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TECHNICAL ADVANCE

Establishment of a patterned GAL4-VP16 transactivation system for discovering gene function in rice

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Summary

A binary GAL4-VP16–UAS transactivation system has been established in rice (*Oryza sativa* L.) in this study for the discovery of gene functions. This binary system consists of two types of transgenic lines, pattern lines and target lines. The pattern lines were produced by transformation of Zhonghua 11, a japonica cultivar, with a construct consisting of the transactivator gene *GAL4-VP16* controlled by a minimal promoter and the GUSplus reporter controlled by the upstream activation sequence (UAS; *cis*-element to GAL4). Target lines were generated by transformation of Zhonghua 11 with constructs carrying the EGFP reporter and target genes of interest, both controlled by the UAS but in opposite directions. Hybrid plants were obtained by crossing target lines of 10 putative transcription factor genes from rice with six pattern lines showing expression in anther, stigma, palea, lemma and leaves. The EGFP and target genes perfectly co-expressed in hybrid plants with the same expression patterns as in the pattern lines. Various phenotypic changes, such as delayed flowering, multiple pistils, dwarfism, narrow and droopy leaves, reduced tillers, growth retardation and sterility, were induced as a result of the expression of the target genes. It is concluded that this transactivation system can provide a useful tool in rice to unveil latent functions of unknown or known genes.

Keywords: *Oryza sativa*, GAL4–UAS, pattern line, target line, ectopic expression, transcription factor.

Introduction

In the past decade, a variety of reverse genetics techniques have been established for the investigation of gene functions. Commonly used techniques currently involve constitutive or inducible overexpression, antisense RNA, double-strand RNA interference (RNAi), dominant-negative mutation and ectopic activation (Case, 2003; Nagy *et al.*, 2003). However, some of these molecular techniques are not always feasible to manipulate genes that are involved in the control of developmental processes such as cell division and differentiation, growth and reproduction, because manipulation, such as constitutive activation or inactivation, of vital genes can lead to cell death and loss of the material for further functional studies. For example, constitutively *WUS*-expressing plants resulted in failure of leaf formation and further development (Schoof *et al.*, 2000; Zuo *et al.*, 2002). Conditional gene expression has been suggested to be very efficient in functional dissection of developmentally important genes (Padidam, 2003; Porter, 1998; Tang *et al.*, 2004).

One of these techniques is the binary transactivation system that was established early in mice (Ornitz *et al.*, 1991) and *Drosophila* (Brand and Perrimon, 1993), and applied to Arabidopsis (Guyer *et al.*, 1998), zebrafish (Koster and Fraser, 2001; Scheer and Camnos-Ortega, 1999), frogs (Hartley *et al.*, 2002) and silkworm (Imamura *et al.*, 2003). In general, a binary transactivation system consists of two lines, a pattern line (or activator line) and a target line (or effector line). The pattern line carries a transcription factor such as *GAL4* from *Saccharomyces cerevisiae* (Fischer *et al.*,

1988) or a chimeric transcription factor such as *GAL4–VP16*, a fusion of the yeast GAL4 DNA binding domain with the strong VP16 transactivation domain of the herpes simplex virus (Sadowski *et al.*, 1988). These transcription factors do not activate or interfere with internal gene expression and are often under the control of an organ- or tissue-specific promoter or enhancer. The target line contains a *cis*-element such as upstream activation sequence (UAS) that can be specifically bound by the cognate activator protein such as GAL4 and the *cis*-element is fused to a reporter gene or a target gene of interest. When a pattern line is crossed with a target line, the hybrid containing the two constructs will express the reporter or target gene in a pattern defined by the pattern line. To generate large numbers of pattern lines with expression of the activator gene in specific tissues or organs, a method called enhancer detection or enhancer trapping (Bellen, 1999) is now widely used in combination with the GAL4-UAS system, in which the *GAL4* or chimeric *GAL4–VP16* gene is under the control of a minimal promoter and randomly inserted into the genome of the target species (Brand and Perrimon, 1993; Gustafson and Boulianne, 1996; Ito *et al.*, 1997; Manseau *et al.*, 1997; Tissot *et al.*, 1997; Wu *et al.*, 2003; Yang *et al.*, 2004). The expression pattern of the *GAL4* or chimeric *GAL4–VP16* gene is then determined by the nature of the enhancer trapped by genomic insertion, and visualized with a reporter, such as LacZ, GUS or GFP, under the control of UAS that is activated by the GAL4 protein. Once the expression patterns of enhancer detection lines (or pattern lines) are identified, these lines can be used as activator lines to transactivate a target gene of interest under the control of UAS (Brand and Perrimon, 1993). Such a system has provided a tool of unprecedented power to explore the function of genes involved in the processes of cell proliferation and differentiation (Asha *et al.*, 2003; Brennecke *et al.*, 2003; Dumstrei *et al.*, 2003; Jeong and Kim-Ha, 2003; Liu *et al.*, 2001), cell death (Marquez *et al.*, 2001; Mirkovic *et al.*, 2002; Shigenaga *et al.*, 1997; Wing *et al.*, 1998; Yoo and McKee, 2004; Zhou *et al.*, 1997) and aging (Aigaki *et al.*, 2001; Chavous *et al.*, 2001), as well as behaviour (Brand and Dormand, 1995; Sokolowski, 2001; Suster *et al.*, 2003, 2004).

Five years after the first report of this technique, a modified GAL4-UAS system involving a chimeric transcription factor *GAL4–C1* was established (Guyer *et al.*, 1998) for functional analysis of Arabidopsis genes. In the same year, Moore *et al.* (1998) developed another binary system LHG4/pOp in Arabidopsis, in which a chimeric transcription factor LHG4 containing the transcription–activation domain-II from GAL4 was fused to a mutant *lac*-repressor that can bind to the *lac*-operator pOp. By using this system, a model of a negative regulatory feedback loop established the interaction between WUSCHEL and CLAVATA which are specifying the stem cells and the organizing centre (OC) respectively (Schoof *et al.*, 2000).

In Arabidopsis, a large number of pattern lines have been developed using GFP as the reporter (Haseloff, 1999), and some of the pattern lines have been applied in functional analysis of unknown genes (Boisnard-Lorig *et al.*, 2001; Gallois *et al.*, 2004; Kim *et al.*, 2002, 2003; Sabatini *et al.*, 2003). For example, the *WUSCHEL* (*WUS*) gene has been well recognized with a master role in specifying and maintaining the shoot apical meristem. The latent role of *WUS* in root was discovered by a exquisite experimental design using the GAL4–VP16-UAS system, in which ectopic expression of *WUS* in Arabidopsis root strikingly resulted in development of leaves on roots without exogenous stimulus (Gallois *et al.*, 2004). This interesting result is reminiscent of the finding that formation of ectopic eye structures was induced on the wings, legs and antennae by switching on a single gene using the binary GAL4-UAS expression system in *Drosophila* (Halder *et al.*, 1995).

Rice is a major crop worldwide and has been established as a monocotyledonous model plant for genome research because of its relative small genome, colinearity with other cereals and high efficiency of genetic transformation. The availability of over 30 000 rice full-length cDNA (Kikuchi *et al.*, 2003) and the genomic sequence of both indica (Yu *et al.*, 2002) and japonica (Goff *et al.*, 2002) subspecies pushed the rice functional genomics study into full swing. Recently the binary GAL4-UAS transactivation system was tested in rice by re-transforming driver lines (with GFP as reporter) with *UAS–uidA* construct and re-transformed plants showed expected GUS expression patterns (Johnson *et al.*, 2005), suggesting that this system may also be useful in discovering gene function in rice.

In our previous report, an enhancer-trapping construct (containing *GAL4–VP16*-UAS–GUS) was transformed into rice to generate an enhancer-trapping mutant library (Wu *et al.*, 2003). In that study, patterned GAL4–VP16-UAS ectopic expression of target genes was established by sexual crossing between target lines and pattern lines. We tested transactivation of 10 transcription factor genes in the hybrids with six representative pattern lines, which induced various phenotypic changes in the hybrid plants resulting from ectopic expression of these target genes. Our data provided solid evidence that this binary system can be employed in rice for the discovery of gene functions.

Results

The activator and effector vectors

The activator vector pSMR-J18R for producing the pattern line (or driver line) contained three components: the transcription factor *GAL4-VP16* under the control of a minimal promoter (MP, -48 CaMV 35S) adjacent to the right border of the T-DNA, a modified GUS (GUSplus) reporter gene under the control of 6 × UAS, and the selection marker gene *Hph*

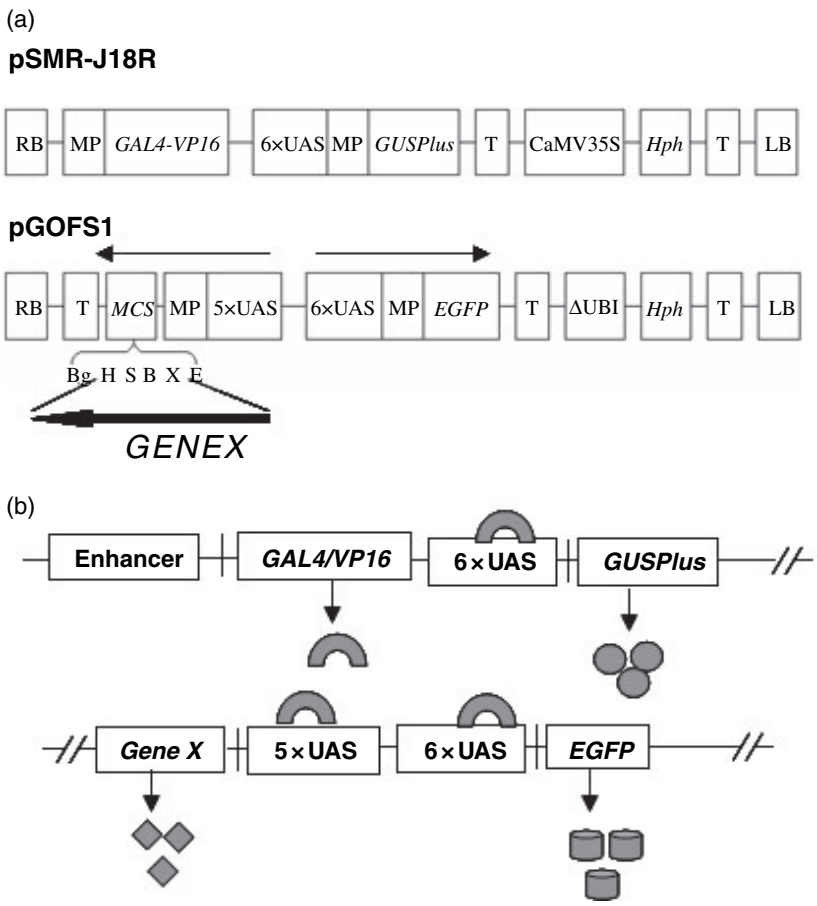

Figure 1. Schematic diagrams of the transactivation system.
(a) T-DNA region of activator vector pSMR-J18R and effector vector pGOFS1.
(b) Transactivation mechanism in the hybrid plant.
GAL4-VP16: a fusion gene of yeast transcriptional activator GAL4 DNA-binding domain with the Herpes simplex virus VP16 activation domain; GUSplus: a modified β-glucuronidase; EGFP: GFP with enhanced activity; 6 × UAS: upstream activator sequence with six repeats; *Hph*: hygromycin phosphotransferase; MP: minimal promoter; ΔUBI: a truncated maize *ubiquitin* promoter; T: terminator; MCS: multiple cloning sites (E: *Eco*RI; H: *Hin*dIII; B: *Bam*HI; X: *Xba*I; S: *Sma*I; Bg: *Bgl*II).

under the control of CaMV 35S (Figure 1a). Similarly to the vector pFX-E24.2–15R (with *GFP* reporter gene) described by Wu *et al.* (2003), the pSMR-J18R vector (without *GFP* reporter gene) could also be used for enhancer trapping.

There were two concerns with the vector (also referred to as effector vector) pUAS3380 (provided by Dr R. Kerstetter) for target line generation. First, the CaMV 35S promoter may function as an enhancer to activate the expression of the target gene. Secondly, efficiency of the *Bar* gene as a selection marker is lower than that of the *Hph* gene in rice transformation. We thus modified the vector such that *Bar* was replaced with *Hph* under the control of a maize *ubiquitin* promoter, which was designated pGOFS1. This effector vector also contained three components: 6 × UAS-MP-*EGFP*, 5 × UAS-MCS (multiple cloning sites for introduction of target gene) and the *Hph* selection marker gene controlled by the maize *ubiquitin* promoter (Figure 1a).

Both *EGFP* and the target gene were controlled by the UAS, but arranged in reverse orientations separated by a stuffer region 280 bp in length so that they could be activated simultaneously by GAL4-VP16. However, the *EGFP* reporter or the target gene would not express until the GAL4-VP16 protein was expressed and bound to the UAS, which would happen only in the cells of a hybrid plant containing both the pGOFS1 and the pSMR-J18R constructs (Figure 1b).

Production of pattern lines

The activator vector pSMR-J18R was previously used to transform rice cultivar Zhonghua 11 to generate an enhancer-trapping library (Wu *et al.*, 2003). In the present study, 4219 T_0 transgenic plants were screened for the expression patterns of the reporter gene in eight organs (Table 1). A total of 931 plants showed specific expression of the reporter gene in one of the eight organs. Specific expression was more frequent in stamen (375) and embryo (362) than in other organs investigated (Table 1).

To increase the opportunity of obtaining specific pattern lines, hygromycin-resistant calli transformed with pSMR-J18R were pre-screened by GUS assay, and calli with no GUS expression were chosen for regenerating plants. From approximately 2800 independent resistant calli screened, 314 calli had no GUS expression. A total of 286 plants were regenerated from these calli, from which 78 plants showed organ-specific GUS expression, covering 14 organs investigated during the rice life cycle (Table 1). The expression patterns can be searched at the Rice Mutant Database (RMD,

Table 1 Identification of pattern lines from enhancer-trapping mutant library and GUS-free callus-derived lines

	Mutant library lines			GUS-free callus-derived lines		
Organs	No. plants tested	Organ- specific expressed lines[a]	Frequency (%)	No. plants tested	Organ-specific expressed lines[a]	Frequency (%)
Root	–	–	–	286	4	1.41
Leaf blade	4169	41	1.08	286	17	5.95
Leaf sheath	–	–	–	286	5	1.75
Ligule	–	–	–	286	1	0.35
Leaf auricle	–	–	–	286	3	1.05
Stem internode	4169	30	0.72	286	4	1.40
Stem node	–	–	–	286	4	1.40
Lodicular	–	–	–	286	3	1.05
Stamen	4169	375	9.00	286	10	3.45
Pistil	4169	14	0.34	286	6	2.13
Lemma/palea	4169	80	1.92	286	8	2.84
Embryo	3559	362	10.2	281	3	1.07
Endosperm	3559	25	0.70	281	9	3.20
Total	4169	931	22.33	286	78	27.27

[a]The specificity was based on the number of organs tested in the column.

Table 2 Features of six selected pattern lines for crossing with target lines

Pattern line no.	Entry ID	GUS expression pattern[a]	Copy number of T-DNA
P1	01Z11AB62	Anther, patched expression	3
P2	03Z11UZ45	Stigma	2
P3	03Z11UZ88	Anther, strong expression	2
P4	03Z11UZ91	Lemma/palea	1
P6	03Z11VA01	Leaf blade and stamen	2
P7	03Z11VA48	Roots, shoots, leaves, culm, palea, lemma, anther and stigma	3

[a]The expression pattern can be viewed at the website (http://rmd.ncpgr.cn/) by a simple search with the pattern line number or entry ID.

http://rmd.ncpgr.cn/) by keywords or entry ID number of mutant.

For establishment of a patterned GAL4/VP16–UAS ectopic activation system, five lines were selected to represent organ- or tissue-specific expression of the GUS gene in anthers, stigma, palea and lemma (or lemma/palea), leaves and stamen respectively (Table 2). In addition, one line showing GUS expression in all these tissues examined was included for reference of activated expression. All these selected pattern lines exhibited normal phenotype, as did the wild-type plant.

Production of target lines and hybrids

Ten putative transcription factor genes, belonging to zinc finger, MADS, MYB and NAC subfamilies, were chosen as the target genes for testing (Table 3). Two of these genes, *OsMADS15* (Lim *et al.*, 2000; Moon *et al.*, 1999) and *OsMYBS3* (Lu *et al.*, 2002), were reported previously, while the functions of the remaining eight genes are currently unknown. The empty vector pGOFS1 (no target gene introduced) was also transformed as the positive control of the EGFP reporter. At least 30 independent transgenic plants were generated for each construct, and only those lines containing single-copy T-DNA and exhibiting normal phenotypes were selected for making crosses with pattern lines (Table 3).

The target lines were crossed individually with the six pattern lines described above, resulting in a total of 50 crosses (Table 4; a few crosses were not made because of limited pollen supply).

Activation of the EGFP *reporter gene in hybrid plants by pattern lines*

To test the effectiveness of the GAL4-VP16–UAS transactivation system, we first checked the expression of the *EGFP* reporter gene in the hybrid plants derived from crosses between the EGFP reporter (ER) line (transgenic plants of the vector pGOFS1 without the target gene) and six pattern lines (Table 2).

In the hybrid between the ER line and P1 (anther-specific pattern as indicated by GUS expression), EGFP expression was detected in the anther of the hybrid but not in the pattern line parent or the ER line parent (Figure 2a). To validate that the EGFP expression is specifically induced by

Table 3 Production of target line plants with single-copy transgenes and normal phenotype for 10 rice transcription factors

Target line vector	Target gene[a]	Accession no.[b]	No. transgenic plants generated	No. plants with single-copy transgene[c]	No. T_0 plants with single copy and normal phenotype	No. families showing no phenotypic segregation in T_1[d]
pGOFS1	–	–	48	5 (22)	5	5
T3	Putative MYB	AL772425	168	5 (20)	5	5
T4	Putative LIM	AP004788	98	7 (22)	6	6
T5	Putative LIM	AK072520	28	12 (23)	10	7
T6	Putative NAC	AK067690	105	3 (12)	3	2
T7	Putative ZF	AK072942	70	6 (24)	6	5
T8	Putative MADS	AK066160	291	6 (24)	6	4
T10	Putative MYB	AP004260	85	4 (20)	4	4
T11	*OsMADS15*	AF058698	31	6 (25)	5	5
T14	CO-like ZF	AK121024	44	4 (14)	3	2
T15	*OsMYBS3*	AY151044	51	5 (20)	5	3

[a]Putative full-length transcription factors were identified from the cDNA library (Chu *et al.*, 2002). Subfamily classification was assigned based on conserved DNA-binding domains.
[b]GenBank accession numbers for full-length cDNA are used for those showing a 100% match with japonica full-length cDNA, and for genomic sequences otherwise.
[c]Numbers in parentheses indicate numbers of T_0 plants examined for copy number using Southern analysis.
[d]T_1 families showing phenotypic segregations were not used for making crosses.

the pattern line, the GFP imaging of the hybrid plants was examined in various tissues during the life cycle (Table 1). No tissues other than anther showed EGFP expression (data not shown), which was in perfect agreement with the GUS expression in the pattern line. This suggested that the anther-specific expression of EGFP in the hybrid was activated by GAL4-VP16 from the pattern line.

In the hybrid between ER line and P2 (stigma-specific expression), EGFP expression was detected in the stigma of the hybrid, not in either of the parents (Figure 2b). No EGFP expression was observed in any other tissues as listed in Table 1, suggesting that EGFP expression was activated in a stigma-specific pattern defined by P2. Similarly, EGFP expression was detected only in the hybrids between ER line and P3 (strong expression in anther specifically), P4 (palea- and lemma-specific pattern) and P6 (leaf- and stamen-specific pattern), with exactly the same patterns as in the pattern lines (Figure 2c–e).

P7 was a pattern line for cross-reference in this study, in which GUS expression was detected in roots, shoots, leaves, culm, palea, lemma, anther and stigma (Table 2). In the hybrid between ER line and P7, EGFP expression was also detected in all these tissues (Figure 2f), again suggesting that EGFP expression was activated following the pattern in P7.

All of these results demonstrate that the *EGFP* is transactivated by GAL4-VP16 in hybrid plants in the same pattern as the GUS expression in the activator parents.

Co-expression of EGFP reporter and target gene

In the target line vector, both the *EGFP* and target gene were controlled by UAS but in reverse directions (Figure 1). To investigate whether the EGFP expression faithfully reflects the expression of target genes, we examined the expression patterns of EGFP and the target genes in a number of crosses.

In the T8/P7 cross, P7 was a pattern line with GUS expressed in roots, shoots, leaves, culm, palea, lemma, anther and stigma (Table 2), and the target gene in T8 encodes a putative MADS protein (Table 3). GUS assay of the leaf tissue of seven F_1 plants from the cross between T_0 plants of T8 (having a single copy of T-DNA) and T_1 plants of P7 (having three copies of T-DNA) identified five plants having GUS activity. Three of the five GUS-positive plants showed GFP signal (Figure 3), in agreement with single locus segregation of the *EGFP* gene. As expected, all the plants showing GFP signal had strong expression of the target gene (Figure 3). We checked the two GFP-negative plants (plants 1 and 2, Figure 3) by PCR and found that they did not have the transgene (data not shown). These two plants were used as the negative control for checking possible background transactivation of the target gene (or expression of the native gene) and also for phenotype reference. In both the negative control and wild-type plants, the transcript level of the target gene in leaves was undetectable (Figure 3), substantiating the idea that the expression of the target gene in the positive plants was the result of transactivation.

To further test co-segregation between the GFP signal and ectopic expression of the target gene, we analysed the expression patterns of GFP and the target genes in GUS-positive hybrid plants of eight additional crosses between pattern lines P6 and P7 and seven target lines (Table 5), all of which had a single copy of the transgene. Of the 76 GUS-positive hybrid plants from the various crosses

Table 4 Crosses between target lines and pattern lines and phenotypic changes in the hybrid plants

Target line	Target gene or predicted protein	Pattern line (male parent)					
		P1	P2	P3	P4	P6	P7
PGOSF1		NC	NC	NC	NC	NC	NC
T3	Putative MYB	Partial sterile	nd	Sterile	NC	Sterile, dwarf	Sterile, dwarf
T4	Putative LIM	NC	nd	NC	NC	NC	NC
T5	Putative LIM	NC	NC	NC	nd	NC	NC
T6	Putative NAC	NC	nd	NC	NC	Delayed flowering	Delayed flowering
T7	Putative ZF	NC	NC	NC	NC	NC	NC
T8	Putative MADS	NC	NC	NC	nd	NC	Delayed flowering, dwarf
T10	Putative MYB	NC	nd	Multiple pistils, fewer anthers	nd	Lesions on leaves	nd
T11	*OsMADS15*	NC	NC	NC	nd	Narrow leaf	Narrow and droopy leaf, fewer tillers
T14	CO-like ZF	NC	nd	NC	NC	NC	NC
T15	*OsMYBS3*	Male sterility	Short stigma	Male sterility	Partial male sterility	Semi-dwarf, early senescence of young leaves, delayed flowering	Retarded growth to death

NC: no change of phenotype; nd: no data. Phenotype observation was repeated in at least two independent F_1 plants of each target/pattern line cross.

investigated, 36 had EGFP expression (Table 5) with the same patterns as the GUS expression in the pattern lines, in agreement with single locus segregation of the *EGFP* gene. Among the 36 GFP-positive plants, 35 showed ectopic expression of the target genes (measured in leaf tissues since both P6 and P7 had GUS expression in leaves: Table 2) with the expression level at least 4.6-fold higher than the control, while the expression level of the remaining one plant (from the T6/P7 cross) was marginally higher (1.47-fold) than the control (Table S1). In contrast, ectopic expression of the target genes was not detected in any of the GFP-negative F_1 plants (data not shown). These results firmly established that ectopic expression of target genes was perfectly correlated with the GFP signal in the hybrid plants.

We also investigated the quantitative relationship of the ectopic expression levels between *GUSplus*, *EGFP* and the target gene by real-time PCR analysis in leaves of hybrid plants derived from crosses of T8 (for a putative MADS gene) with six independent pattern lines (entry IDs: 01Z15AJ47, 01Z15AD61, 01Z15AJ65, 01Z15AF68, 01Z15AH07 and 01Z15AJ46). Two of the six lines (01Z15AD61 and 01Z15AJ65) showed leaf blade-specific expression of GUS and the other four lines had GUS expression in leaf blade plus some other tissues. These pattern lines showed different expression levels of GUS in the leaf tissues, as can be identified by visual assessment (photographs of GUS assay in leaves and the expression pattern of these six lines can be found at http://rmd.ncpgr.cn/ by searching the lines by entry ID). The results of real-time PCR showed that the relative expression levels ($2^{-\Delta\Delta C_T}$) of GUS, GFP and the target genes were highly correlated (Figure 4). The correlation coefficients were 0.99 ($P < 0.01$) between GUS and *EGFP*, 0.98 ($P < 0.01$) between the target gene and GUS, and 0.97 ($P < 0.01$) between the target gene and GFP.

Thus, the GFP reporter in hybrids can faithfully reflect the expression pattern of the driver line and ectopic expression level of target genes both qualitatively and quantitatively in this binary transactivation system.

Phenotypic changes resulting from ectopic expression of transcription factors

To test the potential of this transactivation system in characterizing gene function or finding new functions of known genes, phenotypes of hybrid plants showing expected EGFP expression were carefully examined against their parents, wild-type and the negative control. Six of the 10 transcription factors, when activated in specific tissues as indicated by GFP signal, produced various morphological changes such as delayed flowering, multiple pistils, fewer tillers, narrow and droopy leaf, dwarfism, retarded growth, sterility and lethality (Table 4 and Figure 5). Interestingly, activation of the same transcription factor in different tissues could

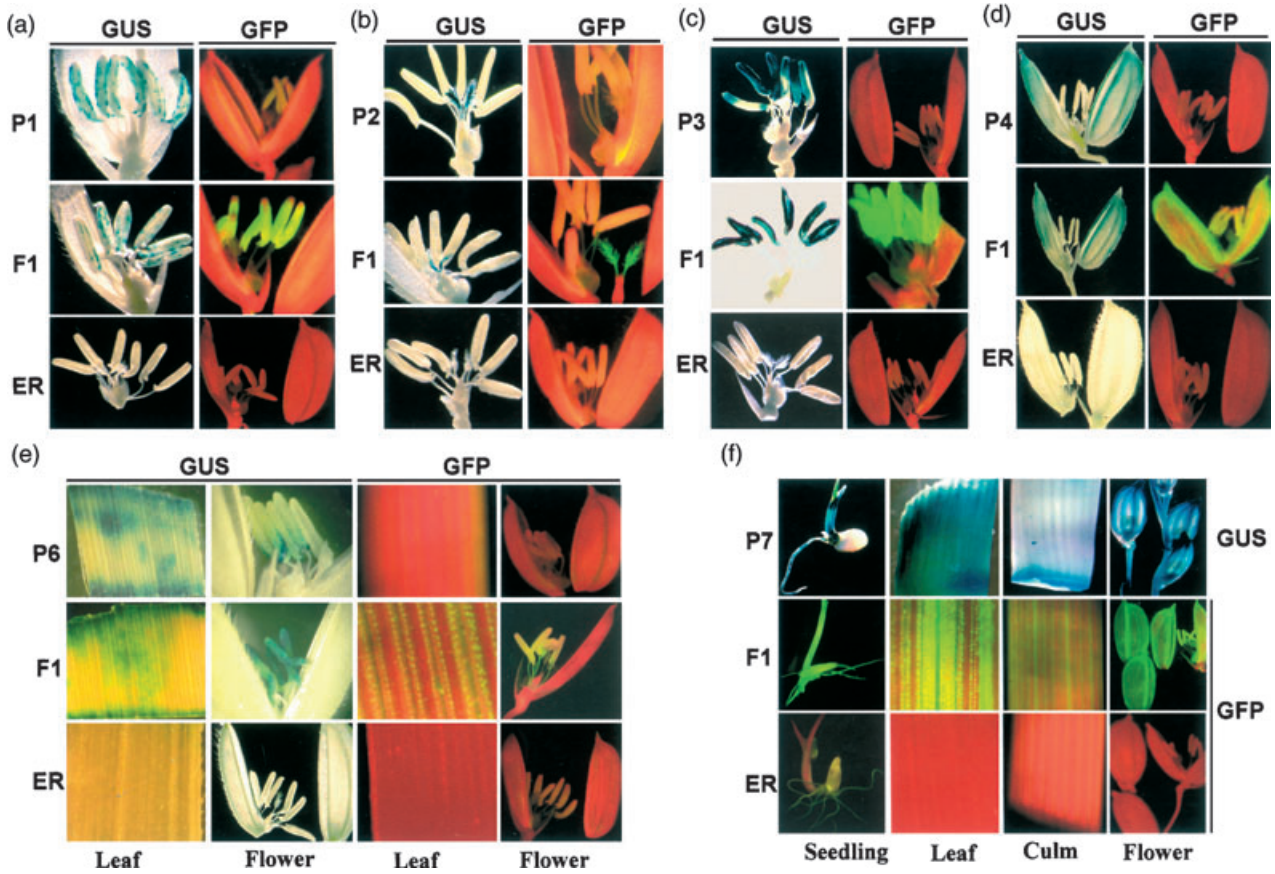

Figure 2. Transactivation of EGFP in the hybrids between pGOFS1-transformed EGFP reporter line (ER) and six pattern lines.
(a) Pattern line P1 (patched expression in anther).
(b) Pattern line P2 (stigma).
(c) Pattern line P3 (strong expression in anther).
(d) Pattern line P4 (palea and lemma).
(e) Pattern line P6 (leaf and stamen).
(f) Pattern line P7. The expected EGFP expressed in various organs (including root, shoot, leaf, stem and flower) of the hybrid of ER with the ubiquitous expression pattern line P7.

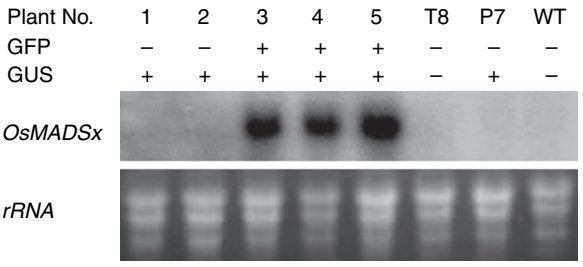

Figure 3. Co-expression of EGFP and the target gene (a putative MADS-box gene) in five sibling plants of T8/P7. Ectopic expression of the target gene was detected by RNA gel blot analysis using the 3′UTR of cDNA as a probe.

result in different phenotypes (Table 4 and Figure 5). Two examples are described in detail in the following paragraphs.

Table 5 Co-segregation of the GUS and GFP signal with ectopic expression of target genes in GUS-positive hybrid plants from eight crosses

Cross	No. GUS$^+$ hybrid plants tested	No. GFP$^+$ plants	No. GFP$^+$*GeneX*$^+$ plants
T3/P7	9	4	4
T3/P6	11	5	5
T4/P6	11	6	6
T5/P6	10	5	5
T6/P7	11	5	4
T7/P6	9	4	4
T11/P7	8	4	4
T14/P6	7	3	3
Total	76	36	35

'+' indicates a positive signal of GUS or GFP, or ectopic expression of target genes determined by real-time PCR, except T11/P7 which was checked by Northern blot (Figure 6a).

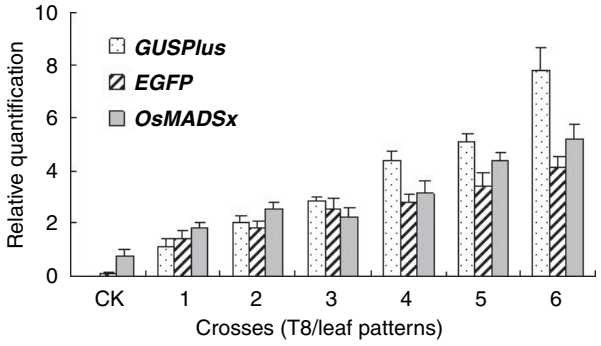

Figure 4. Quantification of reporter and target gene expression.
Relative quantification was determined by real-time PCR analysis (rice *Actin1* as internal control) for the expression level of *GUSplus*, *EGFP* and the target gene (encoding a putative MADS) in the leaves of hybrid plants derived from the crosses of target line T8 with six independent leaf pattern lines. CK is the target line (T8). The error bar is based on repeats of three plants from the same cross. The entry IDs of the six leaf pattern lines are as follows (with the visualized GUS expression level in leaves from low to high): 1, 01Z15AJ47; 2, 01Z15AD61; 3, 01Z15AJ65; 4, 01Z15AF68; 5, 01Z15AH07; 6, 01Z15AJ46.

In the cross of T11/P7, the pattern line P7 showed GUS expression in roots, shoots, culm, leaf and flowers (Table 2) and the target gene *OsMADS15* in the target line encodes a putative MADS transcription factor (Moon *et al.*, 1999). In the F_1, all GUS-positive plants showed expression of EGFP and the target gene as detected in the leaf tissue (Figure 6a). The F_1 plants expressing GUS, EGFP and the target gene had many fewer tillers (3–6 tillers/plant) than the wild-type Zhonghua 11 or the negative segregant (>20 tillers per plant; Figure 6a). In addition, these plants showed narrow leaf blades (Figure 6b) and droopy leaves (Figure 6c). Narrow leaves were also observed in the F_1 plant of T11/P6 (Table 5), in which both GFP and GUS were expressed in the leaves as defined by pattern line P6.

In the cross of T15/P7, with the pattern line the same as in the cross described above but involving a different target gene, *OsMYBS3*, a negative regulator in the starch degradation pathway accumulated in senescent leaves to inhibit the expression of the α-amylase gene (Lu *et al.*, 2002). Of the five F_1 plants from this cross-tested, four showed expression of EGFP in the young shoots (Figure 7a). GUS expression was also detected only in these four plants (data not shown). Interestingly, growth of the four EGFP-expressing plants was severely inhibited (Figure 7b,c), whereas the EGFP-negative plant grew normally. Three of the growth-inhibited shoots failed to develop into green plants and eventually died, while growth of the remaining EGFP-expressing plant was severely inhibited: it exhibited dwarfing, non-tillering and much delayed flowering (it actually never flowered in one rice growing season; Figure 7d). Since EGFP expression was a faithful indicator of target gene expression, the inhibition and retardation of growth and development described above were almost certainly the result of the expression of *OsMYBS3*. F_1 plants derived from the crosses of the *OsMYBS3* target line with pattern lines specifically expressing the reporter gene in reproductive organs (P1, P2, P3 and P4) showed normal vegetative growth. However, all the F_1 plants showing expected EGFP expression patterns were sterile or partly sterile (Table 4).

Discussion

In this report, we have established a binary GAL4–VP16-UAS system allowing co-activation of the EGFP reporter and a target gene. This system can be used to induce phenotypic changes as evidenced by testing a number of transcription factors. Our data have demonstrated that this 'geneticist's Swiss army knife' (Duffy, 2002) can be added to the genetic toolbox for monocotyledonous functional genomics just as it has been applied to mice (Ornitz *et al.*, 1991), Arabidopsis (Guyer *et al.*, 1998), zebrafish (Koster and Fraser, 2001; Scheer and Camnos-Ortega, 1999), frogs (Hartley *et al.*, 2002), and silkworm (Imamura *et al.*, 2003).

More than 1000 organ-specific pattern lines have now been identified in our laboratory and the number is still increasing, as we are continuing with screening of the enhancer trap mutant library currently containing >100 000 lines (Wu *et al.*, 2003; Zhang *et al.*, 2006). It should also be noted that since screening by GUS assay of thousands of transgenic plants in the field was time-consuming and highly costly, we performed a GUS-free pre-screening during rice transformation as a supplementary approach for efficiently generating tissue-specific activator lines. As demonstrated by our results, GUS-free pre-screening eliminated 80% of the positive calli, which increased the chance to obtain tissue-specific pattern lines. This method may be especially useful for establishing the transactivation system in plant species for which the enhancer trap mutant library is currently unavailable.

Co-activation of EGFP and the target gene is essential to the binary transactivation platform to achieve the gain-of-function or loss-of-function mutation caused by activation of the target gene (Nagel *et al.*, 2002). For this, the target gene and EGFP reporter gene must be kept silent in the target line until activated by the GAL4–VP16 protein in the hybrid plant. Our design of the target line vector is aimed at faithfully tracking the target gene expression simply by visualization of the EGFP expression. We did not detect any background EGFP expression in all the target lines generated, suggesting that the system did not have an unexpected effect on EGFP expression. More importantly, we detected perfect co-segregation both qualitatively and quantitatively among the GUS reporter, the EGFP and the target genes. All these suggest that the EGFP signal is a highly reliable indicator of target gene expression in the hybrid plants. In such a system, a

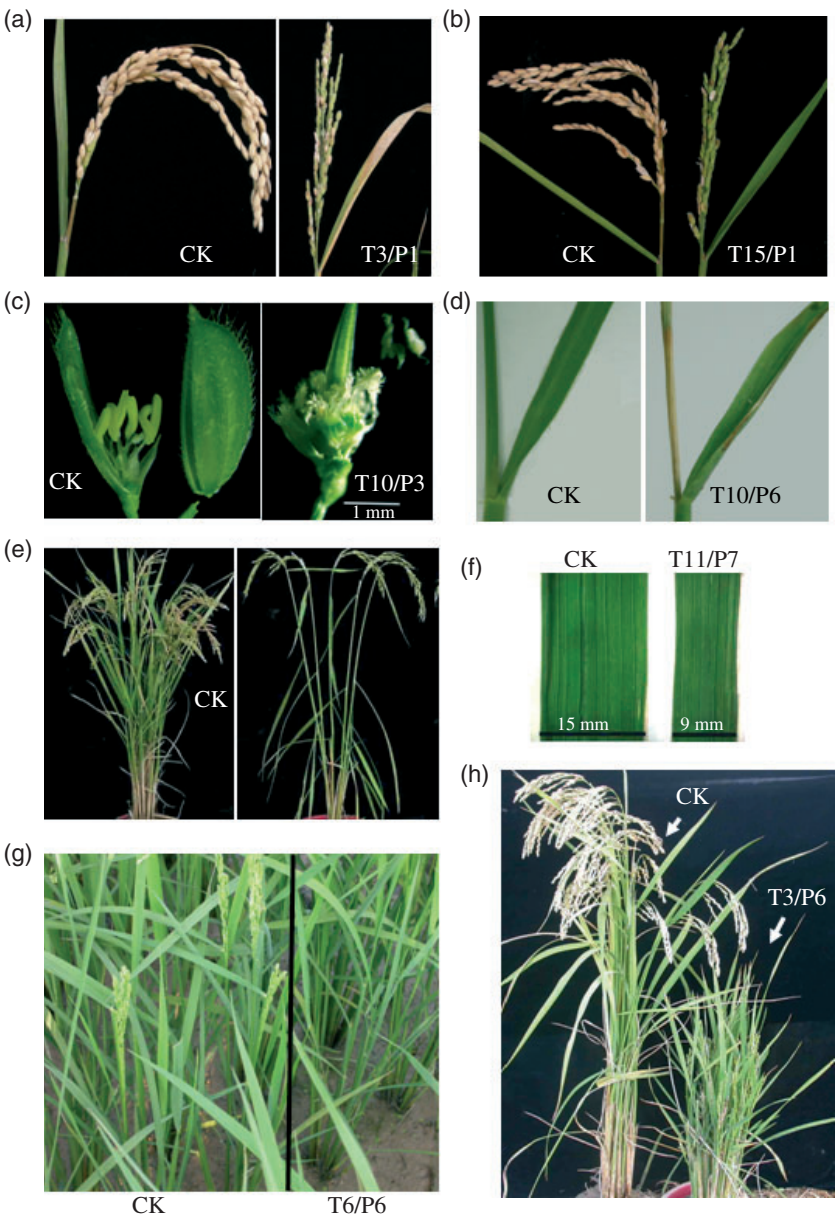

Figure 5. Various morphological changes observed in the F_1 plants between pattern lines and target lines: (a) partial sterility; (b) sterility; (c) multiple pistils (only stamen and pistil were shown for F_1); (d) leaf lesions; (e) fewer tillers and droopy leaf; (f) narrow leaf; (g) delayed flowering; (h) dwarfism. CK refers to either the target line or the pattern line parent.

phenotypic change in an F_1 plant with both GFP and GUS visualized can almost certainly be ascribed to transactivation of the target gene.

The objective for designing such a binary system is efficiently to generate gain- or loss-of-function mutation when the gene of interest is ectopically expressed. To test the effectiveness of the system, 10 transcription factors belonging to different subfamilies were chosen for ectopic expression. Six of them produced various phenotypic changes when mis-expressed in the hybrid plants. Since only six pattern lines were used for testing this system in this study, it can be expected that more phenotypic changes will be found when more pattern lines are used for crossing. Once a full set of the pattern lines is generated, this binary system performs well in discovering functions of genes, especially for those with partially redundant functions such as many transcription factors (Riechmann and Ratcliffe, 2000).

A main objective of developing such a binary system is to discover gene functions. The example provided by *OsMADS15* in this study has demonstrated the usefulness of this system to achieve this objective. OsMADS15 was first isolated by Moon *et al.* (1999) and can interact with OsMADS1 (Lim *et al.*, 2000), but its function is unknown. Our results have shown that the development of flower organs in rice was not affected in the hybrid plants when *OsMADS15* was ectopically expressed in anther, stigma, palea and lemma. This is not likely to be due to the

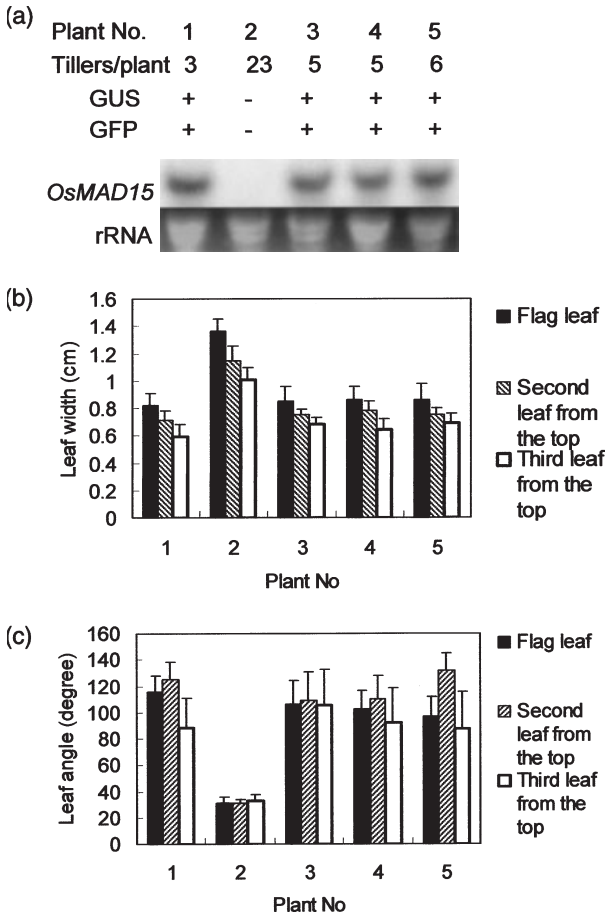

Figure 6. Morphological changes resulting from ectopic expression of *OsMADS15* in the hybrid of T11/P7. Only five sibling plants from one of four independent crosses are shown.
(a) Phenotype of fewer tillers co-segregated with EGFP and transgene expression in hybrid plants.
(b) Significantly (*t*-test, $P < 0.01$) reduced leaf width for all three leaves in the EGFP-expressed hybrid plants. The error bar for each histogram is based on all the major tillers of the plant.
(c) EGFP-expressing hybrid plants showing a larger angle between the leaf blade and the culm. The error bar of each histogram is based on five major tillers.

failure of transactivation of the target genes, since the EGFP expression was a reliable indicator of target gene expression. Rather, this result might suggest that elevated expression of *OsMADS15* in established flower organs had no effect on flower morphology. However, when this gene was ectopically expressed in leaves, leaf morphology and plant size were largely affected as manifested by the narrow and droopy leaves and significantly reduced number of tillers. Kim *et al.* (1998) analysed an Arabidopsis mutant *clf*, showing narrow, small and curled leaves, which was similar to the phenotype caused by constitutive expression of the *AGAMOUS* (*AG*) gene (belonging to the MADS family: Mizukami and Ma, 1992), and found that the *CURLY LEAF* (*CLF*) gene was required for stable repression of the *AG* gene. Goodrich *et al.* (1997) also found that ectopic expression of *AG* in vegetative shoots resulted in the small leaves, whereas the *clf ag* double mutant had large leaves. In addition, two Arabidopsis MADS-box genes, *AP1* and *AP3*, were expressed strongly at the adaxial side of immature lateral organs (Gustafson-Brown *et al.*, 1994; Jack *et al.*, 1992) and showed high homology with *Mcm1*, a yeast transcription factor regulating the expression of cell cycle- and cell division-related genes (Mclnerny *et al.*, 1997). Such phenotypic similarity suggests that *OsMADS15* might be involved in regulating the development of lateral organs (such as leaf and tiller).

Phenotype changes from the ectopic expression of the *OsMYBS3* gene demonstrated another nice feature of this binary system in providing starting materials for identifying gene functions. The *OsMYBS3* gene has been reported as a negative regulator that is predominantly expressed in senescent leaves and can inhibit the expression of α-amylase (Lu *et al.*, 2002). In this study, when the target line of *OsMYBS3* was crossed with the pattern line P7 that showed reporter gene expression in all the tissues screened, growth of the hybrid was severely retarded starting from seed germination (Figure 7). Since α-amylase plays an indispensable role in seed germination and early growth by providing carbohydrates through degradation of starch, the phenotype changes of the ectopic expression plants were likely to be the result of suppression of α-amylase expression, which remains to be characterized in future studies. Ectopic expression of the *OsMYBS3* gene in reproductive organs resulted in partial or complete spikelet sterility, suggesting that ectopic expression of this gene may have a negative effect on the reproductive process of rice.

It should also be noted that this binary system can be applied to test functions of other genes, although we only used transcription factors for assessing the effectiveness of the system. It has been reported in animal studies that ectopic expression of the glutamate–cysteine ligase gene in neuronal tissues can extend the lifespan of flies (Orr *et al.*, 2005); and ectopic expression of the cytochrome P450 gene *Cyp12a4* in the mid-gut and Malpighian tubules of flies results in lufenuron resistance, contrasting to the result that transgenic expression of *Cyp12a4* in a ubiquitous expression pattern results in late embryonic lethality (Bogwitz *et al.*, 2005).

Finally, for more efficient exploitation of the system, a high-throughput plant hybridization method should be useful for individual species to reduce labour intensity. In rice, for example, chemical-induced or photoperiod-sensitive male sterility rice (e.g. Zhang *et al.*, 1994) can be employed for producing target lines, while the wild type is used for producing pattern lines. Once such a high-through-

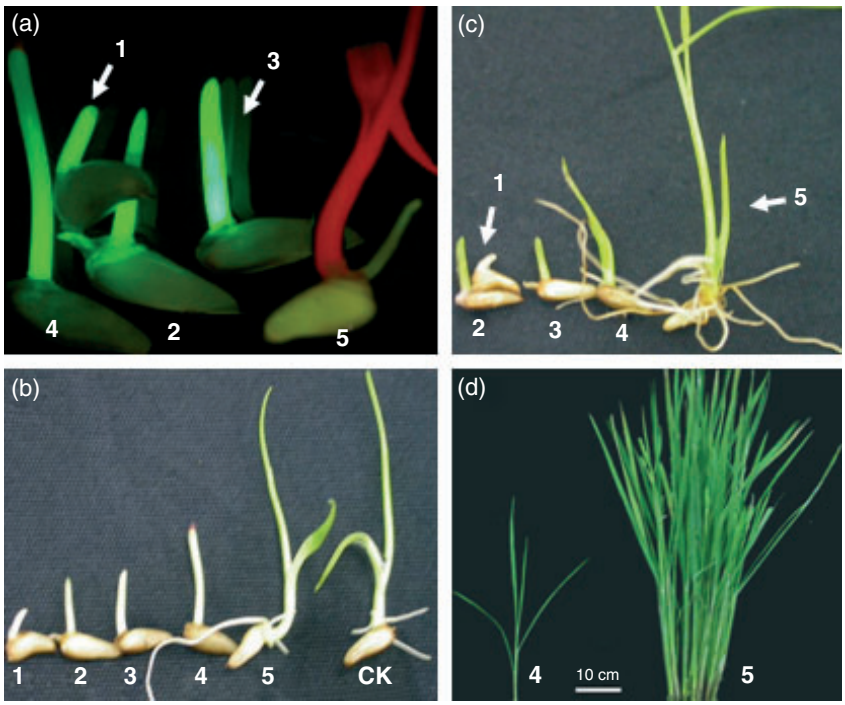

Figure 7. Inhibited growth of hybrid plants of T15/P7. T15 is the target line for the *OsMYBS3* gene.
(a) Growth-inhibited shoots showing EGFP expression.
(b) Growth performance at 1 week after germination. CK is the wild-type (Zhonghua 11).
(c) Growth performance at 2 weeks after germination.
(d) An EGFP-expressing hybrid plant (plant 4) with extremely retarded growth and development with no tillering or flowering in one rice growing season. The scale bar is 10 cm.

put hybridization system has been developed, the genome scale of transactivation experiments can be designed for annotation of thousands of genes.

Experimental procedures

Vector construction

The activator vector pSMR-J18R for producing the pattern line (or driver line) was derived by removing the EGFP reporter gene from the vector pFX-E24.2–15R that was used to generate enhancer-trapping lines in our previous study (Wu *et al.*, 2003). The effector vector pGOFS1 for producing the target line was constructed based on the backbone of pUAS3380 (provided by Dr R. Kerstetter). Briefly, the 35S-*Bar*-LB region in pUAS3380 was removed by *Bst*XI (blunted after cut) and then *Sac*I digestion and replaced with a fragment containing UAS-MP-EGFP-*ubiquitin* promoter-*HPT* gene-NOS terminator-LB (Figure 1a) that was released from an intermediate vector pUHEGFP by *Kpn*I (blunted after cut) and *Sac*II cleavages. The intermediate vector pUHEGFP was constructed based on pCAMBIA1380 (provided by CAMBIA, Canberra, Australia) by introducing the UAS-MP-*EGFP* fragment released from pFX-E24.2–15R, and the ubiquitin promoter-*Hph* gene-NOS terminator fragment released from the vector pUBI1381. The pUBI1381 was constructed based on pCAMBIA1381 by substituting the 35S promoter with a truncated maize *ubiquitin* promoter (589 bp in length) in which the *Eco*RI restriction site was removed but the promoter activity was unaffected (tested by GUS activity, data not shown). The EGFP and target gene were arranged in reverse directions in the vector and each was controlled by tandem repeats of UAS separated by stuffer sequence (280 bp in length from the backbone vector pUAS3380).

To construct target line vectors for individual genes of interest, full-length cDNA encoding putative transcription factors identified from a normalized rice cDNA library (Chu *et al.*, 2002) were released by *Eco*RI and *Bam*HI (or *Hin*dIII) and ligated into the pGOFS1 vector at the multiple cloning sites (Figure 1a).

Production of pattern lines, target lines and hybrid plants

The pattern line construct pSMR-J18R and the pGOFS1 construct containing the target gene were electro-transformed into *Agrobacterium tumefaciens* strain EHA105. *Agrobacterium*-mediated transformation was carried out essentially according to Hiei *et al.* (1994) using vigorous calli derived from mature embryos of cultivar Zhonghua 11 (*Oryza sativa* L. *ssp. japonica*). To obtain GUS-free calli for regenerating plants, one-half of each independent pSMR-J18R-transformed callus was cut off and used for GUS assay (Wu *et al.*, 2003). The other half of the GUS-free callus was subjected to regeneration.

Transgenic plants (T_0) were screened using GUS assay to find plants with expression of reporter gene only in a specific organ or tissue, referred to as pattern lines. For each target gene construct and the pGOFS1 vector, at least 30 independent transgenic lines were generated. Target lines with single-copy T-DNA insertion and showing normal phenotype (independent lines for each construct were shown in the last column of Table 3) were used for making crosses with pattern lines showing normal phenotype. Target lines (T_0 plants) were used as the female parent, and pattern lines (T_1 plants with GUS expression pattern rechecked prior to making the cross) as the male parent. At least 30 hybrid seeds were obtained for each cross.

GUS assay and GFP imaging

GUS assay was performed using the histochemical staining method as described previously (Wu *et al.*, 2003). Rice organ, tissue or young shoots were viewed by fluorescence stereomicroscope (Leica MZ FLIII, Leica Microsystems, Germany). All images were recorded using a CoolPix 5400 digital camera (Nikon, Japan).

Southern and Northern blot analysis

The copy number of integrated T-DNA in the rice genome was checked using Southern blot analysis. Genomic DNA was extracted from rice leaves by using the CTAB method (Murray and Thompson, 1980), digested with *Eco*RI, separated in 0.8% agarose gel, transferred on to nylon membrane, and hybridized using the hygromycin resistant gene (*Hph*) as the probe.

Total RNA was prepared from the transgenic plant using TRIZOL reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. RNA electrophoresis, blotting and hybridization were performed according to the method described previously (Xiong and Yang, 2003) using 3′UTR of the target gene as the gene-specific probe.

Real-time quantitative PCR analysis

Total RNA from plant leaves was pre-treated with RNase-free DNase I (Invitrogen), according to the manufacturer's instructions. The reverse transcriptase (RT) reaction was performed with Super-Script™ II reverse transcriptase (Invitrogen) following the manufacturer's instructions. The efficiency of cDNA synthesis was assessed by real-time PCR analysis of the control rice gene *Actin1* (primers: 5′-TGCTATGTACGTCGCCATCCAG-3′ and 5′-AATGAGTA-ACCACGCTCCGTCA-3′). Only cDNA samples that yielded similar C_T values for *Actin1* were used for real-time PCR of other genes including GUSplus (primers: 5′-GAGCACAAGGGCGGATTC-3′ and 5′-CGGGGTCGTGTAGATTTTC-3′), *EGFP* (primers: 5′-AGTGCTTC-AGCCGCTACCC-3′ and 5′-GTTCACCTTGATGCCGTTC-3′) and putative *MADS* gene (primers: 5′-GTGAAGGGAGTAGGAGCA-3′ and 5′-CTAACAAGCAGAGCCAGAG-3′). Real-time PCR was performed in an optical 96-well plate with an ABI PRISM® 7500 Real-time PCR System (Applied Biosystems, Foster City, CA, USA), using SYBR® Green (Applied Biosystems) to monitor DNA synthesis. All reactions contained 10 μl 2× SYBR® Green Master Mix Reagent (Applied Biosystems), 2.0 ng cDNA and 200 nM of each gene-specific primer in a final volume of 20 μl, using the thermal cycle as follows: 95°C for 3 min; 40 cycles of 95°C for 30 sec; 59°C for 30 sec; 72°C for 1 min. All amplification plots were analysed with an R_n threshold of 0.2 to obtain C_T (threshold cycle) values. The relative expression level of reporter and target genes was determined based on the $2^{-\Delta\Delta C_T}$ method (Livak and Schmittgen, 2001) by using rice *Actin1* as an internal control that had constant C_T (18.2 ± 0.3) values among different samples in this study. The relative ectopic expression level, based on the difference of the $2^{-\Delta\Delta C_T}$ values between hybrid and control (target line) plants, was used for correlation analysis.

The rice pattern line database

The Rice Mutant Database (located at http://rmd.ncpgr.cn/; Zhang *et al*., 2006) has been constructed for the enhancer trap mutant library which contains the images and observations made during the screening of pattern lines – this is being constantly updated as more lines are under screening. The expression pattern of reporter genes and the related information are searchable by entry ID (such as 03Z11UZ88 as listed in Table 2) of mutant or by keyword (such as stamen, stigma, lemma, palea, embryo, leaf blade, P1, P2, etc., as listed in Tables 1 and 2).

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Supplementary Material

The following supplementary material is available for this article online:

Table S1. Ectopic expression level of target genes determined by real time PCR in GUS-positive hybrid plants from seven crosses.

This material is available as part of the online article from http://www.blackwell-synergy.com

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