Running head: A WD40 repeat protein from *Medicago truncatula*

**Correspondence to:** Richard A. Dixon, Plant Biology Division, Samuel Roberts Noble Foundation, 2510 Sam Noble Parkway, Ardmore, OK, 73401, USA.
Phone: 580-224-6601
Fax: 580-224-6692
e-mail: radixon@noble.org

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A WD40 repeat protein from *Medicago truncatula* is necessary for tissue-specific anthocyanin and proanthocyanidin biosynthesis, but not for trichome development.

Yongzhen Pang, Jonathan P. Wenger, Katie Saathoff, Gregory J. Peel, Jiangqi Wen, David Huhman, Stacy N. Allen, Yuhong Tang, Xiaofei Cheng, Million Tadege, Pascal Ratet, Kirankumar S. Mysore, Lloyd W. Sumner, M. David Marks and Richard A. Dixon*

Plant Biology Division, Samuel Roberts Noble Foundation, 2510 Sam Noble Parkway, Ardmore, OK, 73401, USA (Y.P., G.J.P., J.W., D.H., S.N.A., Y.T., X.C., M.T., K.S.M., L.W.S., R.A.D); Department of Genetics and Cell Biology, University of Minnesota, St. Paul, MN, 55108 (J.P.W., K.S., M.D.M.); Institut des Sciences du Vegetale, CNRS, Avenue de la Terrasse, 91198 Gif sur Yvette, France (P.R.)

Present address: Calgene/Monsanto, 1920 5th Street, Davis, CA 95616 (G.J.P).
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* Corresponding author; e-mail: radixon@noble.org; fax +1-580-224-6692

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The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (www.plantphysiol.org) is: Richard A. Dixon (radixon@noble.org).
Abstract
WD40 repeat proteins regulate biosynthesis of anthocyanins, proanthocyanidins (PAs) and mucilagous in the seed, and the development of trichomes and root hairs. We have cloned and characterized a WD40 repeat protein gene from *Medicago truncatula* (*MtWD40-1*) via a retrotransposon tagging approach. Deficiency of *MtWD40-1* expression blocks accumulation of mucilage and a range of phenolic compounds, including PAs, epicatechin, other flavonoids and benzoic acids, in the seed, reduces epicatechin levels without corresponding effects on other flavonoids in flowers, reduces isoflavone levels in roots, but does not impair trichome or root hair development. *MtWD40-1* is expressed constitutively, with highest expression in the seed coat where its transcript profile temporally parallels those of PA biosynthetic genes. Transcript profile analysis revealed that many genes of flavonoid biosynthesis were down-regulated in a tissue-specific manner in *M. truncatula* lines harboring retrotransposon insertions in the *MtWD40-1* gene. *MtWD40-1* complemented the anthocyanin, PA and trichome phenotypes of the Arabidopsis *ttg1* mutant. We discuss the function of MtWD40-1 in natural product formation in *Medicago*, and the potential use of the gene for engineering PAs in the forage legume alfalfa (*Medicago sativa*).

**Keywords:** *Medicago*, WD40 protein, anthocyanin, proanthocyaninidin, transcriptional regulator, trichome development

Introduction
Anthocyanins and proanthocyanidins (PAs, also called condensed tannins) are flavonoids which benefit both plant and human health. Anthocyanins attract pollinators, protect plant tissues from UV damage, and defend plants against predators (Stapleton and Walbot, 1994; Sullivan, 1998). PAs are abundant in beverages such as tea, wine and fruit juice, and exhibit antioxidant activity and cardiovascular protective effects (Bagchi et al., 2000; Cos et al., 2004; Dixon et al., 2005). Moreover, a moderate PA level is an important quality trait in forage crops, because PAs can protect ruminant animals from lethal
pasture bloat by binding proteins and thereby slowing down their fermentation in the rumen (Li et al., 1996; Aerts et al., 1999; Barry and McNabb, 1999; Dixon et al., 2005).

The PA biosynthetic pathway in Arabidopsis thaliana has been studied primarily through the analysis of transparent testa (tt) or transparent testa glabrous (ttg) mutants which exhibit seed coat (tt) or seed coat and trichome (ttg) phenotypes (Shirley et al., 1995; Lepiniec et al., 2006). The mutated genes have been found to encode either pathway enzymes or transcriptional regulators that function alone or in complexes to control the whole or branches of the pathway (Lepiniec et al., 2006). Anthocyanins and PAs share the same upstream phenylpropanoid/flavonoid pathway, and anthocyanidin is the immediate substrate for both glycosylation to anthocyanin or reduction to epicatechin in the biosynthesis of PAs in Arabidopsis (Fig. 1).

We are studying the formation of PAs in the model legume Medicago truncatula (Xie et al., 2003, 2006; Pang et al., 2007, 2008). Four structural genes encoding anthocyanidin synthase (ANS), leucoanthocyanidin reductase (LAR), anthocyanidin reductase (ANR) and epicatechin 3’-O-glucosyltransferase (UGT72L1) were characterized biochemically and/or genetically from this species (Xie et al., 2003; Pang et al., 2007, 2008). However, little is known about the regulatory network involved in anthocyanin/PA biosynthesis in M. truncatula.

A regulatory complex, comprising an R2R3-MYB transcription factor, a basic helix-loop-helix (HLH) domain protein and a WD40 repeat protein, regulates production of anthocyanins in foliar tissues and PAs and mucilage in seed coats; this complex also controls the formation of root hairs and trichomes on aerial tissues in some but not all plants (Baudry et al., 2004; Broun, 2005; Lepiniec et al., 2006; Morita et al., 2006; Serna and Martin, 2006; Gonzalez, 2008; Zhao et al., 2008). In Arabidopsis, these proteins are encoded by Transparent Testa 2 (TT2, Myb), Transparent Testa 8 (TT8, HLH), and Transparent Testa Glabrous 1 (TTG1, WD40 repeat), which together regulate the late flavonoid pathway genes and the PA-specific pathway gene ANR (Baudry et al., 2004). Loss-of-function of either TT2 or TT8 leads to a lack of anthocyanin pigmentation in foliar tissue and a loss of PAs in the seed coat (Nesi et al., 2000, 2001). The presence of TTG1 is essential and irreplaceable in this complex for anthocyanin/PA biosynthesis, trichome formation, seed mucilage production and root hair formation (Koornneef, 1981;
Walker et al., 1999). Several other WD40 repeat proteins functionally orthologous to TTG1 have been described from other species such as petunia, *Perilla frutescens*, cotton and maize; mutation of some affects both anthocyanin/PA and trichome phenotypes, whereas mutation of others only affects the anthocyanin/PA phenotype (Ludwig et al., 1989; Lloyd et al., 1992; de Vetten et al., 1997; Sompompailin et al., 2002; Carey et al., 2004; Humphries et al., 2005).

In an attempt to identify genes involved in the regulation of anthocyanin and PA biosynthesis in *M. truncatula*, we have screened a *Tnt1* retrotransposon insertion population for altered leaf (lack of red pigment) and seed (transparent testa) phenotypes. This led to the cloning and functional characterization of a gene, *MtWD40-1*, with high sequence identity to known WD40 repeat proteins. *MtWD40-1* can complement the Arabidopsis *ttg1* PA and trichome phenotypes, although the *Medicago wd40-1* mutant retained normal trichomes. Loss of *MtWD40-1* function has profound and differential effects on flavonoid biosynthesis in different plant organs. The potential of *MtWD40-1* for engineering the PA pathway in alfalfa (*M. sativa*) was also investigated.

**RESULTS**

**Phenotypic and Genotypic Characterization of *M. truncatula* Retrotransposon Insertion Mutants**

One mutant line (NF0977) drew our attention when screening the *M. truncatula Tnt1* insertion population for visible anthocyanin phenotypes. This line lacked the typical red pigmentation in the stem, the anthocyanin-rich circle at the base of the axial side of the leaflet, and the small red spots on the adaxial side of the leaflet, all of which are seen in wild-type ecotype R108 (Fig. 2A, B). The seed coat of this mutant line was transparent with a yellowish color that contrasted with the brown pigmentation of the wild-type that arises from the presence of oxidized PAs (Fig. 2C). To further confirm the PA phenotype, seeds were stained with DMACA (dimethylaminocinnamaldehyde), a reagent that is specific for PAs and their flavan 3-ol precursors. Mature seeds from the mutant line did not exhibit the typical blue staining characteristic of the reaction of PAs with DMACA (Fig. 2C). The seeds from the mutant also produced less mucilage than those of the wild-type, as seen by the reduced staining of the seed coat with ruthenium red (Fig. 2C). No
other obvious phenotypes, such as altered density of glandular or non-glandular trichomes (Fig. 2D) or root hairs (Fig 2E), were observed in the NF0977 mutant. Root hair density appeared to be unaffected on both young (4 days after germination, Fig. 2E) and mature roots (Supplemental Fig. S1).

One of 12 plants from the NF0977 R2 generation exhibiting the lack of pigmentation phenotype was allowed to undergo self pollination. All 29 plants from the R3 generation were homozygous, as confirmed by PCR with gene-specific primers and a primer for the Tnt1 insert, and retained the visible mutant phenotypes as characterized in Fig. 2A-C. Use of Thermal Asymmetric Interlaced Polymerase Chain Reaction (TAIL-PCR) revealed that all individuals possessed a retrotransposon insertion in a WD40 gene with similarity to the TTG1 gene from Arabidopsis. After sequencing and alignment using the available M. truncatula genome database, this Tnt1 insertion was found to be between the first and second nucleotides of amino acid residue Ser31 of the WD40 protein in the NF0977 mutant (Fig. 2F). A further 20 insertion sites in different regions of the genome were also recovered from NF0977 (Supplemental Table S1), typical for Tnt1 insertional mutagenesis in Medicago (Tadege et al., 2008). None of these insertions was in a gene that would be expected to affect flavonoid biosynthesis, although this does not rule out the possibility that the lack of pigmentation phenotype could have been the result of an insertion in one or more of these genes. A reverse genetic approach was therefore employed to screen the Tnt1 insertion mutant population for additional lines with insertions in the WD40 gene, and another mutant line, NF2745, was obtained. The insertion site in line NF2745 was between amino acid residues S46 and I47 (Fig. 2F). Homozygous NF2745 plants exhibited the same phenotype as NF0977 (Fig 2A-E), strongly suggesting that the loss of function of the WD40 gene is responsible for the pigmentation phenotypes in the two mutants.

Molecular Cloning and Characterization of MtWD40-1
BlastX analysis of the partial WD40 sequence against the GenBank database showed that this gene was located on the M. truncatula BAC clone CR940305. Its full-length sequence was predicted to be 1363 bp in length with a 49 bp 5’UTR and a 285 bp 3’UTR region (designated as MtWD40-1, GenBank Accession No. EU040206). MtWD40-1 is a
single copy gene lacking introns, as confirmed by DNA gel blot analysis and amplification of the *MtWD40-1* open reading frame with genomic DNA as template (data not shown). *MtWD40-1* encodes a predicted protein open reading frame of 343 amino acids, with a calculated isoelectric point of 4.99 and a molecular weight of 38 kDa.

The deduced amino acid sequence of MtWD40-1 showed 77-79% identity to other known WD40 repeat proteins from different plant species, such as TTG1 from Arabidopsis and AN11 from petunia (Fig. 3). The four WD40 repeat domains are highly conserved among all the WD40 repeat proteins including MtWD40-1, and the last two amino acids in each WD40 repeat are identical. Phylogenetic analysis (Fig. 4) showed that MtWD40-1 is most closely related to TTG1 from Arabidopsis. Another *Medicago* WD40-like protein, MtWD40-2, is less than 60% identical to MtWD40-1 at the amino acid level, and somewhat closer to PAC1 from maize.

**MtWD40-1 Complements the Arabidopsis *ttg1* and *Medicago* NF0977 Mutants by Interacting with GL3**

Hairy roots of *M. truncatula* R108 exhibit red anthocyanin pigmentation (Pang et al., 2008), but this was lacking in the NF0977 line. Hairy root transformation was therefore used as a rapid method to confirm that *MtWD40-1* could complement the lack-of pigment phenotype of the NF0977 Tnt1 insertion mutant. Red pigmentation was seen in all 101 phosphinothricin (ppt)-resistant hairy root lines transformed with *MtWD40-1*, but in none of the 30 ppt-resistant NF0977 lines transformed with the *GUS* gene (Fig. 5A). qRT-PCR confirmed that *MtWD40-1*, *ANS* and the anthocyanin-specific glucosyltransferase *UGT78G1* (Modolo et al., 2007; Peel et al., 2008) were expressed at higher levels in hairy roots of the *MtWD40-1* transformed lines than in the *GUS* transformants (Fig. 5B-D), thus accounting for the high levels of extractable anthocyanins in the *MtWD40-1* expressing lines (Fig. 5E). No significant differences were observed in the levels of insoluble PAs (PAs that bind to the cell wall and can not be extracted by organic solvents such as 70% acetone) between the *MtWD40-1*-expressing NF0977 lines compared with the GUS control lines (Fig. 5G), or in the levels of transcripts encoding the PA pathway-specific genes *ANR* and *UGT72L1* (data not shown). In contrast, soluble PA levels
decreased slightly in the mutant line complemented with *MtWD40-1* (Fig. 5F), possibly as a result of flux into soluble PAs being diverted back into the anthocyanin pathway.

To determine whether *MtWD40-1* is a functional ortholog of *TTG1*, the *MtWD40-1* open reading frame under the control of the 35S promoter was transformed into the Arabidopsis *ttg1-9* mutant, and expression of the foreign *MtWD40-1* gene was confirmed by qRT-PCR (Supplemental Fig. S2). 35S:*MtWD40-1* fully complemented the anthocyanin pigmentation, trichome deficiency and seed coat PA phenotypes (Fig. 6A-C). We also tested the ability of *MtWD40-1* to complement the Arabidopsis *ttg1-9* mutant when expressed under control of the Arabidopsis *Glabrous 2* (*GL2*) promoter, which is active in the shoots of *ttg1* mutants (Szymanski et al., 1998). Again, the phenotype was fully rescued (Fig. 6D-F).

To further determine how MtWD40-1 might function to restore the trichome phenotype in *ttg1-9* Arabidopsis, the yeast two-hybrid system was used to test the interaction of MtWD40-1 with Glabrous 3 (*GL3*), a bHLH protein that regulates trichome development in *Arabidopsis* through interaction with *GL1* and *TTG1* (Payne et al., 2000). *GL3* was fused to the activation domain (AD) of GAL4, and MtWD40-1 was fused to the binding domain (BD) of GAL4. Yeast containing empty pGAD424 (AD) and pBridge (BD) vectors in conjunction with MtWD40-1 did not exhibit β-galactosidase activity (Fig. 6G, top), whereas yeast containing *GL3*-AD and MtWD40-1BD exhibited strong activity (Fig. 6G, bottom), suggesting that MtWD40-1 can interact with GL3 for trichome formation in Arabidopsis, even though it is not necessary for trichome formation in *M. truncatula*.

### Tissue- and Developmental-Specific Expression of MtWD40-1

To determine the developmental expression pattern of *MtWD40-1*, normalized data were retrieved from the *M. truncatula* gene expression atlas (Benedito et al., 2008) together with seed coat microarray data (Pang et al., 2008). The expression pattern of two probe sets for *MtWD40-1* (TC105711 and AL372205, probe set locations shown in Fig. 2F) were essentially the same, confirming that, as is also the case for *TTG1* in Arabidopsis (Walker et al., 1999), *MtWD40-1* is expressed in all organs, with highest expression in the seed coat (Fig. 7A). During seed development, *MtWD40-1* showed its highest
expression level at or before 10 days after pollination (dap, Fig. 7B), with a subsequent decline towards seed maturity. This expression pattern parallels the expression of MtANR and UGT72L1 during seed development (Pang et al., 2008).

We also analyzed the expression pattern of MtWD40-2 in the M. truncatula gene expression atlas (Benedito et al., 2008), where it is represented by probe set Mtr. 22605.1.S1_at (http://bioinfo.noble.org/gene-atlas/v2/). The highest expression level is in roots 24 h after salt stress and in developing root nodules, but the expression level in these tissues is nearly two orders of magnitude lower than the maximum expression level of MtWD40-1 (in developing seeds). MtWD40-2 is expressed around 15-fold lower than MtWD40-1 in trichome-containing leaf and petiole tissues, and is only expressed very weakly if at all in isolated non-glandular trichomes from M. truncatula (only called present in one out of three Affymetrix data sets, MDM unpublished results). Furthermore, unlike MtWD40-1, MtWD40-2 is not induced in M. truncatula hairy roots expressing Arabidopsis TT2.

Tissue-Specific Effects of Loss of MtWD40-1 Function on Phenylpropanoid/Flavonoid Pathway Gene Transcripts and Metabolites

To determine the impacts of the loss of WD40-1 function on gene expression in seed, we dissected seeds at 16 dap from both the NF0977 mutant line and the corresponding wild-type control (ecotype R108) for microarray analysis using the Affymetrix Medicago Genechip. We have previously shown that phenylpropanoid/flavonoid biosynthetic pathway genes are highly expressed at 16 dap (Pang et al., 2007). The microarray data showed that 152 probe sets were down-regulated more than 2-fold in the MtWD40-1 mutant line; among these, 3 probe sets were down-regulated by more than 100-fold, 25 by more than 5-fold, with the remainder between 2-5 fold (Supplemental Table 2E). Classification using the GeneBins ontology tool (http://bioinfoserver.rsbs.anu.edu.au/utils/GeneBins/index.php) showed that a high percentage (43.5%) of the down-regulated genes were “unclassified with homology” followed by “biosynthesis of secondary metabolites” (25.9%) (Supplemental Fig. S3). This latter class consisted primarily of phenylpropanoid/flavonoid pathway genes.
Among the 28 probe sets that exhibited a more than 5-fold reduction in expression level in the MtWD40-1 mutant (Table 1), seventeen were associated with the phenylpropanoid/flavonoid pathway, and one had no homology to any known gene. The early phenylpropanoid pathway genes PAL, 4CL, CHS, F3’H and F3’5’H were all down-regulated, almost 200-fold in the case of one CHS probe set (Table 1, Supplemental Table S2). CHS is encoded by a large gene family in Medicago, and nine different CHS probe sets were down-regulated more than 5-fold (Supplemental Table S2). The two later anthocyanin pathway genes, DFR and ANS, were down-regulated by 2.6-fold and 9.2/10.0-fold respectively (Table 1, Supplemental Table S2), suggesting that MtWD40-1 regulates both early and later anthocyanin pathway genes in seeds. Three genes specific for the PA pathway, LAR, ANR and UGT72L1, were down-regulated 3.9-, 34.6- and 14.7-fold, respectively, highlighting the specific involvement of MtWD40-1 in the regulation of PA biosynthesis. MtWD40-1 might also regulate additional branches of the flavonoid pathway, as seen by the 40.6-fold and 2.1-fold reductions in expression of flavonol synthase and a putative isoflavone O-glycosyltransferase in the MtWD40-1 mutant.

Another 271 probe sets were up-regulated in seeds of the mutant, most of them associated with primary metabolism or stress responses, but no phenylpropanoid/flavonoid pathway genes were up-regulated (data not shown).

The large number of changes observed in non-phenylpropanoid/flavonoid pathway genes in the above experiment could potentially occur as a result of the additional retrotransposon insertions in line NF0977. We therefore re-examined changes in key flavonoid pathway gene transcripts in seeds and other organs, in both NF0977 and the independent retrotransposon insertion line NF2745, using quantitative RT-PCR (Table 2). MtWD40-1 transcript levels were more strongly down-regulated in tissues of NF2745 than in NF0977 (Table 2, Supplemental Tables S3, S4). Compared to wild-type R108, PAL and CHI transcript levels were least affected in the two MtWD40-1 retrotransposon insertion mutants. The most consistent changes observed as a result of loss of MtWD40-1 function were strong reductions of CHS expression in flower (but only determined for one probe set corresponding to TC138581) and seed, DFR1 expression in leaf and flower, ANS expression in stem, leaf and seed, LAR and ANR expression in flower and seed, and UGT72L1 expression in seed (Table 2, Supplemental Tables S3, S4). Thus, although
MtWD40-1 is most strongly expressed in the seed (coat), its loss of function can affect flavonoid pathway gene expression in multiple tissues.

To further investigate the impacts of loss of WD40-1 expression on flavonoid biosynthesis, levels of phenylpropanoid-derived secondary metabolites were measured by UPLC-QTOFMS in various tissues of wild-type R108 and the two independent retrotransposon insertion lines (Table 3). The greatest effects were seen in developing seed, where levels of epicatechin and its glucoside (Fig. 8), as well as cyanidin 3-O-glucoside, kaempferol 3-O-rutinoside and two benzoic acid derivatives were reduced to undetectable levels in the insertion lines. In contrast, although epicatechin and its conjugate were likewise undetectable in flowers of the two mutant lines, levels of cyanidin 3-O-glucoside and other flavonoids were increased (Table 3), in spite of the apparently strong reduction in CHS expression in these lines. Loss of function of WD40-1 had little effect on the levels of three flavonoids in leaves, but resulted in reduced isoflavone (biochanin A) and aurone levels in roots (Table 3). Flavonol (kaempferol 3-O-rutinoside) levels were reduced in developing seed of the mutant lines, consistent with the reduction in flavonol synthase expression (Table 1). The less consistent results of MtWD40-1 down-regulation in non-seed tissue could either be because natural product levels are more variable as a result of environmental factors in non-seed tissues, or because of effects of different additional retrotransposon inserts in the two mutant lines.

Over-Expression of MtWD40-1 in Medicago Hairy Roots

Ectopic expression of the Arabidopsis MYB transcription factor TT2 in M. truncatula hairy roots results in a massive induction of PAs accompanied by the up-regulation of several hundred genes, especially those of the anthocyanin/PA biosynthetic pathway (Pang et al., 2008), and TT2, at least in Arabidopsis, functions in a complex with TTG1 and TT8. We therefore introduced MtWD40-1 into hairy roots of wild-type M. truncatula to determine whether over-expression of this gene could modulate PA biosynthesis in the absence of TT2 over-expression. The MtWD40-1 over-expressing root lines did not exhibit obvious phenotypical differences compared with GUS control lines; both exhibited purple pigmentation but neither stained blue with DMACA reagent (data not shown).
Three independent *MtWD40-1* over-expressing hairy root lines were selected for high *MtWD40-1* expression by qRT-PCR along with three *GUS* control lines (Supplemental Fig. S4A), and global transcript levels in these lines were compared by Affymetrix microarray analysis. Only 15 probe sets were up-regulated by at least 2-fold as a result of over-expression of *MtWD40-1* in the hairy roots, and none of these, other than the 28.2-fold induced *MtWD40-1* transcripts, appeared to be associated with the flavonoid pathway (Table 4). The lack of induction by *MtWD40-1* of *ANS* and *ANR* was confirmed by qRT-PCR (data not shown). Consistent with the transcript levels, only a very small change in anthocyanin levels was observed in the *MtWD40-1* over-expressing hairy roots (Supplemental Fig. S4B), and no significant changes in either soluble or insoluble PAs were recorded (Supplemental Fig. S4C,D). Quantitative and qualitative flavonoid profiles, as detected by HPLC, also remained unchanged (data not shown).

**Expression of *MtWD40-1* in alfalfa**

The *MtWD40-1* gene driven by the 35S promoter was introduced into alfalfa by *Agrobacterium tumefaciens*-mediated stable transformation. Fourteen out of 20 independent ppt-positive transgenic lines were further confirmed by qRT-PCR, and the three lines with the highest *MtWD40-1* gene transcript levels (Supplemental Fig. S5A) were selected for global transcript level analysis using the Affymetrix Medicago Genechip. Two hundred and sixty probe sets were up-regulated in leaf tissue from *MtWD40-1* over-expressing alfalfa by at least 2-fold, the top 30 of which are listed in Supplemental Table S5. The two probe sets for *MtWD40-1* itself were up-regulated by 8.2/7.7 fold, respectively. More than half of the probe sets were grouped into the unclassified category when analyzed for gene function classification (Supplemental Fig. S6). No genes up-regulated more than 2-fold appeared to be associated with flavonoid biosynthesis. Eleven of the probe sets that were up-regulated in alfalfa expressing *MtWD40-1* were also up-regulated in *M. truncatula* hairy roots expressing AtTT2 (Pang et al., 2008) (Supplemental Table S6, Supplemental Fig. S7); these include a 51 kDa seed maturation protein precursor that is seed coat preferentially-expressed and down-regulated in the NF0977 mutant, and a glucosyltransferase with yet uncharacterized function.
Anthocyanin level almost doubled in leaf tissue of the MtWD40-1 over-expressing lines (Supplemental Fig. S5B), although the plants showed no visible increase in pigmentation. Only very small changes in soluble and insoluble PAs were detected in leaves of the MtWD40-1 over-expressing lines compared with the GUS control lines (Supplemental Fig. S5C, D).

DISCUSSION

The Role of MtWD40-1 in Anthocyanin/PA Biosynthesis in M. truncatula

In the present study, a M. truncatula gene encoding a WD40 repeat protein necessary for the biosynthesis of anthocyanins/PAs was identified by forward genetic screening of a Medicago Tnt1 insertional mutant population.

In Arabidopsis leaf tissue, anthocyanin/PA biosynthesis is blocked at the DFR step in the ttg1 mutant (Shirley et al., 1995; Pelletier et al., 1997), with expression of upstream genes such as CHS, CHI and F3H being unaffected (Shirley et al., 1995). However, the steps at which the pathways were blocked in other tissues were not determined. MtWD40-1 is expressed in both pigmented (leaf and stem) and non-pigmented (root, flower and seed) tissues, and its expression level is similar in all tissues except the seed coat, where it exhibits the highest expression. However, transcript and metabolite analyses revealed different effects of its down-regulation on pathway genes and/or pathway products in different tissues. For example, the anthocyanin biosynthetic pathway is blocked at the DFR step in pigmented leaf and stem tissue of the NF0977 mutant. In contrast, the pathway was more strongly blocked at the CHS step in flowers (based on qRT-PCR, but targeting only one CHS probe set) and seed (based on both qRT-PCR and microarray). In particular, expression of the PA-specific pathway genes LAR and ANR was very strongly reduced in flower and seed, which in turn led to a deficiency of epicatechin and its glucoside in flowers, and of PAs in seed. The expression pattern of MtWD40-1 during seed development was similar to that of ANR, which encodes the enzyme that catalyzes the first committed step of PA biosynthesis in M. truncatula (Pang et al., 2007).

It is interesting that loss of function of MtWD40-1 expression results in a large reduction in the levels of multiple phenylpropanoid classes (benzoic acids, flavonols, flavan-3-ols, anthocyanins) in seed, whereas only flavan-3-ols were strongly down-
regulated in flowers (where anthocyanin levels were actually increased). Although the qRT-PCR data indicated strong down-regulation of one specific CHS family member in flowers, it is likely that other members of the CHS gene family remain expressed. Additional anthocyanin accumulation would be predicted in flowers in which ANR is strongly down regulated but ANS remains unaffected, since cyanidin is the immediate precursor of epicatechin (Xie et al, 2003).

Although WD40 proteins are known to regulate anthocyanin and PA biosynthesis, their potential involvement in other areas of phenylpropanoid biosynthesis is less clear. Our data indicate that loss of function of MtWD40-1 also results in reduction in the levels of an aurone and an isoflavone glycoside in roots, and complete loss of benzoic acids in seeds. Levels of the latter compounds are likely directly regulated through the action of MtWD40-1, whereas the smaller change in isoflavone levels in roots might be an indirect effect of altered metabolic flux. We did, however, record a 2-fold decrease in isoflavone synthase transcripts in two independent mtwd40-1 alleles by qRT-PCR (data not shown). Together, these data suggest a critical role for MtWD40-1 in the control of seed PA biosynthesis, with additional but less precise (and possibly indirect) effects on the formation of other flavonoid compounds in other tissues.

The Role of MtWD40-1 in Trichome Formation

WD40 repeat proteins are critical for trichome formation in Arabidopsis, but not in all plant species (Serna and Martin, 2006). In Arabidopsis, a regulatory complex consisting of GL1-GL3/EGL3 (Enhance Glabrous 3)-WD40 triggers expression of the downstream GL2 gene by binding to its promoter region, to regulate trichome formation in the epidermal cell layer (Oppenheimer et al., 1991; Payne et al., 2000; Zhang et al., 2003). Lack of TTG1 expression in Arabidopsis leads to the loss of trichomes on aerial tissues (Walker et al., 1999). M. truncatula stems and leaves harbor two types of trichomes; non-glandular hairs and, at a lower density, small glandular structures that generally lie flat against the epidermal surface (Damerval, 1983). MtWD40-1 mutations do not qualitatively affect trichome distribution on young leaves and petioles, even though MtWD40-1 can apparently interact with GL3 to activate GL2 expression and therefore restore (non-glandular) trichome formation in the Arabidopsis ttg1 mutant; however,
subtle changes due to either the direct loss of MtWD40-1 or to secondary effects caused by changes in metabolite production have not been assessed in Medicago. Similar observations with WD40 repeat proteins have been reported in other plant species. For example, neither mutation nor ectopic expression of the single copy AN11 gene caused any obvious change in trichome phenotype in petunia (de Vetten et al., 1997). In maize, the WD40 protein encoded by the pale aleurone colors 1 (PAC1) locus is required for anthocyanin production in the aleurone and scutellum of the seed, and can complement the ttg1 trichome phenotype, although loss of PAC1 expression does not affect trichomes in maize (Carey et al., 2004). PFWD, a WD40 repeat protein from Perilla frutescens, controls both anthocyanin production and trichome initiation in gain-of-function tests (Sompronpailin et al., 2002), and the WD40 repeat genes GhTTG1 and GhTTG3 from cotton can rescue the trichome phenotype of Arabidopsis ttg1 and the anthocyanin deficiency phenotype of the Matthiola incana ttg1 mutant (Humphries et al., 2005). However, it was not shown whether PFWD or GhTTG1/2 control trichome initiation in their host species.

Like AN11 from petunia and PAC1 from maize, MtWD40-1 is also a single copy gene, as determined by DNA gel blot analysis under high stringency (data not shown). Blastn analysis of the M. truncatula genome databases with the MtWD40-1 nucleotide sequence as query recovered no other WD40 repeat protein genes. Furthermore, when the deduced amino acid sequence was queried (by blastp), no other WD40 repeat protein with more then 30% identity was recovered. MtWD40-2, which is only represented as an EST in the Medicago sequence available to date, is less than 60% identical to MtWD40-1 at the amino acid level. MtWD40-1 is related to maize PAC1, which can complement the Arabidopsis ttg1 mutant. It is therefore possible that the absence of a trichome phenotype in the MtWD40-1 mutant is due to genetic redundancy, although the expression level and pattern of MtWD40-2 based on microarray data is not obviously supportive of a primary role in trichome development.

Biotechnological Applications of MtWD40-1

Transcription factors have already been employed for bioengineering of the anthocyanin/PA pathway. Successful examples of engineering anthocyanin production
include ectopic expression of the Myb transcription factors Production of Anthocyanin Pigment 1 (PAP1) in tobacco and Arabidopsis (Borevitz et al., 2000; Xie et al., 2006) and Legume Anthocyanin Production 1 (LAP1) in alfalfa and white clover (Peel et al., 2008), and of the maize bHLH transcriptional regulators *Lc* and *Sn* in alfalfa and *Lotus corniculatus* respectively (Ray et al., 2003; Robbins et al, 2003). Co-expression of *PAP1* and *TT2* led to the accumulation of detectable PA levels in Arabidopsis, although the plants did not survive (Sharma and Dixon, 2006). Co-expression of *ANR* with *PAP1* led to accumulation of PAs in tobacco leaves (Xie et al., 2006). However, none of the components of the TT2-TT8-WD40 transcription complex has been tested for engineering PAs in foliage of forage legumes.

In a previous study, we introduced the *TT2* gene from Arabidopsis into *M. truncatula* hairy roots, and this alone led to massive accumulation of PAs (Pang et al., 2008). Increased transcript levels of both *MtWD40-1* and a *TT8* homolog were observed in these lines, associated with strong induction of flavonoid pathway enzymes which included an over 400-fold increase in *ANR* expression (Pang et al., 2008). In contrast, over-expression of *MtWD40-1* alone did not induce PA formation, or increased expression of flavonoid biosynthetic pathway genes. One explanation could be that basal levels of MtWD40-1 and MtTT8 are sufficient to support PA biosynthesis in the hairy roots, and that TT2 expression is the limiting factor in this tissue. MtWD40-1 is therefore necessary, but not sufficient, for PA biosynthesis. Expression of *MtWD40-1* did not induce either PA-specific genes or PA accumulation in alfalfa foliage. Similarly, expression of *AtTT2* alone does not induce PA biosynthesis in alfalfa foliage (Peel et al., 2008). There are three possible explanations for these observations. Firstly, even though sufficient MtWD40-1 or AtTT2 may be present in the foliar tissue, ANR expression will not be triggered if there is insufficient partner protein present to form the TT2-TT8-WD40 complex. Co-expression of all three genes would address this possibility. Second, low levels of anthocyanidin substrate might limit PA monomer formation in foliar tissues. Finally, we can not rule out the potential existence of suppressors of the anthocyanin/PA biosynthetic pathway in leaves. A protein with a single MYB domain has recently been shown to act as a negative regulator of anthocyanin biosynthesis in Arabidopsis (Matsui et al, 2008), and CAPRICE (CPC), TRIPTYCHON and ENHANCER of TRY, and CPC1 (ETC1) and
ETC2 function as suppressors of the GL1-GL3/EGL3-WD40 complex to repress trichome formation (Schnittger et al., 1999; Schellmann et al., 2002; Kirik et al., 2004a, 2004b) and possibly the anthocyanin/PA-promoting function of the complex. It is clear that the successful bioengineering of PAs in forage crops will depend largely on our gaining a better understanding of the endogenous regulatory controls for PA biosynthesis.

MATERIALS AND METHODS

Insertion Mutant Screening and Molecular Confirmation by TAIL-PCR

Generation of the *M. truncatula* Tnt1 insertional mutant population and growth of R1 seed were as described previously (Tadege et al., 2005). The mutant line NF0977 was selected due to its lack of anthocyanins in the aerial tissues. Genomic DNA from the mutant was isolated using the Dellaporta method (Dellaporta et al., 1983). Tnt1 flanking sequences were recovered using Thermal Asymmetric Interlaced Polymerase Chain Reaction (TAIL-PCR) (Liu et al., 1995, 2005). PCR fragments were purified using a PCR Purification Kit (Qiagen, Valencia, CA) and then cloned into pGEM-T easy vector (Promega, Madison, WI), followed by sequencing with the Tnt1-specific primer Tnt1-F2 (Supplemental Table S7). The sequenced fragments were then analyzed by Blastn against the *M. truncatula* genome at NCBI.

Seeds from the identified Tnt1 insertion lines were scarified with concentrated sulfuric acid, cold-treated for 3 days at 4°C on filter paper, and grown in Metro-mix 350 (Scott Company, OH), with an 18-h light/25°C and 6-h dark/22°C photoperiod in the greenhouse. Genomic DNA from the R2 and R3 progeny was extracted and analyzed as above, using the Tnt1-R1 and MtWD40-1F1 primers (Supplemental Table S7) to confirm the Tnt1 insertion, and the MtWD40-1F1 and MtWD40-1R1 primers to check if an individual plant is homozygous or heterozygous with respect to the mutated *MtWD40-1* gene.

Reverse Genetic Screening for Tnt1 Retrotransposon Insertions in *MtWD40-1*

DNA samples used for mutant screening were 10 superpools of pooled DNA samples from 5,000 Tnt1 insertional mutant lines of *M. truncatula* (Tadege et al., 2005, 2008). A PCR approach was taken for reverse genetic screening to uncover *MtWD40-1* mutants.
Briefly, two rounds of PCR were used to screen the superpools; the primers used for the primary PCR were *Tnt1* reverse primer Tnt1-R and gene specific primer MtWD40-1F. For nested PCR, Tnt1-R1 and MtWD40-1F1 were used (Supplemental Table S7). The PCR products from the final target plants were then purified with a QIAquick PCR Purification Kit (Qiagen) and sequenced with the primer Tnt1-R2.

**Sequence Alignment and Phylogeny Analysis**

A multiple alignment of the deduced amino acid sequences of MtWD40-1 and other WD40 repeat domain proteins was constructed using Clustal X 1.81 (Thompson et al., 1997). For phylogeny analysis, the alignment was performed by using MAFFT (Katoh et al., 2005). The resulting alignment was further edited manually using Mesquite (Maddison and Maddison, 2009). The un-rooted consensus tree was constructed by using PAUP* 4.0b10 with 1000 bootstrap replicates (Swofford, 2003).

**Sample Collection, RNA Extraction, qRT-PCR and Microarray Analysis**

Root, stem, leaf, flower and seed samples from three independent homozygous NF0977 and NF2745 R3 generation and wild-type R108 plants were collected one month after planting in soil. Additional flowers were labeled individually according to pollination date, and seed pods harvested at 16 dap; the seeds were collected and stored at -80°C. RNA was extracted from triplicate biological replicates of the above samples using the CTAB method (Jaakola et al., 2001) followed by treatment with Turbo DNase I (Ambion, Austin, TX), and reverse transcription of 3 μg RNA from each sample. The cDNA samples were used for Quantitative Real-time PCR (qRT-PCR) with technical duplicates. The 10 μL reaction included 2 μL primers (0.5 μM of each primer), 5μL Power Sybr (Applied Biosystems, Foster City, CA), 2 μL 1:20 diluted cDNA from the reverse transcription step, and 1 μL water. The gene-specific primers used for qRT-PCR are listed in Supplemental Table S7. RNA samples from seed collected at 16 dap were further purified with a QIAGEN RNeasy MinElute Cleanup Kit (Qiagen) and 10 μg samples were subjected to microarray analysis. RNA from transgenic hairy roots and alfalfa leaf tissue were extracted with Tri-reagent (Gibco-BRL Life Technologies,
Gaithersburg, MD) for qRT-PCR, and 10 μg purified RNA samples were used for microarray analysis.

qRT-PCR data were analyzed using SDS 2.2.1 software (Applied Biosystems). PCR efficiency (E) was estimated using the LinRegPCR software (Ramakers et al., 2003) and the transcript levels were determined by relative quantification (Pfaffl, 2001) using the *M. truncatula actin* gene (TC# 107326) as a reference.

Probe labeling, hybridization and scanning for microarray analysis were conducted according to the manufacturer’s instructions (Affymetrix, Santa Clara, CA). For each sample, the .CEL file was exported from Genechip Operating System (GCOS) program (Affymetrix). All .CEL files were imported into robust multi-chip average (RMA) and normalized as described by Irizarry et al. (Irizarry et al., 2003). The presence/absence call for each probe set was obtained from dCHIP (Li and Wong, 2001). Differentially expressed genes between wild-type R108 versus NF0977 seed coat sample, and *MtWD40-I* over-expressing hairy roots versus GUS controls, were selected using associative analysis as described (Dozmorov and Centola, 2003). Type I family-wise error rate was reduced by using a Bonferroni corrected P-value threshold of 0.05/N, where N represents the number of genes present on the chip. The false discovery rate was monitored and controlled by calculating the Q-value (false discovery rate) using extraction of differential gene expression (EDGE, http://www.biostat.washington.edu/software/jstorey/edge/) (Storey and Tibshirani, 2003; Leek et al., 2006).

All microarray data have been deposited in ArrayExpress (http://www.ebi.ac.uk/arrayexpress). Accession numbers are: E-MEXP-1757, experiment name “*Medicago truncatula MtTTG1* gene mutant seed transcript profiling”; E-MEXP-1758, experiment name “*Medicago truncatula TTG1* over-expressing hairy root”; E-MEXP-1759, experiment name “*MtTTG1* over-expression transgenic alfalfa gene profiling”.

**Staining Seeds for PA and Mucilage**

To determine the presence of PAs in the seed coat, seeds were soaked in DMACA reagent (0.1% w/v DMACA in methanol-3N HCl) for 1 h and then destained with ethanol/acetic acid (75:25). To stain for mucilage, seeds were imbibed in sterilized
deionized water for 1 h, transferred to 0.01% ruthenium red solution for 10 min, and then washed twice with water.

**Scanning Electron Microscopy**
Young developing leaves with attached petioles were mounted on copper stubs, frozen in liquid nitrogen, sputter coated with gold using an Emitech K1150 cryo preparation system (Emitech, Houston, TX), and imaged with a Hitachi S3500N scanning electron microscope as described by Ahlstrand (1996).

**Analysis of Anthocyanins, PAs and Total Flavonoids**
For extraction of anthocyanins, 2-3 mL 0.1% HCl/methanol was added to 0.1 g ground fresh samples, followed by sonication for 30 min and standing overnight at 4°C. Following centrifugation at 2,500g for 10 min, the extraction was repeated once and the supernatants pooled. An equal volume of water and chloroform was added to remove chlorophyll, and the absorption of the aqueous phase was recorded at 530 nm. Total anthocyanin content was calculated based on the molar absorbance of cyanidin-3-O-glucoside.

For PA analysis, 0.5-0.75 g ground samples were extracted with 5 mL of 70% acetone/0.5% acetic acid (extraction solution) by vortexing, and then sonicated at room temperature for 1 h. Following centrifugation at 2,500g for 10 min, the residues were re-extracted twice as above. The pooled supernatants were then extracted three times with chloroform and once with hexane, and the supernatants (containing soluble PAs) and residues (containing insoluble PAs) from each sample were freeze dried separately. The dried soluble PAs were suspended in extraction solution to a concentration of 3 mg/mL.

Total soluble PA content was determined spectrophotometrically after reaction with DMACA reagent (0.2% w/v DMACA in methanol-3N HCl) at 640 nm, with (+)-catechin as standard. For quantification of insoluble PAs, 2 ml of butanol-HCl (95:5, v/v) was added to the dried residues and the mixtures were sonicated at room temperature for 1 h, followed by centrifugation at 2,500g for 10 min. The absorption of the supernatants was measured at 550 nm; the samples were then boiled for 1 h, cooled to room temperature, and the absorbance at 550 nm was measured again, with the first value being subtracted.
from the second. Absorbance values were converted into PA equivalents using a standard curve generated with procyanidin B1 (Indofine, NJ).

For determination of total flavonoids, 0.1 g batches of ground samples were extracted with 2 ml 80% methanol, sonicated for 1 h, and then kept at 4°C overnight. The extract was centrifuged to remove tissue debris and the supernatant was dried under nitrogen gas, followed by hydrolysis in 2 ml of 5 mg/mL β-glucosidase (34 units) from almond (Sigma, St. Louis, MO). After extracting twice with 2 mL of ethyl acetate, the supernatants were pooled, dried under nitrogen and resuspended in 200 μL of methanol. Fifty μL of the methanolic solution were used for reverse-phase HPLC analysis on an Agilent HP1100 HPLC using the following gradient: solvent A (1% phosphoric acid) and B (acetonitrile) at 1 mL/min flow rate: 0-5 min, 5% B; 5-10 min, 5-10% B; 10-25 min, 10-17% B; 25-30 min, 17-23% B; 30-65 min, 23-50% B; 65-79 min, 50-100% B; 79-80 min, 100-5% B. Data were collected at 254 nm for flavonoid compounds. Identifications were based on chromatographic behavior and UV spectra compared with those of authentic standards.

**Extraction and UPLC-ESI-QTOF-MS Analysis of Flavonoids**

Dried tissues (10.0 ± 0.06 mg) were weighed into a 1 gram glass vial. The samples (biological triplicates) were extracted in 2 mL of 80% methanol containing 2 μg/mL puerarin and 18 μg/mL umbelliferone (internal standards) for 2 h at room temperature with constant agitation. Samples were centrifuged at 2,900 g for 30 min and the supernatants were transferred to LC vials and analyzed with a Waters Acquit UPLC™ system (Waters, Milford, MA) fitted with a hybrid quadropole time-of-flight (QTOF) Premier mass spectrometer (Waters). A reverse phase, 1.7-μm UPLC BEH C18, 2.1 × 150 mm column (Waters) was used for separations. The mobile phase consisted of eluent A (0.1% [v/v] acetic acid /water and Eluent B (acetonitrile) and separations were achieved using a linear gradient of 95% to 30% A over 30 min, 30% to 5% A over 3.0 min, 5% to 95% A over 3.0 min. The flow rate was 0.56 mL/min and the column temperature was maintained at 60°C. Masses of the eluted compounds were detected in the negative electrospray ionization mode from 50-2000 m/z. The QTOF Premier was operated under the following instrument parameters: desolvation temperature of 400°C;
desolvation nitrogen gas flow of 850 L/h; capillary voltage of 2.9 kV; cone voltage of 48 eV; and collision energy of 10 eV. The MS system was calibrated using sodium formate, and raffinose was used as the lockmass. Metabolites were identified based on accurate mass and retention time relative to authentic standards. Mass Lynx version 4.1 - Data Bridge was used to convert the raw data files to NetCDF. Relative abundances were calculated using MET-IDEA (Broeckling et al, 2006) and the peak areas were normalized by dividing each peak area by the value of the internal standard peak area.

Construction of Binary Vectors for MtWD40-1 Expression in Plants

The ORF of the MtWD40-1 gene was amplified from cDNA produced from total RNA isolated from M. truncatula seed coats, using the primers MtWD40-1CF and MtWD40-1R1 and DNA polymerase with proofreading activity. The PCR product was purified and cloned into the Gateway Entry vector pENTR/D-TOPO (Invitrogen, Carlsbad, CA), and the MtWD40-1 ORF in the resulting vector pENTR-MtWD40-1 was confirmed by sequencing.

The primers MtWD40-1NF (with an NcoI site) and MtWD40-1BR (with a BstE II site) (Supplemental Table S7) were used to amplify the ORF region (with added NcoI and BstEII restriction sites) from pENTR-MtWD40-1 template with proofreading DNA polymerase. The resulting fragment was digested, purified and ligated into plasmid pCAMBIA3301-HP (Xiao et al., 2005) digested with NcoI and BstEII, to produce a new construct p3301-MtWD40-1. This construct, as well as a control construct containing the GUS open reading frame in place of WD40-1, was transformed into Agrobacterium rhizogenes strain Arqua1 (Quandt et al., 1993) by electroporation. Single colonies were confirmed by PCR and used for M. truncatula transformation. Both wild-type M. truncatula Jemalong A17 and the mutant line NF0977 (Genotype R108 as background) were transformed using the protocol of Chabaud et al. (2006) with 2.5 mg/L ppt as selection. The generated hairy roots were maintained on B5 agar media in Petri dishes supplied with 2.5 mg/L ppt under fluorescent light (140 μE/m²/s⁻¹) with a photoperiod of 16 h, and were sub-cultured every month onto fresh media.

For stable transformation by Agrobacterium tumefaciens, the MtWD40-1 ORF was first transferred into the Gateway plant transformation destination vector pB2GW7
(Karimi et al., 2002) using Gateway® LR ClonaseTM enzyme mix with pENTR-MtWD40-1 according to the manufacturer’s instructions (Invitrogen). The reading frame of the resulting vector, pB2GW7-MtWD40-1, was confirmed by sequencing. pB2GW7-MtWD40-1 was transformed into *A. tumefaciens* strain AGL1 by electroporation. A single colony containing the target construct was confirmed by PCR and used for genetic transformation of Arabidopsis and alfalfa. The protocol of Austin et al (1995) was used for alfalfa transformation with minor modifications and 10 mg/L ppt-selection.

**Rescue of the Arabidopsis *ttg1-9* Mutant**

The construct used to generate Arabidopsis expressing *GL2::MtWD40-1* was derived from *pGL2::GUS* (Szymanski et al, 1998). This plasmid was modified by removal of the *GUS* coding sequence by *Sma*I/*Sac*I digestion followed by blunt ending with Klenow. The RFA Gateway recombination fragment RFA from Invitrogen was inserted into this site. The coding region of *MtWD40-1* was derived from cDNA using total RNA isolated from *M. truncatula* (Jemalong A17) shoots as a template. Primers flanking the *MtWD40-1* coding region were used to generate a double stranded DNA product via PCR that was first subcloned into pCR8 (Invitrogen) before being moved into the Gateway GL2 promoter vector.

The Arabidopsis *ttg1-9* mutant (Walker et al, 1999) was transformed by the floral dip infiltration method (Clough and Bent, 1998). Selection of transformants was conducted on 0.5 × Murashige and Skoog (MS) medium supplied with 7.5 mg/L ppt. The ppt-resistant seedlings were then transferred into soil to set seed. Progeny from self-fertilized primary transformants were grown in soil for observation of trichome phenotype.

**Yeast Two-Hybrid Assay**

For the yeast two-hybrid assays, PCR was used generate a copy of the *MtWD40-1* coding region with leading and tailing *Eco*RI and *Bam*HI restriction enzyme sites. The coding region was then moved into the corresponding sites of pBridge (Clontech) to create pMtWD40-1DB. The empty vector pGAD424 was from Clontech and pGL3-AD was as described previously (Esch et al, 2003). Beta-galactosidase activity was detected as
adapted from Duttweiler (1996) and further described at (http://www.fccc.edu/research/labs/golemis/betagal/plates_vs_overlay.html).

Supplemental Data
The following materials are available in the online version of this article.

Figure S1. *MtWD40-1* transcript levels in the Arabidopsis *ttg1-9* mutant and two lines complemented with MtWD40-1.

Figure S2. Gene functional categories of probe sets that were down-regulated by more than 2-fold in the NF0977 mutant compared with wild type R108.

Figure S3. Anthocyanin and PA levels in hairy roots of *M. truncatula* A17 over-expressing *MtWD40-1*.

Figure S4. Anthocyanin and PA levels in leaves of *M. sativa* R2336 lines over-expressing *MtWD40-1*.

Figure S5. Gene functional categories of probe sets that were up-regulated by more than 2-fold in leaves of alfalfa expressing *MtWD40-1* compared with a GUS-expressing control line.

Figure S6. Venn diagram showing overlap between probe sets induced by *MtWD40-1* and *AtTT2* in *M. truncatula* hairy roots, and by *MtWD40-1* in alfalfa leaves.

Table S1. BlastN analysis of all *Tnt1* flanking sequences retrieved from the NF0977 mutant.

Table S2. Probe sets that were down-regulated more than 2-fold in developing seed of the *M. truncatula* NF0977 mutant.

Table S3. Changes of flavonoid pathway gene transcripts in different tissues of *M. truncatula* R108 and the NF0977 retrotransposon insertion mutant as determined by qRT-PCR.

Table S4. Changes of flavonoid pathway gene transcripts in different tissues of *M. truncatula* R108 and the NF2745 retrotransposon insertion mutant as determined by qRT-PCR.

Table S5. The top 30 gene probe sets that were up-regulated by expression of *MtWD40-1* in alfalfa leaf tissue.
Table S6. The gene probe sets that were up-regulated by MtWD40-1 in alfalfa leaf and by AtTT2 in hairy roots of *M. truncatula*.

Table S7. The primer sequences used in the present study.

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**LEGENDS TO FIGURES**

**Figure 1.** The flavonoid pathway leading to anthocyanins and PAs. Enzymes are: PAL, L-phenylalanine ammonia-lyase; C4H, cinnamate 4-hydroxylase; 4CL, 4-coumarate: CoA ligase; CHS, chalcone synthase; CHI, chalcone isomerase; CHR, chalcone reductase; F3H: flavanone 3-hydroxylase; DFR: dihydroflavonol reductase; FS, flavone synthase; IFS, isoflavone synthase; LAR, leucoanthocyanidn reductase; ANS, anthocyanidin synthase; ANR, anthocyanidin reductase. GT, glucosyltransferase.

**Figure 2.** Visible phenotypes resulting from insertional mutagenesis of MtWD40-1.
A, Upper panel: Four day-old seedlings of *M. truncatula* R108 (wild-type) and the two insertional mutant lines, showing pigmentation below the cotyledons. Lower panel: Aerial parts of older seedlings, shown in the same order. B, Axial side (lower panels) and adaxial side (upper panels) of leaves from a wild-type plant (left panels), NF0977 (center panels) and NF2745 (right panels). C, Mature seeds of wild-type (left), NF0977 (center), and NF2745 (right), either unstained (upper panels), stained with DMACA reagent to detect PAs (center panels), or stained with ruthenium red to detect mucilage (lower panels). D, SEM analysis of trichomes on young petioles and leaves of wild-type (left panels), NF0977 (center panels) and NF2745 (right panels). Upper panels show non-glandular petiole trichomes, middle panels show non-glandular leaf trichomes, and lower panels show glandular and non-glandular petiole trichomes. Bars in upper and middle panels = 1 mm, and in bottom panel = 200 µm. E, Root hair phenotypes of wild type (left...
panels), NF0977 (center panels) and NF2745 (right panels). Bars represent 2 mm in upper panels, 1 mm in lower panels (showing close up of the hairs just behind the root tip). F, A diagram of the MtWD40-1 gene (1364bp) showing the positions of the independent Tnt1 insertions and the two probe sets on the Medicago Affymetrix genechip.

**Figure 3.** Alignment of deduced amino acid sequences of plant WD40 repeat proteins. The WD40 repeat domains are marked with horizontal bars above the sequence, and the last two amino acids of each repeat domain are marked with stars. Identical residues are highlighted on a black background and similar residues are highlighted on a gray background. The GenBank accession numbers are: BAE94398, InWDR1 from Ipomoea nil; BAE94396, IpWDR1 from Ipomoea purpurea; AAC18914, AN11 from Petunia × hybrida; BAB58883, PFDS from Perilla frutescens; AAM95645, GhTTG3 from Gossypium hirsutum; AAK19614, GhTTG1; ABW08112, MtWD40-1; Q9XGN1, AtTTG1; AAM76742, PAC1 from Zea mays; AC136505_16.4, MtWD40-2.

**Figure 4.** Unrooted phylogram comparison of the amino acid sequences of MtWD40-1 and other functionally characterized plant WD40 repeat proteins. The sequences used are the same as in Fig. 3. The phylogenetic tree was constructed by PAUP* 4.0b10, after alignment using MAFFT software. Node support was estimated using neighbor-joining bootstrap analysis (1000 bootstrap replicates).

**Figure 5.** Genetic complementation of the anthocyanin and PA phenotypes of the NF0977 retrotransposon insertion line.
A, Pigmentation of hairy roots of the NF0977 line expressing GUS (left) and MtWD40-1 (right). B, qRT-PCR analysis of MtWD40-1 transcript levels in hairy roots of NF0977 expressing GUS or MtWD40-1. C, As above, showing ANS transcript levels. D, As above, showing UGT78G1 transcript levels. E, Anthocyanin levels from NF0977 expressing GUS or MtWD40-1 (three independent lines of each). F, As above, showing insoluble PA levels. G, As above, showing soluble PA levels.
Figure 6. Genetic complementation of the Arabidopsis ttg1-9 mutant.
A, Leaves of the ttg1-9 mutant line. B, Leaves of the ttg1-9 mutant expressing 35S::MtWD40-1. C, Seed coat pigmentation of wild-type, ttg1-9 and ttg1-9 expressing 35S::MtWD40-1. D, A single leaf of ttg1-9 showing the glabrous phenotype. E, A single leaf of the ttg1-9 mutant expressing GL2::MtWD40-1, showing the restoration of the trichome phenotype. F, Seed coat pigmentation of wild-type, ttg1-9 and ttg1-9 expressing GL2::MtWD40-1. G, yeast two-hybrid analysis of the interaction between MtWD40-1 and Arabidopsis GL3 (see text for details).

Figure 7. MtWD40-1 transcript levels in M. truncatula ecotype Jemalong A17 as determined by microarray analysis.
A, Tissue-specific expression. B, MtWD40-1 transcript levels during seed development. The data were retrieved from the M. truncatula gene expression atlas (Benedito et al., 2008) and the seed coat microarray dataset (Pang et al., 2008).

Figure 8. UPLC-QTOFMS analysis with selected ion monitoring of epicatechin glucoside in developing M. truncatula seed.
A, Extract of seed of mutant line NF0977. B, Extract of seed of wild type R108. The peak in B corresponds to glucosylated epicatechin (retention time 3.06 min, mass 451.125, 500 ppm). Seed were harvested at 16 dap. Numbers on right indicate absolute intensity of the peaks at RT 3.06 min.

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| Probe sets          | Target Description                                                                 | Ratio (R108/NF0977) | P-Value* | Q-Value** |
|---------------------|--------------------------------------------------------------------------------------|----------------------|----------|-----------|
| Mtr.20567.1.S1_at   | Type III polyketide synthase; Naringenin-chalcone synthase (CHS)                     | 198.05               | 0.000017 | 0.060224 |
| Mtr.20185.1.S1_x_at | Naringenin-chalcone synthase; Type III polyketide synthase (CHS)                     | 105.58               | 0.000147 | 0.089928 |
| Mtr.39897.1.S1_at   | Similar to CPRD12 protein, partial (61%)                                            | 104.58               | 0.000001 | 0.0219   |
| Mtr.20185.1.S1_at   | Naringenin-chalcone synthase; Type III polyketide synthase (CHS)                     | 95.97                | 0.000666 | 0.121225 |
| Mtr.36333.1.S1_at   | Similar to Flavonoid 3'-hydroxylase (Fragment), partial (21%) (F3'H)               | 85.31                | 0.000002 | 0.032849 |
| Mtr.49421.1.S1_at   | 2OG-Fe(II) oxygenase                                                                  | 79.12                | 0.00005  | 0.043799 |
| Mtr.14017.1.S1_at   | Naringenin-chalcone synthase; Type III polyketide synthase (CHS)                     | 40.62                | 0.00018  | 0.060224 |
| Mtr.6517.1.S1_at    | Naringenin-chalcone synthase; Type III polyketide synthase (CHS)                     | 36.77                | 0.000264 | 0.105576 |
| Mtr.44985.1.S1_at   | Anthocyanidin reductase, complete (ANR)                                              | 34.55                | 0.000038 | 0.089283 |
| Mtr.14428.1.S1_x_at | Naringenin-chalcone synthase; Type III polyketide synthase (CHS)                     | 26.53                | 0.000414 | 0.115755 |
| Mtr.51818.1.S1_at   | Predicted protein                                                                     | 23.29                | 0.00086  | 0.124286 |
| Mtr.16432.1.S1_at   | Myb, DNA-binding; Homeodomain-like                                                   | 23.04                | 0.00335  | 0.151484 |
| Mtr.14428.1.S1_at   | Naringenin-chalcone synthase; Type III polyketide synthase (CHS)                     | 22.59                | 0.000774 | 0.124286 |
| Mtr.6511.1.S1_at    | Similar to GTP binding protein, partial (47%)                                        | 6.75                 | 0.00189  | 0.096689 |
| Mtr.41031.1.S1_at   | Homologue to 4-coumarate-CoA ligase (4CL)                                             | 5.26                 | 0.000157 | 0.089928 |

Note: Expression values were obtained from RMA (Irizarry et al., 2003);
*The P-Value was obtained using Associative Analysis (Dozmorov and Centola, 2003);
**The Q-Value was obtained using EDGE (Leek et al., 2006).
Table 2. Fold-change (decrease) of flavonoid pathway gene transcripts in different tissues of the NF0977 and NF2745 retrotransposon-insertion lines compared to wild-type R108.

| Tissue     | Ratio   | PAL   | CHS   | CHI   | F3H   | DFR1  | DFR2  | ANS   | LAR   | ANR   | UGT72L1 | MtWD40-1 |
|------------|---------|-------|-------|-------|-------|-------|-------|-------|-------|-------|---------|----------|
| Root       | R108/NF0977 | 2.66  | 5.98  | 2.24  | 3.10  | 0.10  | 0.57  | 0.68  | 1.43  | 0.92  | 0.34    | 4.25     |
|            | R108/NF2745 | 0.56  | 1.62  | 1.20  | 4.30  | 1.11  | 0.35  | 1.01  | 0.95  | 1.49  | 0.54    | 2132.75  |
| Stem       | R108/NF0977 | 0.98  | 0.52  | 0.58  | ∞     | 105.15| 1.50  | 799.92| 1.48  | 1.80  | 0.65    | 9.32     |
|            | R108/NF2745 | 0.87  | 2.02  | 1.02  | 4.48  | 1.54  | 0.82  | 76.42 | 1.39  | 1.01  | 0.62    | 2454.75  |
| Leaf       | R108/NF0977 | 0.59  | 0.61  | 0.22  | ∞     | 81.06 | 1.00  | 76.22 | 0.17  | 0.16  | 1.55    | 5.14     |
|            | R108/NF2745 | 0.47  | 3.39  | 0.98  | ∞     | 11.07 | 6.13  | 33.21 | 0.17  | 0.64  | 1.68    | 1568.11  |
| Flower     | R108/NF0977 | 0.89  | 67.38 | 0.96  | 0.91  | 5.48  | 1.55  | 2.71  | 20.62 | 216.01| 7.97    | 17.10    |
|            | R108/NF2745 | 0.93  | 46.15 | 0.62  | 0.28  | 8.03  | 1.54  | 1.23  | 16.09 | 119.12| 0.84    | 1521.86  |
| Seed(16 dap)| R108/NF0977 | 0.42  | 240.10| 1.05  | ∞     | 1.73  | 3.11  | 12.25 | 6.47  | 58.83 | ∞       | 23.26    |
|            | R108/NF2745 | 2.12  | 80.22 | 0.72  | ∞     | 1.65  | 3.03  | 7.87  | 8.60  | 33.7  | 4.16    | 2761.54  |

Transcript levels were determined by qRT-PCR with actin as internal reference. Data represent average relative transcript level to actin from biological triplicates, expressed as the ratio of transcript level in R108 to that in the mutants. ∞: numerical ratio set to infinity due to the undetectable transcript level in the mutant line. See Supplemental Tables S3 and 4 for absolute values and standard deviations for each measurement.
Table 3. Levels of selected flavonoid compounds in different tissues of wild-type and mutant *M. truncatula* determined by UPLC-MS analysis

| Compound name | MS ion used | Retention time (min) | NF0977 | R108<sup>a</sup> | NF2745 | R108<sup>a</sup> |
|---------------|-------------|----------------------|--------|-----------------|--------|-----------------|
| **Root**      |             |                      |        |                 |        |                 |
| Pelargonidin-3-O-glucoside | 449.11  | 2.45                 | 28±13  | 32±3            | 1±0    | 6±9             |
| Formononetin-7-O-glucoside | 267.07  | 8.47                 | 9±4    | 14±3            | 35±8   | 139±40          |
| 4,6-Dihydroxy-auroine | 253.04  | 10.06                | 6±2    | 11±2            | 3±1    | 11±2            |
| Biochanin A-7-O-glucoside | 283.06  | 10.68                | 2±1    | 5±0             | 2±0    | 6±2             |
| **Leaf**      |             |                      |        |                 |        |                 |
| Formononetin-7-O-glucoside | 267.07  | 8.47                 | 12±3   | 13±2            | 21±4   | 15±6            |
| Apigenin      | 269.04  | 10.19                | 73±23  | 52±6            | 3±2    | 4±1             |
| **Flower**    |             |                      |        |                 |        |                 |
| Epicatechin 3’-O-glucoside | 451.12  | 3.09                 | ND     | 7±3             | ND     | 3±0             |
| Epicatechin   | 289.07  | 3.22                 | ND     | 4±2             | ND     | 1±0             |
| Cyanidin 3-O-glucoside | 461.07  | 5.43                 | 6±1    | 2±1             | 33±2   | 14±0            |
| Genistein-7-O-glucoside | 431.09  | 5.54                 | 5±2    | 3±2             | 2±1    | 4±1             |
| Apigenin-7-O-glucoside | 431.09  | 7.18                 | 4±2    | 3±2             | 2±1    | 3±1             |
| Luteolin-7-O-glucoside | 579.13  | 5.95                 | 24±2   | 19±4            | 45±5   | 32±1            |
| Kaempferol    | 285.04  | 10.34                | 24±1   | 18±3            | 20±1   | 3±0             |
| **16 day seed** |             |                      |        |                 |        |                 |
| 3,5-Dihydroxybenzoic acid | 153.02  | 1.17                 | ND     | 26±3            | ND     | 40±2            |
| 2,4-Dihydroxybenzoic acid | 151.00  | 1.35                 | ND     | 8±1             | ND     | 10±1            |
| Epicatechin 3’-O-glucoside | 451.12  | 3.09                 | ND     | 69±7            | ND     | 181±22          |
| Epicatechin   | 289.07  | 3.22                 | ND     | 34±5            | ND     | 59±6            |
| Cyanidin 3-O-glucoside | 461.07  | 5.43                 | ND     | 6±2             | ND     | 19±2            |
| Compound                  | Area (std dev) | R108 | R109 | R110 | ND  | R111 |
|---------------------------|----------------|------|------|------|-----|------|
| Kaempferol 3-O-rutinoside | 593.15 (6.25)  | 1±1  | 4±1  | ND   | 4±1 |
| Mature seed               |                |      |      |      |     |      |
| Apigenin                  | 269.04 (10.19) | 18±2 | 24±2 | 40±1 | 19±2 |

The data represent the peak area corresponding to each compound divided by that of the internal standard and multiplied by 1000. Results are presented as mean and standard deviation from biological triplicates. R108 columns to the right of the mutant lines represent independent sets of plants grown in parallel with the corresponding mutants. ND: Not detected.
Table 4. The 15 gene probe sets that were up-regulated by expression of MtWD40-I in M. truncatula hairy roots.

| Probe sets       | Target Description                                                                 | Ratio (HRWD40/HRGUS) | P-value   | Q-value   |
|------------------|------------------------------------------------------------------------------------|----------------------|-----------|-----------|
| Mtr.39774.1.S1_at| TC105711 Ttg1-like protein, partial (46%)                                          | 28.19                | 0         | 0.05      |
| Mtr.11660.1.S1_at| TC110633 /FEA=mRNA /DEF=                                                          | 4.69                 | 3.6E-22   | 0.998392  |
| Mtr.40780.1.S1_at| TC108029 /FEA=mRNA /DEF=                                                          | 4.35                 | 6.4E-22   | 0.998392  |
| Mtr.20158.1.S1_s_at| Zn-finger, CCHC type; Peptidase aspartic                                           | 4.10                 | 6.6E-15   | 0.998392  |
| Mtr.42612.1.S1_s_at| Similar to UP|Q6BGZ6 (Q6BGZ6) Similarity, partial (9%)                                         | 3.90                 | 5.9E-98   | 0.998392  |
| Mtr.5918.1.S1_at| Weakly similar to GB|AAP21357.1|30102878|BT006549 At1g56300 [Arabidopsis thaliana :], partial (60%) | 3.01 | 1.4E-40 | 0.998392 |
| Mtr.50164.1.S1_at| Heat shock protein Hsp20; HSP20-like chaperone                                     | 2.86                 | 1.4E-07   | 0.998392  |
| Mtr.51122.1.S1_at| Hypothetical protein AC126009.22.141 47552 46950  mth2-15c20 01/13/05             | 2.70                 | 6.7E-08   | 0.998392  |
| Mtr.18796.1.S1_s_at| T26F17.17-related                                                                 | 2.69                 | 2.4E-06   | 0.998392  |
| Mtr.40781.1.S1_s_at| Similar to UP|Q6BE36 (Q6BE36) Protein 7, partial (23%)                                            | 2.62                 | 4E-10     | 0.998392  |
| Mtr.37337.1.S1_at| Homologue to UP|HS12_MEDSA (P27880) 18.2 kDa class I heat shock protein, complete                 | 2.53                 | 6.1E-08   | 0.998392  |
| Mtr.40779.1.S1_at| Similar to UP|Q25783 (Q25783) P. falciparum parasite antigen DNA, partial cds. (Fragment), partial (10%) | 2.52 | 2.9E-09 | 0.998392 |
| Mtr.20165.1.S1_at| Hypothetical protein                                                              | 2.48                 | 1E-69     | 0.998392  |
| Mtr.45232.1.S1_at| Similar to UP|DR2A_ARATH (O82132) Dehydration responsive element binding protein 2A, partial (23%) | 2.44 | 4.7E-25 | 0.998392 |
| Mtr.4076.1.S1_s_at| Weakly similar to UP|O24249 (O24249) Methyltransferase, partial (10%)                                    | 2.01                 | 6.5E-12   | 0.998392  |
L-Phenylalanine

PAL

C4H

4CL

CHS

CHI

\[3 \times \text{Malonyl CoA}\]

Flavones

FS

Flavanones

DFR

Dihydroflavonols

LAR

Leucocyanidins

ANR

Anthocyanidins

Anthocyanins

GT

Catechins

Proanthocyanidins

GT

(Epi)catechin glucoside

GT

Isoflavones

FS

Flavanones

GT
Anthocyanin content (µg g⁻¹ FW)

Insoluble PA content (µg B1 equiv. g⁻¹ FW)

Soluble PA content (µg cat equiv. g⁻¹ FW)

Relative transcript level to actin

Transgenic lines

GUS
NF0977

MtWD40-1
NF0977

NF0977

MtWD40-1
