Identification of dynamin as an interactor of rice GIGANTEA by tandem-affinity purification (TAP)

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The transition from vegetative to reproductive development is one of the most important steps in the lives of plants. This transition is regulated by genetic as well as environmental factors such as light and temperature. The photoperiod or day-length is one of the most important factors in the determination of flowering time. Plants are able to measure changes in day-length and use this information to flower during the optimal season. Plants can be classified into three groups depending on their responses to the photoperiod: long-day plants (LDPs), which promote flowering under long-day (LD) conditions, short-day plants (SDPs), which flower under short-day (SD) conditions, and day-neutral plants, which flower independently of the photoperiod.

A number of genes that play important roles in the determination of flowering time were identified in *Arabidpsis*, a LDP. They include *GIGANTEA* (*GI*), *CONSTANS* (*CO*) and *FLOWERING LOCUS T* (*FT*). The rice orthologs of *GI*, *CO*, and *FT* have been identified as *OsGI, Hd1* and *Hd3a* respectively. Under SD conditions *OsGI* activates *Hd1* expression and *Hd1* induces *Hd3a* expression, while under LD conditions, *Hd1* represses *Hd3a* expression. In *Arabidopsis,* the regulation of *FT* expression by *CO* is reversed under LD conditions. Therefore, the difference in the regulation of *FT* by *CO* in *Arabidopsis* and rice was proposed to be the molecular mechanism that explains the difference between LDP and SDP.

Comprehensive analyses of protein-protein interactions have become increasingly important for biological studies in the post-genome era. The tandem affinity purification (TAP) method was developed for the high yield purification of protein complexes formed under native conditions.   The TAP method uses transgenes expressing N-terminal or C-terminal-fused

TAP-tagged versions of the target proteins. The protein complex is isolated from transgenic cells by affinity purification using the TAP tag. The TAP method was originally developed in yeast and has since been used in mammalian cells, insects, and *E. coli*. However, only a few studies of plant protein interactions using the TAP method have been reported. There have been no reports on the purification and identification of novel protein complexes using the TAP method in plants.

In order to study the functions of OsGI, we isolated OsGI complex using TAP method. We generated transgenic rice plants and cell cultures expressing a TAP-tagged version of OsGI. The endogenous OsGI, the TAP-tagged OsGI was also detected in the transgenic SGC line, which carried the C-terminal TAP-tagged construct. This SGC line was used for TAP method. To further investigate the intracellular localization of OsGI, a *35S::OsGI:GFP* construct was produced and introduced into rice leaf sheath cells by particle bombardment and into protoplast by electroporation. GFP signals were detected both in the cytosol and the nucleus in both methods. Before purifying the OsGI interacting proteins by the TAP method, we needed to examine whether the TAP-tagged OsGI protein was functional in rice plants. Hayama have previously reported that overexpression of OsGI delayed flowering in transgenic plants compared with the wild-type under both short day (SD) and long day (LD) conditions. Therefore, we analyzed the flowering times of transgenic plants overexpressing the TAP-tagged OsGI under both SD and LD conditions. As a result, the SGC lines C33 and C53 showed delayed flowering compared with the wild-type under SD conditions. This result suggests that the TAP-tagged OsGI protein (in SGC lines) was functional in transgenic rice plants. After TAP method using SGC line, the OsGI-interacting proteins were analyzed by mass spectrometry. Seven proteins, including dynamin, were identified as OsGI-interacting proteins. The interaction of OsGI with dynamin was verified by co-immunoprecipitation using a myc-tagged version of OsGI. Moreover, an analysis of *Arabidopsis* dynamin mutants indicated that although the flowering times of the mutants were not different from those of wild-type plants, an aerial rosette phenotype was observed in the mutants. These results indicate that the TAP method is effective for the isolation of novel proteins that interact with target proteins in plants

<div align="center">Contents</div>

I. Introduction

The transition from vegetative to reproductive development is one of the most important steps in the lives of plants. This transition is regulated by genetic as well as environmental factors such as light and temperature (Boss et al. 2004, Imaizumi and Kay 2006, Kobayashi and Weigel 2007). The photoperiod or day-length is one of the most important factors in the determination of flowering time. Plants are able to measure changes in day-length and use this information to flower during the optimal season. Plants can be classified into three groups depending on their responses to the photoperiod: long-day plants (LDPs), which promote flowering under long-day (LD) conditions, short-day plants (SDPs), which flower under short-day (SD) conditions, and day-neutral plants, which flower independently of the photoperiod (Fig. 1).

A number of genes that play important roles in the determination of flowering time were identified in *Arabidpsis*, a LDP (Koornneef et al. 1998, Searle and Coupland 2004, Yanovsky and Kay 2003). Flowering time genes can be classified into three groups based on their primary functions. The first group contains genes involved in the circadian clock. They include *CIRCADIAN CLOCK ASSOCIATED 1* (*CCA1*), *LATE ELONGATED HYPOCOTYL* (*LHY*), *TIMING OF CAB EXPRESSION*

1 (*TOC1*), *EARLY FLOWERING 4* (*ELF4*), and *LUX ARRHYTHMO* (*LUX*) /

PHYTOCLOCK 1 (*PCL1*) (Alabadi et al. 2001, Doyle et al. 2002, Hazen et al. 2005,

Onai and Ishiura 2005, Schaffer et al. 1998, Strayer et al. 2000, Wang and Tobin

1998). *CCA1* and *LHY* encode myb-like transcription factors which bind specifically

to the *cis*-element termed the "morning element" in the promoter of *TOC1* and

LUX/*PCL1*, and repress expression of *TOC1* (Harmer et al. 2000) and *LUX*/*PCL1*

(Alabadi et al. 2001, Hazen et al. 2005) during the morning hours. *TOC1*, *ELF4* and

LUX/PCL1 activate the expression of *CCA1* and *LHY* (Alabadi et al. 2001, Doyle et

al. 2002, Hazen et al. 2005). Posttranslational modification is one of the important

mechanisms for the regulation of flowering time, and the phosphorylation of CCA1,

and the degradation of TOC1 by the clock associated F-box protein ZEITLUPE

(ZTL), have been demonstrated.

The second group of flowering time genes in *Arabidpsis* contains the

photoreceptors. They include phytochromes encoded by *PHYA* and *PHYB* and

cryptochromes encoded by *CRY1* and *CRY2* (Lin 2000a, Lin 2000b). Photoreceptors

perceive various light stimuli and regulate expression of flowering time genes, or

alter stability of proteins encoded by flowering time genes. The third group contains

genes which are specifically involved in regulation of the other flowering time genes.

They include *GIGANTEA* (*GI*) (Fowler et al. 1999, Park et al. 1999), *CONSTANS* (*CO*) (Putterill et al. 1995, Suarez-Lopez et al. 2001) and *FLOWERING LOCUS T* (*FT*) (Kardailsky et al. 1999, Kobayashi et al. 1999) (Fig. 2). Transcription of these genes is regulated by the circadian clock. *GI* has been shown to regulate flowering and the circadian clock pathway in *Arabidopsis*, and has been identified in many other plant species (Mizoguchi et al. 2005, Suarez-Lopez et al. 2001). The GI protein interacts with the F-box protein FLAVIN-BINDING, KELCH REPEAT, F-BOX 1 (FKF1) (Nelson et al. 2000) in a blue-light dependent manner, and regulates the stability of CYCLING DOF FACTOR 1 (CDF1), a repressor of *CO* expression (Imaizumi et al. 2005, Sawa et al. 2007). GI also regulates *TOC1* expression, and it regulates the stability of ZTL by interacting with it (Kim et al. 2007).

CO, together with a HAP protein complex which has a CCAAT-box binding function, regulates *FT* expression (Ben-Naim et al. 2006, Cai et al. 2007, Wenkel et al. 2006). *FT* is expressed only in the phloem of the leaf, but the protein FT interacts with FD, a transcriptional factor in the shoot apical meristem (SAM), and the FT-FD complex activates expression of the floral organ identity gene *AP1* (Abe et al. 2005, Wigge et al. 2005). Recently it was shown that proteins encoded by the *Arabidopsis* gene *FT* and its rice homolog *Hd3a* act as florigens, or mobile flowering signals

(Jaeger and Wigge 2007, Mathieu et al. 2007, Tamaki et al. 2007) (Fig. 3). The rice orthologs of *GI*, *CO*, and *FT* have been identified as *OsGI, Hd1* and *Hd3a* respectively (Hayama et al. 2002, Kojima et al. 2002, Yano et al. 2000). Under SD conditions *OsGI* activates *Hd1* expression and *Hd1* induces *Hd3a* expression, while under LD conditions, *Hd1* represses *Hd3a* expression (Hayama et al. 2003). In *Arabidopsis,* the regulation of *FT* expression by *CO* is reversed under LD conditions. Therefore, the difference in the regulation of *FT* by *CO* in *Arabidopsis* and rice was proposed to be the molecular mechanism that explains the difference between LDP and SDP (Hayama et al. 2003) (Fig. 2).

Comprehensive analyses of protein-protein interactions have become increasingly important for biological studies in the post-genome era. Large-scale yeast-two hybrid screening has been used for this purpose (Ito et al. 2000, Uetz et al. 2000). However, more effective methods for analyzing protein-protein interactions under native conditions need to be developed. Improvements in mass spectrometry combined with the development of various protein purification technologies have made it possible to analyze proteins that are present at very low levels in their native states. Therefore, current limitations in the analysis of protein-protein interactions is not in the methods for identifying proteins involved in the interactions, but in the

purification methods available. The tandem affinity purification (TAP) method was developed for the high yield purification of protein complexes formed under native conditions (Puig et al. 2001, Rigaut et al. 1999). The TAP method uses transgenes expressing N-terminal or C-terminal-fused TAP-tagged versions of the target proteins (Fig. 4A). The protein complex is isolated from transgenic cells by affinity purification using the TAP tag. The TAP tag consists of two IgG binding domains of *Staphylococcus aureus* protein A and a calmodulin binding peptide, separated by a TEV protease cleavage site (Rigaut et al. 1999) (Fig. 4B). ProtA binds tightly to an IgG matrix, requiring the use of the TEV protease to elute protein under native conditions. The eluate of this first affinity purification steps is then presence of calcium. After washing, which removes contaminants and the TEV protease remaining after the first affinity purification, the bound proteins are released under mild conditions with EGTA (Fig. 5). In their original study, Rigaut et al. (1999) used this method to identify the U1 snRNP, involved in pre-mRNA splicing, and Mak 3/10/31, which is involved in protein modifications. The TAP method was originally developed in yeast and has since been used in mammalian cells (Knuesel et al. 2003), insects (Forler et al. 2003), and *E. coli* (Gully et al. 2003). However, only a few studies of plant protein interactions using the TAP method have been reported

(Rohila et al. 2004, Rohila et al. 2006, Rubio et al. 2005, Van Leene et al. 2007). There have been no reports on the purification and identification of novel protein complexes using the TAP method in plants.

In order to study the functions of the rice GI protein, we attempted to isolate OsGI-interacting proteins from cultured rice cells expressing a TAP-tagged OsGI protein. We were able to purify seven OsGI-interacting proteins by this method. Moreover, we confirmed the interaction of OsGI with one of these proteins, dynamin, using co-immunoprecipitation. We also characterized *Arabidopsis* plants with mutations in the dynamin genes *ADL3* and *ADL6*.

II. Materials and methods

1. *Plant materials*

Japonica rice (*Oryza sativa*) varieties Norin 8 and OsGI overexpression transgenic plant (OX24) were used as wild type and control of late flowering phenotype, respectively. Plants were grown in climate chamber under SD (10 h light / 14 h dark) and LD (14 h light / 10 h dark) conditions. *Arabidopsis thaliana* ecotype Columbia (Col.) was used as wild-type. *Arabidopsis* wild-type and mutants were used for flowering time and phenotypic analysis. Three T-DNA insertion lines for each gene were obtained from the T-DNA Express (SIGnal Arabidopsis Gene Mapping Tool). *ADL3* (AT1G59610) mutants were SALK_124686, SALK_134887 and SALK_150606, *ADL6* (AT1G10290) mutants were SALK_011319, SALK_071039 and SALK_018859.

2. *Cloning of the OsGI cDNA and plasmid construction*

The *OsGI* cDNA was synthesized from total RNA of rice leaves. Three cDNA

fragments covering three different regions of OsGI were separately amplified using three pairs of PCR primers. The primers were designed using the *OsGI* coding sequence, with alternations to create restriction enzyme sites. The primers used were: *OsGI1*-F-TOPO and *OsGI1*-R for fragment 1, *OsGI2*-F and *OsGI2*-R for fragment 2 and *OsGI3*-F and *OsGI3*-R for fragment 3. Plasmid construction was carried out using the Gateway technology (Invitrogen). The *OsGI* cDNA was inserted into the Gateway destination vector pGWB containing the TAP tag (Nakagawa et al. 2007) using the LR recombination reaction. Primer sequences were described in appendix section.

3. Generation and screening of transgenic plants and cultured cells

Plant transformation vectors carrying the OsGI cDNA were introduced into *Agrobacterium tumefaciens* for rice transformation. *Agrobacterium*-mediated transformation of rice was performed according to a published protocol (Hiei et al. 1994). Transformed callus was selected using hygromycin, and plants were regenerated from the transformed callus.

4. Production of the OsGI antibody

An 868 bps fragment from the 5' end of *OsGI* (from ATG -3 to +865) was amplified by RT-PCR using the primers NdeOsGI (F) and OsGIXhoI (R) and subcloned into the pBluescript SK+ vector (Stratagene). The fragment was re-isolated using *Nde* I and *Xho* I and inserted into the expression vector pET-15b (Novagen). The resultant vector was introduced into BL21 (DE3) competent cells (Stratagene). The antibody was commercially produced (Medical and Biological Laboratories). Primer sequences were described in appendix section.

5. The fractionation of protein and western blot analysis

Rice cultured cells were ground to a powder in liquid nitrogen and the powder was suspended in buffer A (20 mM HEPES, 250 mM sucrose, 10% glycerol, 10% EtOH, 0.5% Triton and the complete protease inhibitor cocktail (Roche)). The suspension was homogenized using a polytron (KINEMATICA; PT-MR 3100) at 15,000 rpm for 1 min on ice then filtered through four layers of miracloth (Calbiochem). The filtered extract was centrifuged at 3,300 x g for 10 min at 4 ˚C and

the supernatant was used as the cytosolic fraction. The pellet was resuspended in

buffer B (20 mM HEPES, 10% sucrose, 10% glycerol, 2.5% EtOH, 0.5% Triton-X

and the complete protease inhibitor cocktail) and centrifuged at 3,300 x g for 10 min

at 4 ˚C. The pellet was resuspended in a modified buffer A, in which the glycerol

concentration was changed to 35%. The suspension was transferred to a beaker and

stirred on ice while 2 µl of 5 M NaCl was added every minute for 1 min to make the

final concentration to 5 mM. Then the suspension was transferred to a new tube and

centrifuged at 15,000rpm for 30 min at 4 ˚C. The supernatant was used as the nuclear

fraction. Protein samples were boiled in sample buffer, run on SDS-PAGE gels (7.5

and 12.5%) and blotted onto polyvinylidene difluoride (PVDF) membranes

(Millipore). The histone H3 antibody (Upstate) was used for detection of the histone

protein.

6. Analysis of OsGI cellular localization by transient assays

The *OsGI* cDNA was subcloned into a modified binary vector carrying the *GFP*

gene, for transient assays. The *OsGI:GFP* and *Ubi::YFP* (Wong et al. unpublished

results) vectors were introduced into protoplasts from Oc cells by electroporation

(Wong et al. 2004) and into rice leaf sheath cells by particle bombardment. After 12 h of incubation, GFP and YFP fluorescence was analyzed using a confocal laser scanning microscope (Zeiss; LSM510)

7. Purification of OsGI interacting proteins by the TAP method

Rice cultured cells (40 g fresh weight) were ground in liquid nitrogen. TAP method was performed essentially as described previously with minor modifications (Rigaut et al. 1999). The AcTEV protease was used instead of TEV to raise the efficiency of the purification. The eluted samples were precipitated using STRATACLEAN (Stratagene).

8. Identification of proteins by mass spectrometry

Mass spectometry was performed as described previously (Fujiwara et al. 2006). Gels containing the proteins to be analyzed were visualized by fluorescent staining and cut into 1 mm slices. The proteins digested by trypsin in the gels and then identified by Q-TOF mass spectrometry (Waters) and a MASCOT database search of

the NCBI (National Center for Biotechnology Information) database. The analyses were performed 3 times and proteins that were detected all 3 times, only in the SGC cell line, were considered to be OsGI-interacting proteins.

9. Immunoprecipitation of OsGI and dynamin

The OsGI cDNA was introduced into the pGWB vector (Nakagawa et al. 2007) to fuse with the myc tag, and transgenic cell lines containing the myc-tagged OsGI protein were produced. Proteins were extracted from the 1 g cultured cells with extraction buffer (25 mM Tris-HCl pH7.5, 5 mM EDTA, 10 mM $MgCl_2$, 10% Sucrose, 150 mM NaCl, 1% NP-40, 1 mM DTT and the complete protease inhibitor cocktail). The protein concentration in the extract was adjusted to 2 mg / ml, and the extract was incubated with 50 µl protein G sepharose 4 Fast Flow (GE Healthcare). The supernatant was transferred to a new tube, 4 µl of myc antibody (Nacalai Tesque) were added, and the solution was incubated for 16 h at 4˚C with gentle rotation. 50 µl of protein G sepharose beads were added and the solution was incubated for a further 3 h with gentle rotation. After centrifugation, the beads were washed 3 times with well chilled extraction buffer. After removing the supernatant, 50 µl of sample buffer

were added to beads. The supernatants from the boiled beads were then applied to

SDS-PAGE. The immunoprecipitated proteins were analyzed on Western blots using

a commercially available plant dynamin antibody (Santa Cruz Biotechnology).

III. Results

1. Generation of transgenic rice plants and cultured cells expressing a TAP-tagged OsGI protein

To purify OsGI interacting proteins, transgenic rice plants and cultured cells expressing TAP-tagged versions of the OsGI protein were generated. A full-length OsGI cDNA was cloned into each of two binary vectors (Nakagawa et al. 2007) by the LR reaction. These binary vectors were designed to produce recombinant proteins carrying the TAP tag at either the N-terminal or C-terminal ends. Expression of the recombinant genes was driven by the CaMV35S promoter. The TAP tag used in this study was the same as the original one used by Rigaut et al. (1999), and consisted of the protein A gene, the TEV protease recognition site, and the calmodulin binding peptide (CBP) (Fig. 4A and 4B). The resulting two vectors, encoding modified versions of OsGI in which the TAP tag was fused either at the N-terminus (*35S::TAP:OsGI*, SGN) or the C-terminus (*35S::OsGI:TAP*, SGC) (Fig. 6A), were introduced into *Agrobacterium tumefaciens* for rice transformation. Transgenic rice plants and cultured cell lines were generated. Since TAP method have not been

successfully used to isolate novel plant proteins and it is not easy to analyze proteins

in rice leaves we thought that cultured rice cells might be suitable materials to apply

TAP for rice. TAP-tagged *OsGI* transcripts were detected by RT-PCR in both he

SGN and SGC cell lines (Fig. 6B). These lines were used for further experiments.

2. *Western blot analysis of TAP-tagged OsGI in transgenic rice cells lines*

To detect the OsGI protein by western blotting, we produced a polyclonal OsGI

antibody using the N terminal (- 3 to + 865) part of the *OsGI* sequence. The

Arabidpsis GI protein was previously shown to localize to the nucleus, by transient

assays using GUS or GFP fusion proteins (Huq et al. 2000). The intracellular

localization of the rice protein OsGI has not been examined previously. Therefore we

used our OsGI antibody to analyze proteins in cytosolic and nuclear fractions isolated

from transgenic cell lines by western blotting. The OsGI protein was detected in both

the cytosolic and the nuclear fractions of wild-type and transgenic cell lines, and

showed the expected size of ca. 130 kDa (Fig. 7). An antibody against histone H3

was used as a control for the nuclear-localized proteins. Interestingly, we observed

repeatedly that the concentration of the OsGI in the cytosol was higher than that in

the nucleus (Fig. 7). In addition to the endogenous OsGI, the TAP-tagged OsGI (ca. 150 kDa) was also detected in the transgenic SGC line, which carried the C-terminal TAP-tagged construct. Even though the transcript levels were similar in both cell lines (Fig. 6B), the TAP-tagged OsGI protein was not detected in the SGN line, carrying the N-terminal construct (Fig. 7) (Fig. 6A). The reason for the lack of protein expression in the SGN line is not clear at this stage. The SGC line was used for purification of the OsGI interacting proteins, and in other experiments.

3. The TAP-tagged OsGI protein was functional in transgenic rice plants

Before purifying the OsGI interacting proteins by the TAP method, we needed to examine whether the TAP-tagged OsGI protein was functional in rice plants. In a study using *Arabidopsis*, Rubio et al. (2005) performed mutant complementation tests and furthermore showed that a TAP-tagged protein was incorporated into the protein complex as was the untagged protein. Although the *Tos-17* insertion lines of *OsGI* have recently become available, they produced few seeds (Abe et al., unpublished results), and *Agrobacterium*-mediated transformation of rice requires seed-derived callus. Therefore, we used another approach to examine whether the TAP-tagged

OsGI protein was functional in rice plants. We have previously reported that overexpression of OsGI delayed flowering in transgenic plants compared with the wild-type under both short day (SD) and long day (LD) conditions (Hayama et al. 2003). Therefore, we analyzed the flowering times of transgenic plants overexpressing the TAP-tagged OsGI under both SD and LD conditions. Wild-type and *OsGI* overexpression plants (the OX 24 line) produced by Hayama et al. (2003) were used as negative and positive controls, respectively. The SGC lines C33 and C53 showed delayed flowering compared with the wild-type under SD conditions (Fig. 8A). The degree of the delay in flowering time in the SGC lines was similar to or higher than that in the OX 24 line. On the other hand, the flowering time of the SGN line N23 was similar to that of the wild-type. Under LD conditions, the flowering time of each transgenic line, including OX 24, was not significantly different from that of the wild-type (Fig. 8B). The reason for this observation is not known. However, these results suggest that the TAP-tagged OsGI protein (in SGC lines) was functional in transgenic rice plants.

4. Intracellular localization of OsGI

Western blot analysis with the OsGI antibody indicated that the OsGI protein was localized in the nucleus as well as in the cytosol (Fig. 7). To further investigate the intracellular localization of OsGI, a *35S::GFP:OsGI* and *35S::OsGI:GFP* construct were produced and introduced into rice leaf sheath cells by particle bombardment and rice protoplasts by electroporation (Fig. 9) (Fig. 10). A *Ubi*:*YFP* vector, which promotes expression of the YFP protein in both the cytosol and the nucleus, was used as a control. Fluorescence signals were observed using a confocal laser scanning microscope 12 h after introduction of the vectors. Both the GFP and YFP signals were detected both in the cytosol and the nucleus (Fig. 9). The plasmolysis will occur in proloplasts, it is easy to observe cytosol. As a result of introduce into protoplast by electroporation, the fluorescence of GFP:OsGI and OsGI:GFP were found in cytosol, but position of nucleus was not clearly found (Fig. 10).These results were consistent with those of the western blot analysis and it was concluded that the OsGI protein was localized in both the cytosol and the nucleus in rice cells.

5. Purification of OsGI interacting proteins by the TAP method

OsGI interacting proteins were purified from SGC rice cell cultures expressing

the TAP-tagged OsGI protein (referred to in this section as OsGI-TAP). In order to follow the progress of the purification, samples were taken during each step of the TAP procedure and analyzed on western blots using both the OsGI and TAP tag antibodies (Fig. 11). A protein extract from wild-type cultured cells was used as a negative control. In the first step, in which the protein A motif of OsGI-TAP was bound to the IgG beads, almost all of the OsGI-TAP in the solution seemed to bind to the beads (Fig. 11, extract after IgG). Since the original TEV protease contained its own cleavage site the efficiency of this step was not very high. Therefore we used AcTEV (Invitrogen), which has no recognition site and is therefore more stable, for cleavage at the TEV site. After digestion with the AcTEV protease the size of the OsGI-TAP protein was reduced by the size of the protein A peptide, which is ca. 15 kDa (Fig. 11, IgG eluate and IgG beads). Although some of the OsGI-TAP protein remained on the IgG beads, most was cleaved by AcTEV (Fig. 11, IgG eluate and IgG beads).

In the second step of the purification, CaCl$_2$ was added to the protein solution to induce calmodulin binding. In the flow through from the CaM beads, the OsGI-TAP signal was not detected, indicating that most of the OsGI-TAP bound to the beads (Fig. 11, CaM beads flow through). Then EGTA was added to chelate the Ca^{2+} and

the OsGI was eluted. Finally, a strong OsGI-TAP signal was detected in the CaM beads eluate (Fig. 11, CaM eluate). Furthermore, the OsGI-TAP signal was not detected in a solution derived from the CaM beads, suggesting that the OsGI-TAP was completely eluted from the beads (Fig. 11, CaM beads). The eluted proteins obtained by this purification method were separated by SDS-PAGE, and visualized by SYPRO-RUBY staining. Some protein bands were specifically found in the SGC lines and were not detected in the wild-type cells (Fig. 12). Fluorescent staining was found to have a similar level of sensitivity to that of silver staining with glutaraldehyde. Thus, fluorescent staining was used to detect protein bands in the mass spectrometric identification of the proteins.

6. Identification of OsGI interacting proteins

Gels containing candidate OsGI-interacting proteins, and corresponding gels from wild-type cells, were cut into 1 mm slices. Then the proteins in each slice were extracted, analyzed by Q-TOF mass spectrometry, and identified in a MASCOT database search (Fujiwara et al. 2006). If a protein was found in gels from both transgenic and wild-type cells, we considered that it was a non-specific protein.

Experiments were repeated three times, and proteins which were identified in all three experiments were considered as candidates for OsGI-interacting proteins. A total of seven proteins were identified (Table 1).

One of the putative OsGI-interacting proteins was a dynamin homolog. There are many more isoforms of dynamin in plants compared with animals, and their functions appear to be redundant. In *Arabidopsis* there exists a family of proteins termed the *Arabidopsis* dynamin-like proteins (ADLs). There are at least 5 forms (A-E) of ADL1. ADL1A, ADL1C, ADL4 and ADL5 are involved in cell-plate formation in dividing cells (Kang et al. 1998, Praefcke and McMahon 2004). ADL2A carries a chloroplast transit peptide at its N terminus, and is found in plastids (Kang et al. 1998). ADL2B is associated with mitochondria in dividing cells. ADL6 has a high level of homology with ADL3, and is the only member of the classical dynamin family. ADL6 is involved in trafficking from the *trans*-Golgi network to the vacuole (Jin et al. 2001).

Amongst the other putative OsGI-interacting proteins, Nup155 is associated with the formation of the nuclear envelope and pore complex (Franz et al. 2005) and is modified by O-GlcNAc (Wells et al. 2002). Another was the S2 subunit of the 26S proteasome, which has been shown in *Arabidopsis* to degrade the GI protein in the

dark (David et al. 2006). The putative vacuolar protein sorting-associated protein (gi|50919153) has homology with VPS35, which recognizes the retrieval signal domains of cargo proteins during their recruitment to vesicles (Nothwehr et al. 2000, Seaman et al. 1997). The multifunctional protein interacts with cortical microtubules and RNA in rice (Chuong et al. 2002, Chuong et al. 2005). The human HP68 (ATP-binding protein ABCE1 or RNase L inhibitor) is a host protein required for formation of the human immunodeficiency virus, type 1 (HIV-1) (Dooher et al. 2007, Lingappa et al. 2006, Zimmerman et al. 2002). However, the functions of plant proteins with homology to HP68 are not known.

7. OsGI interacts with dynamin in vivo

To further examine the interactions between OsGI and the putative OsGI-interacting proteins obtained by the TAP method, rice cell lines (M11 and M12) expressing a myc-tagged OsGI (*OsGI:myc*) were generated (Fig. 13A). After immunoprecipitation with the myc antibody, the OsGI:myc protein was detected in both the M11 and M12 cell lines by western blot analysis, using either the OsGI antibody or the myc antibody (Fig. 13B). Because the OsGI:myc signal was much

stronger in the M12 cell line than in the M11 line, the M12 cell line was used for further experiments. In a western blot analysis of proteins immunoprecipitated using the myc antibody, a specific signal was detected in the M12 line with a plant dynamin antibody (Fig. 13C). Since dynamin is highly conserved in plants we used a commercially available antibody made using the *Arabidopsis* dynamin. These results indicate that OsGI interacts with dynamin *in vivo*.

8. Phenotypic analysis of Arabidopsis dynamin mutants

To study the function of dynamin in relation to OsGI function we searched for available rice lines with insertion mutations in the putative dynamin gene. However, we were not able to obtain any rice dynamin mutants. Therefore, we searched for *Arabidopsis* dynamin mutants. First, to identify the closest *Arabidopsis* homologs of the putative rice dynamin (gi|5092565) a phylogenetic tree was created (Fig. 14). According to the tree, the rice dynamin homolog (gi|5092565) has the highest homology with *ADL3* (AT1G59610) and *ADL6* (AT1G10290). Three T-DNA insertion mutants in each of the *Arabidopsis ADL3* and *ADL6* genes were found by a data base search, and used for further analysis (Fig. 15A and 15B).

We first analyzed the flowering times of these mutants, and found that there were no differences in flowering time between the mutants and wild-type plants. However, the mutants exhibited an aerial rosette phenotype in the main stem (Fig. 15D-F). This phenotype has been described previously in a late flowering ecotype of *Arabidopsis* (Grbic and Bleecker 1996). The frequencies of plants showing the aerial rosette phenotype varied among the insertion lines. The lines SALK_150606 and SALK_071036, showed the highest frequencies amongst the *ADL3* and *ADL6* mutant lines, respectively (Fig. 15F). These results indicate that the dynamin proteins, which may interact with GI in *Arabidopsis*, are required for the suppression of elongation in the main stem. However, the possible role of dynamin in the regulation of flowering time remains to be studied.

IV. Discussion

1. Intracellular localization of OsGI

In this study, the intracellular localization of the OsGI protein was analyzed by two different methods. The first method involved the fractionation of protein extracts into cytosolic and nuclear proteins by centrifugation, and western blot analysis of the separated protein fractions using an OsGI specific antibody. The second method was a transient expression assay using a GFP-fused OsGI protein in rice leaf sheath cells and rice protoplasts. Results from both experiments indicated that OsGI was localized in both the nucleus and the cytosol. It was previously reported that a GFP-fused *Arabidopsis* GI protein was detected in the nucleus of onion epidermal cells when it was transiently expressed (Huq et al. 2000). However, results of a recent fractionation study showed that GI is present in both the nucleus and the cytosol of *Arabidopsis* (Kim et al. 2007).

Recently, the *Arabidopsis* GI was shown to interact with ZTL in the cytosol, and this interaction increased the stability and accumulation of the GI-ZTL complex (Kim

et al. 2007). Accumulation of this complex was shown to regulate a clock protein TOC1, which controls circadian clock oscillation in plants. The *Arabidopsis* GI was also shown to interact with FKF1 in the nucleus, and this complex regulates photoperiodic flowering by controlling the stability of CDF1, a repressor of *CO* transcription (Sawa et al. 2007). Therefore, GI seems to play roles in flowering and circadian clock oscillations by interacting with two different proteins in the two compartments of the cell.

Although our results indicated that OsGI was localized in both the nucleus and the cytosol, the putative OsGI-interacting proteins obtained by the TAP method in this study (Table 1) are primarily cytosolic proteins. This could be because the TAP-tagged OsGI protein was mainly located in the cytosol of the cultured rice cells used for purification (Fig. 7). Alternatively, only strong interactors of OsGI may have been identified in this study. The use of a cross-linker described by Rohila et al. (2004) may be useful for identifying proteins which have weaker interactions with target proteins.

2. Use of the TAP method in plants

Rohila et al. (2004) were the first to apply the TAP method in plants. They introduced a TAP-tagged GVG (*NTAPi:GVG*) construct into *Nicotiana benthamiana* leaves by transient *Agrobacterium* mediated transformation. Proteins extracted from the transgenic cells were used for purification by the TAP method, and HSP90 and HSP70 were identified as GVG-interacting proteins. This confirmed previous reports on the interaction of HSP90 and HSP70 with GVG in humans and plants. By using an optimized purification method, the same group expressed 41 TAP-tagged rice protein kinases in transgenic rice plants, recovered 36 (95%) of the TAP-tagged proteins after purification, and identified interacting proteins for 23 (56%) of the kinases (Rohila et al. 2006). No further studies on the isolated proteins have been reported yet.

The efficiency of protein identification in the study described above was lower than that in yeast, in which endogenous proteins are replaced with the TAP-tagged proteins by homologous recombination (Rigaut et al. 1999). One possible reason for the lower efficiency of the TAP method in previous plant studies may be due to competition for interacting proteins between the untagged endogenous and tagged proteins. In our study the *OsGI:TAP* gene was introduced into wild-type rice, therefore, the TAP-tagged OsGI protein was likely to compete with the endogenous OsGI for interaction. This could have decreased the efficiency of the TAP method in

our experiments. To circumvent this potential problem we used the *35S* promoter to achieve high levels of expression of the TAP-tagged OsGI transgenes. However, no clear quantitative difference was observed between the levels of endogenous OsGI and OsGI:TAP by western blot analysis (Fig. 7).

In our purification, heat shock proteins and elongation factors were identified, but they were shown to be non-specific proteins (data not shown). A similar observation was made in another study (Rohila et al. 2006). Rubio et al. (2005) performed TAP-tagging experiments using CSN3, a component of the COP9 signalosome complex in *Arabidopsis*. They used the rhinovirus C3 protease, which is highly active at low temperatures, instead of the TEV protease. We used AcTEV to increase the efficiency of the procedure, because it has higher stability than TEV. Rubio et al. (2005) used 6 x His and 9 x myc repeats in place of the CBP, to avoid the use of EGTA in the final purification step. The CBP was not changed in our study because we focused on the purification of OsGI interacting proteins. If the objective was to analyze the protein complex functions after purification, then a tag which does not require Ca^{2+} chelation for purification may be advantageous.

Tags might affect the functions of target proteins. In the current study both N-terminal and C-terminal TAP fusions were produced, but the tagged protein was

produced only from the C-terminal fusion (in the SGC lines). SGC plants showed

delayed flowering under SD conditions but not under LD conditions. The reason for

the lack of protein expression in the SGN line is not clear at this stage. OsGI-

overexpression plants were previously shown to be late flowering under both SD and

LD conditions (Hayama et al. 2003). Rubio et al. (2005) generated transgenic plants

expressing 31 TAP-tagged genes, including photomorphogenesis-related and 26S

proteasome-related genes. These transgenic plants exhibited differences in the levels

of gene expression and in their abilities to complement mutant genes (Rubio et al.

2005).

Rubio et al. (2005) and Rohila et al. (2004, 2006) performed pioneering

experiments using the TAP-tagging method in plants. Several other studies have also

applied the TAP method in plants, however the transgenic plants were used only for

western blot analysis of the tagged proteins or for pull down assays. Thus far there

have been no reports on the identification of novel proteins which were shown to

interact with the tagged targets by this method in plants. In this study we were able to

identify seven OsGI-interacting proteins, including a dynamin-like protein, by the

TAP method. Furthermore, we showed that OsGI interacts with dynamin, by

co-immunoprecipitation with the myc antibody from extracts of OsGI:myc cell

cultures.

3. Possible functions of the Arabidopsis dynamin like protein ADL3 and ADL6 in plant development

Dynamin is a high molecular weight GTPase which plays a critical role in vesicle formation on the plasma membrane during endocytosis (Praefcke and McMahon 2004). In *Arabidopsis*, the dynamin-like protein family consists of at least six forms (1-6), and there are five forms of ADL1 (A to E). Of these, ADL6 shows the greatest homology with the animal dynamin 1, and has a conserved GTPase domain at the N terminus, a pleckstrin homology domain at center, and a Pro-rich motif at the C terminus (Jin et al. 2001). ADL6 plays a role in the formation of vesicles for trafficking from the *trans*-Golgi network to the vacuole (Jin et al. 2001). *ADL3* is expressed weakly in most tissues, except for the siliques, in which it is highly expressed (Mikami et al. 2000). Studies of dynamin in *Arabidopsis* have mainly dealt with intracellular localization, and little attention has been given to protein function. In the current study, *dynamin* mutants were grown under LD (16 h light / 8 h dark) conditions and their development was analyzed. Results indicated

that the flowering times of the *dynamin* mutants were not different from those of the wild-type plants, but an aerial rosette phenotype was observed in the main stems of the mutants. This was a diagnostic phenotype found in the *Arabidopsis* ecotype *Sy*-0 (Grbic and Bleecker1996). *ENHANCER OF AERIAL ROSETTE* (*EAR*) and *AERIAL ROSETTE 1* (*ART1*) were identified as the genes required for this phenotype in the *Sy*-0 ecotype (Grbic and Bleecker1996). The flowering time of *Sy*-0 is delayed, because *ART1* activates expression of a floral repressor *FLC* (Poduska et al. 2003). The aerial rosette phenotype has also been observed in other *Arabidopsis* mutants. Overexpression of *GLABROUS INFLORESCENCE STEM* (*GIS*), a C2H2-type transcription factor, caused the aerial rosette phenotype in transgenic plants (Gan et al. 2006). The *GIS* gene was shown to play a role in epidermal cell differentiation during the vegetative phase. An analysis of the *spy gis* double mutant indicated that *SPY* acts upstream of *GIS* and suppresses *GIS* expression (Gan et al. 2006). These functions of *SPY* and *GIS* are related to GA signaling (Gan et al. 2006). Therefore, it is possible that the aerial rosette phenotype was caused by changes in GA signaling. It is also possible that the dynamin mutations analyzed in the current study may have alterations in GA signaling. This remains to be studied.

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Appendix

Primer	Sequence (5'-3')
OsGI1-F-TOPO	caccaagcttATGTCAGCTTCAAATGAG
OsGI1-R	gaattctctagaTGGTGCCTCGAGAAGACC
OsGI2-F	TTGGTCTTCTCGAGGCAC
OsGI2-R	CTTCTAGAGGCTCAGCTT
OsGI3-F	AAGCTGAGCCTCTAGAAG
OsGI3-R	gaattcGCAAGTGAGTGGGCAGCC
NdeOsGI (F)	catATGTCAGCTTCAAATGAGAAGTGGATT
OsGIXhoI (R)	ctcGAGGTAATAGAAGTGCAGGAACAG
Ubq F	CCAGGACAAGATGATCTGCC
Ubq R	AAGAAGCTGAAGCATCCAGC
Hpt F	GGCCTCCAGAAGAAGATGTTGG
Hpt R	GAGCCTGACCTATTGCATCTCC

References

Abe, M., Kobayashi, Y., Yamamoto, S., Daimon, Y., Yamaguchi, A., Ikeda, Y.,
Ichinoki, H., Notaguchi, M., Goto, K., and Araki, T. (2005) FD, a bZIP protein
mediating signals from the floral pathway integrator FT at the shoot apex.
Science. 309: 1052-1056.

Alabadi, D., Oyama, T., Yanovsky, M. J., Harmon, F. G., Mas, P., and Kay, S. A.
(2001) Reciprocal regulation between TOC1 and LHY/CCA1 within the
Arabidopsis circadian clock. *Science.* 293: 880-883.

Ben-Naim, O., Eshed, R., Parnis, A., Teper-Bamnolker, P., Shalit, A., Coupland, G.,
Samach, A., and Lifschitz, E. (2006) The CCAAT binding factor can mediate
interactions between CONSTANS-like proteins and DNA. *Plant J.* 46: 462-476.

Boss, P. K., Bastow, R. M., Mylne, J. S., and Dean, C. (2004) Multiple pathways in
the decision to flower: enabling, promoting, and resetting. *Plant Cell.* 16 Suppl:
S18-31.

Cai, X., Ballif, J., Endo, S., Davis, E., Liang, M., Chen, D., Dewald, D., Kreps, J.,
Zhu, T., and Wu, Y. (2007) A Putative CCAAT-Binding Transcription Factor Is
a Regulator of Flowering Timing in Arabidopsis. *Plant Physiol.* 145: 98-105.

Chuong, S. D., Mullen, R. T., and Muench, D. G. (2002) Identification of a rice

RNA- and microtubule-binding protein as the multifunctional protein, a

peroxisomal enzyme involved in the beta -oxidation of fatty acids. *J.Biol.Chem.*

277: 2419-2429.

Chuong, S. D., Park, N. I., Freeman, M. C., Mullen, R. T., and Muench, D. G. (2005)

The peroxisomal multifunctional protein interacts with cortical microtubules in

plant cells. *BMC Cell Biol.* 6: 40.

David, K. M., Armbruster, U., Tama, N., and Putterill, J. (2006) Arabidopsis

GIGANTEA protein is post-transcriptionally regulated by light and dark. *FEBS

Lett.* 580: 1193-1197.

Dooher, J. E., Schneider, B. L., Reed, J. C., and Lingappa, J. R. (2007) Host ABCE1

is at plasma membrane HIV assembly sites and its dissociation from Gag is

linked to subsequent events of virus production. *Traffic.* 8: 195-211.

Doyle, M. R., Davis, S. J., Bastow, R. M., McWatters, H. G., Kozma-Bognar, L.,

Nagy, F., Millar, A. J., and Amasino, R. M. (2002) The ELF4 gene controls

circadian rhythms and flowering time in Arabidopsis thaliana. *Nature.* 419:

74-77.

Forler, D., Kocher, T., Rode, M., Gentzel, M., Izaurralde, E., and Wilm, M. (2003)

An efficient protein complex purification method for functional proteomics in higher eukaryotes. *Nat.Biotechnol.* 21: 89-92.

Fowler, S., Lee, K., Onouchi, H., Samach, A., Richardson, K., Morris, B., Coupland, G., and Putterill, J. (1999) GIGANTEA: a circadian clock-controlled gene that regulates photoperiodic flowering in Arabidopsis and encodes a protein with several possible membrane-spanning domains. *EMBO J.* 18: 4679-4688.

Franz, C., Askjaer, P., Antonin, W., Iglesias, C. L., Haselmann, U., Schelder, M., de Marco, A., Wilm, M., Antony, C., and Mattaj, I. W. (2005) Nup155 regulates nuclear envelope and nuclear pore complex formation in nematodes and vertebrates. *EMBO J.* 24: 3519-3531.

Fujiwara, M., Umemura, K., Kawasaki, T., and Shimamoto, K. (2006) Proteomics of Rac GTPase signaling reveals its predominant role in elicitor-induced defense response of cultured rice cells. *Plant Physiol.* 140: 734-745.

Gan, Y., Kumimoto, R., Liu, C., Ratcliffe, O., Yu, H., and Broun, P. (2006) GLABROUS INFLORESCENCE STEMS modulates the regulation by gibberellins of epidermal differentiation and shoot maturation in Arabidopsis. *Plant Cell.* 18: 1383-1395.

Grbic, B. and Bleecker, A. B. (1996) An altered body plan is conferred on

Arabidopsis plants carrying dominant alleles of two genes. *Development.* 122: 2395-2403.

Gully, D., Moinier, D., Loiseau, L., and Bouveret, E. (2003) New partners of acyl carrier protein detected in Escherichia coli by tandem affinity purification. *FEBS Lett.* 548: 90-96.

Harmer, S. L., Hogenesch, J. B., Straume, M., Chang, H. S., Han, B., Zhu, T., Wang, X., Kreps, J. A., and Kay, S. A. (2000) Orchestrated transcription of key pathways in Arabidopsis by the circadian clock. *Science.* 290: 2110-2113.

Hayama, R., Izawa, T., and Shimamoto, K. (2002) Isolation of rice genes possibly involved in the photoperiodic control of flowering by a fluorescent differential display method. *Plant Cell Physiol.* 43: 494-504.

Hayama, R., Yokoi, S., Tamaki, S., Yano, M., and Shimamoto, K. (2003) Adaptation of photoperiodic control pathways produces short-day flowering in rice. *Nature.* 422: 719-722.

Hazen, S. P., Schultz, T. F., Pruneda-Paz, J. L., Borevitz, J. O., Ecker, J. R., and Kay, S. A. (2005) LUX ARRHYTHMO encodes a Myb domain protein essential for circadian rhythms. *Proc.Natl.Acad.Sci.U.S.A.* 102: 10387-10392.

Hiei, Y., Ohta, S., Komari, T., and Kumashiro, T. (1994) Efficient transformation of

rice (Oryza sativa L.) mediated by Agrobacterium and sequence analysis of the boundaries of the T-DNA. *Plant J.* 6: 271-282.

Huq, E., Tepperman, J. M., and Quail, P. H. (2000) GIGANTEA is a nuclear protein involved in phytochrome signaling in Arabidopsis. *Proc.Natl.Acad.Sci.U.S.A.* 97: 9789-9794.

Imaizumi, T. and Kay, S. A. (2006) Photoperiodic control of flowering: not only by coincidence. *Trends Plant Sci.* 11: 550-558.

Imaizumi, T., Schultz, T. F., Harmon, F. G., Ho, L. A., and Kay, S. A. (2005) FKF1 F-box protein mediates cyclic degradation of a repressor of CONSTANS in Arabidopsis. *Science.* 309: 293-297.

Ito, T., Tashiro, K., Muta, S., Ozawa, R., Chiba, T., Nishizawa, M., Yamamoto, K., Kuhara, S., and Sakaki, Y. (2000) Toward a protein-protein interaction map of the budding yeast: A comprehensive system to examine two-hybrid interactions in all possible combinations between the yeast proteins. *Proc.Natl.Acad.Sci.U.S.A.* 97: 1143-1147.

Jaeger, K. E. and Wigge, P. A. (2007) FT protein acts as a long-range signal in Arabidopsis. *Curr.Biol.* 17: 1050-1054.

Jin, J. B., Kim, Y. A., Kim, S. J., Lee, S. H., Kim, D. H., Cheong, G. W., and Hwang,

I. (2001) A new dynamin-like protein, ADL6, is involved in trafficking from the

trans-Golgi network to the central vacuole in Arabidopsis. *Plant Cell.* 13:

1511-1526.

Kang, S. G., Jin, J. B., Piao, H. L., Pih, K. T., Jang, H. J., Lim, J. H., and Hwang, I.

(1998) Molecular cloning of an Arabidopsis cDNA encoding a dynamin-like

protein that is localized to plastids. *Plant Mol.Biol.* 38: 437-447.

Kardailsky, I., Shukla, V. K., Ahn, J. H., Dagenais, N., Christensen, S. K., Nguyen, J.

T., Chory, J., Harrison, M. J., and Weigel, D. (1999) Activation tagging of the

floral inducer FT. *Science.* 286: 1962-1965.

Kim, W. Y., Fujiwara, S., Suh, S. S., Kim, J., Kim, Y., Han, L., David, K., Putterill, J.,

Nam, H. G., and Somers, D. E. (2007) ZEITLUPE is a circadian photoreceptor

stabilized by GIGANTEA in blue light. *Nature.* 449: 356-360.

Knuesel, M., Wan, Y., Xiao, Z., Holinger, E., Lowe, N., Wang, W., and Liu, X.

(2003) Identification of novel protein-protein interactions using a versatile

mammalian tandem affinity purification expression system. *Mol.Cell.Proteomics.*

2: 1225-1233.

Kobayashi, Y., Kaya, H., Goto, K., Iwabuchi, M., and Araki, T. (1999) A pair of

related genes with antagonistic roles in mediating flowering signals. *Science.*

286: 1960-1962.

Kobayashi, Y. and Weigel, D. (2007) Move on up, it's time for change mobile signals

controlling photoperiod-dependent flowering. *Genes Dev.* 21: 2371-2384.

Kojima, S., Takahashi, Y., Kobayashi, Y., Monna, L., Sasaki, T., Araki, T., and Yano,

M. (2002) Hd3a, a rice ortholog of the Arabidopsis FT gene, promotes transition

to flowering downstream of Hd1 under short-day conditions. *Plant Cell Physiol.*

43: 1096-1105.

Koornneef, M., Alonso-Blanco, C., Peeters, A. J., and Soppe, W. (1998) Genetic

Control of Flowering Time in Arabidopsis. *Annu.Rev.Plant Physiol.Plant*

Mol.Biol. 49: 345-370.

Lin, C. (2000a) Photoreceptors and regulation of flowering time. *Plant Physiol.* 123:

39-50.

Lin, C. (2000b) Plant blue-light receptors. *Trends Plant Sci.* 5: 337-342.

Lingappa, J. R., Dooher, J. E., Newman, M. A., Kiser, P. K., and Klein, K. C. (2006)

Basic residues in the nucleocapsid domain of Gag are required for interaction of

HIV-1 gag with ABCE1 (HP68), a cellular protein important for HIV-1 capsid

assembly. *J.Biol.Chem.* 281: 3773-3784.

Mathieu, J., Warthmann, N., Kuttner, F., and Schmid, M. (2007) Export of FT protein

from phloem companion cells is sufficient for floral induction in Arabidopsis. *Curr.Biol.* 17: 1055-1060.

Mikami, K., Iuchi, S., Yamaguchi-Shinozaki, K., and Shinozaki, K. (2000) A novel Arabidopsis thaliana dynamin-like protein containing the pleckstrin homology domain. *J.Exp.Bot.* 51: 317-318.

Mizoguchi, T., Wright, L., Fujiwara, S., Cremer, F., Lee, K., Onouchi, H., Mouradov, A., Fowler, S., Kamada, H., Putterill, J., and Coupland, G. (2005) Distinct roles of GIGANTEA in promoting flowering and regulating circadian rhythms in Arabidopsis. *Plant Cell.* 17: 2255-2270.

Nakagawa, T., Kurose, T., Hino, T., Tanaka, K., Kawamukai, M., Niwa, Y., Toyooka, K., Matsuoka, K., Jinbo, T., and Kimura, T. (2007) Development of series of gateway binary vectors, pGWBs, for realizing efficient construction of fusion genes for plant transformation. *J.Biosci.Bioeng.* 104: 34-41.

Nelson, D. C., Lasswell, J., Rogg, L. E., Cohen, M. A., and Bartel, B. (2000) FKF1, a clock-controlled gene that regulates the transition to flowering in Arabidopsis. *Cell.* 101: 331-340.

Nothwehr, S. F., Ha, S. A., and Bruinsma, P. (2000) Sorting of yeast membrane proteins into an endosome-to-Golgi pathway involves direct interaction of their

cytosolic domains with Vps35p. *J.Cell Biol.* 151: 297-310.

Onai, K. and Ishiura, M. (2005) PHYTOCLOCK 1 encoding a novel GARP protein

essential for the Arabidopsis circadian clock. *Genes Cells.* 10: 963-972.

Park, D. H., Somers, D. E., Kim, Y. S., Choy, Y. H., Lim, H. K., Soh, M. S., Kim, H.

J., Kay, S. A., and Nam, H. G. (1999) Control of circadian rhythms and

photoperiodic flowering by the Arabidopsis GIGANTEA gene. *Science.* 285:

1579-1582.

Poduska, B., Humphrey, T., Redweik, A., and Grbic, V. (2003) The synergistic

activation of FLOWERING LOCUS C by FRIGIDA and a new flowering gene

AERIAL ROSETTE 1 underlies a novel morphology in Arabidopsis. *Genetics.*

163: 1457-1465.

Praefcke, G. J. and McMahon, H. T. (2004) The dynamin superfamily: universal

membrane tubulation and fission molecules? *Nat.Rev.Mol.Cell Biol.* 5: 133-147.

Puig, O., Caspary, F., Rigaut, G., Rutz, B., Bouveret, E., Bragado-Nilsson, E., Wilm,

M., and Seraphin, B. (2001) The tandem affinity purification (TAP) method: a

general procedure of protein complex purification. *Methods.* 24: 218-229.

Putterill, J., Robson, F., Lee, K., Simon, R., and Coupland, G. (1995) The

CONSTANS gene of Arabidopsis promotes flowering and encodes a protein

showing similarities to zinc finger transcription factors. *Cell.* 80: 847-857.

Rigaut, G., Shevchenko, A., Rutz, B., Wilm, M., Mann, M., and Seraphin, B. (1999)
A generic protein purification method for protein complex characterization and
proteome exploration. *Nat.Biotechnol.* 17: 1030-1032.

Rohila, J. S., Chen, M., Cerny, R., and Fromm, M. E. (2004) Improved tandem
affinity purification tag and methods for isolation of protein heterocomplexes
from plants. *Plant J.* 38: 172-181.

Rohila, J. S., Chen, M., Chen, S., Chen, J., Cerny, R., Dardick, C., Canlas, P., Xu, X.,
Gribskov, M., Kanrar, S., Zhu, J. K., Ronald, P., and Fromm, M. E. (2006)
Protein-protein interactions of tandem affinity purification-tagged protein kinases
in rice. *Plant J.* 46: 1-13.

Rubio, V., Shen, Y., Saijo, Y., Liu, Y., Gusmaroli, G., Dinesh-Kumar, S. P., and
Deng, X. W. (2005) An alternative tandem affinity purification strategy applied
to Arabidopsis protein complex isolation. *Plant J.* 41: 767-778.

Sawa, M., Nusinow, D. A., Kay, S. A., and Imaizumi, T. (2007) FKF1 and
GIGANTEA Complex Formation is Required for Day-Length Measurement in
Arabidopsis. *Science.* 318: 261-265.

Schaffer, R., Ramsay, N., Samach, A., Corden, S., Putterill, J., Carre, I. A., and

Coupland, G. (1998) The late elongated hypocotyl mutation of Arabidopsis

disrupts circadian rhythms and the photoperiodic control of flowering. *Cell.* 93:

1219-1229.

Seaman, M. N., Marcusson, E. G., Cereghino, J. L., and Emr, S. D. (1997) Endosome

to Golgi retrieval of the vacuolar protein sorting receptor, Vps10p, requires the

function of the VPS29, VPS30, and VPS35 gene products. *J.Cell Biol.* 137:

79-92.

Searle, I. and Coupland, G. (2004) Induction of flowering by seasonal changes in

photoperiod. *EMBO J.* 23: 1217-1222.

Strayer, C., Oyama, T., Schultz, T. F., Raman, R., Somers, D. E., Mas, P., Panda, S.,

Kreps, J. A., and Kay, S. A. (2000) Cloning of the Arabidopsis clock gene TOC1,

an autoregulatory response regulator homolog. *Science.* 289: 768-771.

Suarez-Lopez, P., Wheatley, K., Robson, F., Onouchi, H., Valverde, F., and

Coupland, G. (2001) CONSTANS mediates between the circadian clock and the

control of flowering in Arabidopsis. *Nature.* 410: 1116-1120.

Tamaki, S., Matsuo, S., Wong, H. L., Yokoi, S., and Shimamoto, K. (2007) Hd3a

protein is a mobile flowering signal in rice. *Science.* 316: 1033-1036.

Uetz, P., Giot, L., Cagney, G., Mansfield, T. A., Judson, R. S., Knight, J. R.,

Lockshon, D., Narayan, V., Srinivasan, M., Pochart, P., Qureshi-Emili, A., Li, Y.,
Godwin, B., Conover, D., Kalbfleisch, T., Vijayadamodar, G., Yang, M.,
Johnston, M., Fields, S., and Rothberg, J. M. (2000) A comprehensive analysis of
protein-protein interactions in Saccharomyces cerevisiae. *Nature.* 403: 623-627.

Van Leene, J., Stals, H., Eeckhout, D., Persiau, G., Van De Slijke, E., Van Isterdael,
G., De Clercq, A., Bonnet, E., Laukens, K., Remmerie, N., Henderickx, K., De
Vijlder, T., Abdelkrim, A., Pharazyn, A., Van Onckelen, H., Inze, D., Witters, E.,
and De Jaeger, G. (2007) A tandem affinity purification-based technology
platform to study the cell cycle interactome in Arabidopsis thaliana.
Mol.Cell.Proteomics. 6: 1226-1238.

Wang, Z. Y. and Tobin, E. M. (1998) Constitutive expression of the CIRCADIAN
CLOCK ASSOCIATED 1 (CCA1) gene disrupts circadian rhythms and
suppresses its own expression. *Cell.* 93: 1207-1217.

Wells, L., Vosseller, K., Cole, R. N., Cronshaw, J. M., Matunis, M. J., and Hart, G.
W. (2002) Mapping sites of O-GlcNAc modification using affinity tags for serine
and threonine post-translational modifications. *Mol.Cell.Proteomics.* 1: 791-804.

Wenkel, S., Turck, F., Singer, K., Gissot, L., Le Gourrierec, J., Samach, A., and
Coupland, G. (2006) CONSTANS and the CCAAT box binding complex share a

functionally important domain and interact to regulate flowering of Arabidopsis. *Plant Cell.* 18: 2971-2984.

Wigge, P. A., Kim, M. C., Jaeger, K. E., Busch, W., Schmid, M., Lohmann, J. U., and Weigel, D. (2005) Integration of spatial and temporal information during floral induction in Arabidopsis. *Science.* 309: 1056-1059.

Wong, H. L., Sakamoto, T., Kawasaki, T., Umemura, K., and Shimamoto, K. (2004) Down-regulation of metallothionein, a reactive oxygen scavenger, by the small GTPase OsRac1 in rice. *Plant Physiol.* 135: 1447-1456.

Yano, M., Katayose, Y., Ashikari, M., Yamanouchi, U., Monna, L., Fuse, T., Baba, T., Yamamoto, K., Umehara, Y., Nagamura, Y., and Sasaki, T. (2000) Hd1, a major photoperiod sensitivity quantitative trait locus in rice, is closely related to the Arabidopsis flowering time gene CONSTANS. *Plant Cell.* 12: 2473-2484.

Yanovsky, M. J. and Kay, S. A. (2003) Living by the calendar: how plants know when to flower. *Nat.Rev.Mol.Cell Biol.* 4: 265-275.

Zimmerman, C., Klein, K. C., Kiser, P. K., Singh, A. R., Firestein, B. L., Riba, S. C., and Lingappa, J. R. (2002) Identification of a host protein essential for assembly of immature HIV-1 capsids. *Nature.* 415: 88-92.

Table. 1 OsGI-interacting proteins identified by Q-TOF mass spectometry

Identified proteins	Accession No.	M. W.	Source	score	Peptides*
putative nuclear pore complex protein Nup155	gi|54290722	162656	*Oryza sativa*	103	2
putative dynamin homolog	gi|50912565	100153	*Oryza sativa*	72	2
putative 26S proteasome regulatory subunit S2	gi|53791880	90633	*Oryza sativa*	138	4
putative vacuolar protein sorting-associated protein	gi|50919153	90565	*Oryza sativa*	172	3
multifunctional protein	gi|33338559	78337	*Oryza sativa*	385	8
RNase L inhibitor-like protein	gi|16755057	68940	*Oryza sativa*	106	3
beta-tubulin	gi|303842	4986	*Oryza sativa*	47	1

*Numbers of matching peptides identified in the amino acid sequences of the assigned proteins.
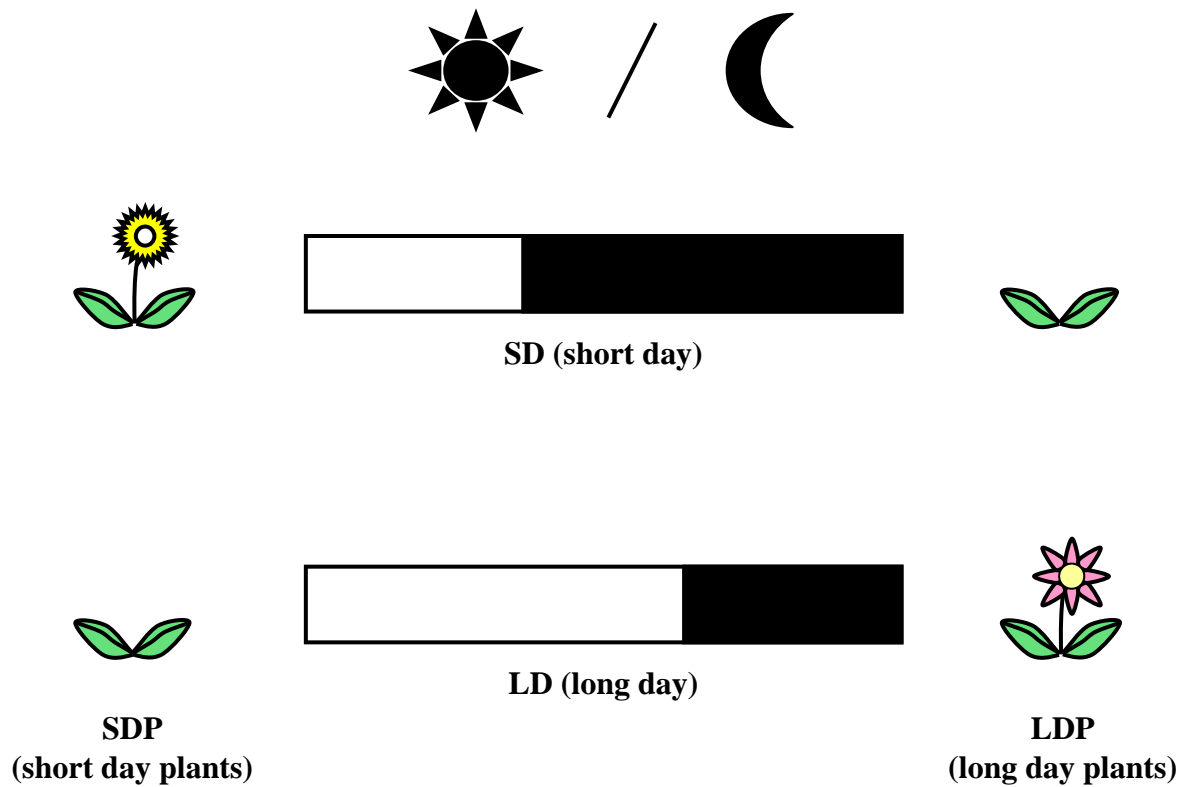
Figure 1. *Classification of plants based on their response to day length.*
Plants are largely classified into short day plants (SDPs) and long day plants (LDPs). SDPs promote flowering when day length become shorter than some critical length whereas LDPs promote flowering when day length become longer than some crucial length. Plants whose flowering is not regulated by photoperiod are classified into day-neutral plants.
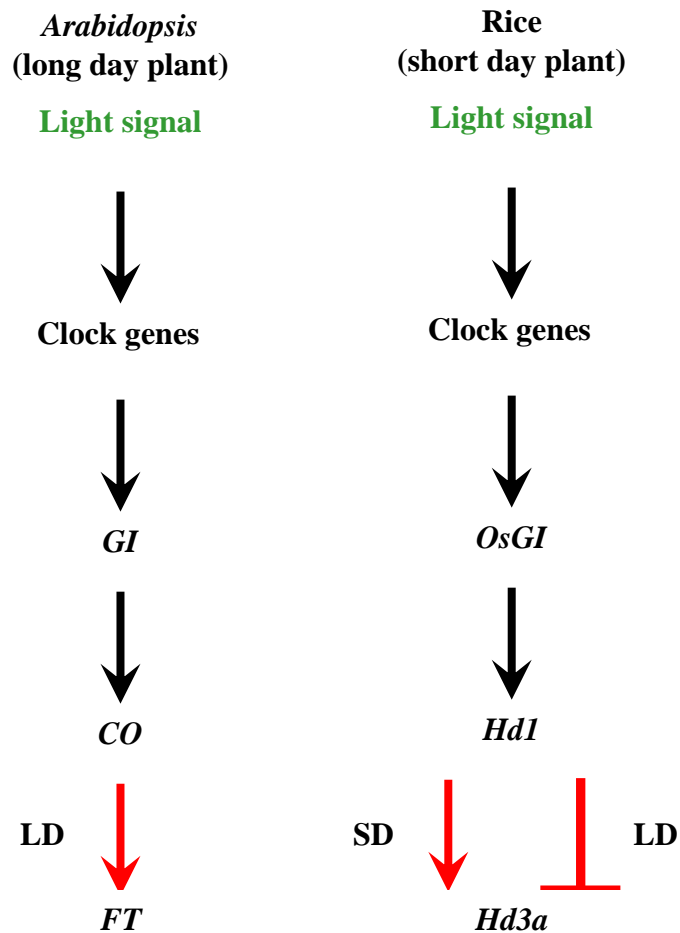
Figure 2. *Hd1 has dual function on the regulation of Hd3a expression in the photoperiodic control flowering in rice.*

In Arabidopsis, under inductive LD conditions, *GI* promotes *CO* expression and, in turn, *CO* induces *FT* expression. However, in rice, under inductive SD conditions, *OsGI* promotes *Hd1* expression and *Hd1* induces *Hd3a* expression, whereas under non inductive LD condition, *Hd1* suppresses *Hd3a* expression, resulting in the delay of flowering. Model is adapted from Yano et al., (2000) and Hayama et al., (2003).
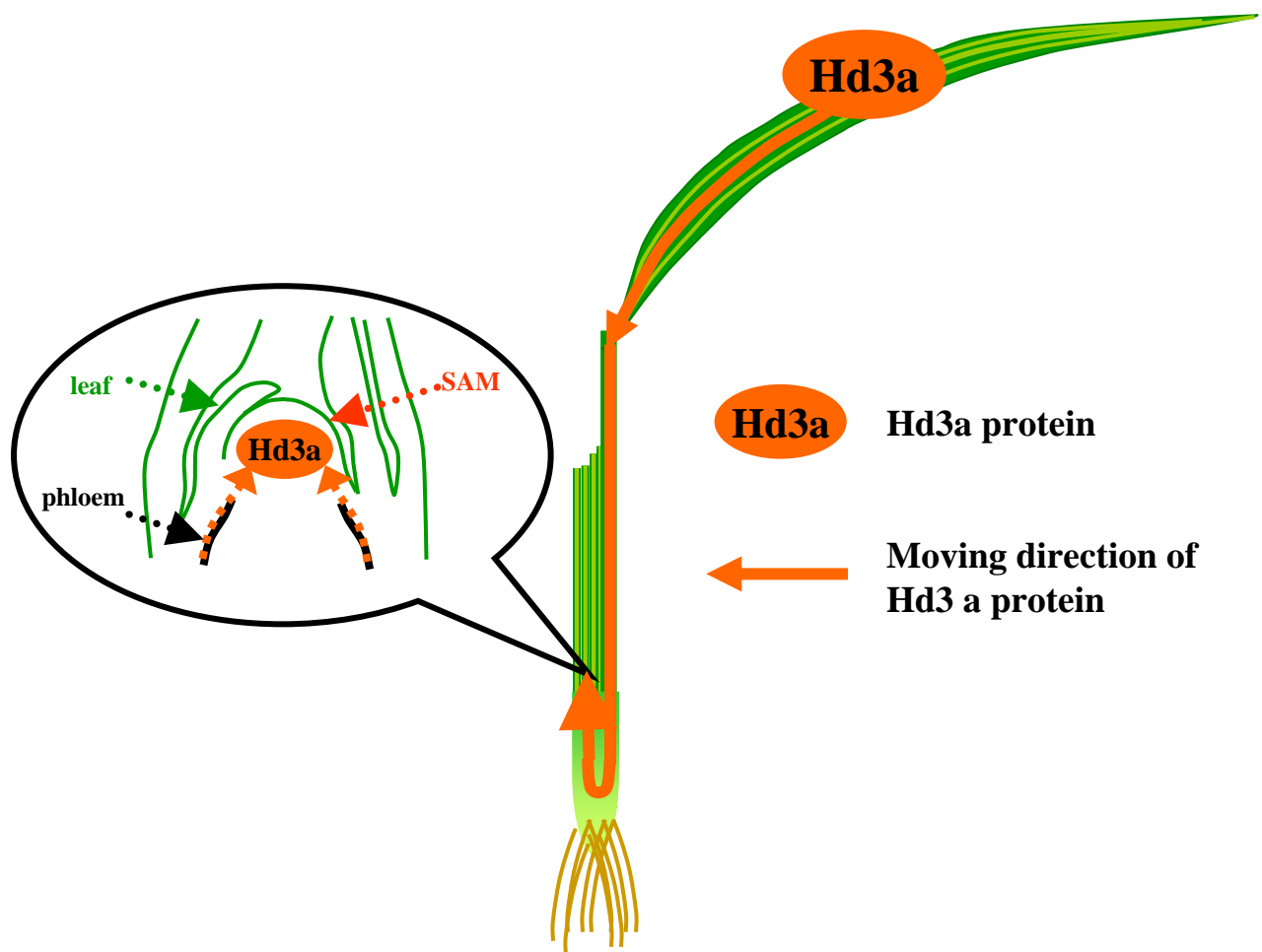
Figure 3. *Rice Hd3a protein is proposed to florigen.*
In rice, *Hd3a* express in leaves under flowering inducement condition.Hd3a protein moves from leaves to shoot apical meristem (SAM).Tamaki et al 2007.
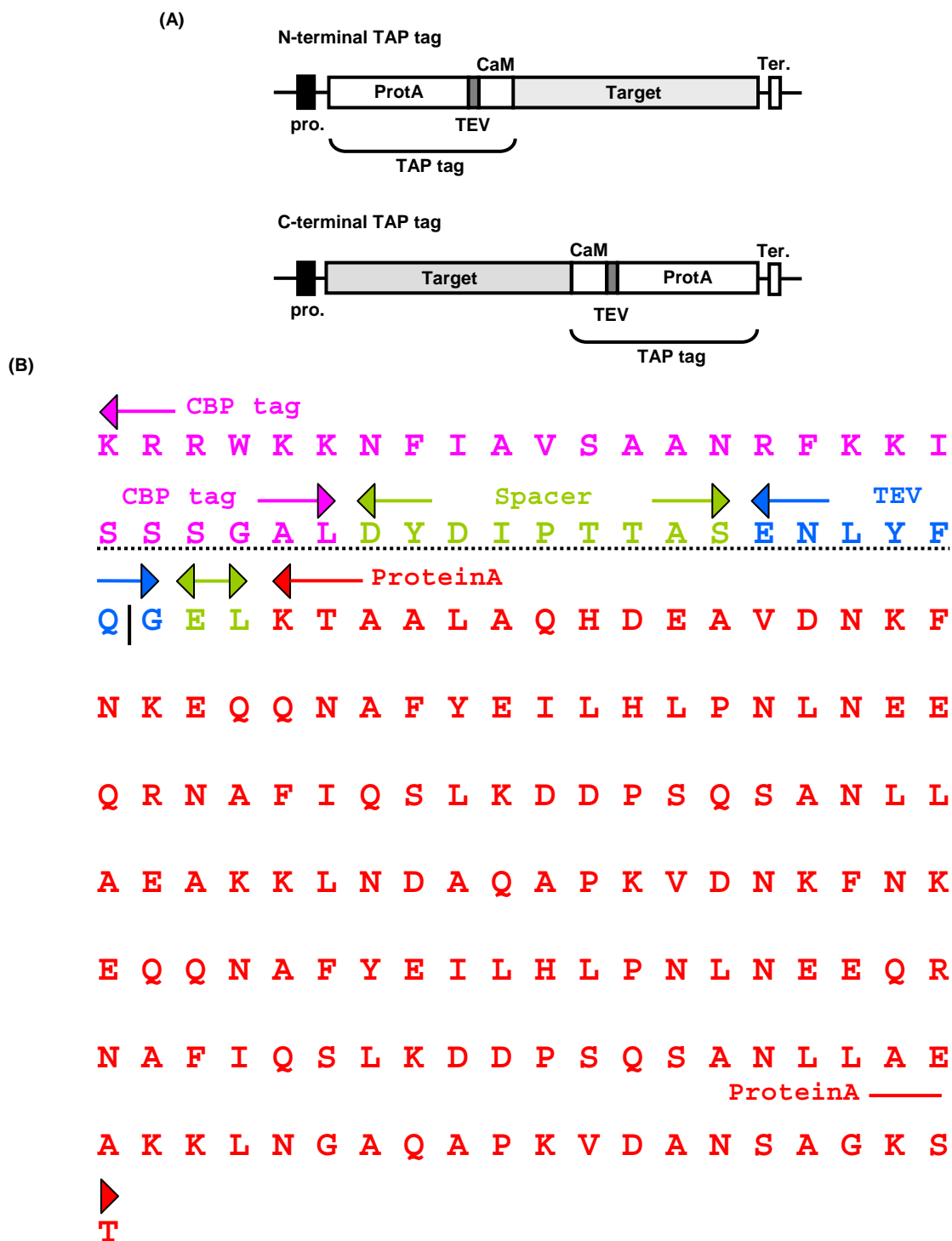
Figure 4. *The structure and sequence of the TAP tag and the recognition site for the SGC TAP tag antibody.*

(A) Schematic representation of the C and N terminal TAP tags. The TAP tag consists of the *S. aureus* protein A gene (ProtA) and the sequences encoding the seven amino acid TEV recognition peptide (TEV) and the calmodulinbinding peptide (CaM). (B) The CBP, TEV, proteinA and spacer sequence were indicated in pink, blue, red and green, respectively. The dotted line indicates the recognition site for the SGC TAP tag antibody. The solid line indicates the TEV cleavage point. The molecular weight of CBP,TEV and proteiA were ca. 3kDa, 1kDa and 15kDa.

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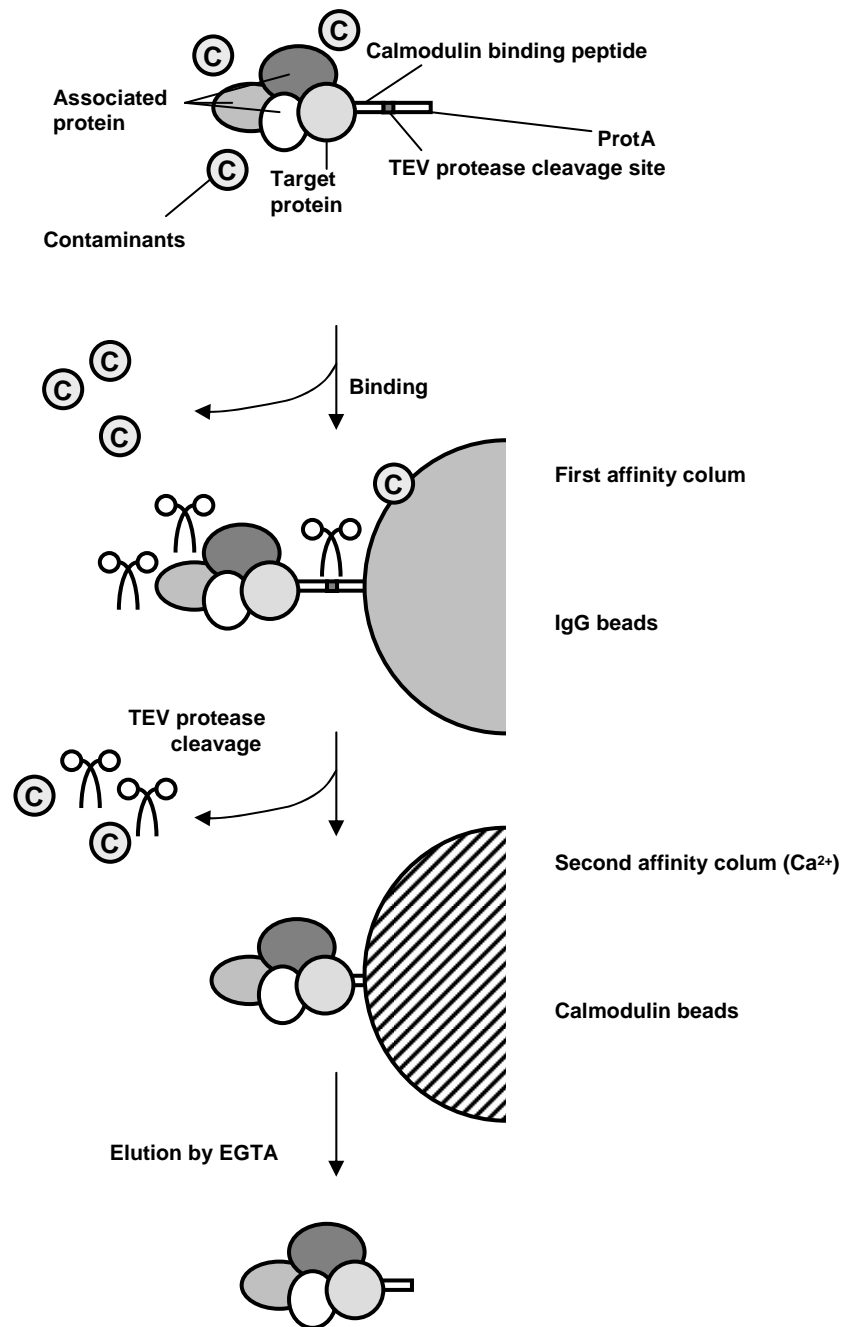
Figure 5. *Steps of TAP method.*
The TAP method involves the fusion of the TAP tag to the target protein and the introduction of the construct into the host cell or organism. The fusion protein and interacting proteins are recovered from cell extracts by affinity selection on an IgG beads. After washing, the TEV protease is added to release the bound proteins. The eluate is incubated with calmodulin beads in the presence of calcium. This second affinity step is required to remove the TEV protease as well as contaminants remaining after the first affinity selection. After washing, the bound proteins are released with EGTA.

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(A)

(B)
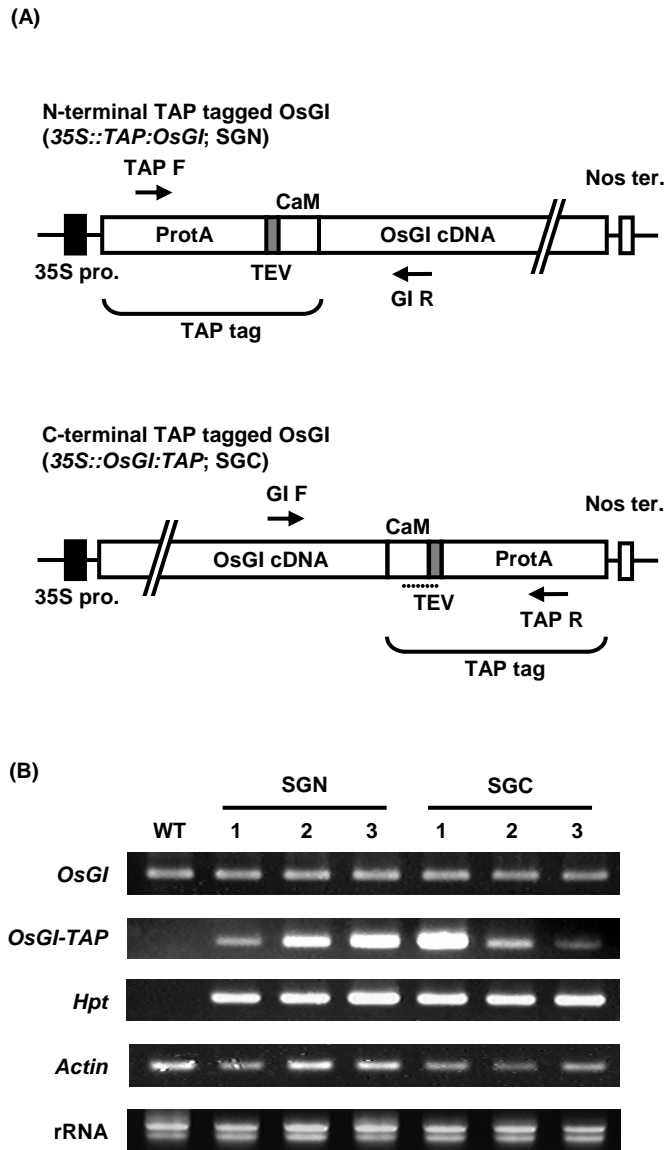
Figure 6. *The TAP-tagged OsGI vectors, and detection of TAP- tagged OsGI transcripts in cultured rice cells.*
(A) Diagrams of the TAP-tagged OsGI constructs. The N-terminal TAP-tagged construct (SGN; *35S::TAP:OsGI*) and the C-terminal TAP-tagged construct (SGC; *35S::OsGI:TAP*) each contain the CaMV 35S promoter for high expression in plants, the TAP tag sequence, the *OsGI* cDNA, and the NOS terminator (Nos ter.). The TAP tag sequence is same as Fig. 2. These constructs are contained within binary vectors for *Agrobacterium*-mediated transformation. The dotted line indicates the recognition site for the SGC TAP tag antibody. The positions of the PCR primers TAP F, TAP R, GI F and GI R are indicated by arrows. (B) Detection of wild-type and TAP-tagged *OsGI* transcripts by RT-PCR. Wild-type (WT) cells and transgenic lines containing SGN and SGC (3 of each) were analyzed. The primer sets shown in (A) were used. A rice *Actin* gene and the hygromycin-resistance gene (*Hpt)* were used as RT-PCR controls. rRNA staining was used as a loading control.
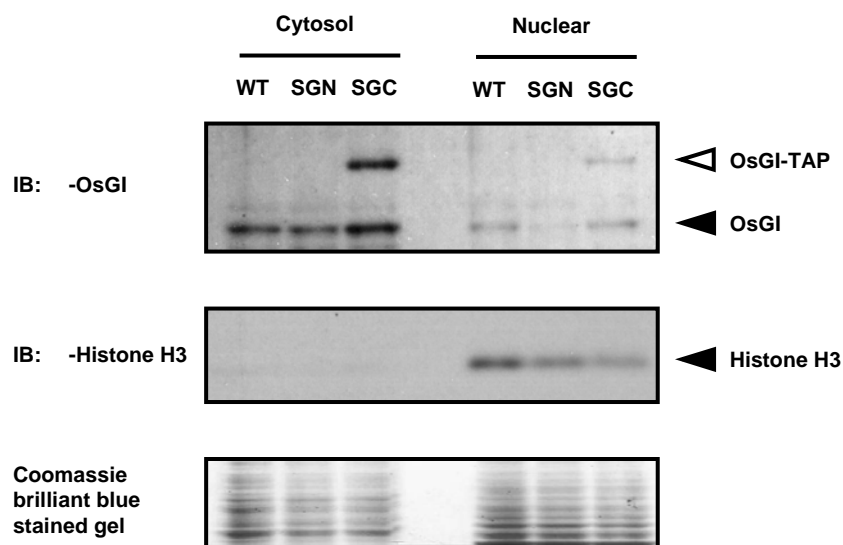
Figure 7. *Western blot analysis of TAP-tagged OsGI protein in transgenic cultured rice cells.*

The OsGI antibody (*a*-OsGI) was used for the detection of TAP-tagged OsGI (open arrowhead) and OsGI (black arrowhead, top panel) in cytosolic and nuclear extracts from wild-type and transgenic cell lines. The TAP-tagged OsGI protein was expressed only in the SGC line. Detection of Histone H3 (black arrowhead, middle panel) using the *a*-Histone H3 antibody was used as a control for the nuclear proteins. The Coomassie brilliant blue stained gel is shown as a loading control.
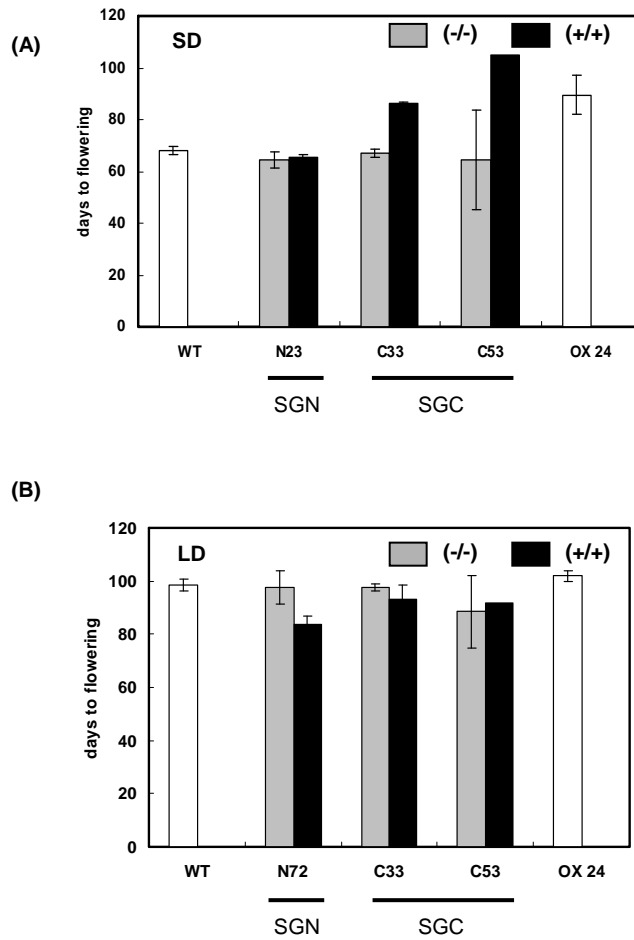
Figure 8. *Flowering times of transgenic rice plants expressing the TAP-tagged OsGI protein.*
The flowering times of wild-type (WT) Norin 8 and transgenic (T1 generation) rice plants were measured under SD (A) and LD (B) conditions. The transgenic lines analyzed were: SGN line N23 (A) or N72 (B), SGC lines C33 and C53, and line OX 24, which over-expresses OsGI. Homozygous (+/+) and segregating (-/-) transgenic plants were analyzed for each treatment. Data are averages for 10-19 plants, and the error bars show the standard deviations.
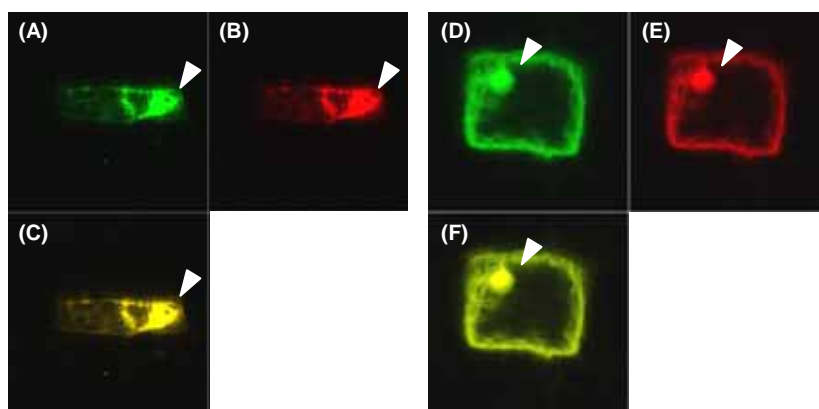
Figure 9. *Intracellular localization of OsGI in rice leaf sheath cell.*
Leaf sheath cells were bombarded with (A-C) *GFP:OsGI or* (D-F) *OsGI:GFP* and *Ubi::YFP*. The *UBI::YFP* vector was localized in both the cytoplasm and the nucleus and used as a control. Fluorescence signals were observed using a confocal laser scanning microscope 12 h after bombardment. (A, D) A single cell showing GFP fluorescence in both the nucleus and the cytosol. (B, E) YFP fluorescence in the same cell. (C, F) Merged image of (A) and (B), (D) and (E), respectively. Arrowheads indicate the nucleus.
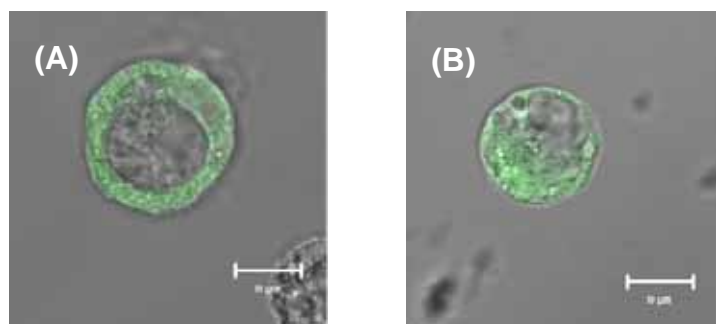
Figure 10. *Intracellular localization of OsGI in rice protoplast.*
The electroporation of rice protoplasts with (A) *GFP:OsGI or* (B) *OsGI:GFP*.
Fluorescence signals were observed using a confocal laser scanning microscope 12 h after electroporation. (A, B) A single cell showing GFP fluorescence in the cytosol.
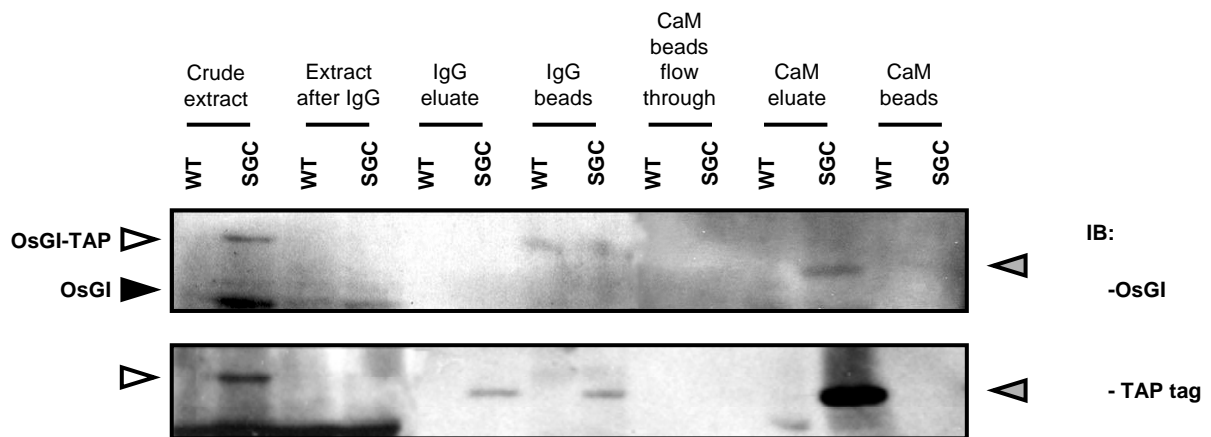
Figure 11. *Purification of OsGI interacting proteins by the TAP method.*

The solution was sampled at each step during the entire TAP procedure. An extract from wild-type (WT) cells was used as a control. The following fractions are shown: Crude protein extract (crude extract), unbound protein after incubation of IgG beads (extract after IgG), eluate from IgG beads after using TEV protease (IgG eluate), IgG beads after TEV treatment (IgG beads), flow through fraction from CaM bead column (CaM beads flow through), final purified protein (CaM eluate) and CaM beads after elution of the protein (CaM beads). Empty arrowheads indicate TAP-tagged OsGI, black arrowheads indicate OsGI and gray arrowheads indicate truncated TAP-tagged OsGI produced by TEV treatment.
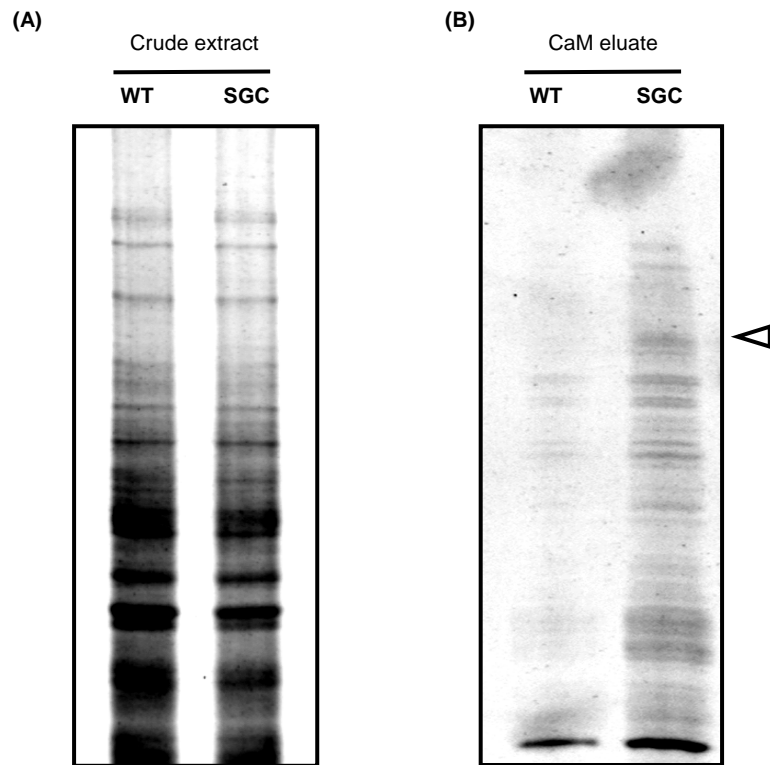
Figure 12. *Separation of OsGI interacting proteins by SDS-PAGE.*
(A) Crude extract and (B) final eluates were pooled and the proteins were precipitated and separated on a 7.5% SDS-PAGE gel, then visualized by fluorescence staining. The empty arrowhead indicates the truncated TAP-tagged OsGI after TEV treatment.
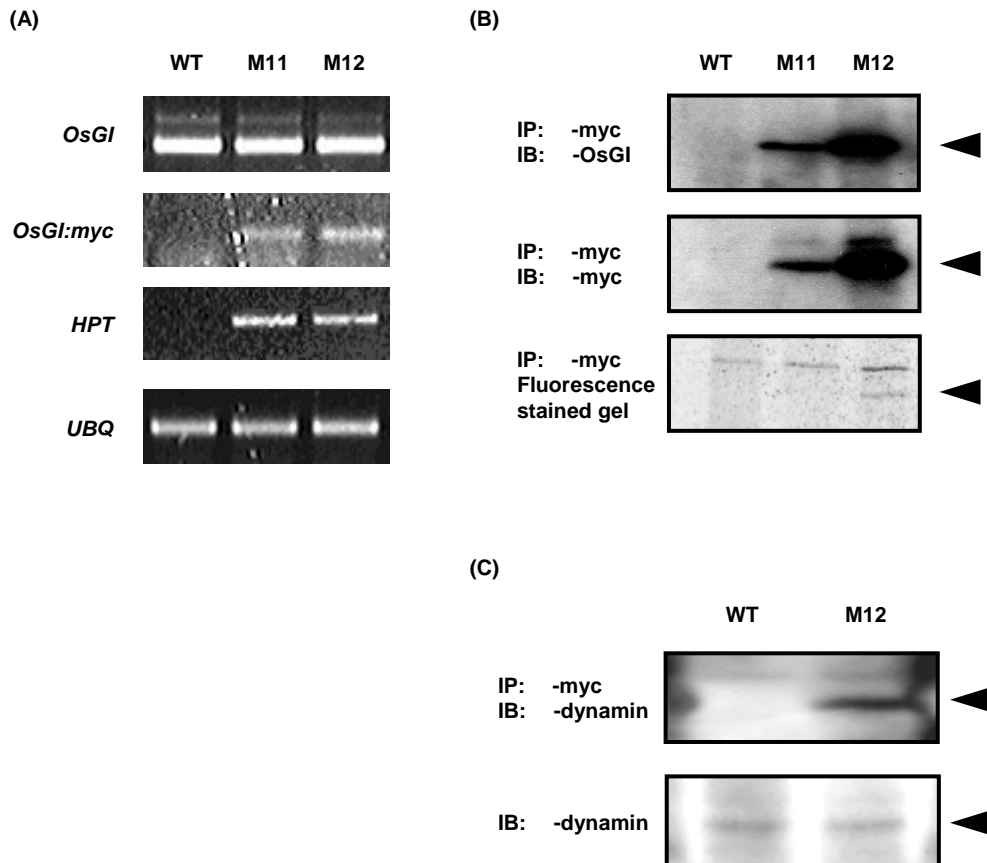
Figure 13. *OsGI interacts with dynamin in vivo.*
Wild-type (WT) and transgenic cell lines expressing a myc-tagged version of OsGI (OsGI:myc) were analyzed. (A) Detection of wild-type and myc-tagged version of *OsGI* lines (M11 and M12) by RT-PCR. Wild-type (WT) cells and transgenic lines containing OsGI:myc were analyzed. The hygromycin-resistance gene (*Hpt*) and a rice *ubq* gene were used as RT-PCR controls. rRNA staining was used as a loading control. (B) OsGI:myc was detected in both the M11 and M12 cell lines by immunoprecipitation using the *a*-myc antibody, followed by western blot analysis (top and middle panels) or fluorescence staining (bottom panel). The protein was detected using both the *a*-OsGI antibody (top panel) and the *a*-myc antibody (middle panel). The arrowheads indicate OsGI. (C) After immunoprecipitating with the *a*-myc antibody, dynamin was detected in the M12 cell line by western blot analysis using the *a*-dynamin antibody (top panel) or fluorescence staining (bottom panel). The arrowheads indicate dynamin.
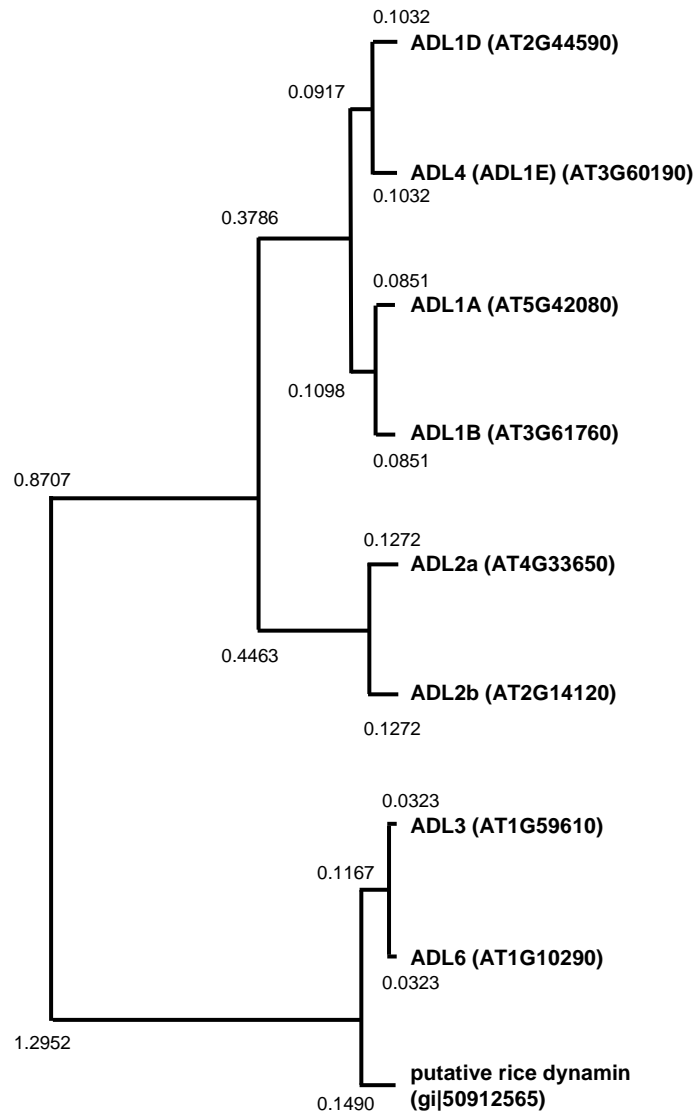
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Figure 14. *Phylogenetic tree of the Arabidopsis dynamin gene family.*
A phylogenetic tree of the *Arabidopsis* dynamin gene family was created using the UPGMA method, to identify the *Arabidopsis* dynamin which is the closest homolog of the putative rice dynamin (gi|50912565) identified by the TAP method. The ADL1C (ADL5) sequence was not included in the analysis because of its low level of homology with other family members. The numbers indicate genetic distance.
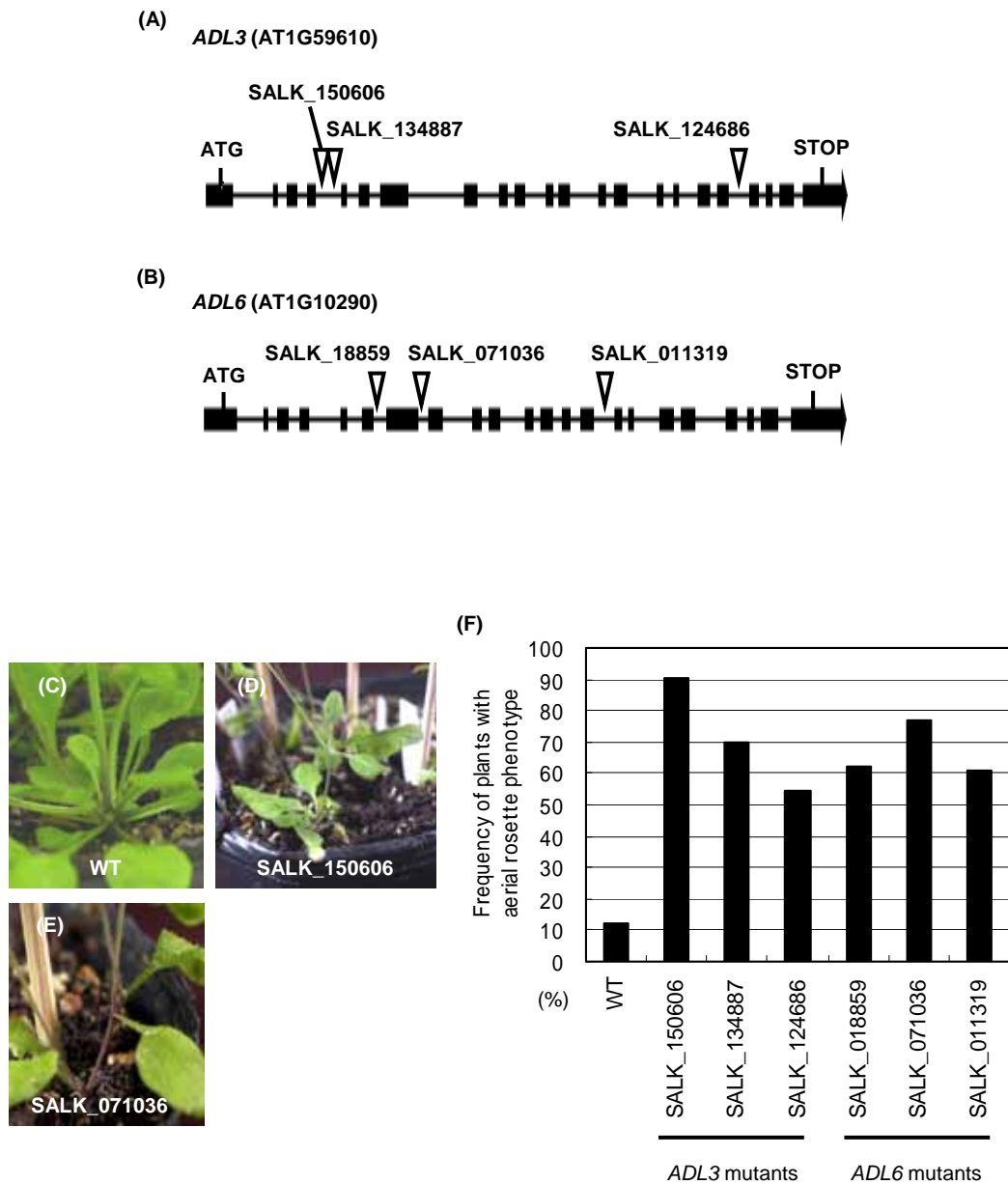
(A) *ADL3* (AT1G59610)

SALK_150606
SALK_134887
SALK_124686
ATG
STOP

(B) *ADL6* (AT1G10290)

SALK_18859 SALK_071036 SALK_011319
ATG
STOP

(C) WT
(D) SALK_150606
(E) SALK_071036

(F)

Frequency of plants with aerial rosette phenotype

ADL3 mutants   *ADL6* mutants

Figure 15. *Phenotypic analysis of Arabidopsis dynamin mutants.*
Diagrams representing the *Arabidopsis ADL3* (AT1G59610) (A) and *ADL6* (AT1G10290) (B) dynamin genes, with T-DNA insertion sites indicated by open arrowheads. Each gene is represented by a line, with exons shown as black boxes. Three T-DNA insertion lines (designated by their SALK line numbers) were obtained for each gene. (C)-(E) Phenotypes of wild-type (WT) plants (C) and dynamin mutants (D), (E) grown under LD (16 h light / 8 h dark) conditions. The mutants have an aerial rosette phenotype. (F) Frequencies (%) of wild-type (WT) and mutant plants showing the aerial rosette phenotype under LD conditions. For each line, 13-21 plants were examined, except for the SALK_134887 mutant, of which 5 plants were examined.