acid-dependent proton leak. Thus, nucleotide inhibition is required to demonstrate specificity of the assay for UCP. Often, ATP is used as an inhibitory nucleotide, which can cause problems in interpretation due to its interactions with other components of the mitochondrial respiratory pathway, particularly activation of succinate dehydrogenase in mitochondria respiring succinate (20, 29). When care is taken to avoid these problems, the fatty acid-stimulated proton leak of potato mitochondria is not nucleotide inhibitable, suggesting that it is not catalyzed by UCP (20). However, we have shown that in the presence of exogenous superoxide there is an additional proton leak that is inhibitable by GTP and is specific to UCP. This contrasts with results obtained when a purified potato mitochondria UCP was reconstituted into liposomes (18). In this instance, the purified UCP catalyzed a fatty acid-dependent proton conductance that was inhibitable by nucleotides without any requirement for superoxide. However, it is not clear whether the purified UCP protein used in this study is the same protein as the *StUCP* gene product, so it is difficult to compare the two experiments. It is possible that different potato UCPs have different properties. Alternatively, the process of reconstituting the UCP protein into an artificial membrane bilayer may have artificially generated sufficient endogenous superoxide (or related molecules) to activate UCP or may have reduced its sensitivity to superoxide or otherwise altered its regulatory properties.

The requirement of a plant UCP for superoxide provides an interesting insight into the biological role of uncoupling proteins in plants. Assuming that there is a functional association between superoxide accumulation and UCP activity, it seems reasonable to argue that UCP may function to reduce reactive oxygen species accumulation. It is known that the mitochondrial electron transport chain is a source of superoxide, mainly as a result of leakage of single electrons to oxygen (34). Conditions that reduce the flow of electrons through the respiratory chain effectively increase the half-life of donor radicals and thereby increase the production of superoxide and associated reactive oxygen species. By facilitating a high rate of respiration, the activity of UCP can reduce the rate of reactive oxygen species production. This role of UCP is consistent with induced expression of UCP genes in dicotyledenous plants by low temperature (12, 14, 15), a stress condition that leads to reactive oxygen species accummulation. An *Arabidopsis* UCP gene has also been shown to be induced by hydrogen peroxide treatment (35). Indirect evidence for the role of UCP in the prevention of reactive oxygen species production by plant mitochondria is provided by the observation that the addition of linoleic acid to isolated potato mitochondria reduces the rate of production of hydrogen peroxide. Conversely, addition of ATP (to inhibit UCP) increases hydrogen peroxide production (36). At this stage, it is not possible to say whether all UCPs will turn out to be involved in reducing reactive oxygen species production. The *Arabidopsis* genome contains four putative UCP genes (37), and it is likely that some of these genes may play a different role. Intriguingly, UCP genes from monocotyledenous plants do not appear to be induced by low temperature (16, 17), which may indicate that UCP is not the main mechanism for avoiding mitochondrial oxidative stress in such plants.

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