Co-option of the SHOOT MERISTEMLESS network regulates protocorm-like body development in Phalaenopsis aphrodite

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Abstract

The protocorm is a structure that is formed upon germination of an orchid seed. It lacks cotyledons and is ovoid in shape. The protocorm-like body (PLB), on the other hand, is a protocorm-like organ induced from somatic tissues. PLBs have been widely used for orchid micropropagation. Because of its unique structure and its application in the orchid industry, PLB development has drawn considerable interest from orchid and developmental biologists. Our previous genome-wide comparative transcriptome study demonstrated that protocorms and PLBs share similar molecular signatures and suggested that SHOOT MERISTEMLESS (STM)-dependent organogenesis is important for PLB development. Here, we show that overexpression of Phalaenopsis aphrodite STM (PaSTM) greatly enhances PLB regeneration from vegetative tissue-based explants of Phalaenopsis orchids, confirming its regulatory role in PLB development. Expression of PaSTM restored shoot meristem function of the Arabidopsis (Arabidopsis thaliana) stm-2 mutant. Moreover, we identified class S11 MYB transcription factors (TFs) as targets downstream of PaSTM. A cis-acting element, TTGACT, identified in the promoters of S11 MYB TFs was found to be important for PaSTM binding and activation. Overexpression of PaSTM or its downstream targets, PaMYB13, PaMYB14, and PaMYB17, enhanced de novo shoot regeneration in Arabidopsis, indicating the active role of the PaSTM-S11 PaMYB module in organogenesis. In summary, our data demonstrate that PaSTM is important for PLB development. The STM-S11 MYB regulatory module is evolutionarily conserved and may regulate shoot or shoot-related organ development in plants.

Introduction

Orchid plants have evolved distinct floral morphology and specialized development programs that have drawn considerable interest from developmental biologists and evolutionary biologists. For example, ovule development in orchids is initiated by pollination, which is distinctly different from other flowering plants whose ovules are fully developed at anthesis and whose egg cells are ready to be fertilized upon pollination. As a result, in orchids, ovule development and fertilization are separated by fairly long time periods ranging from 4 d (e.g. Gastrodia elata) to 10 months (e.g. Vanda suavis; Arditti,
In addition, orchid embryogenesis produces incomplete embryos that are arrested at the globular stages and lack characteristic organogenesis (Raghavan and Goh, 1994; Yu and Goh, 2001; Kull and Arditti, 2002). Even so, orchid embryos that lack cotyledons complete maturation and desiccation processes during seed development. Upon germination, the embryo, which lacks complete organogenesis, emerges from the seed coat and grows into a small ovoid structure that is referred to as a protocorm. Unlike the embryos of other plant species whose shoot and root meristems are established early during embryogenesis, the anterior meristematic domain of the protocorn becomes active after germination, and leaves and roots are produced sequentially (Nishimura, 1981).

Organogenesis is fundamental to the establishment of the basic body plan. De novo organogenesis is initiated by reprogramming somatic cells into pluripotent stem cells, a process that requires broad changes in transcriptional regulatory networks and hormone regulation (Duclercq et al., 2011). The ratio of auxin to cytokinin provides the guiding principle for de novo organogenesis over a wide range of plant species (Skoog and Miller, 1957; Sangwan and Sangwan-Norreel, 1990; De Klerk et al., 1997; Ikeuchi et al., 2016). A high cytokinin-to-auxin ratio often triggers shoot organogenesis, whereas a low cytokinin-to-auxin ratio initiates root development. During de novo organogenesis in Arabidopsis (Arabidopsis thaliana), auxin primes pericycle cells to initiate a lateral root program and activates cell division and subsequent formation of callus mass (Surugimoto et al., 2010; Cheng et al., 2013; Kcurshumova et al., 2014; Kareem et al., 2015). Activation of cell division and formation of callus is a prerequisite for subsequent shoot formation triggered by cytokinin (Cary et al., 2002; Gordon et al., 2007; Atta et al., 2009). The partition of auxin and cytokinin responses guides the cell fate specification and domain formation of the shoot apical meristem (SAM; Gordon et al., 2007, 2009). Localized perception of cytokinin provides a positional cue to establish the WUSCHEL/CLAVATA regulatory module, which is required for organization of the SAM (Gordon et al., 2009; Kareem et al., 2015).

The SAM provides pluripotent stem cells that contribute to organogenesis. Initiation and maintenance of SAM is regulated by the Class I KNOTTED1-like homeobox (KNOX) gene family (Endrizzi et al., 1996; Long et al., 1996; Kerstetter et al., 1997). Expression of the Class I KNOX is associated with organogenic shoot formation in a diverse range of monocot and eudicot plants (Hake et al., 2004; Garceàs et al., 2012). Ectopic expression of the class I KNOX genes triggers the formation of ectopic shoots or organ-related structures (Sinha et al., 1993; Chuck et al., 1996; Williams-Carrier et al., 1997; Golz et al., 2002; Lenhard et al., 2002). During de novo shoot regeneration, expression of the Arabidopsis class I KNOX transcription factor (TF), SHOOT MERISTEMLESS (STM) marks the newly emerging SAMs (Gordon et al., 2007; Atta et al., 2009; Kcurshumova et al., 2014; Iwase et al., 2017). The activity of Arabidopsis STM is essential for de novo shoot formation (Daimon et al., 2003).

Protocorn-like bodies (PLBs) resemble protocorms structurally but are derived from somatic explants via the de novo regeneration pathway (Jones and Tisserat, 1990; Chugh et al., 2009). Similar to protocorms, each PLB has the ability to regenerate into an individual plant. PLB-based micropropagation is therefore commonly used to produce clonal plantlets in the orchid industry (Yam and Arditti, 2009). However, vegetative tissue-based induction of PLBs is not trivial. Choices of explants, growth conditions, culture media, and tissue culture techniques need to be optimized to meet the requirements of the orchid industry (Cardoso et al., 2020). Hence, establishment of a robust PLB-based micropropagation platform is crucial for orchid biotechnology. Although the PLB has been considered to be a somatic embryo (Begum et al., 1994; Chang and Chang, 1998; Ishii et al., 1998; Zhao et al., 2008; Lee et al., 2013), our molecular study provided compelling evidence to argue that the PLB, which has a similar molecular network to the protocorn, is not a somatic embryo (Fang et al., 2016; Chen et al., 2019). With contrasting reports, a clear developmental identity of the PLB still remains elusive.

We are interested in deciphering the molecular origin of the unique PLB structure and exploring its potential to improve clonal propagation in the orchid industry. Based on our previous study, we hypothesized that the Class I KNOX gene PaSTM-based developmental process is involved in PLB regeneration (Fang et al., 2016). The goal of this study was, therefore, to test whether PaSTM functions to regulate PLB development in Phalaenopsis (Phalaenopsis aphrodite) orchids. We were also interested in gaining mechanistic insights into PLB development and reconstructing the regulatory network governed by PaSTM. Toward this end, we generated transgenic Phalaenopsis orchids overexpressing PaSTM and assessed its impact on de novo PLB regeneration. We also identified the PLB-specific TFs and tested their connection to PaSTM. Among the PLB-specific TFs, members of the class S11 MYB TFs (Dubos et al., 2010) were identified as downstream targets of PaSTM. Therefore, our data point to the existence of a STM-S11 MYB regulatory module for PLB development. Furthermore, our results support de novo PLB assembly as being one type of organogenesis, although PLB development is different from de novo shoot development.

Results

PaSTM restores the shoot meristem function of Arabidopsis stm-2

Because PaSTM and Arabidopsis STM forms a paired clade phylogenetically (Fang et al., 2016), we predicted that PaSTM can functionally replace Arabidopsis STM and restore the SAM function of the stm mutant. To test this idea, plants homozygous for 35S:PaSTM (Fang et al., 2016) were crossed to heterozygous stm-2 mutant (Clark et al., 1996). The progeny carrying the stm-2 allele and 35S:PaSTM transgene were allowed to self-pollinate and generate progeny that are homozygous for stm-2 and carry the 35S:PaSTM transgene. Twenty-two kanamycin-resistant progeny carrying the 35S:PaSTM transgene were genotyped (Belles-Boix et al., 2006) for the stm-2 allele. The stm-2 allele segregated in the
expected 1:2:1 ratio (stm-2−/−: stm-2−/+ : wt = 5:12:5). Homozygous stm-2 mutants failed to produce a SAM but formed an axillary shoot meristem that developed into a leaf-like structure (Figure 1A), similar to previous reports (Clark et al., 1996; Endrizzi et al., 1996). The 35S:PaSTM transgene restored the SAM activity of homozygous stm-2 mutant (stm-2−/− 35S:PaSTM) and allowed production of normal-looking leaves 10 d after germination (DAG; Figure 1A). As the plants grew older, the leaf blades of the stm-2−/− 35S:PaSTM plants grew curved and lobed at 15 DAG, showing a phenotype similar to PaSTM overexpressors (Fang et al., 2016). The flowers of stm-2−/− 35S:PaSTM either lacked a carpel in the center of the flower or had unfused carpels with exposed ovule-like tissues and were female sterile (Figure 1B), indicating an incomplete rescue (Grigg et al., 2005). Together, our data demonstrated the shoot meristem function of PaSTM. However, PaSTM was unable to restore the floral meristem defect caused by stm-2−/−.

**Overexpression of eGFP-PaSTM in *P. aphrodite* is sufficient to induce ectopic PLBs**

To test the ability of PaSTM to induce de novo PLB generation in *P. aphrodite*, transgenic Phalaenopsis orchids overexpressing eGFP-PaSTM were generated. We chose the eGFP-PaSTM construct because it has higher activity than PaSTM-eGFP in inducing SAM activity (Fang et al., 2016). Similar to PaSTM overexpressors, overexpression of eGFP-PaSTM induces curved and lobed leaves in Arabidopsis (Fang et al., 2016), a common phenotype observed from overexpressing the class I KNOX TFs (Chuck et al., 1996; Sakamoto et al., 1999; Ori et al., 2000). After genotyping (Supplemental Figure S1A), six out of eight T0 35S:eGFP-PaSTM transgenic orchid plants had expression of the eGFP-PaSTM protein (Supplemental Figure S1B). We chose to examine 35S:eGFP-PaSTM #5, #7, and #8 transgenic lines because they had relatively high protein level.

Because it takes 5–7 years to obtain T1 seedlings (Chen et al., 2019), we decided to use T0 transgenic orchid seedlings for PLB regeneration test. Transgenes have been reported to be distributed homogenously in the T0 protocorm tissues after antibiotic selection (Hsing et al., 2016). Explants from cut tissues of 35S:eGFP-PaSTM seedlings were used for PLB generation. Transgenic plants carrying the empty vector were used as the negative control. After incubating explants in regeneration medium for ~2–3 months, PLBs started to emerge from the cutting surface of explants derived from internodes, young leaves, or root tips of 35S:eGFP-PaSTM #5, #7, and #8 lines (Figure 2A). Explants from completely differentiated tissues such as roots and fully expanded leaves were relatively recalcitrant to PLB induction.

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**Figure 1** Overexpression of PaSTM rescues shoot meristem function of Arabidopsis *stm-2.* A. Overexpression of PaSTM rescues the activity of the SAM. Notice that leaves of *stm-2*, 35S:PaSTM and 35S:PaSTM appeared to be wavy and curved at 15 DAG. B. Overexpression of PaSTM failed to completely rescue the floral meristem activity. White arrowheads indicate lack of carpels in flowers. Arrow indicates unfused carpel with ovule-like structures. Red bar = 1 mm. Blue bar = 500 μm.
Figure 2 Overexpression of PaSTM-eGFP in Phalaenopsis orchids enhances PLB generation. A, PLB induction from explants of T0 35S:PaSTM-eGFP transgenic seedlings. Scale bar, 1 mm. The tissues in the red rectangle of the top were enlarged in the bottom panel. Notice that new shoots (marked by yellow asterisks) were able to grow from explants containing the axillary meristem. White asterisk, individual PLB. Red asterisk, PLB with a developing leaf. B, PLB induction from protocorm explants of T1 35S:PaSTM-eGFP transgenic plants. Scale bar, 1 mm. White asterisk, individual PLB. Red asterisk, PLB with a developing leaf. C, Subcellular localization of PaSTM-eGFP protein in the PLB explant of T1 35S:PaSTM-eGFP #5 transgenic line. The top image is a composite figure. The magnified views of the protocorm explant labeled in red and blue rectangles are shown. Blue bar, 100 μm. Red bar, 50 μm. DIC, differential interference contrast images of cells superimposed with PaSTM-eGFP (GFP). A nonspecific GFP signal was sometimes detected on the surface of the explant (Supplemental Figure S2E). D, The BiFC signal was detected in protoplast cells co-transfected with C-PaSTM-cEYFP and N-nEYFP-PaBEL1 or C-PaSTM-cEYFP and N-nEYFP-PaBEL2. No signal was detected in protoplast cells co-transfected with C-PaSTM-cEYFP and N-nEYFP constructs. mCherry-VirD2NLS was used as a nuclear marker. DIC, differential interference contrast images of cells superimposed with YFP and RFP channels. Scale bar, 10 μm.
In comparison, PLBs were not produced from explants of the transgenic plant carrying the vector, pCAMBIA1300, regardless of where explant tissues were used (Figure 2A; Supplemental Figure S2A). Notably, new shoots were still able to grow from explants carrying the axillary meristems in the control and the eGFP-PaSTM transgenic plants (Figure 2A; Supplemental Figure S2A).

To 35S:eGFP-PaSTM #7 seedlings failed to grow and were omitted from further study. T0 35S:eGFP-PaSTM #5, #8, and pCAMBIA1300 #12 transgenic plants grew into adult plants (Supplemental Figure S2B). The 35S:eGFP-PaSTM #5 plant bloomed after 5 years of cultivation and seeds formed after hand-pollination (Supplemental Figure S2C). Its germinated protocorms survived on hygromycin plates (Supplemental Figure S2D), confirming inheritance of the 35S:eGFP-PaSTM transgene. T1 protocorms were used as explants to test PLB regeneration. It is important to note that PLB induction is relatively easy to achieve from protocorm explants in comparison with explants derived from other vegetative tissues. A few PLBs were produced from protocorm explants of pCAMBIA1300 #1. In comparison, the number of PLBs induced was greatly increased in the 35S:eGFP-PaSTM #5 plant (Figure 2B and Table 1), which agrees with the results from analysis of the T0 transgenic plants. As we examined the expression of the eGFP-PaSTM protein, the GFP signal was predominantly localized in the nucleus of the actively dividing cells on the surface of protocorm explant but not in the differentiated cells of explants (Figure 2C). This indicates that the encoded eGFP-PaSTM is subjected to negative regulation despite being driven by the CaMV 35S promoter. We suspect that excess PaSTM may prevent cells from differentiation and affect development as indicated previously (Scofield et al., 2014; Fang et al., 2016; Liu et al., 2018). Together, our results demonstrated that the eGFP-PaSTM transgene is inheritable and capable of inducing de novo PLB assembly.

The KNOX TFs heterodimerize with BELL1-like (BEL) homeobox proteins to facilitate nuclear localization (Bhatt et al., 2004; Cole et al., 2006), improve DNA-binding affinity (Smith et al., 2002), and control meristem functions (Rutjen et al., 2009; Cao et al., 2020). Coincidently, two BEL homeobox genes, PaBEL1 and PaBEL2, were preferentially expressed in developing PLBs and shared a similar PaSTM expression pattern (Table 2). Bimolecular fluorescence complementation (BiFC) was conducted to test the interaction between PaSTM and PaBEL1 or PaBEL2. Interaction of PaSTM and PaBEL1 or PaSTM and PaBEL2 was confirmed by yellow fluorescence in nuclei (Figure 2D), supporting the presumption of heterodimerization of PaSTM with PaBEL1 or with PaBEL2. As a negative control, no fluorescence was detected when C-PaSTM-cEYFP was co-transformed with the empty vector N-nEYFP. Together, we conclude that PaBEL1 and PaBEL2 are likely to interact with PaSTM in Phalaenopsis orchids.

Identification of PLB-associated genes and TFs

Even though the PLB and protocorm share similar transcriptome dynamics and potentially similar cellular functions, not all of the genes are regulated identically (Fang et al., 2016). We reasoned that genes involved in PLB reprogramming may be specifically upregulated in PLB and sought to identify these genes.

We first categorized PLBs and protocorms to three developmental stages based on tissue morphology. PLBs and protocorms that lack the conspicuous leaf primordia were categorized as Stage I. PLBs and protocorms that display a ridge line at the anterior end were categorized as Stage II. PLBs and protocorms that showed newly emerging leaves (~2–3 mm) were categorized as Stage III (Figure 3A). RNA-sequencing (RNA-seq) was conducted in two biological replicates. To improve the quality of the previously assembled transcriptome (Fang et al., 2016), we used the directional single-end mRNA reads and cDNAs of P. equestris (Cai et al., 2015) to correct the orientation and connect the short reads (see “Materials and methods”). The statistics of this updated transcriptome are summarized in Supplemental Table S1. The mRNA reads were mapped to the updated transcriptome. The mapping read analysis is summarized in Supplemental Table S2.

To search for PLB-specific genes, genes whose expression level was equal to or greater than five-fold in the PLB compared to the protocorm at each categorized stage (e.g. PLB/protocorm ≥ 5) were arbitrarily chosen. After filtering, 928, 562, and 1,108 PLB-specific genes were identified at Stages I, II, and III, respectively (Supplemental Figure S3). To prevent identification of genes whose functions are involved in general meristem functions, only genes whose PLB/protocorm ratio was ≥ 5 in all three categorized stages were isolated for further analysis. A total of 235 genes that passed the described filters were subjected to k-means cluster analysis (Supplemental Figure S3). These PLB-specific genes were separated into three clusters (Figure 3B) and are listed in Supplemental Data Set 1.

The PLB-specific TFs were identified from 235 PLB-specific genes as described in “Materials and methods” and are listed in Table 2. Expression patterns of the PLB-specific TFs were

| Genotype | PLB > 5 (%) | PLB < 5 (%) | Unorganized tissues (%) | Total protocorm explants examined |
|----------|-------------|-------------|-------------------------|----------------------------------|
| pCAMBIA1300 #1 | 10 (8.8) | 104 (91.2) | 0 (0.0) | 114 |
| 35S:eGFP-PaSTM #5 | 37 (37.0) | 46 (46) | 17 (17.0) | 100 |

pCAMBIA1300 #1, transgenic planting carrying the pCAMBIA1300 vector. "PLB > 5." > 5 PLBs were induced from one explant. "PLB < 5." < 5 PLBs were induced from one explant. "Unorganized tissues," tissues with unorganized structures. Number in parentheses represents the percentage of each tissue category.
validated by reverse transcription-quantitative polymerase chain reaction (RT-qPCR) in separate samples except the TFs in Cluster 3 (Figure 3C). It is likely that the peak expression of PaWRKY6 and PabZIP1 spanned across stages of PLBII and PLBIII (Table 2). In agreement with our previous conclusion that PLB is not a somatic embryho, the vast majority of PLB-specific TFs were absent or in low abundance in the ovary tissues containing developing embryos (Supplemental Figure S4). The exceptions were PaERF9 (Cluster 2) whose transcript level was enriched during late embryogenesis, and PaWRKY6 and PabZIP1 (Cluster 3) whose peak expression was also observed in ovary tissue 70 d after pollination (DAP; Supplemental Figure S4) when fertilization occurred (Chen and Fang, 2016).

Overexpression of PaSTM activates Arabidopsis S11 MYB TFs

Given that overexpressing PaSTM greatly enhanced PLB generation (Figure 2A), and its expression marks the initiation of PLB development (Fang et al., 2016), we hypothesized that the PaSTM-dependent regulatory pathway is important for de novo PLB assembly. To investigate whether the PLB-specific TFs were part of the PaSTM-dependent regulatory network, expression of the PLB-specific TFs was monitored in orchid leaves transiently overexpressing PaSTM by Agrobacterium tumefaciens infiltration. Overexpression of PaSTM mRNA was confirmed by RT-qPCR analysis (Figure 4). Overexpression of PaSTM was found to induce expression of PaMYB5, PaMYB13, PaMYB14, PaMYB17, PaNAC2, and PaERF9 in at least three separate experiments (Figure 4), suggesting that they are activated by PaSTM. Interestingly, among the TFs induced by PaSTM, PaMYB13, PaMYB14, and PaMYB17 were found clustered with members of the subgroup 11 (S11) R2R3-MYB TFs (Dubos et al., 2010) in Arabidopsis, maize (Zea mays), and rice (Oryza sativa), and PaMYB5 belongs to the S2 R2R3-MYB TFs (Supplemental Figure S5; Supplemental Methods).

We were intrigued by the ability of PaSTM to upregulate three S11 MYB TFs and decided to investigate this phenomenon further. Interestingly, activating Arabidopsis STM has been shown to increase expression of members of the S11 MYB TFs, MYB74 and MYB102 9 d after induction (DAI) of STM (Scofield et al., 2018). However, no further experiment was carried out to validate their relationship. Considering Arabidopsis STM and PaSTM share a meristematic function (Figure 1A), we tested whether PaSTM could induce Arabidopsis S11 MYB TFs. Expression of members of the S11 R2R3-MYB TFs, MYB41, MYB74, and MYB102 were monitored in transgenic Arabidopsis plants overexpressing PaSTM (Fang et al., 2016). Intriguingly, PaSTM increased expression of AtMYB74 and AtMYB102 mRNAs, but not that of AtMYB41 in the strong overexpressors that had the severe developmental defects (Figure 5A). Hence, similar to Arabidopsis STM, PaSTM was capable of increasing expression of MYB74 and MYB102.

PaSTM binds to the PaMYB13, PaMYB14, and PaMYB17 promoters

Maize KNOTTED1 TF has been reported to regulate its downstream targets through binding to the specific cis-acting element in the promoters (Bolduc and Hake, 2009). We therefore questioned whether a common cis-acting element is shared by members of the S11 MYB TFs. To test this notion, 1-kb DNA sequences upstream of the translational start sites of PaMYB13, PaMYB14, and PaMYB17 and their orthologous counterparts from Arabidopsis, rice, and maize were retrieved and analyzed by MEME ab initio motif finder (Bailey et al., 2009). A TTGACT element (Figure 5B)
**Figure 3** PLB development and PLB-specific genes. A, PLB (PLBI, PLBII, and PLBIII) and protocorm (protocormI, protocormII, and protocormIII) stages were categorized based on morphology. Scale bar, 1 mm. B, k-means analysis was used to categorize PLB-specific genes into three clusters. C, Expression patterns of the indicated TFs in developing PLBs and protocorms categorized in Figure 3A. Samples collected from young leaves were used as a control. In (B) and (C), data are from technical triplicates and the error bars are presented as standard error of the mean (SEM).
shared by most of the S11 MYB TFs from *P. aphrodite*, Arabidopsis, rice, and maize was identified (Supplemental Figure S5). This TTGACT element, carrying the TGAC core is required for binding to KNOTTED1 and its related homeobox proteins (Krusell et al., 1997; Sakamoto et al., 2001; Smith et al., 2002; Bolduc and Hake, 2009). Interestingly, *AtMYB41*, which lacks the TTGACT element, failed to be induced by PaSTM (Figure 5A), suggesting the requirement for this consensus sequence for PaSTM binding. In addition to the TTGACT core, the upstream CGT and ACC elements were also conserved (Figure 5B). Together, our data showed that the TTGACT consensus sequence is evolutionarily conserved in the promoters of the S11 MYB TFs and may be important for PaSTM binding.

To test the function of the TTGACT element, we used an electrophoretic mobility shift assay (EMSA) to examine the ability of PaSTM to bind to the *PaMYB13, PaMYB14*, and *PaMYB17* promoters and whether the binding is TTGACT-dependent. The DNA binding homeodomain of PaSTM (PaSTM\(^{HD}\)) was expressed in frame with a maltose-binding proteins. **Figure 4** Effect of PaSTM overexpression on PLB-specific TFs. PaSTM was overexpressed transiently by agroinfiltration in orchid leaves. The second or third leaf of orchid plants was used for agroinfiltration. Agroinfiltration experiment was repeated 6 times. Upregulation of the TFs was confirmed by at least three independent experiments. Roman numbers represent independent experiments. Data are from three technical replicates and the error bars are presented as SEM.
protein (MBP-PaSTM^{HD}) and purified for EMSA. As predicted, MBP-PaSTM^{HD} recombinant protein bound to the radioisotope labeled PaMYB13, PaMYB14, and PaMYB17 promoters (PaMYB13pro, PaMYB14pro, and PaMYB17pro) carrying the respective 50-nt sequences (Figure 5C). Addition of MBP-specific antibody (Ab) in the binding
promoters by interacting with the TTGACT-containing DNA. Homozygous cauliflower mosaic virus 35S (35S) promoter were generated. PaMYB13 to test this, transgenic Arabidopsis plants overexpressing that they are also capable of enhancing shoot regeneration. During shoot development, the PLB acts as an intermediate structure before leaf development. We, therefore, hypothesized that PLB development may be one type of organogenesis that can subsequently progress to shoot establishment. To test this proposition, we used Arabidopsis hypocotyl explants for de novo organogenesis (shoot induction) assay (Che et al., 2006; Ikeuchi et al., 2017). Regenerated shoots appeared earlier in transgenic 35S:PaSTM plants (Fang et al., 2016) overexpressing PaSTM than in wild-type (wt) or transgenic plants carrying the empty vector (Figure 6A; Supplemental Figure S6A). In addition, expression of PaSTM increased the number of shoots as indicated by the number of emerging leaves (Figure 6B). Thus, PaSTM is able to enhance shoot regeneration ability.

We were interested in investigating the role of PaSTM-dependent regulatory pathway during PLB-related organogenesis. Because PaMYB13, PaMYB14, and PaMYB17 are the direct targets of PaSTM during PLB development, we predict that they are also capable of enhancing shoot regeneration. To test this, transgenic Arabidopsis plants overexpressing PaMYB13, PaMYB14, and PaMYB17 under the control of the cauliflower mosaic virus 35S (35S) promoter were generated. Homozygous 35S:PaMYB13, 35S:PaMYB14, and 35S:PaMYB17 transgenic plants were used for shoot induction assay. Expression of PaMYB13, PaMYB14, and PaMYB17, were verified by RT-qPCR analysis (Figure 6C). Compared to the explants of wt plants and transgenic plants carrying the empty vector, the regenerated shoots represented by emerging leaves appeared earlier in the 35S:PaMYB13, 35S:PaMYB14, and 35S:PaMYB17 transgenic plants than in wt and vector control plants (Figure 6D; Supplemental Figure S6, B–D). Overexpression of PaMYB13 or PaMYB14 increased the number of regenerated leaves significantly in three independent experiments (Figure 6B). Overexpression of PaMYB17, on the other hand, only had marginal effect. Nonetheless, PaMYB13, PaMYB14, and PaMYB17 are capable of promoting growth of regenerated leaves and the PaSTM-S11 PaMYB module may be important for shoot and shoot-related organ development.

Expression of PaSTM, PaMYB13, PaMYB14, and PaMYB17 enhances shoot regeneration in Arabidopsis

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Discussion

PaSTM-dependent organogenesis is important for PLB development

The PLB, which is derived from somatic tissues, resembles the protocorm morphologically. Similar to the protocorm, the leaf primordium is not initiated until the PLB is fully developed and the anterior meristem becomes active. In that sense, the PLB acts as an intermediate organ before the establishment of leaves and roots (Fang, 2021). Our previous study clarified that the PLB is not a somatic embryo and suggested the importance of PaSTM for de novo PLB regeneration (Fang et al., 2016). Here, we showed that PaSTM restored the shoot meristem function of the Arabidopsis sttm mutant, supporting the notion of the central role of PaSTM in shoot meristem function. In addition, PaSTM enhanced de novo PLB regeneration in Phalaenopsis orchid, confirming its role in PLB development. Similar to the class I KNOX TFs, PaSTM enhanced de novo meristem formation and shoot regeneration in Arabidopsis (Sinha et al., 1993; Müller et al., 1995; Chuck et al., 1996; Williams-Carrier et al., 1997; Williams, 1998; Brand et al., 2002; Golz et al., 2002; Lenhard et al., 2002). Even though PaSTM is important for PLB development, it is not a PLB-specific gene because its expression is associated with shoot meristems (Fang et al., 2016) and leaf primordia (Supplemental Figure S7). Consistent with the KNOX/BELL paradigm (Hake et al., 2004; Hay and Tsiantis, 2010; Tsuda and Hake, 2015), PaSTM is likely to interact with PaBEL1 and/or PaBEL2 to exert its functions. Together, our data validate the ability of PaSTM to enhance de novo PLB regeneration in Phalaenopsis orchid and support the notion that PLB development is an organogenesis process. The class I KNOX TFs maintain meristematic cells and promote indeterminate growth during organogenesis (Satterlee and Scanlon, 2019). Organogenesis requires antagonistic regulation that allows cell transition from indeterminate growth to determinate cell identity along the proximal–distal axis (Shin et al., 2020). During leaf development, outgrowth of leaf primordia requires formation of auxin maxima (Reinhart et al., 2000; Benková et al., 2003;
Figure 6 De novo shoot induction of hypocotyl explants of the transgenic Arabidopsis plants. A, Shoot induction assay of the transgenic plants overexpressing PaSTM. Images were taken 9 d after transferring to SIM. B, Quantification of shoot induction assays. The number of leaves ± SEM regenerated from hypocotyl explants was counted after 9 or 10 d on the SIM medium. Each genotype had at least eight explants and the experiment was repeated three times. All P-values come from Student’s t test. *P < 0.05; **P < 0.01; ***P < 0.01. All three experiments (labeled as 1, 2, and 3) are shown. Shoot regeneration capacity was affected by environmental temperature despite all experiments being conducted in a temperature-controlled growth chamber. The second and third replicates, which were conducted in winter, had relative low number of regenerated leaves. Nevertheless, the trends of regeneration capacity of different genotypes were consistent. C, Expression of PaMYB13, PaMYB14, and PaMYB17 mRNAs was validated by RT-qPCR analysis. RNA level of the negative controls was arbitrarily set to 1. PaMYB13, PaMYB14, and PaMYB17 mRNAs were not detected in wt and transgenic plants carrying the pK2GW7 plasmid (V). D, Shoot induction assays of the transgenic plants overexpressing PaMYB13, PaMYB14, or PaMYB17. Images were taken 10 d after transferring to SIM. In A and D: Red scale bar, 1 mm. Transgenic plants carrying the pK2GW7 plasmid and wt plants were used as negative controls for all experiments.
Heisler et al., 2005) and auxin response factor-dependent transcriptional repression of the class I KNOX TFs to activate the lineage-specific program (Chung et al., 2019). Downregulation of class I KNOX TFs in the peripheral zone of the SAM is prerequisite for organogenesis in maize, rice, and Arabidopsis (Smith et al., 1992; Jackson et al., 1994; Long et al., 1996; Sato et al., 1996). Interestingly, PaSTM mRNA is not restricted to SAM (Fang et al., 2016) and its expression domain expanded to the emerging leaf primordia of developing PLB (Supplemental Figure S7), indicating downregulation of PaSTM in the peripheral zone of SAM is not prerequisite for leaf development in Phalaenopsis orchids. Similarly, persistent expression of KNOX in leaf primordia has also been reported in hairy bittercress (Cardamine hirsuta) and tomato (Solanum lycopersicum; Hay and Tsiantis, 2006; Jasinski et al., 2007).

Delineation of distinct populations of cells to form boundaries is fundamental to organogenesis. Meristem-specific expression of class I KNOX TFs define organ boundaries by upregulating expression of CUP-SHAPED COTYLEDON (CUC) genes and leaf primordia-specific genes at peripheral regions of SAM (Aida et al., 1999; Takada et al., 2001; Vroemen et al., 2003; Hribar et al., 2009; Kwon et al., 2006; Spinelli et al., 2011; Bolduc et al., 2012; Johnston et al., 2014; Balkunde et al., 2017; Scofield et al., 2018). Our data showed that PaSTM activates a PLB-specific NAC domain TF PaNAC2 (Figure 4), which is phylogenetically related to CUC genes in Arabidopsis and maize (Supplemental Figure S8). This suggests that PaNAC2 may be the candidate for organ boundary specification in Phalaenopsis orchids. The molecular mechanism underlying establishment of organ boundaries during leaf formation in Phalaenopsis orchids remains to be determined.

The PaSTM-S11 MYB is a regulatory module for organogenesis in Phalaenopsis orchids

Here, we report that the S11 MYB TFs are the direct targets of PaSTM. This is supported by transient expression assays and identification of the promoter binding motif, TTGACT, which contains the conserved KNOTTED1-binding core TGAC (Smith et al., 2002; Bolduc et al., 2014). The presence of this evolutionarily conserved TTGACT consensus in the promotors of members of class S11 MYB TFs from orchids, Arabidopsis, maize, and rice suggests this extended binding core is important for STM binding. This notion is supported by the inability of PaSTM to induce Arabidopsis MYB41, a class S11 MYB TF lacking the TTGACT consensus (Figure 5A). It is intriguing to find that the TTGACT consensus sequence overlaps with the W box TTGACT/C recognized by WRKY TFs (Eulgem et al., 2000; Rushton et al., 2010). Coincidently, a WRKY TF, PaWRKY6, was identified as a PLB-specific gene (Table 2). Whether PaSTM works with PaWRKY6 to coordinate PLB development remains to be determined.

The finding that the S11 MYB TFs are PaSTM targets is quite unexpected because they are reported to be stress-responsive genes and have not been identified as the targets of STM/KNOTTED1/ORYZA SATIVA HOMEobox1 in the previous target hunts in Arabidopsis, maize, and rice (Spinelli et al., 2011; Bolduc et al., 2012, 2014; Tsuda et al., 2014; Scofield et al., 2018). In Arabidopsis, MYB102 is induced by wounding, osmotic stress, and ABA treatment (Denekamp and Smeekens, 2003) and takes part in responses induced by the herbivore Pieris rapae (De Vos et al., 2006) and green peach aphids (Zhu et al., 2018). Arabidopsis MYB74, on the other hand, plays a role in response to salt stress (Xu et al., 2015). Hence, our findings here add a developmental function to members of the S11 MYB TFs.

Even though the class S11 MYB TFs were identified as the targets of PaSTM, our data suggest that this regulatory pathway may be conserved in other plants. It is not clear why the class S11 MYB TFs were not identified as targets of the class I KNOX TFs in previous genome-wide target-search studies. It is possible that the previous studies focused on early-response genes induced by ectopic KNOX proteins (Spinelli et al., 2011; Tsuda et al., 2014; Scofield et al., 2018) or genes enriched at specific developmental stages (Bolduc et al., 2012, 2014). As we re-surveyed these transcriptome datasets, Arabidopsis S11 MYB TFs, MYB74 and MYB102 were found to be increased in abundance 9 d after STM induction when leaves are developing (Scofield et al., 2018). This result supports the premise that the STM-S11 MYB pathway is present in Arabidopsis but only active during a specific developmental window. It is not clear whether the STM-S11 MYB module has a direct role in organogenesis in Arabidopsis. STM has been shown to regulate leaf shape in tomato (Shani et al., 2009) and C. hirsuta (Kierzkowski et al., 2019). Whether PaSTM-S11 MYB has a role in regulating orchid leaf morphology remains to be determined.

PaSTM is co-opted to regulate the PLB in Phalaenopsis orchids

Direct shoot regeneration can be induced from floral nodal buds of Phalaenopsis orchids (Kosir et al., 2004; Tsao et al., 2008; Wu and Chen, 2008; Tsao et al., 2011). This raises an interesting question. If Phalaenopsis orchids have the capacity to assemble de novo shoots, why would they acquire PLBs as intermediate organs before shoot generation? Moreover, our findings reveal that the primary role of PaSTM is PLB development rather than direct shoot development in Phalaenopsis orchids. This apparent functional divergence of PaSTM in Arabidopsis (shoot regeneration) and Phalaenopsis orchids (PLB development) suggests co-option of the PaSTM-dependent pathway for regulating de novo PLB assembly. Further questions, such as what drives the evolution of PLB development in orchids, whether PLBs are only induced by tissue culture, and whether PLB regeneration occurs in nature will require future studies to answer.
Conclusion
In this study, we demonstrated the shoot meristem function of PaSTM and its role during organogenesis. RNA-seq analysis coupled with transient expression assays allowed us to discern the molecular mechanism of PLB development. We propose that the STM-S11 MYB regulatory pathway is important for de novo assembly of PLB in Phalaenopsis orchids and potentially in other orchid species. Interestingly, ectopic PaSTM only induced PLBs but did not activate direct shoot development, suggesting a distinct role for PaSTM in Phalaenopsis orchids. In summary, our work supports the evolutionarily conserved role of PaSTM, but also suggests the co-option of PaSMT in regulating shoot-related organogenesis. De novo organogenesis is an important trait for agriculture and horticulture (Ikeuchi et al., 2019). Considering the ability of PaSTM to induce PLB regeneration, it may be used as a molecular marker to assist screening for progeny that enable PLB-based micropropagation in the orchid breeding program.

Materials and methods
Plant materials and growth conditions
Phalaenopsis aphrodite subsp. formosana (m1663) seedlings in 3- or 3.5-inch pots were purchased from Chain Port Orchid Nursery (Pingtung, Taiwan). Plants were grown in a growth chamber with alternating 12-h light (23°C)/12-h dark (18°C) cycles. Phalaenopsis aphrodite subsp. formosana (m1663), referred to as P. aphrodite, was used in this study except where otherwise mentioned. Arabidopsis (A. thaliana) Columbia ecotype was grown at 22°C under 16-h light/8-h dark cycles in a growth chamber.

To generate the complemented stm-2 strains, pollen of the transgenic plants overexpressing PaSTM (with kanamycin marker) was crossed into heterozygous stm-2/+ plants (Clark et al., 1996). Plants that carried the PaSTM transgene and were homozygous for the stm-2 allele were isolated by genotyping as described previously (Belles-Boix et al., 2006).

Generation of transgenic Phalaenopsis orchid plants overexpressing PaSTM
The ~3.1-kb eGFP-PaSTM expression cassette was isolated by complete XbaI and partial SacI digestion of the p35SCaMV:eGFP-PaSTM plasmid (Fang et al., 2016) and ligated into the pCAMBIA1300 plasmid to make p35SCaMV:eGFP-PaSTM-1300 plasmid. The p35SCaMV:eGFP-PaSTM-1300 and the empty vector pCAMBIA1300 were transformed into A. tumefaciens strain EHA105 and the spectinomycin-resistant colonies were selected.

Protocorms of the commercial tetraploid orchid Phalaenopsis Sogo Yukidian V3 (Tai-Ling Biotech Inc., Tainan, Taiwan) was used for transformation. To obtain protocorms, fully opened flowers were hand-pollinated. After capsules were matured, orchid seeds were sown on a 1/2 Murashige and Skoog (MS) agar plate containing 0.1% (w/v) activated charcoal and allowed to germinate into protocorms. Agrobacterium transformation was applied as described previously (Hsing et al., 2016). Transgenic plants were selected on T2 plates (Chen et al., 2009) containing 20 ppm hygromycin and 40 ppm meropenem 3 times, at 2-week intervals. Potential transgenic plants were allowed to recover and grow on a 1/2 MS agar plate for ~2 months. RNA from leaf tissues of the potential transgenic plants was isolated and reverse transcribed, followed by PCR using the HptII-specific primers HPT-LP (5’-ATCGCCTCGTCCAGTCAATG-3’) and HPT-UP (5’-AGCTGCCCGATGGTTTCTACAA-3’). cDNA of 18S rRNA amplified by primers 18S-F2 (5’-TTAGGGACGGAAATTGCAGG-3’) and 18S-R2 (5’-ACACCTACCGGACCATCTACA-3’) was used as an internal control. The PCR amplification program for 506-bp HPT II was: 94°C for 3 min, 30 cycles at 94°C for 30 s, 58°C for 30 s, and 72°C for 30 s. The amplification program for 28 bp 18S rRNA was: 94°C for 3 min, 28 cycles at 94°C for 30 s, 55°C for 25 s, and 72°C for 50 s.

PLB induction
The seeds of P. aphrodite subsp. formosana (m1663) were allowed to germinate as described previously (Fang et al., 2016). Fifty-five to 80 DAG, protocorms that had grown to ~3 mm in diameter were excised to remove the top meristems and bottom portions. The primary PLBs were induced from protocorm segments after transferring to PLB-inducing medium [0.1% (w/v) tryptone, 2% (w/v) sucrose, 2% (w/v) potato homogenate, 2.5% (w/v) banana homogenate, 0.01% (v/v) citric acid, 0.1% (w/v) charcoal, 1% (w/v) agar], and adjusted to pH 5.5. To induce secondary PLBs, the primary PLBs with top and ~1-2 mm bottom portions removed were allowed to grow in the PLB-induction medium. Secondary PLBs were used for the experiments described below. Cultures were maintained at 25°C under 16-h light/8-h dark cycles under illumination at 45–55 μmol photons m⁻² s⁻¹ in a growth chamber.

Sample collection and RNA purification
Because PLBs did not grow at a synchronized rate, PLBs were collected individually based on the visually categorized size and developmental stage (Figure 3A). Because germination rate varied in seeds collected from different capsules, only the healthy-looking protocorms (light-green in color and relatively large in size) were collected for the described experiments. Protocorms that germinated after 15-, 30-, and 45 d were collected and categorized (Figure 3A). The tissues samples were flash frozen in liquid nitrogen and stored in a freezer at −80°C. Total RNA was isolated by OmicZol RNA Plus extraction reagent (Oomics Bio) or TRizol (ThermoFisher Scientific, Waltham, MA, USA) according to the manufacturer’s instructions. The isolated RNA was treated with RNase-free DNase (Qiagen, Hilden, Germany) followed by RNaseasy mini-column purification according to the manufacturer’s instructions (Qiagen).

RNA-seq, transcriptome assembly, annotation of the assembled unigenes, and identification of TFs
RNA samples purified from protocorms and PLBs collected at the specified stages described above were sent to
Welgene Biotech (Taiwan) for RNA-seq. RNA-seq was conducted in two biological replicates and each replicate contained explants of multiple orchid plants. The 75-nt single-end cDNA library was synthesized and amplified by Agilent SureSelect Strand-Specific RNA Library Prep kit following the manufacturer’s instructions (Illumina, USA). For each sample, ~10-Mb single-end mRNA reads were sequenced by Illumina NextSeq500 (Illumina, USA). The raw sequencing data was filtered by Trimmomatic (Bolger et al., 2014) to remove low-quality (Quality Value ≥20), adaptor, and repeat sequences.

To improve the quality of the transcriptome, directional single-end mRNA reads and cDNAs of *P. equestris* (Cai et al., 2015) were used to correct the orientation and connect the short reads of the assembled transcriptome (Fang et al., 2016) to generate the updated transcriptome. Briefly, qualified reads were de novo assembled by the Trinity program in a strand-dependent manner (Grabherr et al., 2011). After Trinity assembling, cd-hit-est was used to remove redundant sequences (Li and Godzik, 2006). The estimation of unigene abundance was calculated by RNA-seq by expectation maximization (RSEM). The assembled unigenes from 75SE data were further assembled with previously assembled unigenes (Fang et al., 2016) and cDNA sequences of *Phalaenopsis equestris* (Cai et al., 2015) with 75SE sequencing read evidence. The three assemblies were merged by cd-hit-est to form a nonredundant transcriptome. The partitioning algorithm in the khmer software package was used to name unigenes in the final transcriptome. Sequence orientation of unigenes in the final transcriptome was corrected by strand-specific 75SE reads. The total size of the assembly was ~151 Mb.

The qualified 75SE reads were mapped back to the assembled transcriptome using the short read alignment software Bowtie 2 (Langmead and Salzberg, 2012). The relative abundances of the transcripts were quantified by Cufflinks2 (Trapnell et al., 2012). Between 84% and 90% of RNA reads derived from sampled tissues were able to be mapped back to the assembled transcriptome (Supplemental Table S2). The gene expression levels were calculated as fragments per kilobase of transcript per million mapped reads (FPKM).

The unigenes were BLAST searched against the NCBI non-redundant (nr) protein database using the alignment algorithm RAPSearch2 with a cut-off E-value ≤−3 (Zhou et al., 2012). The top alignment hits were used to predict the sequence orientation and gene ontology (GO) accessions of the unigenes.

To identify TFs, TFs annotated or specified under the GO categories “regulation of transcription, DNA-templated (GO:00006355); “transcription, DNA-templated (GO:0006351); “transcription factor activity, DNA binding transcription factor activity (GO:0003700);” and “DNA binding (GO:0003677)” were isolated.

**Co-expression clustering**

To reduce false-positive results caused by low abundance, only transcripts with expression levels equal to or >20 fragments per kilobase of FPKM in at least one sample were selected for clustering analysis. To generate robust groups of coregulated genes, transcripts that showed a five-fold difference in all paired PLB and protocorm tissues (PLB1: protocorm1; PLBII: protocormII; PLBIII: protocormIII) were identified (Supplemental Figure S3). One-way analysis of variance (ANOVA) with a *P* ≤0.05 was applied to the paired samples. Transcripts that were more abundant in PLBs than paired protocorm at all stages were selected for clustering analysis. Identification of co-regulated mRNAs was performed using MultiExperiment Viewer software (Howe et al., 2011). K-means support using Pearson’s correlation was used to separate 235 assembled transcripts into three clusters (groups of coregulated genes, Figure 3B; Supplemental Table S3). TFs falling into the designated clusters were selected.

**BIFC**

The full-length *PaSTM* cDNA was amplified by gene-specific primers (Supplemental Table S4) and cloned into pE3132 plasmid (Tzfira et al., 2005; Citovsky et al., 2006) to make C-PaSTM-cEYFP plasmid. The C-PaSTM-cEYFP allowed the C-terminal of the PaSTM to be tagged with the C terminal half of EYFP. The full-length cDNAs of *PaBEL1* and *PaBEL2* were amplified by gene-specific primers (Supplemental Table S4) and cloned into the pE3136 vector (Citovsky et al., 2006). The encoded proteins of *PaBEL1* and *PaBEL2* were N-terminally tagged with the N terminal half of EYFP to make N-nEYFP-PaBEL1 and N-nEYFP-PaBEL2 plasmids, respectively. Expression of PaSTM, PaBEL1, and PaBEL2 were driven by 2X 35S promoter. The C-PaSTM-cEYFP and N-nEYFP-PaBEL1 or C-PaSTM-cEYFP and N-nEYFP-PaBEL2 plasmids were co-transformed into orchid protoplasts as described previously (Lin et al., 2018). A 514-nm argon laser was used for excitation. Laser intensity was adjusted to 65% and Master Gain was set to 720–750. YFP fluorescence was acquired at the range of 515–599 nm. Fluorescence images were photographed by an LSM 710 Confocal Microscope (Zeiss, Oberkochen, Germany).

**Agroinfiltration and transient expression**

The *A. tumefaciens* strain EHA105 carrying the 35S::GFP-PaSTM construct (Fang et al., 2016) was cultured at 28°C overnight as described previously (Lu et al., 2012). One milliliter of overnight culture was diluted in 10 mL of YEB medium supplemented with 100-µg/mL kanamycin and 100-µM acetosyringone and allowed to grow to OD600 = ~1.0–1.2. The culture was concentrated by centrifugation at 3,000g for 10 min and resuspended in 1 mL of infiltration medium (10-mM MES, 10-mM MgCl2, and 100-µM acetosyringone, pH 5.8). The concentrated culture was incubated at 28°C for 3 h before infiltration. The second or third orchid leaf was infiltrated with 100 µL of *A. tumefaciens* culture using a gage 29G needle.
EMSA
The cDNA encoding PaSTM homeodomain (PaSTM\textsuperscript{HD}, amino acids 244–342) amplified by primers 5'-AATCGAATT CTTAAAGAGAGGAAGAGGG-3' and 5'- GATGTCGCC TCAAAGTAGAGGGGACAGC-3' was digested by EcoRI and SalI and ligated into the pMAL-c2X vector (NEB, Ipswich, MA, USA) to generate pMBP-PaSTM\textsuperscript{HD} construct. The pMBP-PaSTM\textsuperscript{HD} plasmid was transformed into Escherichia coli Rosetta (DE3) cells by electroporation. For protein induction, a single colony was grown in 5 mL LB containing 50-μg/mL carbenicillin overnight. Two and half milliliters of O/N culture was diluted with 50 mL of LB and allowed to grow at 37°C until OD\textsubscript{600} reached ~0.5–0.7. Protein was induced by 0.2 mM Isopropyl β-D-1-thiogalactopyranoside at 24°C for 4 h. Cell culture was harvested by centrifugation at 4,000 g for 20 min. The cell pellet was resuspended in 2-mL buffer (20-mM Tris–HCl, 200-mM NaCl, 1-mM EDTA) containing 1× protease inhibitor cocktail (Sigma, St Louis, MO, USA) and sonicated (Qsonica Q700) with 5 s ON/10 s OFF setting for 5 min. Recombinant MBP-PaSTM\textsuperscript{HD} protein was purified by Amylose Resin (NEB) following the manufacturer’s protocol.

The PaMYB13, PaMYB14, and PaMYB17 promoter fragments and their mutated versions were PCR amplified using the primers listed in Supplemental Table S4. [32P] dCTP was incorporated into PCR-amplified fragments as follows (Green and Sambrook, 2012). Ten microliters of PCR reaction contained 8 pg DNA template, 1× Taq buffer, 0.1-μM of primers, 0.2-mM dATP, 0.2-mM dCTP, 0.2-mM dGTP, 2-μM dTTP, 1 μL [32P] dCTP (10 mCi/μL), 0.4 μL (500 μL/200 μL) Power Tag DNA polymerase (Genomics, Taiwan). The PCR program was: 96°C for 2 min, 11 cycles of 94°C for 20 s, 58°C for 25 s, and 72°C for 20 s. PCR products were purified by G-50 columns (Geneaid, Taiwan). The probe was at ~5,000 cpm after purification.

Purified recombinant protein (1.5 μL) was incubated with an ~500 cpm probe in EMSA binding buffer (20-mM HEPES, pH 7.6, 1-mM EDTA, 10-mM (NH\textsubscript{4})\textsubscript{2}SO\textsubscript{4}, 1-mM DTT, 0.2% (w/v) Tween-20, 30-mM KCl, 100-ng/μL poly(d(A-T))) on ice in a 10-μL reaction for 60 min. For the competition assay, unlabeled probes were added 30 min prior to adding the 32P-labeled probes. Binding reactions were separated on a non-denaturating 4% (w/v) Tris–borate–EDTA (TBE) acrylamide gel in 0.5× TBE buffer. After electrophoresis, gels were fixed in 20% methanol and 10% acetic acid for 15 min, dried on a gel dryer at 70°C for 1 h, and analyzed by Typhoon TRIO imager system (GE).

Arabidopsis shoot induction assay
Shoot regeneration was induced from hypocotyls as described previously (Ikeuchi et al., 2017) with slight modification. Briefly, the plates were placed for 2 d at 4°C and then transferred to a long day (16-h light/8-h dark) growth room at 22°C, where they were placed vertically in the dark for 7 d. The hypocotyls of dark-grown 7-d-old seedlings were dissected and transferred to plates containing Gamborg’s B5 medium (Sigma-Aldrich, St Louis, MO, USA; GS893) with 0.5 g/L MES (Sigma-Aldrich M2933), 20 g/L dextrose, 0.4% Phytagel (Sigma-Aldrich P8169) supplemented with 0.1 mg/L kinetin (Sigma-Aldrich K3253), and 0.5 mg/L 2,4-dichlorophenoxyacetic acid as the callus-inducing medium (CIM) or with 0.894 mg/L 6-γ-dimethylallylamino) purine (2iP, Sigma-Aldrich D7660) and 0.093 mg/L 1-naphthylacetic acid (Sigma-Aldrich; N1641) as the shoot-inducing medium (SIM; Shemer et al., 2015). For shoot regeneration, hypocotyl explants were incubated for 4 d on CIM before transferring to SIM.

Because newly emerging shoots formed clusters at the anterior end of the hypocotyl (proximal to the shoot) that are difficult to discern, leaf numbers were used as an alternative measure of the generated shoots. For quantification analysis, only the developing leaves with lengths larger or equal to 0.25 mm were included. The number of leaves was normalized by the number of explants. All P-values were derived from a two-tailed Student’s t test comparing the indicated genotype to control plant carrying the empty vector pK2GW7. The experiment was conducted in three replicates, and each experiment had at least eight explants. Note that the majority of shoots were generated at the anterior ends, similar to previous observation (Liu et al., 2018).

Plasmid construction and Arabidopsis transformation
Total RNA was isolated as previously described (Lin et al., 2014). Five micrograms of total RNA were used for cDNA synthesis. The full-length cDNA of PaMYB14, PaMYB17, and PaMYB13 were BP recombined into pDONR221 vector to make pDONR_PaMYB13, pDONR_PaMYB14, and pDONR_PaMYB17 respectively. pDONR_PaMYB13, pDONR_PaMYB14, and pDONR_PaMYB17 were then LR recombined with pK2GW7 (Karimi et al., 2002) to make p35ScaMV:PaMYB13, p35ScaMV:PaMYB14, and p35ScaMV:PaMYB17. The nomenclature of MYB genes follows a recent phylogenetical study of orchid MYB genes (Fan et al., 2020). The corresponding IDs in Orchidstra version 2.0 (Chao et al., 2017) and P. equestris (Tian et al., 2020) are listed in Supplemental Table S5.

The resulting plasmids were transformed into A. tumefaciens strain GV3101. Arabidopsis Columbia ecotype plants were transformed with A. tumefaciens GV3101 by the floral dipping method (Clough and Bent, 1998). In addition to antibiotic selection, the transformants were verified by PCR amplification of 525-bp PaSTM cDNA fragment by primers 5'-GGGATTGAGACTGCAGGAGA-3' and 5'-GGGATTGAGACTGCAGGAGA-3'.

RT-qPCR
DNA-free RNA was reverse transcribed in the presence of a mixture of oligo dT and random primers (9:1 ratio) using

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\text{RT-qPCR}
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the GoScript Reverse Transcription System (Promega, Madison, WI, USA) according to the manufacturer’s instructions. Ten microliters of RT-PCR reaction contained 2.5 μL of 1/20 diluted cDNA, 0.2 μM of primers, and 5 μL of 2 × KAPA SYBR FAST master mix (KAPA Biosystems, Wilmington, MA, USA). The following program was used for amplification: 95°C for 1 min, 40 cycles at 95°C for 5 s and 58°C to 62°C for 20 s. PCR was performed in triplicate, and the experiments were repeated with RNA isolated from independent samples. Primer pairs and the specified annealing temperature used are listed in Supplemental Table S6. Ubiquitin (PaUBI) was used as an internal control (Lin et al., 2014).

**Fluorescence imaging of orchid samples**
Explants were first fixed in 4% paraformaldehyde in 1 × PBS (pH 7.4) and vacuum infiltrated overnight. Fixed explants were sectioned and 150- or 200-μm tissue sections were cleared in ClearSee solution (Kurihara et al., 2015) overnight at room temperature. For confocal imaging, a 488-nm argon laser was used for excitation. Laser intensity was adjusted to 90% and Master Gain was set to 640. Z-stack images were acquired using a 20 x or 63 x objective lens (Plan-Apochromat 20x/0.8 DIC M27 or Plan-Apochromat 63x/1.40 oil DIC M27; Zeiss). Images were acquired in the ranges 505–535 nm for eGFP and 657–797 nm for autofluorescence. Fluorescence images were photographed by a LSM 710 Confocal Microscope (Zeiss).

**Accession numbers**
Raw sequenced reads are available in the National Center for Biotechnology Information (NCBI) Sequence Read Archive repository: SRR13039217–SRR13039228. The nucleotide assembled unigenes are available in the NCBI Transcriptome Shotgun Assembly repository: GJHX00000000. GenBank accession numbers for TFs mentioned in this manuscript are PaMYB14 (MZ504996), PaMYB17 (MZ504997), and PaMYB13 (MZ504998).

**Supplemental data**
The following materials are available in the online version of this article.
- **Supplemental Figure S1.** Generation of transgenic orchid plants overexpressing eGFP-PaSTM protein.
- **Supplemental Figure S2.** Characterization of the transgenic orchid plants.
- **Supplemental Figure S3.** Venn diagram showing PLB-enriched genes at stage I (red), stage II (blue), and stage III (green).
- **Supplemental Figure S4.** Expression profiles of selected TFs in developing ovaries collected at 30–200 DAP and developing PLBs and protocorms.
- **Supplemental Figure S5.** Phylogenetic relationship of MYB TFs of *A. thaliana* (AT) and selected members from *P. aphrodite* (Pa), *O. sativa* (Os), and *Z. mays* (GRMZM).

**Supplemental Figure S6.** De novo shoot induction of hypocotyl explants of the transgenic Arabidopsis plants.
**Supplemental Figure S7.** In situ hybridization with an antisense *PaSTM* probe on longitudinal sections through the center of a large PLB.
**Supplemental Figure S8.** Phylogenetic relationship of NAC TFs of *A. thaliana* (AT) and selected NAC genes from *P. aphrodite* (Pa) and *Z. mays* (ZmCUC2, Zm00001d031463).

**Supplemental Table S1.** Statistics of the de novo transcriptome assembly.
**Supplemental Table S2.** Statistics of RNA-seq reads.
**Supplemental Table S3.** Statistics of three gene clusters in Supplemental Data Set 1.
**Supplemental Table S4.** List of primers used for promoter and cDNA amplification.
**Supplemental Table S5.** List of gene IDs in this study and their corresponding IDs in *P. aphrodite* and *P. equestris* databases.
**Supplemental Table S6.** List of primers used for RT-qPCR.
**Supplemental methods and references.**
**Supplemental Data Set 1.** FPKM values and annotations of genes in Clusters 1, 2, and 3 in Figure 3B.

**Acknowledgments**
We thank Ms Pei-Miao Chien, Ms Miao-Ju Wei, Mr Chin-Lin Chung, and Dr Yen-Ling Lin for technical support. We also thank the Orchid Transformation, Confocal Microscopy, Green House, and In-Situ Hybridization Core Facilities at Academia Sinica Biotechnology Center in Southern Taiwan for their services; and Ms. Miranda Loney for English editing.

**Funding**
This work was supported by the Innovative Translational Agricultural Research Program grants 2014CP02s4, 2015CP02s4, 2016CP02s4, 2017CP01s3, 2018PRE033, and AS-KPQ-108-ITAR-033 (to S.C.F.); and in part by a grant (to S.C.F.) from the Biotechnology Center in Southern Taiwan, Academia Sinica.

**Conflict of interest statement.** None declared.

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**Supplemental Data Set 1.** FPKM values and annotations of genes in Clusters 1, 2, and 3 in Figure 3B.
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