Role of photosynthesis and analysis of key enzymes involved in primary metabolism throughout the lifespan of the tobacco flower

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Abstract

Although the physiological and economical relevance of flowers is recognized, their primary metabolism during development has not been characterized, especially combining protein, transcript, and activity levels of the different enzymes involved. In this work, the functional characterization of the photosynthetic apparatus, pigment profiles, and the main primary metabolic pathways were analysed in tobacco sepals and petals at different developmental stages. The results indicate that the corolla photosynthetic apparatus is functional and capable of fixing CO₂ with its photosynthetic activity mainly involved in pigment biosynthesis. The particular pattern of expression, across the tobacco flower lifespan, of several proteins involved in respiration and primary metabolism, indicate that petal carbon metabolism is highest at the anthesis stage; while some enzymes are activated at the later stages, along with senescence. The first signs of corolla senescence in attached flowers are observed after anthesis; however, molecular data suggest that senescence is already onset at this stage. Feeding experiments to detached flowers at anthesis indicate that sugars, but not photosynthetic activity of the corolla, are capable of delaying the senescence process. On the other hand, photosynthetic activity and CO₂ fixation is active in sepals, where high expression levels of particular enzymes were detected. Sepals remained green and did not show signs of senescence in all the flower developmental stages analysed. Overall, the data presented contribute to an understanding of the metabolic processes operating during tobacco flower development, and identify key enzymes involved in the different stages.

Key words: Flower, metabolism, photosynthesis, tobacco.

Introduction

The petal, which is one of the two non-reproductive organs of the flower, serves to protect the floral reproductive organs and to attract pollinators ensuring pollination and fertilization. The maintenance of petals is costly in terms of respiratory energy, nutrients, and water loss (Stead et al., 2006). Carbohydrates are usually produced by photosynthesis in the green source tissues and transported to the flowers buds, and the distribution among the various floral organs depends on the phase of developmental stage (Clément et al., 1996). On the other hand, some reports indicate that photosynthesis in floral organs is important in reducing the cost of reproduction (Aschan and Pfanz, 2003); however, the flowers per se have been largely ignored in photosynthesis research. There are only a few cases in which the photosynthesis of corollas has been reported, and even in those, studies were conducted

Abbreviations: DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; AOX, alternative oxidase; F₀, basal fluorescence; COX, cytochrome c oxidase; ef, elongation factor; ETC, electron transport chain; FBPase, fructose-1,6-bisphosphatase; G6P-DH, glucose-6-P dehydrogenase; il, isocitrate lyase; MDH, malate dehydrogenase; Fₘ, maximum fluorescence; MS, Murashige–Skoog medium; NADP-ME, NADP-malic enzyme; QNP, non-photochemical quenching; PEPC, phosphoenolpyruvate carboxylase; PRK, phosphoribulokinase; qₚ, photochemical quenching; PSI1, Photosystem I; PCD, programmed cell death; PK, pyruvate kinase; PPDK, pyruvate orthophosphate dikinase; PPFD, photosynthetic photon flux density; QRT-PCR, quantitative real-time RT-PCR; RuBisCO LSU, ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit; SBPase, sedoheptulose-1,7-bisphosphatase; VPE, vacuolar processing enzyme.

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on flowers which have green corollas, or on young flower buds (Weiss and Halevy, 1991).

After pollination, petals undergo senescence, in which the term ‘senescence’ is considered to be a synonym of developmental programmed cell death (PCD; van Doorn and Woltering, 2008). Senescence is a highly regulated developmental process that is tightly controlled by multiple genes. At the cellular level, PCD is characterized by an increase in the activity of a variety of degradative enzymes, a decrease in lipid, protein, and nucleic acid levels, rupture of the tonoplast, and degradation of the cell walls (Yamada et al., 2006). In the corolla, many senescence-related genes that regulate PCD have been identified using differential screening and microarray analysis (Panavas et al., 1999; Eason et al., 2002; Hunter et al., 2002; van Doorn et al., 2003; Breeze et al., 2004; Yamada et al., 2007). However, it is unclear whether the decrease in carbon levels during both petal and leaf senescence, is the result of C recycling or tissue respiration (Himelblau and Amasino, 2001; Verlinden, 2003).

The visual and textural quality of flower petals is of commercial significance, with international trade in cut flowers worth more than 4–4.5 billion dollars annually (O’Donoghue et al., 2009). Thus, it is surprising that the primary metabolism of flowers has not been analysed in depth. Only anthocyanin biosynthesis has been intensely studied as an integral part of the development of many flowers and its accumulation is accompanied by petal growth. In addition, the role of light in flavonoid synthesis has largely been explored with respect to the regulation of flavonoid gene expression (i.e. light is required for chalcone synthase gene expression; Moscovicici et al., 1996; Schuurink et al., 2006). However, a complete understanding of flower primary metabolism is lacking. Multilevel analysis of primary flower metabolism is necessary in order to identify key enzymes involved in each developmental stage; which may help in the identification of cultivars with improved flower quality or better post-harvest performance. Moreover, there is virtually no information on sepal metabolism. In this work, a multilevel approach was adopted to analyse transcript profiles, enzyme content or activity of the main primary metabolic pathways and pigment profiles of tobacco flowers at different developmental stages. The objectives of this study were (i) to assess the gas exchange and the photosynthetic electron transport capacity of sepals and petals throughout the developmental stages of the flower, (ii) to investigate the relevance of photosynthetic properties to flower metabolism, and (iii) to obtain an insight into the metabolic changes occurring during flower development.

**Materials and methods**

**Plant growth conditions and treatments**

Plants of *Nicotiana tabacum* (L. cv. Petit Havana SR1) were grown from seeds in a compost:sand:perlite mixture (2:1:1 by vol.). Seedlings were transferred to a greenhouse with a 30/18 °C 16/8 h day/night period and 200 μmol m⁻² s⁻¹ photosynthetic photon flux density (PPDF). Whole corollas were collected at different developmental stages (1, 3, 6, 7, and 9; Fig. 1A, B) as described by Serafini-Fracassini et al. (2002) by observing corolla size, shape, and colour. These stages are divided from closed corolla (stage 1) through anthesis (stage 6) to death (stage 9) and are morphologically and physiologically distinguished (Serafini-Fracassini et al., 2002).

For incubation experiments, flower at anthesis were excised from the plant at the base of the pedicel. Cut flowers were placed in Murashige–Skoog medium (MS) as the control or in MS solutions containing 200 mM sucrose or fructose or glucose or mannitol or 60 μM 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) or with 200 mM sucrose plus 60 μM DCMU. Samples were taken at 24, 48, and 72 h after immersion. Cut flowers were kept under the same light conditions specified above.

Entire sepals and petals, and young leaves from unflowered plants, were immediately frozen in liquid N₂ and stored at –80 °C for further analysis, with the exception of Evans Blue staining, gas exchange, and chlorophyll fluorescence experiments for which fresh tissue was used.

**Evans Blue staining**

Cell integrity was determined by the Evans Blue Staining method described by Delledonne et al. (2001) with some modifications. Tissues were incubated during 2 h with 0.1% (w/v) Evans Blue solution and washed with distilled water. After washing with abundant water, chlorophylls were removed by washing with 96% (v/v) ethanol. Dead cells were visualized by the blue staining. Quantitative measurements were carried out by solubilization of the fixed colorant in 50% (v/v) ethanol, 1% (w/v) SDS solution at 60 °C for 30 min. Solutions were spectrophotometrically measured at 600 nm.

**Protein extraction**

Total soluble protein from the different tobacco tissues was extracted using a buffer containing 100 mM TRIS-HCl, pH 7.8, 1 mM EDTA, 10 mM MgCl₂, 15 mM β-mercaptoethanol, 20% (v/v) glycerol, and 1 mM phenylmethylsulphonylfluoride, in the presence of 33 μl of protease inhibitor cocktail (Sigma, St Louis, MO, USA) mg⁻¹ of fresh tissue. The supernatant of crude extracts was desalted in a cold Sephadex G-25 column pre-equilibrated with the above buffer according to Penefsky (1977). This extract was used for activity measurements or, alternatively, diluted in 0.25 M TRIS-HCl, pH 7.5, 2% (w/v) SDS, 0.5% (v/v) β-mercaptoethanol, and 0.1% (v/v) bromophenol blue and boiled for 2 min for SDS-PAGE. In the case of D1 protein analysis, protein thylakoids were extracted as in Tambussi et al. (2004).

**Protein concentration**

Protein concentration was determined by the method of Bradford (1976) using the Bio-Rad protein assay reagent (Bio-Rad, Hercules, CA, USA) and bovine serum albumin as standard.

**Enzyme assay**

The activity of enzymes was measured spectrophotometrically in a final volume of 1 ml at 30 °C and 340 nm using a UNICAM Helios β spectrophotometer (UNICAM instruments, Cambridge, England). The reaction mixtures used for each enzyme were as follows.

- **Glucose-6-P dehydrogenase (G6P-DH):** 50 mM TRIS-HCl, pH 8.0, 10 mM MgCl₂, 0.15 mM NADP, and 3 mM Glc-6-P (Esposito et al., 2005).

- **NADP-malic enzyme (NADP-ME):** 50 mM TRIS-HCl, pH 7.3, 0.5 mM NADP, 10 mM l-malate, and 10 mM MgCl₂. The reaction was started with malate (Lara et al., 2003).
**Phosphoenolpyruvate carboxylase (PEPC)**: 100 mM TRIS-HCl, pH 8.0, 20% (v/v) glycerol, 10 mM MgCl₂, 10 mM NaHCO₃, 4 mM PEP, 0.15 mM NADH, and 10 U of malate dehydrogenase (MDH; Lara et al., 2003).

**SDS-PAGE and Western blot analysis**

SDS-PAGE was carried out using 10% or 15% (w/v) polyacrylamide gels according to Laemmli (1970). Proteins were visualized with Coomassie blue or electroblotted onto a nitrocellulose membrane for immunoblotting according to Burnette (1981). Bound antibodies were located by linking to alkaline phosphatase-conjugated goat anti-rabbit IgG according to the manufacturer’s instructions (Bio-Rad).

The antibodies used for detection were the following: 1:200 anti-*Amaranthus viridis* PEPC (Colombo et al., 1998); 1:10 000 against spinach RuBisCO large subunit (RuBisCO LSU, provided by Dr A Viale; National University of Rosario, Argentina); 1:2000...
against D1 protein of Solanum nigrum (Guiaumet et al., 1991); 1:1000 anti-Zea mays L. pyruvate orthophosphate dikinase (PPDK, Chastain et al., 2000); 1:100 against Brassica napus fructose-1,6-bisphosphatase (FBPase, Moorhead et al., 1994); 1:2000 against pyruvate kinase (PK) from Brassica napus (Plaxton et al., 1989).

The kinetic masses of the polypeptides were estimated from a plot of the log of molecular mass of marker standards versus migration distance. Densitometric analysis of the bands detected in at least three independent blots was performed for each enzyme.

**Chlorophyll quantitation**

Total chlorophyll was extracted in 80% (v/v) acetone. After clarification, chlorophyll a and b contents were determined as described by Wintermans and Mots (1965).

**Pigment extraction and analysis**

**Anthocyanins**: Anthocyanins were extracted in methanol containing 1% (v/v) HCl for 24 h in the dark and estimated by measuring the absorbance at 330 nm (Weiss et al., 1988).

**Flavonoids**: Frozen petals (250 mg) were ground in liquid nitrogen and then extracted with 1 ml methanol. For the hydrolysis of flavone glucosides, 4.5 ml 10% (v/v) HCl was added before incubation at 100 °C for 90 min. The hydrolysis solution was extracted twice with ethyl acetate. The ethyl acetate fraction was evaporated and redissolved in 500 ml methanol. This solution was filtered through a 0.22 μm filter (Millipore) and analysed by high performance liquid chromatography (HPLC; Äkta, GE). The HPLC was carried out with a reverse phase column Luna 5 μm, 5 μm, which was in the region joining the tube and the limb in corollas, as performed in petunia (Weiss et al., 1988). Measurements were performed using a Chlorophyll Fluorescence Spectrum (HPLC) analysis were performed as described previously Nishihara et al. (2005).

**Chlorophyll fluorescence**

Measurements were performed using a Chlorophyll Fluorescence Package containing a Fluorometer F1, from Qubit Systems Inc. (Kingston, Ont., Canada). The beam was aimed at an area of 5×5 mm, which was in the region joining the tube and the limb in corollas, as performed in petunia (Weiss et al., 1988). At the beginning of each measurement, plant material was dark-adapted for 20 min. Basal fluorescence with open PSII centres (F0 and Fm) was measured with modulated weak red light. An saturating white light pulse (3000 μmol m−2 s−1; duration 0.8 s) was applied to determine the maximum chlorophyll fluorescence at closed PSII centres in the dark (maximum fluorescence, Fm0) and during actinic light illumination Fm′. The steady-state chlorophyll fluorescence level (F0) was recorded during actinic light illumination (150 μmol m−2 s−1). Fm/F0 was calculated as (Fm−F0)/Fm. Photochemical quenching qP was calculated as (Fm−F0)/Fm−F0) and non-photochemical quenching QNP was estimated as Fm−F0/Fm. PSII efficiency was calculated using the following equation: Fm′−F0/Fm′.

**Gas exchange analysis**

CO2 exchange was measured with an infrared gas analyser (IRGA) from Qubit Systems Inc., Kingston, On., Canada (Lara et al., 2003). Detached young leaves, petals, or sepal were weighed and sealed in the gas exchange chamber with a PPFD of 150 μmol m−2 s−1 provided by a LED source and 25 °C. Data are expressed as nmol CO2 se−1 g−1 FW.
Each cDNA sample was run in triplicate and repeated in at least three independent set of treatments.

Statistical analysis

Data from the quantitative real-time experiments were tested using one-way analysis of variance (ANOVA). Minimum significance differences were calculated by the Bonferroni, Holm-Sidak, Dunett, and Duncan tests ($\alpha=0.05$) using the Sigma Stat Package.

Results

Identification of different developmental stages in tobacco flowers

By using morphological markers (Fig. 1A, B), different developmental stages of tobacco flowers were identified (Serrafini-Fracassini et al., 2002). Whole corollas and sepals were collected separately at stages 1, 3, 6, 7, and 9, which are representative of the main process in tobacco flower lifespan (Fig. 1A, B). In order to develop a better description of the stages, pigments such as chlorophylls and anthocyanins, and water content status (fresh: dry weight mass ratio) were measured (Fig. 1E, F). From stages 1–3 the corollas are mostly green, which is supported by the highest chlorophyll levels in the first stage (Fig. 1A, B, E). The transition from stage 3 to 4 is characterized by the beginning of anthocyanin accumulation (Fig. 1A, B, E). Accumulation of anthocyanins is largely in the upper part of the flower (the limb) which becomes pink, while the lower part (the tube) is white with a light greenish tint. Anthesis occurs at the end of stage 6. Stage 7 shows the first signs of senescence; the corolla starts losing turgidity (Fig. 1F) and, in some cases, fading is observed. The very basal part exhibits a brown ring that marks the future abscission zone (Fig. 1A). At stage 9, which is characterized by cell death, the corolla is dry and it is easily detached from the receptacle. Regarding sepals, water content was constant during flower development (Fig. 1F), while chlorophyll increased, reaching the highest levels in the last stages (Fig. 1E). Chlorophyll content in sepals was similar to that in leaves, while it was more than six-times lower in petals (Fig. 1E).

Petals showed intense Evans Blue accumulation at stage 9 in the area defined between the distal petal and the basal tube (Fig. 1C). With the exception of coloration in the cut area, sepals in all stages did not show signs of cell death, evaluated by Evans Blue staining (Fig. 1D).

In addition, the levels of expression of two transcripts that were previously suggested as senescence markers (van Doorn and Woltering, 2008), vacuolar processing enzyme 3 (vpe3) involved in macroautophagy and isocitrate lyase (il) participating in the glyoxylate cycle, were quantified by QRT-PCR (Fig. 1G, H). Petals exhibited maximum il and vpe3 expression at anthesis and the following stage. There was a 100-fold increase in vpe3 compared with the first stage. In the case of sepals, il levels were also highest at stage 6 while vpe3 reached maximum amounts at stage 9 (Fig. 1G, H).

Photosynthetic traits of tobacco flowers

Chlorophyll fluorescence parameters and photosystem II analysis: The photochemical quenching coefficient ($q_p$) provides a measure for the oxidative state of the primary acceptor of photosystem II (PSII). Both sepals and petals showed practically the same values for flowers collected at the different developmental stages, with values similar to $q_p$ of green leaves (Fig. 2A). In addition, non-photochemical quenching (NPQ) was similar in petals and sepals at the different developmental stages, and similar to the value of leaves (Fig. 2B). By contrast, PSI efficiency of the flower organs was lower than that of leaves (Fig. 2C). At anthesis (stage 6), both sepals and petals displayed maximum efficiencies. While, in sepals, the values were lower prior to anthesis, in petals, this parameter decreased after anthesis (Fig. 2C). $F_v/F_m$ values during development followed a similar trend to PSII efficiency (Fig. 2D). In addition, on a soluble protein basis, the amount of D1, a PSII reaction centre protein, was lower during the first developmental stages in both petals and sepals compared with leaves (Fig. 3A). However, petals showed similar levels of immunoreactive protein to those in the leaf following anthesis (Fig. 3A).

CO$_2$ assimilation, gas exchange rates, and analysis of Calvin cycle enzymes: In sepals, net rates of CO$_2$ assimilation on a fresh weight basis were observed in flowers in the light in all stages analysed but to a lower extent than that of green leaves (Fig. 4A). By contrast, a net release of CO$_2$ was measured in petals collected during the day (Fig. 4A), which was more pronounced during anthesis and the stages thereafter (stages 6 and 7, respectively). In the case of petals, analysis of the rates of respiration was also performed in the dark for flowers collected during the day. The difference in the CO$_2$ exchange in the light and dark for samples collected during the light period was calculated (Fig. 4B). In this way, even though net CO$_2$ release was observed in petals during the day (Fig. 4A), CO$_2$ fixation by photosynthesis occurred in petals in stages 1 and 3 (Fig. 4B). On the other hand, after anthesis, no CO$_2$ fixation was observed (Fig. 4B). In addition, a net CO$_2$ release was observed in flowers collected during the night period and analysed in the dark (night-time dark respiration), which was similar in both sepals and petals in all developmental stages, similar to that of leaves (Fig. 4C).

Regarding enzymes of the Calvin Cycle, Western blot analysis revealed the presence of the RubisCO large subunit (RubisCO LSU) in both tobacco petals and sepals; nevertheless, the expression in these organs on a soluble protein basis was considerably lower than in leaves, with the lowest amount observed in petals (Fig. 3A; note the different amounts of total proteins for flower and leaf samples). In petals, the RubisCO LSU was lower in stage 9, while in sepals the amount of immunoreactive protein decreased at anthesis and thereafter (Fig. 3A). Transcripts encoding sedoheptulose-1,7-bisphosphatase (sbpase) and phosphoribulokinase (prk), both enzymes exclusively participating in carbon assimilation, were also detected in treatment.
flowers (Fig. 3B). In sepals, both transcripts displayed the highest levels during the time of anthesis, which were similar to the levels detected in leaves. In the case of petals, the highest amounts were found in the first stage (Fig. 3B).

**Analysis of enzymes involved in carbon and oxidative-reductive metabolism**

In order to get an insight into flower primary metabolism, the level of a number of enzymes was evaluated in the petals and sepals of flowers at different developmental stages. This was accessed by the semi-quantification of Western blot analysis or by activity assays (Fig. 5A, C). In addition, the levels of transcripts encoding some of these proteins were quantified by QRT-PCR (Fig. 5B).

Pyruvate kinase (PK) and pyruvate orthophosphate dikinase (PPDK), both involved in pyruvate production from PEP, were semi-quantified. PK levels in both the sepals and petals were lower than in the leaves and remained practically constant during the different stages of flower development. In the case of petals, two immunoreactive bands were detected (Fig. 5A). PPDK was not detected in tobacco sepals and barely detected in tobacco leaves. On the other hand, with the exception of the first stage, levels of PPDK in petals were more than 20 times higher than those in tobacco leaves (Fig. 5A).

Malic enzymes also catalyse pyruvate production using malate as the substrate. Transcripts encoding a plastidic (Nt-nadp-me1) and two cytosolic (Nt-nadp-me2 and 3) NADP-MEs were quantified (Fig. 5B). Nt-nadp-me3, which is mainly expressed in tobacco vegetative tissues (Müller et al., 2008) was detected neither in petals nor in sepals. Nt-nadp-me1 and Nt-nadp-me2 showed a different pattern of expression through flower development in both tissues studied. On the one hand, the level of Nt-nadp-me1 was almost constant in sepals but variable in petals at the different developmental stages. On the other hand, Nt-nadp-me2 was variable in sepals and constant in petals. Expression of Nt-nadp-me1 was notably high, with the levels in petals in stage 3 almost 300-times higher than in leaves (Fig. 5B), and about 40-times higher in sepals. Regarding Nt-nadp-me2, the level of this transcript in sepals was also higher than in leaves, but lower in petals—with the exception of stage 3. NADP-TE activity was constant in sepals during flower development and similar to that in the leaf. By contrast, in petals, higher levels were measured, especially in stages 6 and 7 (Fig. 5C).

Phosphoenolpyruvate carboxylase (PEPC) content, evaluated either at the protein as well as at the transcript level, did not vary among sepals at different stages, while a higher PEPC expression was observed in petals at stages 6 and 7 (Fig. 5). The change in the level of PEPC protein correlated with enzyme activity in both the tissues analysed, with increases in PEPC activity in petals at stage 7 (Fig. 5C).

The cytosolic fructose-1,6-bisphosphatase (FBPase) immunoreactive protein level was practically constant in all the sepal samples analysed and similar to that in leaves,
while in petals, higher amounts were semi-quantified from stages 3 to 7 (Fig. 5A). With respect to glucose-6-P dehydrogenase (G6P-DH), in sepals the activity was similar to that in leaves and remained constant in all the stages analysed. By contrast, in petals, the activity was similar to that in leaves in stages 1 and 9 and increased in stages 3 to 7 (Fig. 5C).

With the aim of exploring respiration in flowers, transcripts encoding cytochrome c oxidase (cox) and alternative oxidase (aox1 and aox 2), involved in the ‘classic’ and ‘alternative’ electron transport pathways, respectively, were also quantified at different stages (Fig. 5B). In sepals, cox levels were higher than those in leaves, showing an expression peak at anthesis. In petals, cox expression was also highest in stage 6; nevertheless, the levels in the other stages were similar to those in the leaf (Fig. 5B). A high level of expression was observed for aox1 in both the sepals and petals in comparison to the leaf. Aox1 was greatly increased in sepals at stage 7 while in petals the increase was observed during anthesis (Fig. 5B). With respect to aox2, the highest expression was observed at stage 9, in both sepals and petals (Fig. 5B).
Effect of sugar feeding and inhibition of photosynthesis on flower senescence

To understand the connection between photosynthesis, sugar levels, and senescence, two inhibitors of the photosynthetic electron transport, DCMU and atrazine, which block the electron flow toward plastoquinone in PSII, were applied to cut flowers kept in MS medium at anthesis (stage 6). In addition, flowers were fed with different sugars in the absence or presence of the photosynthesis inhibitors. Since both compounds produced essentially the same effects on tobacco flowers, only the results using DCMU are presented. In all cases, flowers were visually inspected for visible signs of senescence (see Supplementary Fig. S1 at *JXB* online) and Evans Blue accumulation as an indicator of cell death was measured spectrophotometrically (Fig. 6A).

After 24 h of incubation in MS or MS+DCMU, in both treatments an increase in the Evans Blue staining was observed, while symptoms of petal senescence, such as loss of turgidity, were visible at 48 h (see Supplementary Fig. S1 at *JXB* online). Thereafter, death was extended in petals and in both cases, a complete phenotype of senescence was observed after 72 h (see Supplementary Fig. S1 at *JXB* online). On the other hand, exogenous sugars (sucrose, glucose or fructose) in the presence or absence of DCMU, delayed senescence, which was visible after 72 h and very apparent after 96 h of incubation (see Supplementary Fig. S1 at *JXB* online; Fig. 6A). On the other hand, incubation with mannitol delayed senescence to a lesser extent than sugar feeding (see Supplementary Fig. S1 at *JXB* online), suggesting that the osmotic effect is not the only reason for this phenomenon. Photosynthetic efficiency was evaluated in all samples, after 24 h and 48 h of incubation. After 48 h of keeping the cut flowers in MS a decrease in this parameter was observed in both petals and sepals (not shown). DCMU significantly decreased the PSII quantum yield in both petals and sepals, independently of the presence of sucrose, indicating that this inhibitor entered the cells (not shown).

Pigment content was also evaluated in the presence of photosynthesis inhibitors (Fig. 6B, C). In cut flowers kept either in MS or supplemented with sucrose, an increase in the absorbance at 530 nm at 48 h with respect to time 0 was observed in petals (Fig. 6B). The same trend was observed in flowers in planta, with increases at stage 6 (Fig. 1E). On the other hand, DCMU not only prevented the increase, but also caused a decrease in the anthocyanin levels after 48 h (Fig. 6B). Moreover, the addition of sucrose could not prevent the decrease observed in the presence of DCMU (Fig. 6B). Flavonoid content in petals was also analysed by HPLC-UV after 48 h of incubation of the cut flowers in MS, and in the presence of DCMU or sucrose (Fig. 6C). In the presence of DCMU, a 20% decrease in the main peak of absorbance at 330 nm with respect to the peak observed in flowers kept either in MS or supplemented with sucrose was observed (Fig. 6C). The main flavonoids detected in tobacco petals, quercetin and kaemferol (Nakatsuka *et al.*, 2007), were used as controls, which displayed practically the same retention time as the peak observed in cut flowers by this technique (Fig. 6C).
Fig. 5. Analysis of proteins involved in carbon metabolism and respiration, in tobacco flowers at different developmental stages. (A) Western blot analysis using antibodies against phosphoenolpyruvate carboxylase (PEPC), pyruvate orthophosphate dikinase (PPDK), fructose-1,6-bisphosphatase (FBPase), and pyruvate kinase (PK). Twenty-five micrograms of total soluble protein from sepals (upper panel) and petals (lower panel) were downloaded in each lane. As a reaction control, proteins from a green tobacco leaf were used.
**Discussion**

Photosynthesis is carried out in petals and sepals of tobacco flowers

Data presented in the present work indicate that, although to a lesser extent than in leaves, tobacco sepals and petals are capable of fixing CO₂ in the early developmental stages (Fig. 4). In petals, the maximal chlorophyll concentration, achieved at an early developmental stage was about 20% of those of leaves (Figs 1E, 2D). In petals, although net CO₂ release was observed in the light, light minus dark rates of CO₂ exchange indicate substantial fixation of CO₂ by photosynthesis during the first stages (Fig. 4B). In agreement, RuBisCO LSU protein, and prk and sbpase transcripts were also higher during the first stages (Fig. 3). In sepals, net carbon uptake by photosynthesis was observed during all the stages analysed (Fig. 4A), and higher levels of RuBisCO LSU protein, prk, and sbpase than in petals were observed, with increases in prk and sbpase during anthesis (Fig. 3). Overall, the results obtained indicate that, although the flower organ functions mainly as a sink, the photosynthetic apparatus is operative and contributes to the supply of organic C in some developmental stages. Carbon gain by the flower through photosynthesis during the day could offset carbon losses by respiration at night.

Photosynthetic electron transport in tobacco petals is required for pigment biosynthesis

After anthesis, no net CO₂ uptake was detected in tobacco flowers (Fig. 4B); however, the photosynthetic electron transport chain is still operating (Fig. 2). Inhibitors of the electron transport chain (ETC) were used at this stage to analyse its role in pigment content (Fig. 6). In cut flowers kept either in MS or supplemented with sucrose, an increase in the anthocyanin content was observed after 48 h, indicating that endogenous compounds were enough to support pigment biosynthesis which was not interrupted due to detachment (Fig. 6B). However, DCMU produced a decrease in anthocyanin content, which could not be prevented by the presence of sucrose (Fig. 6B). Moreover, DCMU also induces a decrease in flavonoid content in relation to the levels observed in the presence of sucrose (Fig. 6C). Therefore, a plausible hypothesis is that electrons absorbed by the photosynthetic ETC in petals are, at least in part, sustaining pigment biosynthesis.

Role of photosynthesis and sugars after anthesis

Feeding cut tobacco flowers with sugars in MS media at stage 6 delayed senescence signs by about 1 d (see Supplementary Fig. S1 at JXB online; Fig. 6A). Since mannitol exerted an intermediate effect between the presence or absence of sugars (see Supplementary Fig. S1 at JXB online), it suggests sugars have an effect through metabolism that is not only osmotic. On the other hand, the presence of DCMU did not significantly affect the time of cell death establishment with respect to flowers in MS (Fig. 6A). In addition, in flowers incubated with sucrose, DCMU did not affect the senescence delay, besides differences in pigment concentrations (Fig. 6). Overall, these results indicate that corolla photosynthetic ETC is not involved in the senescence process in tobacco flowers; but conversely, sugars have a role in delaying this process. This result reinforces the idea that the photosynthetic ETC after anthesis in petals is not involved in sugar biosynthesis, with no net CO₂ fixation (Fig. 4B), and that its principal role is related to pigment biosynthesis. Previous studies on cut flowers have also shown that exogenous sugars delay flower senescence; but, it was not established if they produced a specific effect or if they simply improved water relations (van Doorn, 2008). As sugars regulate the ethylene response by inhibiting the expression of genes involved in its synthesis and signalling (Wingler and Roitsch, 2008), it is possible that exogenous sugars could inhibit the ethylene response and thus, delay the senescence process.

Anthesis is characterized by active metabolism and respiration in petals

After anthesis, there is an increase in the respiration rate in petals both during the day and at night (Fig. 4). In addition, proteins participating in mitochondrial ETC are also

Molecular mass of immunoreactive proteins are shown on the left and expressed in kDa. The quantification of the immunoreactive bands is expressed in relation to the amount in green leaves (reference dashed line) shown below each Western blot (n = 2 or 3). (B) Expression analysis of aox, nadp-me, pepc, and cox, determined by QRT-PCR. Analyses were made with RNA isolated from flowers at different stages of development. The means of the results obtained, using three independent RNAs as a template, are shown. Y axis refers to the fold difference in a particular transcript level relative to its amount found in green leaves (dashed lines). (C) PEPC, NADP-ME, and G6P-DH activity measurement. Total activity is expressed in International Units mg⁻¹ of total soluble protein. Values obtained in tobacco leaves are shown as a reference dashed line. Standard deviations are shown. For each tissue, values with the same letters are not significantly different (P < 0.05). Sepals, black bars; petals, gray bars.
increased at this stage, as accounted for by maximum expression levels of cox, the terminal electron carrier, and a dramatic increase of aox1, which bypasses COX (Fig. 5). AOX1 may participate in thermogenesis since free energy released during electron flow is not used for proton translocation (Siedow and Umbach, 1995; Vanlerberghe, 1997). Heat production in flowers such as lilies has been related to the release of volatile compounds, which attract insects (Meeuse and Raskin, 1988) as well as in the generation of a warm environment for them (Seymour, 2004; Seymour and Gibernau, 2008).

Other enzymes involved in cytosolic carbon metabolism such as FBPase, PPDK and PEPC are highest at anthesis (Fig. 5). PPDK levels are remarkably high in petals, in comparison to leaves, probably denoting the participation of this enzyme in PEP metabolism. The shikimate pathway uses PEP and erythrose-4-P and provides precursors for the biosynthesis of various primary and secondary metabolites such as anthocyanins. During the anthesis stage, a major rate of increase in the anthocyanin content is observed, although the levels start increasing at stage 3 (Fig. 1E). Higher levels of PPDK could support PEP provision for pigment biosynthesis. Since part of the pigment biosynthesis takes place in the plastids (Saito et al. 2006), the plastidic NADP-ME1, which transcripts levels are highest at this stage, could work in a concerted manner with PPDK providing pyruvate for PPDK and NADPH for flavonoids biosynthesis (Casati et al., 1998). In addition, G6P-DH activity, which produces NADPH, is also high during anthesis (Fig. 5C).

Onset of senescence and metabolism in tobacco petals

In the present work, flower anthocyanin content is maximum at stage 7 but dehydration is already observed (Fig. 1E, F). The following biochemical and molecular results are indicative of petal senescence at stage 7: (i) maximum il levels (Fig. 1G), (ii) increased respiration rate (Fig. 4), (iii) increased PEPC activity, which contributes to the supply of carbon skeleton compounds for nitrogen compounds synthesis (Fig. 5C), and (iv) a high level of FBPase expression enabling gluconeogenesis (Fig. 5A). On the other hand, PPDK was proposed as an earlier marker of senescence in A. thaliana (Lin and Wu, 2004), and enhanced PPDK expression in senescing broccoli florets (Page et al., 2001) and Brassica napus (King and Morris, 1994; Buchanan-Wollaston, 1997) has also been described. However, in tobacco petals, PPDK is not specifically induced at this stage, probably because the levels are already high. On the other hand, since il and vpe3 increase at anthesis, it raises the question whether senescence has already begun at this stage (Fig. 1H). Petal senescence in Ipomoea nil starts before the flowers open, with a few mesophyll cells showing ultrastructural features of senescence (Yamada et al., 2009). This could also be true for tobacco flowers where at least some senescence-related processes may start before the anthesis.

At stage 9, there is extended cell death in the corolla (Fig. 1C). However, not all the cells were stained with Evans Blue. On the one hand, there is a moderate to large decline in some proteins, transcripts, and activities (Figs 2–5). However, other transcripts, proteins, and metabolites are found at their highest levels, with remarkable increases in
Rubisco level were detected in sepals than in leaves during senescence. These transcripts could be used as earlier markers of senescence before signs of senescence are observed; suggesting that aox2 and D1 protein (Figs 1, 3, 5). Therefore, at this stage there is a high metabolic activity, probably due to carbon mobilization. Nevertheless, impairment of the pentose phosphate pathway (G6P-DH decrease, Fig. 5C), together with a decrease in photosynthesis (Fig. 2) and NADP-ME (Fig. 5), could result in insufficient production of NADPH for membrane repair processes and for the maintenance of cellular redox states.

**Sepal metabolism**

Sepals are modified leaves, which remain green in all the flower developmental stages analysed, with the highest level of chlorophyll in the latter stages, similar to those of the leaf (Fig. 1E). Although a lower PSII efficiency and Rubisco level were detected in sepals than in leaves (Figs 2C, 3A), net CO₂ uptake in sepals was measured during all the developmental stages analysed (Fig. 4A). On the other hand, sbpase and prk levels were similar in petals and leaves, especially at stage 6 (Fig. 3B). G6P-DH, PEPC, and NADP-ME activities were similar in leaves and sepals; however, differences in the levels of transcripts encoding enzymes involved in the respiration process such as aox1, aox2, and cox were found (Fig. 5), indicating active mitochondrial processes in the sepals. It is also notable the higher levels of nadp-me2 in sepals than in petals and leaf (Fig. 5B). Specific roles for each NADP-ME isoform have been suggested in Arabidopsis, and it is likely that the protein encoding nadp-me2 may have a particular physiological role in this tissue (Maurino et al., 2009).

During the period of time analysed, neither dehydration (Fig. 1A) nor cell death was detected in sepals by Evans Blue staining (Fig. 1D). In addition, even after corolla abscission sepals remained green (not shown). Nonetheless, il and vpe3 are greatly increased in stages 7 and 9, respectively (Fig. 1G, H). Therefore, the timing of senescence is different in sepals and in petals. Nevertheless, it is clear that in both petals and sepals vpe3 and il increases before signs of senescence are observed; suggesting that these transcripts could be used as earlier markers of senescence.

**Supplementary data**

Supplementary data are available at *JXB* online.

**Supplementary Fig. S1.** Effects of sugar feeding and photosynthetic transport inhibition on flower senescence.

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