Mitochondrial Uncoupling Protein 1 Overexpression Increases Yield in Nicotiana Tabacum under Drought Stress by Improving Source and Sink Metabolism

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Running title: UCP1 protects from stress and increases yield

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Abstract
Mitochondrial uncoupling proteins (UCPs) sustain mitochondrial respiration independent of intracellular ATP concentration. Uncoupled respiration is particularly beneficial under stress conditions, during which both photosynthesis and respiration may be impaired. Sustaining carbon fixation during the reproductive phase is essential for plants to develop viable pollen grains and for seed setting. Here, we examined whether UCP1 overexpression (UCP1-oe) would help tobacco plants cope with drought stress during reproductive development. We observed that WT and UCP1-oe plants lost water at the same rate under moderate drought stress, but that UCP1-oe lines regained water faster upon rewatering. UCP1-oe plants maintained higher levels of respiration and photosynthesis and decreased H2O2 content in the leaves during the drought stress period. We examined whether UCP1-oe impacts reproductive tissues and seed production by monitoring the progress of flower development, focusing on the early stages of pollen formation. UCP1-oe lines induced the expression of mitochondrial genes and increased mtDNA content in reproductive tissues, which increased the consumption of carbohydrates and reduced H2O2 content and pollen disturbances. Finally, the beneficial impact of UCP1-oe on the source and sink organs resulted in an increased seed size and number under both control conditions and drought stress.

Keywords: UCP1, drought stress, mitochondria, photosynthesis, yield
Plants face a range of adverse conditions that impose metabolic constraints during their lifecycle. Among these stresses, water deficit is perhaps the most important because it affects both cell energy supply and demand. The consequences of drought stress are not limited to a cellular context but extend to whole plant physiology. The effect of water deficit on plant energy status is especially harmful during reproduction, where there is competition for nutrients between newly established sink tissues (flowers) and roots from source tissues (leaves). Because of that water deficit is the major abiotic stress factor affecting crop development and yield. In the past few years, the frequency and intensity of drought have increased in many regions around the globe, and there are predictions of further increases as the result of global climate change.

Drought stress inhibits photosynthesis, causing reduced production of photoassimilates. During drought stress, leaves close their stomata to prevent water loss, which in turn reduces CO₂ intake from the atmosphere and consequently affects carbon assimilation. Most of the carbon fixed in mature autotrophic leaves (source tissues) is used to synthesize sucrose for loading into phloem to be exported to roots and reproductive tissues (sink tissues). Sucrose loading is essential for reproductive tissue development, where flowers become the major sink of sugars to sustain the energy requirements for pollen production and initial seed setting. During flower formation, mitochondrial biogenesis increases twenty fold in the pollen mother cells and the tapetum layer compared to that of vegetative tissues. The increased mitochondrial activity makes these tissues more prone to reactive oxygen species (ROS) production under stress because the electron leakage to form ROS at the ETC increases at higher membrane potentials.

Because mitochondria and chloroplasts are highly interconnected organelles, the maintenance of mitochondrial respiration would help to alleviate the intracellular effects of water limitation. Under drought stress, while carbon assimilation is impaired, plants continue to receive light and produce reducing power in the form of NADPH at the photosystems. The excess reducing power accumulated in the chloroplasts might be exported and oxidized inside the mitochondria, which would alleviate photooxidation of chloroplast components and reduce the impairment of the Calvin cycle. Photorespiration would also be improved because it is limited by the oxidation of glycine to serine in the mitochondria in a reaction dependent on NAD⁺ regeneration by the mitochondrial electron transport chain. The problem with this equation is that mitochondrial respiration in
mature leaves is also inhibited during drought stress\textsuperscript{2,14,15,16}. One of the hypotheses relies on the fact that most of the ATP demands in mature leaves are used for sucrose synthesis and phloem loading; therefore, intracellular ATP content may increase during drought and restrict mitochondrial respiration adenylate\textsuperscript{2}. It is also possible that mitochondrial respiration decreases because of a limitation on substrates provided by photosynthesis or a reduction in activity of mitochondrial enzymes\textsuperscript{1}.

Nevertheless, plant mitochondria possess mechanisms to sustain respiration independently of adenylate control. The mitochondrial uncoupling proteins\textsuperscript{17} (UCPs) and alternative oxidases\textsuperscript{18} (AOXs) sustain mitochondrial respiration that is uncoupled from ATP synthesis. The overexpression of AOX protects tobacco plants from drought stress by maintaining mitochondrial respiration\textsuperscript{16,19}. In durum wheat, a drought-tolerant cereal plant, mitochondria exhibit high UCP activity, suggesting this protein may be part of the tolerance mechanism\textsuperscript{20}. Overexpression of UCP1 (UCP1-oe) protects plants from abiotic\textsuperscript{21-25} and biotic stresses\textsuperscript{24}. Increased stress tolerance is associated with the maintenance of mitochondrial biogenesis, respiration,\textsuperscript{25} and carbon assimilation\textsuperscript{23}. In contrast, knockouts of UCP1 in \textit{Arabidopsis thaliana} decrease carbon assimilation due to impaired photorespiration\textsuperscript{12}. UCP1-oe also alters the expression of chloroplast genes and induces the expression of a large subset of stress-responsive genes, which may explain the broad abiotic and biotic stress tolerance\textsuperscript{26}.

In this work, we combined physiological assessments with gene expression and metabolomics analyses to evaluate the impact of UCP1 overexpression in flower development and seed production during control and water-limiting conditions. The data suggest that UCP1-oe increases respiration and photosynthesis in leaves under control conditions and maintains these processes during drought, which allows maintenance of normal flower metabolism and results in increased yields. The results are discussed in the context of the role of mitochondria in the coordination of source/sink metabolism as a prerequisite for sustained yield.

\textbf{Materials and Methods}

\textbf{Plant Material and Growth Conditions}

Tobacco plants (\textit{Nicotiana tabacum}, ecotype SR1) (WT) and three transgenic lines (P07, P30, and P49) overexpressing \textit{Arabidopsis thaliana} UCP1\textsuperscript{21} were used in this study. These lines have been previously used by our group\textsuperscript{25,26} and others\textsuperscript{23}. Seeds were sown in
trays containing a soil:vermiculite (1:1) mixture; 15 days after planting, the seeds were transferred to 0.2-L pots. When plants reached the 5-leaf stage, they were transferred to 5-L pots and grown to maturity. Plants were grown in the greenhouse with a photoperiod of 16 h of light and 8 h of dark at 25-28°C at 400 μmol m⁻² s⁻¹.

The experimental design consisted of three conditions (control, drought, and recovery), each composed of 20 plants for a total five biological replicates for each genotype (Supplementary Figure 1A). The drought stress treatment consisted of withholding irrigation for 15 days at the beginning of the reproductive stage, which was defined as the moment the first inflorescence became visible (T = 0) (Supplementary Figure 1B). Leaves and flower buds were sampled at day zero (T = 0) and 15 days after drought stress imposition (T = 15). At T = 15, plants were again irrigated and visually monitored for recovery, and leaf and flower bud samples were collected 24 h after rewarming on day 16 (T = 16). Flower buds and the youngest expanded leaf were sampled for all plants, frozen in liquid nitrogen and stored at -80°C. We defined the first expanded leaf as the younger mature leaf that was fully developed before the beginning of stress. As large variations are expected when plants develop outside the growth chamber, the whole experiment was repeated twice under the same conditions. This additional experiment was designed to confirm the results by using important proxies, such as physiological parameters, RWC, and seed yield. All the data presented in this manuscript were obtained from a single trial.

**Water Status**

The relative water content (RWC) was determined for evaluating plant water status. For each plant, twenty leaf discs (0.5 cm diameter) were sampled from the youngest expanded leaf. Leaf discs were weighed to determine the leaf fresh weight (FW). The turgid weight (TW) was determined by floating the leaf discs on water for 4 h, after which they were surface-dried and weighed. The dry weight (DW) was determined after drying the leaf discs at 80°C for 48 h. The percentage of leaf RWC was calculated as \[
\left(\frac{FW - DW}{TW - DW}\right) \times 100.
\]

**Hydrogen Peroxide Content**

Hydrogen peroxide (H₂O₂) content was measured in flower buds and leaves using the potassium iodide (KI) method. Approximately 100 mg of tissue was collected, frozen in liquid nitrogen and ground into a fine powder. The powder was homogenized with 0.5 ml of a solution containing 0.25 ml of 0.1% w:v trichloroacetic acid (TCA), 0.5 ml 1 M KI and
0.25 ml of potassium phosphate buffer (10 mM, pH 5.8) at 4°C for 10 min. The homogenate
was centrifuged at 12,000 × g for 15 min at 4°C, and 200 µl of the supernatant was
transferred to UV microplate wells and incubated at room temperature for 20 min, after
which the optical density (OD) measured at 350 nm. Samples and blanks were analyzed in
triplicate. A calibration curve was obtained using H2O2 standard solutions prepared in 0.1%
TCA. A pool of flower buds (8-12 mm in length) and the youngest expanded leaves from
WT and UCP1-oe plants grown under control, drought and recovery conditions were
evaluated. Five biological replicates were analyzed for each genotype and condition.

**Gas Exchange and Chlorophyll Fluorescence Measurements**

Gas exchange was measured in the youngest completely expanded leaf using an infrared
gas analyzer (IRGA - LCpro+, ADC Bioscientific, UK) at growth light intensity (400 µmol
m⁻² s⁻¹). Stomatal conductance (Gₛ), net photosynthetic rate (A_N), respiration in the light
(R_L) and respiration in the dark (R_D) were measured for each plant/treatment. R_L was
estimated by the Kok method as described¹⁶,²⁹. A_N was evaluated in a range varying from 0
to 120 µmol m⁻² s⁻¹ “PPFD” (increases of 10 µmol m⁻² s⁻¹ PPFD at each point). The
measurements resulted in a Kok break point at 20 µmol m⁻² s⁻¹ PPFD. R_D was evaluated
after 3 h of acclimation in the dark. A photosynthetic CO₂ response curve was estimated by
measuring A_N at saturating irradiance (1,500 µmol m⁻² s⁻¹ PPFD) during which CO₂ was
supplied at a range of concentrations (50, 100, 150, 200, 300, 400, 500, 750 and 1,000 µmol
mol⁻¹). The Gₛ values obtained from this curve were used to calculate the Gₛ inhibition by
CO₂ by comparing the values obtained at 400 and 1,000 µmol CO₂ mol⁻¹. We estimated
the maximal velocity of Rubisco carboxylation (V_cmax) as the slope of the linear phase of
the CO₂ response curve³⁰. The linear phase was defined as the maximum range where the
R² was above 0.95 (0 - 300 µmol mol⁻¹ CO₂).

Chlorophyll fluorescence was measured using a photosynthetic yield analyzer (MINI-
PAM-II, Walz, GE). When necessary, a dark-adaptation period of 40 min was
imposed. Initial (minimum) PSII fluorescence in the dark-adapted state (F₀), maximum PSII
fluorescence in the dark-adapted state (Fₚₚₚₚ), maximum PSII fluorescence in the light-adapted
state (Fₚₚₚₚ), steady-state fluorescence (Fₛ), and initial (minimum) PSII fluorescence in the
light-adapted state (F₀) were determined as described previously³¹. The maximal quantum
yield of PSII (F_v/F_m) was estimated as [(F_m - F₀) / F_m] and the effective PSII quantum yield
during illumination Yₚₚₚₚ as ΔF / F_m. Nonphotochemical energy quenching (NPQ), which is
a measure of heat dissipation of absorbed light energy, was calculated as NPQ = (F_m - Fₚₚₚₚ)
The photochemical energy quenching ($q_P$) was estimated as $(F_{m'} - F_s) / (F_{m'} - F_{0'})$ and used to determine the ratio of reduced PSII reaction centers $(1 - q_P)$.

**Metabolic Profiling**

Metabolites were isolated from flower buds as described, with minor modifications. A total of 100 mg of frozen tissue was powdered in liquid nitrogen, incubated with 1 ml of extraction solution containing a chloroform:methanol:water solution (2:4:1) for 30 min on ice, and vigorously homogenized for an additional 15 min. After the addition of 1 ml of water, samples were centrifuged at 12,200 x g for 5 min for phase separation. The methanol:water phase was collected and vacuum-dried for 8 h. Metabolites were analyzed using a 1H-nuclear magnetic resonance (NMR) spectrometer as described. Spectral phase and baseline corrections and the identification and quantification of the metabolites were performed using Chenomx NMR Suite 7.6 software. Five biological replicates for each genotype under control, drought and recovery conditions were evaluated.

**DAPI Staining of Pollen Grains and TUNEL Assay**

Flower buds were collected and fixed in a 3:1 ethanol:acetic acid solution. Anthers were removed from flower buds, placed on a glass slide and gently crushed to release pollen grains. Thirty microliters of DAPI staining solution (0.1 M sodium phosphate; 1 mM EDTA; 0.1% Triton X-100; 0.4 µg ml$^{-1}$ DAPI; pH 7.0) was applied to each anther. Slides were kept in the dark for 3 h before visualization in a fluorescence microscope (361 nm absorption, 461 nm emission). Three biological replicates for each genotype were analyzed for each flower bud size.

For the TUNEL assay, fixed anthers were removed from the flower buds and embedded in paraffin as described previously. Anthers were washed with PBS (pH 7.2) and dehydrated using a graded ethanol series (30, 50, 70, 90 and 100%) at room temperature. The samples were then cleared by washing with ethanol/histoclear (2:1, 1:1, and 1:2 v/v) for 1 h each and then three times in 100% histoclear for 30 min each at room temperature. Tissues were embedded in paraffin for sectioning. Ten µm sections were cut using a Microm HM315 microtome attached to polylysine-coated slides, deparaffinized with histoclear, and hydrated in a graded ethanol series (20% increments). In situ nick end labeling of nuclear DNA fragmentation was performed in a humidity chamber for 1 h in the dark at 37°C with an In Situ Cell Death Detection Kit (Roche, CH) according to the manufacturer’s instructions. For each experiment, a positive control was prepared by treating the sections
with 1 U µl⁻¹ DNase I for 10 min at 37°C before labeling as above. The negative controls were labeled in parallel, except for the absence of the enzyme terminal deoxynucleotidyl transferase (TdT). The reaction solution was removed by rinsing the slides 3 times with PBS (pH 7.2). Samples were analyzed under a fluorescence confocal scanning microscope (Leica TCS SP2 Confocal). The fluorescence filter was set to view the green fluorescence of fluorescein at 520 nm.

**Gene Expression and mtDNA Quantification**

Total RNA was isolated from flower buds using a Spectrum Plant Total RNA Kit (Sigma-Aldrich). The first-strand cDNA was synthesized using a RevertAid First Strand cDNA Synthesis kit (Fermentas, US) according to the manufacturer’s protocol. Mitochondrial DNA was quantified from total DNA isolated from flower buds using the Plant DNAzol reagent (Invitrogen, US). Real-time PCR was performed using the ABI PRISM 7500 system (Applied Biosystems, US) with SYBR Green dye (Applied Biosystems, US). At least triplicate reactions were performed with four biological replicates, and the results were expressed relative to the expression level of the *N. tabacum* PP2A gene in each sample using the 2⁻ΔΔCT method. Some of the primers were designed for *Nicotiana sylvestris* because reference data for *N. tabacum* were unavailable in the NCBI database (Supplementary Table 1). The PCR products for primers designed for *N. sylvestris* were sequenced after amplification of *N. tabacum* for validation. All values are presented as fold changes of transgenic lines relative to those of WT. Student’s t-test was performed to determine significant changes (*P < 0.1, **P < 0.05, ***P < 0.01).

**Western Blot**

Total soluble proteins were isolated from WT and transgenic lines by grinding frozen flower tissue in protein extraction buffer (50 mM Tris, pH 8.5; 1 mM EDTA; 1 mM DTT; 5 mM PMSF; 0.2% Triton X-100; 5 mM benzamidine; 5% w/v PVPP). A cOmplete™, EDTA-free Protease Inhibitor Cocktail (Roche, SW) was added to the protein extraction buffer before use. The proteins were separated on 12% SDS-PAGE gels, transferred to nylon membranes, and blotted using UCP1 (1:3000) and Actin11 (1:1500) polyclonal antibodies raised against Arabidopsis (Agrisera, SE). Bands were detected using an ImageQuant LAS500 (GE Healthcare, UK) system with the SuperSignal West Pico chemiluminescent substrate (Thermo-Scientific, US).

**Yield and Seed Size Quantification**
Five flower buds from each plant were tagged at the end of the drought stress treatment. The tagged buds were kept for seed set, the mature pods were collected at the same time for all genotypes, and their entire seed content weighed. The yield was estimated as the mass of seeds per pod. For seed size analysis, an image field containing approximately 100 seeds was imaged, and seed area was measured using ImageJ software. Six siliques were analyzed for each plant from five biological replicates. Approximately 2,500 seeds were imaged to estimate the average seed size for each condition.

**Statistical Analysis**

ANOVA with Tukey-Kramer test was used to assess the significance of changes in this research. The analyses were conducted using PRISM 6.0 (GraphPad Software Inc., US). The only exception was for principal component (PCA) and correlation analysis, on which we used the Metaboanalyst webportal\(^\text{33}\). The metabolite and physiological data were normalized by median centering before running the analysis. The Pearson correlation coefficient was used for correlation analysis.

**Results**

**UCP1-o.e Accelerates Recovery Upon Rehydration After Drought Stress**

WT and three independent UCP1-o.e lines (P07, P30, and P49) were grown until the beginning of the flowering stage (Figure 1A), when the irrigation was withdrawn (IW). Plants started to show symptoms of water deficit after 9 days of IW (Figure 1B) and showed severe wilting after 15 days of IW (Figure 1C). After 15 days of IW, plants were rewatered; 24 h after irrigation resumption, it was evident that UCP1-o.e lines rehydrated faster than the WT plants based on the plant turgidity (Figure 1D) and RWC (Figure 1E). The RWC at 15 days of IW decreased to 65%, which is considered moderate drought stress\(^\text{16,19}\). The decrease in RWC did not differ between WT and UCP1-o.e lines. UCP1-o.e lines also presented increased dry weight after the drought period compared to their non-stressed counterparts (Figure 1F). WT plants under drought stress presented 42% as much dry mass as did their well-watered counterparts, whereas the UCP1-o.e lines showed 58-62% as much dry weight as did their well-watered controls. We also examined the presence of the heterologous Arabidopsis thalianaUCP1 mRNA (Figure 1G) and UCP1 protein (Figure 1H) in flower buds. The results confirmed that heterologous UCP1 is being expressed in tobacco
flower buds. Interestingly, UCP1 protein accumulation is induced by drought in flower buds of the WT.

The increase in dry weight suggests that the UCP1-oe lines maintained physiological activities such as respiration and photosynthesis under drought stress. This agrees with the higher mitochondrial respiratory capacity of UCP1-oe$^{25}$ and the lower respiration of UCP1 knockout tomato lines$^{34}$. Additionally, increased mitochondrial respiratory capacity is associated with enhanced tolerance to drought stress$^{16,19}$. Mitochondrial respiration in the dark ($R_D$) did not differ between WT and UCP1-oe lines under control conditions (Figure 2A), whereas UCP1-oe lines presented a slight but significant increase (12%) in mitochondrial respiration in the light ($R_L$) (Figure 2B). Although there is a tendency of UCP1-oe cause increased $R_D$, this trend was only significant after drought treatment or during recovery (Figure 2A). In contrast, $R_L$ significantly increased in UCP1-oe lines during the whole course of the experiment, but this increase was more pronounced at the end of the drought treatment (38%) and during recovery (25%) (Figure 2B).

There was an increase in net carbon assimilation ($A_N$) under control conditions and water limitation as well as during the recovery period (Figure 2C). The $A_N$ values after recovery increased 45% in UCP1-oe lines compared to that of WT plants. Stomatal conductance ($G_S$) (Figure 2D) was also higher in UCP1-oe lines under control conditions and during recovery; nevertheless, $G_S$ severely decreased under drought in a similar extent to that of WT plants. In a similar manner to the $A_N$, $G_S$ values after recovery were 95% higher in UCP1-oe lines as compared to the WT plants. As a larger variation is expected when growing plants in the greenhouse, we performed a second trial of this experiment where we could confirm the effect of UCP1-oe upon the physiological parameters (Supplementary Figure 2).

Chlorophyll fluorescence was used to evaluate the impact of drought stress on $PS_{II}$. Excitation pressure ($1-q_P$) (Figure 2E), photosynthetic yield ($Y_{II}$) (Figure 2F), the maximum photochemical efficiency of $PS_{II}$ in the dark-adapted state ($F_v/F_m$) (Figure 2G) and nonphotochemical energy quenching (NPQ) (Figure 2H) were similar in WT and UCP1-oe throughout most of the experiment. There were only differences between WT and UCP1-oe after 15 days of IW, where $Y_{II}$ and $F_v/F_m$ increased in UCP1-oe lines while NPQ and $1-q_P$ decreased by 10% and 23%, respectively.

We next examined which of the physiological parameters evaluated present higher correlation with the observed increased in $A_N$ (Figure 3A-C). When comparing the correlation between $R_D$ (Figure 3A), $R_L$ (Figure 3B) and $G_S$ (Figure 3C) with $A_N$ it is clear
that $G_S$ is the one with the highest correlation values in both WT and transgenic lines. Interestingly both the intercept and slope of the WT $G_S \times A_N$ correlation differ from the transgenic lines (Supplementary Table 2). This fact is also true for $R_L \times A_N$ correlation but not for $R_D \times A_N$. In the opposite direction, both the correlations $R_D$ or $R_L \times G_S$ differ at intercept and slope between WT and transgenic lines (Figure 3D, E and Supplementary Table 2). As previously observed, there is a high correlation between $1-q_o$ and $R_L^{35}$ but its slope and intercept did not differ between WT and transgenic lines (Figure 3F and Supplementary Table 2).

Due to the high impact of UCP1-oe on photosynthesis, we next examined $A_N$ and $G_S$ as a response to varying $CO_2$ concentrations (Figure 4). The slopes of the linear portion of the curves were used to estimate the maximum velocity of Rubisco carboxylation ($V_{\text{cmax}}$) (Supplementary Table 3). There were no differences between transgenic lines and WT $V_{\text{cmax}}$ under control and recovery conditions, while transgenic lines showed higher $V_{\text{cmax}}$ under drought stress. In the opposite direction, $A_N$ was found increased in transgenic lines at RuBP-regeneration-limited condition under control (Figure 4A), drought (Figure 4B) and recovery (Figure 4C) conditions. Differences were more pronounced in stressed plants than in control or recovery plants, where $A_N$ in the WT represents 61% of that of the transgenic lines at the saturation point. As previously observed (Figure 2) the $G_S$ values for UCP1-oe plants were higher as compared with WT at the entire range of $CO_2$ concentration in all the conditions evaluated (Figure 4). In addition, $G_s$ was inhibited by $CO_2$ to the same extent as the UCP1-oe plants under control and recovery conditions (Figure 4A and C). On the other hand, $G_S$ was less inhibited by $CO_2$ in UCP1-oe during drought stress (Figure 4B).

The maintenance of physiological parameters during the drought might result in a less stressed cellular environment. As UCP1-oe plants have been shown to reduce $H_2O_2$ production$^{22-24}$ and as the levels of $H_2O_2$ increase in response to drought$^{36}$, measuring $H_2O_2$ content represents a good proxy to infer the degree of stress imposed during the period of water withholding. As expected, UCP1-oe lines accumulated 28% less $H_2O_2$ in leaves under control, drought and recovery conditions than did WT (Figure 5). Under drought stress, WT plants showed a 60% increase in $H_2O_2$ content compared to only a 32% increase in UCP1-oe lines. During the first day of recovery, the $H_2O_2$ content in the UCP1-oe lines decreased to a level that was only 7% higher than that in the well-watered control. After the same recovery time, the WT $H_2O_2$ content increased 26% compared to that of the control.

**Impact of UCP1-oe on Flower Development During Drought Stress**
During the reproductive stage, leaves act as source tissues to satisfy the high energy demand of flowers, especially during pollen development. Therefore, it would be expected that the positive impact of UCP1-oe on photosynthesis in the leaves would be reflected in the reproductive tissues of the plants. We first characterized how flower development progresses in tobacco from early formed flower buds (4 mm) to fully developed flowers (50 mm) (Figure 6A). Anthers were isolated from WT flower buds ranging from 4 to 14 mm under both control (Figure 6B) and drought stress conditions (Figure 6C). Expression profiling of the two key transcription factors (TFs) ABORTED MICROSPORES (AMS) and MALE STERILITY 1 (MS1) that act during tapetum maturation was evaluated in WT plants under both control and drought stress conditions. While AMS expression was inhibited approximately two-fold (Figure 6D), MS1 was induced and expressed at an earlier stage (Figure 6E) in drought-stressed plants than in their control counterparts. The faster progression of pollen development was confirmed by DAPI staining of pollen grains from WT and UCP1-oe P07 flower buds, ranging from 6 to 12 mm (Figure 6F). There were no differences in the progression of pollen development between WT and P07 plants, but it seemed that both were equally affected by drought stress. Pollen grains seemed mostly uninuclear in 10-mm flower buds in control plants, whereas most pollen grains were binuclear in plants under drought stress. Flower development was accelerated under drought stress – especially anther development, which was noticeable in anther size and stamen elongation (Figure 6B, C, and F). For these reasons, we decided to further analyze 10-mm flower buds from control plants and 8-mm flower buds from drought-stressed and recovery plants. This would allow us to equalize the stages analyzed in control, drought-stressed and recovered plants.

We first determined whether the increased mtDNA content and induction of mitochondrial gene expression that was previously shown in leaf tissues was present in flowers (Figure 6A-C). UCP1-oe lines showed a 1.6-fold increase in mtDNA content in flowers under control conditions compared to those of WT plants (Figure 7A). Under drought stress in WT plants, mtDNA content decreased to 56% of the control level, while in UCP1-oe lines it decreased to 68%. However, the mtDNA content of UCP1-oe buds was restored faster than that of their WT counterparts (76% compared to 62%) during the first 24 h of recovery. The expression of both nuclear-encoded mitochondrial NADH-dehydrogenase (NADH-D) (Figure 7B) and mitochondrial-encoded cytochrome oxidase II (COXII) (Figure 7C) was more than 2-fold higher in UCP1-oe under control conditions than
in WT. The expression of both genes remained elevated in UCP1-o.e lines in both drought stress and recovery conditions.

Due to their higher mitochondrial content, flower tissues are much more prone to oxidative stress than are leaf tissues. In this context, UCP1-o.e might benefit flower tissues by counteracting increased ROS production. The H$_2$O$_2$ content in UCP1-o.e flower buds corresponded to 72% of the level in those of WT (Figure 8). Under drought stress, H$_2$O$_2$ levels in WT plants increased by 82%, whereas in UCP1-o.e, the levels increased by only 65%. As previously observed in leaves, the H$_2$O$_2$ content during recovery was much closer to that of the control conditions in UCP1-o.e lines (123%) than in WT (155%).

**Modeling of Primary Metabolism and Physiological Response**

The adaptation to drought stress is often associated with the accumulation of compatible osmolytes. We were interested in whether UCP1-o.e lines would alter osmolyte profiles under both control and drought stress conditions. Nevertheless, alanine was found to be 30% higher in WT plants under drought stress when compared to the transgenic lines. In contrast to what was expected, choline, glycine, and ornithine were slightly but significantly decreased in UCP1-o.e lines under control conditions.

We also examined the possible impact on intermediates of flower energy metabolism (Figure 9). The photosynthetic assimilates sucrose and glucose were reduced in the UCP1-o.e lines compared to WT under both control and drought stress conditions. Pyruvate increased in UCP1-o.e lines only during recovery conditions. Intermediates of the Krebs cycle have distinct patterns of accumulation. Citrate was found to be reduced in UCP1-o.e under drought stress, whereas succinate differs to that of WT under control conditions. Fumarate accumulated to high levels in UCP1-o.e lines during recovery, and malate levels were unchanged in all conditions evaluated.

We asked how the flower primary metabolism is linked to the physiological responses observed in WT and UCP1-o.e lines exposed to drought stress (Figure 10). A principal component analysis (PCA) separate primary metabolism data into three distinct groups in accordance to the treatments (Figure 10A). When we combined primary metabolism, and physiological parameters, well-watered and recovery plants did not statistically differ from each other at 95% confidence level (Figure 10B). Also, it is important to notice that transgenic lines group together and differ from the WT across treatments. To obtain a global view of how significant alterations in primary metabolism is associated with the observed
physiological response during the drought stress, we analyzed changes in correlations in WT and transgenic lines across treatments (Figure 10C). Pearson correlation coefficient values were calculated (Supplementary Table 4) for the 16 metabolites (Figure 9) and 8 physiological parameters (Figure 2). In general, metabolites correlated positively among themselves. The same pattern was observed for physiological parameters. Exceptions were found for succinate, fumarate, 1-α-P and NPQ. Interestingly, sugars content and succinate, which are decreased in UCP1-oec flower buds under control conditions, correlate negatively with the physiological parameters. Although fumarate increases drastically in UCP1-oec during recovery it seems not correlate strongly with most of physiological parameters and metabolites.

Expression Profiling of Flower-Specific Transcription Factors and the TUNEL Assay

Pollen development demands high levels of energy, which is provided by the mitochondrial metabolism in the tapetum cells to provide nutrients for the increased rate of cell division to produce pollen grains. This process is accompanied by a 20-fold increase in mitochondrial numbers compared to that of vegetative tissues. By boosting mitochondrial biogenesis, the UCP1-oec lines sustain high metabolic activity during drought stress that is accompanied, due to increased uncoupled respiration, by decreased production of ROS. Therefore, we determined whether the expression of the transcription factors DYSFUNCTIONAL TAPETUM 1 (DYT1) and DEFECTIVE IN TAPETUM DEVELOPMENT AND FUNCTION 1 (TDF1), which are both important regulators of pollen development, were altered in UCP1-oec. These TFs are involved in early, intermediary and late tapetum development. Tapetum development is initiated by DYT1 and TDF1, which were both severely inhibited by drought stress (Figure 11A-B). While DYT1 (Figure 11A) expression was maintained at high levels in UCP1-oec lines under drought stress, no differences were observed for TDF1 (Figure 11B). Additionally, while DYT1 expression was 50% higher in UCP1-oec under control conditions, that of TDF1 was 30% lower. DYT1 interacts with three bHLH TFs (bHLH010, bHLH89, and bHLH91) to regulate the expression of its targets in Arabidopsis. The tobacco ortholog of bHLH89 was inhibited under drought stress in WT plants compared with that in UCP1-oec lines (Figure 11C). Interestingly, the expression of bHLH89 under control conditions was also 30% lower in UCP1-oec lines than in WT plants. These early regulators coordinate the expression of AMS and MSI, which are involved in the maturation of the tapetum cells, pollen wall
formation, and tapetum programmed cell death (PCD). During drought stress, AMS expression was 50% lower in WT but was practically unaltered in UCP1-oe lines (Figure 11D). The MS1 expression pattern changed in the opposite direction; MS1 expression after drought stress was 37-fold higher in WT and more than 70-fold higher in UCP1-oe lines (Figure 11E). During recovery, MS1 expression was maintained at high levels in the UCP1-oe lines compared to the levels of WT. After tapetum PCD, there is a final stage of dehiscence during which the TFs that regulate the endothecium and secondary thickening are expressed. MYB26, which is expressed at this later stage, was also similarly inhibited under drought stress between WT and UCP1-oe lines (Figure 11F). Interestingly, MYB26 expression was positively affected in the UCP1-oe lines in control conditions.

To verify if the impact of UCP1-oe on the expression of pollen-specific TFs would result in tapetum PCD, we performed a TUNEL assay on the anthers of WT and UCP1-oe line P07 under control and drought stress conditions (Figure 11G). There was no staining of the WT tapetum and P07 anthers under control conditions, but after 15 days of IW, tapetum PCD was observed via the consistent staining of the entire tapetum layer that surrounds the anther (Figure 11G).

UCP1-oe Increases Yield and Seed Size of Tobacco Plants Under Drought Stress

The impact of UCP1-oe on seed yield under drought stress was evaluated (Figure 12). WT plants that were subjected to drought stress presented 25% sterile siliques, whereas none of the UCP1-oe siliques were sterile (Figure 12A). The mass of seeds per silique increased in the control and drought-stressed UCP1-oe lines compared to that of WT (Figure 12B).

To determine if the increased yield resulted from the increase in seed number or mass, the individual seed area for WT and UCP1-oe lines was analyzed (Figure 12C, D). Images of seeds were taken, and the image backgrounds were filtered to compute the area of individual seeds (Figure 12C). The UCP1-oe lines presented 5% increase in seed area as compared to the WT under control conditions (Figure 12D). Under drought stress, both WT and transgenic lines showed increased seed size compared to that in control conditions; either way, UCP1-oe still presented 5% higher seed area than did WT (Figure 12B). Despite the increase in seed area, most of the difference in yield is due to an increase in seed number (40% increase in silique mass) rather than an increase in seed size (5% increase in seed area).
Discussion

Although the mechanistic effects of UCP1-oie are not yet clearly understood\textsuperscript{26}, it has been observed that UCP1-oie confers tolerance to multiple stresses\textsuperscript{21-25}. Nevertheless, all these studies thus far have been conducted on plants during the vegetative phase of development. Here, we assessed the performance of UCP1-oie lines under drought stress during flower development as well as seed yield under water deficit conditions. The data obtained support the notion that UCP1-oie during the reproductive phase of development allows sustained cellular metabolism and counteracts $H_2O_2$ production in leaves and flowers under moderate drought stress. UCP1-oie produces a less toxic cellular environment in anthers, which contributes to maintaining proper pollen development and seed production.

UCP1-oie lines were previously evaluated under an artificial drought stress simulated by watering plants with a hyperosmotic mannitol solution\textsuperscript{23}. The use of this setup offers experimental advantages, such as the control of both the environment and the stress level as well as low variability, although it is inherently imperfect at mimicking the drought stress conditions plants are exposed to in the field\textsuperscript{41-44}. In this work, we grew plants in an environment that was more similar to field conditions to evaluate the reproductive phase of development, especially seed production. We could observe for the first time that our transgenic lines present high UCP1 expression and protein content in flower buds (Figure 1G-H). Also, UCP1 protein accumulates in WT under drought stress, which reinforces the importance of UCP1 for stress tolerance.

The transport of photoassimilates during plant development depends on source supply and sink demand\textsuperscript{3,45}. Thus, the inhibition of photosynthesis by drought stress is extremely harmful, as the leaf supply of energy for flower and seed development is disturbed. We observed that UCP1-oie does not reduce water losses compared to WT under drought stress, which suggests that the beneficial effect on yield is not a direct result of plant moisture content. The drought stress imposed by withholding water in this work was considered moderate based on the RWC reduction to 65\%\textsuperscript{16,19} and the fact that $V_{cmax}$ is similar between control and recovered plants (Supplementary Table 3). At first, we intended to impose severe drought stress to evaluate the UCP1 effect when the RWC drops below 60\%, but we observed that lower RWCs resulted in many aborted flower buds, which would interfere with our analysis. Although the RWC did not change between WT and transgenic plants, UCP1-oie lines recovered faster than the WT plants after rewatering and presented a decreased loss of biomass. Drought stress is often associated with a reduction in the biomass
of aboveground organs in monocots\textsuperscript{46,47} and dicots\textsuperscript{23}. In this scenario, the beneficial effects of UCP1-oep could be attributed to the preservation of physiological parameters and decreased H$_2$O$_2$ production. We previously hypothesized that the increase in A$_N$ might be a consequence of stomatal function\textsuperscript{26} and, in fact, there is a high correlation between G$_S$ and A$_N$ in both WT and UCP1-oep lines (Figure 3C). Nevertheless, the intercepts of the transgenic A$_N$ x G$_S$ curves were increased (Figure 3A and Supplementary Table 2). In theory, this means that when there is no gas exchange (G$_S$ = 0) there is an increase in photosynthesis in UCP1-oep plants, and consequently, G$_S$ is not the only responsible for the increased A$_N$\textsuperscript{49}. In the same direction, G$_S$ is inhibited by CO$_2$ in a similar manner in WT and UCP1-oep under control and recovery conditions. We hypothesize that the increased mitochondrial respiration, by stimulating TCA cycle, provides CO$_2$ for the chloroplast and increases photosynthesis independently of G$_S$. Nevertheless, the maintenance of A$_N$ in the UCP1-oep plants under drought could be at least partially attributed to the maintenance of G$_S$ since it is less sensitive to the environment under drought stress (Figure 4B). We could also observe from the data obtained from the CO$_2$ response curves that V$_{\text{cmax}}$ did not differ between genotypes under control conditions, but there is a difference in A$_N$ under high CO$_2$ concentration where carboxylation is limited by Ribulose 1,5-bisphosphate (RuBP) regeneration\textsuperscript{50} (Figure 4 and Supplementary Table 3). Under this condition A$_N$ is limited by the availability of inorganic phosphate released during the synthesis of sucrose and starch\textsuperscript{49-51}. We have previously shown that UCP1-oep lines accumulate sucrose and starch in the leaves under control conditions\textsuperscript{26}, therefore it could be hypothesized that there is a decreased impact on A$_N$ under RuBP-limiting conditions in these plants.

It has been shown that G$_S$, G$_M$, and RuBP regeneration have a direct impact on photosynthesis\textsuperscript{49}. Nevertheless, the only direct impact of UCP1 overexpression is on respiration\textsuperscript{17}. It is then counterintuitive that a process that contributes negatively to A$_N$ would, in a global picture, result in an increased A$_N$. It is important to point out that high UCP1 activity should result in high uncoupled respiration (high oxygen consumption) but low ATP production. This means that it is not only respiration that is increased but the pattern of respiration is modified (coupled to uncoupled). We observe that a hypoxic response is triggered in these conditions, which leads to an accumulation of metabolites in the leaves that are markers of hypoxic adaptation such as glucose, sucrose, starch, fumarate, and succinate\textsuperscript{26}. These metabolites are also markers of photosynthetic activity\textsuperscript{49}, suggesting a strong interplay between hypoxia, carbon fixation and mitochondrial metabolism. It is known that down regulation of succinate dehydrogenase in tomato and Arabidopsis results
in the accumulation of succinate and enhancement of photosynthesis\textsuperscript{49,52,53}. The increased synthesis of succinate in Arabidopsis is also strongly correlated with $A_N$ and $G_S$\textsuperscript{54}. In this context, the accumulation of succinate in the UCP1-oe leaves due to increased mitochondrial respiration and TCA activity play a role in the signaling for increased photosynthetic capacity. Nevertheless, sucrose, glucose, and succinate decreased in flower buds, suggesting an inverse correlation between the amounts of these metabolites in leaves and flower buds.

Also, it was found a strong correlation between the maintenance of respiration during drought, and enhanced photosynthetic performance in plants overexpressing AOX has been observed\textsuperscript{16,19}. The inhibition of both UCPs and AOX has a direct effect on photosynthetic performance. The inhibition of AOX decreases photosynthesis in wheat plants suffering from drought stress\textsuperscript{55,56}, and mutants lacking Arabidopsis UCP1 showed reduced photosynthetic performance under control conditions\textsuperscript{12}. Drought stress induces both UCP1 and AOXs in Durum wheat\textsuperscript{20}; therefore, the beneficial effects of UCP1-oe when plants are subjected to drought might be similar to those observed for AOX-oe in tobacco\textsuperscript{16,19}. Based on our observation that UCP1 protein content increases under drought stress in WT plants, we can infer that UCPs might have a role in counteracting drought in tobacco flower buds. These observations suggest there could be a connection between UCP1 and AOX function in plants.

The expressions of these proteins are at least partially correlated when either UCP1 is knocked out\textsuperscript{12} or UCP1 is overexpressed\textsuperscript{25}. It seems that both proteins have the same function since they ultimately reduce ATP yield\textsuperscript{57}. We believe it is more appropriate to say that both the UCP1 and AOX proteins lower the amount of $O_2$ and substrate consumed per molecule of ATP generated (i.e., respiration efficiency). A more in-depth look at how both proteins influence this reduction in efficiency reveals how different they are and how they could complement each other. Increased AOX activity will decrease both the ratio of NADH consumption and generation of the proton gradient, indicating that AOX would have to consume more substrate to produce the same amount of proton gradient because AOX bypasses proton-pumping complexes III and IV\textsuperscript{56}. The fundamental fact is that AOX does not directly influence the dissipation of the proton gradient – only its production. On the other hand, an increase in UCP activity will consume the proton gradient without directly affecting the efficiency in the consumption of reducing power\textsuperscript{17}. Thus, only together can these proteins result in less efficient respiration both in the supply (substrate consumption per proton pumped) and the demand (proton dissipation per ATP yield) parts of
mitochondrial respiration. In a situation where there is excess reducing power because photosystems continue generating NADPH and mitochondrial respiration would be adenylate restricted, such as that which can occur during drought stress, the activity of both proteins would complement each other.

The other side of the plant energy balance relies on sink demand for energy, which is known as sink strength or sink dominance\(^3\). The basis of sink strength is the ability to lower the concentration of photoassimilates in the sinks to establish a favorable hydrostatic pressure gradient between the source and the sink\(^7\). We observed in this study a decreased concentration of sugars and increased expression of mitochondrial genes in flower buds of UCP1-o lines compared to WT plants. In contrast, we previously reported increased carbohydrate contents in transgenic leaves\(^26\). Drought stress is often associated with increased concentrations of sugars in flower pods and further reductions in yield\(^58-60\), which suggest that the utilization of flower resources rather than sugar concentration is the limiting factor for yield\(^60\). These data in conjunction with a higher respiration rate of isolated mitochondria\(^25\) may result in an increased sink strength of UCP1-o flowers both under control and drought stress conditions, which would be sustained by enhanced photosynthesis and increased carbohydrate content in the leaves. Our data suggest that while in source tissues there is a positive correlation of carbohydrate content and \(A_N\), in sink tissues this correlation is negative (Figure 10C). Apparently, this is not true for succinate, which is positively correlated with \(A_N\) in both leaves\(^26,46\) and flower buds (Figure 10C). The capacity of source tissues to provide energy for a strengthened sink resulted in less accumulation of \(H_2O_2\) in UCP1-o lines. We also observed that compatible osmolytes that are broadly associated with increased drought tolerance, such as proline, did not differentially accumulate between WT and UCP1-o plants.

The impact of drought stress on tapetum development, which is a high energy-demanding process in plants for sustaining pollen formation, was analyzed by profiling the expression of DYT1, TDF1, bHLH89, AMS, MS1, and MYB26. We found that DYT1, bHLH89, AMS, and MS1 were less inhibited under drought stress in UCP1-o lines than in WT plants. Some of these TFs were affected under control conditions, which indicates that UCP1-o impacts tapetum metabolism. There were no apparent differences in tapetum PCD between UCP1-o and WT plants when drought stress conditions were compared to control conditions. Thus, it is not clear how UCP1-o affects the expression of these TFs and what the consequence of the downstream processes are during tapetum development.
The positive effect of UCP1-oe on leaf photosynthesis and flower bud metabolism resulted in a higher yield compared to that of WT under both control and drought stress conditions. Drought stress affects reproductive tissue development by affecting pollen grain viability, ovary development and turgor maintenance of floral organs. This study focused on the early stages of anther development because of the importance of mitochondria in this process. Since pollen development depends on a high energy supply, disturbances in the function of mitochondria might result in dramatic effects on male fertility. Mitochondria play a fundamental role in triggering the death of male reproductive organs, not only by affecting the levels of ATP but also by their involvement in increased ROS production. In this context, we believe that the positive effects observed on yield are because UCP1-oe can maintain mitochondrial respiration and photosynthesis in source leaves independent of the consequences of water limitation compared with WT plants; UCP1-oe, therefore, does not limit the ATP requirement of reproductive tissues, avoiding ROS production.

Nevertheless, from the observations made in this study, it is important to note that the effect of UCP1 was not drought specific because it also improved plants in well-watered conditions. At this point, it’s hard to establish the basis of the resistance of UCP1-oe plants to biotic and abiotic stresses. UCP1 activity per se is beneficial to mitochondrial metabolism itself as well as to the whole cell because it reduces ROS production by stimulating respiration when needed. We believe that this fact alone contributes to the phenotype, but we are not able to conclude its extent, mainly because there are some side effects of constitutive UCP1-oe. The first is the fact that mitochondrial biogenesis is induced in transgenic lines. The expression of multiple mitochondrial components such as AOX, SOD, catalases and Krebs cycle enzymes amplifies the UCP1 effect by improving the whole mitochondrial antioxidant activity, steps of the Krebs cycle and oxidative phosphorylation. We also cannot determine the share of the mitochondrial biogenesis effect on the UCP1 phenotype. Also, there is an interesting secondary side effect of a hypoxic response that is triggered in these plants. We found that when UCP1 is overexpressed, it triggers a hypoxic response that induces components of the N-end rule pathway of the hypoxic response, leading to accumulation of specific metabolites that are markers of the hypoxic response. Beneficial effects of the activation of the N-end rule pathway upon stress tolerance constitute a widespread and a trending topic in plant sciences; therefore, we also cannot conclude how the N-end rule pathway contributes to the UCP1 phenotype. The evaluation of how each of these effects contributes to stress tolerance and of the interplay between UCP1 and AOX would have a significant impact on the understanding of
how plants manage metabolic imbalances and might be a valuable tool to improve crops for increased yield under stresses.

Conflict of Interest

The authors declare no conflict of interest.

Authors Contributions

PB, JTCY, ZAW and PA designed the experiments. PB and JTCY performed experiments. PB, JTCY, ZAW and PA analyzed the data. PB and PA wrote the paper.

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Coordinated regulation of photosynthetic and respiratory components is necessary to maintain chloroplast energy balance in varied growth conditions. *J. Exp. Bot.* 68, 657-671.


**Supplementary Material**

**Supplementary Figure 1.** Experimental design for assessment of the impact of drought stress in UCP1-oe tobacco plants. (A) WT and UCP1-oe lines (P07, P30, and P49) were grown in 3 different groups (control, drought and recovery) containing 20 plants each (5 per genotype). (B) Plants were grown for 12 weeks until they reached the reproductive phase of development. Samples were collected from the control group (C) before we stopped irrigation (T = 0). Drought (D) samples were collected after 15 days of withholding water (T = 15), after which irrigation was resumed. Plants were allowed to recover under normal irrigation for 24 h, after which the recovery (R) samples were collected (T = 16). Plants from all groups were maintained until harvest.

**Supplementary Figure 2.** Effects of drought stress on physiological and chlorophyll fluorescence parameters of WT and *AtUCP1*-overexpressing transgenic plants on the second trial of the experiment. The measurements were taken at growth light intensity (400 µmol m⁻² s⁻¹) using the first expanded leaf during the whole course of the experiment. For this experiment, plants were allowed to recover from drought stress for 3 days. (A) Respiration in the dark: (R_D), (B) respiration in the light (R_L), (C) net photosynthetic rate (A_N) and (D) stomatal conductance (G_S).

**Supplementary Table 1.** Primers designed for mRNA and mtDNA quantification.

**Supplementary Table 2.** Slopes and intercepts of the physiological parameters correlations.

**Supplementary Table 3.** The impact of drought stress on the maximum velocity of Rubisco carboxylation (*V_{cmax}* ) in WT and transgenic lines.
**Supplementary Table 4.** Pearson correlation coefficients of primary metabolites and physiological parameters used in Figure 10C

**Figure Legends**

**Figure 1.** UCP1-oe lines recover faster from drought stress than do WT upon rewatering. Plants were grown until (A) the reproductive phase of development, after which irrigation was stopped. Plants started to show symptoms of drought stress after (B) 9 days without irrigation, and they were maintained without irrigation for (C) 15 days after the start of the drought treatment. (D) Transgenic plants recovered faster after 1 day of recovery. (E) Relative water content (RWC) was monitored on the first expanded leaf during the whole course of the experiment. (F) The absolute dry mass of the first fully expanded leaf (20 leaf discs) was compared between control and drought-stressed plants. (G) The heterologous UCP1 was overexpressed in flower buds of all three transgenic lines (P07, P30 and P49) as previously described for the leaves. (H) Immunoblot analysis of the flower buds of WT and transgenic lines P07, P30 and P49 under control and drought conditions was performed using an anti-UCP1 antibody. The gel loading control was estimated using an anti-β-actin antibody. Bars and points represent the means ± SD. Bars with *** differ significantly from the WT (P < 0.01). Points with ** mean that all transgenic lines differ from the WT (P < 0.05). D: Drought, R: Recovery.

**Figure 2.** Effects of drought stress on physiological and chlorophyll fluorescence parameters of WT and AtUCP1-overexpressing transgenic plants. The measurements were taken at growth light intensity (400 µmol m⁻² s⁻¹) using the first expanded leaf during the whole course of the experiment. For this experiment, plants were allowed to recover from drought stress for 3 days. (A) Respiration in the dark: (RD), (B) respiration in the light (RL), (C) net photosynthetic rate (AN), (D) stomatal conductance (GS), (E) excitation pressure (1-qP), (F) effective PSII quantum yield during illumination (YII), (G) maximal quantum yield of PSII (Fv/Fm) and (H) nonphotochemical energy quenching (NPQ). Points represent the means of 5 plants ± SD. Points with *, ** and *** mean that all three transgenic lines differ significantly from the WT (P < 0.1, P < 0.05 and P < 0.01, respectively). D: Drought; R: Recovery.

**Figure 3.** Correlation of physiological parameters in WT and transgenic lines. The relationships of (A) RD, (B) RL and (C) GS with AN was determined for WT (dashed line)
and transgenic lines (plain lines). (D, E) \( G_S \) as a function of \( R_D \) and \( R_L \), respectively. (F) 1-qP as a function of \( R_L \). The curves were calculated for each line individually. Control, drought and recovery data were pooled together for the linear regression analysis.

Figure 4. The impact of water limitation on net carbon assimilation and stomatal conductance response to \( \text{CO}_2 \) in WT (dashed line) and transgenic plants (plain lines). The measurements were taken using the first expanded leaf during the whole course of the experiment in (A) control, (B) drought-stressed and (C) recovery plants at growth light intensity. Measurements of the drought-stressed plants were taken at the end of the stress period, whereas recovery measurements were taken after 3 days of recovery under normal irrigation. Points represent the means of 5 plants ± SD. Points with *, ** and *** mean that all three transgenic lines differ significantly from the WT (\( P < 0.1 \), \( P < 0.05 \) and \( P < 0.01 \), respectively).

Figure 5. Quantification of \( \text{H}_2\text{O}_2 \) in leaves of WT and \( \text{AtUCP1} \) transgenic tobacco plants. The first expanded leaf was collected from WT and transgenic plants under control (D = 0), drought (D = 15) and recovery (D = 16) conditions. Bars represent the means of 5 plants ± SD. Bars with *, ** and *** differ significantly from the WT (\( P < 0.1 \), \( P < 0.05 \) and \( P < 0.01 \), respectively).

Figure 6. Flower and anther development in tobacco plants. (A) Flower development was assessed in tobacco plants based on bud size. Focusing on the early stages of pollen development, anthers was isolated from 4-14mm buds from (B) control and (C) drought-stressed WT plants. The expression of (D) \( \text{AMS} \) and (E) \( \text{MSI} \) was analyzed using qRT-PCR in control and drought-stressed WT plants to identify the key initial stages in pollen development. (F) Pollen grains of WT and transgenic line \( \text{P07} \) were stained with DAPI to determine correlations between bud size and pollen stage in control and drought-stressed plants. Scale bars = 10 µm.

Figure 7. Overexpression of \( \text{AtUCP1} \) results in increased expression of mitochondrial transcripts in flower buds. (A) The mtDNA content is higher in transgenic lines in control, drought stress and recovery conditions. The expressions of (B) nuclear-encoded \( \text{NADH-D} \) and (C) mtDNA-encoded \( \text{COXII} \) were also higher in transgenic plants in all conditions analyzed. Bars represent the means of 5 plants ± SD. Bars with *, ** and *** differ significantly from the WT (\( P < 0.1 \), \( P < 0.05 \) and \( P < 0.01 \), respectively).
**Figure 8.** Quantification of H$_2$O$_2$ in flower buds of WT and *AtUCP1* transgenic tobacco plants. Flower buds (8-12 mm) were collected from WT and transgenic plants under control (D = 0), drought stress (D = 15) and recovery (D = 16) conditions. Bars represent the means of 5 plants ± SD. Bars with *, ** and *** differ significantly from the WT (P < 0.1, P < 0.05 and P < 0.01, respectively).

**Figure 9.** Heatmap showing the distribution of metabolites that were significantly affected by drought stress. Metabolites were isolated from WT and transgenic plants (P07, P30 and P49) under well-watered (C), drought (D) and recovery (R) conditions for metabolomics using H¹-NMR. The average fold-change is displayed inside the enclosed cells as relative to those of WT under control conditions. Cells represent the means of 5 plants ± SD. Points with *, ** and *** mean that the transgenic line differ significantly from the WT (P < 0.1, P < 0.05 and P < 0.01, respectively).

**Figure 10.** Data integration of metabolomics combined with physiological parameters of WT and transgenic lines. Principle component analysis (PCA) scores plot representation of (A) metabolomics or (B) metabolomics combined with physiological parameters from WT and transgenic lines (P07, P30 and P49). Dashed lines represent 95% confidence intervals for well-watered (C), drought (D) and recovery (R) conditions. (C) Pearson correlation analysis of metabolomics combined with physiological parameters.

**Figure 11.** Overexpression of *AtUCP1* impacts the expression of anther-specific transcription factors but does not affect tapetum PCD during drought stress. We evaluated the expression of transcription factors that regulate the beginning of tapetum development, tapetal maturation and anther maturation. The values of (A) DYT1, (B) TDF1, (C) bHLH89, (D) AMS, (E) MSI and (F) MYB26 are displayed as the amounts of relative expression compared to those of WT under control conditions. (G) A TUNEL assay was performed in WT and P07 lines under both control and drought stress conditions. A TUNEL signal can be seen in the tapetum cells; autofluorescence/TUNEL signal was observed in the pollen grains. V: vascular bundle; PG: pollen grains; T: tapetum. Bars represent the means of 5 plants ± SD. Points with * and ** differ significantly from the WT (P < 0.1 and P < 0.05, respectively). Scale bars = 150 µm.

**Figure 12.** Estimation of the yield of WT and transgenic lines under drought stress. Flower buds (8-12 mm) from control and drought-stressed plants (WT and transgenic) were tagged and maintained under normal irrigation until the end of plant life cycle. (A) Transgenic
plants did not produce any sterile pods after being subjected to a 15-day drought stress, whereas 25% of WT siliques were sterile. (B) WT plants produced fewer seeds under control conditions and were more affected by drought stress compared to transgenic lines. (C) Seeds were collected from the pods as described in the Materials and Methods, and a field image containing approximately 100 seeds was collected for each pod. We removed the background and adjusted the contrast to measure the individual seed size using ImageJ. (D) Transgenic plants presented larger seeds under control conditions than did WT plants. The seed size increased equally by approximately 5% in all genotypes after drought treatment. Bars represent the means of 25 pods and 2,500 seeds ± SD for seed mass and area, respectively. Bars with ** and *** differ significantly from the WT (P < 0.05 and P < 0.01, respectively).