

Nitrogen deficiency enhances expression of specific MYB and bHLH transcription factors and accumulation of end products in the flavonoid pathway

Unni S. Lea · Rune Slimestad · Pål Smedvig · Cathrine Lillo

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Abstract Expression of regulators of the flavonoid pathway was examined in *Arabidopsis thaliana* wild type and *pap1D* plants, the latter being a T-DNA activation-tagged line over-expressing the *PAP1/MYB75* gene which is a positive regulator of the pathway. Anthocyanin accumulation was induced in plants grown in soil, on agar plates, and hydroponics by withdrawing nitrogen from the growth medium. The agar-grown seedlings and rosette stage plants in hydroponics were further explored, and showed that nitrogen deficiency resulted in the accumulation of not only anthocyanins, but also flavonols. The examination of transcript levels showed that the general flavonoid pathway regulators *PAP1* and *PAP2* were up-regulated in response to nitrogen deficiency in wild type as well as *pap1D* plants. Interestingly, *PAP2* responded much stronger to nitrogen deficiency than *PAP1*, 200- and 6-fold increase in transcript levels, respectively, for wild-type seedlings. In rosette leaves the increase was 900-fold for *PAP2* and 6-fold for *PAP1*. At least three different bHLH domain transcription factors promote anthocyanin synthesis, and transcripts for one of these, i.e. *GL3* were found to be sixfold enhanced by nitrogen deficiency in rosette leaves. The MYB12 transcription factor, known to regulate flavonol synthesis, was

slightly induced by nitrogen deficiency in seedlings. In conclusion, four out of eight regulators involved in the flavonoid pathway showed an enhanced expression from 2 to 1,000 times in response to nitrogen deficiency. Together with MYB factors, especially *PAP2*, *GL3* appears to be the BHLH partner for anthocyanin accumulation in response to nitrogen deficiency.

Keywords Anthocyanins · *Arabidopsis* · BHLH · Flavonoids · MYB · Nitrogen deficiency

Abbreviations

BHLH	Basic helix-loop-helix domain
EGL3	Enhancer of GLABRA3
GL3	GLABRA3
MYB	DNA-binding domain
PAL	Phenylalanine ammonium lyase
PAP1(2)	Production of anthocyanin pigment 1 (2)
TTG1	Transparent TESTA GLABRA1
TT8	Transparent TESTA8
WT	Wild type

Introduction

Flavonoids and other polyphenolic compounds have a wide range of functions in plants related to, for instance, UV-absorption, pathogen attack, low temperature and nutrient stress. It is clear that some polyphenolic compounds like sinapoyl esters and flavonols are important protectants against UV-stress, but often the biological functions of flavonoids are not known (Kliebenstein 2004). Flavonoids have also inspired the development of medical treatments, and are recognised as important for human nutrition. Growth conditions

U. S. Lea · P. Smedvig · C. Lillo (✉)
Faculty of Science and Technology,
University of Stavanger, 4036 Stavanger, Norway
e-mail: cathrine.lillo@uis.no

R. Slimestad
PlantChem, Sæverheim Research Center,
4353 Klepp stasjon, Norway

can be manipulated in order to produce crops rich in various flavonoids; for instance, it has been shown that nutrient stress increased the concentrations of flavonols in tomato leaves. Although the flavonol levels in tomato fruits did not change, nutrient stress as a way of directing the plant to accumulate flavonoids in vegetative crops seems feasible (Stewart et al. 2001), as is also metabolic engineering as a tool to change the content of flavonoids (Martens et al. 2003; Schijlen et al. 2004). A combination of genetic engineering and environmental factors may also synergistically promote the accumulation of certain compounds as was shown for the *pap1D* mutant of *Arabidopsis* for which anthocyanin accumulation was strongly stimulated by sucrose or UV light/high light intensity (Borevitz et al. 2000; Wade et al. 2003).

The synthesis of flavonoids, including anthocyanins, is governed by regulatory proteins of different families. In *Arabidopsis* the WD repeat protein TTG1 appears to be on top of the hierarchy and controls the network by interacting with several BHLH and MYB proteins (Zimmermann et al. 2004; Baudry et al. 2004). In the *ttg1* mutant anthocyanins are not synthesised, neither are proanthocyanidins. Three BHLH genes especially important for flavonoid synthesis have been identified: *EGL3*, *GL3* and *TT8*, and the triple mutant *egl3-1 gl3-1 tt8-1* are essentially phenotypically indistinguishable from *ttg1* mutants (Zhang et al. 2003). Ectopic co-expression of *GL3/EGL3* suppresses the *ttg1* phenotype with respect to trichome defect and seed coat pigment (Zhang et al. 2003). The positive regulation of anthocyanin synthesis by MYB proteins has been shown in several species (Koes et al. 2005), and for *Arabidopsis* this was demonstrated by activation tagging, quantitative-trait locus analysis (QTL), and transposon insertion in the *PAP1* gene (Borevitz et al. 2000; Teng et al. 2005). Furthermore, the BHLH proteins interact with MYB proteins PAP1 and PAP2 (Zimmermann et al. 2004). Recently another MYB factor, MYB12, was shown to be important for flavonol synthesis in *Arabidopsis* (Mehrtens et al. 2005). Often expression of a gene in the pathway is regulated by more than one of these transcription factors. It is not clear as to why many transcription factors are involved, but it may be anticipated that they contribute to a complex regulation where different transcription factors can respond to different stimuli, for instance different nutrients, UV-light, and pathogens.

There are reports showing that nitrogen deficiency induces increased levels of anthocyanins and other flavonoids in *Arabidopsis* (Hsieh et al. 1998; Martin et al. 2002; Scheible et al. 2004), but how nitrogen deficiency promotes high concentration of certain flavonoids is not

clear. Phenylalanine is a key amino acid at the interphase of primary and secondary metabolism. Phenylalanine ammonium lyase (PAL) is directing flux into secondary metabolism, and at the same time releases ammonium which is, however, generally re-assimilated. PAL is a highly regulated gene/enzyme influenced by various environmental factors (Weaver and Herrmann 1997). Possibly, nitrogen deficiency could partly act through regulation of PAL, but this was not tested in the present work. In this work we examined nitrogen effects on expression of the various regulators in the flavonoid pathway, i.e. down-stream of PAL. The results revealed that nitrogen deficiency resulted in enhanced transcript levels of certain MYB and BHLH genes important for accumulation of anthocyanins and flavonols, while other regulators did not appear to be involved in sensing of nitrogen status.

Materials and methods

Plant material

Seeds of *Arabidopsis thaliana* ecotype Columbia (Col1) and *pap1-D* in the Columbia background were obtained from the Nottingham Arabidopsis Stock Centre. Seeds were surface-sterilised by immersion for 5 min in 0.1% Ca-hypochlorite and 0.01% Triton-X-100 in 70% ethanol. The solution was discarded and the seeds washed twice with 95% ethanol and left to dry over-night in the hood. Seeds were sown on agar medium in Petri dishes on half-strength Murashigie and Skoog (MS) salts (Stewart et al. 2001) containing 3% sucrose, or this same medium lacking nitrogen and/or sucrose, or sown directly into soil. Plates/pots were left at 5°C in the dark for 3 days before placed under constant incandescent light (50 cm below Osram Fluora L33W/77) at 25°C. Samples were harvested after 4 days for analysis of transcripts, and after 7 days for analysis of both transcripts and flavonoids. Plants were also grown in hydroponics. Seeds were sown on half-strength Hoagland (Hoagland and Arnon 1950) containing 0.75% agar in Eppendorf tubes in continuous light. After approximately 3 weeks, plants were transferred to complete Hoagland solution for further cultivation in a 12 h light/dark regimen. One week later plants were divided into two groups and placed in either complete Hoagland or Hoagland without nitrogen. The first sample was harvested before solution was changed, and then 4 and 7 days after the change. Samples for transcript and flavonoids analysis were harvested at all three points of time.

Anthocyanin measurement

Plant tissue (0.05 g) was extracted overnight at 4°C under shaking in 300 µl of 1% (v/v) hydrochloric acid in methanol. Distilled water (200 µl) was added to the extract and mixed. Chloroform (500 µl) was added, mixed and centrifuged at 15,000g for 2 min. The top layer (400 µl) was transferred to a fresh microtube, and 600 µl of 1% (v/v) hydrochloric acid in methanol was added and centrifuged to remove particles. The supernatant was used for absorbancy measurements at 530 and 657 nm. Relative anthocyanin concentrations were calculated as absorbancy at 530 nm minus absorbancy at 657 nm (modified after Martin et al. 2002). Alternatively (Fig. 3a, b), anthocyanins were identified after separation by HPLC as described below for other flavonoids.

Measurements of phenolic compounds

About 0.1 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 HPLC analyses. A liquid chromatograph (Agilent 1100-system, Agilent Technologies, Cheshire, UK) supplied with an autosampler and a photodiodearray detector was used for the analysis of individual phenolics. The phenolics were separated on an Eclipse XDB-C8 (4.6 × 150 mm, 5 µm) column (Agilent Technologies) by using a binary solvent system consisting of (A) 0.05% TFA in water and (B) 0.05% TFA in acetonitrile. The gradient (%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, 10 µl samples were injected on the column, and separation took place at 30°C. Detection was made over the interval 230–600 nm in steps of 2 nm in order to obtain full absorbance spectrum of the compounds of interest, whereas quantitative data were obtained at 370 nm.

The individual peaks were characterised as derivatives of kaempferol, quercetin, sinapic acid ester and anthocyanins according to their UV absorbance spectra (Mabry et al. 1970; Wintersohl et al. 1979; Veit and Pauli 1999; Lehfeltdt 2001). In addition, acid hydrolysis of the compounds was performed according to Markham (1982). The hydrolysed products were determined by co-chromatography with authentic kaempferol, quercetin and sinapic acid (Carl Roth GmbH, Karlsruhe,

Germany). Peak areas were adjusted to exact sample weights, and pooled together as kaempferols, quercetins, sinapic acid esters or anthocyanins for the individual samples, respectively.

RT-PCR

Total RNA was isolated using RNeasy® Plant Mini Kit (Qiagen, Chatsworth, CA, USA). RNA was quantified by the spectrophotometer and cDNA synthesised 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 (Applied Biosystems). The reaction volume was 25 µl containing 12.5 µl TaqMan buffer (includes ROX as a passive reference dye), 8.75 µl H₂O, 2.5 µl cDNA and 1.25 µl primers. Primers were pre-designed TaqMan® Gene Expressions assays (Applied Biosystems) 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_g1), *TTG1* At5g24520 (At02333810_m1), *TT8* At4g09820 (At02298669_m1), *ACT8* At1g49240 (At02270958_gH), *MYB12* At2g47460 (At02264273_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 C_T method for relative quantification has been used with ubiquitin as endogenous control and WT grown for 4 days in half-strength MS medium with 3% sucrose as calibrator (Fig. 2), and WT on the day of change of nutrient solution (day 0, Fig. 3). Relative quantity (RQ = 2^{-ΔΔCT}) of any gene is given as percent.

Results

Plants in soil

When seeds had been germinated and grown in soil watered with a complete nutrient solution (Hoagland) in 12 h dark/12 h light for 14 days, anthocyanin content was low in WT, but considerably higher in *pap1D* (Fig. 1 a, black bars). When nitrogen was omitted from the nutrient solution anthocyanins accumulated in both

genotypes (Fig. 1a, grey bars). When nitrogen was omitted, but plants were kept in dim light, anthocyanins were not detected in WT, and the concentration in *pap1D* was very low (Fig. 1a, for WT not above baseline, for *pap1D* white bar). Hence nitrogen deficiency alone was not sufficient to induce anthocyanin accumulation but high-intensity light was a prerequisite for anthocyanin accumulation.

Seedlings on agar plates

Although sucrose induced high anthocyanin content in 7-day-old seedlings grown on MS medium without nitrogen (Fig. 1b), sucrose could not fully replace the requirement for light because WT seedlings grown in the dark on these agar plates had no anthocyanins even in the absence of nitrogen (data not shown). Also, *pap1D* had very low levels of anthocyanins when grown in the dark, i.e. less than 10% of values for plants in the light (data not shown). Figure 1c, d shows the relative content of flavonols in WT and *pap1D* seedlings grown on agar plates in the light with MS salts or MS salts minus nitrogen. An addition of 3%

sucrose to the growth medium was essential for high concentrations of anthocyanins, as previously shown by Teng et al. (2005), and flavonols in WT as well as *pap1D* seedlings (Fig. 1b–d). In WT seedlings nitrogen deficiency in combination with sucrose was necessary for the accumulation of anthocyanins, whereas nitrogen deficiency had no effect on anthocyanin accumulation in *pap1D* seedlings under these conditions (Fig. 1b). Nitrogen deficiency apparently did increase the level of flavonol conjugates in both genotypes, and the effect was more pronounced for quercetin compared with kaempferol-type compounds. According to the *t* test at the 0.05 level the effect of nitrogen deficiency was, however, only significant for quercetin in WT, whereas the sucrose effect was very clear for both WT and *pap1D* for both types of flavonols.

Transcript levels of recognised regulator genes of flavonoid synthesis as well as the enigmatic *PII* gene thought to be involved in nitrogen and sucrose sensing or regulation were tested in WT and *pap1D* seedlings grown on agar (with sucrose) with or without nitrogen (Fig. 2). Seedlings were tested on day 7 as in Fig. 1, and also at an earlier stage, on day 4, because changes in

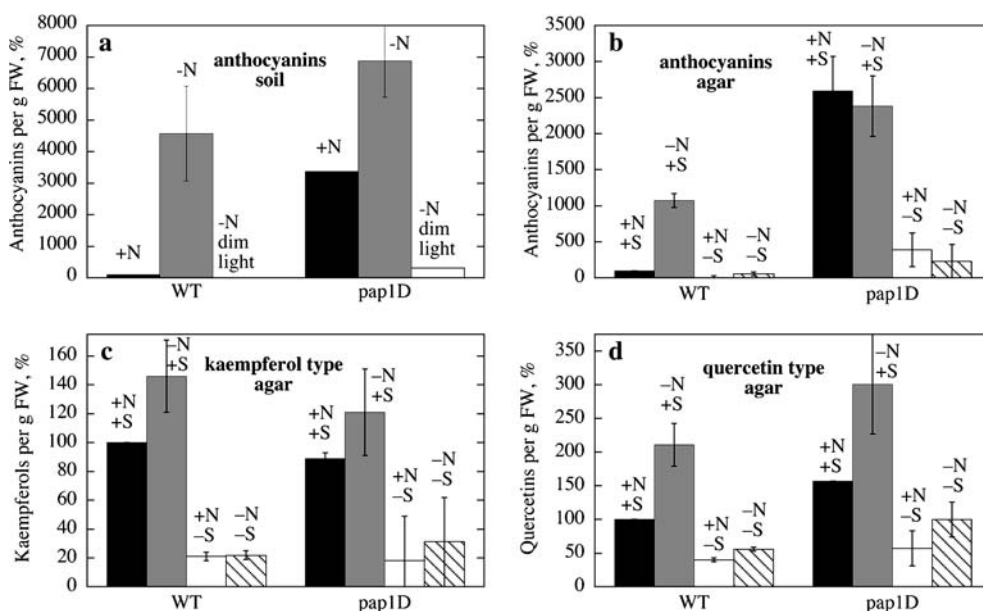


Fig. 1 a–d Flavonoid content in *Arabidopsis* WT and *pap1D* seedlings grown in soil or on agar plates. **a** Relative content of anthocyanins in seedlings grown in soil. Seedlings were grown for 2 weeks in soil in a growth chamber under fluorescent lamps, and watered with a complete nutrient solution (black bars) or nutrient solution lacking nitrogen (grey bars), or nutrient solution lacking nitrogen and kept under dim light (white bars). Data are presented relative to values obtained for WT growing in soil and given complete nutrient solution (100%). There were two biological parallels. The range is given. **b–d** Relative content of flavonoids in seedlings grown on agar plates: anthocyanins (**b**), kaempferol

type (**c**), and quercetin type flavonols (**d**). Seedlings were grown for 1 week in continuous light on half-strength MS salts with 3% sucrose, or medium where nitrogen and/or sucrose was omitted. Complete MS salts plus sucrose (black bars), plus sucrose but without nitrogen (grey bars), complete MS salts without sucrose (white bars), without sucrose and without nitrogen (hatched bars). Data (mean values \pm SE) are presented relative to values obtained for WT growing on complete salts plus sucrose (100%). The number of biological parallels was six for anthocyanins, five for flavonols

gene expression can be expected to occur before changes in the concentrations of compounds which accumulate as a result of gene expression. The *PII* transcript decreased under nitrogen deficiency in both WT and *pap1D* (Fig. 2a, b). The transcript level of *TTG1* was not significantly influenced by nitrogen content in the growth medium (Fig. 2a, b). The *BHLH* gene *TT8* was tested, but apparently expression was below detection limit, and withdrawal of nitrogen did not lead to detectable levels of *TT8* transcript. Transcript levels of the *BHLH* gene *EGL3* were not influenced by nitrogen deficiency. On the contrary *GL3*, also a bHLH domain transcription factor, showed increased expression in response to nitrogen deficiency in both WT and *pap1D* seedlings (Fig. 2a, b). Expression of the *MYB12* transcription factor increased in response to nitrogen deficiency as seen especially on day 4 for WT as well as *pap1D* seedlings. Generally, the response to nitrogen deficiency on the transcript levels was stronger in 4-day-old compared to 7-day-old plants, and for *MYB12* the effect was no longer significant in 7-day-old seedlings.

The *PAP1* transcript level was higher in *pap1D* than WT plants as expected. On day 4 the *PAP1* transcript level was 6 and 1.7 times higher in nitrogen-deficient WT and *pap1D* seedlings, respectively, compared with seedlings on complete MS medium. On day 7 there were not much difference in *PAP1* transcripts related to nitrogen. *PAP2* transcripts were clearly induced by nitrogen deficiency in both WT and *pap1D* (Fig. 2c, d). In fact *PAP2* was the one gene tested that was most strongly enhanced by withdrawal of nitrogen. At day 4 the *PAP2* transcript level was enhanced about 200-fold in WT as well as *pap1D*. The expression level of *PAP1* was generally higher than for *PAP2* when plants were growing in a complete nutrient medium as seen in WT by the C_T values 29.0 and 31.2 for *PAP1* and *PAP2*, respectively. (C_T is the cycle number at which the fluorescence passes the threshold; a low C_T value is related to a high number of mRNA copies of the gene in question.) However, when nitrogen was withdrawn, these values were 26.8 and 25.4 showing that *PAP2* was not only strongly induced but also more strongly expressed than *PAP1* in nitrogen-deficient seedlings.

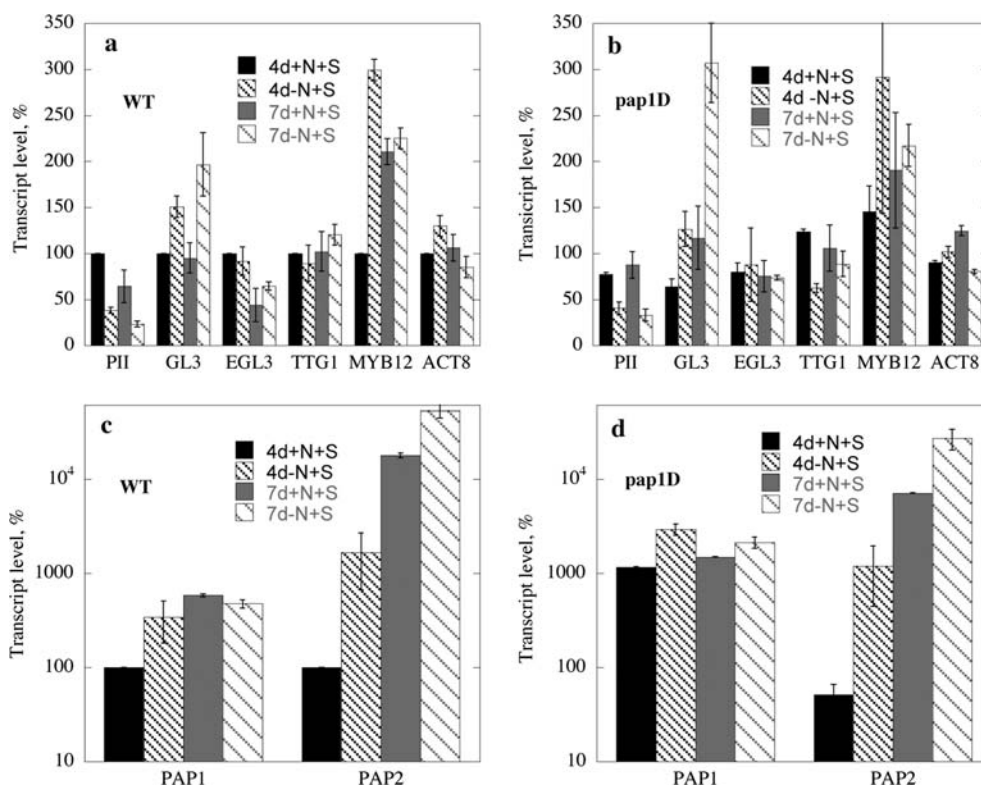


Fig. 2 a–d Relative levels of transcripts in *Arabidopsis* WT and *pap1D* seedlings grown on agar with sucrose, and with or without nitrogen. Transcript levels of *PII*, *GL3*, *EGL3*, *TTG1*, *MYB12* and *ACT8*, in WT (a) and *pap1D* (b). Transcript levels of *PAP1* and *PAP2* in WT (c) and *pap1D* (d). Seedlings were grown on half-strength MS salts with 3% sucrose for 4 days with nitrogen (black bars) or without nitrogen (narrow hatched bars), and for

7 days on half-strength MS salts with 3% sucrose with nitrogen (grey bars) or without nitrogen (hatched bars). Data (mean values \pm SE) are presented as percent of the RQ-value ($RQ = 2^{-\Delta\Delta CT}$) where WT grown for 4 days with nitrogen is used as calibrator and ubiquitin as endogenous control. Transcript assays were analysed on three biological parallels. Note logarithmic scale in c and d

Leaves from rosette stage plants in hydroponics

To test if the response to nitrogen deficiency in agar-grown seedlings was of a more general type, leaves from plants at the rosette stage growing in hydroponics were also tested. In contrast to the seedlings, these plants were not fed sucrose. The data confirmed that anthocyanins accumulated in WT and *pap1D* in response to nitrogen withdrawal, and that anthocyanin content was always relatively high in the *pap1D* mutant also in the presence of nitrogen as expected (Fig. 3a, b). A positive response to nitrogen deficiency was seen also for kaempferol and quercetin levels (Fig. 3a, b). Leaves from WT plants did not show measurable amounts of quercetin.

Generally, the pattern of transcript changes was similar in the leaves from rosette stage plants and seedlings. For the rosette stage plants the increase in *GL3* transcript in response to nitrogen withdrawal was even more pronounced, and transcript levels increased sixfold in WT as well as *pap1D* plants. For the *PAP2* gene the increase was 900 and 1,100 times (averaged for day 4 and 7) for WT and *pap1D*, respectively, and confirmed that the largest change was found for the *PAP2*, not *PAP1* which showed at most sixfold increase. A strong induction of *PAP2* contra *PAP1* transcripts, although to a smaller extent, in response to nitrogen deficiency was also previously shown by Scheible et al. (2004). The increase for the *MYB12* transcript was only about 30%. The *PII* transcript was the only transcript not confirming the trend found in seedlings because for rosette stage leaves the *PII* transcript increased in response to nitrogen deficiency.

Discussion

Nitrogen deficiency increased the levels of both anthocyanins, and flavonol conjugates in *Arabidopsis* (Figs. 1, 3) showing that nitrogen starvation influence different branches of the flavonoid pathway. Interestingly, the nitrogen effect on anthocyanin accumulation was over-ridden by the *pap1D* phenotype when tested in 7-day-old seedlings on agar plates (Fig. 1b). This indicates that nitrogen deficiency may exert its effects (partly) through *PAP* gene(s). Although changes in flavonol content was observed in response to nitrogen limitation, the effects on anthocyanins were more pronounced; hence nitrogen appears to influence the anthocyanin synthesis branch of the pathway most strongly. The effects were recorded for plants at the seedling stage as well as rosette leaves stage. (Figs. 1, 3a, b).

Sucrose-induced anthocyanin accumulation has been observed in many plant species, and sucrose was clearly very important for inducing anthocyanin accumulation in agar-grown seedlings in WT as well as *pap1D* seedlings (Fig. 1). However, in WT seedlings sucrose alone was not sufficient for the accumulation of anthocyanins, but was triggered by additional lack of nitrogen. Teng et al. (2005) recently identified the *PAP1* gene as an important QTL for sugar-induced anthocyanin induction in *Arabidopsis*, and found that *PAP1* only, not *PAP2*, was essential for sucrose-induced anthocyanin induction. Furthermore, the work by Solfanelli et al. (2006) using Affymetrix chips for testing sugar effects on gene expression in *Arabidopsis* seedlings showed *PAP1* to be strongly induced whereas *PAP2* was not induced by sucrose. *PAP2* was induced in the *pho3* mutant which accumulate sucrose, but *pho3* accumulates also other compounds that could be the signal for *PAP2* induction (Lloyd and Zakhleniuk 2004). Both *PAP1* and *PAP2* were induced in senescent *Arabidopsis* leaves and in response to glucose/low nitrogen treatment (Pourtau et al. 2006). Two of the same transcription factors, *PAP1* and *PAP2*, tested in the present work were also analysed by Scheible et al. (2004) using RT-PCR. Scheible et al. found that nitrogen deficiency in seedlings in liquid culture resulted in 166 times increase in *PAP2* and 28 times increase for *PAP1* transcripts. In the present work the effect of nitrogen deficiency on *PAP1* and *PAP2* expression was consistent in seedlings and rosette leaves with the stronger effect in rosette leaves where *PAP2* transcripts increased about 1,000-fold in WT as well as *pap1D* plants, whereas *PAP1* increased sixfold in WT, and very little in *pap1D*. Altogether from the recent literature and the experiments presented here, *PAP1* and *PAP2* appear to respond differently to sugar and nitrogen signals. Apparently, both genes respond positively to glucose, but *PAP1* is much more strongly induced by sucrose whereas *PAP2* is much stronger induced by nitrogen deficiency.

The ratio between nitrogen and carbohydrates is often suggested to be an important parameter for regulation of gene expression although the mechanisms behind maintenance of the C/N balance is still unclear (Krapp and Truong 2006; Pourtau et al. 2006). In prokaryotes the PII protein has been found to be a sensor for N/C status (Krapp and Truong 2006), and a homologous protein is found in plants (Smith et al. 2003). *PII* was included in the present work because it has also been shown to be involved in the regulation of anthocyanin synthesis by inducing higher levels of anthocyanins under certain conditions when ectopically expressed (Hsieh et al. 1998). *PII* was the one

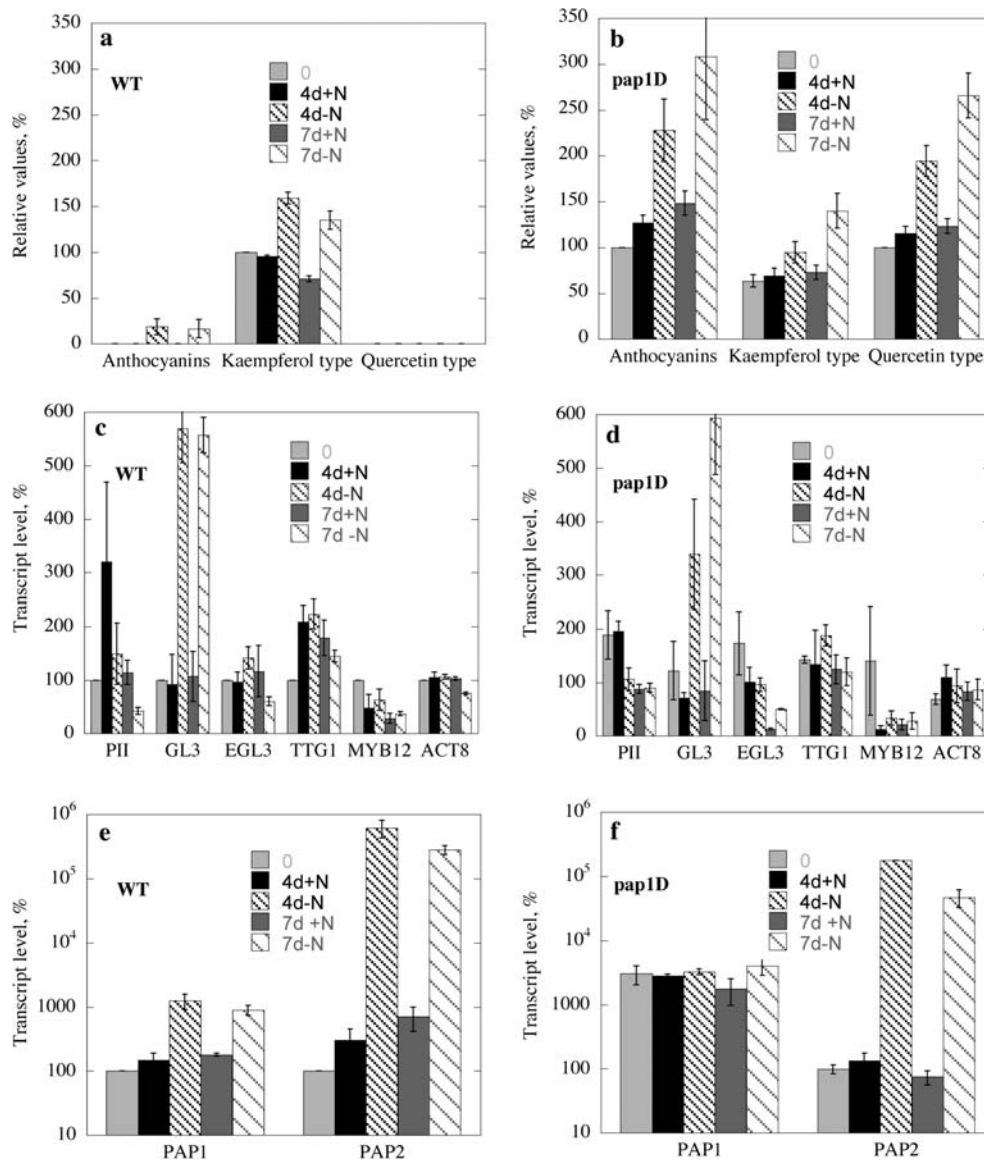


Fig. 3 a–f Relative levels of flavonoids and transcripts in the leaves of *Arabidopsis* WT and *pap1D* rosette stage plants grown in hydroponics. Content of anthocyanins, kaempferol type, and quercetin type flavonols in WT (**a**) and *pap1D* (**b**). Transcript levels of *PII*, *TTG1*, *EGL3*, *GL3*, *MYB12* and *ACT8* in WT (**c**) and *pap1D* (**d**). Transcript levels of *PAP1* and *PAP2* in WT (**e**) and *pap1D* (**f**). Leaves from plants growing in Hoagland solution were harvested about 4 weeks after sowing (day 0), and then 4 and 7 days later. At day 0 the nutrient solution was changed, and nitrate was withdrawn from half of the batch. Samples taken at day 0 (light grey bars), after another 4 days on complete nutrient solu-

tion (black) or without nitrogen (narrow hatched), after 7 days on complete nutrient solution (grey) or without nitrogen (hatched). Data (mean values \pm SE) are presented relative to values obtained for WT growing in complete nutrient solution (day 0). Since anthocyanins and quercetin type flavonols were below detection limit in WT on day 0, these data are presented relative to measurements for *pap1D* on day 0. For transcript analysis data are presented as percent of the RQ-value ($RQ = 2^{-\Delta\Delta CT}$) where WT at day 0 is used as calibrator and ubiquitin as endogenous control. Note logarithmic scale in **e** and **f**. The number of biological parallels was three

gene acting differently in seedlings and rosette leaves, responding to nitrogen withdrawal with a decrease in transcript level in seedlings on agar, but increase in rosette leaves, hence not revealing any clear trends in expression.

In *Arabidopsis* the WD repeat protein TTG1, the bHLH domain (GL3, EGL3, TT8), and MYB (PAP1,

PAP2) transcription factors are connected in a hierarchical network to regulate flavonoid synthesis (Zhang et al. 2003). Other MYBs are also involved, like MYB12, which clearly influence flavonol synthesis (Mehrtens 2005). The TTG1 is on top of this hierarchy and no anthocyanins or proanthocyanins are synthesised in *ttg1* mutants. We did not find any significant

influence of nitrogen deficiency on *TTG1* expression in WT or *pap1D* plants (Figs. 2a, b, 3c, d), indicating that nitrogen exerts its effect on a different level in the hierarchy, which appears reasonable since there are several BHLH and MYB proteins involved, opening the possibility for complex responses to external factors. Expression of *TT8* was apparently too low for detection in these plants regardless of nitrogen supply, hence *TT8* did not appear to be essential for the response to nitrogen deficiency. *TT8* is generally found to be more important in seeds than leaves, and is more likely to be regulated by developmental, not external, factors (Shirley 1995). Only *GL3* expression, not *EGL3*, was influenced by nitrogen deficiency indicating different roles for these transcription factors in response to external factors. The enhancement of *GL3* transcript levels was found in WT as well as *pap1D*, six times enhancement in rosette leaves and 2 times enhancement in seedlings (Figs. 2a, b, 3c, d). *GL3* as a nitrogen deficiency responding gene has not previously been reported, which may be explained because *GL3* (At5g41315) is not present on the ATH1 array used by many researchers.

PAP1 over-expression is known to increase the expression of genes coding for at least 11 enzymes of anthocyanin synthesis (Tohge et al. 2005). The *pap1D* mutant was originally included to test the possibility that nitrogen effects would be wiped out when *PAP1* was over-expressed; however, the regulators we tested were hardly influenced by *PAP1* over-expression showing that *PAP1* is not directing transcription of these other regulators. The *pap1D* mutant generally confirmed the results found in WT.

The concentration of flavonols was shown to be slightly increased in nitrogen-deficient plants. The MYB12 factor was recently shown to be a regulator of flavonol synthesis by enhancing expression of specific flavonol synthase gene(s) as well as some general flavonoid pathway genes (Mehrtens et al. 2005). Expression of *MYB12* was enhanced by nitrogen deficiency in both WT and *pap1D* seedlings (Fig. 2a, b), but not significantly enhanced in rosette leaves (Fig. 3c, d), hence increased *MYB12* expression could not generally explain the increased accumulation of flavonols in response to nitrogen deficiency (Figs. 1, 3). When both types of flavonols were present, quercetin type was slightly more enhanced by nitrogen withdrawal than kaempferol type. This could be explained by regulation of a gene/enzyme specific for synthesis of one of the flavonol types being influenced by nitrogen. A candidate would be flavonoid 3'-hydroxylase (F3'H). However, specific changes in glycosylation and degradation could also contribute to a difference in accumulation.

This has not yet been investigated. In WT rosette leaves quercetin was not present in measurable amounts whereas the compound was found in *pap1D* plants showing that the *pap1D* phenotype influenced quercetin content.

Although analysis of nitrogen effects using the ATH1 chip had revealed only small changes in transcripts of *PAL* genes (Scheible et al. 2004; Wang et al. 2004) studies of *pal* mutants have shown that blocking *PAL* leads to higher accumulation of amino acids, whereas the level of secondary metabolites are decreased (Rohde et al. 2004). It was especially shown that the *pal1* mutant had lower concentrations of kaempferol-type glycosides. For a more complete picture of effects of nitrogen deficiency on flavonoid content, expression and activity of *PAL* should be a subject for further investigations into nitrogen effects.

In conclusion, this work shows that three transcription factors, *PAP1*, *PAP2* and *GL3*, known to be involved in the regulation of flavonoid synthesis respond to nitrogen deficiency by enhanced expression levels, and in 4-day-old WT seedlings also a fourth transcript *MYB12* is significantly enhanced. Other transcription factors tested, *TTG1*, *EGL3* and *TT8*, were not influenced. Interestingly, *PAP2* mRNA levels were 30- to 200-fold more strongly increased than *PAP1*. *GL3* and *PAP2* transcripts showed the most profound responses to nitrogen depletion, and their genes are therefore likely targets in the signal transduction chain responding to nitrogen deficiency and resulting in increased accumulation of flavonoids. In further work we will establish how different nutrients in combination with developmental stages will influence expression of these regulators, and examine how *gl3*, *pap1* and *pap2* mutant lines will influence the nutrient effects.

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