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Functional characterization of PETIOLULE-LIKE PULVINUS (PLP) gene in abscission zone development in Medicago truncatula and its application to genetic improvement of alfalfa

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Summary

Alfalfa (Medicago sativa L.) is one of the most important forage crops throughout the world. Maximizing leaf retention during the haymaking process is critical for achieving superior hay quality and maintaining biomass yield. Leaf abscission process affects leaf retention. Previous studies have largely focused on the molecular mechanisms of floral organ, pedicel and seed abscission but scarcely touched on leaf and petiole abscission. This study focuses on leaf and petiole abscission in the model legume Medicago truncatula and its closely related commercial species alfalfa. By analysing the petiolule-like pulvinus (plp) mutant in M. truncatula at phenotypic level (breakstrength and shaking assays), microscopic level (scanning electron microscopy and cross-sectional analyses) and molecular level (expression level and expression pattern analyses), we discovered that the loss of function of PLP leads to an absence of abscission zone (AZ) formation and PLP plays an important role in leaflet and petiole AZ differentiation. Microarray analysis indicated that PLP affects abscission process through modulating genes involved in hormonal homeostasis, cell wall remodelling and degradation. Detailed analyses led us to propose a functional model of PLP in regulating leaflet and petiole abscission. Furthermore, we cloned the PLP gene (MsPLP) from alfalfa and produced RNAi transgenic alfalfa plants to down-regulate the endogenous MsPLP. Down-regulation of MsPLP results in altered pulvinus structure with increased leaflet breakstrength, thus offering a new approach to decrease leaf loss during alfalfa haymaking process.

Keywords: abscission, alfalfa, forage improvement, Medicago truncatula, Medicago sativa, PETIOLULE-LIKE PULVINUS (PLP).

Introduction

Alfalfa (Medicago sativa L.) is one of the most widely cultivated forage species in the world and the fourth most valuable crop in the United States after corn, soybean and wheat (Zhang et al., 2005). Alfalfa has high biomass yield, excellent nutritional quality, wide adaptation and nitrogen fixation ability (Putnam et al., 2008). In the United States, it is estimated that more than 95% alfalfa is used for hay production. Leaf loss during the alfalfa haymaking process is considerable, which is in the range of 0.84% to 15.5% of hay dry matter (Pitt, 1990). Based on alfalfa production and price data from USDA National Agricultural Statistics Service (2018 data), haymaking process led to 0.5-8.6 million tons of leaf loss and economic loss of 0.07 to 1.16 billion dollars in the United States per year. The current methods to decrease mechanical leaf loss include optimization of alfalfa harvesting conditions, mechanical or chemical conditioning and utilization of advanced machinery (Summers & Idowu et al., 2013; Putnam et al., 2008). However, such management practice requires extensive experience and is difficult to control due to unpredictable weather conditions. Based on our observation during alfalfa hay making process, over 95% leaf loss occurred specifically at the leaflet and petiole pulvinus region, which is a joint-like thickening located at the base of the petiole or petiolule.
The weakness of leaflet and petiole pulvinus region might due to the pulvinus structure or the progressive degradation of middle lamella under abscission process at that region. Therefore, strengthening pulvinus structure and manipulating leaflet and petiole abscission are promising targets in alfalfa genetic improvement.

Abscission is a developmental process allowing a plant to shed its unwanted organs in response to various developmental, hormonal or environmental cues (Addicott, 1982; Estornell et al., 2013; Roberts et al., 2002). Abscission occurs at functionally specialized cell layers, called abscission zone (AZ), which is developed at the junction between the leaving organ and the main plant body. AZ is composed of a few layers of small and dense cytoplasm cells (Addicott, 1982; Osborne and Morgan, 1989; Sexton and Roberts, 1982). The abscission process has been divided into four major phases according to studies of floral organ abscission in Arabidopsis thaliana: (i) abscission zone differentiation; (ii) acquisition of the competence to respond to abscission signals; (iii) activation of abscission; and (iv) protective layer formation (Estornell et al., 2013; Kim, 2014; Patharkar and Walker, 2018; Patterson, 2001; Taylor and Whitelaw, 2001). All of these steps are important for the fate of organs that will shed.

The prerequisite of abscission is AZ differentiation; a set of genes participating in this process has been identified in Arabidopsis, tomato and rice (Lewis et al., 2006). In Arabidopsis, BLADE-ON-PETIOLE1 (BOP1)/BOP2, ARABIDOPSIS THALIANA HOMEobox GENE1 (ATH1), BREVIPEDICELLUS/ KNOTTED-LIKE FROM ARABIDOPSIS THALIANA (BPNK1) and ASYMMETRIC LEAVES 1 (AS1) are critical for floral organ AZ differentiation (Gómez-Mena and Sablowski, 2008; Gubert et al., 2014; McKim et al., 2008; Shi et al., 2011). Several genes regulating pedicel AZ differentiation have been identified, including JOINTLESS, LATERAL SUPPRESSOR (Ls), MACROCALYX (MC) and SLMBP2 (Liu et al., 2014; Mao et al., 2000; Nakano et al., 2012; Schumacher et al., 1999). In rice, SHATTERING4 (SH4), qSH1, OsSH1 and SHATTERING ABORTION 1 (SHAT1) function as positive regulators to modulate pedicel AZ development, while OsCPL1 represses pedicel abscission zone differentiation (Li et al., 2010; Konishi et al., 2006; Li et al., 2006; Lin et al., 2012; Zhou et al., 2012b).

After AZ is differentiated, the AZ cells will be staying at a quiescent state, until they perceive abscission-promoting signals to initiate the abscission process (Nakano et al., 2013). Abscission signals will activate the expression of genes such as cell wall hydrolytic enzymes (e.g. polygalacturonases) to act on structural polysaccharides leading to the hydrolysis of the middle lamella and cell walls of the AZ cells (Estornell et al., 2013). Both ethylene and auxin (IAA) were previously proposed as important signals for abscission activation (Taylor and Whitelaw, 2001). However, ethylene-insensitive mutants ethylene resistant 1-1 (etr-1) and ethylene-insensitive 2 (ein2) showed delayed instead of abolished floral organ abscission, indicating that ethylene is not critical for abscission, and thus, auxin has been proposed as a key player in the regulation of abscission process (Patterson and Bleecker, 2004; Taylor and Whitelaw, 2001). Auxin response factors (ARFs) are transcription factors that mediate responses to auxin. In Arabidopsis, arf2 mutant delayed floral organ abscission, while arf1 enhanced arf2 phenotype (Okushima et al., 2005). The mutation of NPH4/ARF7 and ARF19 enhanced arf2 phenotype (Ellis et al., 2005). It has been shown that indoleacetic acid (IAA) signalling is a prerequisite for floral organ shedding in Arabidopsis (Basu et al., 2013). Microarray analysis of gene alterations in response to auxin depletion in tomato and Mirabilis jalapa revealed that acquisition of ethylene sensitivity in leaf and flower AZ is associated with differential expression of auxin-related genes (Meir et al., 2006; Meir et al., 2010). In tomato, KD1, a KNOTTED1-LIKE HOMEOBOX (KNOX) family gene, was identified to modulate pedicel and petiole abscission by regulating genes involved in the auxin signalling pathway (Ma et al., 2015).

However, the transcriptional regulation of hormonal homeostasis in the leaf AZ is still unclear. To date, a wealth of valuable information has been accumulated based on the studies of floral organ abscission in Arabidopsis, pedicel abscission in tomato and seed shattering in rice. However, our understanding of molecular mechanisms on leaf abscission is still limited. Recently, it has been revealed that both drought- and pathogen-triggered calamine leaf abscission in Arabidopsis might be regulated through salicylic acid signalling (Patharkar and Walker, 2016; Patharkar et al., 2017). HAESA/HAESA-like 2 (HAES/HASL2), INFLORESCENCE DEFICIENT IN ABSCISION (IDA), NEVERSHEDED (NEV) and MAPK KINASE4/5 (M KK4/5) are required for drought-induced leaf abscission (Patharkar and Walker, 2016). HAESA/HAESA-like 2 (HAES/HASL2), IDA and NEVERSHEDED (NEV) are required for the initiation of pathogen-triggered leaf abscission (Patharkar et al., 2017). BOP1/BOP2 in Arabidopsis and its ortholog NODULE ROOT (NOOT) in Medicago truncatula have been shown to be involved in leaf abscission zone formation (Couzigou et al., 2016; McKim et al., 2008). Recent transcriptome studies of leaf abscission have provided insights into the regulatory mechanism of abscission (Kim et al., 2016; Li et al., 2017; Li et al., 2016; Liao et al., 2015b, 2016c).

As a model plant for legume species, Medicago truncatula is also suitable for leaf abscission study. It has compound leaves and its leaflets and petioles abscise at the senescing stage at the base of pulvinus. Over the years, with the development of genomic resources, particularly the generation of large-scale insertional mutagenesis (Tadege et al., 2005) and the sequencing of the M. truncatula genome (Young et al., 2011), many genes with novel functions have been identified (Chai et al., 2016; Kang et al., 2016; Zhao et al., 2019; Zhou et al., 2019). Leguminous plants open their leaves during the day and close them at night, and this kind of nyctinastic movement is induced by volume change in motor cells in the pulvinus (Ueda and Nakamura, 2007). The PETIOLULE-LIKE PULVINUS (PLP) gene was identified by forward screening from the retrotransposon-tagged mutant population of M. truncatula; the loss of function of PLP caused the alteration of pulvinus to petiololes and affected the nyctinastic movement of leaflets (Zhou et al., 2012a). In this study, we report novel findings that the loss of function of PLP results in an absence of AZ formation and PLP plays an important role in leaflet and petiole AZ differentiation, thus providing new information on molecular mechanism of leaf and petiole abscission. Furthermore, down-regulation of the PLP gene (MsPLP) in alfalfa results in the alteration of pulvinus to petioloile-like structure with increased leaflet breakstrength, offering a new approach to minimize leaf loss during alfalfa haymaking process.

Results

Mutation in PLP abolishes shedding of leaflet and petiole in M. truncatula

The PLP gene has one exon with 579 nucleotides containing a LOB domain (Zhou et al., 2012a). To characterize the role of PLP in leaflet and leaf abscission, three alleles plp-1 (a Tnt1 inserted after base 178), plp-2 (a Tnt1 inserted after base 130) and plp-4

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(a Mere1 inserted after base 246) were used for analysis (Figure 1a). The loss of function of PLP altered the leaflet pulvinus into petiolule-like structures (Figure S1a-d) and the petiole pulvinus into petiole-like structures (Figure S1e-h).

In wild-type M. truncatula, organ detachment started with leaflet at the base of pulvinus (Figure 1b, d) and then followed by petiole at the stem-petiole junction (Figure 1f, h). Leaflet and petiole started to shed at the leaf age of 36 days (67% remaining) and 37 days (87% remaining), respectively (Table S1). The percentage of plants with remaining leaves and petioles decreases with the increase of leaf ages until no leaflets and petioles were left on the plant, when the leaf age reaches 43 and 45 days (Table S1). In contrast, both leaflets and petioles of the plp mutant remain attached to the plant even after 45 days (Table S1). In plp mutants, shedding of leaflet and petiole was abolished even when the whole plant was dead.

The abscission defects of plp were further characterized by shaking assay. After shaking vigorously for 2 min, almost all the senescing leaves of wild type shed, while for plp mutants, most leaflets and petioles were still attached to the plant body (Figure S2a). Markedly reduced leaf shed was observed for plp with respect to wild-type plants (Figure S2b-g). The weight of dropped leaves of wild type was 5 times more than that of plp (Figure S2h). The shaking assay further confirmed that plp has a defect in leaflet and petiole abscission at senescing stage.

In order to further characterize the leaflet and petiole abscission defects of plp, a breakstrength assay was performed. The wild-type leaflet and petiole breakstrength gradually decreased after 33 days old and 35 days old, respectively, indicating a progressive degradation of the middle lamellae in leaflet and petiole abscission zone (Figure 2). However, leaflet and petiole breakstrength of plp showed no obvious change from 33- to 39-day-old leaf and 35- to 39-day-old leaf (Figure 2). After 39 days, the leaflet and petiole breakstrength of plp gradually decreased with the increase of leaf ages (Figure 2). In all cases, both leaflet breakstrength and petiole breakstrength of plp mutants were significantly higher than wild type (Figure 2).

PLP plays an important role in controlling leaflet abscission zone (LAZ) and petiole abscission zone (PAZ) differentiation

To further determine whether normal LAZ and PAZ were developed in plp, we used scanning electron microscopy (SEM) to examine the leaflet pulvinus–petiolule boundary and petiole pulvinus–stem boundary of wild type and plp mutant (Figure 3).

In wild type, scars were left after manual removal of leaflet and petiole from the plant. Ruptured cells were observed upon removal of 31-day-old leaflet and 35-day-old leaflet at the fracture plane (Figure 3b,c). Owing to the weakening of the middle lamellae, removal of 39-day-old leaflet only caused very few cell ruptures (Figure 3d). Leaflets were already abscised at 43 days and 47 days, and there was a protective layer of rounded cells formed at the leaflet abscission zone (Figure 3e,f). By contrast, the leaflet fracture surface of plp mutant sample

![Figure 1](https://example.com/figure1.png)

*Figure 1* Petiolule-like pulvinus (plp) mutant of M. truncatula showing defects in leaflet and petiole abscission. (a) Schematic representation of the gene structure of PLP. The arrows indicate the location of Tnt1 and Mere1 retrotransposons in the plp alleles. (b, c) Senescing leaf (39-day-old) of wild type and plp mutant. Wild-type leaflet shed at this stage. (d, e) Close-up view of leaflet base at leaflet abscission zone (LAZ) in wild type and plp mutant. Red arrow indicates the LAZ in wild type. The leaflet has already abscised from wild type, while the AZ left at the base of pulvinus, while for plp mutant, leaflet is still attached to petiole. (f, g) Senescing leaf (47-day-old) with petiole abscission zone. Wild-type leaflet shed at this stage. (h, i) Close-up view of petiole abscission zone of wild type and plp mutant. Red arrow indicates petiole abscission zone (PAZ). The petiole of wild type has already abscised from stem with the PAZ left at the base of pulvinus, while plp petiole is still attached to stem, although the leaflets are nearly dry. PE, petiolule; P, petiole; PLP: petiolule-like pulvinus; S, stem; D39, 39-day-old; D47, 47-day-old. Scale bars: (b, c, f, g) 1 cm; (d, e, h, i) 1 mm.
showed no evidence of AZ activation at any age and cells ruptured during leaflet removal (Figure 3g-k).

Broken cells were observed upon removal of petiole (from 31 to 43 days old) in wild type (Figure 3l-o). Due to the abscission of petiole in 47-day-old leaf, the petiole fracture surface of wild type showed a layer composed of spherical elongated cells (Figure 3p). However, in plp mutant, ruptured cells at fractured plane were observed at all leaf ages (Figure 3q-u).

Phenotype characterization and SEM analysis revealed the important role of PLP in LAZ and PAZ development. To determine more precisely the differences in cellular morphology between wild type and plp, longitudinal sections of leaflet and petiole pulvinus region at 25 and 35 days were examined and compared (Figure 4). The 25-day-old leaf (mature leaf) represents the stage before abscission process occurs, whereas the 35-day-old leaf (senescing leaf) represents the stage the abscission process was undergoing at both leaflet and petiole abscission zones based on the observation in Table S1. In wild type, a layer of small cytoplasmic dense cells at the junction between pulvinus and petiolule was differentiated at 35 days (Figure 4e, red arrow), and the morphological differences were apparent when compared with the 25-day-old section (Figure 4c). The edge of the pulvinus and petiolule junction was detached due to the dissolution of middle lamella of LAZ cells (Figure 4e). Unlike the wild-type control, no differentiated small cells were detected in the plp region which is marked by red asterisk indicative of the region corresponding to wild-type LAZ (Figure 4f). Compared with 25-day-old section at petiole pulvinus region, cytoplasmic dense AZ cell layers marked by red arrow were visible between petiole pulvinus and stem in wild type at 35 days (Figure 4k). However, plp did not show such a structure differentiated at the corresponding location marked by a red asterisk at 35-day-old leaf (Figure 4l). Therefore, the distinct cell layers that differentiated at the junctions between pulvinus and petiolule and between petiole and stem were absent in plp.

PLP expression pattern during the leaf senescing process

The expression pattern of MtPLP in wild-type plants was analysed by M. truncatula Gene Expression Atlas (Benedito et al., 2008). The expression of MtPLP was detected in almost all the organs at various developmental stages, with a relatively high expression level in vegetative buds and nodules and relatively low levels in seed and leaf (Figure S3).

To determine whether there is a correlation between the PLP expression pattern and leaflet and petiole abscission, we analysed transgenic M. truncatula plants that expressed GUS under the control of PLP promoter. We examined GUS expression during the time course of abscission in three stages as shown in Table S2: stage I, prior to organ separation; stage II, during abscission; and stage III, after abscission when the remaining cells form protective
scar tissue. The expression of the proPLP::GUS construct was observed in both leaflet and petiole pulvinus regions and at the base of leaflet–petiolule junction and stem–petiole junction (Figure 5a,e). Prior to organ abscission, the expression of PLP was restricted to the abscission zones, in which the leaflet and petiole had not detached (Figure 5b,f). During abscission, a strong signal was detected at the base of leaflet and petiole pulvinus region (Figure 5c,g). After abscission, at the AZ scars following leaflet and petiole detachment, no GUS signal was detected (Figure 5d,h). Taken together, the proPLP::GUS expression pattern showed that PLP is strongly expressed in the abscission zone prior to abscission and during abscission but not observed after abscission, confirming that PLP contributes to AZ differentiation.

Gene expression analysis identified genes regulated by PLP in the abscission process

To explore the transcriptional mechanisms underlying the loss of function of AZ development in plp mutants, gene expression in 25-day-old leaflet AZ tissues in wild type and three independent mutant lines was analysed using Affymetrix Medicago GeneChips (Affymetrix, CA, USA). The ratio between mutant and wild type above twofold was considered up-regulated, while below 0.5 was considered down-regulated. We identified 826 up-regulated and 1216 down-regulated genes (Tables S3 and S4). The differentially expressed genes were mapped in pathways including photosynthesis, hormone metabolism, signalling, stress, RNA, secondary metabolism, mitochondrial electron transport and DNA pathway (Figure S4a). Genes mapped in the hormone metabolism and cell wall pathways are shown in Figure S4b, c. In the hormone metabolism pathway, 67 auxin-related genes were down-regulated, including 53 SMALL AUXIN-UP RNA (SAUR) like family genes, 4 Gretchen Hagen3 (GH3) genes and 1 AUX/IAA gene. For ethylene-related genes, 3 ethylene response factors (ERFs), 3 1-amino-cyclopropane-1-carboxylic acid synthases (ACSs) and the ethylene-insensitive 4 (EIN4) were down-regulated in the plp mutant (Figure S4b). In the cell wall pathway, the expression of 64 cell wall-related genes were significantly altered in plp mutant, including genes for polygalacturonase (PG), xyloglucan endotransglucosylase/hydrolase (XTH), glycosyl hydrolase (GH), pectin methyl esterase (PME) and expansin (EXP) (Figure S4b). Twenty auxin-related genes were selected for PCR validation (Table S5), and they showed the same expression pattern as microarray data (Figure S5).

Microarray analysis revealed 152 putative abscission-related transcription factors (TFs). They belong to 23 different families of

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**Figure 3** Abscission phenotype and scanning electron micrographs of leaflet and petiole abscission zones in *M. truncatula*. (a) Leaves of wild type and plp at different ages. In wild-type plants, 39-day-old leaflet started to abscise from the petiolule and petiole of 47-day-old leaf had already abscised from stem, while in plp plants, leaflets and petiole did not abscise. (b-u) Scanning electron micrographs of leaflet and petiole fracture planes in wild type and plp from leaf age of 31 days to 47 days. (b-f) Leaflet fracture planes of wild type showing progression from broken cells (31-, 35- and 39-day-old leaflets) to rounded AZ cells (43- and 47-day-old leaflets). (g-k) Leaflet fracture planes of plp mutant showing broken cells (all ages). (l-p) Petiole fracture planes of wild type showing progression from broken cells (31-, 35-, 39- and 43-day-old leaves) to rounded AZ cells (47-day-old leaves). (q-u) Petiole fracture planes of plp mutant showing broken cells (all ages). D31, 31-day-old leaf; D35, 35-day-old leaf; D39, 39-day-old leaf; D43, 43-day-old leaf; D47, 47-day-old leaf. Scale bars: 100 μm.
Figure 4  Anatomical comparisons between leaflet abscission zone (LAZ) and petiole abscission zone (PAZ) in wild type and plp. (a, b) Close-up view of leaflet pulvinus region. (c-f) Microscopic analysis of longitudinal sections across the LAZ stained by toluidine blue at leaflet pulvinus region. (c, d) Mature leaflet (25-day-old) pulvinus region in wild type and plp. No leaflet AZ was formed in either wild type or plp mutant. (e, f) Senescing leaflet (35-day-old) pulvinus region in wild type and plp. LAZ of wild type is shown in the picture, and no LAZ is formed in plp mutant. (g, h) Close-up view of petiole pulvinus region. (i-l) Microscopic analysis of longitudinal sections across the PAZ stained by toluidine blue at the petiole pulvinus region. (i, j) Mature leaf (25-day-old) petiole pulvinus region in wild type and plp. No AZ was formed in either wild type or plp. (k, l) Senescing leaf (35-day-old) petiole pulvinus region in wild type and plp. Small cytoplasmic cells were observed along the petiole AZ region in wild type, and no AZ cells were observed in plp. D25, 25-day-old leaf; D35, 35-day-old leaf. Red arrows indicate LAZ and PAZ; red asterisks in (f) and (l) indicate the region where the LAZ and PAZ should be present. Scale bars: (a, b) 5 mm; (g, h) 1 mm; (c-f, i-l) 500 μm. L, leaflet; PE, petiolule; PLP: petiolule-like pulvinus; PU, pulvinus; P, petiole; S, stem.

Figure 5  GUS staining of leaflet pulvinus region and petiole pulvinus region at different abscission zone developmental stages of transgenic M. truncatula plants carrying the PLP promoter-GUS construct. (a, e) GUS expression in the leaflet and petiole pulvinus region. (b, f) Prior to organ abscission, GUS expression was detected at the base of leaflet pulvinus and at the base of leaf pulvinus in 25-day-old leaf. (c, g) During organ abscission, GUS activity was detected at the base of 35-day-old leaflet and 43-day-old leaf petiole. (d, h) After abscission, no GUS expression was observed at the base of leaflet pulvinus or petiole pulvinus after the abscission of leaflet and petiole. L, leaflet; PE, petiolule; PU, pulvinus; P, petiole; S, stem; LAZ, leaflet abscission zone; PAZ, petiole abscission zone. Scale bars: (a, e) 5 mm; (b–d, f–h) 1 mm.
TFs mainly including bHLH (8.6%), WRKY(8.6%), zinc fingers (8.6%), Aux/IAA family (7.9%), MYB (7.9%) and Homeobox (6.6%), suggesting a complex regulation of leaflet abscission in *M. truncatula* (Figure S6). In addition, there were several hormone-related TFs represented by AUX/IAA (7.9%), ARR (4.6%) and ARF (4.6%). We performed a Venn diagram analysis using our identified 152 putative abscission-related TFs and the 188 soybean abscission-specific TFs selected by Kim (Kim et al., 2016). Totally 26 overlapped candidates are potentially involved in both soybean and *M. truncatula* leaf abscission (Figure S7; Table S6). These results suggest that *plp* affects auxin-related genes which, in turn, affect LAZ development in *M. truncatula*

Expression of the NOOT gene is down-regulated in *plp*

In order to examine whether there are any overlaps between published AZ-related genes and our microarray results, homologous genes of published genes related to AZ development were identified in *M. truncatula* by blast on phytozome (https://phytozome.jgi.doe.gov/pz/portal.html) (Table. S7). Except for the NOOT gene, none of the genes listed in Table S7 were significantly up- or down-regulated, indicating the regulatory pathway of floral organ abscission in *Arabidopsis* might be different from that of leaflet and petiole abscission in *M. truncatula*.

We investigated the relationship between PLP and NOOT. The NOOT gene is involved in leaflet, petiole, petal and seed abscission (Cougizou et al., 2016). For wild type, a 39-day-old leaflet was already abscised from petiolule, while a 39-day-old leaflet of noot mutant was still attached to petiolule (Figure S8a, b). The 47-day-old petiole of wild type had already abscised from stem, while the petiole of *plp* was still attached to stem tightly (Figure S8a,b). The expression analysis of NOOT in wild type and *plp* mutants showed that the NOOT gene was significantly down-regulated for more than twofold compared with wild type (Figure S8e).

Isolation and suppression of *MsPLP* gene in alfalfa

Because of the high degree of sequence identity between *M. truncatula* and alfalfa, the alfalfa PLP (*MsPLP*) gene was cloned using the primers derived from *MtPLP*. Sequence comparison analysis showed that *MsPLP* and *MtPLP* share 97.4% similarity (Figure S9). Analysis of deduced amino acid sequences revealed that *MsPLP* protein contained 193 amino acids, showing 99% identity to *MtPLP* (Figure S10). The *MsPLP* protein is also highly similar to its putative orthologs in *Glycin max*, *Lotus japonicas*, *Pisum sativum* and *Trifolium medium*, indicating *PLP* is highly conserved in legume species (Figure S10). The PLP orthologs in various species were obtained by BLASTN through NCBI and Phytozome and used for phylogenetic analysis (Figure S11). Phylogenetic analysis showed that *MsPLP* and *MtPLP* were closest to each other and were clustered close to *PsAPU*, *TmELP1* and *TtLOB* (Figure S11).

To suppress the activity of the endogenous *MsPLP* in alfalfa, *MsPLP*-RNAi vectors (Figure S12) were constructed and introduced into alfalfa by *Agrobacterium tumefaciens*-mediated transformation (Figure S13a-e). Only one plant was selected from each callus, representing an independent line. Fourteen independent transgenic lines were identified by PCR analysis (Figure S13f). Quantitative RT-PCR analysis revealed that 12 transgenic lines (*S1-S12*) had *MsPLP* expression levels reduced by more than 75% when compared with the wild type (Figure S14a). Three transgenic lines, *S1, S2* and *S3*, were used for further analysis.

*MsPLP*-RNAi transgenic alfalfa shows defects in pulvinus development

Plant height and fresh weight showed no significant differences between wild type and transgenic lines, suggesting that knocking down *MsPLP* had no effects on biomass yield (Figure S14b-d). The RNAi alfalfa plants showed defects in leaflet and petiole petiole development (Figure 6a-h) similar to the *M. truncatula* *plp* mutant.

Suppression of *MsPLP* strengthens the leaflet and petiole pulvinus region

Leaflet and petiole breakstrengths were measured when plants reached blooming stage, at which alfalfa is normally harvested. In the field, alfalfa plants are generally harvested 10 cm above ground. Therefore, we evaluated the leaflets above the height of 10 cm. Breakstrengths of 3rd leaflets to 12th leaflets were evaluated (Figure 7a). For both terminal and lateral leaflets, leaflet breakstrengths of transgenic lines were significantly higher than that of wild type (Figure 7b,c). For petiole breakstrength, 3rd to 5th petiole and 11th to 12th petiole of the transgenic lines were significantly higher than that of wild type (Figure 7d).

In order to further characterize whether the pulvinus region was strengthened, the frequency of the breaking positions during breakstrength assay was recorded. In wild type, the terminal leaflet, lateral leaflet and petiole were detached specifically at the pulvinus region during the breakstrength assay, indicating the pulvinus region is the weakness area (Figure S15a,c). In transgenic lines, the detachment of terminal leaflet occurred at three positions, *D1*: leaflet (8%), *D2*: petiulike pulvinus (22%) and *D3*: petiole (70%); the detachment of lateral leaflet occurred at three positions, *D1*: leaflet (2%), *D2*: petiulike pulvinus (57%) and *D3*: base of petiulike pulvinus (41%); and the detachment of petiole occurred at two positions, *D1*: petiulike pulvinus (95%) and *D2*: petiole (5%) (Figure S15b,c). The various detachment position demonstrates that the pulvinus region was strengthened by genetically knocking down the *PLP* gene in alfalfa.

Analysis of nutritive value of *MsPLP* down-regulated alfalfa plants

In order to evaluate whether the knockdown of *MsPLP* will affect alfalfa quality under greenhouse non-senescing conditions, acid detergent fibre (ADF), neutral detergent fibre (NDF), crude protein (CP), in vitro true dry matter digestibility (IVTDMD), total digestible nutrients (TDN) and relative feed value (RFV) were analysed. No significant change was found in these forage quality parameters between wild type and transgenic lines for the whole plant, leaf and stem (Figure S16a-c). The results show that down-regulation of *MsPLP* has no impact on alfalfa nutritive value when the vegetative plants were gently harvested without leaf loss.

Discussion

*PLP* is required for AZ differentiation

Abscission zone is a unique anatomical structure which is essential for the abscission process. Several genes involved in *Arabidopsis* floral organ (*BOP1*BOP2, *ATH1* and *AS1*), tomato pedicel (*JOINTLESS*, *MC* and *SLMBP21*) and rice pedicel (*SH*, *QSH1*, *SHAT1*, *Ox5* and *OxCP1*) abscission zone differentiation have been identified (Gómez-Mena and Sablowski, 2008; Gubert et al., 2014; Ji et al., 2010; Konishi et al., 2006; Li et al.,
Figure 6 MsPLP-RNAi transgenic lines showed defects in pulvinus development. (a, b) Leaf of wild type and MsPLP-RNAi alfalfa. (c, d) Close-up view of leaflet base in wild type and transgenic alfalfa. Transgenic alfalfa showed defects in pulvinus development, pulvinus was altered to petiolule-like structure. (e, f) Petiole of wild type and MsPLP-RNAi alfalfa. (g, h) Close-up view of petiole base in wild type and transgenic alfalfa. Transgenic alfalfa showed defects in petiole pulvinus development. L, leaflet; PE, petiolule; PU, pulvinus; P, petiole; S, stem; PLP, petiolule-like pulvinus. Scale bars: (a, b) 1 cm; (c, d) 1 mm; (e, f) 1 cm; (h, 1) 1 mm.

Figure 7 Terminal and lateral leaflet breakstrength assay and petiole breakstrength assay of wild type and transgenic alfalfa. (a) Schematic leaflet distribution on alfalfa stem. The evaluation of leaflet and petiole breakstrength was started from the third leaflet because alfalfa is normally harvested 10 cm above ground under field conditions. (b) Terminal leaflet breakstrength measurements at blooming stage. (c) Lateral leaflet breakstrength measurements at blooming stage. (d) Petiole breakstrength measurements at blooming stage. Error bars indicate SD (n = 5). P3, the 3rd leaflet; P4, the 4th leaflet; ... P12, 12th leaflet.
2006; Lin et al., 2012; Liu et al., 2014; Mao et al., 2000; McKim et al., 2008; Nakano et al., 2012; Zhou et al., 2012b). In *M. truncatula*, the *NOOT* gene, a *BOP* ortholog, has been shown to modulate petal, leaflet and seed abscission zone differentiation (Couzigou et al., 2016). Interestingly, in both *plp* and *noot* mutants, simplified stipules were observed, while the pulvinus structure of *noot* mutant is not modified as the *plp* mutant, suggesting that the absence of *PLP* activity modifies the cellular structure of the tissue where leaflet and petiole AZs are differentiated. Until now, the molecular mechanism of leaflet and petiole AZ differentiation is still largely unknown. The present study focused on leaflet and petiole abscission. We discovered that the *PLP* gene plays an important role in regulating leaflet and petiole abscission zone differentiation in *M. truncatula*. The loss of function of *PLP* results in a complete absence of leaflet and petiole abscission during plant senescence. Microscopic observation of cross sections and SEM analysis further confirmed that the *plp* mutants have no leaflet and petiole abscission zone differentiation, suggesting the importance of *PLP* in initiating AZ differentiation.

Lateral organ boundary formation and abscission zone development

Night closure of leaflets is a common feature of legumes, and such nucinastic movement is generated by the pulvinus. It has been proposed that *PLP* regulates pulvinus establishment through controlling boundary formation for the correct production of compact and convoluted motor cells to mediate leaflet movement (Zhou et al., 2012). PLP is a LOB domain protein, and it has 75% identity to *Arabidopsis* ASL4/LOB which belongs to the LBD protein family (Zhou et al., 2012a). LBD proteins play important roles in modulating plant development (pollen, embryo, root, leaf and inflorescence development), hormone response (cytokine, ABA, auxin, JA, gibberellin), anthocyanin and nitrogen metabolism, plant regeneration (callus formation), disease susceptibility, photomorphogenesis, secondary growth and pulvinus development (Majer and Hochholdinger, 2011; Xu et al., 2016). LBDs are specifically expressed at organ boundaries regulating plant lateral organ development (Yang et al., 2016). In wild type, there is a clear boundary region at the base of leaflet and petiole pulvinus, whereas no boundary was formed at the base of leaflet and petiole in the *plp* mutant (Figure 4). The wild-type leaflet and petiole AZs were differentiated after the development of leaflet and petiole pulvinus (Figure 4c,e,i); thus, they are adventitious AZs according to Addicott (1982). In the *plp* mutant, the loss of cell fate in leaflet and petiole AZs led to the failure of AZ cell placement and differentiation. These observations indicate that the existence of organ boundaries influences AZ formation. Several genes that are involved in organ boundary formation have been shown to be involved in AZ development including *BOP1/2*, *ATH1*, *AS1* and *HW5* (Gómez-Mena and Sablowski, 2008; González-Carranza et al., 2007; Gubert et al., 2014; Hepworth et al., 2005; Jun et al., 2010; McKim et al., 2008; Norberg et al., 2005; Xu et al., 2008). Our study provides more evidence to support the potential link between organ boundary formation and AZ development.

In *Arabidopsis*, *BOP1/BOP2* have been demonstrated as the earliest regulators associated with AZ differentiation, which are required for abscission in both wild-type and *35S::IDA* plants (McKim et al., 2008). The homologous genes of *BOP* in tobacco (*Nicotiana tabacum*) as well as in a few legume species (*Pisum sativum*, *Lotus japonicus*, *Lupinus angustifolius* and *Lupinus luteus*) are also necessary for the abscission of lateral organs (Couzigou et al., 2016; Frankowski et al., 2015; Kucko et al., 2019; Wu et al., 2012). In *M. truncatula*, the *noot* mutant has no specialized small condensed cell layer along its leaflet and leaf abscission zone region, indicating the role of *NOOT* gene in regulating the establishment of AZ (Couzigou et al., 2016). In the current study, we found that the *NOOT* gene was down-regulated in the *plp* mutant, suggesting a possible relationship between *PLP* and *NOOT*. It has been shown that the LBD domain genes *ASYMMETRIC LEAVES2* and LOB were up-regulated in *35S* *BOP* and down-regulated in *bop*1/2 mutant, therefore indicating a potential link between these genes in regulating lateral organ cell fate and polarity in *Arabidopsis* (Ha et al., 2007). In addition, the overexpression of *LBD15* activated the transcription of *BOP1* and *BOP2*, which were critical in controlling lateral organ development and inflorescence architecture (Zhu et al., 2014).

Since *PLP* is an LBD protein, we propose a potential link between *PLP* and *NOOT* in defining the AZ cells at organ boundaries by modulating lateral organ polarity and cell fate. Further research on genetic interactions between *PLP* and *NOOT* may provide more details about AZ differentiation.

Transcriptional regulation of hormonal homeostasis, cell wall degradation and remodelling by *PLP* in the abscission process

The conventional model suggests that auxin gradient plays a central role in abscission (Addicott, 1982). The sensitivity of AZ to ethylene is regulated by the alteration of auxin gradient (Abeles and Rubenstein, 1964; Meir et al., 2006). Previous research on *PLP* used a DR5rev: GFP auxin response reporter to detect the role of PLP in auxin signalling (Zhou et al., 2012a). A GFP signal was detected specifically in the wild-type pulvini, while no signal was observed in the *plp*-1 mutant (Zhou et al., 2012a). This result was used to confirm the alteration of the unique structure of pulvini in the mutant without realizing its implications in abscission (Zhou et al., 2012a). Here, when abscission is considered, the result also suggests that auxin gradient across AZ was affected due to *PLP* mutation. Our microarray results showed that 137 genes involved in hormone metabolism were affected by the mutation of *PLP*. Among them, 70 auxin-related genes and 12 ethylene-related genes were significantly affected, indicating a possible role of *PLP* in modulating abscission process through auxin and ethylene biosynthesis and signalling.

Auxin response factors (ARFs) are transcription factors that mediate responses to auxin. In *Arabidopsis*, ARF2 was reported to regulate floral organ abscission (Ellis et al., 2005). ARF1 or NPH4/ARF7 and ARF19 were reported to enhance the delayed abscission of arf2 mutant (Ellis et al., 2005; Okushima et al., 2005). The tomato homologue of the *Arabidopsis* ARF19 was down-regulated in *KD1* antisense transgenic plants with delayed pedicel abscission phenotype (Ma et al., 2015). In tomato, *miR160* was found to regulate floral organ abscission and fruit abscission (Damodharan et al., 2016). The sly-miR160 depletion was associated with the up-regulation of ARFs including *SIARF10A*, *SIARF10B* and *SIARF17* which are homologues of *Arabidopsis* *ARF10* and *ARF17* (Damodharan et al., 2016). The expression pattern of ARFs during tomato flower pedicel abscission under auxin and ethylene treatment suggested important roles of ARFs in regulating pedicel abscission (Guan et al., 2014). Studies on *LBD18*, *LBD16* and *LBD30* revealed the function of LBDs in
regulating auxin signalling pathway. LBD18 functions in the initiation and emergence of lateral roots, in conjunction with LBD16, downstream of ARF7 and ARF19 (Lee et al., 2009). ILO (LBD30) is expressed in boundaries and regulates both auxin transport and meristem fate by promoting the expression of the KNOX genes SHOOTMERISTEMLESS (STM) and BPKNAT1 (Bureau and Simon, 2008). In our study, eight ARFs were affected in plp mutant, including ARF8, ARF10, ARF16 and ARF19, indicating a possible role of PLP in regulating auxin response to control the abscission process.

Abscission is activated after perceiving abscission signals, such as auxin and ethylene in the AZ. The microarray analyses of 25-day-old leaflet abscission zone region showed that several genes for cell wall degrading and remodelling factors were down-regulated in the plp mutant. These include PGs, XTHs, GHS, PMEs and EXPs. The roles of these cell wall modifying enzymes have been associated with abscission in Arabidopsis and tomato (Estornell et al., 2013; Hong et al., 2000; Jiang et al., 2008; Lashbrook and Cai, 2008; Ogawa et al., 2009). In our study, abscission was not yet detected in 25-day-old leaflets (Table S1). The expression of PLP gene was detected in both prior and during the abscission process in Figure 5 (a-c, e-g), suggesting that PLP might play a role not only in AZ differentiation but also in the competence to respond to abscission signals and the activation of abscission. Both HAE and HLS2 regulate programmed separation of cells during abscission (Cho et al., 2008). Interestingly, AtLOB, the homologous gene of MtPLP in Arabidopsis, is up-regulated in floral receptacles of the Arabidopsis double mutant hae hls2, indicating that the AtLOB is closely related to abscission execution (Niederhuth et al., 2013). This reinforces the regulatory role of MtPLP on hormonal- and cell wall-related genes during leaflet abscission. Taken together, the above evidence consistently suggests that PLP affects abscission execution through transcriptional regulation of hormonal homeostasis, cell wall degradation and remodelling. Here we propose a model of PLP in regulating leaflet and petiole abscission (Figure S17).

Promising candidate genes in leaf abscission studies

To analyse genes involved in leaf abscission in soybean, Kim et al. (2016) performed RNA sequencing (RNA-seq) using RNA isolated from the leaf abscission zones and petioles (Non-AZ, NAZ) of soybean after treating stem/petiole explants with ethylene for 0, 12, 24, 48 and 72 h. 188 abscission-specific TFs were identified, which include TFs belonging to homeobox, MYB, Zinc finger, bHLH, AP2, NAC, WRKY, YABBY and ARF families (Kim et al., 2016). In the present study, we identified 26 overlapped candidates that are involved in both soybean and M. truncatula leaf abscission (Figure S7). These genes are promising candidates for future leaf abscission studies (Table S6).

PLP has potential for alfalfa improvement

In the present study, we explored a new way to minimize leaf loss during the haymaking process through genetically modifying alfalfa leaflet and petiole structures. The leaflet pulvinus and petiole pulvinus regions were strengthened with increased breakstrength and decreased detachment frequency by RNAi suppression of MsPLP without negative impacts on biomass yield and nutritive value. The abscission of leaflet and petiole was abolished in transgenic alfalfa. In future studies, a complete knockout of PLP in alfalfa can be achieved by genome editing (Wolabu et al., 2020). A large-scale field test is needed to evaluate leaf loss and forage quality of the materials using commercial machines under field conditions.

In summary, our study revealed a new function of PLP in regulating AZ development in the model legume M. truncatula and we have successfully applied this knowledge to alfalfa improvement. This study demonstrated that loss of function of PLP resulted in an absence of AZ differentiation. The regulation of abscission by PLP is associated with auxin-related genes and NOOT. Furthermore, the alteration of pulvinus to petiolule-like pulvinus has significantly increased the breakstrength of leaflets and petioles in alfalfa, which offers a new approach to decrease leaf loss during the haymaking process. This study demonstrates how knowledge gained from a model plant can be applied to the genetic improvement of a commercial crop.

Experimental procedures

Plant materials and growth conditions

The Medicago truncatula Gaertn. Ecotype R108 was used as the wild type in this study. The source of the three plp alleles (Zhou et al., 2012a) and the root allele (Couzigou et al., 2016) is described in Table S8. Mutant and wild-type seeds were scarified with sandpaper and treated at 4°C for 7 days on filter paper and then transferred into soil. An alfalfa genotype, Regen SY-4D, was used for Agrobacterium tumefaciens-mediated transformation to produce transgenic plants following the protocol described by Fu et al. (2015). All the plants were grown in Metro-Mix 830 soil in the greenhouse with the condition 22°C day/20°C night temperature, 16-h light/8-h dark photoperiod and 70-80% relative humidity.

Isolation of MsPLP and creation of MsPLP modified transgenic alfalfa plants

The alfalfa PLP (MsPLP) gene was cloned using the following primers designed based on MtPLP: MsPLPFLF2: ATTTGCTTTGTTGGAGGAA and MsPLPFLR2: CAAGAGACATAACATAAGTAAACACCTT. To knockdown the expression of MsPLP in alfalfa, 262-bp and 205-bp MsPLP fragments were amplified from alfalfa (genotype Regen SY-4D) cDNA using primers shown in Table S9. Each fragment was independently cloned into the PENTRTM/D-TOPO cloning vector (Invitrogen, Chicago, IL, USA) and transferred into the pANDA35HK vector (Miki & Shimamoto, 2004) by attL × attR recombination reactions (Invitrogen). Sonication-assisted Agrobacterium-mediated transformation and tissue culture were performed for transgenic alfalfa generation (Fu et al., 2015). PCR analysis of the regenerated plants was performed using primers from the GUS linker of the pANDA35HK vector: GUS4F: CATGAAGATGCGGACTTACG and GUS5R: ATCATATGGAATTGGACTTACG and GUS5R: ATCATATGGAATTGGACTTACG. A large-scale field test is needed to evaluate leaf loss and forage quality of the materials using commercial machines under field conditions.

In summary, our study revealed a new function of PLP in regulating AZ development in the model legume M. truncatula and we have successfully applied this knowledge to alfalfa improvement. This study demonstrated that loss of function of PLP resulted in an absence of AZ differentiation. The regulation of abscission by PLP is associated with auxin-related genes and NOOT. Furthermore, the alteration of pulvinus to petiolule-like pulvinus has significantly increased the breakstrength of leaflets and petioles in alfalfa, which offers a new approach to decrease leaf loss during the haymaking process. This study demonstrates how knowledge gained from a model plant can be applied to the genetic improvement of a commercial crop.

Protein alignment, phylogenetic analysis and expression pattern of MtPLP

Predicted PLP amino acid sequences from various plant species were obtained by protein blast in GenBank (http://www.ncbi.nlm.nih.gov/) or Phytozome (https://phytozome.jgi.doe.gov/). All sequences were subsequently aligned using the ClusterW by DNASTAR. A phylogenetic tree was built using the neighbour-joining method by employing the DNASTAR software. The DNA sequence of MtPLP was used for the analysis of expression pattern through BLAST function in Gene Expression Atlas (https://mtgea.noble.org/V2/).
Microscopic examination of leaflet and petiole abscission zone

Leaflet pulvinus region and petiole pulvinus region of M. truncatula mutants and transgenic alfalfa were carefully examined under a dissecting microscope.

In M. truncatula, 25-day-old leaves (mature leaves) represent the stage that prior to leaflet abscission and petiole abscission. Thirty five-day-old leaves (senescing leaves) represent the stage when leaflet and petiole are under the abscission process. The 25-day-old and 35-day-old AZ regions of petiole and leaflet were collected and fixed with 4% paraformaldehyde, 2.5% glutaraldehyde and 0.01% (v/v) Triton X-100 dissolved in PBS (pH 7.4) at 4°C for overnight. All specimens were then dehydrated in a series of ethanol and embedded in LR White resin (London Resin Co. Ltd., Berkshire, UK) using gelatin capsules. The resin was polymerized at 60°C for two days. In order to closely examine the abscission zones from wild type and plp mutant, serial 0.25-µm-micrometre longitudinal sections were cut with a diamond knife with a Leica EM UC7 ultramicrotome (Leica Microsystems GmbH, Vienna, Austria) or RMC MT-X ultramicrotome (Boeckeler Instruments, Inc., Tucson, Arizona, USA). Semi-thin sections (0.25 µm in thickness) placed onto the slide glasses (Superfrost Plus; VWR International, LLC, Radnor, Pennsylvania, USA) were stained with 1% (w/w) Toluidine Blue O (with 1% (w/v) sodium borate) for 5 min and observed under a Nikon Microphot-2 (Nikon Corporation, Tokyo, Japan).

For Zeiss NEON 40 EsB scanning electron microscopy (SEM) examination of leaflet and petiole AZs, leaflets and petioles (31, 35, 39, 43 and 47 days old) were removed at the pulvinus if not yet abscised from the plant in wild type and plp mutant. Samples were fixed, dried and sputter-coated as reported previously (Gou et al., 2019; Zhou et al., 2012a). The fracture planes on petiolule and stem created by removing leaflets and petiole or naturally abscised were observed.

Shaking assay for abscission

M. truncatula plants were staked in a similar manner in the greenhouse. Both mutant and wild-type plants at senescence stage (108-day-old plant) were shaken vigorously for 2 min by hand. The leaves dropped onto ground were collected and weighed.

Breakstrength assay

Leaflet breakstrength is the force in gram equivalents required to remove a leaflet from the petiulome. Petiole breakstrength is the force to remove a whole leaf from the stem. The leaflet and petiole breakstrengths were measured by using the CT3 Texture Analyzer (BROOKFIELD, MA) in tension mode. Samples were clamped at the same position with dual grip assembly (TA-DGF001) and extended at a constant speed of 2.0 mm s⁻¹ until breakage and the breakstrength was recorded. For both wild type and the plp mutants in M. truncatula, we started to mark fully expanded leaves as one-day-old leaves when plants were 48 days old. Afterwards, leaf samples at different ages from 25 to 49 days were collected in two-day intervals for leaflet and petiole breakstrength measurements (eight replicates). For alfalfa materials, we evaluated the terminal, lateral leaflet and petiole breakstrengths from the 3rd to 12th leaflets, which represent leaflets that are normally harvested during alfalfa haymaking process. The breakstrength data were analysed by SPSS software.

β-Glucuronidase (GUS) staining

To determine whether there is a correlation between the PLP expression pattern and leaflet and petiole abscission, we examined transgenic plants that express β-glucuronidase (GUS) under the control of PLP promoter. GUS expression was analysed during the time course of abscission in three stages as shown in Table S2: stage I, prior to organ separation; stage II, during abscission; and stage III, after abscission when the remaining cells form a protective scar tissue. At stage II, the organs were separated manually.

RNA extraction, qRT-PCR and microarray analysis

Pulvinus regions (abscission zones included) of 25-day-old leaflets from wild type and three plp alleles of M. truncatula (85-day-old plants) and of wild type and 14 transgenic alfalfa lines were collected for RNA isolation, cDNA reverse transcription and qRT-PCR as described previously (Gou et al., 2019; Zhou et al., 2012a). Primers for quantifying the expression levels of auxin-related genes and the NOOT gene are listed in Table S10.

For microarray analysis, 10 µg of purified RNA was extracted from the pulvinus region of 25-day-old leaves of Medicago truncatula. RNA purification, probe labelling, hybridization, scanning and microarray data analysis were performed following the manufacturer’s instructions (Affymetrix; http://www.affymetrix.com). Functional enrichments were visualized with MAPMAN (Thimm et al., 2004).

Forage analysis of transgenic alfalfa lines

Three transgenic alfalfa lines S1, S2, S3 and control wild-type alfalfa plants were grown in the greenhouse and propagated by cuttings. At early blooming stage, plants were harvested and fresh biomass yield was measured immediately. Forage quality was analysed using near-infrared reflectance spectroscopy (NIRS) as previously described (Gou et al., 2018).

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Conflicts of interest

The authors declare no conflict of interest.

Author contribution

ZYW, YZ and JD designed and planned the experiments; JD, SL, MC, CZ, JN, JK, ZW, MB, JS and TZ performed research; JD, YT and LS analysed the data; and JD, YZ and ZYW wrote the manuscript.
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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

**Figure S1** Petiole-like pulvinus (plp) mutant of *M. truncatula* shows developmental defects in leaflet and petiole pulvini.

**Figure S2** Leaf dropping after shaking assay in *plp* mutant of *M. truncatula*. 

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**Figure S3** Expression pattern of MtPLP in wild type, generated from the *M. truncatula* Gene Expression Atlas.

**Figure S4** Differential expression of genes in pulvinus region of 25-day-old leaves of wild type and *plp* mutant of *M. truncatula*.

**Figure S5** Validation of microarray data by qPCR of auxin-related genes in AZ region of *M. truncatula*.

**Figure S6** Pie chart of leaflet abscission-related transcription factors (TFs) differentially expressed in *M. truncatula* during leaflet abscission.

**Figure S7** Venn diagram analysis of the overlap of abscission-specific TFs in soybean and TFs that are differentially expressed in *plp* mutant of *M. truncatula*.

**Figure S8** Loss of leaflet and petiole abscission phenotype in *noot* mutant and the expression of NOOT in wild type and *plp*.

**Figure S9** Sequence comparison of MsPLP (*M. sativa* SY4D) with PLP (*M. truncatula* R108).

**Figure S10** Amino acid alignment of MsPLP (*M. sativa* SY4D) with PLP (*M. truncatula* R108) and their orthologs from Arabidopsis thaliana (At), Glycine max (Gm), Lotus japonicas (Lj), Pisum sativum (Ps) and Trifolium medium (Tm).

**Figure S11** Phylogenetic analysis of PLP-related orthologs in different plant species.

**Figure S12** Construction of MsPLP-RNAi binary vectors for alfalfa transformation.

**Figure S13** Generation of MsPLP-RNAi transgenic alfalfa plants.

**Figure S14** Molecular and phenotypic characterization of alfalfa MsPLP-RNAi transgenic lines.

**Figure S15** Frequency of detachment occurs at marked positions in both wild type and transgenic alfalfa lines.

**Figure S16** Evaluation of nutritive quality of transgenic alfalfa lines.

**Figure S17** A proposed model of PLP in regulating leaflet and petiole abscission.

**Table S1** Characterization of leaflet and petiole abscission in wild type and *plp* mutant of *M. truncatula*.

**Table S2** Abscission stages of leaflet and petiole according to leaf ages.

**Table S3** List of genes that are up-regulated in *plp* mutant.

**Table S4** List of genes that are down-regulated in *plp* mutant.

**Table S5** List of auxin-related genes that were down-regulated in LAZ for qPCR validation.

**Table S6** List of transcription factors in *M. truncatula* that overlapped with abscission-specific TFs in soybean.

**Table S7** List of homologous genes of already published genes related to AZ development in *M. truncatula*.

**Table S8** List of *M. truncatula* lines used in this study.

**Table S9** List of primers used for MsPLP-RNAi vector construction.

**Table S10** Primers for qPCR analysis of auxin-related genes and the NOOT gene.
Figure S1 PetioluleA-like pulvinus (plp) mutant of *M. truncatula* shows developmental defects in leaflet and petiole pulvini. (a, b) Leaf of wild type and *plp* mutant. (c, d) Close-up view of leaflet base of wild type and *plp* mutant. (e, f) Petiole of wild type and *plp* mutant. (g, h) Close-up view of petiole base of wild type and *plp* mutant. L, Leaflet; PE, Petiolule; PLP, Petiolule-like pulvinus; PU, Pulvinus; P, Petiole; S, Stem. Scale bars: (a, b, e, f) 1 cm; (c, d, g, h) 1 mm.
Figure S2 Leaf dropping after shaking assay in *plp* mutant of *M. truncatula*. Whole plant phenotype of the *plp* mutant compared to wild type after shaking assay of senescing plants. (a) Leaf dropping of wild type and *plp* plants after shaking; (b-e) Close view of wild type and *plp* plants after shaking. (f-g) Close view of the leaves dropped onto the ground after shaking. (h) Weight of dropped leaves after shaking assay of wild type and *plp* mutants.
Figure S3 Expression pattern of MtPLP in wild type, generated from the *M. truncatula* Gene Expression Atlas. The data for MtPLP (probe set Mtr.17172.1.S1_at) are accessible at [https://mtgea.noble.org/v3/probeset.php?id=Mtr.17172.1.S1_at&print=true](https://mtgea.noble.org/v3/probeset.php?id=Mtr.17172.1.S1_at&print=true).
Figure S4 Differential expression of genes in pulvinus region of 25-day-old leaves of wild type and plp mutant of *M. truncatula*. (a) Transcriptome regulation of *PLP*. MapMan classification of differentially expressed genes in plp mutant lines compared with those in wild type. (b) MapMan classification of differentially expressed genes in the hormone metabolism pathway. (c) MapMan general overview of differentially expressed genes related to the cell wall pathway. Each square represents a gene. Red and blue indicate higher and lower expression in plp mutant compared to wild type. The figure is shown in log2-transformed ratios of plp mutant divided by wild-type.
Figure S5 Validation of microarray data by qPCR of auxin-related genes in AZ region of *M. truncatula*.
Putative abscission related TFs in leaf abscission in *M. truncatula*

**Figure S6** Pie chart of putative abscission related transcription factors (TFs) differentially expressed in *M. truncatula* during leaflet abscission. The chart displays gene family classification of 152 abscission related TFs that were up-regulated or down-regulated in the microarray assay.
Figure S7 Venn diagram analysis of the overlap of abscission-specific TFs in soybean and TFs that are differentially expressed in plp mutant of *M. truncatula*. 

| List names                  | number of elements | number of unique elements |
|-----------------------------|--------------------|--------------------------|
| Soybean TFs                 | 174                | 134                      |
| *Medicago truncatula* TFs   | 152                | 152                      |
| Number of overlapped elements | 26                 |                          |
**Figure S8** Loss of leaflet and petiole abscission phenotype in *noot* mutant and the expression of *NOOT* in wild type and *plp*. (a,b) Loss of leaflet abscission in *noot*. (c,d) Loss of petiole abscission in *noot*. (e) Relative expression of *NOOT* in wild type and *plp* in the 25-day-old leaflet abscission zone region. PE, petiole; S, stem; P, petiole. Scale bars: (a-d) 1 mm.
Figure S9 Sequence comparison of MsPLP (M. sativa SY4D) with PLP (M. truncatula R108). MtPLP and MsPLP have 97.4% identity.
Figure S10 Amino acid alignment of MsPLP (M. sativa SY4D) with PLP (M. truncatula R108) and their orthologs from Arabidopsis thaliana (At), Glycine max (Gm), Lotus japonicas (Lj), Pisum sativum (Ps) and Trifolium medium (Tm). Sequences were aligned using ClustalW with DNASTAR. Arrows indicate lateral organ boundaries domain. MtPLP and MsPLP have 99% identity.
Figure S11 Phylogenetic analysis of PLP related orthologs in different plant species. PLP orthologs in various species include: *Theobroma cacao* (Tc), *Manihot esculenta* (Me), *Ricinus communis* (Rc), *Salix purpurea* (Sp), *Populus trichocarpa* (Pt), *Eucalyptus grandis* (Eg), *Carica papaya* ( Cp), *Vigna unguiculata* (Vu), *Vigna angularis* (Va), *Phaseolus vulgaris* (Pv), *Cajanus cajan* (Cc), *Spatholobus suberectus* (Ss), *Glycine max* (Gm), *Trifolium pratense* (Tp), *Trifolium medium* (Tm), *Pisum sativum* (Ps), *Medicago sativa* (Ms), *Lotus japonicas* (Lj), *Cucumis sativus* (Cs), *Zea mays* (Zm), *Oryza sativa* (Os), *Brassica rapa* (Br), *Arabidopsis thaliana* (At), *Citrus sinensis* (Csi), *Citrus clementine* (Ccl), *Prunus persica* (Pp), *Fragaria vesca* (Fv), *Malus domestica* (Md) and *Amaranthus hypochondriacus* (Ah). The analysis indicated that MtPLP is close to MsPLP. It is also close to PsAPU, TmELP1 and TpLOB.
**Alfalfa CDS**

1. **pANDA35HK-MsPLP-RNAi-1**

   ![Diagram of pANDA35HK-MsPLP-RNAi-1]

   **MsPLPF1**: CAACGGATCTTCCACCAGAAGACCTCA  
   **MsPLPR1**: CGAGAATTCTATTCAATTGTAAGCAAGCGA

2. **pANDA35HK-MsPLP-RNAi-2**

   ![Diagram of pANDA35HK-MsPLP-RNAi-2]

   **MsPLPF2**: CAACGGATCTTCCCTTACACCTCTCTCTCTCA  
   **MsPLPR2**: ACAGAAATTCTCCCTTACATCTCTCTCTTACA

**Figure S12** Construction of *MsPLP*-RNAi binary vectors for alfalfa transformation. Two *PLP* target sequences RNAi-1 and RNAi-2 of the *MsPLP* gene were amplified using two pairs of primers *MsPLPF1/R1* and *MsPLPF2/R2* and then independently cloned in both sense and anti-sense orientations into the pANDA35HK binary vector. LB, left border; RB, right border; NptII, kanamycin gene selectable marker; 35SPro, CaMV35S promoter; HYG, hygromycin gene selectable marker; nosT, nopaline synthase gene terminator.
Figure S13 Generation of *MsPLP*-RNAi transgenic alfalfa plants. (a) Leaf explants from alfalfa were cultured on SH3a medium for co-cultivation with *Agrobacteria* carrying the gene constructs. (b) Explants were cultured on selection medium (SH3a) containing hygromycin. (c) Hygromycin resistant calluses on regeneration medium (MSBK). (d) Shoots formed from embryonic calluses on shooting medium (SH9a). (e) Rooted alfalfa plants on rooting medium (MSO). Only one plant was selected from each callus, representing an independent line. (f) PCR analysis of regenerated transgenic alfalfa plants. The size of the fragment of GUS linker is 636bp. Marker, 100bp.
Figure S14 Molecular and phenotypic characterization of alfalfa *MsPLP*-RNAi transgenic lines. (a) Quantitative RT-PCR analysis of *MsPLP* gene expression in transgenic lines. All values were normalized using the wild-type control. Error bars indicate SE (n=3). (b) Phenotype of wild type and three transgenic alfalfa lines. (c) Plant height of wild type and transgenic alfalfa plants. Error bar indicates SD (n=3). (d) Fresh weight of wild type and transgenic alfalfa plants. Error bars indicate SD (n=12).
Figure S15 Frequency of detachment occurs at marked positions in both wild type and transgenic alfalfa lines. (a) In wild type, both terminal leaflet and lateral leaflet were detached at pulvinus region (100%), and petiole was detached at pulvinus region (100%). (b) In transgenic line, the detachment of terminal leaflet occurred at three positions, D1: leaflet (8%), D2: petiolule-like pulvinus (22%) and D3: petiolule (70%); the detachment of lateral leaflet occurred at three positions, D1: leaflet (2%), D2: petiolule-like pulvinus (57%) and D3: at the base of petiolule-like pulvinus (41%); the detachment of petiole occurred at two positions, D1: petiole-like pulvinus (95%) and D2: petiole (5%). The measurement of both leaflet and petiole detachment used 30 replicates.
Figure S16 Evaluation of nutritive quality of transgenic alfalfa lines. (a) Nutritive quality of whole plant in wild type and transgenic lines (S1, S2, S3). (b) Nutritive quality of stem in wild type and transgenic lines. (c) Nutritive quality of leaf in wild type and transgenic lines. ADF: acid detergent fiber, NDF: neutral detergent fiber, CP: crude protein, IVTDMD: In vitro true dry matter digestibility, TDN: total digestible nutrients, RFV: relative feed value. Error bars indicate SD (n = 5) (P < 0.05).
**Figure S17** A proposed model of *PLP* in regulating leaflet and petiole abscission.