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Nutrient depletion as a key factor for manipulating gene expression and product formation in different branches of the flavonoid pathway

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ABSTRACT

The content of flavonoids increases in response to nitrogen and phosphorus depletion in plants. Manipulation of these macronutrients may therefore be used to control the levels of desirable compounds and improve plant quality. Key enzymes in the shikimate pathway, which feeds precursors into the flavonoid pathway, are regulated post-translationally by feedback from aromatic amino acids, and possibly by redox control through photosynthesis. Use of microarrays for global transcript analysis in *Arabidopsis* has revealed that transcript levels are less influenced by mineral nutrients in the shikimate pathway compared with the flavonoid pathway. The responses in the shikimate pathway appear complex, whereas in the flavonoid pathway, a single gene often responds similarly to mineral depletion, high light intensity and sucrose. MYB [production of anthocyanin pigment 1 (PAP1)/production of anthocyanin pigment 2 (PAP2)] and bHLH [GLABRA3 (GL3)] transcription factors are important for the nutrient depletion response. PAP1/2 stimulate gross activation of the flavonoid pathway, and different investigations support merging signal transduction chains for various abiotic treatments on PAP1/2. Flavonol synthase is not part of the PAP1/2 regulon, and expression is mainly enhanced by high light intensity and sucrose, not mineral depletion. Nevertheless, both cyanidin and flavonol derivatives increase in response to nitrogen depletion. Kaempferols are the dominating flavonols in *Arabidopsis* leaves under normal cultivation conditions, but quercetin accumulation can be triggered by nitrogen depletion in combination with other abiotic factors.

Key-words: abiotic stress; anthocyanins; bHLH; flavonoids; flavonols; macronutrients; MYB; nitrogen; phosphorus; sucrose.

INTRODUCTION

Secondary compounds like flavonoids (Fig. 1) are of special interest for plant quality because they contribute to the colour and taste of fruits and vegetables, and are believed to have health-beneficial effects. Flavonoids have various functions in plants, for instance, as protectors against ultraviolet (UV) light and pathogens, and their concentrations generally increase in response to such factors. Although the functions of increased flavonoid concentration in response to nutrient limitation is obscure, these effects are striking, and especially the responses to nitrogen and phosphorus are profoundly documented during the past years (Stewart *et al.* 2001; Scheible *et al.* 2004; Wang *et al.* 2004; Misson *et al.* 2005; Lea *et al.* 2007; Morcuende *et al.* 2007; Müller *et al.* 2007).

The shikimate pathway is present in both plants and microorganisms, but never in animals. In plants, the shikimate pathway provides phenylalanine not only for protein synthesis, but also for secondary metabolites such as lignin and flavonoids. The pathway is strongly affected by various stimuli like light, pathogens and wounding (Weaver & Herrmann 1997). Some genes in the shikimate pathway have also been pointed out as activated, although moderately, by nitrogen deficiency (Scheible *et al.* 2004). The supply from the shikimate pathway is important for subsequent flux through the flavonoid pathway. The flavonoids are synthesized from phenylalanine (occasionally tyrosine) in a special pathway that is only present in plants. Because flavonoid synthesis is strongly influenced by environmental factors, profound knowledge of these effects should enable prediction and selection of growth conditions to achieve a desirable content of these secondary metabolites. Manipulation of environmental factors should, at least to some degree, represent an alternative to genetic engineering for achieving special effects on the level of plant components. In greenhouse crops, manipulation of macronutrient supply is generally easy, and could enhance the quality of fruits, vegetables or ornamental plants if responses to different fertilizer regimens are thoroughly known. In this review, we describe control of the shikimate and flavonoid pathways with special emphasis on how nitrogen and phosphorus depletion influence gene expression and content of flavonoids in vegetative parts of the model plant *Arabidopis*. The most important post-translational regulation in these pathways is also highlighted.

MICROARRAY DATA

Microarray data from different laboratories are not always easily comparable because growth conditions, plant age and tissue used vary extensively. There are several studies of phosphorus depletion using the Affymetrix ATH1 chip, and

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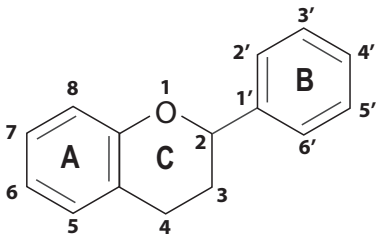

Figure 1. A general structure and numbering system for flavonoids.

as pointed out by Morcuende *et al.* (2007), some of these studies show disappointingly little overlap in their conclusions on which genes are responsive to phosphorus. In addition, sugar effects on gene expression are reported to be very different by various research groups (Lloyd & Zakhleniuk 2004; Price *et al.* 2004; Solfanelli *et al.* 2006; Osuna *et al.* 2007). Time is a very important parameter in these experiments, and the genes responding within a short time period to various treatments are likely to be different from the set of genes responding after a longer period of time (Misson *et al.* 2005; Morcuende *et al.* 2007). The data in Table 1 are all taken from experiments where nitrogen and phosphorus depletion lasted for at least 2 d, sugar addition for 6 h and high light intensity exposure for 8 h. Table 1 presents the change in transcript levels of genes in the shikimate and flavonoid pathways, and relevant transcription factors expressed in leaves and seedlings. The table summarizes the results from different research groups, and ratios are presented on a log 2 scale for each gene. For better visual assessment, a colour code is associated to each value. White fields represent changes equal to or less than twofold (–1 to 1 on the logarithmic scale). Light blue to dark blue fields represent increased, while yellow to red fields represent decreased transcript levels. For example, value x represents a ratio between treated and control plants equal to 2^x. Brief information on growth conditions, plant age, treatment, etc. is listed in Table 2, and more detailed information is presented in Supplementary Table S1. Absolute signal values are given in Supplementary Tables S2–S8. Overviews of the pathways and genes in *Arabidopsis* are presented in Figs. 2 and 3.

THE SHIKIMATE PATHWAY

3-Deoxy-D-arabino-heptulosonate 7-phosphate synthase (DAHPS) is a crucial control point linking synthesis of aromatic amino acids to photosynthesis, possibly by redox regulation

The first enzyme of the shikimate pathway (Fig. 2), DAHPS, catalyses the condensation of phosphoenolpyruvate (PEP) and erythrose-4-phosphate (E4P), yielding 3-deoxy-D-arabino-heptulosonate 7-phosphate and inorganic phosphate. DAHPS is an important target for regulation of the pathway in microorganisms, as well as in plants, although regulated in response to different signals in these organisms. In microorganisms, regulation is by negative feedback from the aromatic amino acids, but this has not been observed in plants for this reaction step (Herrmann & Weaver 1999). Plants are likely to have evolved a different mechanism for regulation because the pathway is not only involved in formation of amino acids for protein synthesis, but also in the formation of lignin, flavonoids and other secondary compounds. DAHPS appears to be regulated transcriptionally as well as post-translationally in plants.

Arabidopsis has three genes coding for DAHPS. One gene (*DHS1*) is characterized functionally; two others (*DHS2* and *DHS3*) are identified by homology (Keith *et al.* 1991; Ehlting *et al.* 2005). *DHS1* transcripts were enhanced three- to fivefold by wounding or by pathogen attack (Keith *et al.* 1991). Possibly, *DHS2* and *DHS3* are present for a more constitutive assurance of sufficient flow into the aromatic amino acids for protein synthesis. The three *DHS* transcripts were all present in *Arabidopsis* seedlings according to microarray analysis, and *DHS2* and *DHS3* were as strongly or more strongly expressed than *DHS1* (Scheible *et al.* 2004; Wang *et al.* 2004). Interestingly, *DHS1* and *DHS3* responded positively by a factor of 4.2 and 3.3, respectively, to sucrose (Solfanelli *et al.* 2006), and *DHS2* transcripts increased by a factor 2.3 in response to high light intensity (Vanderauwera *et al.* 2005). *DHS3* responded to phosphorus depletion by a threefold increase in transcript levels in two reports (Misson *et al.* 2005; Morcuende *et al.* 2007).

The shikimate pathway is localized in chloroplasts; hence, regulation coupled to photosynthesis is anticipated. Heterologously expressed *DHS1* requires reduced thioredoxin for activity, thereby suggesting a link between carbon flow into the shikimate pathway and electron flow from photosynthesis (Entus, Poling & Herrmann 2002). Thus, synthesis of Phe, and subsequently flavonoids, may be redox activated by light through the thioredoxin system. It is still an open question which role the other two genes, *DHS2* and *DHS3*, have in the pathway, and to our knowledge, it has not been investigated if they also encode redox-regulated enzymes.

The next two steps in the shikimate pathway are catalysed by 3-dehydroquinate synthase (DQS) and 3-dehydroquinate dehydratase/shikimate dehydrogenase (DHQD/SD), both encoded by single genes in *Arabidopsis*. These genes apparently do not represent important regulatory steps, and their response to nitrogen or phosphorus deficiency according to microarray analysis was always less than twofold (Scheible *et al.* 2004; Misson *et al.* 2005; Morcuende *et al.* 2007; Müller *et al.* 2007). DHQD/SD transcripts increased 2.5-fold in response to high light intensity (Vanderauwera *et al.* 2005).

Shikimate kinase (SK) and 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) expression is not consistently influenced by macronutrients

In tomato, one gene coding for SK has been identified, and two homologs of this gene (At2g21940 and At4g39540) are present in *Arabidopsis* (Herrmann & Weaver 1999).

Table 1. Expression of genes involved in shikimate and flavonoid synthesis in *Arabidopsis* seedlings and leaves in response to various treatments

Name	Gene		1. pap 1D leaves	2. pap 1D roots	3. low N seedlns	4. low P seedlns	5. low P leaves	6. low P leaves	7. hv leaves	8. suc seedlns	9. pho3 seedlns
DAHPS DHS1	At4g39980	252831_at			–0.2	1.2			0.7	2.1	0.4
DHS2	At4g33510	253333_at			–0.6	–0.1			1.2	0.1	–0.2
DHS3	At1g22410	261933_at			0.6	1.5	1.6		0.3	1.7	0.5
DQS	At5g66120	247138_at			0.1	0.3			0.8	0.7	0.1
DHQD/SD	At3g06350	258908_at			0.1	0.1			1.3	0.8	–0.2
SK1	At2g21940	263897_at			1.0	0.3			0.5	0.3	0.3
SK2	At4g39540	252900_at			–0.1	0.2			0.7	0.7	0.5
SK3	At3g26900	258281_at			–1.9	–1.1			–0.1	–0.9	–0.5
SK4	At2g35500	266608_at			–2.4	–1.6			0.6	0.0	–1.1
SK putative	At5g47050	248819_at			0.7	1.2			0.8	0.0	0.9
SK putative	At1g06890	260818_at			0.4	0.2			1.3	0.1	0.3
EPSPS1	At2g45300	246627_s_at			0.1	0.4			0.9	1.1	0.2
CS	At1g48850	245832_at			0.2	0.3			1.9	1.5	0.3
CM1	At3g29200	257746_at			0.7	0.4			2.6	0.2	–0.3
CM2	At5g10870	250407_at			0.1	–0.2			0.3	–0.3	–0.6
PNT	At2g38400	267035_at			0.3	0.5			1.0	–4.5	0.3
PNT	At2g20610	263714_at			–0.1	0.0			0.5	1.9	–0.5
ADT1	At1g11790	262825_at			–0.4	0.0			–0.5	1.9	–0.5
ADT2	At3g07630	259254_at			–0.1	–0.3			1.5	0.8	0.2
ADT3	At2g27820	266257_at			0.1	0.2			2.1	1.2	0.4
ADT4	At3g44720	252652_at			0.8	1.4	1.4	0.7	0.8	0.8	0.4
ADT putative	At5g22630	249910_at			1.5	1.7			0.9	0.6	2.2
ADT putative	At1g08250	261758_at			0.6	1.1		0.6	1.2	0.4	–0.9
PAL1	At2g37040	263845_at	1.6	0.1	1.5	1.4	1.7		2.3	1.6	1.6
PAL2	At3g53260	251984_at	0.5	0.0	–0.3	0.5			2.0	1.1	1.3
PAL3	At5g04230	245690_at	–0.1	–0.3	–1.0	–0.9				–0.2	0.9
PAL4	At3g10340	259149_at			1.1	1.0				0.5	–0.5
C4H	At2g30490	267470_at	0.2	–0.3	0.3	0.5			1.9	1.2	1.4
4CL1	At1g51680	256186_at		0.0	0.7	0.8			0.5	0.7	0.2
4CL2	At3g21240	258047_at	0.5	–0.2	0.9	1.2				1.2	–0.1
4CL3	At1g65060	261907_at	1.2	0.4	1.2	0.5	1.9	1.3	4.5	3.3	2.9
4CL5	At3g21230	258037_at			1.1	1.8			–2.0	1.1	0.3
4CL putative	At1g20490	259568_at	2.4	0.3	1.1	0.7			2.6	0.7	1.0
CHS	At5g13930	250207_at	4.0	0.8	0.9	1.1	1.8	2.1	2.5	8.2	5.1
CHI	At3g55120	251827_at	1.8	0.4	0.2	0.2			4.0	2.4	1.0
F3H	At3g51240	252123_at	2.8	1.3	1.7	0.9	2.2		4.3	7.2	4.7
F3'H	At5g07990	250558_at	4.1	2.4	1.7	1.3	1.9		6.5	2.0	2.3
DFR	At5g42800	249215_at	4.2	4.0	4.3	2.9	2.6		7.1	5.5	4.9
ANS	At4g22870	254283_s_at	4.8	4.0	4.6	2.3	2.3		7.5	5.4	7.6
FLS1	At5g08640	250533_at	–0.2	0.0	0.8	0.1			4.4	4.1	3.3
FLS putative	At3g50210	252213_at			0.9	0.1			2.7	–0.5	1.2
FLS putative	At5g63580	247358_at			0.3	0.4			1.4		
FLS putative	At5g63590	247354_at			1.1	0.8					0.5
AGT/FGT UGT78D2	At5g17050	246468_at	2.3	1.0	1.0	1.0			4.2	6.0	2.8
AGT UGT75C1	At4g14090	245624_at	4.0	3.2	4.1	1.8	3.5		8.2	5.4	6.7
AGT UGT79B1	At5g54060	248185_at	6.7	3.5	3.0	2.3			7.3	5.7	4.7
UGT UGT84A2	At3g21560	258167_at	1.4	0.0	1.5	2.4	3.0		3.6	1.2	3.3
UGT UGT71C1	At2g29750	266669_at			2.8	1.0				1.6	2.6
UGT UGT72B1	At4g01070	255622_at			1.4	0.1			1.0	1.5	0.1
UGT UGT85A5	At1g22370	255943_at			0.2	–0.6			3.1	–0.9	0.7
FGT UGT89C1	At5g17050	260955_at			1.5	1.3			2.8	6.0	3.7
FGT UGT78D1	At1g30530	261804_at			–0.1	0.7			3.0	5.2	1.8
FGT UGT73C6	At2g36790	265200_s_at			2.2	1.4			1.2	–0.4	0.2
UGT UGT91A1	At2g22590	265290_at									0.0
UGT UGT84A1	At4g15480	245560_at							5.7		–0.1
UGT UGT73B2	At4g34135	253268_s_at			2.7	1.8		0.6	2.7	0.0	2.8
UGT UGT78D3	At5g17030	246419_at			0.9	0.3			4.5	0.4	0.3
AAT1	At1g03940	265091_s_at	5.1	4.3	5.7	2.7	3.2	2.3	8.6	5.7	6.8
AAT	At3g29590	256924_at	3.9	3.2	1.9	1.2	1.9		8.5	2.8	2.8
GST	At5g17220	250083_at	4.6	3.1	4.5	3.4	3.3		10.5	4.0	7.1
GST	At1g02930	262119_s_at	4.6	0.1	–0.7	1.3		0.8	–0.5	1.5	2.1
GST	At1g02940	262103_at	2.0	2.0	0.0	0.2			1.4		0.3
PAP1/MYB75	At1g56650	245628_at	5.1	2.9	4.1	2.7	3.2	1.8	3.1	5.1	3.3
PAP2/MYB90	At1g66390	260140_at	1.2	0.9	7.5	4.8	4.8		10.0		8.1
MYB11	At3g62610	251223_at								1.0	0.0
MYB12	At2g47460	245126_at			0.6	0.5			2.8	1.0	0.5
MYB111	At5g49330	248596_at				1.0	1.8	0.8		3.2	1.9
MYB32	At4g34990	253219_at			1.3	0.7		0.4	1.2	0.4	–0.1
MYB4	At4g38620	252958_at			0.5	0.2			1.5	0.9	–0.6
TTG1/WD40	At5g24520	249739_at			0.1	0.2			–0.6	–0.2	–0.5
ANL2/HD	At4g00730	255636_at			–0.2	–0.1			1.1	0.1	–0.2
HY5/bZIP	At5g11260	250420_at			–0.1	0.2		0.5	1.8	–0.7	0.2
HYH/bZIP	At3g17609	258349_at			–0.4	–0.6			0.1	3.5	–0.4

Scale
log2
>4
>3
>2
>1
–1 to1
<–1
<–2
<3
<–4

The ratio between treated (low N, low P, high light, +sucrose) and control plants, or between mutants (*pap1D*, *pho3*) and WT is presented on a log 2 scale, and visualized by different colours. WT Columbia was generally used, but for pho3 Wassilewskija was used. All ratios given in Table 1 are on a log 2 scale. White represents changes equal to or less than twofold (–1 to 1 on the log 2 scale); light blue represents changes larger than twofold but smaller or equal to fourfold [1 < log 2(ratio) ≤ 2]; darker blue represents further increased expression, and yellow to red represents decreased expression levels as indicated on the bar. Details of growth conditions and treatments, and references are given in Table 2 and further details in Supplementary Table S1. Original data are given in Supplementary Tables S2–S8, including some genes for the lignin pathway and genes that turned out not to be significantly expressed.

DAHPS, 3-deoxy-D-arabino-heptulosonate 7-phosphate synthase; DHQD/SD, 3-dehydroquinate dehydratase/shikimate dehydrogenase; SK, shikimate kinase; EPSPS, 5-enolpyruvylshikimate-3-phosphate synthase; CS, chorismate synthase; CM, chorismate mutase; PNT, chorismate mutase; ADT, arogenate dehydratase; PAL, phenylalanine ammonia-lyase; C4H, cinnamate 4-hydroxylase; CHI, cinnamate 4-hydroxylase; F3H, flavanone 3-hydroxylase; F3′H, flavonoid 3′-hydroxylase; DFR, dihydroflavonol 4-reductase; ANS, anthocyanidin synthase; FLS, flavonol synthase; AGT, anthocyani(di)n glycosyltransferase; FGT, flavonol glycosyltransferase; UGT, UDP-dependent glycosyl transferases; AAT; anthocyanin acyltransferase; GST, glutathione S-transferase; TTG1, transparent testa glabra1; ANL2, anthocyaninless2; PAP, production of anthocyanin pigment.

Table 2. Specification of growth conditions and treatments of plants described in Table 1 (further details in Supplementary Table S1)

	1	2	3	4	5	6	7	8	9
Notation in Table 1	pap1D	pap1D	Low N	Low P	Low P	Low P	hv	suc	pho3
Treatment or mutant examined	pap1D relative to WT	pap1D relative to WT	2 d N starvation	2 d P starvation	2 d low P (5 μM)	P starved 3 weeks followed by 1 week +/–P	High light intensity (1600–1800 μmol m^{-2} s^{-1}) for 8 h	90 mM suc for 6 h	pho3 (high sugar mutant) relative to WT
Material	Leaves	Roots	Seedlings	Seedlings	Leaves	Leaves	Leaves	Seedlings	Leaves
Age at harvest	3 weeks	3 weeks	9 d	9 d	9 d	5 weeks	6 weeks	3 d	First flower opened
Growth medium	Agar GM 1.0% suc	Agar GM 1.0% suc	Liquid MS 0.5% suc	Liquid MS 0.5% suc	Agar MS 0.5% suc	Soil/rockwool	Not given	Liquid MS	Compost
Light/dark	16 h/8 h	16 h/8 h	24 h/0 h	24 h/0 h	Not given	8 h/16 h	12 h/12 h	24 h/0 h	16 h/8 h
Light intensity	4500 lux	4500 lux	50 μmol m^{-2} s^{-1}	50 μmol m^{-2} s^{-1}	Not given	120 μmol m^{-2} s^{-1}	100–140 μmol m^{-2} s^{-1}	90 μmol m^{-2} s^{-1}	140 μmol m^{-2} s^{-1}
Temperature	22 °C	22 °C	22 °C	22 °C	Not given	20 °C	22 °C/18 °C	22 °C	22 °C
Number of experiments	2	1	3	3	3	4	1 (+2 similar)	2	2
Reference	Tohge *et al.* 2005	Tohge *et al.* 2005	Scheible *et al.* 2004	Morcuende *et al.* 2007	Misson *et al.* 2005	Müller *et al.* 2007	Vanderauwera *et al.* 2005	Solfanelli *et al.* 2006	Lloyd & Zakhleniuk 2004

Additionally, four other genes (Fig. 2) in *Arabidopsis* have SK and chloroplast signatures [The Arabidopsis Information Resource (TAIR) annotation]. One of these genes (*SK1*, Table 1) showed a twofold increase in transcript level in response to nitrogen depletion, while a putative SK (At5g47050) showed a more than twofold positive response to phosphorus depletion. SK3 and SK4 both decreased in response to nitrogen and phosphorus depletion (Scheible *et al.* 2004; Morcuende *et al.* 2007; Table 1). It is not known if all these genes contribute to SK activity, but the relatively large number of genes certainly points to SK as a likely regulatory step with possible differentiated and complex expression of involved genes. The SK enzyme has been reported to be negatively regulated by products of the reaction (ADP, shikimate-3-P); hence, both transcriptional and post-transcriptional regulation appear to be involved in this step (Herrmann & Weaver 1999).

EPSPS catalyses a readily reversible reaction, and is therefore not a likely target for specific regulation at the protein level. The step is, however, very important as a target of the glyphosate herbicide as the WT enzyme is famous for binding glyphosate, which blocks transport into the chloroplasts, as well as activity of the protein.

Chorismate synthase (CS) – another enzyme regulated by redox state?

Synthesis of chorismate is catalysed by CS. The enzyme requires a reduced flavin nucleotide cofactor ($FMNH_2$), although a net change in the oxidation state of the reactants does not take place. The regulation of the enzyme by $FMNH_2$ is still poorly understood (Macheroux *et al.* 1999), but represents another step in the pathway, in addition to DAHPS, which is influenced by redox compounds. Inspection of microarray data did not reveal any influence of nitrogen or phosphorus on this step.

Chorismate mutase (CM), the committing step in synthesis of Phe and Tyr, is feedback regulated by aromatic amino acids

Feedback regulation in the pathway to aromatic amino acids occurs at the branching point where CM and anthranilate synthase (AS) compete for the common substrate chorismate (Voll *et al.* 2004). CM is the first enzyme in the branch leading to Phe and Tyr, and CM is positively regulated by Trp, but is inhibited by Phe and Tyr (Eberhard *et al.* 1996). This implies that when the level of Trp is sufficiently high, flow can be directed towards the synthesis of the other two aromatic amino acids. AS (leading to Trp), on the other hand, is inhibited by Trp (Voll *et al.* 2004, references therein) *Arabidopsis* has three isoenzymes CM1, CM2 and CM3. Both CM1 and CM3 are located in the chloroplasts and are feedback regulated by amino acids, while CM2 is not (Mobley, Kunkel & Keith 1999). The enigmatic CM2 is located in the cytosol, and for a long time, this has nourished

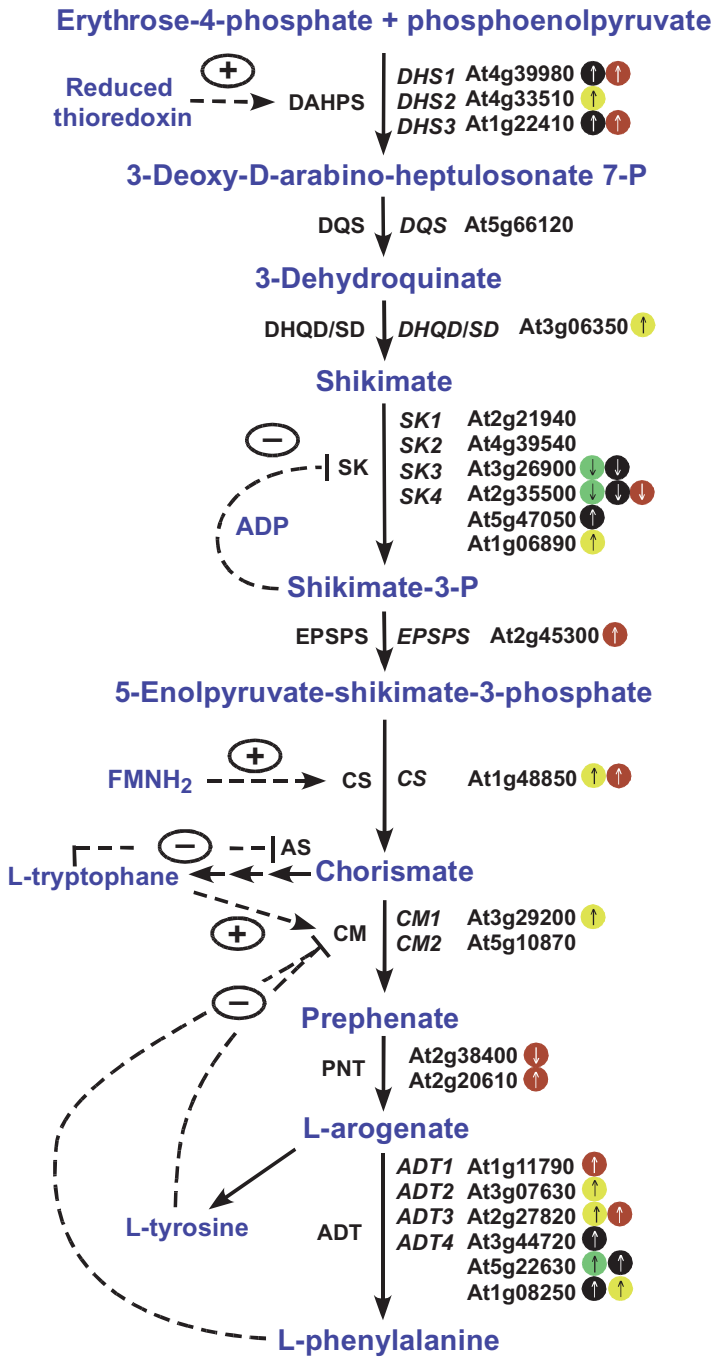

Figure 2. Simplified shikimate pathway and influence of nutrients on transcript levels in *Arabidopsis*. Symbols are added to indicate transcripts that were increased or decreased more than twofold in plants with *PAP1* over-expression relative to WT (Tohge *et al.* 2005), by nitrogen depletion (Scheible *et al.* 2004) or phosphorus depletion in at least one report (Misson *et al.* 2005; Morcuende *et al.* 2007; Müller *et al.* 2007), and high light intensity (Vanderauwera *et al.* 2005) or sucrose in at least one report (Lloyd & Zakhleniuk 2004; Solfanelli *et al.* 2006). Increased gene expression in *PAP1* over-expressor plants is marked by a red AGI number. The green circle represents N depletion; the black circle is P depletion; the yellow circle is high light intensity, and the orange circle is sucrose. Arrows pointing up show increased expression; arrows pointing down show decreased expressions. The steps are catalysed by the indicated enzymes 3-deoxy-D-arabino-heptulosonate 7-phosphate synthase (DAHPS), 3-dehydroquinate synthase (DQS), 3-dehydroquinate dehydratase/shikimate dehydrogense (DHQD/SD), shikimate kinase (SK), 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS), chorismate synthase (CS), anthranilate synthase (AS), chorismate mutase (CM), prephenate aminotransferase (PNT) and arogenate dehydratase (ADT). Genes listed are annotated by The Arabidopsis Information Resource (TAIR) and Ehlting *et al.* (2005).

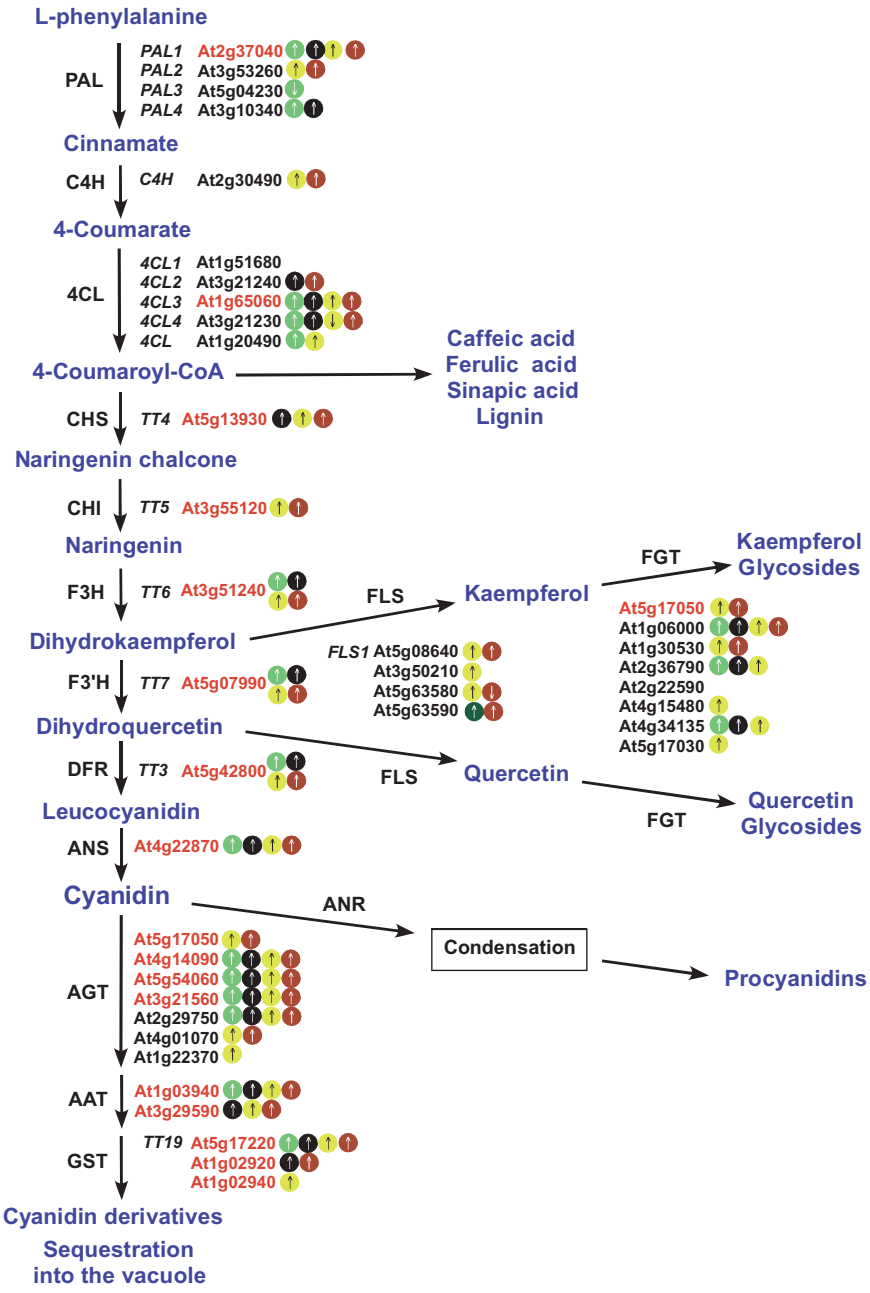

Figure 3. Simplified scheme for flavonoid and phenylpropanoid synthesis and influence of nutrients exceeding a twofold change in *Arabidopsis*. Symbols and explanations are as in Fig. 2. The steps are catalysed by phenylalanine ammonia lyase (PAL), cinnamate 4-hydroxylase (C4H), 4-coumarate-CoA ligase (4CL), chalcone synthase (CHS), chalcone isomerase (CHI), flavanone 3-hydroxylase (F3H), flavonoid 3′-hydroxylase (F′3H), flavonol synthase (FLS), flavonol glycosyltransferase (FGT), dihydroflavonol 4-reductase (DFR), anthocyanidin synthase (ANS) (LDOX), anthocyani(di)n glycosyltransferase (AGT), flavonoid glycosyltransferase (FGT) and glutathione S-transferase (GST). The step catalysed by anthocyanidin reductase (ANR) (BAN) is also shown, but is generally only expressed in seeds.

speculation about a putative cytosolic pathway for Phe and Tyr synthesis. However, this has not been confirmed. Generally, changes in transcript levels in response to macronutrients are small for *CM1* and *CM2* (*CM3* is not on the ATH1 chip), and most likely, feedback regulation at the enzyme level by amino acids is the important regulatory mechanism by nitrogen compounds at this step.

Function and regulation of prephenate aminotransferase (PNT) and arogenate dehydratase (ADT) is largely unknown

PNT transfers the amino group from glutamate or aspartate for synthesis of arogenate. The gene(s) are not yet cloned for any plant, but Ehlting *et al.* (2005) highlighted candidate

genes in *Arabidopsis* for the transferase function on the basis of coordinated expression with other genes in aromatic amino acid synthesis. Regulation by macronutrients does not appear to be important. Under nitrogen starvation, one may assume that the amino-donating substrates (glutamate, aspartate) will become limiting factors for this reaction.

ADT then catalyses the formation of Phe from L-arogenate. Ehlting *et al.* (2005) listed six candidate genes for *ADT* in *Arabidopsis* based on structural similarity with genes in microorganisms. Three of these genes were induced two- or threefold by nitrogen and phosphorus deficiency (Table 1) (Scheible *et al.* 2004; Misson *et al.* 2005; Morcuende *et al.* 2007). Especially, the *ADT4*/*At3g44720* gene was robustly responsive to phosphorus depletion because this gene responded not only in seedlings, but also in experiments set up quite differently with leaves or leaf segments of older tissue (Misson *et al.* 2005; Müller *et al.* 2007). Synthesis of Phe by the help of arogenate is, however, not firmly established, and an alternative route through phenylpyruvate has been proposed (Warpeha *et al.* 2006). This would involve an enzyme prephenate dehydratase (PD). At2g27820 is listed as PD1 in the TAIR database, but expression of this gene has not been reported to be enhanced by nitrogen or phosphorus depletion.

Altogether, the response to various treatments was very complex in the shikimate pathway; genes encoding different isoenzymes being up-regulated or down-regulated by the same treatment. Furthermore, in contrast to the flavonoid pathway, not many genes were stimulated by both nitrogen and phosphorus depletion, high light intensity or sucrose. This complex way of responding may have evolved because the many different products derived from the shikimate pathway have very diverse functions in the plant.

THE FLAVONOID PATHWAY

Variations in phenylalanine ammonia-lyase (PAL) levels influence different branches of the phenylpropanoid pathway in a diversified manner

PAL commits the flux of primary metabolism into the phenylpropanoid pathway (Fig. 3). The enzyme is long known to be highly regulated, at the transcriptional level in response to abiotic factors, pathogen attack or due to a demand for lignin in special tissues (Anterola & Lewis 2002). The existence of several PAL isozymes and genes, four in *Arabidopsis*, inspires the assumption that these genes may give rise to different pools of metabolites, which are channelled into specific pathways. The hypothesis that different PAL genes should feed cinnamate into different pathways is not well underpinned by experimental data. However, the *PAL4* gene appears to be a good candidate for having a special role in lignin synthesis because its expression is high, especially in stem tissue (Raes *et al.* 2003; Rohde *et al.* 2004). The *PAL3* gene has so far been shown only to have very low expression levels. Taking into account that the K_m value for Phe is 20 times higher for PAL3 than PAL1 (Cochrane, Davin & Lewis 2004), the importance of the *PAL3* gene in the phenylpropanoid pathway is questionable. Expression studies show that generally, *PAL1* and *PAL2* are the two *PAL* genes most strongly expressed in *Arabidopsis*. These genes are enhanced in tissues with high lignin synthesis (Raes *et al.* 2003), and also respond positively under conditions that give increased flavonoid synthesis (Provart *et al.* 2003; Olsen *et al.* 2007). Nitrogen or phosphorus deficiency led to increased *PAL1* transcript levels by a factor 3 (Scheible *et al.* 2004; Misson *et al.* 2005; Morcuende *et al.* 2007). *PAL1* also responded positively to all other treatments depicted in Table 1, that is, sucrose, high light intensity and production of anthocyanin pigment 1 (*PAP1*) over-expression (Lloyd & Zakhleniuk 2004; Tohge *et al.* 2005; Vanderauwera *et al.* 2005; Solfanelli *et al.* 2006).

The influence of *PAL* gene expression on flavonoid accumulation has been confirmed by analyzing *Arabidopsis pal1* and *pal2* mutants and the double mutant *pal1 pal2* (Rohde *et al.* 2004). The decrease in phenolics correlated with a decrease in total PAL activity. The most pronounced effect was seen for kaempferol glycosides, which were present at very low levels in the *pal1 pal2* double mutant. Other UV light-absorbing metabolites of the phenylpropanoid pathway, such as sinapoyl glucose and sinapoyl malate were not altered (Rohde *et al.* 2004). The differentiated effects on accumulation of products from phenylpropanoid metabolism by decreased PAL activity may be understood as a balance between the branches of the phenylpropanoid pathway and properties of enzymes involved downstream of PAL. In WT plants, the induction of *PAL* genes in response to different external factors apparently assures that this enzyme does not become limiting. To fully understand and predict this phenomenon, a systems biology approach describing fluxes as influenced by gene expression and kinetics of the various enzymatic steps in the pathway is required.

Cinnamate 4-hydroxylase (C4H)

A P-450-linked monooxygenase, C4H, catalyses the reaction where cinnamic acid is hydroxylated to p-coumaric acid. Transcript levels were only very moderately influenced by nitrogen or phosphorus depletion (Table 1).

Expression of all 4-coumarate coenzyme A ligase (*4CL*) genes is, to some extent, stimulated by nutrient deficiency

4CL converts 4-coumaric acid and other substituted cinnamic acids such as caffeic acid and ferulic acid into CoA thiol esters used for synthesis of various phenylpropanoids, including lignin and flavonoids. 4CL depends strictly on ATP, and the reaction resembles the activation of fatty acids. The *Arabidopsis* genome has 14 genes annotated as (putative) *4CLs*; however, after heterologous expression, only four genes were revealed to code for proteins with 4CL

activity (Costa *et al.* 2005). All enzymes had a broad substrate specificity converting different derivatives of cinnamate into its CoA thiol ester. K_m values for p-coumarate varied from 4 to 45 μM, with 4CL1 and 4CL3 being the most efficient enzymes for binding p-coumarate. The *At1g20490* gene could possibly also contribute to 4CL activity, but was not tested by Costa *et al.* (2005). *At1g20490* is a clear candidate gene for 4CL because it was induced along with the anthocyanin regulon in response to *PAP1* over-expression, nutrient deficiency, high light intensity and sucrose treatment. *4CL3* was the gene most responsive to nutrient depletion in the investigations among all research groups (Table 1). *4CL3* was also found to be differently expressed in various tissues compared to *4CL1* or *4CL2*. This is in agreement with *4CL3* being more important in flavonoid synthesis, whereas the other two genes are important for lignin synthesis (Ehlting *et al.* 1999). When the promoter regions of these genes were linked to *GUS* expression, GUS was seen in vascular tissue, but only *4CL3::GUS* showed expression outside vascular tissue (Soltani *et al.* 2006). This particular regulation of *4CL3* may therefore have evolved to assure the presence of precursors to make light-protecting compounds like flavonols.

Chalcone synthase (*CHS*) expression is regulated by many environmental factors including nutrients

CHS is the first committed enzyme in flavonoid synthesis and produces an intermediate used in the synthesis of all flavonoids. The enzyme catalyses stepwise the synthesis of naringenin chalcone (tetrahydroxychalcone) from one molecule of p-coumaroyl-CoA and three acetate units derived from three molecules of malonyl-CoA. CHS from parsley is the first gene in flavonoid metabolism being isolated (Kreuzaler *et al.* 1983), and it has later been isolated from species as petunia, maize and *Arabidopsis*. The number of *CHS* genes varies between species. In *Arabidopsis*, one *CHS* gene is known, whereas 12 are identified in the petunia genome (Holton & Cornish 1995 and references therein).

Light quality as well as light intensity is well known to influence *CHS* expression (Wade *et al.* 2001). In several species, there is a change of photoreceptor usage during development, and phytochrome control is diminished in older tissue (Jenkins *et al.* 2001). Expression of CHS is stimulated by UV and blue light, and this can be seen as a protective mechanism promoting the accumulation of UV-absorbing flavonoids (Jenkins *et al.* 2001). A positive response on *CHS* transcription was also found for high light intensity and sugars in microarray experiments (Lloyd & Zakhleniuk 2004; Vanderauwera *et al.* 2005; Solfanelli *et al.* 2006).

Expression of *CHS* is influenced by the availability of mineral nutrients. Nitrogen depletion resulted in increased expression by a factor close to two (Scheible *et al.* 2004). In tomato leaves, transcripts of CHS gene(s) were also enhanced by nitrogen depletion (Bongue-Bartelsman & Phillips 1995). Several microarray studies showed a two- to threefold increase in the expression of *CHS* under phosphorus depletion in *Arabidopsis* (Misson *et al.* 2005; Morcuende *et al.* 2007; Müller *et al.* 2007).

Chalcone isomerase (CHI) regulation can account for accumulation of naringenin chalcone

CHI catalyses the isomerization of naringenin chalcone to the flavanone naringenin. Most plants do not accumulate chalcones, and naringenin chalcone is normally rapidly isomerized to naringenin by CHI. Regulation of CHI depends strongly on species, and tomato is an interesting example where *CHI* expression is undetectable in peel tissue, although other genes of the flavonoid pathway are expressed. In tomato, genes of the anthocyanin pathway, *CHS* and dihydroflavonol 4-reductase (*DFR*), were found to be induced by nitrogen depletion in leaves. *CHI*, on the contrary, was repressed (Bongue-Bartelsman & Phillips 1995). Tomato clearly has evolved a special type of regulation that enables the accumulation of naringenin chalcone in certain tissue (Muir *et al.* 2001; Slimestad & Verheul 2005). Although naringenin chalcone can isomerize spontaneously, the uncatalysed reaction rate is 10^6 times lower, and also, pH influence favours that the innate physiological reaction most likely proceeds as an enzyme-catalysed ring closure (Jez & Noel 2002). Naringenin chalcone has interesting effects on the human immune system (Yamamoto *et al.* 2004), and it may be desirable to have a high level of this compound in the diet. According to microarray data (Table 1), CHI is not much influenced by nitrogen or phosphorus depletion in *Arabidopsis*.

Flavonoid 3′-hydroxylase (F3′H) and flavonoid 3′5′-hydroxylase (F3′5′H) are important for the colour of anthocyanins

F3H is a 2-oxoglutarate-dependent dioxygenase (2-ODD) catalyzing the 3-hydroxylation of flavanones. In *Arabidopsis*, this means hydroxylation of naringenin to give dihydrokaempferol. Expression of *F3H* in *Arabidopsis* is enhanced in response to nitrogen or phosphorous depletion (Scheible *et al.* 2004; Misson *et al.* 2005; Morcuende *et al.* 2007).

F3′H catalyses 3'-hydroxylation (in the B ring, Fig. 1) of flavonoids, and this enzyme belongs to the P-450 superfamily. Hydroxylation of the B-ring is important for the colour of anthocyanins, and another hydroxylase, F3′5′H, is found in, for example, petunia, and gives rise to a blue colour. These genes are sometimes informally called the red (*F3′H*) and blue (*F3′5′H*) genes (Schwinn & Davies 2004). The P450 enzymes are generally found to be anchored to the endoplasmic reticulum, and in *Arabidopsis*, F3′H, CHI, CHS and DFR are known to interact. The F3′H enzyme may therefore anchor a large enzyme complex and may facilitate channeling of synthesis into anthocyanins (Burbulis & Winkel-Shirley 1999). A positive response to nitrogen and

phosphorus depletion was also found for expression of *F3′H* in *Arabidopsis* (Scheible *et al.* 2004; Misson *et al.* 2005; Morcuende *et al.* 2007).

Flavonol synthase 1 (*FLS1*) is strongly expressed in response to high light intensity and sucrose

Flavonol synthases (FLSs) are 2-ODDs, and convert dihydroflavonols to flavonols by introducing the C2C3-double bound. FLS enzymes from *Arabidopsis* and *Citrus unshiu* have been shown to exhibit substantial F3H activity in addition to their primary enzymatic activity, at least *in vitro* (Prescott *et al.* 2002; Winkel 2006). However, a knock-out mutant of *F3H* in *Arabidopsis* showed no flavonoids to be present in these plants; hence, FLS did not appear to be able to substitute for F3H *in vivo* (Wisman *et al.* 1998).

A special MYB12 transcription factor has been found to regulate *FLS1* (Mehrtens *et al.* 2005), and recently, two close homologs of MYB12, MYB11 and MYB111, were also shown to activate *FLS1* transcription (Stracke *et al.* 2007). MYB12 was found to be mainly expressed in roots, while MYB111 was mainly expressed in leaves. Seedlings of the triple mutant clearly showed that these MYB factors were essential for flavonol synthesis because the mutant did not form flavonols, whereas anthocyanins still accumulated. A striking feature of *FLS1* expression is its strong induction by high light intensity and sucrose, while it is little affected by nitrogen or phosphorus depletion. This is in agreement with *FLS1* not being part of the PAP1 regulon that appears to be influenced by both nutrient depletion as well as high light intensity and sucrose (Table 1). However, flavonols were found to increase in *Arabidopsis* leaves in response to nitrogen or phosphorus deficiency (Stewart *et al.* 2001; Lea *et al.* 2007). In seedlings, especially, quercetin levels increased strongly in response to nitrogen deficiency, that is, twofold in the experiments by Lea *et al.* (2007), and as much as 18-fold in the experiments by Stewart *et al.* (2001). Stewart and co-workers also tested phosphorus depletion, and found a three- to fourfold increase for both kaempferols and quercetins. The increased accumulation of flavonols in response to mineral depletion could be caused by changes in stability and degradation of the flavonols as a result of increased activity of glycosylases (Fig. 3). Furthermore, putative FLSs, which are not yet characterized, could be involved. In the experiments with seedlings (Stewart *et al.* 2001; Lea *et al.* 2007), sucrose was present in the growth medium. When seedlings were grown without sucrose, flavonol content was low, and nitrogen effects were not found (Lea *et al.* 2007). In leaves of rosette-stage *Arabidopsis* grown hydroponically, nitrogen depletion clearly resulted in increased kaempferol concentration. However, quercetin was not detectable in these leaves, whether deprived of nitrogen or not (Lea *et al.* 2007). As previously noticed, quercetin is generally not present in *Arabidopsis* leaves under normal greenhouse conditions (Veit & Pauli 1999). Accumulation of quercetin appears to require a second trigger such as sucrose (Stewart *et al.* 2001; Scheible *et al.* 2004; Lea *et al.* 2007) or low temperature (Olsen *et al.* 2007), and together with this second factor, nitrogen depletion synergistically leads to quercetin accumulation. For *Arabidopsis*, these findings are summarized in Fig. 4. Very similar responses to nitrogen depletion and sucrose were also seen in tomato seedlings and leaves (Stewart *et al.* 2001). Bongue-Bartelsman & Phillips (1995) found, however, that quercetin did increase in tomato leaves in response to nitrogen depletion in hydroponics, although sucrose was not added. Clearly, details in experimental conditions or plant variety used are critical for quercetin accumulation.

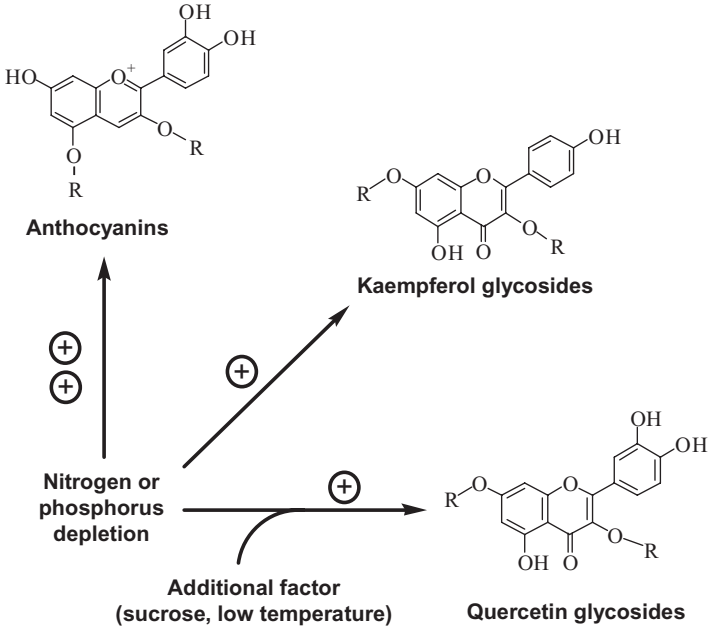

Figure 4. General scheme for effects of nitrogen and phosphorus depletion on main classes of products of the flavonoid pathway in *Arabidopsis*. Nutrient deficiency strongly increased accumulation of anthocyanins, and also positively influenced kaempferol accumulation. In normal greenhouse conditions, nutrient deficiency alone did not lead to accumulation of quercetin, but nutrient deficiency acted synergistically with other factors to stimulate quercetin accumulation. This scheme is based on Stewart *et al.* (2001), Lea *et al.* (2007) and Olsen *et al.* (2007).

'Late genes' dihydroflavonol 4-reductase (*DFR*) and anthocyanidin synthase (*ANS*) are strongly induced by nutrient deficiency

DFR catalyses the reduction of the carbonyl group in position 4 in dihydroflavonols to an OH-group by the help of NADPH, giving the product leucoanthocyanidin, and more specifically in *Arabidopsis*, leucocyanidin. The substrate specificity varies in different plants and is important for the colour of the anthocyanins formed. *Arabidopsis* is known to accumulate cyanins (Bloor & Abrahams 2002) in agreement with dihydroquercetin being the substrate for DFR.

ANS is a 2-ODD and catalyses the conversion of leucoanthocyanidin to coloured anthocyanidin. Anthocyanidin is extremely unstable, and hardly detected in plants. *In vivo*, it is the anthocyanidin in pseudobase form that is made, and 2-flaven-3,4-diol is an intermediate (Davies & Schwinn 2006). ANS was also shown to produce dihydroquercetin and quercetin *in vitro* (Turnbull *et al.* 2004). Such a function for ANS *in vivo* could explain the differentiated accumulation pattern often found for kaempferol and quercetin (Borevitz *et al.* 2000; Tohge *et al.* 2005; Lea *et al.* 2007; Olsen *et al.* 2007). The possibility that ANS catalyses conversion of leucocyanidin to quercetin *in vivo* needs investigation. Moreover, several other explanations for differentiated accumulation of flavonols are possible, like different affinities of substrates for FLSs involved, or differences in glycosylation pattern and stability of the derivatives of kaempferol and quercetin.

In *Arabidopsis* and other dicotyledons, light activation of flavonoid production first results in production of CHS, CHI, F3H, F3′H and FLS (early genes); somewhat later, *DFR* and *ANS* (late genes) are activated, which also requires transparent testa glabra1 (TTG1) (Rauscher 2006 and references therein). Especially, the late genes were strongly induced by nitrogen and phosphorus depletion; a 15–20 times increase of transcript levels was observed (Scheible *et al.* 2004; Misson *et al.* 2005; Morcuende *et al.* 2007).

Genes for derivatization of flavonoids are also strongly induced by nitrogen and phosphorus depletion

The UDP-dependent glycosyl transferases (UGTs) use UDP-activated sugars as the sugar donor, and glycosylate flavonoids as well as other compounds. Glycosylation serves to stabilize and change the solubility of flavonoids. Not many of the hundred UGTs present in *Arabidopsis* have been well characterized, but some are examined after heterologous expression and reverse genetics. Recombinant UGT78D2 (At5g17050) could use cyanidin, kaempferol and quercetin as substrates and transferred glucose to the 3 position of the heterologous ring. Glycosylation at the 3 position is especially important for stability of anthocyanins, and in the *ugt78d2* mutant, anthocyanin content was reduced to 20% compared to WT. Flavonols with glucose at the 3 position were also reduced (Jones *et al.* 2003; Tohge *et al.* 2005). Transcripts for UGT78D2 were induced twofold by nitrogen and phosphorous depletions (Scheible *et al.* 2004; Morcuende *et al.* 2007), and very strongly by high light intensity and sucrose (Lloyd & Zakhleniuk 2004; Vanderauwera *et al.* 2005; Solfanelli *et al.* 2006). The UGT75C1 (At4g14090) was found to be responsible for 5-O-glycosylation *in vivo*. In the *ugt75c1* mutant, the cyanidin derivatives but not the flavonols were altered. Other potential anthocyanin glycosylases listed in Fig. 3 are based on co-expression patterns (Tohge *et al.* 2005; Vanderauwera *et al.* 2005; Solfanelli *et al.* 2006).

UGT89C1 (At1g06000) was found to be essential for formation of flavonol-7-O-rhamnosyl, because the *ugt89c1* mutant had no 7-O-rhamnosylated flavonols (Yonekura-Sakakibara *et al.* 2007). UGT78D1 (At1g30530) and UGT73C6 (At2g36790) were found to transfer rhamnose and glucose to flavonols *in vitro* at positions 3 and 7, respectively (Jones *et al.* 2003). Three UGTs, At5g17040, At2g22590 and At4g15480, were suggested as flavonol glycosylases on the basis of the effect of the triple mutation (*myb11/12/111*) on these genes (Stracke *et al.* 2007; see further). One of these glycosylases, At4g15480, was expressed in response to high light intensity (Vanderauwera *et al.* 2005). None of them responded to nitrogen or phosphorus depletion. Other glycosylases are included in Table 1 and Fig. 3 on the basis of homology with known flavonol glycosylases and co-expression (Jones *et al.* 2003; Vanderauwera *et al.* 2005).

Many of the UGTs known, or assumed, to glycosylate flavonoids were highly inducible by mineral nutrient depletion. The activity levels of many of the glycosylases are essential for accumulation of flavonoids. On the basis of their specificity and regulation of expression, UGTs are likely to be key enzymes in differential accumulation of the main classes of products, cyanins, kaempferols and quercetins. However, their properties and regulation are still little explored.

Anthocyanin acyltransferase (AAT) and glutathione S-transferase (GST) also modify flavonoids, and GST is important for transportation of flavonoids into the vacuole. A GST (At5g17220, *TT19*) was shown to be essential for accumulation of cyanins in the vacuole (Kitamura, Shikazono & Tanaka 2004); other genes were pointed out by co-expression (Tohge *et al.* 2005). These genes were also strongly induced by nitrogen or phosphorus deficiency.

Regulators

The regulation of the phenylpropanoid biosynthetic pathways by MYB proteins in combination with bHLH and WD40 proteins seems to be conserved throughout the plant kingdom. The WD40 proteins appear to be ubiquitously expressed (Koes, Verweij & Quattrocchio 2005). TTG1 is a WD40 repeat protein necessary for cyanin and procyanidin production, and also influences epidermal traits in *Arabidopsis*. TTG1 interacts with the bHLH transcription factors GLABRA3 (GL3), enhancer of GLABRA3 (EGL3) and TT8 as shown in yeast two-hybrid assays (Broun 2005).

TTG1 interacts also with the R2R3 MYB transcription factors PAP1, PAP2 and TT2. TTG1 is important for expression of the late genes in *Arabidopsis*, that is, *DFR*, *ANS* and anthocyanidin reductase (*ANR*). *TTG1* is constitutively expressed, and is not influenced by nutrient deprivation (Table 1).

Anthocyaninless2 (*ANL2*) is a homeobox gene important for cyanin accumulation in vegetative tissue in *Arabidopsis* (Kubo *et al.* 1999). It is not clear how this gene acts on flavonoid synthesis, and the gene was not much influenced by mineral nutrient depletion (Table 1). The bZIP transcription factors HY5 and HYH are important for light-dependent processes in plants, and recently, several genes important in the flavonoid pathway were found to be positively regulated by HY5: *CHS*, *FLS1*, *F3H* and *MYB12 (*Lee *et al.* 2007). These bZIP factors were not much influenced by mineral depletion (Table 1). Many transcription factors are regulated in a tissue-dependent manner, as, for example, the transcription factors necessary for procyanidin formation in seeds (Lepiniec *et al.* 2006). Others have been found to be important in pollen (Preston *et al.* 2004). However, these genes (*MYB21*, *TT2*, *EGL3*, *TTG2*, *TT16*, *TT1*, *TT8*) were not significantly expressed in seedlings or leaves, and therefore were not included in Table 1 (confer Supplementary Tables S2–S8).

PAP1 and PAP2 are induced by nitrogen and phosphorus deficiency, high light intensity and sucrose

The *Arabidopsis* PAP1/MYB75 is a transcription factor known to globally enhance expression of genes in the flavonoid pathway. Twenty structural genes of the flavonoid pathway were reported to be up-regulated in *PAP1* over-expressor plants (Tohge *et al.* 2005). *PAL1*, *4CL3* and genes throughout the anthocyanin pathway including glycosyl transferases, acyl transferases and GSTs showed enhanced expression. Expression of nearly all these genes was also enhanced by nitrogen and/or phosphorus deprivation (Table 1, Fig. 3). This points to the likelihood that nitrogen and phosphorus deficiency acts on these structural genes through the PAP transcription factors. PAP2 is a homolog of PAP1 and is generally found to have the same effect on the flavonoid pathway as PAP1 (Borevitz *et al.* 2000). In many investigations, PAP2 has been pointed out as especially strongly enhanced by nitrogen and phosphorus depletion (Scheible *et al.* 2004; Misson *et al.* 2005; Lea *et al.* 2007; Morcuende *et al.* 2007), whereas PAP1 is important for the sucrose-meditated activation of genes in the flavonoid pathway (Teng *et al.* 2005; Diaz *et al.* 2006; Solfanelli *et al.* 2006). *PAP2* is clearly differently regulated from *PAP1*, being expressed at a lower level than *PAP1* under non-stressful conditions. However, under nitrogen deficiency, *PAP2* was not only highly induced, but also the overall amount of *PAP2* transcripts became higher than for *PAP1* as shown by RT-PCR in both seedlings and rosette leaves of *Arabidopsis* (Scheible *et al.* 2004; Lea *et al.* 2007).

It is certainly also striking that in addition to nitrogen or phosphorus deficiency, high intensity light or sucrose exposure led to increased levels of both *PAP1* and *PAP2* transcripts (Lloyd & Zakhleniuk 2004; Vanderauwera *et al.* 2005; Solfanelli *et al.* 2006). Mostly the same structural genes in the flavonoid pathway were enhanced in response to all these treatments. The overall impression is, therefore, that signal transduction from various environmental treatments converges at two common MYB transcription factors, PAP1 and PAP2, which induce structural genes, and lead to an amplification in flavonoid synthesis.

The close homologs MYB12 and MYB111 are induced by high light intensity and sucrose

MYB12 was the first specific regulator described for the flavonol pathway (Mehrtens *et al.* 2005). MYB12 interacts with the target genes through a specific cis-acting element, Myb recognition element (MRE). *MYB12* expression levels and concentration of flavonols in *Arabidopsis* were clearly correlated; hence, *MYB12* is a target for manipulation of flavonol content in plants (Mehrtens *et al.* 2005). Recently, the close homologs of MYB12, MYB11 and MYB111 have also been shown to activate *FLS1* transcription. In seedlings, *MYB12* was found to be mainly expressed in roots, while *MYB111* was mainly expressed in cotyledons. According to microarray data, *MYB12* and *MYB111* can be expressed at about the same levels in rosette leaves (Supplementary Table S6, Vanderauwera *et al.* 2005). Generally, *MYB11* was expressed at a lower level than *MYB12* and *MYB111*. In addition to activating *FLS1*, these MYB factors also enhanced transcription of other early genes, that is, *CHS*, *CHI* and *F3H* as analysed with promoters linked to the GUS reporter gene (Stracke *et al.* 2007).

Evidently, high light intensity has a profound influence on flavonol synthesis. In addition, mineral nutrient depletion leads to increased accumulation of flavonols, but as can be seen from the list of FLS genes and glycosylases assumed to be involved, a signal transduction chain different from the PAP-dependent signal transduction appears to be active because most of the genes for flavonol synthesis and derivatization were not enhanced in the *pap1D* over-expressor plants.

GL3 is important for the nutrient depletion response in Arabidopsis

The bHLH transcription factors GL3 and EGL3 are both known to interact with the MYB transcription factors PAP1 and PAP2 (Zhang *et al.* 2003; Zimmermann *et al.* 2004a). EGL3 or GL3 in combination with PAP1 or PAP2 activated transcription of the *GUS* reporter gene coupled to the *DFR* promoter, whereas the MYBs or bHLHs alone could not activate the *DFR* promoter (Zimmermann *et al.* 2004). *GL3* transcripts were sixfold enhanced in response to nitrogen depletion in rosette leaves, and are an important component in the response to nutrient deficiency in addition to *PAP2* (Lea *et al.* 2007). Induction of anthocyanins by nitrogen

depletion was found to be absent in cotyledons and rosette leaves of the *gl3* mutant; hence, GL3 was not replaced by EGL3 or TT8 in leaves. The WT and *egl3* mutant both showed normal anthocyanin accumulation in response to nitrogen depletion in these experiments (Olsen, Lea & Lillo, unpublished results).

CONCLUDING REMARKS

In the shikimate pathway, regulation on the post-translational level is crucial for positive feedforward by photosynthesis as well as negative feedback by amino acids. (Herrmann & Weaver 1999; Entus *et al.* 2002). The effects of nitrogen or phosphorus depletion on transcript levels of genes in this metabolic pathway are complex, giving both positive and negative responses (Fig. 2, references in figure text). Thirteen genes in the shikimate pathway were up-regulated greater than twofold by sucrose, or high light intensity, but only two of these genes by both (Fig. 2, Lloyd & Zakhleniuk 2004; Vanderauwera *et al.* 2005; Solfanelli *et al.* 2006), excluding a general mediator for these treatments. This may reflect the fact that the shikimate pathway provides precursors for many products having very diverse functions in the plant.

As opposed to genes in the shikimate pathway, genes throughout the flavonoid pathway showed a consistent response to nutrient depletion (Table 1, Fig. 3, references in Table 2). Transcript levels were enhanced by factors 2–3 (*PAL*, *C4H, 4CL*, *CHS*, *F3H*, *F3′H*), and even as high as 20–40 times for genes towards the end of the pathway that are involved in anthocyanin synthesis only (*DFR*, *ANS*, *AGT*, *AAT*, *GST*). Most of these genes, which were responsive to mineral nutrient depletion, also responded to sucrose and high light intensity and were induced in *PAP1* over-expressors. This points to PAP transcription factors as a component in common for mediating the mineral nutrient responses as well as high light intensity and sucrose responses. The components in the signal perception and transduction upstream of PAP1/PAP2 still need to be revealed.

Flavonol content increased when plants were depleted of nitrogen or phosphorus. Especially quercetins showed increased levels in response to nitrogen depletion provided that a second trigger was present such as sucrose or below optimal growth temperature (Stewart *et al.* 2001; Lea *et al.* 2007; Olsen *et al.* 2007). In contrast to the effect of mineral depletion on anthocyanin synthesis, the mechanism for increased quercetin levels cannot easily be explained through enhanced *PAP* expression. This is because *PAP* over-expression did not lead to activation of *FLS* or *FGT* genes, which are proposed to be involved in flavonol synthesis, except for At5g17050, which transfers glucose to the 3 position in flavonoids. Very little is known concerning mineral nutrients and regulation of UGTs, and this will be an interesting topic for future research.

The differentiated response of kaempferol as opposed to quercetin accumulation is also not well understood. Kaempferols appear to be more constitutively present, and quercetin accumulation appears to depend on the interaction of several factors. The ability of ANS to produce quercetin from leucocyanidin could possibly contribute to the specific increase in quercetins (Turnbull *et al.* 2004), but establishing or excluding this function of ANS *in vivo* needs investigation. The synergy of important growth conditions such as mineral nutrients, temperature and light needs to be further explored to be able to manipulate plant components in agreement with desirable criteria for quality. Furthermore, agricultural plants need thorough explorations. Synthesis of flavonoids is far better investigated than catabolism; the latter also needs to be taken into account when considering mechanisms for accumulation of flavonoids. Although many details have been elucidated during the past years, it is still largely unclear how a cell determines which class and derivatives of flavonoids will accumulate. A systems biology approach taking into account both expression levels and enzymatic properties is necessary to fully understand and predict accumulation kinetics in the shikimate and flavonoid pathways.

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SUPPLEMENTARY MATERIAL

The following supplementary material is available for this article:

The genes shown in Table 1 were extracted and sorted from various studies (Supplementary Table S1) using a Perl script that was written for this purpose. In addition to the data shown in Table 1, the extracted data in their original format and sorted in the order of Table 1 are also provided (Supplementary Tables S2–S8).

Table S1. Summary of the data used in this study, description of plant material.

Table S2. Extracted data from Scheible *et al.*'s (2004) supplementary table S2 (see also Supplementary Table S1, column D). The values in Table 1 were calculated by determining the average of the signals of starved plants (columns B, D, F), the average of signals of plants that received full nutrition (columns T, V, X) and then by calculating the log 2 values of the ratios between the averaged signals of starved and non-starved plants.

Table S3. Extracted data from Morcuende *et al.*'s (2007) supplementary table S1 (see also Supplementary Table S1, column E). The values in Table 1 were calculated by determining the average of the signals of Pi-starved plants (columns C, E), the average of signals of plants that received full nutrition (columns O, Q, S) and then by

calculating the log 2 values of the ratios between the averaged signals of Pi-starved and non-starved plants.

Table S4. Extracted data from Misson *et al.*'s (2005) supplementary table 5 (see also Supplementary Table S1, column F). The values in Table 1 were calculated by determining first the three ratios between Pi-starved and non-starved plants by calculating the ratios between columns C and F, I and L, and O and R. Then, the average ratio was determined, and the log 2 value from the average ratio was calculated.

Table S5. Extracted data from Müller *et al.*'s (2007) supplementary table S2 (see also Supplementary Table S1, column G). The values for Pi starvation in Table 1 were taken directly from column AV.

Table S6. Data from Vanderauwera *et al.* (2005) (see also Supplementary Table S1, column H) downloaded using Genvestigator ATH1 chip 52 (http://www.genevestigator.ethz.ch/) (Zimmermann *et al.* 2004b). The values for the effect of high light in Table 1 were taken from column K.

Table S7. Extracted data from Solfanelli *et al.*'s (2006) supplementary table S1 (see also Supplementary Table S1, column I). The values in Table 1 were calculated by determining first the two ratios between sucrose-treated plants and control plants by calculating the ratios between columns K and G, and M and I. Then, the average ratio was determined, and the log 2 value from the average ratio was calculated.

Table S8. Extracted data from Lloyd & Zakhleniuk's (2004) Table NASCARRAY-49, http://arabidopsis.info/ (see also Supplementary Table S1, column J) The values in Table 1 were calculated by determining the ratio between *pho3* and WT plants (columns K and L). Column M contains the log2 values of the ratio.

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