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Temperature and nitrogen effects on regulators and products of the flavonoid pathway: experimental and kinetic model studies

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ABSTRACT

The flavonoid pathway is known to be up-regulated by different environmental stress factors. Down-regulation of the pathway is much less studied and is emphasized in the present work. Flavonoid accumulation was induced by exposing plants for 1 week to nitrogen depletion at 10 °C, giving high levels of anthocyanins and 3-glucoside-7-rhamnosides, 3,7-di-rhamnosides and 3-rutinoside-7-rhamnosides of kaempferol and quercetin. Flavonol accumulation as influenced by temperatures and nitrogen supply was not related to the glycosylation patterns but to the classification as quercetin and kaempferol. When nitrogen was re-supplied, transcripts for main regulators of the pathway, *PAP1/GL3* and *PAP2/MYB12*, fell to less than 1 and 0.1% of initial values, respectively, during 24 h in the 15–30 °C temperature range. Anthocyanins showed a half-life of approximately 1 d, while the degradation of flavonols was much slower. Interestingly, the initial fluxes of anthocyanin and flavonol degradations were found to be temperature-independent. A kinetic model for the flavonoid pathway was constructed. In order to get the observed concentration-temperature profiles as well as the temperature compensation in the flavonoid degradation flux, the model predicts that the flavonoid pathway shows an increased temperature sensitivity at the end of the pathway, where the up-regulation by *PAP/GL3* has been found to be largest.

Key-words: *Arabidopsis*; anthocyanins; flavonoids; flavonols; glycosylation; kinetic model; nitrogen; temperature.

INTRODUCTION

To get further insight into the conditions and mechanisms promoting high flavonoid levels, the accumulation and decrease of specific flavonoids and expression of pathway regulators were studied over a wide range of temperatures at different nitrogen supplies. Temperature influences vegetative growth, flowering, formation of storage organs and seasonal acclimation (Thingnaes *et al.* 2003; Hasdai *et al.* 2006). Within a certain temperature range, organisms will generally try to adapt their metabolism and growth to variations in temperature. Although not well understood, the accumulation of flavonoids appears to protect plants against various stressful conditions, for example, freezing (Winkel-Shirley 2002; Hannah *et al.* 2006) or nutrient limitation (Peng *et al.* 2008), and flavonoids can reach high levels in response to cold treatment or nitrogen depletion (Lillo, Lea & Ruoff 2008).

The phenylalanine ammonia-lyase (PAL) enzyme is a link between primary metabolism and secondary metabolism. High *PAL* expression is often found in parallel with high levels of flavonoids (Lillo *et al.* 2008). PAL is, however, important not only for flavonoid metabolism, but for the synthesis of other secondary compounds, especially lignin (Fig. 1). PAL is of special interest in relation to nitrogen nutrition because it releases nitrogen from phenylalanine, and thereby makes nitrogen available for redistribution, which may be of importance for survival in response to severe nitrogen limitation.

Activation of the flavonoid pathway is a result of various interacting environmental and developmental factors – light very often being a prerequisite for allowing synthesis of flavonoids. In several investigations, cold treatment was performed in darkness or in dim light, which would restrict induction of the flavonoid pathway (Leyva *et al.* 1995; Vogel *et al.* 2005). In the present study, plants were subjected to the various temperatures under standard growth-light.

Kaempferols and quercetins are synthesized from dihydroflavonols, apparently by the same enzymes (Fig. 1). Flavonol synthase (FLS) is a 2-oxoglutarate-dependent dioxygenase, and converts dihydroflavonols to flavonols by introducing the C2C3-double bound in the C-ring. Various uridine 5′-diphosphate (UDP)-dependent glycosyl transferases will then glycosylate the flavonols before accumulation. Although the flavonoid pathway is subjected to gross regulation by two homologous MYB factors, PAP1 and PAP2 (production of anthocyanin pigment), the accumulation of products is responding also in a differential manner

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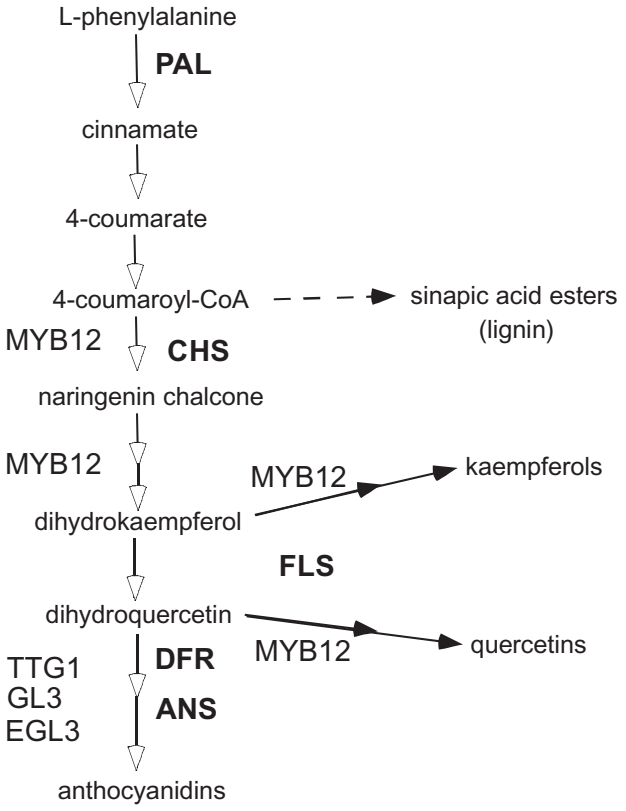

Figure 1. Simplified scheme of phenylpropanoid synthesis in *Arabidopsis* with pathways leading to lignin and major soluble phenolic compounds (sinapic acid esters, kaempferols, quercetins, anthocyanidins). Enzymes named are phenylalanine ammonia-lyase (PAL), chalcone synthase (CHS), flavonol synthase (FLS), dihydroflavonol-4-reductase (DFR), anthocyanidin synthase (ANS). The pathway is regulated by PAP1/2, which enhances expression of genes coding for enzymes marked by arrows with white arrowhead. MYB12 stimulates expression of FLS, and the three steps anterior to FLS. TTG1 and GL3 stimulate specifically steps towards anthocyanidin synthesis, i.e. DFR and ANS. 4-coumaroyl-CoA is established as a precursor for sinapic acid esters and lignin, but additional routes may exist (Raes *et al.* 2003; Gachon, Langlois-Meurinne & Saindrenan 2005; Besseau *et al.* 2007).

in response to various external factors, indicating that each branch of the flavonoid pathway is also regulated specifically. Only (derivatives of) kaempferols, not quercetins, are generally present in *Arabidopsis* leaves under normal greenhouse conditions (Veit & Pauli 1999). Various factors may contribute to a differential accumulation such as different stability/degradation rates of flavonols and properties of the FLS, for example, possible different substrate affinities may be important. Furthermore, increased flux to the anthocyanin branch may influence the balance between pathways and decrease the kaempferol/quercetin ratio.

Degradation of flavonoids *in planta* is studied very little, although many investigations concerning degradation of flavonoids, particularly anthocyanins, in beverages, preserves or in the intestine have been carried out (Heim, Tagliaferro & Bobilya 2002; Cheynier 2006). In attached pears, anthocyanin content was much higher at 10 °C compared with 20 or 30 °C, and when wrapped in light impermeable bags the content of anthocyanins decreased by 50% during 2 weeks (Steyn *et al.* 2004). To test how degradation rates might differ for kaempferols, quercetins and anthocyanins, *Arabidopsis* plants were first treated to induce a high level of flavonoids, i.e. by nitrogen depletion at 10 °C for 1 week. Thereafter, plants were placed in non-inductive conditions by giving full nutrient solution. Transcript levels of essential regulators, MYB and bHLH (basic helix-loop-helix domain) transcription factors, were followed under the non-inductive conditions and the decrease in flavonoid concentrations was also followed. To understand and predict flavonoid accumulation and degradation in the different branches related to temperature, a minimal kinetic model based on the data presented was constructed.

MATERIALS AND METHODS

Plant material

Seeds of *Arabidopsis thaliana* ecotype Columbia (Col1) were sown on soil with complete Hoagland solution (Hoagland & Arnon 1950). After approximately 3 weeks, seedlings were transferred into rock wool cubes and grown in a growth chamber at 20 °C in a 12 h light/12 h dark regimen (standard growth-light conditions). Light was provided by fluorescent lamps (Osram L58W/21) with a photon flux density (PPFD) of 100 μmol m^{-2} s^{-1} PAR (photosynthetically active radiation). Plants were watered when needed using a complete Hoagland solution. After one week plants were transferred randomly to growth chambers at temperatures of 5, 10, 15, 20, 25 or 30 °C. Plants were given either complete Hoagland or Hoagland without nitrogen. A water vapour pressure deficit of 3.5–1.0 g m^{-3} and a CO_2 level of 350 μmol mol^{-1} were maintained at all temperatures. Temperature fluctuations in the growth chambers were kept within $\pm$0.5 °C. The first samples were harvested before exposure of plants to different treatments (day 0), and then 4 and 7 d after the change.

For studying down-regulation of the flavonoid pathway, plants were first grown for 2 weeks in rock wool at normal growth conditions, then exposed to higher light, 200 μmol m^{-2} s^{-1} PAR, 10 °C and given Hoagland without nitrogen (previous Hoagland solution was washed out) to induce the pathway. After one week plants were transferred randomly to growth chambers at temperatures of 5, 10, 15, 20, 25 or 30 °C. Half were placed at 100 μmol m^{-2} s^{-1} PAR in a 12 h light/12 h dark regimen (standard growth-light), and the other half in darkness. All plants were given complete Hoagland solution. The first samples were harvested before exposure of plants to different treatments (day 0), and then every day to 5 d after the change.

Measurements of flavonoids

About 0.100 g of plant tissues were exactly weighted and transferred to Eppendorf tubes. One millilitre of methanol [1% trifluoroacetic acid (TFA), v/v] was added to each tube,

and phenolics were extracted for 18 h at ambient temperature and in darkness. The extracts were filtered through 45 μm nylon filters prior to high-performance liquid chromatography analyses. A liquid chromatograph (Agilent 1100-system, Agilent Technologies, Palo Alto, CA, USA) supplied with an autosampler and a photodiodearray detector was used for the analysis of individual flavonoids. The flavonoids were separated on an Eclipse XDB-C8 (4.6×150 mm, 5 μm) column (Agilent Technologies) by use of a binary solvent system consisting of (A) 0.05% TFA in water and (B) 0.05% TFA in acetonitrile. The gradient (% of B in A) was linear from 5 to 10 in 5 min, from 10 to 25 for the next 5 min, from 25 to 85 in 6 min, from 85 to 5 in 2 min and finally, recondition of the column by 5% in 2 min. The flow rate was 0.8 mL min^{-1}, 10 μL samples were injected on the column, and separation took place at 30 °C. Detection was made over the interval of 230–600 nm in steps of 2 nm in order to obtain full absorbance spectrum of the compounds of interest, whereas quantitative determinations were done at 370 nm (flavonols) and 520 nm (anthocyanins), and given as peak area per 100 mg sample.

The individual peaks were characterized by liquid chromatography coupled with mass spectrometry using an Acquity UPLC (Waters, Milford, MA, USA) connected to a Q-TOF micro (Waters) with Lockspray mass calibration. Reversed phase separations were achieved on a custom-made, 0.5×225 mm HotSep column (G&T Septech, Norway) packed with PLRP-S particles (Polymer Laboratories, UK) of 3 μm in diameter and 1000 Å pore size. The mobile phase gradient was a mixture of (A) 0.1% acetic acid and (B) acetonitrile, starting with 5% B for 2 min, then a linear increase to 60% B in 15 min. The flow rate was 20 μL min^{-1}. Electrospray ionization in the positive mode was used with a capillary voltage at 3 kV and a cone voltage at 35 V.

PAL assay

Approximately 50 mg leaf tissue was thoroughly ground with cold mortar and pestle in 2 mL of 100 mM Tris-HCl (pH 8.8) with 12 mM β-mercaptoethanol and transferred to a centrifuge tube. The samples were centrifuged at 4 °C for 5 min at 16 000 *g*. The supernatant was passed through a Sephadex G25 column (GE Healthcare, Uppsala, Sweden). The eluate was used for PAL assay according to Saunders & McClure (1974). The PAL assay was performed at 37 °C for 1 h in an assay mixture containing 500 μL enzyme extract, 450 μL 100 mM Tris-HCl (pH 8.8) and 50 μL 100 mM L-phenylalanine. Assays were run in triplicate. The reaction was terminated by adding 50 μL 5 M HCl, centrifuged at 16 000 *g* for 15 min and absorbance recorded at 290 nm against blanks made in the same way as the assays, except that 50 μL 5 M HCl was added before L-phenylalanine. The activity was expressed as nmol trans-cinnamic acid formed per gram of plant tissue, per hour.

RT-PCR

Total RNA was isolated using RNeasy Plant Mini Kit (Qiagen, Chatswort, CA, USA). RNA was quantified by spectrophotometer and cDNA synthesized using the High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA, USA), following the manufacturer's instructions (concentration of RNA in the reaction tube was 4.6 μg mL^{-1}). Real-time PCR reactions were assayed using an ABI 7300 Fast Real-Time PCR System. The reaction volume was 25 μL containing 12.5 μL TaqMan buffer (Applied Biosystems, includes ROX as a passive reference dye), 8.75 μL H_2O, 2.5 μL cDNA and 1.25 μL primers. Primers were pre-designed TaqMan Gene Expressions assays obtained for the following genes (TaqMan identification number is given in parenthesis). *Arabidopsis thaliana PII* At4g01900 (At02207948_g1), *PAP1* At1g56650 (At02213787_gH), *PAP2* At1g66390 (At02334068_g1), *GL3* At5g41315 (At02327731_g1), *EGL3* At1g63650 (At02217883_1g), *TTG1* At5g24520 (At02333810_m1), *ACT8* At1g49240 (At02270958_gH), *MYB12* At2g47460 (At02264273_m1), *PAL1* AT2G37040 (At02323251_g1), *PAL2* AT3G53260 (At02188099_g1), *PAL3* AT5G04230 (At02180826_m1), *PAL4* AT3G10340 (At02291526_m1) and *UBQ* At3g02540 (At02163241_g1) (Applied Biosystems). Standard cycling conditions (2 min at 50 °C, 10 min at 95 °C and 40 cycles altering between 15 s at 95 °C and 1 min at 60 °C) were used for product formation. Real-time PCR products were analysed by Sequence Detection Software version 1.3. Comparative CT (cycle treshold) method for relative quantification has been used with ubiquitine as endogenous control and the sample taken before change of nutrient solution/light/temperature (day 0) as calibrator. Relative quantity ($RQ = 2^{-CT}$) of any gene is given as fold change related to day 0. This method which is based on a light signal from each transcript copy being formed also allows comparing expression levels between the genes.

RESULTS

Inductions of the flavonoid pathway and accumulation of flavonoids

The six major flavonols detected in all samples were assigned to be the 3-*O*-rutinoside-7-*O*-rhamnoside, the 3-*O*-glucoside-7-*O*-rhamnoside and the 3-*O*-rhamnoside-7-*O*-glucoside of kaempferol and quercetin according to their mass spectrometric data (Supporting Information Table S1) and in accordance with previous reports (Graham 1998; Veit & Pauli 1999; Tohge, Nishiyama & Hirai 2005). The major anthocyanin was tentatively assigned to be cyanidin 3-*O*-[2-*O*-(2-*O*-(sinapoyl)-β-D-xylopyranosyl)-6-*O*-(4-*O*-(β-D-glucopyranosyl)-p-coumaroyl-β-D-glucopyranoside] 5-*O*-[6-*O*-(malonyl) β-D-glucopyranoside] according to Bloor & Abrahams (2002).

The general flavonoid pathway activators *PAP1* and *PAP2* responded strongly to a decrease in temperature and depletion of nitrogen. For *PAP2*, the increase in transcript level was more than 10 000-fold in the 5–15 °C temperature range. *PAP1* increase was about 200-fold in response to the same treatment (Fig. 2a,b). *GL3* transcript levels also increased strongly at low temperatures and in response to

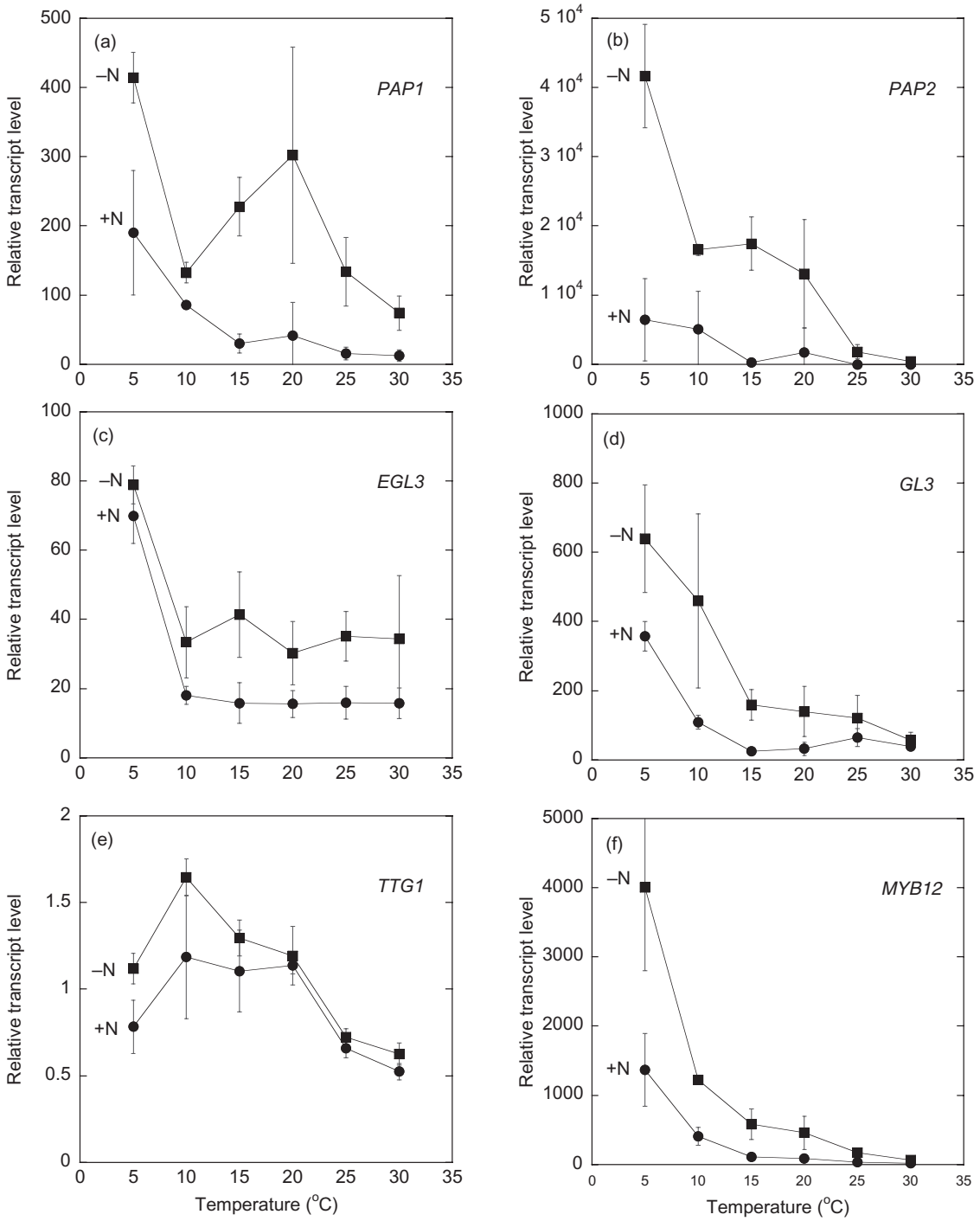

Figure 2. Effects of nitrogen depletion and temperature on transcript levels of regulators involved in the flavonoid pathway. Plants were grown in rock wool with Hoagland solution containing 15 mM KNO_3 at 20 °C. Rosette stage *Arabidopsis* plants were placed at various growth temperatures between 5 and 30 °C (day 0), and exposed to Hoagland solution with (●) or without (■) nitrogen. Leaves were harvested on days 4 and 7 after the variable treatments had started. There were no clear differences between results obtained at days 4 and 7, therefore the data were pooled. $n = 4$; standard error is given.

nitrogen depletion (Fig. 2d). Interestingly, *EGL3* (*enhancer of GLABRA3*) showed a pronounced increase in transcript levels at 5 °C only (Fig. 2c). Together with *PAP2*, *GL3* is known to stimulate *DFR* (*dihydroflavonol 4-reductase*) and *ANS* (*anthocyanidin synthase*) expression hence increased accumulation of anthocyanins can be expected, as was indeed observed (Fig. 3) (and Fig. 5 in Olsen *et al.* 2008). Also, the expression of the *MYB12* transcription factor increased strongly at low temperatures and in response to N-depletion (Fig. 2f). This is in accordance with the general increase seen in flavonol accumulation, but does not explain a differential increase in kaempferols against quercetins (Fig. 3). The three quercetin derivatives showed increased levels at 5 and 10 °C, and a very strong effect of nitrogen depletion after 4 d and at 7 d (Fig. 3). All quercetins showed a peak at 10 °C under N-depletion. For the kaempferol derivatives, there was no sharp peak at 10 °C, and levels were high also at 15 °C. The difference between the plus/minus nitrogen treatments, although significant, was not as pronounced for kaempferols as for quercetins. Because identical glycosylation patterns did not lead to the same accumulation pattern for kaempferols and quercetins, these point to a regulation related to the hydroxylation of the B-ring being essential for the differential accumulation of quercetins and kaempferols. Anthocyanins accumulated most at 10 and 15 °C. Only the data for the major anthocyanin is presented (Fig. 3g,h).

Down-regulation of the flavonoid pathway and decrease of specific flavonoids

PAL activity and transcript levels

PAL activity decreased rapidly after addition of full nutrient solution. After 2 d, activity was between 4 and 22% of the start value for all treatments, except for 5 °C in standard growth-light, which apparently preserved PAL activity as 61% was still left. Activity decreased in both darkness and normal growth-light, although more rapidly in darkness (Fig. 4). Transcript levels of *PAL1* and *PAL2* decreased to less than 1% after a re-supply of nitrogen in the darkness, except for the 5 and 10 °C treatments where PAL transcript levels stayed higher (Fig. 5a,b). Transcript levels also decreased rapidly under standard growth-light conditions, but were higher than in darkness (Figs 5a,b & 6a,b).

Transcript levels of regulators

After a re-supply of full nutrient solution, transcript levels of the main regulators of the pathway *PAP1* and *PAP2* fell to less than 1 and 0.1%, respectively, during 24 h in the temperature range 15–30 °C in darkness (Fig. 5c,d). Transcripts for specific regulators of late steps in anthocyanin synthesis, *GL3* (Fig. 5f) and flavonol synthesis, *MYB12* (Fig. 5h), fell to less than 1 and 0.1%, respectively, in the 15–30 °C range in darkness. Transcript levels of *PAP2*, *MYB12*, *GL3*, *PAP1*, *PAL2* and *PAL1* showed the strongest decrease after addition of nitrogen. However, a relatively large decrease was also observed for *EGL3*. The *TTG1* regulator, which is important for the late steps in anthocyanin synthesis, did not respond to nitrogen re-supply (Figs 5g & 6g). Other genes tested (Supporting Information Fig. S1), like the enigmatic *PII* gene, which is important for nitrogen-sensing in prokaryotes and nitrite transport in plants (Templeton & Moorhead 2004; Ferrario-Mery, Meyer & Hodges 2008), did not respond to nitrogen addition. The control gene *ACT8* was also constant throughout all treatments. The *PAL3* gene showed a small increase in response to nitrogen re-supply, whereas *PAL4* showed a small decrease, except for 5 °C. *ACT8* and *PII* were not influenced by temperature, whereas transcript levels of all other genes tested appeared to reach a steady state level dependent on temperature, with higher levels at low temperatures, i.e. at 5 and 10 °C. *TTG1*, *PII* and *ACT8* transcripts were not influenced by darkness against standard light conditions, but all other genes generally showed slightly lower levels in the darkness as opposed to standard growth-light (Figs 5 & 6, and Supporting Information Fig. S1).

Flavonoids

Flavonoids were measured in leaves of nitrogen-starved plants at day 0, and 1 and 2 d after a re-supply of full nutrient solution and exposure to temperatures from 5 to 30 °C. Activation energies for the decrease in flavonoid content were calculated from Arrhenius plots. The Arrhenius plots showed a bend at 15 °C. Two different values were therefore calculated, one for the range of 15–30 °C and one for the range of 5–15 °C (Supporting Information Table S2). Activation energies, Ea, were small in the higher temperature range for all compounds tested. The decrease in flavonoids was almost temperature-compensated in the 15–30 °C range. Because there was very little difference in the degradation of flavonoids in the range of 15–30 °C, these data were pooled. For the three quercetins tested – quercetin-3-glucoside-7-rhamnoside, quercetin-3-rhamnoside-7-rhamnoside and quercetin-3-rutinoside-7-rhamnoside – 45 ± 6, 29 ± 2 and 32 ± 2%, respectively, were left after 2 d in normal daylight conditions (data in Fig. 7a,c,e). For the corresponding kaempferols (same glycosylation patterns), 55 ± 3, 53 ± 2 and 61 ± 3% were left. The results showed that these kaempferols were slightly more stable than the corresponding quercetins. Decreases were very similar in standard growth-light (open symbols) and darkness (closed symbols; Fig. 7a,c,e). Decrease in flavonoids was significantly slower in the 5–10 °C range (Fig. 7b,d,f). At days 3–5, the decrease levelled off and variations among samples indicated that flavonol content started to fluctuate (not shown). The major anthocyanin decreased rapidly after a re-supply of full nutrient solution, and after 2 d, the amount left was approximately 25% at 15–30 °C (Fig. 7g) and 45% at 5–10 °C. Decrease in anthocyanin content was the same in darkness and standard growth-light conditions.

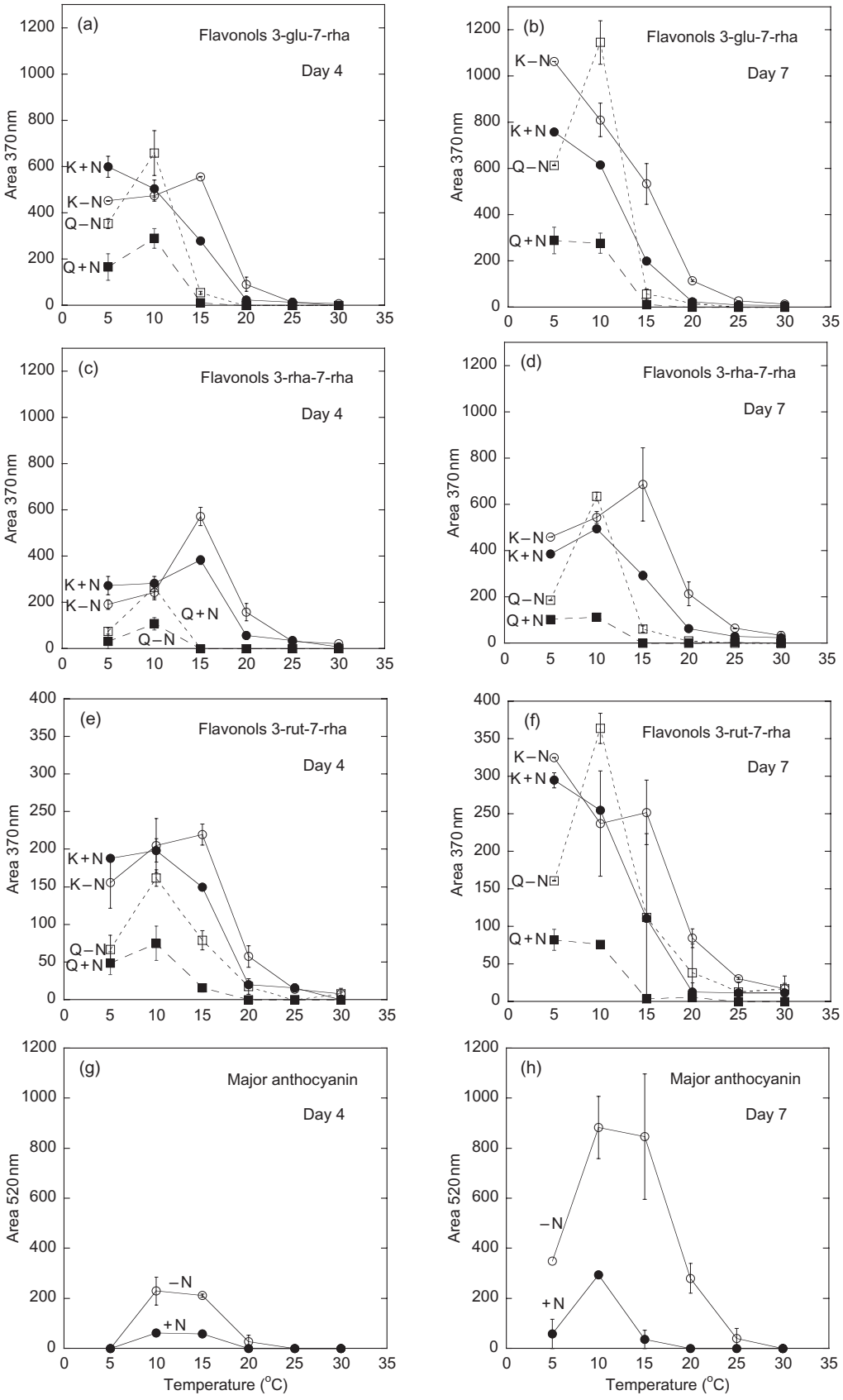

Figure 3. Concentration of flavonols 3-glucoside-7-rhamnoside (a, b), flavonols 3-rhamnoside-7-rhamnoside (c, d), flavonols 3-rutenoside-7-rhamnoside (e, f), and the major anthocyanin (g, h), as influenced by nitrogen depletion and growth temperature. Plants were grown in rock wool with Hoagland solution containing 15 mM KNO_3 at 20 °C. Rosette stage *Arabidopsis* plants were placed at various growth temperatures between 5 and 30 °C (day 0), and exposed to Hoagland solution with (filled symbols) or without (open symbols) nitrogen. Circles/dots represent kaempferols (K), and squares represent quercetins (Q). The figure shows data for samples harvested on day 4 after start of treatment (a, c, e, g) from the first batch of plants and on day 7 (b, d, f, h) for the second batch of plants. Except for day seven 10 °C minus nitrogen and 5 °C plus nitrogen which represent only one sample, there were two samples for each treatment, and the spread is given as vertical bars when exceeding the size of the symbol.

Analysing temperature adaptation of the flavonoid pathway by using a kinetic model

To investigate the occurrence of the temperature profiles as well as the temperature compensation of the degradation fluxes in the kaempferol, quercetin and anthocyanin branches of the flavonoid pathway after the addition of nitrogen, we constructed a minimal kinetic model (Fig. 8). The model contains a precursor I_1 leading into the flavonoid pathway with the intermediates dihydrokaempferol and dihydroquercetin forming side reactions to K (kaempferols) and Q (quercetins), respectively, and their degradations. Variable I_2 represents (lumped) intermediates leading to A (anthocyanin with its degradation). The greyed products are not explicitly considered in the model, but represent other products or degradation products formed during the degradation of intermediates when nitrogen is added after starvation. The influence (up- and down-regulation) by *PAP* is indicated by arrows to the individual rate constants. After the addition of nitrogen, *PAP* transcript levels decrease with increasing temperature, which can approximately be described by a 50% reduction of *PAP* levels for each increase in temperature by 5 °C (Fig. 2a,b).

The rate equations of the model are:

$$\frac{dI_1}{dt} = k_1 - (k_2 + k_3) I_1 \tag{1}$$

$$\frac{dDHK}{dt} = k_3 I_1 - (k_4 + k_6) DHK \tag{2}$$

$$\frac{dK}{dt} = k_4 DHK - j_K \tag{3}$$

$$\frac{dDHQ}{dt} = k_6 DHK - (k_7 + k_9) DHQ \tag{4}$$

$$\frac{dQ}{dt} = k_7 DHQ - j_Q \tag{5}$$

$$\frac{dI_2}{dt} = k_9 DHQ - (k_{10} + k_{11}) I_2 \tag{6}$$

$$\frac{dA}{dt} = k_{11} I_2 - j_A \tag{7}$$

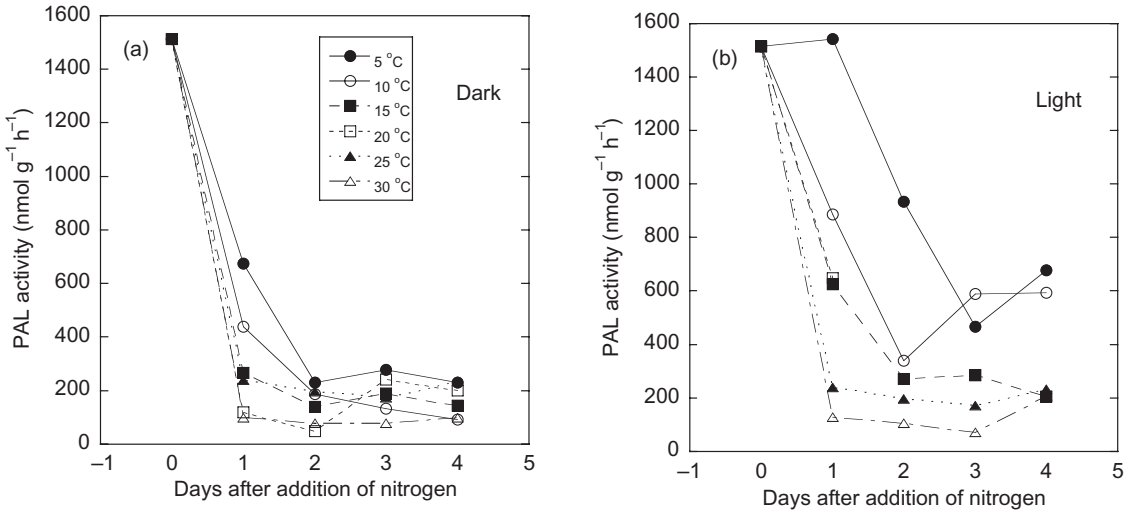

Figure 4. Decrease of phenylalanine ammonia-lyase (PAL) activity in N-starved *Arabidopsis* plants after re-supply of full nutrient solution and placement at various temperatures from 5 to 30 °C. Plants were grown in rock wool with Hoagland solution, then plants were placed at 10 °C, and the growth medium was deprived of nitrogen for 1 week with high light intensity to induce high levels of flavonoids. Nitrogen was then re-supplied (day 0), and plants were placed at six different growth temperatures in darkness (a), or standard growth-light conditions (100 μmol m^{-2} s^{-1} PAR) (b). PAL activity was followed during 4 d. Data are means of three assay parallels, but only one biological sample was tested for each point as the different temperatures systematically confirmed the results obtained.

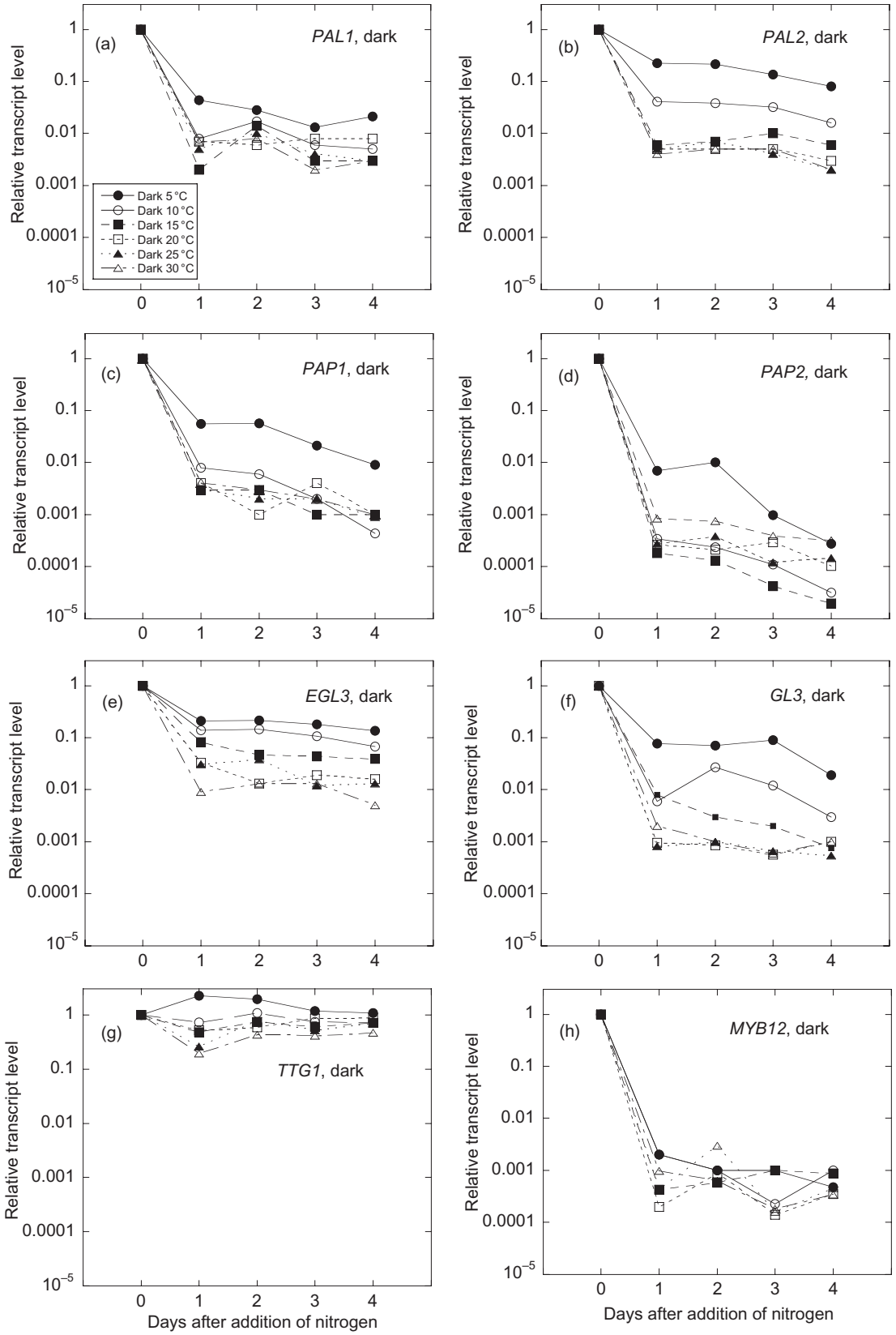

Figure 5. Transcript levels of *phenylalanine ammonia-lyase 1* (*PAL1*) and *phenylalanine ammonia-lyase 2* (*PAL2*) and different regulators of the flavonoid pathway after a re-supply of full nutrient solution to nitrogen-starved *Arabidopsis*. Plants were placed at various temperatures from 5 to 30 °C in darkness, and development in transcript levels were recorded during 4 d. Growth conditions are as in Fig. 4.

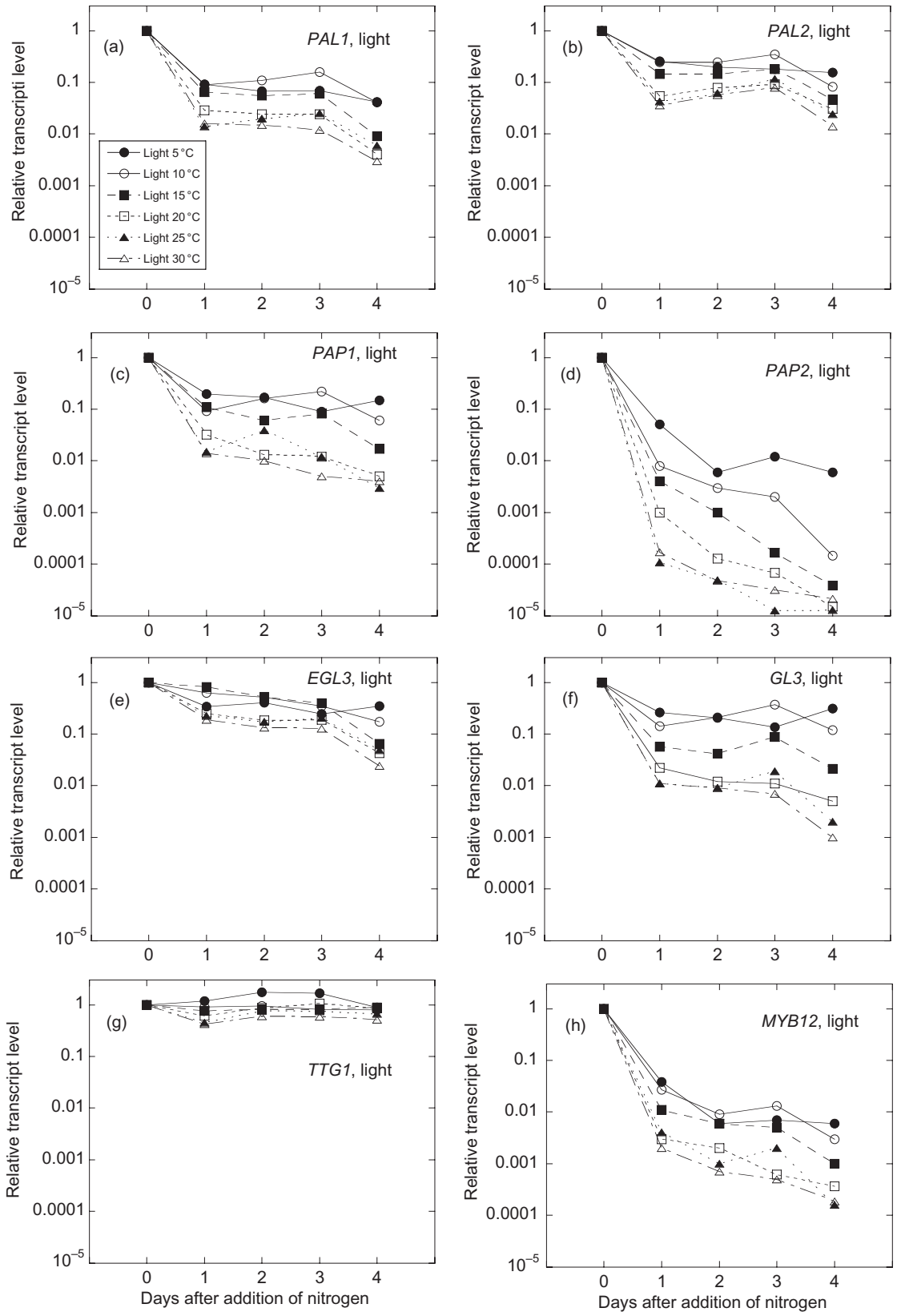

Figure 6. Transcript levels of *phenylalanine ammonia-lyase 1* (*PAL1*) and *phenylalanine ammonia-lyase 2* (*PAL2*) and different regulators of the flavonoid pathway after a re-supply of full nutrient solution to nitrogen-starved *Arabidopsis*. Plants were placed at various temperatures from 5 to 30 °C under standard growth-light conditions, and development in transcript levels were recorded during 4 d. Growth conditions are as in Fig. 4.

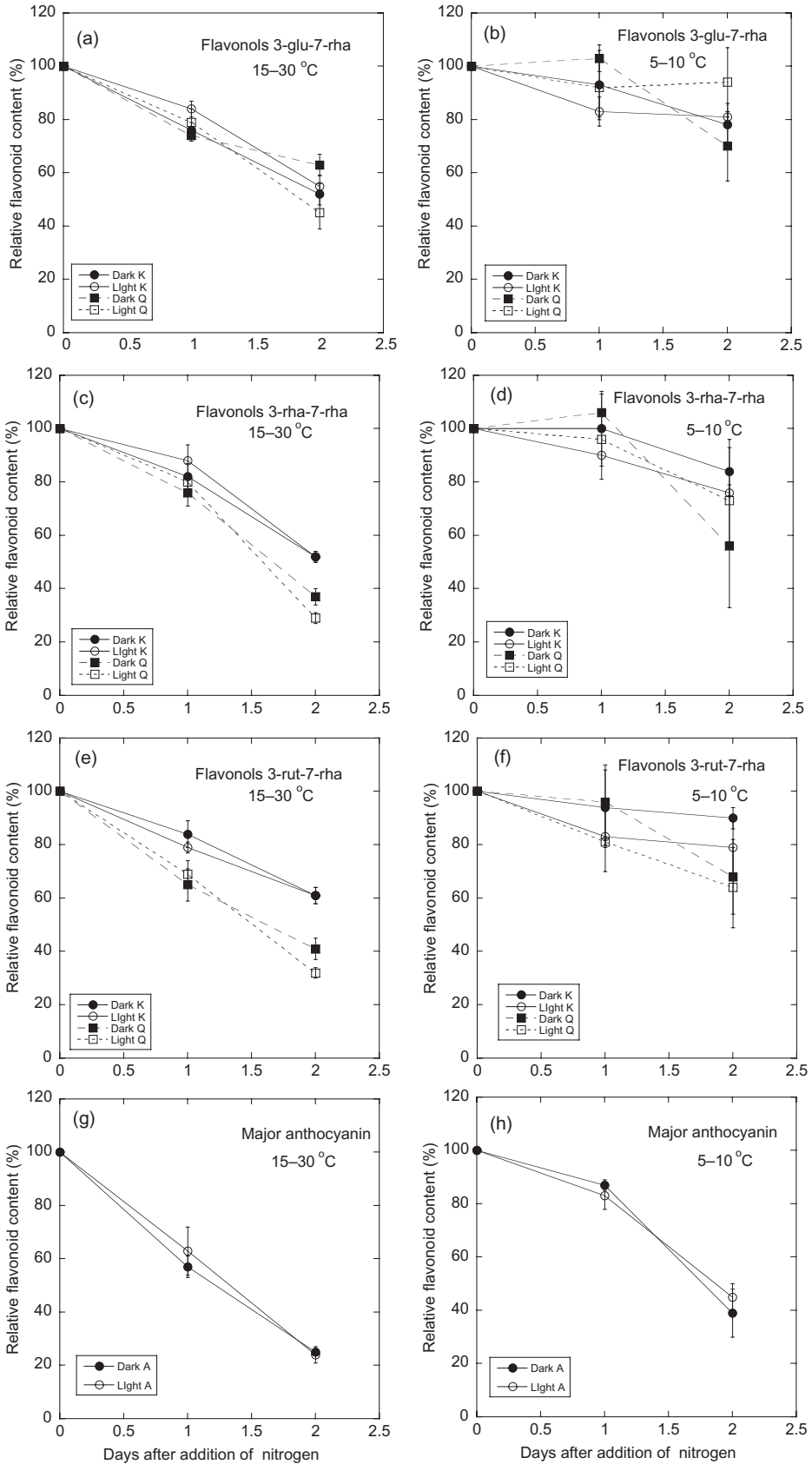

Figure 7. Degradation of different flavonoids after re-supply of full nutrient solution to nitrogen-starved *Arabidopsis*. The data were averaged for the temperature range 15–30 °C (a, c, e, g), and 5–10 °C (b, d, f, h), because variations within these temperatures were not significant. Closed symbols represent plants in continuous darkness, and open symbols plants in standard growth-light conditions. Growth conditions are as in Fig. 5. Standard error is given, $n = 4$ (a, c, e, g), or the spread is given, $n = 2$ (b, d, f, h).

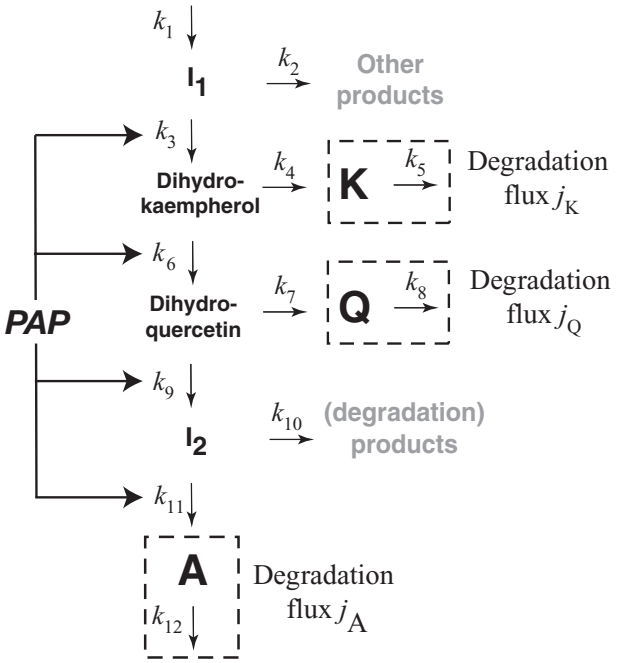

Figure 8. Minimal kinetic model representation of the flavonoid pathways with production and degradation of K (kaempferols), Q (quercetins) and A (anthocyanins).

where I_1, *DHK*, *K*, *Q*, *DHQ*, I_2 and A denote concentrations of intermediate I_1, dihydrokaempferol, kaempferol, quercetin, dihydroquercetin, intermediate I_2 and anthocyanin, respectively. The fluxes for the degradation of K, Q and A are given as $j_K = k_5 K$, $j_Q = k_8 Q$ and $j_A = k_{12} A$, respectively. The temperature dependence of the rate constants k_i are given by the Arrhenius equation $k_i = A_i \exp(-E_i/RT)$, where E_i is the activation energy, and R and T are the gas constant and temperature (in Kelvin), respectively. The pre-exponential factors A_i and the activation energies E_i are assumed to be temperature-independent. To describe the influence of the decrease in *PAP* levels on the rate constants with increasing temperature (Fig. 2a,b), rate constants k_3–k_{11} are formulated as $k_j = f_{PAP}\, A_j \exp(-E_j/RT)$, where $f_{PAP} = 1$ at 5 °C, and decreases by a factor of 0.8706 for each 1 K increase in temperature. This leads to a 50% reduction in k_3, k_6, k_9 and k_{11} for every 5 K increase [i.e. $f_{PAP}(T) = \exp(-\alpha_i T)$ with $\alpha_i = 0.1386\ K^{-1}$] as indicated by the experimentally determined *PAP* levels when nitrogen is supplied (Fig. 2a,b).

The model was analysed under steady-state conditions by setting the rate Eqns 1–7 to zero, and studying the temperature behaviours of the resulting steady-state concentrations K_{ss}, Q_{ss}, A_{ss} and steady-state fluxes $j_{K,ss}$, $j_{Q,ss}$ and $j_{A,ss}$ (a spreadsheet file containing the steady-state orders of the model is available upon request). Although it was not difficult to get rate constant and activation energy combinations that showed a good fit to the experimental steady-state temperature profiles, we were interested in whether the model would also be able to show both a fit to the temperature profiles of the steady-state concentrations as well as to the observed temperature compensation of the degradation fluxes in K, Q and A. Considering the relative simplicity of the model, we found good qualitative/semi-quantitative agreements with experimental results. Figure 9 shows that the model's steady-state temperature profiles for K, Q and A have a close resemblance to the experimental data. Experimental findings (Fig. 7) also described by the model is the presence of temperature compensation in the degradation fluxes of K, Q or A, meaning that these fluxes are approximately constant at different but constant temperatures over a large temperature range. For A, however, temperature compensation in its degradation flux is observed only for a rather narrow temperature window (Supporting Information Table S3). Supporting information Table S4 shows the activation energies and the rate constants at 10 °C. Interestingly, to get a fit to the experimental temperature profiles of the K_{ss}, Q_{ss} and A_{ss} levels, the model predicts that the pathway's temperature sensitivity is largest at its end (where A is made), which appears to be correlated by the fact that at this position in the pathway, its regulation by *PAP* is strongest (see Fig. 3 in Lillo *et al.* 2008).

DISCUSSION

PAP2 and *GL3* were previously found to show increased expression in response to N-depletion in seedlings and leaves of rosette stage *Arabidopsis* plants at standard growth temperature (20 °C) (Lea *et al.* 2007). These same genes showed strong positive responses to lowering the temperature (Fig. 2). Interestingly, the flavonol synthesis regulator *MYB12* also showed a very strong positive response to lowering the temperature. The effect of nitrogen depletion on *MYB12* was previously found to be ambiguous at normal growth temperature (Lea *et al.* 2007), but at the lower temperatures, the positive effects of nitrogen depletion on *MYB12* expression was clear (Fig. 2). *EGL3* showed an abrupt increase in transcript levels when the temperature was lowered to 5 °C. This was in contrast to the other regulators for which a gradual increase in transcript levels was seen when the temperature was lowered through the range of 25–5 °C. This indicates different mechanisms for temperature regulation of EGL3 compared with the other transcription factors tested. It should be noted that in contrast to PAP1 and PAP2, which specifically influence genes involved in flavonoid synthesis (Tohge *et al.* 2005), EGL3 and GL3 transcription factors are also essential for trichome formation and epidermal cell fate (Bernhardt *et al.* 2003).

The three most abundant kaempferols were glycosylated in the same manner as the three most abundant quercetins. The glycosylation pattern did not determine the shape of the curves as a function of growth temperature. Apparently, the essential chemical difference for temperature responses was the hydroxylation of the B-ring, as quercetins generally showed a peak at 10 °C irrespective of glycosylation pattern (Fig. 3). The kaempferols showed a more gradual response to temperature, and a clear increase was seen also at 15 °C compared with 20–30 °C for all three kaempferols analysed (Fig. 3).

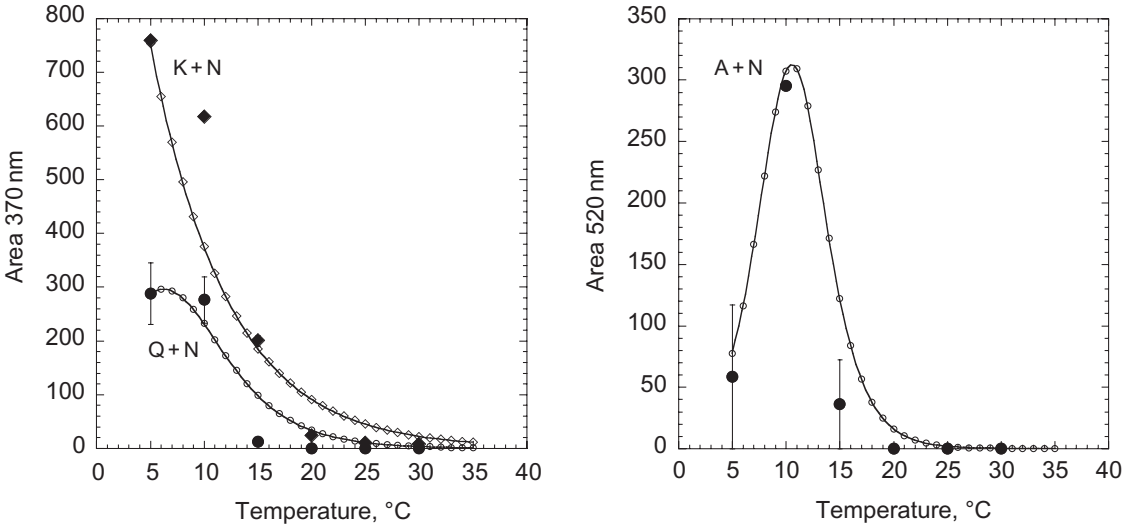

Figure 9. Experimental results (solid symbols with error bars) and calculated steady-state levels (small open symbols with solid lines) for K (kaempferols), Q (quercetins) and A (anthocyanins) as a function of temperature. For parameter values see Supporting Information Table S3.

Synthesis of anthocyanins passed the early seedling stage in *Arabidopsis*, is known to be light-dependent and induction of key enzymes, PAL1 and chalcone synthase, are light-dependent as well during cold acclimation (Leyva *et al.* 1995). The degradation rates observed for anthocyanin did not, however, differ between complete darkness and normal growth-light. Although light is necessary for induction and prolonged synthesis of anthocyanins, there was very little difference in concentration of flavonoids and transcript levels in standard light conditions against darkness after reapplication of nitrogen. Addition of nitrogen, therefore, appeared to be sufficient enough to halt flavonoid synthesis in these experiments.

The few studies on flavonoid degradation that exist are from different plants and tissues, and the results found are strikingly different. For example, in mustard seedlings, when anthocyanin levels reached a high steady-state level, turnover was about 3–6% per day. This was shown by pulse chase experiments with ^{14}C-labelled phenylalanine (Zenner & Bopp 1987). In grape berries, turnover of anthocyanins was measured at three different temperatures by help of ^{13}C-labelling, and showed less than 10% daily turnover at 15 and 25 °C for total anthocyanins, but a 50% turnover rate at 35 °C (Mori *et al.* 2007). In the *Brunfelsia* (yesterday-today-tomorrow) flowers, the colour turns from deep blue into almost white within 2 d, and daily anthocyanin turnover is as high as 70% (Vaknin *et al.* 2005). In our experiments with leaves of rosette stage *Arabidopsis* plants growing in rock wool, the turnover rate for anthocyanins was also very high. In the timespan between 24 and 48 h after reapplication of nitrogen, there was a 60% decrease in the major anthocyanin (Fig. 7g). Most likely, the synthesis of flavonoids is completely halted after addition of nitrogen as PAL activity and regulators of the pathway rapidly reached low levels. However, some remaining synthesis cannot be excluded, which means that a 60% turnover could be a slight underestimation. Whether such a high turnover rate is a steady-state catabolism that may be found under various growth and developmental conditions in *Arabidopsis*, or if the addition of nitrogen influences both synthesis and catabolism, is not yet clarified. The results from *Brunfelsia*, grapes, and *Arabidopsis* clearly show that in different plants, a daily turnover rate of 50% or higher can be found under certain environmental or developmental conditions in different species. At lower temperatures (5 and 10 °C), the decrease in anthocyanins was still rapid in *Arabidopsis* leaves, however, the decrease in flavonols were markedly slower at these temperatures. Kaempferols decreased by only 6–16% and quercetins by 12–35% at 5–10 °C (Fig. 7). This likely reflects slower degradation at these temperatures, but also some remaining activity for synthesis of flavonols may be present.

One of the remarkable findings is the presence of perfect temperature adaptation in the degradation fluxes of kaempferols, quercetins or anthocyanins (Supporting Information Table S3), meaning that these fluxes are approximately constant at different but constant temperatures within a certain temperature interval. Why these degradation fluxes are constant is not understood. Temperature compensation of physiological processes have been observed in a variety of instances such as for the oxygen consumption in inter-tidal organisms (Hazel & Prosser 1974), the CO_2 uptake in plants adapted to cool climates (Berry & Björkman 1980) or in circadian or other clock-related rhythms (Rensing & Ruoff 2002).

The molecular mechanisms (Hastings & Sweeney 1957; Hazel & Prosser 1974) behind temperature compensation are not well understood, although some general statements within the context of metabolic control theory (Kacser & Burns 1973; Heinrich & Schuster 1996; Fell 1997) can be made (Ruoff 1992; Ruoff, Zakhartsev & Westerhoff 2007) and have been applied to the biological clock (Ruoff, Loros

& Dunlap 2005). If j_X represents any of the degradation fluxes for kaempferols, quercetins or anthocyanins, its temperature dependence can be described in the form of equation

$$\frac{d\ln j_X}{dT} = \frac{1}{RT^2}\sum_i C_{k_i}^{j_X}(E_i - RT^2\alpha_i) \quad (8)$$

where the α_i's describe the temperature dependence of the f_{PAP}'s by

$$k_i = f_{PAP}A_i\exp(-E_i/RT) = \exp(-\alpha_i T)A_i\exp(-E_i/RT) \quad (9)$$

where $\alpha_i = 0.1386\ K^{-1}$ for $i \in (3, 6, 9, 11)$ describing a 50% reduction in these rate constants by an increase of five degrees, while $\alpha_i = 0$ for all other i's. The $C_{k_i}^{j_X}$s are the control coefficients (Heinrich & Schuster 1996; Fell 1997) defined as $C_{k_i}^{j_X} = \partial\ln j_X/\partial\ln k_i$ and given in Supporting information Table S5. Because the control coefficients are both positive and negative, certain combinations in the activation energies will allow that positive and negative contributions in Eqn 8 to balance, such that $d\ln j_X/dT = 0$, leading to temperature compensation in flux j_X. Another mechanistic way to explain the temperature compensation of the degradation fluxes is that the apparent activation energies for each of the temperature compensated degradation fluxes are zero. This may be achieved by an enzymatic process where the degradation flux is described by a Michaelis–Menten-type of degradation kinetics

$$j_X = \frac{V_{max}X}{K_m + X} \quad (10)$$

but where the temperature dependences of V_{max} and K_m balance each other in a similar way as for the negative and positive contributions in Eqn 8 (Ruoff, Vinsjevik & Rensing 2000).

Degradation of flavonoids is largely unexplored, and it appears that the set-up with nitrogen depletion followed by re-supply can be chosen as a useful experimental system for further examining and understanding the regulation of degradation of flavonoids in *Arabidopsis*.

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REFERENCES

Bernhardt C., Lee M.M., Gonzalez A., Zhang F., Lloyd A. & Schiefelbein J. (2003) The bHLH genes *GLABRA3* (*GL3*) and ENHANCER OF GLABRA3 (EGL3) specify epidermal cell fate in the *Arabidopsis* root. *Development* **130,** 6431–6439.

Berry J. & Björkman O. (1980) Photosynthetic response and adaptation to temperature in higher plants. *Annual Review of Plant Physiology* **31,** 491–543.

Besseau S., Hoffmann L., Geoffroy P., Lapierre C., Pollet B. & Legrand M. (2007) Flavonoid accumulation in *Arabidopsis* repressed in lignin synthesis affects auxin transport and plant growth. *The Plant Cell* **19,** 148–162.

Bloor S.J. & Abrahams S. (2002) The structure of the major anthocyanin in *Arabidopsis thaliana*. *Phytochemistry* **59,** 343–346.

Cheynier V. (2006) Flavonoids in wine. In *Flavonoids. Chemistry, Biochemistry and Applications* (eds Ø.M. Andersen & K.R. Markham), pp. 263–318. CRC Taylor & Francis, Boca Raton, FL, USA.

Fell D. (1997) *Understanding the Control of Metabolism*. Portland Press, London, UK and Miami, FL, USA.

Ferrario-Mery S., Meyer C. & Hodges M. (2008) Chloroplast nitrite uptake is enhanced in *Arabidopsis* PII mutants. *FEBS Letters* **582,** 1061–1066.

Gachon C.M.M., Langlois-Meurinne M. & Saindrenan P. (2005) Plant secondary metabolism glycosyl transferases: the emerging functional analysis. *Trends in Plant Science* **10,** 542–549.

Graham T.L. (1998) Flavonoid and flavonol glycoside metabolism in *Arabidopsis*. *Plant Physiology and Biochemistry* **36,** 135–144.

Hannah M.A., Wiese D., Freund S., Fiehn O., Heyer A.G. & Hincha D.K. (2006) Natural genetic variation of freezing tolerance in Arabidopsis. *Plant Physiology* **142,** 98–112.

Hasdai M., Weiss B., Levi A., Samach A. & Porat R. (2006) Differential responses of *Arabidopsis* ecotypes to cold, chilling and freezing temperatures. *Annals of Applied Botany* **148,** 113–120.

Hastings J.W. & Sweeney B.M. (1957) On the mechanism of temperature independence in a biological clock. *Proceedings of the National Academy of Sciences of the United States of America* **43,** 804–811.

Hazel J.R. & Prosser C.L. (1974) Molecular mechanisms of temperature compensation in *poikilotherms.* Physiological Reviews **54,** 620–677.

Heim K.E., Tagliaferro A.R. & Bobilya D.J. (2002) Flavonoid antioxidants: chemistry, metabolism and structure-activity relationships. *Journal of Nutritional Biochemistry* **13,** 572–584.

Heinrich R. & Schuster S. (1996) *The Regulation of Cellular Systems*. Chapman and Hall, New York, NY, USA.

Hoagland D. & Arnon D. (1950) The water-culture method for growing plants without soil. *California Agricultural Experimental Station Circular* **347,** 1–32.

Kacser H. & Burns J.A. (1973) The control of flux. *Symposium of the Society for Experimental Biology* **27,** 65–104.

Lea U.S., Slimestad R., Smedvig P. & Lillo C. (2007) Nitrogen deficiency enhances expression of specific MYB and bHLH transcription factors and accumulation of end products in the flavonoid pathway. *Planta* **225,** 1245–1253.

Leyva A., Jarillo J.A., Salinas J. & Martinez-Zapater J.M. (1995) Low temperature induces the accumulation of phenylalanine ammonia-lyase and chalcone synthase mRNAs of *Arabidopsis* thaliana in a light-dependent manner. *Plant Physiology* **108,** 39–46.

Lillo C., Lea U.S. & Ruoff P. (2008) Nutrient depletion as a key factor for manipulating gene expression and product formation in different branches of the flavonoid pathway. *Plant, Cell & Environment* **31,** 587–601.

Mori K., Goto-Yamamoto N., Kitayama M. & Hashizume K. (2007) Loss of anthocyanins in red-wine grape under high temperature. *Journal of Experimental Botany* **58,** 1935–1945.

Olsen K.M., Lea U.S., Slimestad R., Verheul M. & Lillo C. (2008) Differential expression of the four *Arabidopsis PAL* genes; *PAL1* and *PAL2* have functional specialization in abiotic environmental triggered flavonoid synthesis. *Journal of Plant Physiology* **165,** 1491–1499.

Peng M., Hudson D., Schofield A., Tsao R., Yang R., Gu H., Bi Y.M. & Rothstein S.J. (2008) Adaptation of *Arabidopsis* to nitrogen limitation involves induction of anthocyanin synthesis which is

controlled by the NLA gene. *Journal of Experimental Botany* **59,** 2933–2944.
Raes J., Rohde A., Christensen J.H., Van de Peer Y. & Boerjan W. (2003) Genome-wide characterization of the lignification toolbox in Arabidopsis. *Plant Physiology* **133,** 1051–1071.
Rensing L. & Ruoff P. (2002) Temperature effect on entrainment, phase shifting, and amplitude of circadian clocks and its molecular bases. *Chronobiology International* **19,** 807–864.
Ruoff P. (1992) Introducing temperature-compensation in any reaction kinetic oscillator model. *Journal of Interdisciplinary Cycle Research* **23,** 92–99.
Ruoff P., Vinsjevik M. & Rensing L. (2000) Temperature compensation in biological oscillators: a challenge for joint experimental and theoretical analysis. *Comments on Theoretical Biology* **5,** 361–382.
Ruoff P., Loros J.J. & Dunlap J.C. (2005) The relationship between FRQ-protein stability and temperature compensation in the Neurospora circadian clock. *Proceedings of the National Academy of Sciences of the United States of America* **102,** 17681–17686.
Ruoff P., Zakhartsev M. & Westerhoff H.V. (2007) Temperature compensation through systems biology. *FEBS Journal* **274,** 940–950.
Saunders J.A. & McClure J.W. (1974) The suitability of a quantitative spectrophotometric assay for phenylalanine ammonia-lyase activity in barley, buckwheat, and pea seedlings. *Plant Physiology* **54,** 412–413.
Steyn W.J., Holcroft D.M., Wand S.J.E. & Jacobs G. (2004) Anthocyanin degradation in detached pome fruit with reference to preharvest red color loss and pigmentation patterns of blushed and fully red pears. *Journal of the American Society for Horticultural Science* **129,** 13–19.
Templeton G.W. & Moorhead G.B. (2004) A renaissance of metabolite sensing and signaling: from modular domains to riboswitches. *The Plant Cell* **16,** 2252–2257.
Thingnaes E., Torre S., Ernstsen A. & Moe R. (2003) Day and night temperature responses in Arabidopsis: effects on gibberellin and auxin content, cell size, morphology and flowering time. *Annals of Botany (London)* **92,** 601–612.
Tohge T., Nishiyama Y., Hirai M.Y., *et al.* (2005) Functional genomics by integrated analysis of metabolome and transcriptome of *Arabidopsis* plants over-expressing an MYB transcription factor. *The Plant Journal* **42,** 218–235.
Vaknin H., Bar-Akiva A., Ovadia R., Nissim-Levi A., Forer I., Weiss D. & Oren-Shamir M. (2005) Active anthocyanin degradation in *Brunfelsia calycina* (yesterday – today – tomorrow) flowers. *Planta* **222,** 19–26.
Veit M. & Pauli G.F. (1999) Major flavonoids from *Arabidopsis* thaliana leaves. *Journal of Natural Products* **62,** 1301–1303.
Vogel J.T., Zarka D.G., Van Buskirk H.A., Fowler S.G. & Thomashow M.F. (2005) Roles of the CBF2 and ZAT12 transcription factors in configuring the low temperature transcriptome of *Arabidopsis*. *The Plant Journal* **41,** 195–211.
Winkel-Shirley B. (2002) Biosynthesis of flavonoids and effects of stress. *Current Opinion in Plant Biology* **5,** 218–223.
Zenner K. & Bopp M. (1987) Anthocyanin turnover in *Sinapsis albas* L. *Journal of Plant Physiology* **126,** 475–482.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Figure S1. Transcript levels after re-supply of full nutrient solution at day 0.
Table S1. Chromatographic and spectrometric values used to characterise major flavonols in *Arabidopsis* leaves.
Table S2. Activation energies for degradation of specific flavonoids.
Table S3. Model calculations of K, Q and A degradation fluxes and their temperature-compensated regions.
Table S4. Rate constant values (a.u.) and activation enthalpies (in kJ mol^{-1}) used in the model[b].
Table S5. Control coefficients for steady-state fluxes $j_{k,ss}$, $j_{Q,ss}$ and $j_{A,ss}$.

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