SHORT TITLE
TMS inhibits protein secretion by binding to SYP1s

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Overexpressed Tomosyn binds syntaxins and blocks secretion during pollen development

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ONE-SENTENCE SUMMARY
Overexpressed Tomosyn protein inhibits secretion in pollen by competitively binding to syntaxins, the proteins that control secretory vesicle fusion with its target compartment.

FOOTNOTES

AUTHOR CONTRIBUTIONS
Y.B., and B.L. designed the research; B.L., YB.L., Q.R., X.T., L.Q., G.L., Y.L., R.S. and R.G. performed experiments; Y.B., B.L., and F.L. analyzed and interpreted the data; Y.B. and B.L prepared and wrote the article. All authors read and approved the manuscript.

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ABSTRACT

SNARE (soluble N-ethylmaleimide-sensitive factor attachment protein receptors) complex formation is necessary for intracellular membrane fusion and thus has a key role in processes such as secretion. However, little is known about the regulatory factors that bind to Qa-SNAREs, which are also known as syntaxins (SYPs) in plants. Here, we characterized *Arabidopsis thaliana* Tomosyn protein (AtTMS) and demonstrated that it is a conserved regulator of SYPs in plants. AtTMS binds strongly via its R-SNARE motif-containing C-terminus to the Qa-domain of PM-resident, pollen-expressed SYP1s (SYP111, 124, 125, 131 and 132), which were narrowed down from twelve SYPs. AtTMS is highly expressed in pollen from the bicellular stage onwards, and overexpression of AtTMS under the *UBIQUITIN10*, *MSP1* or *LAT52* promoter all resulted in defective pollen after the microspore stage in which secretion was inhibited, leading to the failure of intine deposition and cell plate formation during pollen mitosis I (PMI). In tobacco (*Nicotiana benthamiana*) leaf epidermal cells, overexpression of AtTMS inhibited the secretion of SecGFP (secreted green fluorescent protein). The defects were rescued by mCherry-tagged SYP124, 125, 131, or 132. *In vivo*, SYP132 partially rescued the *pMSP1:AtTMS* phenotype. In addition, AtTMS lacking a transmembrane domain, was recruited to the plasma membrane by SYP124, 125, 131 and 132, and competed with VAMP721/722 (vesicle associated membrane protein 721/722) for binding to, for example, SYP132. Together, our results demonstrated that AtTMS might serve as a negative regulator of secretion, whereby active secretion might be fine-tuned during pollen development.
INTRODUCTION

Male gametophyte development is a key process during plant reproduction. A number of events, such as the deposition of the intine, the expansion of the cell plate during pollen mitosis I and pollen tube growth, require active membrane traffic to the plasma membrane (PM) (Kato et al., 2010; Twell, 2011; Shi et al., 2015). It is generally recognized that SNARE (soluble N-ethylmaleimide-sensitive factor attachment protein receptors) proteins control the ultimate fusion of a secretory vesicle with its target compartment (Jahn and Scheller, 2006). They have been grouped into Q-SNAREs (Qa, Qb, Qc, or Qbc) or R-SNAREs based on the conserved residues within the SNARE domain (Fasshauer et al., 1998). Different sets of Q- and R-SNAREs in two opposing membranes associate into trans-SNARE complexes through SNARE domains, which drive membrane fusion and cargo secretion (Jahn and Scheller, 2006).

In recent years, several SNARE proteins have been shown to play roles in male gametophyte development. The PM-resident Qa-SNAREs SYP124/125/131 (Uemura et al., 2004) are expressed specifically in pollen (Enami et al., 2009), and a syp124 syp125 syp131 triple mutant shows defective pollen tube growth (Slane et al., 2017). SYP111 and SYP132 are expressed in the early stages of pollen development and are possible candidates to mediate fusion processes during the mitotic divisions that give rise to tricellular pollen (Enami et al., 2009; Slane et al., 2017). Unlike SYP111, SYP132 was essential for secretory trafficking to the PM (Karnahl et al., 2018; Park et al., 2018). R-SNARE SEC22 localized at endoplasmic reticulum (ER) and sec22-2 pollen development was defective (El-Kasmi et al., 2011). Overexpressing a pollen-specific R-SNARE PiVAMP726 in Petunia inflata inhibited growth and caused the formation of novel membrane compartments within the tip of pollen tube (Guo and McCubbin, 2012). Apparently, more components of the vesicle fusion machinery and regulators remain to be studied in pollen.

Compared to the characterization of fusion machinery itself, little is known about factors modulating SNARE complex assembly that ensure the correct vesicle fusion spatially and temporally (Toonen and Verhage, 2003; Pobbati et
In plants, the best-studied SNARE regulator is the SM/SEC1 family protein KEULE. KEULE binds to the open conformation of Qa-SNARE SYP111/KNOLLE, thus stabilizing KNOLLE and promoting trans-SNARE formation (Park et al., 2012). As a result, keule, knolle single and keu knolle double mutants had abnormal vesicle accumulation and defective cell plate formation during somatic cytokinesis (Jürgens, 2005).

Tomosyn was first identified as a binding protein for Qa-SNARE domain of Syntaxin1 from rat cerebra (Fujita et al., 1998) and is conserved in mammals. Overexpression of Tomosyn inhibits exocytosis in a number of neuroendocrine secretory cells (Widberg et al., 2003; Gladycheva et al., 2007), and in neurons (Yizhar et al., 2004; Yizhar et al., 2007). Moreover, genetic studies with a C. elegans tom-1 mutant (Gracheva et al., 2007) along with a tomosyn knock-out mouse (Sakisaka et al., 2008) have demonstrated enhanced synaptic transmission.

In this study, we provide evidence that AtTMS might act as a negative regulator of secretion in pollen development by competitively binding to a set of SYP1s.
RESULTS

AtTMS is an R-SNARE motif-containing protein homologous to animal Tomosyns

AtTMS (AT5G05570) encodes a 1124-amino acid protein containing a small C-terminus harboring a coiled-coil R-SNARE motif and a large N-terminus containing WD40 repeats (Fig. 1A). A maximum-likelihood phylogenetic tree analysis showed that Tomosyns are highly conserved in animals and plants (Fig. 1A). The R-SNARE motif in each Tomosyn homolog bears 16 fully conserved hydrophobic amino acids and the conserved arginine in the layer 0 of the central heptad repeat of the α-helix (Fig. 1B). This motif shows very high sequence similarity to that of the R-SNARE synaptobrevin and serves as the main interaction site with syntaxin1a (Hatsuzawa et al., 2003; Pobbati et al., 2004). These data indicate that AtTMS is a conserved Tomosyn protein in plants and may have a Qa-SNARE binding capacity like its animal counterparts.

AtTMS localizes to the trans-Golgi network, PM and Cytosol

To narrow down the possible AtTMS interacting Qa-SNAREs, subcellular localization of eGFP tagged AtTMS was studied in Arabidopsis protoplasts. eGFP-AtTMS partially overlapped with the trans-Golgi network (TGN) (Lam et al., 2007) (Fig. 2A) and PM (Hou et al., 2014) (Fig. 2B), but not with the Golgi (Tse et al., 2004) (Fig. 2C), and the prevacuolar compartment PVC (Miao et al., 2006) (Fig. 2D). A similar AtTMS localization pattern was demonstrated in tobacco (Nicotiana benthamiana) leaf epidermal cells (Supplemental Figure S1). These data demonstrated that AtTMS disperses in the cytosol, and also localizes to the TGN and the PM.

AtTMS interacts with PM-resident SYP111/124/125/131/132 and TGN-resident SYP41/43

The subcellular localization pattern (Fig. 2) suggested that AtTMS interacting Qa-SNAREs should be of TGN and/or PM origin. There are nine PM (SYP1 subfamily) and three TGN (SYP4 subfamily) Qa-SNAREs in Arabidopsis (Uemura
et al., 2004). The interaction between AtTMS and the Qa-domain of twelve individual SYPs was then tested. In a yeast-two-hybrid assay, AtTMS interacted strongly with the Qa-domain of SYP124/125/131/132/41/43 (forward slash means ‘and’), weakly with that of SYP111, but not at all with SYP112/121/122/123/42 (Fig. 3A, 3B).

Next, each of the twelve SYPs without a transmembrane domain (SYP^ΔTM)
was tested for their binding capacity to AtTMS full-length (AtTMSFL, 1-1124 a.a), N-terminus containing WD40 repeats (AtTMSNNT, 1-921 a.a), and C-terminus containing R-SNARE motif (AtTMSCT, 922-1124 a.a) (Fig. 3C) using a firefly luciferase complementation imaging (LCI) assay (Chen et al., 2007). Only SYP111/124/125/131/132/41/43△TM-nLUC, but not SYP112/121/122/123/42△TM-nLUC, reconstituted a high Luciferase activity with either cLUC-AtTMSFL or cLUC-AtTMSCT, respectively (Fig. 3D). Similarly, GST-SYP111/124/125/131/132/41/43△TM, but neither GST alone nor GST-SYP112/121/122/123/42△TM, were able to pull down His-tagged AtTMSCT (Fig. 3E).

Taken together, these results demonstrated that among twelve SYPs, AtTMS binds specifically to only seven of them (named as TMS interacting SYP, SYPsTI), i.e., PM-resident SYP111/124/125/131/132 (SYP1sTI) and TGN-resident SYP41/43 (SYP4sTI). Furthermore, the Qa-domain of each SYPsTI and the R-
SNARE motif containing AtTMS\textsuperscript{CT} were demonstrated to mediate the reciprocal bindings. Notably, AtTMS interacted only weakly with SYP111 in two assays (Fig. 3B, 3E). These interaction studies are summarized in a schematic drawing (Fig.
Overexpression of AtTMS impairs pollen development after the microspore stage

To analyze the function of AtTMS, attms-1/+ and attms-2/+ mutants were generated by CRISPR/Cas9-mediated gene editing, and homozygotes were obtained. Sequence analysis revealed a single base A and T deletion at the eighth exon in attms-1 and attms-2, respectively, and both mutations caused premature translational termination (Supplemental Figure S2). However, attms-1 and attms-2 mutants exhibited no obvious abnormality in growth under the standard conditions.

The UBIQUITIN10 promoter (pUBQ10), which facilitates moderate gene expression in nearly all tissues, including pollen (Norris et al., 1993), was first used to drive the expression of AtTMS. Three independent T0 lines of pUBQ10:AtTMS showed significantly increased shriveled and non-viable pollen grains (Fig. 4A). To verify this result, two additional pollen-specific promoters, MICROSPORERE-SPECIFIC (pMSP1) (Honys et al., 2006) and LAT52 (Twell et al., 1989; Yao et al., 2018), were used. Similar pollen defects were noticed in ten pMSP1:AtTMS and nine pLAT52:AtTMS T0 lines, respectively (Fig. 4A). This phenotype could be inherited stably, T2 generation plants were used for further observation. In two randomly selected lines of each transformation, the level of AtTMS expression was found to correlate positively with the ratio of pollen abortion (Fig. 4B). Consistently, endogenous AtTMS was highly expressed in pollen and pollen tube, as indicated by β-glucuronidase (GUS) assays performed with pAtTMS:GUS plants (pAtTMS: 1965 bp in relation to ATG) (Supplemental Figure S3). It is worth noting that the abnormal pollen grains in three AtTMS overexpression (AtTMS-OE) transgenics were not caused by the possible variations of growth conditions, since other pLAT52 bearing transgenic plants cultured in the same growth room developed normal pollen (Tan et al., 2016; Li et al., 2017).

Phenotypic analysis was performed in two randomly chosen pMSP1:AtTMS
lines: #21 and #22. Wild-type pollen development proceeds through well-defined stages that can be easily followed by counting 4',6-diamidino-2-phenylindole (DAPI) stained nuclei (Backues et al., 2010). pMSP1:AtTMS pollen was normal at the microspore stage (Fig. 4C), however, some pollen began to display only one or no nucleus from the bicellular stage (Fig. 4C, 4D), associated to its endogenous expression pattern (Fig. 4E). Our results demonstrated that AtTMS-OE affected pollen development after the microspore stage.
**AtTMS-OE** leads to defective pollen mitosis I (PMI) cell plate and intine formation

Ectopic callose deposition was noticed in *pMSP1:AtTMS* bicellular pollen (Fig. 5A) indicating a defective membrane trafficking and cell wall deposition (Backues et al., 2010). During PMI, a distinct hemispherical cell plate forms around the generative nucleus in wild type pollen (Twell, 2011; Fig. 5B). However, in *pMSP1:AtTMS*, ~9% of PMI pollen displayed incomplete cell wall stubs between two separating nuclei (Fig. 5B). In *pMSP1:AtTMS* tricellular pollen, cellulose was barely detectable by calcofluor white staining (Fig. 5C). Further examination of *pMSP1:AtTMS* bicellular stage pollen showed that the secretion of JIM7-labeled highly methylesterified homogalacturonan was blocked (Fig. 5D).

Consistent with this, as revealed by transmission electron microscopy (TEM), the intine was not deposited properly in the same stage pollen (Fig. 5E to 5J), in some cases, irregular membrane structures were seen to accumulate just beneath the PM (Fig. 5G and 5H). However, Golgi stacks looked largely normal (Fig. 5K to 5M), indicating a post-Golgi trafficking defect. In summary, **AtTMS-OE** leads to defective cell plate and intine formation by inhibiting secretion of cell wall components.

**AtTMS-OE** causes SecGFP blockage in tobacco leaf epidermal cells that is rescued by overexpression of SYP124/125/131/132

The *syp41 syp43* double mutant displayed normal plant and pollen development (Uemura et al., 2012), indicating that the **AtTMS-OE** effect might not be due to an inhibition of TGN resident SYP4sTI, therefore, they were not included in the following assays performed in tobacco leaf epidermal cell.

In **AtTMS-OE** cells, vacuolar transport of sporamin was not affected (Supplemental Figure S4). SecGFP, the secreted form of green fluorescent protein, was then used as a secretory marker that accumulates poorly and exhibits weak fluorescence in the apoplast (Zheng et al., 2005). Notably,
in intracellular SecGFP signals accumulated when *AtTMS* was coexpressed (Fig. 5).
6B), whereas cells exhibited very weak SecGFP fluorescence with an empty vector (Fig. 6A). This provided an excellent system to determine which SYP1sTI (Fig. 3) could rescue the AtTMS-OE defects, thus acting as potential AtTMS-regulated SYP1s in vivo.

Firstly, we showed that individual mCherry-SYP1sTI under their respective native regulatory sequence (Enami et al., 2009) imparted no inhibition on SecGFP secretion (Supplemental Figure S5). Different mCherry-SYP1sTI were then coinfiltrated with SecGFP and Cerulean-AtTMS into the cells. Our results showed that SYP124, SYP125, SYP131, SYP132 (Fig. 6C to 6F), but not SYP111 (weakly interacting with AtTMS) (Fig. 6G) or SYP112 (not interacting with AtTMS) (Fig. 6H), rescued SecGFP secretion defects.

AtTMS is recruited to the plasma membrane by SYP124/125/131/132

AtTMS bears no signal peptide and lacks a transmembrane domain.
(http://octopus.cbr.su.se/), thus it might be recruited by PM-localized SYP1sTI (Uemura et al., 2004). Indeed, AtTMS displayed a sharp PM-localization when coexpressed with full length SYP124/125/131/132-FL, and a largely cytosolic pattern when coexpressed with SYP124/125/131/132-ΔTM (Fig. 3A; Fig. 7A to 7D). In addition, AtTMS could not be recruited by non-interacting SYP112 (Fig. 7E). These data suggested that AtTMS could be recruited to the PM specifically by SYP124/125/131/132.

**AtTMS competes with VAMP721/722 for the binding to SYP132, and SYP132 partially rescues pollen defects in pMSP1:AtTMS plants**

As AtTMS and R-SNAREs VAMP721/722 all bind to the Qa-domain of SYP132 (Fig. 3; Park et al., 2018), we wanted to know if AtTMS and VAMP721/722 bind to SYP132 sequentially, or if their association with SYP132 is mutually exclusive. For this purpose, equal amounts of GST-SYP132ΔTM immobilized on GST-Bind resin were incubated with His-VAMP721ΔTM (~30 kDa) in the presence of increasing concentrations of HIS-AtTMSCT1 (793 a.a.-1124...
a.a., including R-SNARE motif, ~46 kDa), or HIS-SYP31Δ™ (~41 kDa) which is a
Golgi resident Qa-SNARE. As shown in Fig. 8A and 8B, the interaction between
VAMP721Δ™ and SYP132Δ™ was substantially significantly reduced in the
presence of increasing amount of HIS-AtTMSCT1, but not HIS-SYP31Δ™.
Therefore, AtTMS and VAMP721 specifically compete for the binding site in
SYP132. Moreover, the binding of AtTMS and VAMP722 to SYP132 is mutually
exclusive as well (Fig. 8C and 8D).

The syp124 syp125 syp131 triple mutant showed normal male
gametogenesis, but pollen tube growth arrest (Slane et al., 2017). Therefore, we
choose SYP132 among SYP124/125/131/132 to see whether SYP132 could rescue the *AtTMS-OE* defects in Arabidopsis. Two independent transgenic lines bearing *pSYP132:mCherry-SYP132* and *pMSP1:AtTMS* were generated by crossing, and we found that SYP132 could reduce the amount of defective pollen by about 15%, thereby partially rescuing the *AtTMS-OE* phenotype (Fig. 8E).

**Dex-induced *AtTMS* expression causes pleiotropic phenotype related to secretion blockage**

To see the possible effects of *AtTMS-OE* in sporophytic growth, plants expressing *AtTMS* under the dexamethasone (DEX)-inducible promoter were generated. Upon DEX treatment, *AtTMS-OE* seedlings became dwarfed with short root hairs. Root cells showed cytokinesis defects with multiple nuclei and cell wall stubs, and JIM7 antigen secretion was blocked in the root hairs (Supplemental Figure S6). These phenotypes suggested a negative role of *AtTMS* in secretory vesicle fusion during vegetative growth as well.
DISCUSSION

AtTMS-OE inhibits exocytosis in developing pollen

AtTMS is a Tomosyn homolog that contains the highly conserved R-SNARE motif (Fig. 1). In this study, the physiological function of AtTMS in plant development was explored. Overexpression of AtTMS driven by three different promoters, pUBQ10, pMSP1 and pLAT52, caused high percentage of shriveled mature pollen. Notably, the ratio of abnormal pollen is positively correlated with the AtTMS expression level in all three independent transgenic events (Fig. 4). The abnormal pollen development was only detectable from the bicellular stage onwards, when the endogenous AtTMS starts expressing.

Pollen development involves intensive secretion which is required for intine development and cytokinesis during PMI (Shi et al., 2015). In pMSP1:AtTMS bicellular pollen, the highly methylesterified homogalacturonan was retained inside the cell (Fig. 5), and the intine structures were not detected. Instead, large amounts of irregular membranes accumulated beneath the PM (Fig. 5), meanwhile, the expansion of the cell plate during PMI, a redirected secretion event (Jürgens, 2015), was affected (Fig. 5). These data implied that AtTMS plays a predominantly inhibitory role in secretory trafficking in developing pollen.

The mutants of attms have no detectable phenotype (Supplemental Fig. S2). There is an AtTMS-like (AtTMSL) protein in Arabidopsis which bears an R-SNARE-like motif without the conserved Arginine residue. It will be interesting to explore the attmsl mutants in the future.

AtTMS interacts with pollen-expressed PM-localized SYP1sTI, and four SYP1sTI could rescue AtTMS-OE secretion defects, respectively

In the present study, we found several AtTMS binding partners. AtTMS binds to pollen-expressed PM-localized SYP1sTI which is consistent with its subcellular localization (Fig. 2 and Fig. 3). These interactions are highly specific, since AtTMS did not interact with non-pollen expressed SYP112/121/122/123 (Fig. 3), implying a specific, endogenous role of AtTMS in pollen development.

SYP124/125 were first detected during PMI, while SYP131 was detected at
advanced stages of floral development (Ichikawa et al., 2015). Although the
syp124 syp125 syp131 triple mutant showed normal male gametogenesis (Slane
et al., 2017), they might have an overlapping function with SYP132 which
displayed a clear PM localization in many tissues, including developing pollen
(Enami et al., 2009; Karnahl et al., 2018). In agreement with this, overexpressing
SYP124, SYP125, SYP131, or SYP132 rescued SecGFP trafficking defects
caused by AtTMS-OE (Fig. 6). In addition, SYP132 transgene could partially
rescue the pMSP1:AtTMS pollen phenotype (Fig. 8). Although SYP111 localizes
to the PMI cell plate and maybe participates in cell plate expansion (Lee et al.,
2007; Slane et al., 2017), it only weakly interacted with AtTMS (Fig. 3), and
SYP111 could not rescue SecGFP defects (Fig. 6). Therefore, in pollen, AtTMS-
OE inhibits secretion probably through binding to the SNARE domain of
SYP124/125/131/132.

Brefeldin A (BFA) blocks the functions of BFA-sensitive ARF-GEFs and this
aggregates Golgi and post-Golgi compartments, such as the TGN and
endosomes (Dettmer et al., 2006). SYP124/125 mainly localized to the BFA
compartment, suggesting that SYP124/125 functions in both recycling and
secretion (Slane et al., 2017), whereas SYP131/132 mainly serves the secretion
of newly synthesized proteins (Karnahl et al., 2018; Park et al., 2018). As AtTMS
also presents at the TGN, AtTMS might be able to inhibit the recycling pathway
as well during pollen development.

Dex-inducible AtTMS-OE seedlings display a range of phenotypes that could
be attributed to secretion defects, suggesting AtTMS plays a similar role in
vegetative growth.

**AtTMS might participate in the formation of nonfusogenic complexes**

Animal Tomosyn contains an R-SNARE motif which shares sequence
similarity with that of the R-SNARE synaptobrevin and has been shown to bind
Syntaxin and SNAP-25, forming a SNARE-like complex precluding synaptobrevin
(Ashery et al., 2009). Our data suggested that a similar model of AtTMS action
might hold true in plants. AtTMS lacks a membrane anchor and cannot act as a
true, fusion-active R-SNARE. In the *AtTMS-OE* plants, each pair of AtTMS-SYP1 (AtTMS with SYP124, 125, 131, or 132) is nonfusogenic, preventing genuine R-SNAREs (VAMP721 or VAMP722) from entering the SNARE complexes whereby the secretion events are blocked, resulting in defective pollen development. Therefore, AtTMS might serve to fine-tune the active secretion events in pollen development.
**MATERIALS AND METHODS**

**Plant growth conditions**

*Arabidopsis thaliana* (L.) Heynh ecotype Columbia-0 (Col-0) was used in the experiments. Seeds were surface sterilized for 1 min in 70% (v/v) ethanol, followed by 10 min incubation in 2% (v/v) NaClO with occasional mixing, and washed five times with sterile distilled water. Seeds were germinated and grown on Murashige and Skoog (MS) plates containing 1% (w/v) agar. *AtTMS-OE* seeds were germinated on the same medium supplemented with $2.5 \times 10^{-2}$ mg ml$^{-1}$ Hygromycin. After 7 days, the seedlings were transferred to soil and grown at 22°C with a 16 h : 8 h light : dark photoperiod in a growth room. Tobacco plants (*Nicotiana benthamiana*) were grown at 25°C with a 16 h : 8 h light : dark photoperiod in a growth chamber.

**Phylogenetic analysis**

Homologs of Arabidopsis AtTMS were identified using the BLASTP search program of the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov). The phylogenetic tree was constructed using MEGA 7.0 (http://www.megasoftware.net), based on the neighbor-joining method with the following parameters: p-distance model, pairwise deletion, and bootstrap (1000 replicates; random seed).

**Subcellular localization assay in Arabidopsis protoplasts**

To study the subcellular localization of AtTMS protein, two transient expression systems were used. First, the CDS of *AtTMS* was cloned into pAN580 to create p35S:eGFP-AtTMS, which was transformed into Arabidopsis protoplasts with or without fluorescent markers (Lee et al., 2017). Second, the coding sequence of *AtTMS* were cloned into pCambia1305 to obtain a pUBQ10:eGFP-AtTMS construct, which was introduced into *Agrobacterium tumefaciens* (EHA105), infiltrated into tobacco (*Nicotiana benthamiana*) leaves, and observed after 72 h (Wu et al., 2017). To study the recruitment of AtTMS by various SYPs, the coding sequence of SYP112/124/125/131/132, and the
SYP124/125/131/132-△TM was cloned into pAN583 vector, respectively, to obtain N-terminal mCherry fusion constructs. Pairs of constructs were transformed into Arabidopsis protoplasts for observation.

**Yeast Two-Hybrid assay**

Yeast-two hybrid analysis was performed using the Matchmaker GAL4 Two-Hybrid System according to the supplier’s instructions (Clontech), and was performed as described previously (Tan et al., 2016). In the assay, the open reading frame, NT and CT of AtTMS were subcloned into pGBK7, while the Qa-domain of SYP111/112/121/122/123/124/125/131/132/41/42/43 was cloned into pGAD7, respectively.

**Firefly LCI assay**

For the firefly LCI assay, the ORF, NT and CT of AtTMS were cloned into vector pCAMBIA1300-cLuc to generate cLUC-AtTMS, respectively. Meanwhile, the cytoplasmic fragments of SYP111/112/121/122/123/124/125/131/SYP132/41/42/43 were ligated into pCAMBIA1300-nLUC to generate SYPs-△TM-nLUC, respectively. The firefly LCI assay were performed as previously described (Chen et al., 2007; Wu et al., 2017).

**GST pull-down assay**

The C-terminus of AtTMS containing the R-SNARE motif was cloned into the pET28a to generate HIS-AtTMS-CT (2766-3372 bp), The cytoplasmic portions of SYP/112/121/122/123/124/125/131/132/41/42/43 were ligated into pGEX4T-1, to generate GST-SYPs-△TM. The GST pull-down assays were performed as previously described (Wu et al., 2013).

**Generation of AtTMS-OE and knockout lines**

To overexpress AtTMS gene, the coding sequence (CDS) of AtTMS was cloned into pCambia1300 (which containing either an UBQ10 or MSP1 or Lat52
promoter) at the XbaI sites to generate pUBQ10:AtTMS, MSP1:AtTMS or Lat52:AtTMS, and the CDS of AtTMS was also cloned into pTA7002 (which containing a DEX-inducible promoter) at XhoI and SpeI. To create AtTMS knockout mutants, a RNA targeting the sequence within the 8th exon (ATCTATGGTGGTGATATCAT) was cloned into the pAtU6-26:sgRNA-2x35S:Cas9 pBlunt vector. The resulting constructs were transformed into Col-0 using floral dipping (Clough and Bent, 1998).

**RT-qPCR Analysis**

Total RNA was isolated using the RNA prep pure plant kit (Tiangen). The first-strand cDNA was synthesized using oligo(dT)$_{18}$ as the primer and PrimeScript reverse transcriptase (TaKaRa). Arabidopsis gene Tubulinβ8 (TUB8) was used as an internal control. Quantitative analysis was performed using an ABI 7500 real-time qPCR system with the SYBR Green Mix (Bio-Rad) and three biological repeats (Tan et al., 2016).

**Phenotype characterization in pollen**

To visualize nuclei, pollen grains were collected into a 4',6-diamidino-2-phenylindole (DAPI) staining solution (0.1 M sodium phosphate pH 7.0, 1 mM EDTA, 0.1% (v/v) Triton X-100, and 0.5 mg/ml DAPI) and examined 15 min after staining. Pollen viability was examined using Alexander staining (Tan et al., 2016). Cell plate and deposited callose in pollen were revealed using aniline blue staining. To observe cellulose deposition, anthers were fixed in a formalin-acetic acid-alcohol solution overnight, embedded and sectioned to 1 µm-thick slices with a Leica EM UC7 microtome. After 10 mg/ml Propidium iodide (PI), 35 mg/mL Calcofluor white staining the sections were observed using confocal microscopy (LSM780, Zeiss, Germany). To observe JIM7 antigen distribution, wild-type and pMSP1:AtTMS anthers were fixed in 0.1 M Phosphate Buffer (PB), embedded in LR White resin. 1 µm-thick sections were blocked in a buffer [3% (w/v) BSA in PBS, 100 mM potassium phosphate, 138 mM NaCl, and 2.7 mM KCl, pH 7.3] for 30 min, washed with PBS, and incubated with the JIM7 antibodies (Carbo souse)
for 1 h. After washing, the sections were labeled with Alexa 488 conjugated secondary antibodies (Life technologies; 1322327) for 30 min before analysis.

**GUS staining**

Histochemical staining for GUS expression in transgenic plant materials was performed according to the method by (Tan et al., 2016).

**Transient expression in tobacco leaf epidermal cells**

Each construct for transient expression was transformed into *Agrobacteria*. For subcellular localization studies, individual bacterial strains (OD$_{600} = 0.1$) bearing *AtTMS* or various fluorescent markers were infiltrated together into tobacco leaf epidermal cells. For functional studies, different combinations of an *Agrobacteria* strain bearing *Cerulean-AtTMS, SecGFP, Spo-mCherry, mCherry-SYP111, mCherry-SYP124, mCherry-SYP125, mCherry-SYP131, mCherry-SYP132* and *mCherry-SYP112* constructs were infiltrated. The OD$_{600}$ of bacteria used for these infiltrations was 0.05 for *SecGFP*, 0.5 for *Cerulean-AtTMS*, 0.1 for *Spo-mCherry*, 0.08 for *mCherry-SYP111, mCherry-SYP124, mCherry-SYP125*, and 0.5 for *mCherry-SYP131, mCherry-SYP132* and *mCherry-SYP112*. After 72 h of infiltration, the leaves were observed using confocal microscopy.

**Scanning and transmission electron microscopy**

For scanning electron microscopy (SEM) observation, mature pollen grains were coated with gold particles (EIKOIB-3) and observed using a HITACHI S-2000N scanning electron microscope. For TEM observation, anthers were immediately frozen in a high-pressure freezer (EM HPM100, Leica, Wetzlar, Germany), followed by subsequent freeze substitution in 100% (v/v) acetone containing 0.1% (w/v) uranyl acetate at -85°C in an AFS freeze substitution unit (Leica), and substituted with 2% (w/v) OsO$_4$ in 100% acetone and infiltrated with Epon resin as previously described (Tan et al., 2016). Sections were examined using a transmission electron microscope (H-7650, Hitachi, Tokyo, Japan) with a charge-coupled device camera (Hitachi High-Technologies) operating at 80 kV.
All primers used in this study were listed in Supplemental Table 1.

**ACCESSION NUMBERS**

Sequence data from this article can be found in the Arabidopsis Information Resource (TAIR) under the following accession numbers: *AtTMS* (AT5G05570), *SYP111/KNOLLE* (At1g12360), *SYP112* (AT2G18260), *SYP121* (AT3G11820), *SYP122* (AT3G52400), *SYP123* (AT4G03330), *SYP124* (AT1G61290), *SYP125* (AT1G11250), *SYP131* (AT3G03800), *SYP132* (AT5G08080), *SYP41* (AT5G26980), *SYP42* (AT4G02195), *SYP43* (AT3G05710), *AtTMS-like* (AT4G35560).

**SUPPLEMENTAL DATA**

**Supplemental Figure S1.** The localization of *AtTMS* in tobacco leaf epidermal cells.

**Supplemental Figure S2.** Generation of *attms* mutants by CRISPR/CAS9-mediated gene editing.

**Supplemental Figure S3.** The expression pattern of *AtTMS:GUS* in Arabidopsis.

**Supplemental Figure S4.** Overexpression of *Cerulean-AtTMS* does not inhibit vacuolar trafficking of Sporamin in tobacco leaf epidermal cells.

**Supplemental Figure S5.** mCherry-tagged *SYP111*, *SYP124*, *SYP125*, *SYP131*, *SYP132* and *SYP112* alone under their respective promoter does not affect SecGFP secretion in tobacco leaf epidermal cells.

**Supplemental Figure S6.** Overexpression of *AtTMS* under the control of a (DEX)-inducible promoter affected vegetative growth of Arabidopsis.

**Supplemental Table 1.** The list of primers used in this study.

**Supplemental Data Set 1.** The text file of sequence alignment used for the phylogenetic analysis in Figure 1A.

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FIGURE LEGENDS

Figure 1. AtTMS is homologous to animal Tomosyns.
(A) Left panel: phylogenetic relationship among AtTMS, other plant Tomosyn
homologs, and animal Tomosyn proteins. The phylogenetic tree was constructed
using MEGA version 7.0. Species abbreviations: Mt, Medicago truncatula; Gm,
Glycine max; Br, Brassica rapa; At, Arabidopsis thaliana; Os, Oryza sativa; Zm,
Zea mays; Sb, Sorghum bicolor; Si, Setaria italica; Smo, Selaginella
moellendorfii; Mp, Marchantia polymorpha; Ppa, Physcomitrella patens; Cre,
Chlamydomonas reinhardtii; Mm, Mus musculus; Rn, Rattus norvegicus; Hs,
Homo sapiens; Brv, Brachydanio rerio var; Dm, Drosophila melanogaster; Ce,
Caenorhabditis elegans; The sequence alignment used for this analysis is
available as Supplemental Data Set 1. Right panel: Domain structure of each
Tomosyn family member is presented next to the corresponding protein.
(B) Sequence alignment of R-SNARE motif of Tomosyn proteins. Numbers at the
top indicate helical layers formed during SNARE complex assembly. Arrow
indicates the Arginine residue (R) at the layer 0.

Figure 2. Subcellular localization of AtTMS.
(A) to (D) eGFP-AtTMS was co-expressed with known organelle markers in
Arabidopsis protoplasts. Images were collected by confocal microscopy after 16
to 18 h incubation. The nonlinear Spearman’s rank (rs) correlation coefficient
(PSC) between eGFP-AtTMS and each marker are shown in the rightmost panel.
Bars = 10 µm.

Figure 3. C-terminus of AtTMS and the SNARE-domain of SYPs mediate
mutual binding between SYPs11 and AtTMS.
(A) Schematic presentation of truncations for SYP proteins.
(B) A yeast two-hybrid assay showed that AtTMS interacts with the Qa-domain of
SYP111/124/125/131/132/41/43. Transformed yeast cells able to grow and turn blue on TDO + 5 µM 3AT + 200 ng/ml X-α-gal plate indicates positive interactions. DDO: Double dropout medium (-Leu-Trp), TDO: Triple dropout medium (-Leu-Trp-His).

(C) Schematic representation of the size and position of each AtTMS truncation. WD40 domain (blue) and R-SNARE motif (red) were indicated.

(D) SYP111/124/125/131/132/41/43-\(\Delta\)TM interact with both the AtTMS\(^{FL}\) and the AtTMS\(^{CT}\) in firefly luciferase complementation imaging assays. Different pairs of indicated plasmids were co-transformed in tobacco leaves. A pair of cLUC and nLUC empty vectors were used as negative controls (Empty). The luciferase signal was captured using a low-light cooled CCD camera and the color scale on the right-hand side of the panel shows the range of luminescence intensity.

(E) Only SYP111/124/125/131/132/41/43-\(\Delta\)TM can interact with the AtTMS\(^{CT}\). GST and each GST-SYP\(\Delta\)TM protein coupled to beads were incubated with His tagged AtTMS\(^{CT}\). Bound proteins were detected using mouse anti-His antibody. The blot was stripped and re-blotted with mouse anti-GST antibody to ensure the quality and coupling of the bait proteins. * indicates nonspecific or degraded protein bands.

(F) Diagram of the mode of interaction between AtTMS and SYPs\(\text{Tl}\). Gray text for SYP111 indicates a weak interaction.

Figure 4. AtTMS-OE impairs pollen development after the microspore stage.

(A) Scanning electron microscopy (top panel) and Alexander-staining (bottom panel) micrographs of pollen grains from WT and AtTMS-OE plants, using different promoters. The arrows indicate the shrunken and nonviable pollen grains.

(B) #1, #3 of pUBQ10:AtTMS; #21, #22 of pMSP1:AtTMS and #4, #5 of pLAT52:AtTMS transgenic lines were chosen randomly for analysis. Left-hand graph, mRNA used for RT-qPCR was extracted from flower buds, AtTMS expression relative to that of the wild type was shown for each line. Right-hand
graph, n > 200 pollen was counted for each line. Values represent the means ± SD of three independent experiments.

(C) pMSP1:AtTMS-#22 pollen was stained with DAPI and visualized under fluorescent (top row) or bright field (bottom row) optics. Arrows indicate abnormal pollen.

(D) Quantification of abnormal DAPI-stained pollen. n > 200 pollen was counted for each line. Values represent the means ± SD of three independent experiments, **indicates P < 0.001 by Student’s t-test.

(E) AtTMS expression starts from the bicellular stage. GUS-stained pollen from pAtTMS:GUS plants in the lower panel was further stained with DAPI (upper panel) before observation.

Bars = 10 µm in (A), (C) and (E).

Figure 5. The phenotype of pMSP1:AtTMS pollen.

(A) Ectopic callose deposition found in bicellular pMSP1:AtTMS pollen. The color scale under the panel shows the range of callose intensity.

(B) Cell wall stubs were detected at PMI in pMSP1:AtTMS pollen. DAPI (red) and aniline blue (blue) stained nuclei and callose, respectively. The quantification of abnormal cell plate formation is shown underneath the micrographs. n > 100. Values represent the means ± SD of three independent experiments.

(C) Cellulose deposition is defective in pMSP1:AtTMS bicellular pollen. Anthers were fixed in a formalin-acetic acid-alcohol solution overnight, embedded and sectioned in 1 µm-thick slices. The blue fluorescence represents cellulose labeled by calcofluor white (CW). Red fluorescence signals denote cytoplasm stained by propidium iodide (PI). Arrows indicate the arrested pMSP1:AtTMS pollen.

(D) JIM7 positive signals were blocked inside pMSP1:AtTMS bicellular pollen. Highly methylesterified homogalacturonan labeled by mouse JIM7 monoclonal antibodies (green, left panel), exine autofluorescence (red, middle panel), merged images (right panel).
(E) TEM micrograph of a normal bicellular pollen in *pMSP1:AtTMS* anthers.

(F) A magnified view of blue boxed area in (E). Intine was indicated between two triangles. In: intine. (G) TEM micrograph of abnormal bicellular pollen in *pMSP1:AtTMS* anthers. V: vacuole.

(H) A magnified view of red boxed area in (G). Purple arrows indicate abnormal membrane structures.

(I) TEM micrograph of a shriveled bicellular pollen in *pMSP1:AtTMS* anthers.

(J) A magnified view of black boxed area in (I). In?: abnormal intine formation.

(K) to (M) Golgi stacks from a normal (K), and defective (L and M) bicellular stage pollen in *pMSP1:AtTMS*. Two triangles point to normal or abnormal intine. The blue arrows indicate the Golgi stacks. V: vacuole; In?: abnormal intine formation; In: intine. The blue arrows indicate the Golgi stacks.

Bars = 5 µm in (A), (B) and (D); 10 µm in (C); 2 µm in (E), (G) and (I); 1 µm in (F), (H) and (J); 500 nm in (K) to (M).

**Figure 6.** Intracellular SecGFP accumulation caused by *AtTMS-OE* is released by coexpression of *SYP124/125/131/SYP132*.

(A) and (B) Overexpression of *Cerulean-AtTMS* (B), but not *Cerulean* alone (A), inhibits the trafficking of SecGFP to the apoplast of tobacco leaf epidermal cells. *Cerulean* fluorescent protein was used to create *Cerulean-AtTMS* fusion protein. In panel A, *Cerulean* highlights nonspecifically the nucleus as a bright blue spot.

(C) to (H) Different combinations of plasmids were coinfiltrated into the tobacco leaf epidermal cells, and assayed after 72 hr. *Cerulean* protein alone (A) or *Cerulean-AtTMS* fusion protein (B-H) were presented in cyan, SecGFP protein in green, and mCherry-SYP1s in red.

Bars = 50 µm in (A) and (B), 20 µm in (C) to (H).

**Figure 7.** *AtTMS* is recruited to the PM by *SYP124/125/131/132*.

(A) *AtTMS* displayed a sharp PM localization when coexpressed with mCherry-

*SYP124FL* (1st row), and a largely cytosolic pattern with mCherry-

*SYP124ΔTM* (2nd row). In the far-right panel, the fluorescence intensity along the...
white lines was measured by a plot profile plugin for ImageJ. PM localization was indicated by arrows. (B) to (E) AtTMS could be recruited by SYP125 (B), SYP131 (C), and SYP132 (D), but not by SYP112 (E). Bars = 5 µm.

**Figure 8.** AtTMS competes with VAMP721/722 for the binding to SYP132, SYP132 partially rescues defects of pMSP1:AtTMS pollen

(A) and (B) AtTMSCT1 competes with VAMP721\(^{\Delta TM}\) for binding to SYP132\(^{\Delta TM}\). In each lane, 6 µg GST-SYP132\(^{\Delta TM}\) proteins coupled to beads were incubated with 2 µg VAMP721\(^{\Delta TM}\) and increasing amount (1 µg, 3 µg, 6 µg, 9 µg) of HIS-AtTMSCT1, respectively (A) 6 µg GST-SYP132\(^{\Delta TM}\) proteins, 2 µg VAMP721\(^{\Delta TM}\) with increasing amount of Golgi resident Qa-SNARE HIS-SYP31\(^{\Delta TM}\) (B). After washing, Bound proteins were detected using anti-His antibodies. (C) and (D) The same method used in (A) and (B) were adopted. Similarly, AtTMSCT1 was shown to compete with VAMP722\(^{\Delta TM}\) for the binding to SYP132\(^{\Delta TM}\).

(E) Confocal images of pollen grains from WT, pMSP1:AtTMS and pMSP1:AtTMS + pSYP132:mCherry-SYP132. Arrows and asterisks indicate collapsed pollen and mCherry-SYP132-expressing pollen, respectively. Note that collapsed pollen grains display specific red fluorescence. Left-hand graph, mRNA used for RT-qPCR was extracted from flower buds, AtTMS expression relative to that of wild type was presented. Right-hand graph, One pMSP1::AtTMS line showed approximately 45% abnormal pollen grains, whereas two lines of pSYP132::mCherry-SYP132 + pMSP1::AtTMS showed about 30% abnormal pollen grains. n > 200 pollen was counted for each line. Values represent the means ± SD of three independent experiments. Statistical significance using Duncan (D) ANOVA is indicated by lowercase letters (P < 0.05). Bars = 10 µm.
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