Conserved and None-Conserved Functions of the Rice Homologs of the Arabidopsis Trichome Initiation-Regulating MBW Complex Proteins

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Abstract

**Background:** Trichome initiation in Arabidopsis is regulated by a MYB-bHLH-WD40 (MBW) transcriptional activator complex formed by the R2R3 MYB transcription factor GLABRA1 (GL1), MYB23 or MYB82, the bHLH transcription factor GLABRA3 (GL3), ENHANCER OF GLABRA3 (EGL3) or TRANSPARENT TESTA8 (TT8), and the WD40-repeat protein TRANSPARENT TESTA GLABRA1 (TTG1). However, the functions of the rice homologs of the MBW complex proteins remained uncharacterized.

**Results:** Based on amino acid sequence identity and similarity, and protein interaction prediction, we identified OsGL1s, OsGL3s and OsTTG1s as rice homologs of the MBW complex proteins. By using protoplast transfection, we show that OsGL1D, OsGL1E, OsGL3B and OsTTG1A were predominantly localized in the nucleus, OsGL3B functions as a transcriptional activator and is able to interact with GL1 and TTG1. By using yeast two hybridization and protoplast transfection assays, we show that OsGL3B is able to interact with OsGL1E and OsTTG1A, and OsGL1E and OsTTG1A are also able to interact with GL3. On the other hand, we found that OsGL1D functions as a transcription activator, and it can interact with GL3 but not OsGL3B. These results suggest that rice homologs of the Arabidopsis MBW complex proteins are able to form MBW complexes, but may have conserved and none-conserved functions. Furthermore, we found that expression of OsTTG1A in the ttg1 mutant restored the phenotypes including alternations in trichome and root hair formation, seed color, mucilage production and anthocyanin biosynthesis, indicating that OsTTG1A and TTG1 may have similar functions.

**Conclusion:** These results aid in our understanding of the mechanism of trichome initiation in rice.

**Background**

Trichomes are appendages on the surfaces of the aerial parts of the plants, they are developed from epidermal cells and are very diverse in appearance. Trichomes could prevent plants from excessive heat and water loss, and protect plants from insect or pathogen attacks by increasing the boundary layer thickness between the epidermal tissues and the environment [1, 2].

Available evidence suggest that trichome initiation in Arabidopsis is regulated by a MYB-bHLH-WD40 (MBW) complex formed by a R2R3 MYB transcriptional activator, a bHLH transcription factor, and a WD40-repeat protein [3–7]. The R2R3 MYB transcription factor in this MBW complex is GLABRA1 (GL1) [8], the bHLH transcription factor is GLABRA3 (GL3), ENHANCER OF GLABRA3 (EGL3) [9, 10], or TRANSPARENT TESTA8 (TT8) [11], and the WD40-repeat protein is TRANSPARENT TESTA GLABRA1 (TTG1) [12]. MYB23 and MYB82 have also been shown to be able to interact with GL3 and/or EGL3, and to regulate trichome formation [13, 14]. The MBW transcriptional activator complex is able to induce the expression of the homeodomain protein gene GLABRA2 (GL2) [15], leading to the promotion of trichome initiation[3–7, 16].

This MBW complex is also able to induce the expression of some R3 MYB genes including TRYPHTCHON (TRY), CAPRICE (CPC), ENHANCER OF TRY AND CPC1 (ETC1) and ETC3 [17–22]. These R3 MYB
transcriptional factors, together with the ones that are not regulated by the MBW complex, including ETC2, TRICHOMELESS1 (TCL1) and TCL2 [22–25], are able to move to their neighboring cells, where they can compete with GL1 for binding GL3, therefore inhibiting the formation of the MBW complex, leading to the inhibition of trichome initiation [3–7, 26–28].

At least in some plants, functions of MBW complex proteins in regulating trichome initiation is conserved, for example, *Brassica napus* plants expressing Arabidopsis GL3 produced ectopic trichomes [29], cotton homologs of GL1 and GL2 regulate trichome initiation in Arabidopsis [30, 31], and trichome phenotypes in the *ttg1* mutants were restored by expressing a *Freesia hybrida* TTG1 homolog gene [32].

Even though trichomes could provide protection for plants [1, 2], glabrous has been considered to be a favorite agronomic trait in rice (*Oryza sativa*), because grains of glabrous rice have greater packing capability, and glabrous rice produces less itchy causing dust [33, 34]. Therefore, great efforts have been devoted to addressing the regulation mechanisms under the control of trichome initiation in rice.

So far, several regulator of trichome initiation have been indentified in rice, some of them are homologs of Arabidopsis trichome initiation regulators, whereas some others are not. For example, OsWOX3B, a homolog of GL2, regulates trichome initiation in rice [33–35]. SPL9 is a squamosa promoter binding type protein that has been shown to regulate trichome initiation in Arabidopsis via directly regulating the expression of *TCL1* [36], OsSPL10, a homolog of SPL9 is also able to regulate trichome initiation in rice [37]. These results suggest that trichome initiation in rice may be regulated by similar mechanisms as in Arabidopsis.

On the other hand, SDG714, a histone H3K9 Methyltransferase, HL6 (Hairy Leaf 6), an AP2/ERF transcription factor have been shown to be involved in the regulation of trichome initiation in rice [38, 39], but none of them are homologs of known Arabidopsis trichome initiation regulators. Our previous studies have also shown that OsTCL1, a homolog of TCL1, is able to regulate trichome initiation in Arabidopsis, but not in rice [40]. These results indicate that trichome initiation in rice may also be regulated by different mechanisms.

Here we report the identification and characterization of rice homologs of the trichome initiation-regulating MBW complex proteins. Based on amino acid sequence identity and similarity, and protein interaction prediction, we identified OsGL1A - OsGL1E, OsGL3A - OsGL3C, and OsTTG1A and OsTTG1B as homologs of GL1, GL3 and TTG1, respectively. By using Arabidopsis protoplast transfection assays, we found that these proteins may have conserved and none-conserved functions in forming MBW complexes. By generating transgenic plants expressing *OsTTG1A* in the *ttg1* mutants, we found that OsTTG1A and TTG1 may have similar functions in regulating trichome initiation as well as root hair formation and secondary metabolism in Arabidopsis.

**Results**

**Homologs of the MBW complex proteins in rice**
In previous experiments, we have identified OsGL1A, OsGL1B and OsGL1C as rice homologs of Arabidopsis GL1, OsGL3A, OsGL3B and OsGL3C as rice homologs of Arabidopsis GL3, and OsTTG1A, OsTTG1B and OsTTG1C as rice homologs of Arabidopsis TTG1[40].

To examine if these MBW homologs in rice can form MBW complexes, we first analyzed their interaction relationship on STRING (https://string-db.org/). We found that OsGL1D and OsGL1E were predicted as potentially interaction proteins with OsGL3B. As shown in Fig. 1A, OsGL1 proteins showed a 29.4%~36.3% identity, and a 43.9%~54.6% similarity with GL1 at amino acid level (Fig. 1A). Phylogenetic analysis showed that OsGL1A is closely related to OsGL1B, whereas OsGL1D is closed related to OsGL1E. Together with OsGL1C, these five OsGL1s formed a clade (Fig. 1B). On the other hand GL1 is closed related to MY23, and they formed another clade together with MYB82 (Fig. 1B). Sequence alignment showed that the most conserved region of the OsGL1s is the R2R3 MYB domain (Fig. S1). The [D/E]L×2[R/K]×3L×6L×3R amino acid signature required for the interaction of MYB transcription factors with R/B-like bHLH transcription factors [41], and the S residue has been shown to be required for the activation of GL2 [42], are fully conserved in all the five OsGL1s (Fig. S1).

As for the OsGL3s, both OsGL3A and OsGL3B showed a more than 33% identify and a more than 51% similarity with GL3 and EGL3, whereas that for OsGL3C are only about 25% and 40%, respectively (Fig. 1C). Phylogenetic analysis showed that OsGL3A is closely related to OsGL3B, and they formed a clade with GL3 and EGL3 pair (Fig. 1D). Sequence alignment showed that the most conserved regions of the OsGL3s are the N-terminal and C-terminal domains (Fig. S2). At both the first 97 amino acids of GL3 that are required to interact with GL1 [9], and the HLH domain region, OsGL3A and OsGL3B, but not OsGL3C showed high similar to GL3 and EGL3 (Fig. S2).

Among the MBW complex protein homologs in rice, OsTTG1s are the most conserved ones when compared with their Arabidopsis homologs. OsTTG1A and OsTTG1B showed a 60.5% and 49.3% identity, and a 75.4% and 63.7% similarity, respectively to TTG1 (Fig. 1E). Phylogenetic analysis showed that OsTTG1A is closely related to TTG1 (Fig. 1F). Sequence alignment showed OsTTG1s and TTG1 are highly conserved at full-length amino acid sequence level (Fig. S3), including the 25 amino acid sequence that is required for interaction of TTG1 with GL3 [9].

In order to get a better pictures on the relations that exists between the Arabidopsis MBW complex component proteins and their rice homologs, we identified MBW complex component protein homologs, i.e., proteins with highest amino acid similarity with GL1, GL3 and TTG1, respectively, in the Brassicaceae family plants Brassica rapa, Capsella grandiflora and Capsella rubella, the Fabidae family plant Glycine max, the Malpighiales family plant Populus trichocarpa, and the Panicoideae family plants Zea mays, Setaria italica and Panicum hallii, and expended the phylogenetic analysis. We found that for OsGL1s and the Arabidopsis GL1, MYB23 and MYB82 are still in two different clades (Fig. S4). The Arabidopsis GL1, MYB23 and MYB82 are closely related to homologs from the three Brassicaceae plants and the Malpighiales family plant P. trichocarpa, whereas OsGL1s are closely related to homologs from the three Panicoideae family plants and the Fabidae family plant G. max (Fig. S4). On the other hand, OsGL3C and
TT8 formed a clade, whereas GL3, EGL3, OsGL3A and OsGL3B formed another clade with homologs from all the eight plants mentioned above, in which OsGL3A and OsGL3B formed a sub-clade with homologs from the three Panicoideae family plants, and GL3 and EGL3 formed another sub-clade with homologs from the three Brassicaceae plants, *P. trichocarpa* and *G. max* (Fig. S5). For the WD40 proteins, OsTTG1B alone formed a clade, whereas OsTTG1A, TTG1 and homologs from all the eight plants formed another clade, in which the OsTTG1A and homologs from the three Panicoideae family plants formed a sub-clade, and TTG1 and homologs from the three Brassicaceae plants, *P. trichocarpa* and *G. max* formed another sub-clade (Fig. S6).

**Subcellular localization of the MBW complex homolog proteins**

Previous reports have shown that GL3, GL1 and TTG1 are all localized in the nucleus [43]. Based on the above bioinformatics analysis, OsGL1A, OsGL1B, OsGL1C, OsGL1D, OsGL1E, OsGL3B and OsTTG1A were chosen for subcellular localization assays. OsGL1A and OsGL1B were chosen because they showed relative high amino acid identify and similarity to GL1, whereas OsGL1D and OsGL1E are potential interactors of OsGL3B according to STRING assays. OsGL3B was chosen because both OsGL3A and OsGL3B showed relative high amino acid identify and similarity to GL3, OsGL3B was predicted to interact with OsGL1D and OsGL1E on STRING, whereas OsGL1C is not paired with other OsGL1 proteins. OsTTG1A was chosen because it showed relative high amino acid identify and similarity to TTG1.

We examined their subcellular localization in Arabidopsis protoplasts. GFP fused constructs of the MBW complex homolog genes were transfected into Arabidopsis protoplasts, and GFP fluorescence was observed under a confocal microscope. We found the OsGL1D, OsGL1E, OsGL3B and OsTTG1A were predominantly localized in nucleus, whereas OsGL1A and OsGL1B may be localized in nucleus and likely some other organelles such as cell membranes and chloroplasts (Fig. 2).

**OsGL3B is a transcriptional activator and it interacts with GL1 and TTG1 in Arabidopsis protoplasts**

Preciously we have shown that GL3 functions as a transcription activator in transfected Arabidopsis protoplasts [44]. To examine if the MBW complex homologs in c rice can indeed form MBW complexes, we then examined if OsGL3B may also functions as a transcription activator. Plasmids of effector gene GD, GD-OsGL3B or GD-GL3, together with the reporter gene Gal4-GUS were co-transfected into Arabidopsis protoplasts, and GUS activities were examined by using a microplate reader. We found that, similar to GD-GL3, cotransfection of GD-OsGL3B activated the reporter gene expression (Fig. 3A).

Having shown that OsGL3B functions as a transcriptional activator, we examined if OsGL3B may form a MBW complex with GL1 and TTG1 by examining their interactions in yeast cells and Arabidopsis protoplasts. As shown in Fig. 4, OsGL3B interacts with GL1 and TTG1 in yeast cells. Cotransfection of OsGL3B with GD-GL1 and GD-TTG1, respectively activated reported gene expression in protoplasts (Fig. 3B), indicating that OsGL3B may able to interact with GL1 and TTG1 in plant cells. Yet this result may not indicate a direct interaction.
Interactions of OsTTG1A and OsGL1s with OsGL3B and GL3

The above results suggest that OsGL3B is able to form a MBW complex with GL1 and TTG1. We then further examined if it may form MBW complex with OsGL1s and OsTTG1A. To do that, we examined interaction of OsGL3B with OsGL1s and OsTTG1A in yeast cells and Arabidopsis protoplasts. As shown in Fig. 4, OsGL3B is able to interact with OsGL1D, OsGL1E and OsTTG1. Similar, cotransfection of OsGL3B with GD-OsTTG1 activated reporter gene expression in protoplasts, whereas cotransfection of OsGL3B with GD-OsGL1A or GD-OsGL1B failed to do so (Fig. 5A). However, cotransfection of OsGL3B with GD-OsGL1E activated reporter gene expression (Fig. 5B). These results suggest that OsGL1E, OsGL3B and OsTTG1A may able to form a MBW complex.

Our protoplast transfection assays also suggest that both OsTTG1A and OsGL1E may able to interact with GL3 (Fig. 5). MBW complex proteins in Arabidopsis and rice are interchangeable in forming MBW complex.

Surprisingly, we found that transfection of GD-OsGL1D activated reporter gene expression (Fig. 5B), suggesting that unlike GL1 and other OsGL1A examined, OsGL1D is able to function as a transcription activator. We also found that GUS activities were increased when GL3, but not OsGL3B was cotransfected with confection of GD-OsGL1D (Fig. 5B), indicating that OsGL1D is able to interact with GL3, but not OsGL3B.

Ectopic expression of OsTTG1A rescued ttg1 phenotypes

After showing that OsGL1E, OsGL3B and OsTTG1A are able to form a MBW complex, we wanted to further examine if they may have similar functions as their Arabidopsis homologs. Considering that the ttg1 mutant has a variety of obvious phenotypes relate to trichome and root hair cell fate determination and secondary metabolism including seed color, mucilage production and anthocyanin biosynthesis [12, 45, 46], and OsTTG1A showed high amino acid identity and similarity to TTG1, we decided to examine if OsTTG1A is a functional analogue of TTG1 by examine if ectopic expression of OsTTG1A could rescue the ttg1 mutant phenotypes.

Transgenic plants were generated in the ttg1 mutant plants by expressing OsTTG1A under the control of the 35S promoter (35S:OsTTG1A/ttg1). Two independent homozygous lines were used for phenotypic analysis. As shown in Fig. 6A, transcript of TTG1 was only detectable in the Ler wild type plants, whereas transcript of OsTTG1A was only detectable in the 35S:OsTTG1A/ttg1 transgenic plants, and relative high transcript level of OsTTG1A was observed in seedlings of the 35S:OsTTG1A/ttg1 #1 line. We observed that plants of both 35S:OsTTG1A/ttg1 transgenic lines produced trichomes on rosette leaves and stems (Fig. 6B). Quantitative analysis showed that plants of the 35S:OsTTG1A/ttg1 #1 line produced more trichomes on rosette leaves (Fig. 6C), consistent with the relative high transcript level in seedlings of this line. On the other hand, reduced root hairs formation was observed in both of the 35S:OsTTG1A/ttg1 transgenic lines when compared with the ttg1 mutants (Fig. 7A), and quantitative analysis showed that root hair density in the 35S:OsTTG1A/ttg1 transgenic seedlings is similar to the Ler wild type (Fig. 7B).
We also found that seed color phenotype of the ttg1 mutant was recovered in the 35S:OsTTG1A/ttg1 transgenic plants, but also to different degree in the two different lines (Fig. 8A). Whereas mucilage production in the 35S:OsTTG1A/ttg1 #1 line was recovered nearly to the Ler wild type, but that in #2 line was largely similar to the ttg1 mutants (Fig. 8B), anthocyanin biosynthesis was also largely recovered in the 35S:OsTTG1A/ttg1 #1 line, but not #1 line (Fig. 8C). These results indicate that OsTTG1A is likely the functional analogue of TTG1.

**Discussion**

A MYB-bHLH-WD40 (MBW) complex formed by the R2R3 MYB transcriptional activator GL1, the bHLH transcription factor GL3, EGL3 or TT8, and the WD40-repeat protein TTG1 regulates trichome initiation in Arabidopsis [3, 4, 6, 7, 24]. By identifying and characterizing rice homologs of the trichome initiation-regulating MBW complex proteins, we found that similar MBW may present in rice and at least some components in the complex may have similar function as the one in Arabidopsis.

First, the rice homologs shared similar features as the Arabidopsis MBW complex, i.e., The [D/E]L × 2[R/K] × 3L × 6L × 3R amino acid signature required for the interaction of MYB transcription factors with bHLH transcription factors [41], and the S residue required for activation of GL2 [42], are full conserved in all the five OsGL1s (Fig. S1); The first 97 amino acids region in GL1 that is required for its interaction with GL3 [9], is highly conserved in both OsGL1A and OsGL1B (Fig. S2); and the 25 amino acids region TTG1 that is required for its interaction with GL3 [9], is also highly conserved in OsTTG1A and OsTTG1B (Fig. S3). Second, it has been shown that GL3 can function as a transcription activator, but GL1 and TTG1 can not [41, 44], similar, we found that OsGL3B activated reporter gene expression in transfected protoplast, but OsGL1E and OsTTG1 failed to do so (Fig. 3). Third, OsGL3B is able to interact with OsGL1E and OsTTG1A, respectively (Fig. 3, Fig. 4), indicating that they can form a MBW complex. In addition, OsGL1E and OsTTG1A can interact with GL3 (Fig. 3, Fig. 4), and OsGL3B is able to interact with GL1 and TTG1 (Fig. 5), suggested that rice homologs are interchangeable with their Arabidopsis MBW complex proteins in forming MBW complexes. Last but not least, ectopic expression of OsTTG1A restored phenotypes in the ttg1 mutant plants (Fig. 6, Fig. 7, Fig. 8), indicating that OsTTG1A is likely the functional analogue of TTG1 in controlling trichome formation in Arabidopsis. Considering that OsGL1E and OsGL3B are able to form MBW complex with OsTTG1A, it may be worthwhile to examine if they have similar function with GL1 and GL3, respectively in regulating trichome formation in Arabidopsis. Since OsTTG1A and OsTTG1B show high amino acid sequence similarly, it will be of interest to examine if OsTTG1B also have similar functions.

The MBW complex formed by GL1, GL3/EGL3 and TTG1 is able to regulate trichome initiation via activating GL2 [3–5, 7], but the same MBW complex is also able to activate the R3 MYB trichome initiation inhibitor genes [17–22]. Even though our previously study showed that OsTCL1 can regulate trichome initiation in Arabidopsis, but not in rice [40], considering that trichome initiation in rice can be regulated by OsWOX3B, a homolog of GL2 [33–35], it is worthwhile to examine if MBW complex formed by OsGL1E, OsGL3B and OsTTG1A is able to regulate trichome initiation in rice via activating OsWOX3B.
As discussed above, some of our experiments support that rice homologs and the Arabidopsis MBW complex proteins may have conserved functions, but some others also suggest that they may also have none conserved functions. First, OsGL1A and OsGL1B shared higher amino acid sequence identity and similarity with GL1 when compared with OsGL1D and OsGL1E (Fig. S1), however, they can not interact with GL3 or OsGL3B (Fig. 3, Fig. 4). Considering that the [D/E]L × 2[R/K] × 3L × 6L × 3R amino acid signature and the S residue required are conserved in all the five OsGL1s (Fig. 1S), it is very likely the some other amino acid residues may also be critical for the interaction of OsGL1s/GL1 with GL3/OsGL3B. According to the amino acid sequence alignment, there are only a few amino acids in the R3 MYB domain of OsGL1A and OsGL1B are different from these in GL1 and OsGL1E, it will be interest to examine if any of them may be critical for the interaction of OsGL1s/GL1 with GL3/OsGL3B. Second, unlike GL1, OsGL1D functions as a transcription activator (Fig. 3). It is unlike that OsGL1D contains an activation domain, whereas other OsGL1s do not, since our previously we have shown that the C-terminal domain of GL1 confer transcription activation activities, but as a whole, GL1 can not function as a transcription activator [44]. Therefore, it is worthwhile to Fig. out why OsGL1D as a whole, is able to show its transcription activation activities, whereas other GL1 and OsGL1s can not. Third, OsGL1D is able to interact with GL3, but not OsGL3 (Fig. 3, Fig. 4). Considering that OsGL1D is closely related to the OsGL1E (Fig. 1), it will also interest to examine why OsGL1D can interact with GL3 but not OsGL3B.

**Conclusions**

In this study, our results show that OsGL1E, OsGL3B and OsTTG1A are able to form a MBW complex, and they are interchangeable with the Arabidopsis MBW complex proteins. Further more, OsTTG1A is interchangeable with TTG1 in regulating trichome initiation in Arabidopsis. Our results show that OsGL1D is a transcription activator, and it can interact with GL3, but not OsGL3B. There results suggest that rice homologs and the Arabidopsis MBW complex proteins have conserved and none-conserved functions.

**Methods**

**Bioinformatics analysis**

Identification of GL1 and GL3 homologs in rice (*Oryza sativa*), including OsGL1A, OsGL1B, OsGL1C, OsGL3A, OsGL3B, OsGL3C, OsTTG1A, and OsTTG1B has been described previously [40]. OsGL1D (Loc_Os03g29614) and OsGL1E (Loc_Os06g10350) were identified as potentially interaction proteins of OsGL3B on STRING (https://string-db.org/).

Full-length amino acid sequences were used for phylogenetic analysis and sequence alignment. Phylogenetic analysis was performed on Phylogeny (www.phylogeny.fr) by using “One Click” mode with default settings, and sequence alignment was generated using BioEdit. Percentage of amino acid identity and similarity were calculated using MatGAT (v2.02) [47].

**Plant materials and growth conditions**
The Japonica rice variety *Nipponbare* was used for gene cloning, the Columbia-0 (Col) Arabidopsis was used for protoplasts isolation, and the Landsberg erecta (Ler) Arabidopsis was use for as a control for phenotype analysis. The *ttg1* mutant was in the Ler ecotypic background [12].

For trichome phenotypic analysis, protoplast isolation and plant transformation, seeds of indicated Arabidopsis materials were germinated and grown in soil pots as described previously [48]. For root hair phenotypic analysis, seeds were surface-sterilized and sown on solidified 1/2 MS (Murashige and Skoog) medium and grown vertically as described previously [40].

For RNA isolation from Arabidopsis to examine the expression of *TTG1* and *OsTTG1A*, seeds of the Ler wild type, the *ttg1* mutant, and the 35S:*OsTTG1A/*ttg1 transgenic plants were surface-sterilized and sown on solidified 1/2 MS medium as described previously [48].

For RNA isolation from rice to clone related genes, seeds of *Nipponbare* rice were generated and grown in water for 10 days. The photoperiod in the growth rooms was 16 h light/8 h dark, the light density was \(~120 \, \mu\text{mol} \, \text{m}^{-2} \, \text{s}^{-1}\), and the temperature were 22 °C for Arabidopsis, and 28 °C for rice [48].

**RNA isolation and RT-PCR**

Ten-day-old Arabidopsis and rice seedlings were used for RNA isolation. Total RNA was isolated using an EasyPure™ Plant RNA Kit (Transgene Biotech) and following the manufacturer’s instructions. cDNA was synthesized using an EasyScript First-Strand DNA Synthesis Super Mix (TransGen Biotech) following the manufacturer’s procedures, and used for RT-PCR amplification. For expression analysis of *TTG1* and *OsTTG1A*, the expression of *ACTIN2* (*ACT2*) was used as a control.

The primers used for amplification of *OsGL1A* are 5’-CAACATATGATGGGGAGGTCGCCGTGC-3’ and 5’-CAACTTAAGTCATTTTCATGGGGAGGCTTCTG-3’, for *OsGL1B* are 5’-CAACATATGATGGGGAGGTCACCG-3’ and 5’-CAACTTAAGTCATTTTCATGGGGAGGCTTCTG-3’, and for *OsTTG1A* are 5’-CAACATATGAGCAGCCCAAGCCG-3’ and 5’-CAACTTAAGTCAGACCCTGAGAAGCTGGA-3’. Other primers used for RT-PCR have been described previously [48].

**Constructs**

The nuclear indicator construct *NLS-RFP*, the reporter construct *Gal4:GUS* and the effector constructs *GD* (Gal4 DNA Binding Domain), *CAT*, *GL3*, *GD-GL3*, *GD-GL1*, *GD-TTG1* used for protoplasts transfection have been described previously [44, 49, 50].

To make HA (Human influenza hemagglutinin)-tagged *OsGL3B* construct, and GFP (Green fluorescent protein) tagged or GD-tagged *OsGL1s*, *OsGL3B* and *OsTTG1A* constructs for protoplast transfection, the full-length ORF (open-reading frame) of these corresponding genes were amplified by RT-PCR using RNA isolated from rice seedlings, or synthesized (for *OsGL3B*, *OsGL1D* and *OsGL1E*) by Sangon Biotech Co., Ltd, and cloned in-frame with an N-terminal HA, GFP or GD tag and under the control of the double 35S promoter into *pUC19* vector [50, 51].
To make 35S:OsTTG1A construct for plant transformation, the HA tagged OsTTG1A construct in pUC19 was digested with NdeI and AflII and subcloned into the binary vector pPZP211 vector [52].

To generate bait and pray constructs for yeast-two-hybrid assays, OsGL3B was cloned into pGBK7 vector (Oebiotech), and GL1 and TTG1 and their homologs in rice were cloned into pGADT7 vector (Oebiotech).

**Plant transformation and transgenic plants selection**

For phenotype rescue experiment, the 35S:OsTTG1A construct was introduced into Agrobacterium tumefaciens GV3101, and used to transform the ttg1 mutant plants by using the floral dip method [53]. The ttg1 mutant plants used for transformation were ~5-week-old, and have several mature flowers on the main inflorescence.

To select transgenic lines, T1 seeds were sown on 1/2 MS medium containing 50 μg/ml Kanamycin and 100 μg/ml Carbenicillin. More than 20 transgenic plants were obtained, and three trichome bearing T1 plants were chosen to isolate transgenic plants with a single T-DNA insertion locus in T2, and homozygous lines in T3 by germinating on 1/2 MS medium containing 25 μg/ml Kanamycin. Seeds from two homozygous lines were used for phenotypic analysis.

**Yeast two hybridization**

Yeast two hybridization assay was performed by using Yeast Transformation System 2 (Clontech) according to the manufacturer's instructions.

**Plasmid DNA isolation, protoplast transfection and GUS activity assay**

Plasmid DNA used for protoplast transfection was isolated using a GoldHi EndoFree Plasmid Maxi Kit (CWBO) according to the manufacturer's instructions.

Protoplasts were isolated from rosette leaves of 3~4-week-old Col plants, and transfected with plasmids of the reporter and effector genes by using the procedure described previously [50].

To examine subcellular localization of rice homologs of GL1, GL3 and TTG1, the plasmids of GFP fused constructs and nuclear indicator construct NLS-RFP were cotransfected into protoplasts. The transfected protoplasts were incubated at room temperature and under darkness for 20~22 h. GFP and RFP florescence were examined and photographed under a florescence microscope.

To examine the transcriptional activity of OsGL3B, plasmids of the reporter gene Gal4:GUS and the effector gene GD-OsGL3B were cotransfected into protoplasts. Cotransfections of GD and GD-GL3 were used as negative and positive controls, respectively. To examine the possible interaction of OsGL3B with GL1 and TTG1 in plant cells, plasmids of the reporter gene Gal4:GUS, the effectors gene GD-GL1 or GD-TTG1, and OsGL3B or CAT were cotransfected into protoplasts. To examine the possible interaction of OsTTG1A or OsGL1s with OsGL3B or GL3, plasmids of the reporter gene Gal4:GUS, the effectors gene GD-
OsGL1s or GD-OsTTG1A, and OsGL3B, GL3 or CAT were cotransfected into protoplasts. Cotransfections of GD were used as controls. The transfected protoplasts were incubated at room temperature and under darkness for 20~22 h. GUS activities were measured using a microplate reader (Synergy™ HT, BioTEK). Transfection of each combination contains three biological replicates, and the experiments were repeated at least twice with similar results.

**Mucilage production assays**

Seed were stained and mounted as described previously [54], and mucilage was viewed examined under a Motic K dissecting microscope.

**Anthocyanin biosynthesis assays**

Anthocyanin biosynthesis was assayed as described previously [55], except that 5% rather than 3% sucrose was used for the experiment.

**Microscopy**

GFP fluorescence in transfected protoplast was observed and photographs were taken under an Olympus FV1000 confocal microscope. Leaf trichomes, root hair, mucilage, anthocyanin and seeds color were examined under a Motic K microscope, and photographs were taken using an EOS 1100D digital camera connected to the microscope. Photographs of stem trichomes were taken using an EOS 1100D digital camera.

**Abbreviations**

Col: Columbia-0; GD: Gal4 DNA Binding Domain; GFP: Green fluorescent protein; HA: Human influenza hemagglutinin; Ler: Landsberg erecta; MBW: MYB-bHLH-WD40; MS: Murashige and skoog; ORF: open-reading frame; PEG: Polyethyleneglycol; SD-TL : SD-Trp-Leu; SD-TLH: SD-Trp-Leu-Hi; SD-TLHA: SD-Trp-Leu-His-Ade; WT: Wild type

**Declarations**

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Not applicable.

**Author contributions**

S.W. conceived the study. K.Z. and S.W. designed the experiments. K.Z., X.W. and Y.W. did the experiments. K.Z. and S.W. analyzed the data and drafted the manuscript. All the authors participated in the revision of the manuscript.

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**Availability of data and materials**

Rice genes sequences in this research were downloaded from Phytozome (www.phylogeny.fr). The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

**Ethics approval and consent to participate**

Not applicable.

**Consent for publication**

Not applicable.

**Competing interests**

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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