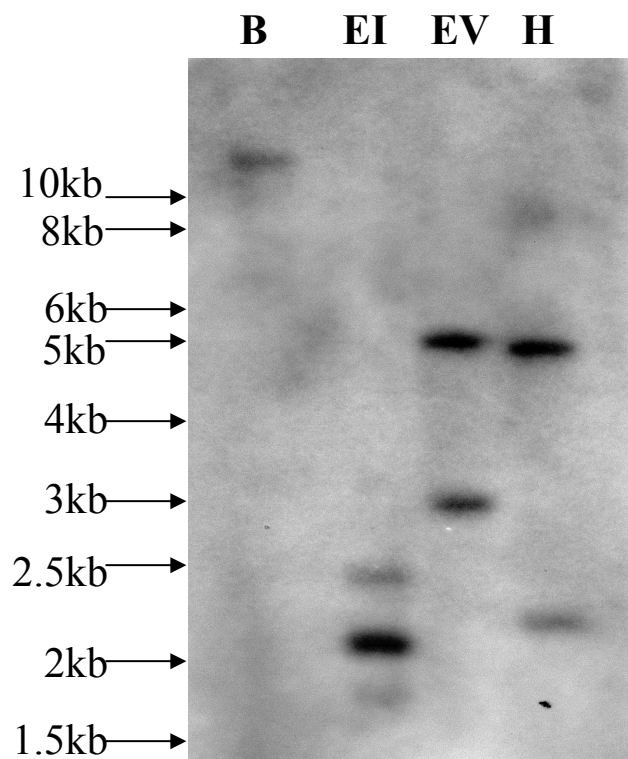


Early steps in proanthocyanidin biosynthesis in the model legume *Medicago truncatula*

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SUPPLEMENTAL MATERIAL

Supplemental Figure 1. DNA gel blot analysis of *LAR* gene copy number in *Medicago truncatula*. Genomic DNA was digested with *Bam*HI (B), *Eco*RI (EI), *Eco*RV (EV), or *Hind*III (H), and size-fractionated on a 0.8% agarose gel, blotted, and hybridized with *MtLAR* ORF probe.

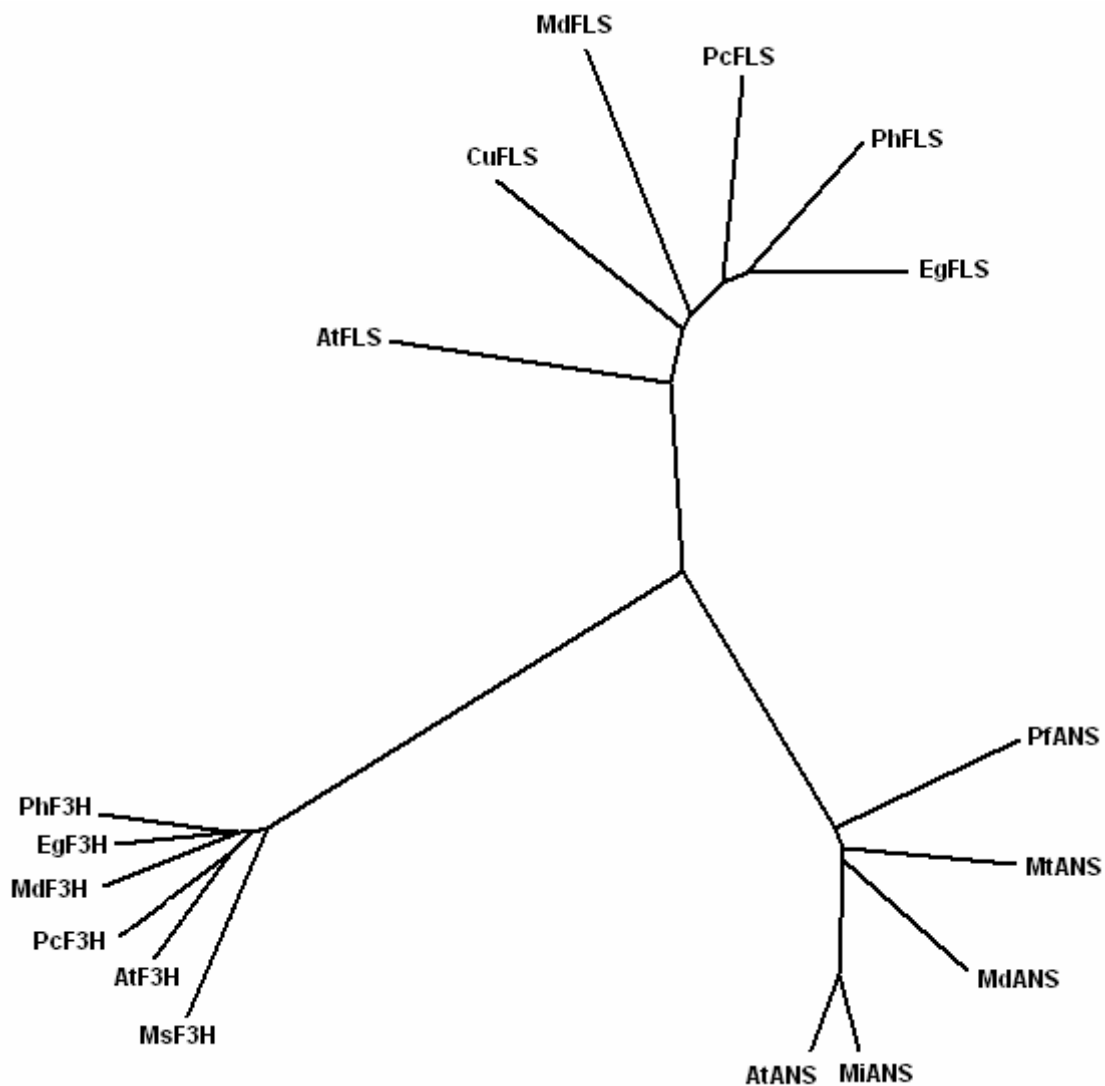


Supplemental Figure 2. Alignment of the deduced amino acid sequences of *ANS* genes. The sequences are from *M. truncatula*, *Arabidopsis thaliana* (Q96323), *Malus domestica* (BAB92998.1) and *Perillia frutescens* (O04274). Identical amino acids are indicated by white letters on a black background, conservative amino acids by white on a dark gray background, and similar amino acids by black on a light gray background. The four amino acid residues required for coordinating ferrous-iron and binding of 2-oxoglutarate are indicated by stars.

		1	60
MtANS	(1)	----MGTVAQRVESLALSGISSIPKEYVRPKEELANIG-NIFDEEKK-EGPOVPTIDLKE	
AtANS	(1)	----MVAVERVESLAKSGTISIPKEYIRPKEELESIN-DVFLEEKKEGPOVPTIDLKN	
MdANS	(1)	-MVSSDSVNSRVETLAGSGTISTIPKEYIRPKDELVNIG-DIFEOEKNNEGPOVPTIDLKE	
PfANS	(1)	MVTSAMGPSFRVEELARSGLDTIPKDYVRPEEELKSLIGNILAEKSSSEGPOVPTIDLKE	
Consensus	(1)	M SAMSVA RVESLAKSGISSIPKEYIRPKEEL SIG NIFDEEKK EGFQVPTIDLKE	
		61	120
MtANS	(55)	INSSDEIVRGKCREKLLKAAEEWGMHLVNHGISDDLINRLKKAGETFFELPVVEEKKEYA	
AtANS	(55)	IESDDEKIRENCIEELKKASLDWGMHLINHGIPADLMERVKKAGEEFSLSVVEEKKEYA	
MdANS	(59)	IESDNEKVRRAKCREKLLKAAVDWGMHLVNHGISDELMDKVRKAGKAFDFLPIEOKKEYA	
PfANS	(61)	MDSRDEEGKPKCHEELKKAATDWGMHLINHGIPPELIDRVKAAGKEFFELPVVEEKKEYA	
Consensus	(61)	IESDDEKVRRAKCREKLLKAAALDWGMHLINHGISDDLIDRVKKAGKEFFELPVVEEKKEYA	
		121	180
MtANS	(115)	NDOSSGKIQGYGSKLANNASGOLEWEDYFFHCIFPEDKRDLSIWPKTPADYTKVTSEYAK	
AtANS	(115)	NDOATGKIQGYGSKLANNASGOLEWEDYFFHLAYPEEKRDLSIWPKTPSDYIEATSEYAK	
MdANS	(119)	NDOASGKIQGYGSKLANNASGOLEWEDYFFHCVPYEDKRDLSIWPKTPADYIEATAEYAK	
PfANS	(121)	NDQAAGNVQGYGSKLANNASGOLEWEDYFFHCVPYEPKTDLSIWPKTPEDYIPATSEYAK	
Consensus	(121)	NDQASGKIQGYGSKLANNASGOLEWEDYFFHCVPYEDKRDLSIWPKTPADYIEATSEYAK	
		181	* *
MtANS	(175)	ELRVLASKIMEVLSIELGLEGRLEKEAGGMEELLIOMKINYYPICPOPELALGVEAHTD	
AtANS	(175)	CLRLLATKVEKALSVGLGLEPDRLEKEVGGIEELLIOMKINYYPKCPOPELALGVEAHTD	
MdANS	(179)	OLRELATKVLKVLVSLGLGLEGRLEKEVGGIEELLIOMKINYYPKCPOPELALGVEAHTD	
PfANS	(181)	QLRALATKILSVLSIGLGLEKGRLEKEVGGAEIDLIVOMKINYYPKCPOPELALGVEAHTD	
Consensus	(181)	QLRLLATKILKVLVSLGLGLE GRLEKEVGGIEELLIOMKINYYPKCPOPELALGVEAHTD	
		241	* 300
MtANS	(235)	VSSLTFLHNMVPGLOLFYEGKQVVTAKCVDSILMHIGDTIEILSNGKYKSILHRGLVNK	
AtANS	(235)	VSALTFILHNMVPGLOLFYEGKQVVTAKCVDSIVMHIGDTIEILSNGKYKSILHRGLVNK	
MdANS	(239)	VSALTFILHNMVPGLOLFYEGKQVVTAKCVENSIVMHIGDTIEILSNGKYKSILHRGLVNK	
PfANS	(241)	VSALTFILHNMVPGLOLFYEDKQVVTAKCVENSIVMHIGDTIEILSNGKYKSILHRGLVNK	
Consensus	(241)	VSALTFILHNMVPGLOLFYEGKQVVTAKCVFNSIVMHIGDTIEILSNGKYKSILHRGLVNK	
		301*	360
MtANS	(295)	EKVRISWAVFCEPPKKEKILKPLPELVTEKEPARFPPRTFAOHIHKKLFRKDEEEKKDDF	
AtANS	(295)	EKVRISWAVFCEPPKDKIVLKLPEMVSVEVSPAKFPPRTFAOHIHKKLFRKDEEEKKDDF	
MdANS	(299)	EKVRISWAVFCEPPKKEKILKPLPETVSEDEPAMFPPRTFAOHIHKKLFRKDEEEKKDDF	
PfANS	(301)	EKVRISWAVFCEPPKKEKIVLQPLPETVSEVEPPRFPPRTFAOHLKHLFRKDEEEKKDDF	
Consensus	(301)	EKVRISWAVFCEPPKKEKILKPLPETVSEDEPARFPPRTFAOHI HKKLFRKDEEEKKDDF	
		361	
MtANS	(355)	KK	
AtANS	(355)	ND	
MdANS	(357)	K-	
PfANS	(361)	TY	
Consensus	(361)	K	

Supplemental Figure 3. Phylogenetic tree of several classes of 2-ODDs (flavonol synthases [FLS], ANSs and flavanone 3- β -hydroxylases [F3H]) involved in flavonoid biosynthesis.

The reliability of the tree was measured by bootstrap analysis with 1000 replicates. The deduced FLS protein sequences are from: *Eustoma grandiflorum* (AAF64168.1), *Petroselinum crispum* (AAP57395), *Petunia* \times *hybrida* (CAA80264), *Malus* \times *domestica* (AAD26261), *Citrus unshiu* (BAA36554.1) and *A. thaliana* (Q96330). The deduced ANS protein sequences are from *P. frutescens* (O04274), *A. thaliana* (Q96323), *M. domestica* (BAB92998.1), *Matthiola incana* (AAB82287) and MtANS in the present report. The F3H sequences are from *Medicago sativa* (CAA57410), *Petroselinum crispum* (AAP57394.1), *A. thaliana* (AAC49176), *P. hybrida* (A42110), *E. grandiflorum* (BAD34459) and *M. domestica* (Q06942).

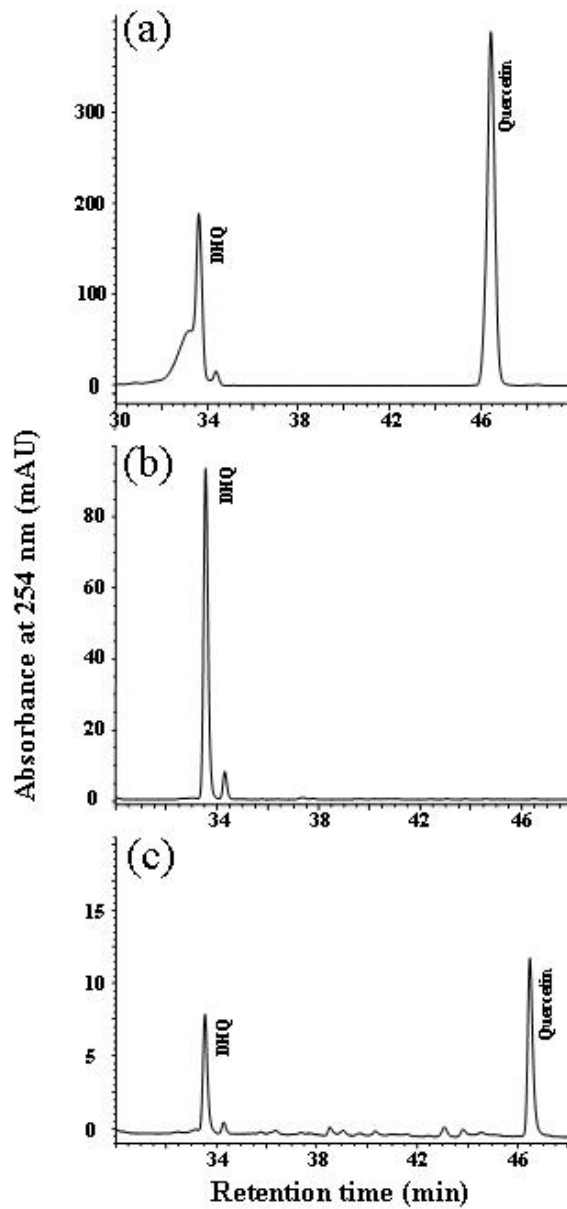


Supplemental Figure 4. HPLC analysis of the products from incubation of recombinant MtANS protein with dihydroquercetin.

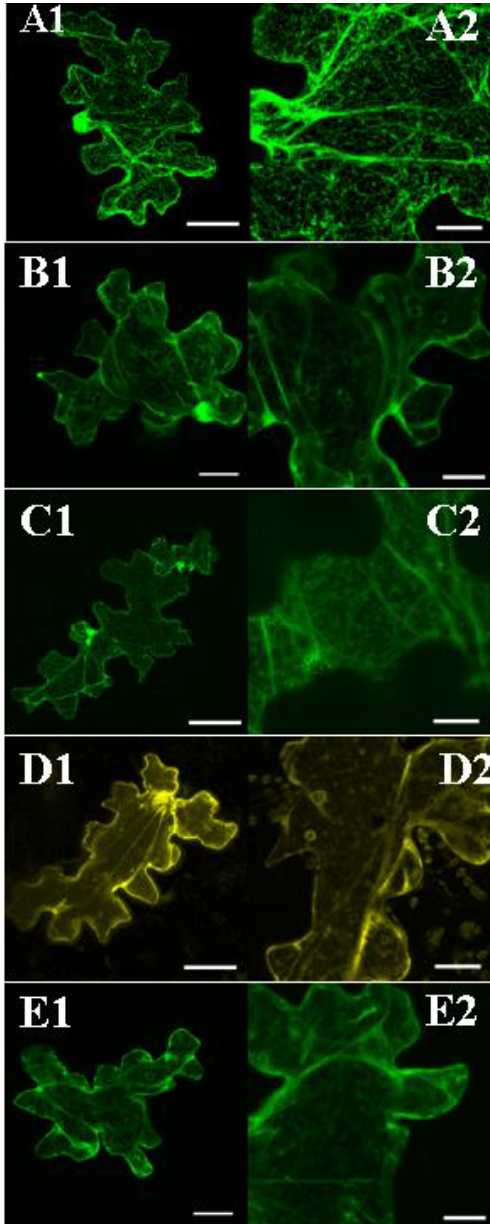
(a) Dihydroquercetin (DHQ) and quercetin standards.

(b) Product from incubation of dihydroquercetin with crude protein extract from vector control.

(c) Product from incubation of dihydroquercetin with purified recombinant MtANS.



Supplemental Figure 5. Subcellular localization of MtANS, MtLAR and MtANR in tobacco leaf epidermal cells by particle bombardment. Representative images of whole cells (left column) and part of a cell (right column) transiently expressing the various EGFP/YFP fusions. Cells expressing the cinnamate 4-hydroxylase transmembrane domain (C4H MA)-EGFP fusion display the reticulate ER localization (A1-2). Cells transiently expressing EGFP control (B1-2), MtLAR-EGFP (C1-2), MtANS-YFP (D1-2) and MtANR-EGFP (E1-2) show diffuse labeling, which is indicative of cytoplasmic localization. All images are projections of 20-30 optical sections taken at 0.5 μm intervals. Bars = 40 μm (left column) and 20 μm (right column).

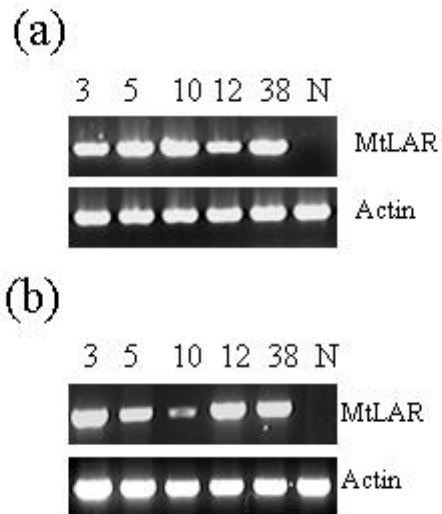


Supplemental Figure 6. RT-PCR analysis of MtLAR transcript levels in transgenic tobacco plants constitutively expressing MtLAR.

(a) Leaf tissue.

(b) Flower tissue.

Numbers refer to independent transgenic lines, N is empty vector control.



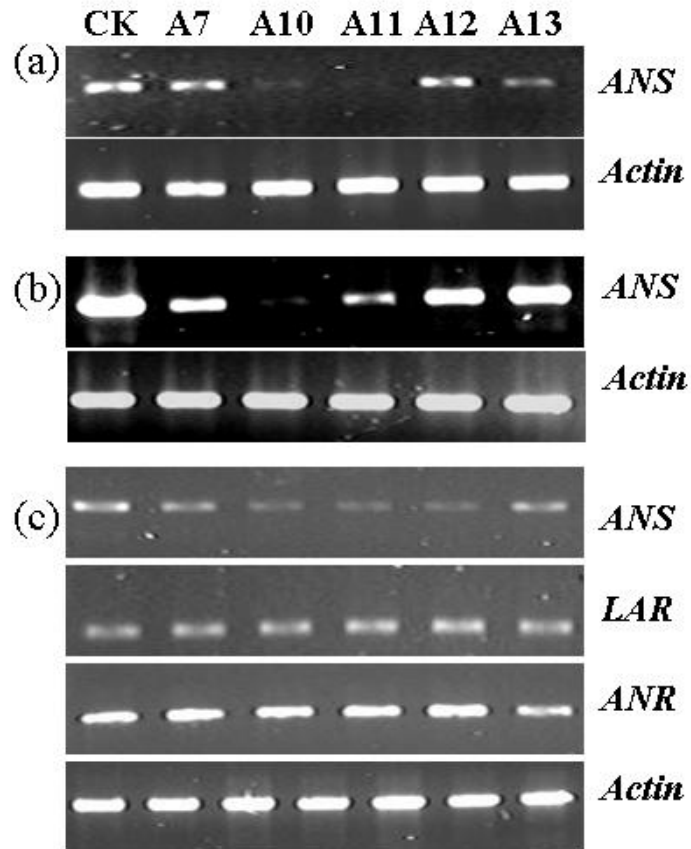
Supplemental Figure 7. RT-PCR analysis of transcripts encoding PA biosynthetic genes in transgenic *M. truncatula* R108 plants expressing an antisense *MtANS* transgene (A7-A13) and empty vector control (N).

(a) ANS transcript levels in leaf tissue.

(b) ANS transcripts in flower tissue;

(c) ANS, LAR and ANR transcripts in seeds.

Actin is used as constitutive control gene.



Supplemental Figure 8. HPLC analysis of flavonoids from control *M. truncatula* (a-c) and transgenic MtANS antisense line A10 (d-f).

(a, d) Flavonol standards- M, myricetin, Q, quercetin, K, kaempferol.

(b, e) Total flavonoids from leaf tissue.

(c, f) Total flavonoids from flower tissue.

