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Overexpression of Plant Uncoupling Mitochondrial Protein in Transgenic Tobacco Increases Tolerance to Oxidative Stress

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An *Arabidopsis thaliana* cDNA clone encoding a plant uncoupling mitochondrial protein (AtPUMP1) was overexpressed in transgenic tobacco plants. Analysis of the AtPUMP1 mRNA content in the transgenic lines, determined by Northern blot, revealed variable levels of transgene expression. Antibody probing of Western blots of mitochondrial proteins from three independent transgenic lines showed significant accumulation of AtPUMP1 in this organelle. Overproduction of AtPUMP1 in transgenic tobacco plants led to a significant increase in tolerance to oxidative stress promoted by exogenous hydrogen peroxide as compared to wild-type control plants. These results provide the first biological evidence for a role of PUMP in protection of plant cells against oxidative stress damage.

KEY WORDS: Transgenic tobacco; plant uncoupling protein; oxidative stress; constitutive overexpression.

INTRODUCTION

Energy dissipation in plant mitochondria can be mediated by two processes: a redox potential dissipating system, the quinol alternative oxidase (AOx) branched from the main respiratory chain at the level of ubiquinone; and a proton electrochemical potential dissipating system, the *p*lant *u*ncoupling *m*itochondrial *p*rotein (PUMP; Vercesi et al., 1995). PUMP functions similarly to the uncoupling protein (UCP; for a review see Ricquier and Bouillaud, 2000) of mammalian brown adipose tissue that exports anionic free fatty acids from the mitochondria, which can subsequently return by flipping back in a protonated form through the membrane bilayer (Ježek et al., 1996, 1997).

PUMPs are found either in climacteric or nonclimacteric, as well as in thermogenic and nonthermogenic plants (Ježek *et al.*, 1998). Genes encoding PUMP have been isolated and characterized from several plant species, including *Solanum tuberosum* (Laloi *et al.*, 1997), *Arabidopsis thaliana* (Maia *et al.*, 1998; Watanabe *et al.*, 1999), *Symplocarpus foetidus* (Ito, 1999), *Triticum aestivum* (Murayama and Handa, 2000), and *Oryza sativa* (Watanabe and Hirai, 2002).

The finding of uncoupling proteins in plants raised a question of the physiological significance of such a system, originally involved in adaptational thermoregulatory heat production in mammals. Recent hypotheses favor a more general function (Almeida *et al.*, 1999; Jarmuszkiewicz *et al.*, 1998), implicating PUMP in the regulation of energy metabolism in mitochondria, thus preventing situations where extremely high $\Delta_{\mu H+}$ would lead to excess of reactive oxygen species (ROS) formation (Møller, 2001). Accordingly, it has been reported that PUMP inhibition strongly correlates with increased hydrogen peroxide (H_2O_2) production in potato mitochondria (Kowaltowski *et al.*, 1998). Conversely, in the same study, activation of PUMP by linoleic acid

Key to abbreviations: AOx, alternative oxidase; AtPUMP1, *Arabidopsis thaliana* uncoupling protein 1; CSPD, disodium 3-(4-methoxyspiro {1,2-dioxetane-3,2′-(5′-chloro) tricyclo [3.3.1.1^{3,7}]decane}-4-yl) phenyl fosfate; EST, expression sequence tag; PUMP, plant uncoupling mitochondrial protein; ROS, reactive oxygen species; UCP, uncoupling protein.

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decreased mitochondrial ROS formation. Moreover, results obtained by Pastore *et al.* (2000) suggest that PUMP activity is modulated by ROS production in respiring durum wheat mitochondria. In 2mammals, evidence for a role of the ubiquitous UCP2 in the limitation of ROS production was recently provided using UCP2-deficient mice (Arsenijevic *et al.*, 2000). Recently, an EST encoding a putative PUMP was identified among *Arabidopsis* genes induced by exogenous H_2O_2, using microarray analysis (Desikan *et al.*, 2001). Altogether, these data strongly support the notion that mild uncoupling of respiration by UCP/PUMP has a major importance in regulating ROS generation in different organisms.

In normal mitochondria, most of the oxygen is reduced to water by cytochrome c oxidase in four consecutive one-electron steps. Nevertheless, a small fraction (about 1–2%) is converted to superoxide anion ($O_2^{\cdot-}$) at intermediate steps of the respiratory chain (Boveris and Chance, 1973; Liu, 1997; Møller, 2001; Turrens, 1997). This production may occur at the level of the NAD(P)H dehydrogenase (Turrens and Boveris, 1980) or at the level of coenzyme Q (Cadenas *et al.*, 1977). During respiration, electrons are transferred from NADH to the oxidized form of coenzyme Q (UQ), to yield its reduced form (UQH_2). UQH_2 then transfers electrons to cytochrome c reductase (Complex III) or AOx and is converted again into UQ, passing firstly through the free-radical semiubiquinone anion species ($UQ^{\cdot-}$). Accumulation of this species during resting respiration favors electron donation from $UQ^{\cdot-}$ to O_2 producing superoxide anion. Because the $O_2^{\cdot-}$ generation and its conversion to H_2O_2 and hence to the hydroxyl radical ($OH^{\cdot}$) are continuous processes during respiration, plant mitochondria possess an efficient antioxidant system (Møller, 2001; Scandalios, 1990) composed by superoxide dismutase, glutathione peroxidase/reductase system, NAD(P)-transhydrogenase, NADPH, vitamins E and C, dehydroascorbate reductase, thiol peroxidases (Watabe *et al.*, 1997), cytochrome c (Halliwell and Gutteridge, 1989; Skulachev, 1998; Sutton and Winterbourn, 1989), and catalase.

In an attempt to investigate the physiological significance of the uncoupling pathway in cell defense against mitochondrial oxidative stress, we generated transgenic tobacco plants constitutively expressing PUMP from *A. thaliana* (AtPUMP1; Maia *et al.*, 1998). Leaf discs from these plants showed significant tolerance against high concentration of exogenous H_2O_2, compared to control nontransgenic plants. Our findings indicate a prevalent role of PUMP in controlling oxidative damage in plants.

MATERIALS AND METHODS

Construction of the AtPUMP Expression Cassette and Plant Transformation

The AtPUMP1 coding region was amplified by polymerase chain reaction (PCR) from a full-length cDNA clone, using primers AtPUMP-F (5′-CCGGGA TCCATGGTGGCGGCTGGT-3′; start codon in bold) and AtPUMP-R (5′-CGCAAAGCTTTCAGTTTCTTTTGG ACG-3′; stop codon in bold). The primers were supplemented with restriction sites for *Nco*I and *Hind*III (underlined) for direct cloning of the PCR product into the *Nco*I- and *Hind*III-digested plasmid pDMC200. A fragment obtained by digestion of the pDMC200 derivative with *Nco*I and partially with *Sst*I was subcloned into a pBI426 derivative digested by *Nco*I/*Sst*I. In this plasmid, the AtPUMP1 coding region was placed under the control of an enhanced CaMV 35S promoter (2 × 35S) and a nopaline synthase transcriptional terminator (Fig. 1(A)). The resulting expression cassette was excised as a *Hind*III

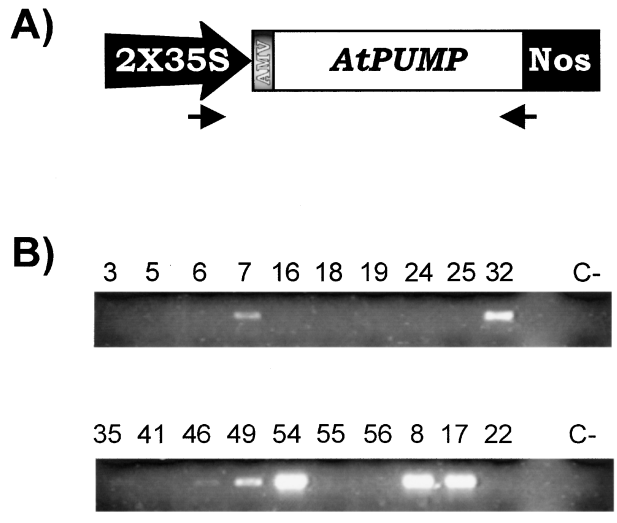

Fig. 1. (A) Schematic representation of the gene construct used for transformation of tobacco. The AtPUMP1 ORF (open box) was placed under the control of an enhanced CaMV 35S promoter (2 × 35S) and a nopaline synthase terminator (Nos). Additionally, the alfalfa mosaic virus (AMV) enhancing sequence (gray box) was inserted upstream from the start codon of the cDNA to strength its translation efficiency. The resulting cassette was inserted into a binary vector (pBI121) and then transferred to Agrobacterium. (B) Agarose gel electrophoresis of AtPUMP1-PCR fragments amplified from genomic DNA samples isolated from different transgenic tobacco lines. The numbers indicate different transformation events. Genomic DNA from a nontransgenic control plant was also included (C−). The location of the 35S CaMV- and AtPUMP1-specific primers used to generate the PCR fragments are indicated (black arrows in A).

partial/*Eco*RI fragment and inserted into the binary vector pBI121 (Clontech) at *Hind*III/*Eco*RI sites. The correctness of the construct was verified by sequencing in an ABI 310 automatic sequencer (Perkin-Elmer).

The recombinant plasmid was then transferred into *Agrobacterium tumefaciens* (strain LBA4404) for transformation of tobacco (*Nicotiana tabacum* cv SR1) leaf discs as described (Horsch *et al.*, 1985). The transformed plants were analyzed for integration of the transgene into the genome by PCR with a sequence from the 3′ end of the 35S CaMV promoter (5′-ACAATCCCACTATCCTTCG-3′) and a sequence from the AtPUMP1 coding region (5′-GAATCTCCCATCATTCTTGAC-3′; complementary to nucleotides 867-887) as primers and genomic DNA as a template.

Northern and Southern Analysis

For Southern blot analysis, samples of genomic DNA (10 μg) isolated from leaves of transgenic tobacco plants (Sambrook *et al.*, 1989) were digested overnight at 37°C with *Eco*RI. The resulting DNA fragments were fractionated in a 0.8% agarose gel and transferred onto a nylon membrane (Hybond-N; Amersham). For Northern blot analysis, total RNA was isolated from 100 mg of leaf tissue of transgenic plants using Trizol reagent (Gibco BRL) according to the supplier's protocol. Total RNA from each sample was separated in denaturing 1.0% agarose gels and blotted to positively charged nylon membranes (Hybond-N^+; Amersham). Hybridization procedures were carried out as previously described (Maia *et al.*, 1998) using the uniformly ^{32}P-labeled AtPUMP1 cDNA as probe.

Western Blot Analysis

Total soluble protein extracts were obtained by grounding leaf discs directly in 3x protein sample buffer (187 mM Tris, pH 6.8; 6% SDS; 30% glycerol; 3% 2-mercaptoethanol; 0.06% bromophenol blue). Mitochondria from leaves of transgenic and control plants were isolated using a standard protocol (Day *et al.*, 1985). Protein profiles were analyzed by 0.1% sodium dodecylsulfate-12% polyacrylamide gel electrophoresis (SDS-PAGE). Western blot analysis was carried out as described using polyclonal antibodies raised against *E. coli* expressed AtPUMP1 (1:1000 dilution; Borecký *et al.*, 2001) and an antirabbit IgG alkaline phosphatase conjugate (1:5000 dilution). The bands were detected by autoradiography using the CSPD chemiluminescent substrate (Tropix Inc.).

Oxidative Stress Treatment and Chlorophyll Determination

Oxidative stress was induced essentially as described by Blaszczyk *et al.* (1999). Fully expanded leaves from the transgenic line AtPUMP-07 grown for 8–10 weeks in a growth chamber were used. Leaf discs (approx. 1-cm diameter) were cut at random and then floated on distilled H_2O (control) or H_2O_2 at 0.05, 0.1, 0.2, 0.4, and 0.8 M concentrations for 12, 24, 36, and 48 h at 25°C and constant light photoperiod in four independent experiments. Leaf discs from nontransformed tobacco plants were used as control in the same manner. The degree of oxidative stress in treated leaf tissues was determined spectrophotometrically as a content of Chlorophyll A and B in discs following extraction in acetone at 4°C as described by Arnon (1949).

RESULTS AND DISCUSSION

AtPUMP1 Expression in Transgenic Tobacco Plants

A binary vector (pBI121) carrying a fusion of the enhanced *Cauliflower mosaic virus* (CaMV) 35S promoter (2×35S) and the AtPUMP cDNA (Fig. 1(A)) was used for *Agrobacterium*-mediated transformation of tobacco. After regeneration, several kanamycin-resistant tobacco plants, which were shown to contain the introduced gene by PCR analysis (Fig. 1(B)), were obtained and self-pollinated. The insertion of one to three copies of the transgene was observed in Southern blot analysis (not shown).

Analysis of the AtPUMP1 mRNA content in the kanamycin-resistant R1 transgenic lines, determined by Northern blot, revealed variable levels of transgene expression (Fig. 2). The AtPUMP1 protein content in these lines, determined by Western blot analysis of protein extracts from leaf tissues with anti-AtPUMP1 polyclonal antibodies (Fig. 3(A)), was proportional to the level of the AtPUMP1 mRNA. Subsequent immunodetection of AtPUMP1 in isolated mitochondria showed significant accumulation of the AtPUMP1 protein in these organelles (Fig. 3(B)). No apparent phenotypic alteration could be observed in the transgenic plants constitutively expressing the *AtPUMP1* gene.

Constitutively Overexpressed AtPUMP Enhances Tobacco Tolerance Against H_2O_2-Induced Oxidative Stress

Current evidences indicating that PUMP activity is modulated by ROS production prompted us to investigate

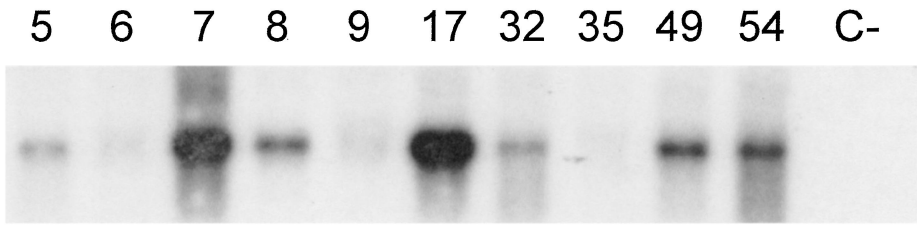

Fig. 2. Northern blot analysis of total RNA (20 μg) isolated from leaf tissues of several AtPUMP1-transgenic tobacco lines or from a nontransgenic control plant (C−). The blot was hybridized with a radioactive probe specific for the AtPUMP1 sequence.

the involvement of this protein in the oxidative stress response in plants. For that, leaf discs from wild type and transgenic line AtPUMP-07, exhibiting the highest expression of AtPUMP1 (cf. Fig. 3), were employed in a resistance test against H_2O_2, an oxidative stress-inducing agent. H_2O_2-induced damage was monitored by determining the chlorophyll content after treatment.

Figure 4 illustrates the phenotypic differences between nontransgenic and transgenic leaf discs after treatment with various concentrations of H_2O_2 for 24 and 48 h, respectively. A visible delay in tissue damage, as determined by leaf disc yellowing, was observed in the transgenic samples as compared to control ones. Pronounced yellowing of the control leaf discs (P-WT) was observed at 0.4 and 0.8 M H_2O_2 concentrations after 24 h (Fig. 4, upper left plate), whereas the discs from the transgenic line AtPUMP-07 (P-7) were clearly less sensitive to damage at this point (Fig. 4, upper right plate). After 48 h, the observed differences became also evident at lower H_2O_2 concentrations (at 0.1 M for example; cf. Fig. 4, lower plates).

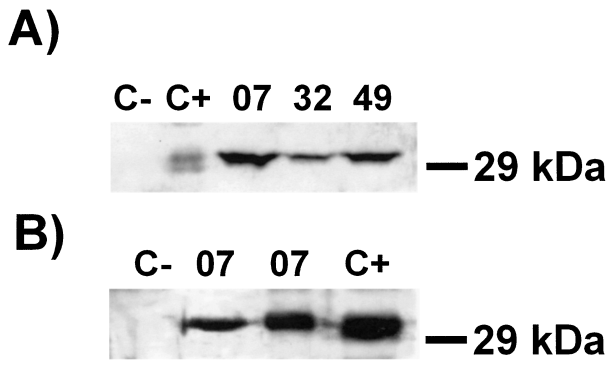

Fig. 3. Western blot analysis showing the AtPUMP1 content in three independent transgenic lines. (A) Total protein isolated from leaves of the transgenic lines AtPUMP-07, AtPUMP-32 and AtPUMP-49 was fractionated, electroblotted, and probed with an AtPUMP1 polyclonal antiserum. (B) Immunodetection of PUMP in mitochondria isolated from two different plants of the transgenic line AtPUMP-07. In both panels, an extract from a nontransformed tobacco plant and 20 ng of a recombinant *E. coli* expressed AtPUMP1 were used as negative (C−) and positive (C+) controls, respectively. The position of a protein molecular weight standard (in kDa) is indicated to the right of the figure.

Measurement of the chlorophyll content in treated leaf discs further confirmed the phenotypic differences depicted in Fig. 4. Spectrophotometric results from water-treated leaf discs confirmed that experiment set-up did not alter total chlorophyll content (Fig. 5; Panels A, D, and G). In contrast, the chlorophyll content was severely decreased in the presence of H_2O_2 (Fig. 5; Panels B-C, E-F, and H-I). However, chlorophyll oxidation in leaf discs obtained from transgenic plants was significantly reduced in comparison to wild-type control ones. This was clearly noticeable over 24 h of treatment.

These results support previous observations (Kowaltowski *et al.*, 1998; Pastore *et al.*, 2000) indicating mitochondrial uncoupling as a potential way to reduce oxidative damage. Upregulation of PUMP expression has been observed in response to low temperature (Ito, 1999; Laloi *et al.*, 1997; Maia *et al.*, 1998; Nantes *et al.*, 1999), aging (Nantes *et al.*, 1999), wounding (our unpublished results), and pathogen attack (Vercauteren *et al.*, 2001), stresses that are normally associated with increased cellular ROS formation. Although being indirect evidences, these results further strengthen the concept implicating PUMP in the response to oxidative stress. Moreover, it was recently demonstrated that superoxide activates nucleotide-sensitive mitochondrial proton transport through the UCPs (Echtay *et al.*, 2002a, 2002b).

The underlying mechanism of how overproduction of PUMP confers tolerance to oxidative stress promoted by exogenous H_2O_2 remains to be demonstrated. However, a mechanism could be proposed on the basis of current knowledge on how ROS are generated in cells and the conditions that either stimulate or inhibit their production, and how the cellular antioxidant system protects cells against oxidative stress (see also Kowaltowski and Vercesi, 1999). ROS are naturally generated in aerobic cells as by-products of many metabolite reactions

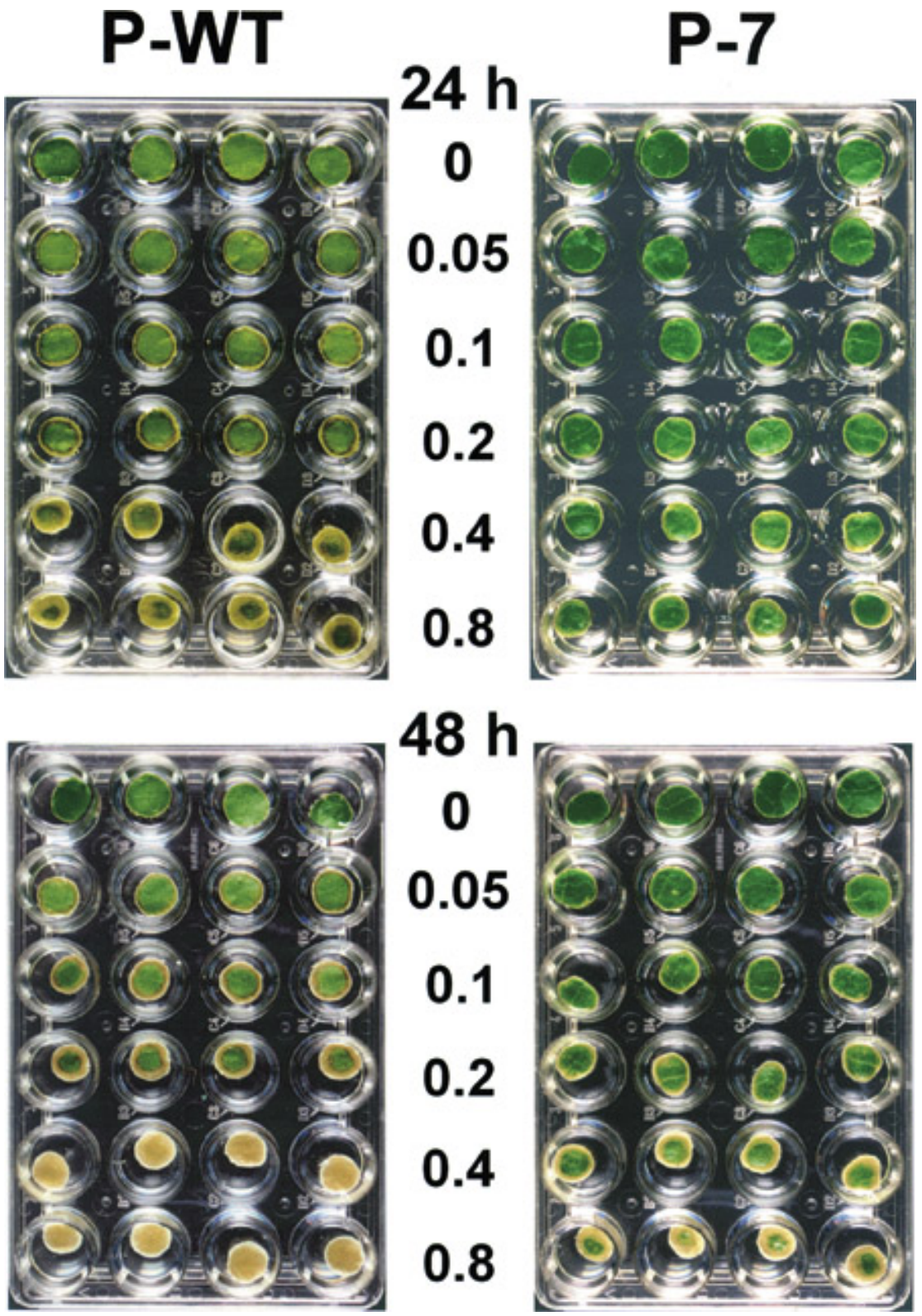

Fig. 4. Resistance of AtPUMP1 transgenic plants to oxidative stress induced by exogenous H_2O_2. Sets of tobacco leaf discs from four different nontransgenic control plants (P-WT) and from four different plants of transgenic line AtPUMP-07 (P-7) were treated with either water (control) or H_2O_2 at 0.05, 0.1, 0.2, 0.4, and 0.8 M concentrations (as indicated between the panels) for 24 h (upper two plates) and 48 h (lower two plates). In comparison to the controls, leaf discs from transgenic plants were less sensitive to H_2O_2-damage, indicating enhanced defense of AtPUMP1 overexpressing plants against oxidative stress.

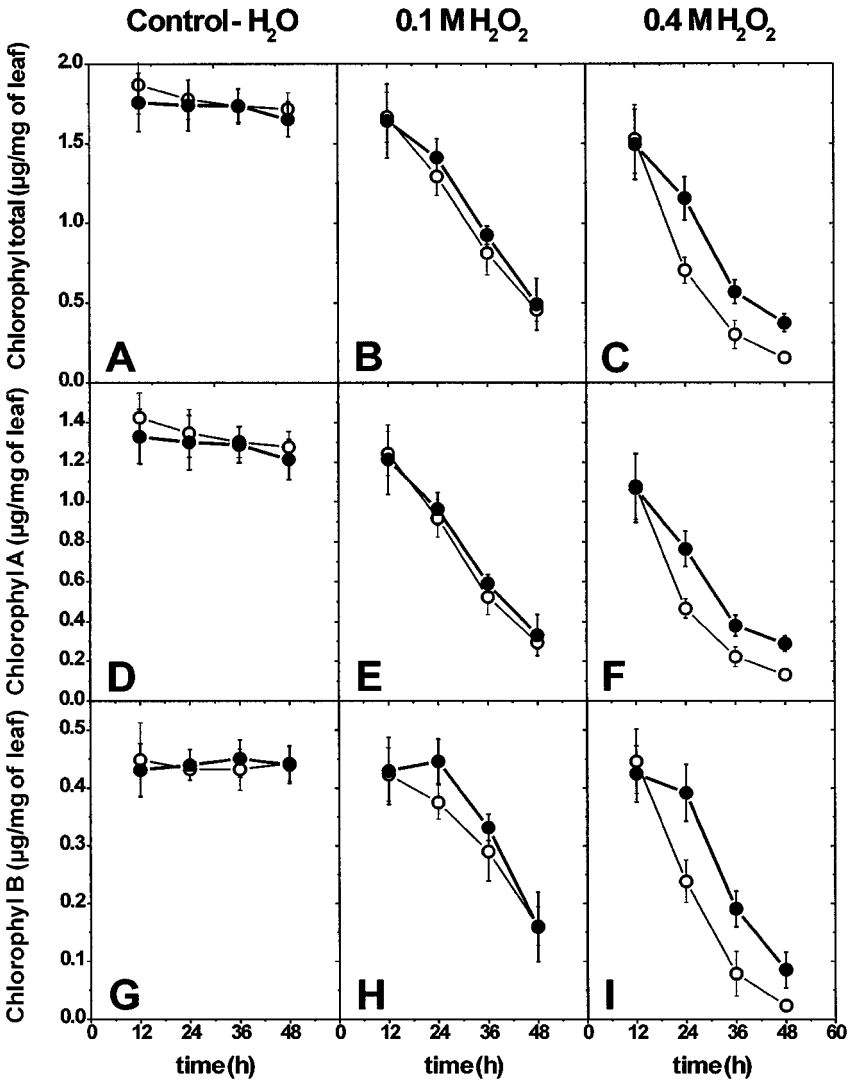

Fig. 5. Spectrophotometric quantification of chlorophyll content in AtPUMP1 transgenic plants exposed to oxidative stress induced by exogenous H_2O_2. Leaf discs from transgenic line AtPUMP-07 (filled circles) and nontransgenic control plant (open circles) were treated by water (control; Panel A, D, and G), 0.1 M H_2O_2 (Panel B, E, and H), and 0.4 M H_2O_2 (Panel C, F, and I). Total chlorophyll content and consequently chlorophyll A and B were evaluated spectrophotometrically in acetone extracts of treated leaf discs. Error bars were calculated from four experiments.

and respiration is thought to be a major source. Electron transport inhibitors, particularly the bc_1 complex inhibitor antimycin A, enhance ROS production by inducing the accumulation of semiubiquinone, the main source of superoxide generation in mitochondria (Kowaltowski and Vercesi, 1999; Møller, 2001; Turrens, 1997). In contrast, protonophores and respiratory dissipative pathways, such as the UCPs and the plant AOx, decrease the rate of ROS production by increasing the rate of respiration. Faster respiration shortens the semiubiquinone lifetime and lowers the oxygen tension in the tissues, resulting in the decrease of electron donation from $UQ^{\cdot -}$ to O_2. Therefore, if less endogenous ROS are being produced, the cell antioxidant defense system should be more available/efficient to protect against exogenous ROS. Cytochrome c is another mitochondrial component that can act as an antioxidant. The stimulated respiration keeps cytochrome c in the oxidized form that can receive one electron from ROS, converting the electron back into the electron transport chain (Kowaltowski and Vercesi, 1999) and inactivating ROS.

Our results maintain that these mechanisms are more efficient in transgenic plants constitutively overproducing

the uncoupling protein. Pronounced uncoupling of their mitochondria maintains the respiration rate high and could therefore either decrease the production or enhance the defense against ROS. In conclusion, the degree of plant cell tolerance to exogenous applied H_2O_2 can be used to determine a PUMP role or contribution to the defense against oxidative stresses in plant tissues. It is known that H_2O_2 is highly diffusible through biological membranes, a property that allows it to be removed by the mitochondrial antioxidant system, thus protecting the whole cell against the oxidative stress generated by H_2O_2 present in the incubation medium.

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