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Control of leaf blade outgrowth and floral organ development by LEUNIG, ANGUSTIFOLIA3 and WOX transcriptional regulators

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Summary

- Plant lateral organ development is a complex process involving both transcriptional activation and repression mechanisms. The WOX transcriptional repressor WOX1/STF, the LEUNIG (LUG) transcriptional corepressor and the ANGUSTIFOLIA3 (AN3) transcriptional coactivator play important roles in leaf blade outgrowth and flower development, but how these factors coordinate their activities remains unclear. Here we report physical and genetic interactions among these key regulators of leaf and flower development.
- We developed a novel *in planta* transcriptional activation/repression assay and suggest that LUG could function as a transcriptional coactivator during leaf blade development.
- MtLUG physically interacts with MtAN3, and this interaction appears to be required for leaf and flower development. A single amino acid substitution at position 61 in the SNH domain of MtAN3 protein abolishes its interaction with MtLUG, and its transactivation activity and biological function. Mutations in *lug* and *an3* enhanced each other's mutant phenotypes. Both the *lug* and *an3* mutations enhanced the *wox1 prs* leaf and flower phenotypes in *Arabidopsis*.
- Our findings together suggest that transcriptional repression and activation mediated by the WOX, LUG and AN3 regulators function in concert to promote leaf and flower development, uncovering novel mechanistic insights into the complex regulation of plant lateral organ development.

Key words: ANGUSTIFOLIA3 (AN3), *Arabidopsis*, leaf blade, LEUNIG (LUG), *Medicago truncatula*, STENOFOLIA (STF), transcription, WUSCHEL-related homeobox (WOX).

INTRODUCTION

During organogenesis and development, transcriptional activation and repression are crucial mechanisms to achieve the coordination of cell division, differentiation and expansion in both plants and animals. In plants, the Gro/Tup1 family of corepressors are key regulators of shoot apical meristem (SAM) maintenance (Kieffer *et al.*, 2006; Stahle *et al.*, 2009), leaf and flower development (Liu & Meyerowitz, 1995; Cnops *et al.*, 2004; Krogan *et al.*, 2012; Tao *et al.*, 2013; Zhang *et al.*, 2014), embryogenesis (Long *et al.*, 2006; Sitaraman *et al.*, 2008) and hormone signaling (Szemenyei *et al.*, 2008; Pauwels *et al.*, 2010; Oh *et al.*, 2014; Flores-Sandoval *et al.*, 2015). Based on their unique domains and additional features, the plant Gro/Tup1 family can be grouped into two subfamilies: the TOPLESS (TPL) subfamily and the LEUNIG (LUG) subfamily. The TPL family corepressors have a conserved CTLH domain which is implicated in protein-protein interactions, and an additional WD40 repeats in the middle. The LUG family corepressors have additional conserved amino acids together with the LiSH motif to form a protein-protein interaction domain, designated as LUFS domain (Sridhar *et al.*, 2004). In the middle part, LUG family corepressors have several "Q" rich motifs, which are together named as QR domain (Liu & Karmarkar, 2008). Both TPL and LUG family corepressors form complexes with RPD3-type HDACs and Mediator components, suggesting conserved mechanisms in transcriptional repression (Long *et al.*, 2006; Gonzalez *et al.*, 2007; Krogan *et al.*, 2012). In *Arabidopsis*, around 130 members from at least 17 diverse families of transcription factors are found to interact with TPL proteins in yeast two-hybrid assays. Direct recruitment of TPL has been confirmed for several DNA binding transcription factors (Causier *et al.*, 2012) including Auxin Response Factors (ARFs), STENOFOLIA (STF) and APETALA2 (AP2) in embryo, leaf and flower development, respectively (Szemenyei *et al.*, 2008; Krogan *et al.*, 2012; Zhang *et al.*, 2014). Transcription factors, however, may recruit LUG for repressive activity through

adaptors, SEUSS (SEU) and SEU-LIKE (SLK) proteins (Sridhar *et al.*, 2004). These coregulators also do not directly bind to DNA but physically interact with LUG via the LUFS domain to form a LUG-SEU corepressor complex (Sridhar *et al.*, 2004; Bui *et al.*, 2011).

In addition to the transcriptional corepressors, transcriptional coactivators are also conserved in animals and plants. ANGUSTIFOLIA3 (AN3)/GRF-INTERACTING FACTOR1 (GIF1) is a member of the AN3/GIF transcriptional coactivator family in *Arabidopsis* (Kim & Kende, 2004; Horiguchi *et al.*, 2005). The N-terminal region of GIF coactivators shows over 50% identity to the SNH domain of human SYNOVIAL TRANSLOCATION (SYT) coactivator (Kim & Kende, 2004). Both SYT and AN3 coactivators interact with the SWITCH/SUCROSE NONFERMENTING (SWI/SNF) chromatin remodeling complexes to regulate transcription (Nagai *et al.*, 2001; Vercruyssen *et al.*, 2014). AN3 is recruited by DNA-binding factors, such as Growth Regulator Factors (GRFs) to activate target gene expression (Kim & Kende, 2004). In *Arabidopsis*, GIFs are found to redundantly regulate leaf blade development through regulation of cell proliferation (Lee *et al.*, 2009), and act in a non-cell autonomous manner (Kawade *et al.*, 2013). Genetic relationship between Gro/Tup1 corepressors and GIF coactivators has not been established.

The plant specific WUSCHEL-RELATED HOMEOBOX (WOX) transcription factor *STENOFOLIA* (*STF*) and its homolog *BLADELESS*/*LAMINALESS* (*LAM1)* regulate blade outgrowth in *M. truncatula* and *Nicotiana sylvestris*, respectively, by promoting cell proliferation (Tadege *et al.,* 2011a,b;Tadege, 2016). Both the *stf* and *lam1* mutants are characterized by narrow leaves and severely affected flower phenotypes; the strongest leaf phenotype being displayed by *lam1* in which nearly all of the blade tissue is absent (McHale, 1992; Tadege et al., 2011a). In *Arabidopsis*, *WOX1* (homologue of *STF*) acts redundantly with *WOX3/PRESSED FLOWER* (*PRS*) to control blade outgrowth (Vandenbussche *et al.*, 2009; Nakata *et al.*, 2012). The regulation of blade outgrowth is conserved across these three species since the *STF* coding region under the control of the *STF* promoter can rescue not only *stf*, but also *lam1* and *wox1 prs* mutants in *M. truncatula* (*Mt*), *N. sylvestris* (*Ns*) and *A. thaliana* (*At*), respectively (Zhang et al., 2014; Zhang and Tadege, 2015). STF mainly acts as a transcriptional repressor in both leaf and

flower development (Lin, *et al.*, 2013a,b) and recruits MtTPL for its repressive activity. In this study, we report that MtLUG, unlike MtTPL, exhibits a transcriptional activation activity in an *in planta* assay and enhances the *stf* and *lam1* mutant phenotypes when fused to the C-terminal end deleted form of STF (STFdel). We uncovered that LUG physically interacts with AN3 and we show that *LUG*, *AN3*, *WOX1* and *PRS* genetically interact with each other in *Arabidopsis*, indicating complex regulatory mechanisms involving transcriptional repressors, corepressors, and coactivators during leaf and flower morphogenesis and elaboration.

Materials and Methods

Plant Materials, Genetic Crossing and Plant Transformation

Plant Materials: *Medicago truncatula* ecotype R108 and mutant *stf* (Tadege et al., 2011a), *N. sylvestris* and mutant *lam1*(McHale, 1992), *Arabidopsis* Col and mutant *wox1 prs* (Vandenbussche et al., 2009), *lug-444* (Stahle *et al.*, 2009) and *an3/gif1* (SALK_150407) mutant (Lee *et al.*, 2009) were used in this study. To make *lug an3* double mutant, the pollen from *an3* mutant was crossed to *lug* +/- mutant, and the F1 double heterozygous plants were genotyped by primers listed in Table S1. Seeds from double heterozygous plants were harvested and the double mutants were identified in this generation. Seeds harvested from *lug* +/- *an3*-/- mutant were grown on plates, and the phenotype of double mutants were further confirmed by comparison with *an3* mutant. To make *wox1 prs an3* triple mutant, *wox1 prs* double mutant was pollinated by *an3* pollen. Seeds harvested from F1 generation were grown on MS medium and the phenotypes of *wox1 prs* were identified and these plants were further genotyped by *an3* primers for heterozygous or homozygous *an3* mutation. Seeds harvested from *wox1 prs an3*+/- plants were further grown and the *wox1 prs an3* triple mutant phenotypes were re-confirmed. To make *wox1 prs lug* triple mutant, pollen from *lug*+/- mutant was transferred to the *wox1 prs* double mutant, and seeds were harvested from F1 plants genotyped as *lug* mutation positive. Seeds from *wox1*+/- *prs*+/- *lug*+/- plants were grown on MS medium and the *wox1 prs* or *wox1 prs lug*+/- plants were identified by genotyping. The fertility of *wox1 prs lug*+/- is greatly reduced, and the triple mutant was

identified from its progeny by both phenotyping (cotyledon vein) and genotyping. Transformation of *Medicago*, tobacco and *Arabidopsis* was performed as described previously (Zhang *et al.*, 2014).

Plasmid Construction

The gateway compatible plasmid pSTF-pMDC32 which contains a 2663-bp upstream promoter of *STF* was used to make all constructs for *lam1* complementation assays. Fusion of MtLUG, MtAN3 or VP16 to the STFdel mutant was constructed as previously described for STFdel-MtTPL fusion (Zhang *et al.,* 2014). Briefly, STFdel (1-900 bp) was cloned to the *Sal* I and *Eco*R I sites of pRS300 to make pRS300-STFdel construct. *MtLUG*, *MtAN3* or *VP16* were cloned in-frame to the *Eco*R I and *Bam*H I or *Not* I sites of pRS300-STFdel construct. The gene fusions were amplified by *STF* forward primer and *MtLUG*, *MtAN3* or *VP16* reverse primers listed in Table S1, and cloned to pSTF-pMDC32 by a using gateway system. *SRDX* fusions were constructed by using sense *STF* primer containing *SRDX* sequence. The mutations of *MtAN3* or *MtLUG* were introduced using appropriate mutagenic primers listed in Table S1.

Yeast Assays

Yeast two-hybrid (Y2H) assays were performed as previously described (Zhang *et al.,* 2014). Yeast transactivation assay was performed as previously described (Kim & Kende, 2004).

BiFC Analysis and Confocal Microscopy

Bimolecular fluorescence complementation (BiFC) assays were conducted according to Lu et al. (Lu *et al.*, 2010). Briefly, *STF, MtAN3, MtAN3 N61A* and *MtSWP73B* were cloned to pEARLEYGATE201-YN, while *MtTPL, MtLUG, MtLUGS164F, MtAN3* were cloned to pEARLEYGATE202-YC by LR reaction. Constructs were introduced into *Agrobacterium tumefaciens* strain GV2260. Pairs of combinations were co-infiltrated into 4-wk old *N. benthamiana* leaves. The YFP signal was observed by a Leica TCS SP2 AOBS confocal laser scanning microscope (Leica Microsystems). Results were verified in at least three repeats.

Leaf Growth Analysis

Growth analysis was performed on the first leaf pair of mutants and wild type. For this purpose, leaves were harvested at 14 days after stratification and were fixed and cleared

with 70% ethanol for 24 hrs, and subsequently stored and mounted in 100% lactic acid for subsequent microscopic observations. Whole leaf dark field images were obtained with a binocular microscope (Olympus SZX16) fitted with a digital camera (Olympus DP72). Leaf surface area was measured using ImageJ v 1.48 (http://rsbweb.nih.gov/ij/) whereas abaxial epidermal cells of 3-5 leaves were imaged using DIC optics on a Nikon TE300 at 20x and 40x magnification. The outlines of the cells were hand drawn on an LCD tablet (Microsoft surface Pro) running ImageJ. Morphometric analysis of the drawn cells was performed with automated image analysis software that measures total area of the drawn cells and counts the number of pavement and guard cells (Andriankaja *et al.*, 2012). From these data we calculated the average cell area and estimated the number of cells per leaf by dividing leaf blade area by cell area.

Transient Expression Assay

A GAL4-reporter based transient expression system was utilized for plant cell trans-activation assay. *MtAN3* and its mutation forms were first cloned to pGBKT7 plasmid and the BD-MtAN3/mutant fusions were amplified by BD-F and MtAN3-R primers. These fusions were cloned into p2GW7 vector using gateway system and serve as effectors. A GAL4-LUC plasmid as described was used as reporter while pRLC plasmid was used as internal control. For the *Arabidopsis* protoplast isolation, transfection and dual luciferase analysis were performed as previously described (Zhang *et al.*, 2014).

Co-Immunoprecipitation (co-IP)

Nicotiana benthamiana leaves co-transfected with agrobacteria harboring constructs pGWB521-MtLUG and pEarley104-MtAN3 or pEarley104-MtAN3 N61A, were harvested after 3 days. Total protein was extracted in 1 mL protein lysis buffer (50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 150 mM NaCl, 10% glycerol, 25 mM β-glycerophosphate, 10 mM NaF, 0.1% Tween20 and protease inhibitor cocktail), and before incubation with antibodies, 10 µL of protein extract was used as an input. 500 µg protein was added to 1 mL lysis buffer with 2 µL GFP antibody (Abcam, AB290), incubated at 4℃ for 2-4 hours with rotator, 20 µL protein A-agarose beads was subsequently added and incubated for 2 hours at 4℃. After incubation, the beads were washed at least 2 times with ice-cold lysis buffer containing 200 mM NaCl, and 2 times with lysis buffer containing 150 mM NaCl to remove impurities. The specifically bound

proteins were eluted by boiling the beads in 40 μL 1×SDS loading buffer and subjected to 12% SDS–PAGE, followed by Westernblot. MtAN3-GFP or MtAN3(N61A)-GFP and MtLUG-MYC were detected with anti-GFP and anti-MYC (Invitrogen, MA1-980) antibodies, respectively.

Gene Expression Analysis Assays

For quantitative RT-PCR, total RNAs from *Arabidopsis* inflorescences of WT and mutants (include flower buds before stage 13) were extracted using TRIzol reagent (Invitrogen) and pretreated by DNase using TURBO DNA-free™ Kit (Ambion). One microgram of total RNA was reverse transcribed using TaqMan reverse transcription reagent (Invitrogen) in a reaction volume of 50 μL. Quantitative PCR was performed using SYBR Green real-time PCR Master Mix (Bio-Rad) with three biological repeats. The relative expression level of each gene was calculated using the ddCt method, and the *Arabidopsis PP2A* gene was used as an internal control.

Sequence Alignment and Phylogenic analysis

Alignment of the amino acid sequences was performed using Clustal W program (http://www.mbio.ncsu.edu/bioedit/bioedit.html). Phylogenic analysis was performed using MEGA5.2, neighbor-joining method with 1000 bootstrap replications) (http://www.megasoftware.net/).

Histological analysis

Tissue fixation, dehydration and embedding were performed as previously described (Lin, *et al.*, 2013a). A Leica RM2145 microtome was utilized to cut the tissues into 10 μm sections. Specimens were mounted to slides and stained using 0.1% Toluidine blue as previously described (Tadege, *et al.*, 2011a). Images were captured by an Olympus BX-51 microscope.

Accession Numbers

Sequence data from this article can be found in the *Arabidopsis* Information Resource (http://www.arabidopsis.org), *M. truncatula* Information Resource (http://jcvi.org/medicago/) or GenBank/EMBL databases under the following accession numbers: STF*,* JF276252; Mt TPL, KC525957; Mt LUG, Medtr3g107910; Mt LUGL1,

Medtr1g011610; Mt LUH, Medtr1g050340; Mt LUHL1, Medtr7g058460;Mt AN3, Medtr1g080590; Mt AN3L1, Medtr7g115410; Mt AN3L2, Medtr8g024650; Mt SWP73B, Medtr6g023835; Ns LUG, LOC104235190; Ns AN3, XP_009801724; At LUG, AT4G32551; At LUH, AT2G32700; At AN3/GIF1, AT5G28640; GIF2, AT1G01160; GIF3, AT4G00850.

RESULTS

Fusion of MtLUG or MtAN3 to STFdel enhances *stf*, *lam1* and *wox1 prs* mutant leaf blade phenotypes

We previously reported that the *M. truncatula WOX1* gene, *STF* regulates lateral outgrowth of the leaf blade, floral organs and vascular patterning by primarily promoting cell proliferation at the adaxial-abaxial organ boundary through a transcriptional repression mechanism (Tadege, *et al.*, 2011a; Lin, *et al.*, 2013a,b; Zhang *et al.*, 2014). The repressive activity of STF in leaf and flower development requires the recruitment of the *M. truncatula* TOPLESS (MtTPL) corepressor through protein-protein interaction (Zhang *et al.*, 2014). Given that the Gro/Tup1 family of corepressors LUG, and the AN3/GIF1 coactivator family member AN3 also have roles in leaf and flower development (Sitaraman *et al.*, 2008; Stahle *et al.*, 2009), we wondered whether the MtLUG and MtAN3 can also act with STF in leaf and flower development. Y2H assays showed that STF interacted with both MtLUG and MtAN3 (Fig. S1a). We further carried out bimolecular fluorescence complementation (BiFC) assays using split YFP to confirm the interaction in living cells. While no YFP signal was detected in negative controls where the YN or YC half of YFP was fused in-frame to STF or MtAN3 with the other half alone in a vector, clear YFP signal was observed for the combination of STF-YN and MtLUG-YC or STF-YN and MtAN3-YC, demonstrating interactions in living plant cells (Fig. S1b). Domain deletion assay showed that deleting the C-terminal domain (CTD) of STF (STFdel) nearly abolished its interaction with MtAN3 and reduced the interaction strength with MtLUG (Fig. S1c), suggesting that the CTD of STF is important for its interaction with MtLUG and MtAN3. Since LUG is supposed to be a corepressor, the MtLUG interaction with STF may be expected analogous to the STF and MtTPL interaction, but that with MtAN3 was not.

We have previously shown that the CTD of STF is important for recruiting the corepressor MtTPL. The STF mutant protein with CTD deletion (STFdel), is unable to complement both the *stf* and *lam1* mutants, but when fused directly to MtTPL, it can rescue the narrow leaf phenotype of both mutants (Zhang *et al.*, 2014). To evaluate if a similar situation exists between STF and MtLUG or MtAN3 function, we fused the full-length MtLUG protein or MtAN3 protein to the STFdel in a way that was similar to that of the STFdel-MtTPL fusion. We also fused the activator domain VP16 protein to STFdel as negative control. All of these fusions were introduced into *stf* mutants under the control of the *STF* promoter (Fig. 1a). While the *stf* mutant transformed with STFdel is identical to the untransformed *stf* mutant, we found that unlike STFdel-MtTPL, the STFdel-MtLUG construct not only failed to rescue *stf* mutant phenotype, but enhanced the narrow leaf phenotype to such a degree that the resulting transgenic plants displayed a bladeless phenotype. These lines were similar in appearance to those expressing the STFdel-MtAN3 fusion (Fig. 1b,c). And both phenotypes resemble phenotypes arising in lines expressing the STFdel-VP16 fusion (Fig.1b,c). Microscopic examination of transverse sections confirmed that the blades of STFdel-MtLUG or STFdel-MtAN3 transgenic plants were severely affected compared to the *stf* mutant (Fig. 1d,e,f). The observed growth defects apparent in the vegetative tissue of these lines were extended into the flower, where the outer petals displayed reduced lateral extension when compared to the *stf* mutant transformed with STFdel alone or the wild type (Fig. S2). These phenotypic similarities between the STFdel-VP16, STFdel-AN3, and STFdel-MtLUG transformed *stf* lines, suggest that MtLUG may be acting as a transcriptional activator when fused to STFdel.

To show that this activity of MtLUG and MtAN3 fusion proteins is not limited to *M. truncatula* but has a wider occurrence, we introduced the STFdel-MtLUG and STFdel-MtAN3 constructs into the *lam1* mutant of *N. sylvestris* (Fig. 2b). *lam1* mutants have an even stronger leaf blade reduction phenotype compared to the *stf* mutant which resulted from a deletion of the *N. sylvestris STF* homologue (Tadege, *et al.*, 2011a). In comparison to *lam1* mutant leaves, which had severe defects in medial-lateral growth but near normal proximal-distal growth (Fig. 2b), all transgenic *lam1* lines expressing the STFdel-MtLUG, STFdel-MtAN3, and STFdel-VP16 fusions had severe growth defects

along both axes in which blades were narrower and shorter than the *lam1* mutant (Fig. 2d,e,f; Fig. S3). *lam1* mutants transformed with the STFdel construct alone, on the other hand, appeared identical to the untransformed *lam1* mutant (Fig. 2b,c), consistent with the findings in the *stf* mutant. Similarly, expression of *STFdel-MtLUG* and *STFdel-MtAN3* under *STF* promoter in *Arabidopsis wox1 prs* mutants also enhanced the narrow leaf mutant phenotype (Fig. S4). These similar responses to the introduced STFdel protein fusions suggest that the corepressor MtLUG and the coactivator MtAN3 are behaving in a similar manner with respect to transcriptional activation, and these activities are conserved among *M. truncatula*, *N. sylvestris* and *A. thaliana*.

To demonstrate that the enhancement of the *lam1* phenotype was due to the activation activity of the chimeric proteins (STFdel-MtLUG, STFdel-MtAN3, and STFdel-VP16), we added an exogenous ethylene-responsive element binding factor-associated amphiphilic repression (EAR) motif, SRDX, to these constructs (Fig. 2a). We reasoned that if the fusion of an activator to STFdel enhances the defects in leaf blade growth, then addition of a repressor domain may counter the activation activity and restore leaf growth. We found that the SRDX-STFdel-VP16 partially rescued the *lam1* mutant phenotype (Fig. 2i), suggesting that the SRDX repression activity is capable of suppressing the activation activity of VP16. Similarly, the SRDX-STFdel-MtLUG construct fully suppressed the severe leaf growth defects induced by the STFdel-MtLUG fusion (Fig. 2j). In both the VP16 and MtLUG fusions, addition of the SRDX fusion not only reversed the enhanced phenotype by these fusions but also complemented the *lam1* mutant leaf blade to a limited extent (Fig. 2b,i,j). This complementation was even more exaggerated in the case of SRDX-STFdel-MtAN3 fusion where the *lam1* blade defect was restored, albeit with distorted veins and leaf margins (Fig. 2h). The positive control SRDX-STFdel fusion alone without the activator domain, on the other hand, fully rescued the *lam1* mutant equivalent to wild type at least at the early stages of development (Fig. 2g, k). These results together indicate that MtLUG and MtAN3 activities are opposite to the activity of the SRDX repressor domain and are consistent with MtLUG and MtAN3 functioning as activators when fused to STFdel.

MtLUG physically interacts with MtAN3

Since our data suggested that both MtLUG and MtAN3 could function as activators and both LUG and AN3 are known to affect leaf and flower development in *Arabidopsis*, we investigated whether there is a biochemical link between these two proteins. Such a link has recently been suggested by studies in maize where the LEUNIG_HOMOLOG (LUH) was found to co-purify with the AN3 associated SWI/SNF complex (Nelissen *et al.*, 2015). In Y2H assays, MtLUG but not MtTPL interacted with MtAN3 (Fig. 3a). We confirmed this interaction with BiFC assays in plant cells**.** MtLUG and MtTPL were fused in frame to the C terminal half of YFP while MtAN3 was fused in frame to the N terminal half of YFP. Only MtLUG-YC but not MtTPL-YC reconstituted the YFP signal in tobacco cells with MtAN3-YN (Fig. 3b), confirming the Y2H result that MtAN3 physically interacts with MtLUG but not with MtTPL. Because of functional redundancy in the LUG family and AN3 family, we further tested this interaction in other members of the LUG and AN3 families. The Results revealed that both MtLUG and MtLUH interact with MtAN3, MtAN3-LIKE1 (MtAN3L1), and MtAN3L2 (Fig. S5; Fig. S6). We also confirmed that the physical interactions between LUG and AN3 are conserved in *N. sylvestris* and *A. thaliana*, as we found interaction between NsAN3 and NsLUG, and between AtAN3 and both AtLUG and AtLUH (Fig. 3c). These findings suggest that the LUG and AN3 interactions are conserved at least in the three species tested and can be extended to other members of the two gene families.

The *Arabidopsis* AN3 protein recruits SWI/SNF chromatin remodeling complexes to regulate leaf development (Vercruyssen *et al.*, 2014). The subunit of this complex, SWI/SNF ASSOCIATED PROTEIN 73B (SWP73B), is reported to be recruited to several promoters of regulatory factors during leaf development (Sacharowski *et al.*, 2015a). We tested whether MtLUG can recruit MtSWP73B directly. Yeast two-hybrid assay detected interaction between MtLUG and MtSWP73B (Fig. S7). A phenylalanine substitution of a serine residue at position 123 of *Arabidopsis* LUH protein results in the loss-of-function allele *luh-2* (Sitaraman *et al.*, 2008). Introducing an analogous mutation into the MtLUG protein (S164F) fully abolished the capacity of MtLUG to interact with MtSWP73B, without affecting interactions with MtSEU or MtAN3 (Fig. S7). We further confirmed the interactions between MtLUG and MtSWP73B using BiFC assays, which showed that intact MtLUG but not the mutated MtLUG S164F version interacted with

MtSWP73B (Fig. S7). Thus, in addition to its interaction with MtAN3, MtLUG directly interacts with the transcription activator SWI/SNF chromatin remodeling factor subunit MtSWP73B.

A conserved amino acid (N61) in the SNH domain of MtAN3 is required for the physical interaction with MtLUG and MtAN3 function

To better understand the nature of the MtLUG and MtAN3 interaction, we made a series of deletions in both MtLUG and MtAN3 proteins and performed Y2H assays mapping the interacting domains. MtLUG proteins lacking either the LUFS domain (1-80) or the WD40 repeats (471-end) were unable to interact with MtAN3 (Fig. S8), suggesting that both of these domains are needed for interactions with MtAN3, but that neither is sufficient alone. In contrast, the MtLUG LUFS domain was sufficient for its interaction with MtSEU (Fig. S8). The MtAN3 protein has two domains: The N-terminal SNH domain and the C-terminal QG domain, which has a high frequency of Gln (Q) and Gly (G) residues (Fig. 4a and Fig. S9). Analysis of MtAN3 mutated proteins in Y2H assays revealed that the SNH domain of MtAN3 is required and sufficient for interactions with MtLUG (Fig. 4b).

The loss-of-function *Arabidopsis an3-2* mutant has a six-base deletion in the second exon, which leads to two amino acid deletion in the SNH domain (NKS to -K-) of AN3 (Horiguchi *et al.*, 2005). We introduced an analogous mutation into *MtAN3* (*Mt an3-2*, 'NKS' to '-K-' in the SNH domain) and tested whether this mutation can affect the MtAN3-MtLUG interaction (Fig. 4a). Y2H analysis indicated that Mtan3-2 protein retained the capacity to interact with MtLUG, suggesting that the N-terminus region of SNH domain may not be involved in the MtAN3-MtLUG interaction (Fig. 4b,c). We then examined the C-terminus region of SNH domain, which is conserved from animals to plants (Fig. 4a). Substitution of a conserved asparagine with alanine (N61A) in MtAN3 abolished or greatly reduced the interaction with MtLUG in Y2H and BiFC assays (Fig. 4b,c). However, the MtAN3(N61A) mutation did not affect the interaction with MtSWP73B in Y2H assays (Fig. S10), indicating the specificity of N61 residue for the interaction with MtLUG. We further tested the capacity of the N61A mutation of MtAN3 to interact with MtLUG *in vivo* using co-immunoprecipitation (co-IP) assays. Myc-

tagged MtLUG protein can pull down GFP-tagged MtAN3, indicating strong interaction between MtLUG and MtAN3, but the signal for this interaction when using GFP-tagged MtAN3(N61A) mutant protein was drastically reduced (Fig. 4d), further confirming the importance of the MtAN3N61 residue for *in vivo* interaction with MtLUG.

We further tested whether the N61A mutation affects the transactivation activity of MtAN3 using the yeast GAL4 transactivation-reporter system. MtAN3 showed strong activation activity in yeast, while the N61A mutation fully abolished this activation activity (Fig. 5a). We next tested whether the N61A mutation affects MtAN3 activation activity in plant cells using transient protoplast dual-luciferase assay. MtAN3 showed approximately eightfold activation activity compared to the BD control, while the N61A mutation in MtAN3 reduced this activation activity to the level of the BD control (Fig. 5b), confirming that the N61A mutation affects MtAN3 activation activity in yeast and protoplasts.

Since STFdel-MtAN3 fusion enhances the *lam1* mutant leaf phenotype, we reasoned that if N61A mutation abolished the activation activity, the STFdel-MtAN3 (N61A) fusion should not enhance the *lam1* phenotype. To test this directly, we introduced STFdel-MtAN3(N61A) into the *lam1* mutant under the control of the *STF* promoter and compared this with *lam1* transformed with the same fusion construct using wild type MtAN3. Unlike the STFdel-MtAN3 fusion, which greatly enhanced the *lam1* phenotype (Fig. 5c), all 18 out of 18 N61A transgenic plants failed to enhance the *lam1* mutant phenotype and appeared indistinguishable from the *lam1* mutant transformed with STFdel alone (Fig. 5c), further confirming that the N61 residue at the SNH domain of MtAN3 is required for the activation activity of MtAN3 protein *in planta*. We next examined if the N61A mutation can affect the function of MtAN3 by complementing the *Arabidopsis an3* mutant. MtAN3 or MtAN3(N61A) mutant constructs were driven by *35S* promoter and introduced into *an3* mutant plants. Our results revealed that overexpression of MtAN3 in *Arabidopsis an3* mutant rescued the narrow leaf phenotype of *an3* (11 out of 32 lines showed complete rescue), but overexpression of MtAN3(N61A) failed to rescue any (0 out of 48 lines) (Fig. 5d; Fig. S11), demonstrating that the N61 residue is critical for MtAN3 function as transcriptional coactivator in leaf blade outgrowth. Taken together, these observations suggest that the MtAN3(N61)

residue is critically required for the MtLUG and MtAN3 physical interaction *in planta*, and this interaction may be important for MtAN3 coactivator function in leaf blade outgrowth.

LUG and AN3 interact genetically

To gain insight into the biological significance of the LUG-AN3 physical interaction, we conducted genetic analysis in *Arabidopsis*, where both *lug* and *an3* mutants are available. We generated a *lug an3* double mutant through crossing, and subsequently analyzed leaf and inflorescence phenotypes. The *an3* mutant has a narrow leaf phenotype (Kim & Kende, 2004), but the *lug* mutant leaf width appeared similar to wild type Col under our growth conditions, albeit with serrated leaf margins as previously reported (Cnops *et al.*, 2004). However, the leaves of the *lug an3* double mutant appeared narrower than the *an3* single mutant (Fig. 6a,b), suggesting that the *lug* mutation enhances the *an3* mutant leaf phenotype. The inflorescence and flower phenotypes observed in the *lug* mutant were also enhanced in the *lug an3* double mutants. From node 5 to node 10, both Col and the *an3* mutant showed same 4 petals per flower, but nearly half of *lug* mutant flowers have only 3 petals per flower. However, in the *lug an3* double mutant, 49% of the flowers contained no petals at all, leading to an average of 1 petal per flower (Fig. 6c; Fig. S12a). In addition, approximately 8.3% of the *lug an3* mutants appeared more fascinated compared to the *lug* single mutant (Fig. 6d), enhancing the *lug* mutant inflorescence phenotype. Some of the siliques of the *lug an3* mutants were coiled or not elongated, and the pedicel length of the siliques appeared shorter than that in the *lug* or *an3* single mutants (Fig. S12b). Similarly, the *an3* mutation also enhanced the *lug luh*/+ leaf phenotype in the *an3 lug luh*/+ mutant (Fig. S13). These observations together indicated stronger *lug an3* double mutant phenotypes in the leaves, flowers, inflorescences and siliques that were more than simple addition of the individual mutant phenotypes, suggesting overlapping activities and functions of LUG and AN3 in lateral organ development.

LUG and AN3 genetically interact with WOX transcription factors in leaf and flower development

Since STF/WOX1 physically interacted with MtLUG and MtAN3, we conducted genetic analysis to see if there is any functional link between these regulators. First, we constructed the *wox1 prs lug* triple mutant in *Arabidopsis* through crossing. The 7-day old *wox1 prs lug* mutant showed a narrow cotyledon phenotype that was not apparent in either the *wox1 prs* or *lug* mutants (Fig. 7a). Detailed analysis of vein patterning showed that the *wox1 prs* mutant had a weak vein defect, while the *lug* mutant had obvious defect on vein closure. The *wox1 prs lug* triple mutants, however, usually had only a single vein at 7-day old (Fig. 7b), indicating strong vascular differentiation defects. The leaves of the triple mutant also appeared narrower than the *wox1 prs* double mutant (Fig. 7c,d; Fig. S14), suggesting that the *lug* mutation enhanced the *wox1 prs* narrow leaf phenotype. This enhancement was mainly due to reduction in cell size rather than cell number (Fig. 7d,e) as no significant difference was observed in cell number per leaf between *wox1 prs* and the *wox1 prs lug* triple mutant (Fig. 7e) . In addition to the cotyledon and leaf phenotypes, the flower and inflorescence phenotypes of the *wox1 prs lug* triple mutant also appeared stronger with deep splitting of the styles and significantly reduced or abolished petals (Fig. 7f,g). These observations are again suggestive of a synergistic role for WOX1/PRS and LUG during leaf and flower development.

Next, we analyzed genetic interaction between *wox1 prs* and *an3* mutants. The *wox1 prs an3* triple mutant displayed leaf blades that are narrower than either of *wox1 prs* or *an3* mutants. The first pair of leaf width of *wox1 prs* mutant and *an3* mutant measured at the middle was approximately 76% and 62%, respectively, while the *wox1 prs an3* triple mutant leaf width was only around 41% of that of the wild type (Fig. 8a,b). We conducted morphometric analysis of leaf cells using automated image analysis software (Andriankaja *et al.*, 2012) in the first pair of leaves of wild type, *wox1 prs*, *an3,* and *wox1 prs an3* mutants. The cell number of *wox1 prs* mutant and *an3* mutant was found to be approximately 67% and 51%, respectively, while that of the *wox1 prs an3* triple mutant was only around 25% of that of the wild type control (Fig. 8c), suggesting a potential additive effect. The cell size did not show significant change in *wox1 prs an3*, (Fig. S15), suggesting primary defects in cell proliferation, consistent with the *wox1 prs* and *an3* mutant defects (Kim & Kende, 2004; Horiguchi *et al.*, 2005; Nakata *et al.*, 2012). The *wox1 prs* double mutant has unfused petals and the *an3* mutant has a mild

over-proliferation phenotype in the inflorescence, while the *wox1 prs an3* triple mutant displayed a combination of the *wox1 prs* and *an3* mutant phenotypes (Fig. 8d). In addition, unlike the fertile flowers in *wox1 prs* and *an3* mutants, the *wox1 prs an3* triple mutant flowers were totally infertile (Fig. 8e) due to the absence of pollen in the anther (Fig. 8f), suggesting that *WOX1, PRS* and *AN3* may have some redundant role in pollen production. Taken together, our biochemical and genetic analyses uncover a novel LUG-AN3 transcriptional coactivator interaction, which may have a synergistic or additive interaction with the *WOX1/PRS* transcriptional repressor to promote leaf blade outgrowth and development of reproductive organs, suggesting a complex interaction between transcriptional activation and repression programs in plant lateral organ development.

DISCUSSION

LEUNIG has a dual function as transcriptional coactivator and corepressor in leaf and flower development

Plant lateral organs develop post embryonically by a well-regimented cell proliferation/differentiation pattern through transcriptional activation and repression gene regulatory programs. The Groucho (Gro)/Tup1 family of transcriptional corepressors bridge transcription factors with chromatin remodeling complexes (Lee & Golz, 2012; Martin-Arevalillo *et al.*, 2017) to facilitate repression. One of the two major Gro/Tup1 corepressor sub families in plants, TPL and its family members (TPRs) can directly interact with a multitude of repressor transcription factors including ARF, WOX, AP2, and histone modifying enzyme HDA19 to repress the transcription of a wide range of target genes involved in embryogenesis, meristem maintenance and lateral organ development (Kieffer *et al.*, 2006; Szemenyei *et al.*, 2008; Causier *et al.*, 2012; Krogan *et al.*, 2012; Tao *et al.*, 2013; Zhang *et al.*, 2014). The second Gro/Tup1 corepressor subfamily, LUG and its homolog LUH, regulate leaf development, floral organ identity, gynoecium and ovule development through interaction with several transcription factors usually via an adaptor SEU (Franks *et al.*, 2006; Sridhar *et al.*, 2006; Liu & Karmarkar, 2008; Stahle *et al.*, 2009). For example, MADS-box transcription factors AP1 and SEP3

recruit LUG through SEU to repress *AG* expression in the outer whorls during flower development (Sridhar *et al.*, 2006). AP2 also recruits LUG through SEU, to negatively regulate the expression of *miRNA172* in the outer whorls of *Arabidopsis* flowers (Grigorova *et al.*, 2011). Thus, the corepressor function of LUG when in complex with SEU is well established (Conner & Liu, 2000; Franks *et al.*, 2002; Sridhar *et al.*, 2004; Gonzalez *et al.*, 2007; Sitaraman *et al.*, 2008), and parallels that of TPL (Sridhar *et al.*, 2004; Sitaraman *et al.*, 2008; Szemenyei *et al.*, 2008; Krogan *et al.*, 2012; Zhang *et al.*, 2014) to the extent that both TPL and LUG could be recruited even by the same transcription factor, such as AP2, for repression with specific or similar outcomes.

Here we described a novel function for LUG in which it acts as a coactivator together with a known transcriptional coactivator AN3/GIF1 to promote leaf blade outgrowth and floral organ development. Using the *lam1* mutant of *N. sylvestris* and the C-terminal domain truncated *STF* (STFdel) which has lost its repressive activity, we developed a novel system for assaying transcriptional repression and activation activity *in planta*. Fusion of a transcriptional repressor to STFdel such as SRDX recapitulates the intact STF function when introduced into the *lam1* and *stf* mutants, while fusing a transcriptional activator such as VP16 enhances the *lam1* mutant phenotype (Lin, *et al.*, 2013a; Zhang *et al.*, 2014). We used this system to assess if MtLUG functions as a repressor or an activator, and our data suggests that MtLUG can act as a coactivator in the context of leaf development, enhancing the *lam1* and *stf* mutant phenotypes similar to MtAN3 and VP16 but opposite to SRDX (Fig. 1; Fig. 2). Importantly, we demonstrated that the coactivator MtAN3 can physically interact with MtLUG using Y2H, BiFC and co-IP assays (Fig, 3; Fig. 4). A single amino acid change at the SNH domain of MtAN3 (N61A) is sufficient to disrupt the MtLUG-MtAN3 interaction and MtAN3 function (Fig. 4; Fig. 5), suggesting a critical role for LUG as a coactivator partner of AN3. The identification of direct association between MtLUG and MtAN3 and the requirement of MtLUG-MtAN3 interaction for MtAN3 coactivator function are consistent in MtLUG being in a coactivator complex with MtAN3. In support of this, tandem affinity purification (TAP) followed by mass spectrometry (MS) analysis identified that the LUG homologue (LUH) co-purifies with AN3 in maize (Nelissen et al., 2015). This shows that LUH is in complex with AN3 in maize although LUG itself escaped detection perhaps

due to low expression. Through Y2H and BiFC assays, MtLUG was also found to interact with another member of the AN3 complex, SWI/SNF ASSOCIATED PROTEIN 73B (MtSWP73B), a subunit of the SWITCH/SUCROSE NONFERMENTING (SWI/SNF) chromatin remodeling complex (Fig. S7). The binding of AN3 to the SWI/SNF complex that includes the ATPases BRAHMA (BRM) and SPLAYED (SYD) in growing *Arabidopsis* and maize leaves, and the recruitment of SWP73B by AN3 to the promoters of various growth regulators has been well documented (Vercruyssen *et al.,* 2014; Nelissen *et al.,* 2015). It is known that the SWI/SNF chromatin remodeling complex is primarily associated with transcriptionally accessible chromatin (Clapier & Cairns, 2009; Sacharowski *et al.*, 2015). Thus, our data suggests that MtLUG may be associated with active chromatin when in complex with MtAN3 and MtSWP73B, consistent with a coactivator role. However, LUG also associates with repressed chromatin through interaction with histone deacetylase HDA19 and Mediator proteins MED14 and CDK8 (Gonzalez *et al.*, 2007) when in complexes with SEU. The emerging picture from our data and previous reports suggests that LUG has a dual function depending on its non-DNA binding interaction partners; acting as a coactivator in the LUG-AN3 complex and as a corepressor in the LUG-SEU complex, but both the activation and repression functions may be required for cell proliferation and differentiation during leaf blade outgrowth and floral organ development.

WOX1 and PRS interplay with transcriptional coactivators to regulate leaf and flower development

In *Arabidopsis WOX1* and *PRS* function redundantly in regulating leaf blade outgrowth (Vandenbussche *et al.*, 2009; Nakata *et al.*, 2012) similar to the *M. truncatula WOX1* homolog *STF,* which recruits MtTPL and functions as a transcriptional repressor in promoting cell proliferation during leaf blade outgrowth and floral organ development (Lin, *et al.,* 2013a; Zhang *et al.*, 2014). The *M. truncatula loose flower* (*lof*) mutant (homolog of *PRS*) has no leaf blade phenotype but functions as a transcriptional repressor in petal fusion (Niu *et al.*, 2015). Nevertheless, both *WOX1* and *PRS* driven by the *STF* promoter complement the tobacco *lam1* mutant (Lin, *et al.*, 2013a). Although the founding member of the WOX family, WUSCHEL (WUS), has been reported to be

bifunctional acting as repressor and activator in vegetative and reproductive tissues, respectively (Ikeda *et al.*, 2009), direct activation activity for STF or WOX1 and PRS in lateral organ development has not been shown, and the main mechanism of their function in both the leaves and flowers involves repression. Despite this, we identified genetic interaction between *WOX1*, *PRS* and *LUG* (Fig. 7), as well as between *WOX1*, *PRS* and *AN3* (Fig. 8) in which the *wox1 prs* mutant leaf and flower phenotypes of *Arabidopsis* were enhanced by either *lug* or *an3* mutants.

The exact molecular mechanism of this genetic interaction is unclear at this stage and requires detailed investigation in the future. One possibility is that WOX1, like WUS, retains context dependent activation activity and requires AN3 for transcriptional activation. Although we consider such a scenario unlikely based on what we know so far, the additive/synergistic interactions with LUG and AN3 would make sense under such circumstances. In analogous scenario, AP2 recruits LUG for its repression activity, and the *lug* mutant enhances the *ap2-1* mutant phenotype in the *lug ap2-1* double (Liu & Meyerowitz, 1995). In a similar example, *Arabidopsis* growth regulating factor1 (GRF1) recruits GIF1/AN3 for its activation activity in promoting leaf blade outgrowth, and the *grf1 gif1* mutants have a synergistic phenotype (Kim & Kende, 2004). A second possibility is that WOX1/PRS and LUG/AN3 share an ultimate common target (s) for the repressing and activating pathways. In effect, the demonstrated functions of the WOX1-TPL and GRF-AN3 pathways independently achieve a similar goal, promoting lateral outgrowth of the leaf blade via cell proliferation, albeit repression and activation mechanisms, respectively (Kim & Kende, 2004; Horiguchi *et al.*, 2005; Lee *et al.*, 2009; Tadege, *et al.*, 2011a; Nakata *et al.*, 2012; Lin, *et al.*, 2013a; Zhang *et al.*, 2014). A likely scenario is that AN3 may activate a target while WOX1 may repress a repressor of such a target or conversely, AN3 may activate repressor of a WOX1 target, such that both AN3 and WOX1 in the end converge promoting the same activity. These potential scenarios are depicted in a hypothetical cartoon (Fig. S16). Anther development may serve as a good system to test this possibility, as *wox1 prs* and *an3* mutants produce pollen, while the *wox1 prs an3* triple mutant totally lack pollen production (Fig. 8e,f). Gene expression analysis showed that *SPL/NZZ*, a pivotal regulator for early anther development (Yang *et al.*, 1999), was greatly reduced in the triple mutant, while such expression in the *wox1 prs*

or *an3* mutants appeared normal (Fig. S17). This suggests that WOX1/PRS and AN3 act early in anther development, and positively regulate a common target *SPL/NZZ*. Interestingly, a recent report showed that WOX1 from cucumber positively regulates *SPL*/*NZZ*, as this gene and genes in the *SPL*/*NZZ*-mediated pathway including *AMS*, *MS1*, *MYB103* were all downregulated in the cucumber *wox1* mutant (Niu *et al.*, 2018). This indicates that *WOX1* indeed activates *SPL/NZZ* (probably indirectly by repressing the *SPL/NZZ* repressor) although this role is redundant with *AN3* in *Arabidopsis*. Such a role for *AN3/GIF1* was revealed by a higher order *grf gif* mutation in *Arabidopsis* showing a similar phenotype to the *spl*/*nzz* mutant (Lee *et al.,* 2018). These observations suggest that the potential exists for the WOX1/STF complex and LUG and/or AN3 complex to influence common target gene expression directly or indirectly.

Taken together, our study has unveiled a novel LUG-AN3 interaction, and the involvement of WOX transcription factors sharing genetic pathways with this interaction in the control of leaf blade and floral organ development in *Arabidopsis* and other species. This points to the complexity of plant transcriptional regulatory mechanisms and uncovers a dual role for LUG acting as transcriptional coactivator or corepressor depending on its interaction partners, providing a context dependent flexible module for coordinating transcriptional activation and repression mechanisms in tissue specific plant developmental programs.

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Author Contributions

F.Z. designed research, performed research, analyzed data, and wrote the article. H.W., S.K., T.W.W. performed research. J.N. contributed analytical tools. J.F.G. Performed research, analyzed data and edited the article. M.T. designed research, analyzed data and wrote the article.

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Supporting Information

Fig. S1 STF interacts with both MtLUG and MtAN3

Fig. S2 Expression of *STFdel-MtAN3* or *STFdel-VP16* fusions under *STF* promoter worsen the *M. truncatula stf* mutant flower phenotypes.

Fig. S3 Expression of *STFdel-MtLUG* fusion worsens the *lam1* mutant leaf phenotype.

Fig. S4 Phenotypes of *Arabidopsis* cotyledon and leaves expressing *STFdel*-Mt *LUG* and *STFdel-MtAN3* fusion transgenes in *wox1 prs* background.

Fig. S5 Phylogenetic analysis of the LUG and AN3 families in *Medicago truncatula*, *Arabidopsis thaliana* and *Nicotiana sylvestris.*

Fig. S6 Conservation of protein-protein interaction between LUG family and AN3 family members of *Medicago truncatula* in Y2H assays.

Fig. S7 MtLUG interacts with MtSWP73B in Y2H and BiFC assays.

Fig. S8 Domain deletion analysis of protein-protein interaction between MtLUG and MtAN3 using Y2H assay.

Fig. S9 MtAN3 protein domain arrangement and amino acid sequence alignment of AN3 family proteins.

Fig. S10 MtAN3 directly interacts with MtSWP73B in Y2H assays.

Fig. S11 The MtAN3 N61A mutation abolishes its ability to complement the *Arabidopsis an3* mutant.

Fig. S12 Effect of the *lug* and *an3* mutation on petal number and silique length.

Fig. S13 Leaf phenotypes of 20-day old mutant plants grown under short day conditions.

Fig. S14 The *lug* mutant and *wox1 prs* mutant enhance each other in leaf development.

Fig. S15 Morphometric analysis of leaf growth in the first two leaves of 14-day old *wox1 prs*, *an3* and *wox1 prs an3* mutants.

Fig.S16 The interaction of WOX and LUG/AN3 complex in plant development.

Fig. S17 The *SPL*/*NZZ* mediated pathway is regulated by *WOX1*, *PRS* and *AN3*.

Table S1 Sequences of primers used in this study.

Figure legends:

Fig.1 MtLUG acts as a co-activator in STFdel fusion *in planta* assay in *stf* mutant.
(a) Constructs used in the transformation assay for complementation of the *Medicago truncatula stf* mutant. All constructs were driven by the *M. truncatula STF* promoter. NTD, the N-terminal domain (amino acids 1 to 90), HD, homeodomain (amino acids 91

to 163), MD, middle domain (amino acids 164 to 300), and STFdel was devoid of the C-terminal domain (amino acids 301 to 358).

(b) Wild type R108 and the *stf* mutant transformed with *STFdel* alone (STFdel), *STFdel-MtLUG* (+LUG), *STFdel-MtAN3* (+AN3) and *STFdel-VP16* (+VP16) constructs.

(c) Individual leaf phenotypes of the transgenic plants shown in **(b)** compared to wild type R108. Scale bar = 1 cm.

(d-f) Transverse sections through the leaves of *stf* mutant transformed with *STF::STFdel* **(*d*)**, *STF::STFdel-MtLUG* **(e)**, and *STF::STFdel-MtAN3* **(f)**. Scale bars = 200 μm.

Fig. 2 MtLUG functions opposite to the SRDX repression domain in STFdel fusion *in planta* assay in *Nicotiana sylvestris lam1* mutant.

(a) Constructs used in the complementation of the *N. sylvestris lam1* mutant. All constructs were driven by the *Medicago truncatula STF* promoter.

(b-k) Plant phenotypes of untransformed *lam1* mutant **(b)** and *lam1* mutant transformed with the constructs of *STF*::*STFdel* alone **(c),** *STF::STFdel-AN3* **(d)**, *STF::STFdel-VP16* **(e)**, *STF::STFdel-LUG* **(f)**, *STF::SRDX-STFdel* **(g)**, *STF::SRDX-STFdel-AN3* **(h)**, *STF::SRDX-STFdel-VP16* **(i),** *STF::SRDX-STFdel-LUG* **(j)** and **(k)** Untransformed WT *N. sylvestris.* Scale bars = 5 cm.

Fig.3 MtLUG physically interacts with the coactivator MtAN3 in Y2H and BiFC assays.

(a) MtLUG but not MtTPL interacts with MtAN3 in Y2H assay. Interaction was examined by the presence or absence of yeast growth on a quadruple dropout medium (QDO) in the presence of X-gal.

(b) Interaction between MtAN3 and MtLUG in tobacco epidermal cells using split YFP BiFC assay. MtAN3 can form homodimer with itself or heterodimer with MtLUG but does not interact with MtTPL in living plant cells. Scale bars = 50 μm.

(c) Conservation of LUG and AN3 interaction in *Nicotiana sylvestris* and *Arabidopsis thaliana.* Interaction was also observed between AtLUH and AtAN3.

Fig. 4 A conserved Asparagine at position 61 of the MtAN3 SNH domain is required for interaction with MtLUG.

(a) Multiple alignment of the SNH domain based on the human synovial translocation (SYT) protein SNH domain. Black arrows point to Mtan3-2 mutation while red arrow points to MtAN3N61.

(b) Yeast two-hybrid assay showing that the SNH domain of MtAN3 is sufficient for interaction with MtLUG and the conserved Asparagine at position 61 is required for the MtLUG interaction.

(c) BiFC assay showing substitution of Asparagine residue at position 61 of the MtAN3 SNH domain with Alanine abolishes the interaction with MtLUG. Scale bars = 50 μm.

(d) Co-IP assay demonstrating MtAN3 and MtLUG physical interaction *in vivo*. Note that MtLUG-MYC can pull-down MtAN3-GFP but not MtAN3 N61A-GFP mutant protein as the signal is drastically reduced.

Fig. 5 The conserved Asparagine at position 61 of the MtAN3 SNH domain is required for the trans-activation activity and biological function of MtAN3.

(a) Yeast trans-activation assay using the GAL4 system. MtAN3 or its mutant MtAN3-N61A were fused in-frame to the yeast GAL4-DNA binding domain in the BD vector. Constructs were introduced into yeast with AD vector. Growth in the QDO plates indicates trans-activation activity.

(b) Upper panel, schematic representation of reporter and effector constructs used in dual luciferase assay. Lower panel, relative luciferase activities using MtAN3 or MtAN3-N61A as effectors compared with GAL4-DB control. Error bars indicate SD (n=3). Asterisks indicate significance (**P<0.01, *t* test).

(c) Upper panel, constructs used for complementation of *lam1* mutant; lower panel, transgenic *lam1* showing the STFdel-MtAN3N61A transgene (right) failed to worsen the lam1 narrow leaf phenotype (left) compared to STFdel-MtAN3 (middle**).**

(d) Complementation of *an3* mutant by MtAN3 or MtAN3N61A, showing MtAN3 but not MtAN3N61A transgene complementing the *an3* narrow leaf phenotype. Three homozygous T3 lines were analyzed for each construct. Error bars represent SE (n=12).

Asterisks indicate significant difference from the *an3* mutant (**P<0.01, *t* test). n.s: non-significant difference.

Fig. 6 Genetic interaction between *LUG* and *AN3* in *Arabidopsis*.

(a) Leaf phenotypes showing enhancement of the *an3* mutant narrow leaf in the *lug an3* double mutant (14-day old plants were shown). Scale bars = 2 mm.

(b) Relative leaf width of mutants shown in (a). Error bars represent SE (n=20). Asterisks indicate significance (**P<0.01, *P< 0.05, *t* test). n.s: non-significant difference.

(c) Floral phenotypes showing enhancement of the *lug* mutant phenotype in the *lug an3* double mutant with petals reduced or abolished. Scale bars = 1 mm.

(d) Top view of the *lug an3* double mutant inflorescence phenotype compared to the *lug* or *an3* single mutant. Inflorescence of 5-wk-old plants were shown. Scale bars = 1 mm.

Fig.7 Genetic interaction between *WOX* transcription factors and *LUG* in *Arabidopsis*.

(a) Cotyledon phenotype of Col, *wox1 prs, lug* and *wox1 prs lug*. Cotyledons were from 7-day old seedlings grown on plates.

(b) Cotyledon phenotype of *Arabidopsis* 7-day old mutants showing the *lug* mutation enhancing the vein phenotype of *wox1 prs*.

(c) *Arabidopsis* mutants showing the *lug* mutation enhances *wox1 prs* narrow leaf phenotype. Plants were 26-day old.

(d) Relative leaf width of *wox1 prs, lug* and *wox1 prs lug* mutants of 10-day old leaves 1 and 2. Error bars represent SE (*n* = 5). Asterisks indicate significant difference from the wild type (Col-0) or *wox1 prs* (**P<0.01, *t* test).

(e) Pavement cell number of 10-day old leaves 1 and 2. Error bars represent SE (*n* = 5). Asterisks indicate significant difference from the wild type (Col-0) or *wox1 prs* (**P < 0.01, *t* test). n.s: non-significant difference.

(f) Inflorescence phenotype of *wox1 prs, lug* and *wox1 prs lug* mutants at 35-day old. Scale bars = 1 mm.

(g) Floral phenotype of *wox1 prs, lug* and *wox1 prs lug* mutants. Flowers are from nodes 5-10 of the main stem. Scale bars = 1 mm.

Fig. 8 Genetic interaction between *WOX* transcription factors and *AN3* in *Arabidopsis.*

(a) *Arabidopsis* mutants showing the *an3* mutation enhancing the narrow leaf phenotype of *wox1 prs* in 22-day old plants.

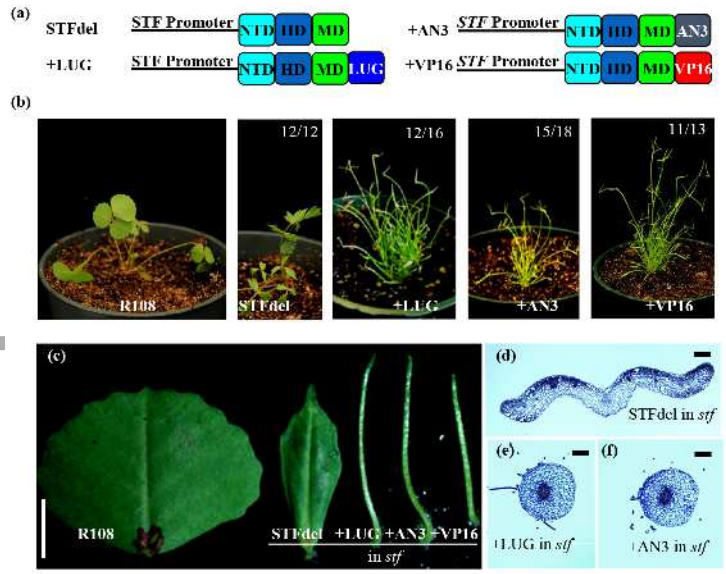
(b) Quantitative measurement of leaf width in wild type Col, *wox1 prs*, *an3* and *wox1 prs an3* mutants. The first pair of leaves of 10-day old seedlings grown on media were measured for leaf width. Error bars represent SE ($n \geq 10$). Asterisks indicate significant difference from the wild type (**P < 0.01, *t* test).

(c) Pavement cell number of 10-d old leaves 1 and 2 of Col-0 and mutants in **(b)**. Error bars represent SE ($n = 5$). Asterisks indicate significant difference from the wild type (**P < 0.01, *t* test).

(d) Representative images of 35-day old inflorescence of Col, *wox1 prs*, *an3,* and *wox1 prs an3* mutants. Scale bars = 1 mm.

(e) Siliques of Col, *wox1 prs*, *an3* and *wox1 prs an3*. Note that *wox1 prs an3* is infertile.

(f) Pollen production on the anthers of Col, *wox1 prs*, *an3*, and *wox1 prs an3*. Note that *wox1 prs an3* fails to produce any pollen.



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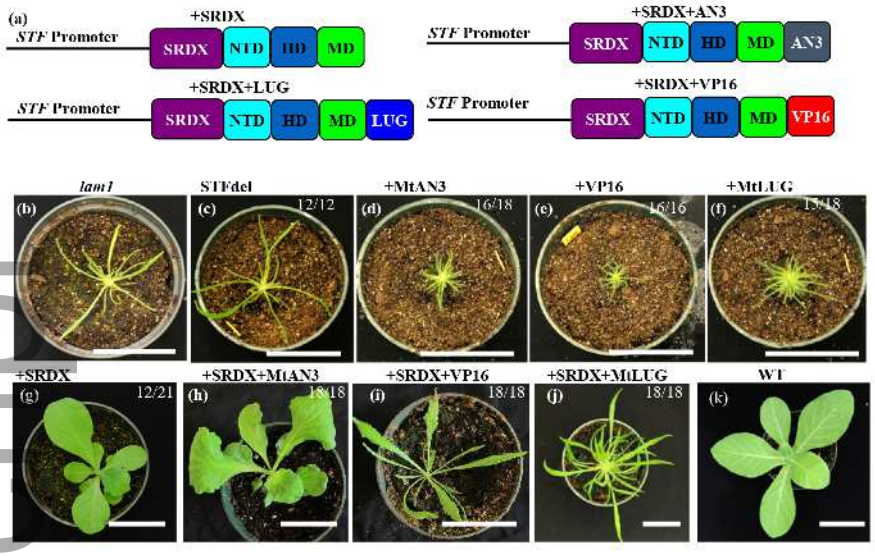

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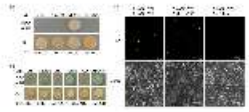

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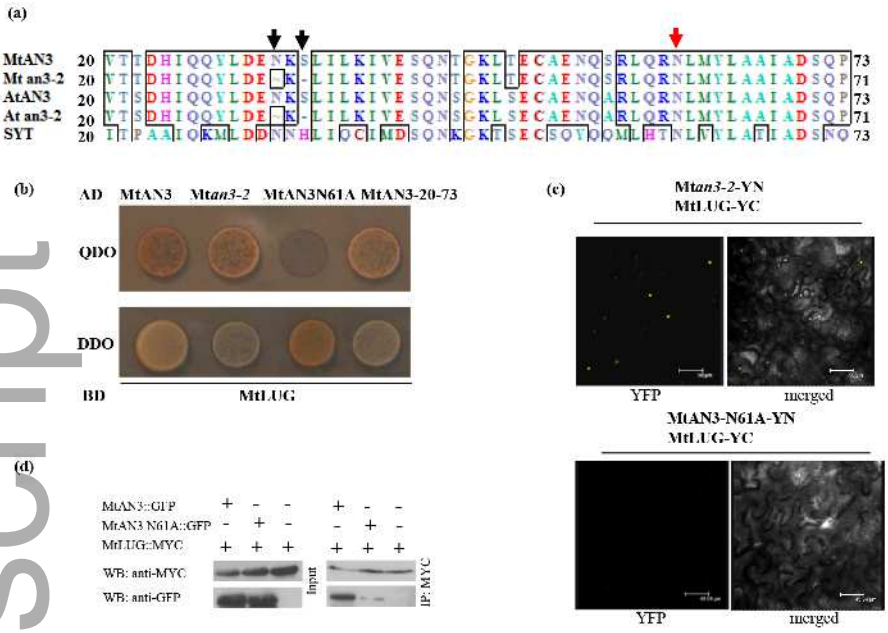

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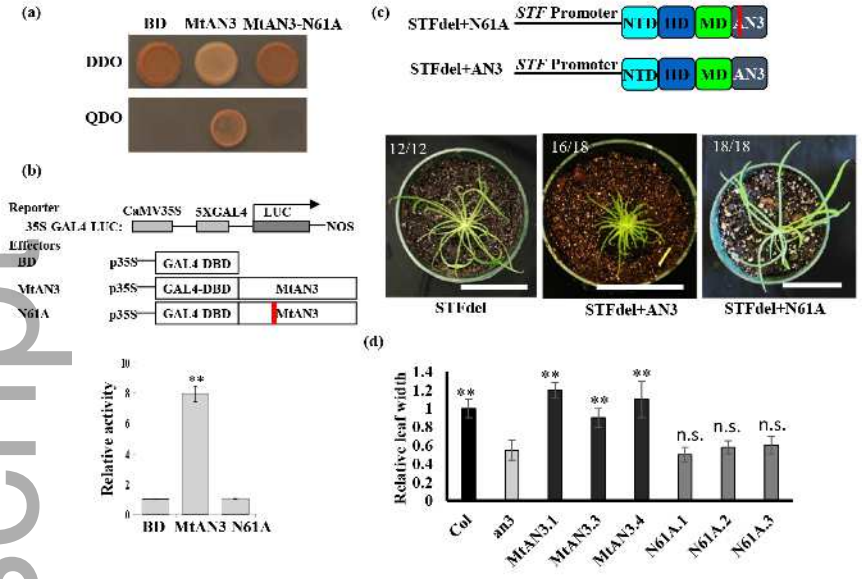

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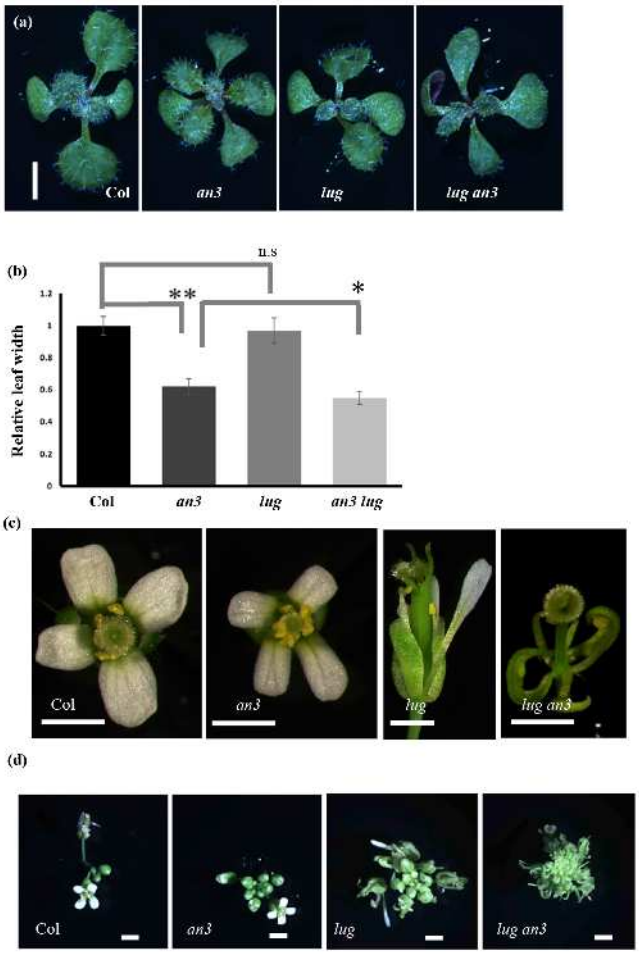

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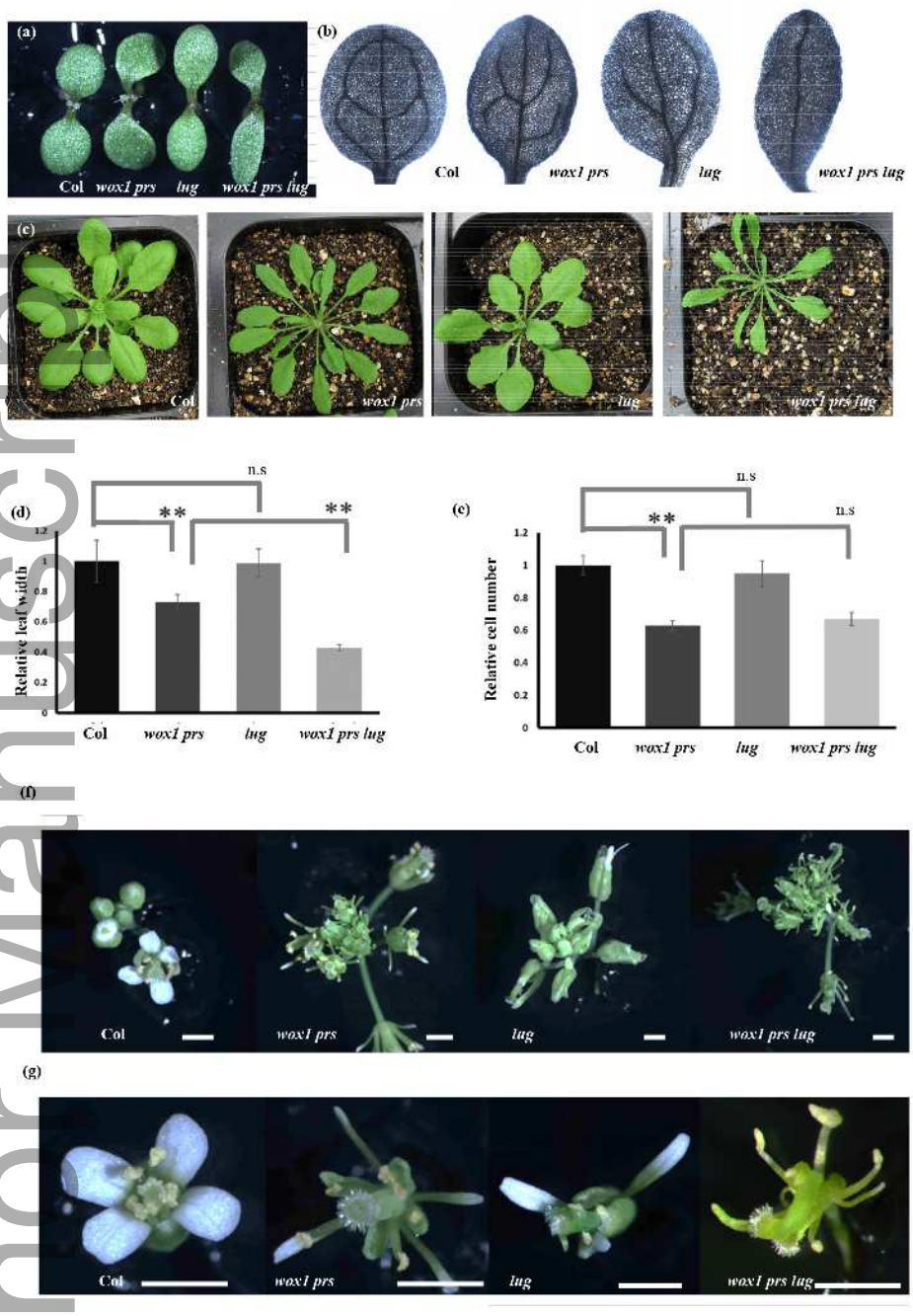

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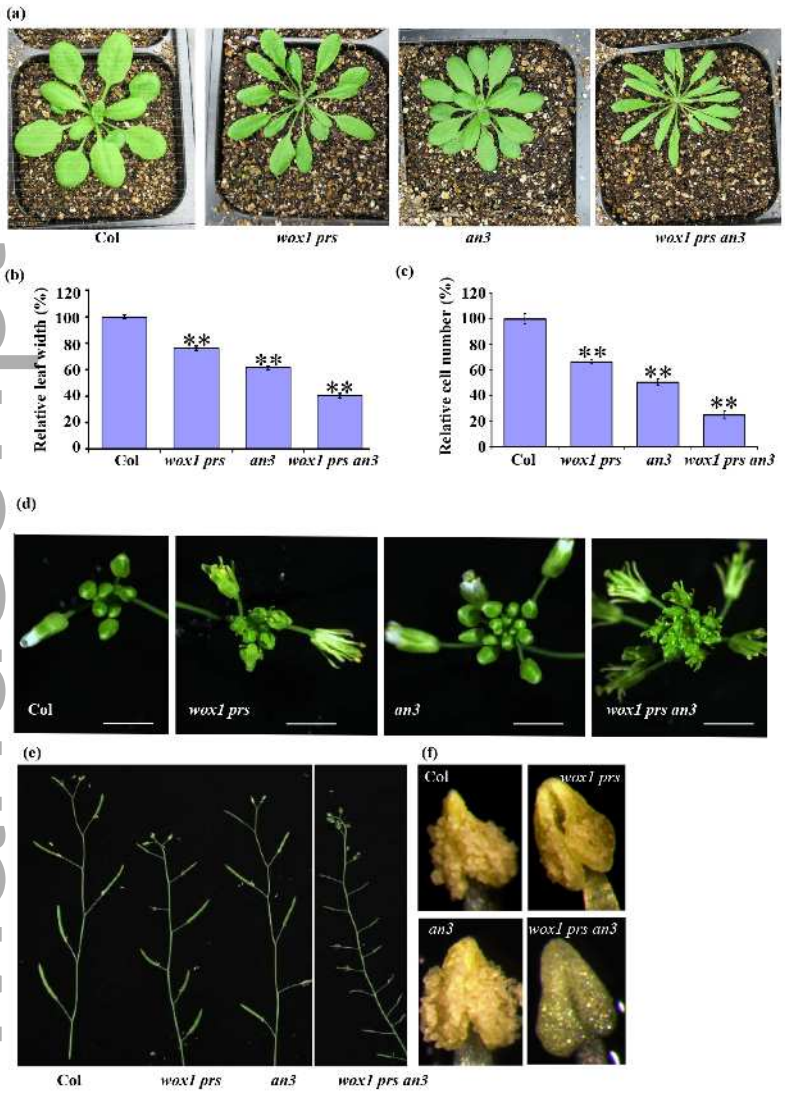

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