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Palmate-like pentafoliata1 encodes a novel Cys(2)His(2) zinc finger transcription factor essential for compound leaf morphogenesis in *Medicago truncatula*

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As the primary site for photosynthetic carbon fixation and the interface between plants and the environment, plant leaves play a key role in plant growth, biomass production and survival, and global carbon and oxygen cycles. Leaves can be simple with a single blade or compound with multiple units of blades known as leaflets. In a palmate-type compound leaf, leaflets are clustered at the tip of the leaf. In a pinnate-type compound leaf, on the other hand, leaflets are placed on a rachis in distance from each other. Higher orders of complexities such as bipinnate compound leaves of the "sensitive" plant, *Mimosa pudica*, also occur in nature. However, how different leaf morphologies are determined is still poorly understood. *Medicago truncatula* is a model legume closely related to alfalfa and soybean with trifoliate compound leaves. Recently, we have shown that *Palmate-like Pentafoliata1* (*PALM1*) encodes a putative Cys(2)His(2) zinc finger transcription factor essential for compound leaf morphogenesis in *M. truncatula*. Here, we present our phylogenetic relationship analysis of *PALM1* homologs from different species and demonstrate that *PALM1* has transcriptional activity in the transactivation assay in yeast.

Key words: Compound leaves, Medicago truncatula, PALM1, LFY/UNI/SGL1, transcription factor

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Leaf development is divided into three continuous phases, organ initiation, primary morphogenesis and secondary morphogenesis (or histogenesis).[1-4] Initiation of leaf primordia occurs along the periphery of the shoot apical meristem (SAM), a pluripotent structure capable of self renewable. Downregulation of the class I Knotted-like homeobox transcription factors (KNOXIs) at sites of incipient leaf primordia (P_0, P for Plastochron) is essential for the initiation of leaf primordia.[5-7] KNOXI proteins remain downregulated in simple leaf primordia. In contrast, they are reactivated in leaf primordia in most compound-leafed eudicot species studied.[8-12] Thus, development of a compound leaf requires a transient phase of indeterminacy along the margin of the leaf primordium, or called marginal blastozones.[13]

Legume (Fabaceae) represents the third largest family of flowering plants with significant economic importance.[14] The diverse array of leaf forms found in legume species presents legume as an ideal system for genetic and evolutionary studies of plant forms.[15] In garden pea (*Pisum sativum*) and *Medicago truncatula*, both belonging to the inverted repeat lacking clade (IRLC) of legume, the role of KNOXI proteins in compound leaf development is replaced by the FLORICAULA (FLO)/LEAFY (LFY) orthologs UNIFOLIATA (UNI) and SINGLE LEAFLET1 (SGL1), respectively.[16-20] This is mainly because (1) KNOXI proteins are not reactivated in compound leaf primordia in these plants and (2) loss-of-function mutants of *UNI* and *SGL1* develop simplified or simple leaves. Interestingly, leaf developmental programs remain responsive to ectopically expressed KNOXI proteins in these species.[17] Both *UNI* and *SGL1* are similarly expressed in young leaf primordia.[16,20-22]

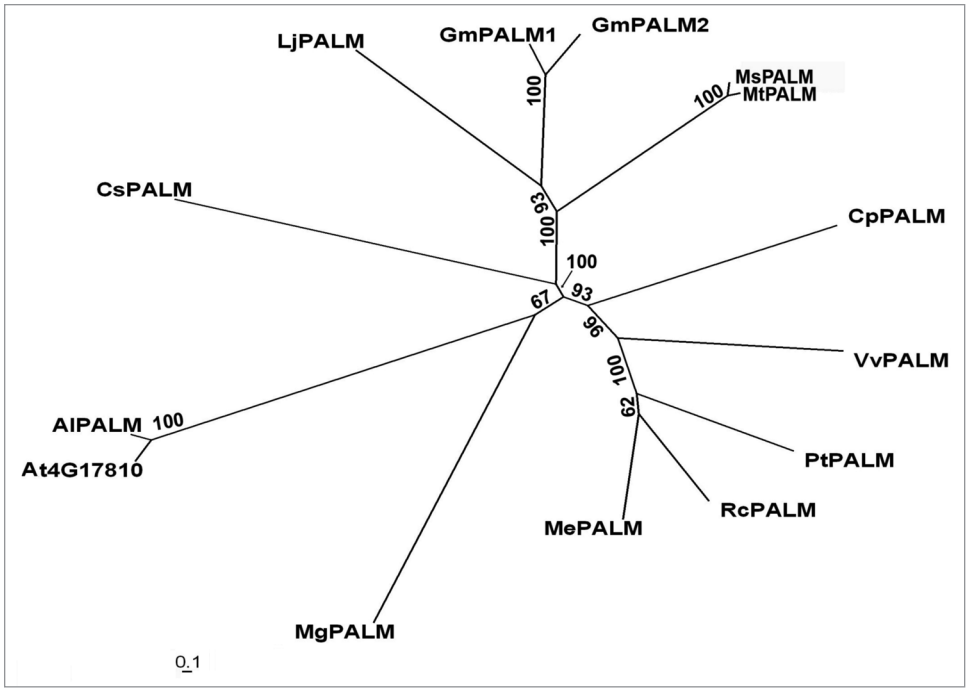

Figure 1. Phylogenetic relationships of *PALM1* homologs. A neighbor joining phylogenetic tree was reconstructed using PAUP4.0 with 1,000 bootstrap repeats. *MtPALM1, Palmate-like pentafoliata1* from *Medicago truncatula*; *Ms, M. sativa; Gm, Glycine max; Lj, Lotus japonicus; At, Arabidopsis thaliana; Al, A. lyrata; Vv, Vitis vinifera; Cs, Cucumis sativus; Me, Manihot esculenta; Mg, Mimulus guttatus; Pt, Populus trichocarpa; Rc, Ricinus comunis; and Cp, Carica papaya.*

The expression was greatly reduced in older leaf primordia, consistent with their role in promoting a transient phase of indeterminacy required for leaflet initiation in these species. Although both *UNI* and *SGL1* play a similar role in compound leaf development in pea and *M. truncatula*, leaflet primordia develop acropetally in pea with pinnate compound leaves and tendrils[19] but basipetally in *M. truncatula* with ternate leaves.[16] This suggests that differences in compound leaf development in closely related species may explain some differences in compound leaf phenotypes of loss-of-function *uni* mutants in pea and *sgl1* mutants in *M. truncatula*.

In *M. truncatula palmate-like pentafoliata1* (*palm1*) mutants, each compound leaf is consisted of five leaflets clustered at the tip with two distally oriented lateral leaflets subtended by rachis.[23] This is in contrast with the morphology of the WT compound leaf with three leaflets at the tip and only the terminal leaflet is subtended by a rachis.[16,23] In *palm1* mutants, petiole is slightly longer and the central rachis is slightly shorter than the WT counterparts, indicating that *PALM1* also plays a role in the proximal-distal axis development of compound leaves. The proliferation of lateral leaflets in loss-of-function *palm1* mutants requires the activity of *SGL1* because (1) the expression level of *SGL1* is upregulated by 2.7-folds in *palm1* mutants and (2) *palm1 sgl1* double mutants exhibit the simple leaf morphology similarly as the *sgl1* mutants.

The *PALM1* gene encodes a putative transcription factor with a single Cys(2) His(2) zinc finger DNA binding domain at the N-terminus and an EAR transcription repressor domain at the C-terminus.[23] Cys(2)His(2) zinc finger transcription factors belong to a large divergent family of transcription factors in eukaryotic organisms. Using synteny analyses in plants with available genome sequences, we uncovered candidate *PALM1* orthologs from closely related legume species such as alfalfa (*M. sativa*), soybean (*Glycine max*) and *Lotus japonicus*, and from remotely related species such as *Arabidopsis thaliana*, *A. lyrata*, *Vitis vinifera*, *Cucumis sativus*, *Manihot esculenta*, *Mimulus guttatus*, *Populus trichocarpa, Carica papaya* and *Ricinus comunis* (**Fig. 1**). A duplication event results in two closely related *PALM1* orthologs in the soybean genome (**Fig. 1**). The observation that *PALM1* homologous sequences exist in lower land plants (our unpublished results) and in species with simple leaves (**Fig. 1**) suggests a diverged function or recruitment of the Cys(2) His(2) zinc finger protein in dissected leaf morphogenesis in some compound-leafed lineages.

PALM1 is localized to nucleus in onion epidermis cells, consistent with its role as a putative transcription factor.[23] To provide evidence that *PALM1* encodes a transcription factor, we carried out a transactivation assay in yeast. **Figure 2** shows that the full-length PALM1 protein is able to activate transcription of reporter genes in the yeast system, supporting its role as a transcription factor. To delineate domains required for the transactivation activity, we tested several truncated fragments of the *PALM1* gene in yeast. The experiment showed that the N-terminal Cys(2) His(2) zinc finger DNA binding domain is essential for the transactivation activity in yeast, whereas the C-terminal EAR domain is not.

Our study identifies PALM1 as a key regulator of compound leaf development in *M. truncatula*, an IRLC legume. Our studies show that PALM1 binds specifically to the promoter sequence and regulates the spatial-temporal expression

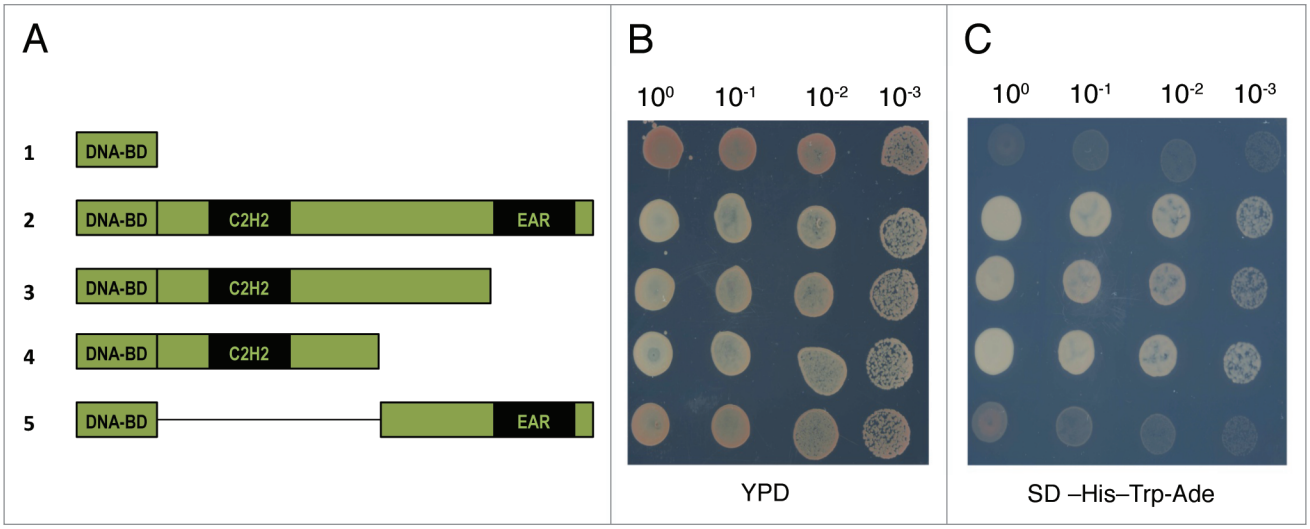

Figure 2. Yeast transactivation assay. The full-length and various truncated fragments of PALM1 were cloned into the pGBKT7 DNA-BD vector (Clonetech). The resulting plasmids were transformed into the yeast (*Saccharomyces cerevisiae*) Y2HGold strain (Clonetech). (A) pGBKT7 DNA-BD PALM1 fusion constructs. (1) BD, empty vector; (2) BD-PALM1FL, BD-full-length PALM1 fusion; (3) BDPALM1CΔ1, BD-PALM1 C-terminal deletion 1 fusion; (4) BD-PALM1CΔ2, BD-PALM1 C-terminal deletion 2 fusion; (5) BD-PALM1NΔ, BD-PALM1 N-terminal deletion fusion. (B) Yeast cultures at $O.D._{600}$ = 0.5 were diluted as specified and grown at 30°C on YPD medium for 48 hrs. (C) Yeast cultures at $O.D._{600}$ = 0.5 were diluted as specified and grown at 30°C on SD (synthetically defined) medium without tryptophan, histidine and adenine for 48 hrs. Table 1 lists primers used.

of *SGL1* in developing leaf primordia.[23] Together, they define the trifoliate morphology of WT leaves. In future, identification of the role of PALM1 orthologs in non-IRLC legumes and elucidating its mode of regulation in compound leaf development promise to provide new insights in the evolution of complex leaf forms in legume.

GenBank Database

The sequences reported in this addendum have been deposited in the GenBank database [accession nos. HM038482 (PALM1); HM038483 (MsPALM1); HM038484 (LjPALM1); HM038485 (GmPALM1); HM038486 (GmPALM2); HM453333 (VvPALM1); HM453334 (AlPALM1); HM453335 (CsPALM1); HM453336 (MePALM1); HM453337 (MgPALM1); HM453338 (PtPALM1); HM453339 (RcPALM1); and HM453340 (CpPALM1)].

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Table 1. Primers used in the study

PALM1_F1	aaaGAATTCATGGCTACAGATATTGGCC
PALM1_R1	aaaGGATCCTCAAGTTGGTGTTGGCTTGTTCCC
PALM1_R2	aaaGGATCCTCATTGCTGGTCCACTTTGC
PALM1_R3	aaaGGATCCTCACGGTGGTTGGGTTTGATGG
PALM1_F2	aaaGAATTCTCTCATCCTTCATCACC

Nucleotides underlined are introduced restriction sites.

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