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Technical advance

A highly efficient transient protoplast system for analyzing defence gene expression and protein–protein interactions in rice

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SUMMARY

The transient assay system based on mesophyll or cultured cell-derived protoplasts has been exploited in several plant species and has become a powerful tool for rapid gene functional analysis and biochemical manipulations. However, the system has not been widely used in rice owing to the difficulties in large-scale isolation of viable rice protoplasts from leaves or suspension-cultured cells. Here, we describe a significantly improved method to isolate a large number of protoplasts from stem and sheath tissues of both young and mature plants. High-level coexpression of multiple constructs and efficient suppression of exogenous and endogenous genes were observed in the stem- and sheath-derived protoplasts. A transient green fluorescent protein and luciferase-based reporter system for defence-related genes expression analysis has been established, which is useful for screening and characterizing genes involved in rice defence signalling pathways. Furthermore, a protoplast-based bimolecular fluorescence complementation (BiFC) system for the detection of protein–protein interactions in living rice cells was developed. The YFP complementation of two split-YFP halves mediated by homodimerization of the GUS and SPIN1, a cell-death related protein, was observed in transfected protoplasts. In combination with genetic, genomic and proteomic approaches, the established versatile protoplast transient assay system will facilitate large-scale functional analysis of defence-related genes in rice.

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INTRODUCTION

The generation of transgenic lines has become routine in many plant species and has offered a powerful research tool for investigating gene function in plants. However, the relatively expensive and time-consuming process for obtaining transgenic lines still limits the utilization of this approach for large-scale analyses of plant genes. Transient assays provide a convenient alternative system to analyse the functional expression of plant genes (Dekeyser *et al.*, 1990; Sheen, 2001). Compared with the transgenic approach, transient assays have a great advantage that gene activity can be measured facilely and shortly after the DNA delivery (Kapila *et al.*, 1997). This approach has been widely used in plants for a long time for measuring promoter activity, examining subcellular localization and targeting of proteins (Abel and Theologis, 1994; Bruce *et al.*, 1989). Moreover, the great advances in plant genomics and proteomics have stimulated renewed interest in the transient assay approach for high-throughput analysis of gene function in recent years. The transient assay based on virus infection or *Agrobacterium* infiltration of leaves is an effective method to study genes of interest, and has been proposed for large-scale functional analysis of genes in both dicot (Liu *et al.*, 2002; Ratcliff *et al.*, 2001) and monocot plants (Holzberg *et al.*, 2002). Transient assay utilizing protoplasts is another refined method to analyse plant gene activity (Abel and Theologis, 1994), and has been established in several plant species, such as Arabidopsis, maize and tobacco. In addition to showing a high transformation efficiency, plant protoplasts have also proven to show similar reactions to that of intact tissues and plants to hormones, metabolites, environmental cues and pathogen-derived elicitors (Sheen, 2001), providing a powerful and versatile cell system for high-throughput dissection of plant signal transduction pathways. Based on the protoplast transient assay systems, Sheen and colleagues (Asai *et al.*, 2002; Kovtun

et al., 1998, 2000) successfully uncovered the mitogen-activated protein (MAP) kinase cascades involved in oxidative stress, auxin and defence signalling pathways. De Sutter *et al.* (2005) recently developed an automated protoplast transient assays system based on robotic platforms, and identified two transcription factors involved in tobacco jasmonate signalling.

Traditionally, the yeast two-hybrid system has been the choice for *in-vivo* screening of protein–protein interactions. Although this system has been valuable in detecting protein–protein interactions (Chern *et al.*, 2005), it is not without limitations such as the high rate of false positive clones and the lack of plant-specific post-translational protein modifications (Walter *et al.*, 2004). Direct read-out systems to detect protein–protein interactions in plant cells are appealing and have been used in conjunction with transient assay approaches. For example, Seidel *et al.* (2005) used fluorescence resonance energy transfer (FRET) to visualize the interaction of the VHA-c and VHA-a subunits of the V-ATPase complex in Arabidopsis protoplasts. However, FRET detection is technically challenging and is of low efficiency (Chapman *et al.*, 2005). In the last few years, new protein–protein interaction detection methods such as bimolecular fluorescence complementation (BiFC) and luciferase complementation imaging (LCI) have been developed in animal and plants cells (Bracha-Drori *et al.*, 2004; Hu *et al.*, 2002; Luker *et al.*, 2004; Walter *et al.*, 2004). The BiFC method is based on the complementation of the YFP fluorescence activity by the N-terminal (YFPN) and C-terminal (YFPC) halves of the YFP protein when brought together by two interacting proteins that are fused to each half (Bracha-Drori *et al.*, 2004; Walter *et al.*, 2004). The method has been successfully used to detect the homodimerization of the Arabdopsis basic leucine zipper transcription factor bZIP63 and the interaction between the α and β units of the Arabdopsis protein farnesyltransferase (Bracha-Drori *et al.*, 2004; Walter *et al.*, 2004). The LCI method is a similar method that has been developed for protein–protein interactions in living animal cells (Luker *et al.*, 2004). The two split-protein complementation methods have different advantages. The BiFC method can visualize protein interactions that take place in different cellular compartments, and the LCI method is better for quantitatively detecting the protein–protein interaction. Up to now, an efficient BiFC or LCI method for protein–protein interaction assays has not been reported in rice.

Rice (*Oryza sativa* L.) is one of the most important food crops in the world. As a result of the many rice genomics projects initiated in several countries in the last decade, the rice genome has been completely sequenced (International Rice Genome Sequencing Project, 2005), global gene expression profiles have been obtained (Jantasuriyarat *et al.*, 2005; Li *et al.*, 2006), and a large number of T-DNA and transposon-based tagged mutants have been produced (Hirochika *et al.*, 2004). These lines of progress have provided a wealth of information and resources for functional genomics studies in rice. However, the function of the majority of rice genes has not been experimentally identified (Zhang *et al.*, 2006). By contrast, although many techniques, such as stable transformation, are currently available for studying gene function in rice, there is a lack of versatile transient assay systems that can be use for large-scale analyses of gene function.

We are focusing on studying the defence gene expression and signal transduction in rice. By using both genetic and genomic approaches, a number of cell death-related and disease resistance genes (Qu *et al.*, 2006; Zeng *et al.*, 2004), and many novel genes from a large-scale expressed sequence tag (EST) sequencing project (Jantasuriyarat *et al.*, 2005), have been obtained. Here we describe the development of a transient expression assay system using protoplasts isolated from rice stem and sheath tissues for functional analysis of rice defence genes. High levels of coexpression of multiple constructs and efficient suppression of exogenous and endogenous genes in rice protoplasts were observed. A transient GFP and luciferase-based reporter system for defence-related gene expression analysis has been established that will be useful for screening and characterizing genes involved in rice defence signalling pathways when combined with genetic, genomic and proteomic approaches. Furthermore, a protoplast-based BiFC system for the detection of protein–protein interactions in living cells was developed. The stem- and sheath-derived protoplast transient assay approach might also be applied easily to other plants from which sufficient protoplasts cannot be isolated from leaves or suspension-cultured cells.

RESULTS

Isolation of a large number of rice protoplasts from stem and sheath tissues of young seedlings

Mesophyll protoplasts freshly isolated from leaves have proven to be versatile systems for studying gene activity in several plant species. Compared with the isolation of mesophyll protoplasts from maize and Arabidopsis (Sheen, 2001), or tobacco (Tao *et al.*, 2002), the isolation of mesophyll protoplasts from rice is difficult owing to its leaf structure and size. To establish an efficient protocol for rice protoplast isolation, we compared the number of protoplasts isolated from leaves and from both stems and sheaths of aetiolated seedlings. The result indicated that a large number of rice protoplasts could be isolated from stem and sheath tissues but not from leaves. From the stems and sheaths of 105 2-week-old Taipei 309 seedlings with approximately 12 h of enzyme digestion, about 14×10^6 protoplast cells were obtained, approximately 41-fold higher than the yield of protoplasts isolated from leaves of the same seedlings (Fig. 1A). The rice seedling tissues were then used for optimizing the digestion time. The protoplast cells obtained increased significantly and in

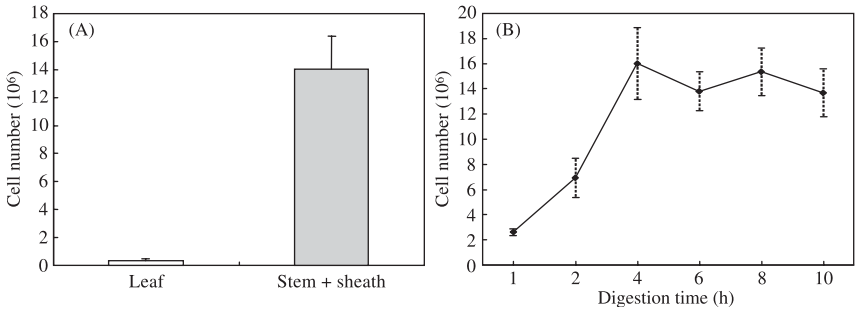

Fig. 1 Isolation of a large number of rice protoplasts from stem and sheath tissues of young seedlings. (A) Comparison of the yield of protoplasts isolated from leaves and from stems and sheaths. (B) Effect of digestion time on the yield of protoplasts.

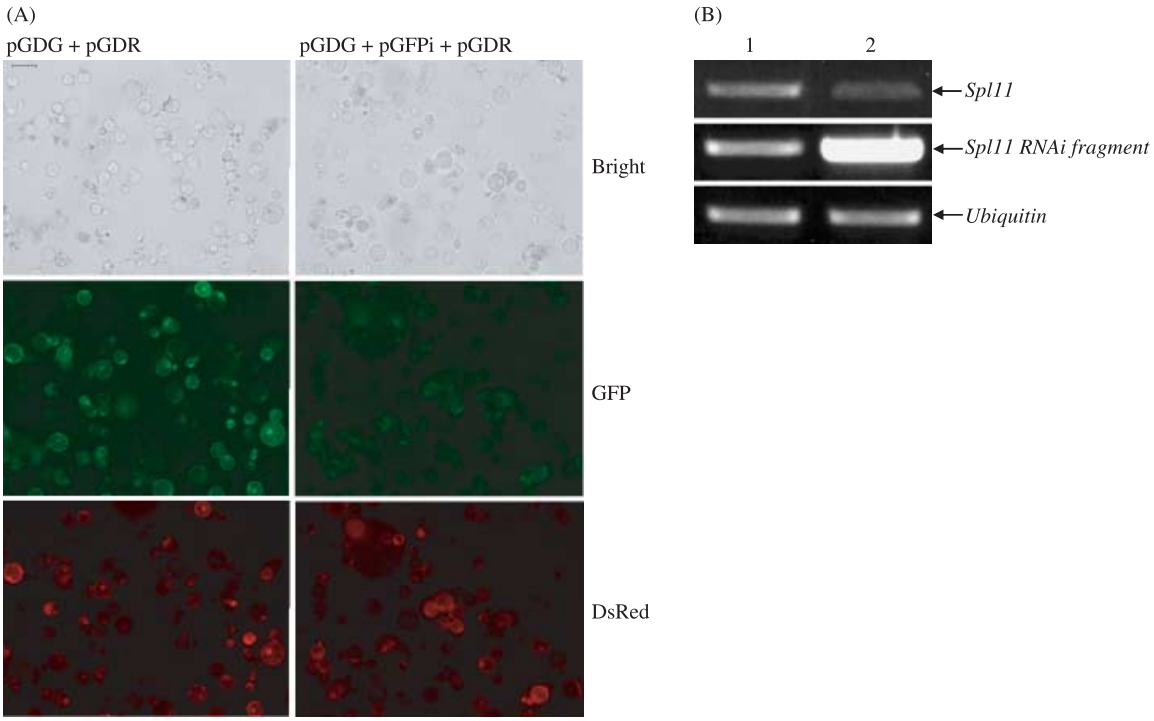

Fig. 2 Transient suppression of gene expression in rice protoplasts. (A) High-efficiency co-expression of different genes, *gfp* and *DsRed*; and suppression of exogenous *gfp* expression by co-transfecting pGFPi with pGDG. Fluorescence images of GFP- or DsRed-expressing protoplasts were taken with the same exposure time. Scale bar, 50 μm. (B) RNA silencing of endogenous gene *Spl11* by transient expression of the *Spl11* RNAi construct pSPL11i: 1, non-transfected sample; 2, pSPL11i-transfected sample. Semi-quantitative PCR was performed with 23 cycles.

proportion to the digestion time, but finally reached a stable yield level when the tissues were digested up to 4 h (Fig. 1B). An average yield of 10–16 × 10^6 protoplasts was routinely obtained from ~105 2-week-old rice seedlings and the viability of the protoplasts was up to 90% as judged by the Evans blue staining assay (data not shown).

High-efficiency transient expression and suppression of exogenous and endogenous genes in protoplasts

The PEG-mediated transfection procedure (Tao *et al.*, 2002) was applied to establish the protoplast transformation. The transformation efficiency of the protoplasts was measured by using green fluorescent protein (GFP) and red fluorescent protein (DsRed) as detecting markers (Goodin *et al.*, 2002). The percentage of transfected cells was routinely scored in the range between 50 and 70%. Furthermore, fluorescence microscopy of GFP and DsRed revealed that the rice protoplasts have roughly the same high efficiency for coexpressing multiple constructs in the same cells (Fig. 2A, left-hand panels).

Gene silencing has become a powerful tool for gene functional analysis. To investigate the effectiveness of transient silencing of gene expression in rice protoplasts, two RNAi vectors, pGFPi and pSPL11i, carrying inverted repeats of a 426-bp fragment of the

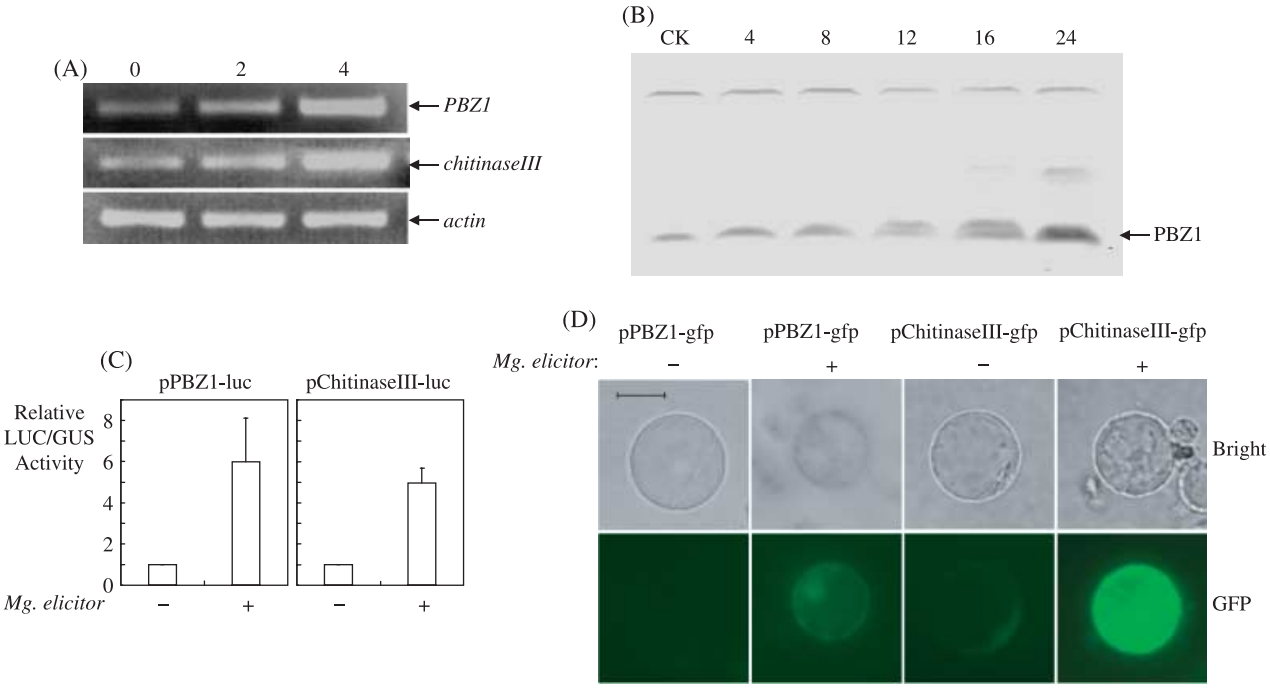

Fig. 3 Transient reporter system of defence-related genes in *M. grisea* elicitor-treated rice protoplasts. (A) RT-PCR analysis of two defence-related genes, *PBZ1* and *chitinaseIII*, 0, 2 and 4 h after elicitor treatment. (B) Western blot analysis of PBZ1 at 0, 4, 8, 12, 16 and 24 h after elicitor treatment. (C) Transient expression assay of promoter activities of *PBZ1* and *chitinaseIII* genes using the reporter *luc* gene. Transfected protoplasts were treated with elicitor for 10 h. Promoter activities are represented as relative LUC/GUS activity, and normalized to the value obtained with transfected protoplasts without elicitor treatment. (D) Transient expression assay of *gfp* driven by *PBZ1* or *chitinaseIII* promoters. Fluorescence was detected 20 and 16 h after transfetction with pPBZ1-gfp or pChitinaseIII-gfp, respectively. Scale bar, 20 μm.

gfp gene and a 300-bp fragment of the *Spl11* gene (Zeng *et al.*, 2004), respectively, were generated based on the Gateway RNAi vector pANDA-Mini (Miki and Shimamoto, 2004). Compared with the GFP expression of the protoplasts transfected with only the 35S-gfp vector pGDG, GFP fluorescence decreased drastically when the protoplasts were co-transfected with both pGDG and pGFPi (Fig. 2A, right-hand panels). When the pSPL11i was transfected into the rice protoplasts, accumulation of endogenous *Spl11* mRNA was significantly reduced 6 h after transfection (Fig. 2B). These results indicated that the rice protoplast system can be used to induce efficiently the specific RNA silencing of both exogenous and endogenous genes.

Transient reporter system for defence-related gene expression analysis

To develop a protoplast-based system for monitoring the expression activity of the defence genes involved in rice defence responses, the rice protoplasts were tested for their reaction to the cell-wall extract of *Magnaporthe grisea*, an elicitor used previously for the defence response in suspension-cultured rice cells (Matsumura *et al.*, 2003). Two defence-related genes, *PBZ1* (D38170, Midoh and Iwata, 1996) and *chitinaseIII* (AU062762), were chosen to test for the induction by elicitor treatment, as they were dramatically induced in suspension cells by *M. grisea* infection (Matsumura *et al.*, 2003) and the *N*-acetylchitooligosaccharide elicitors (Akimoto-Tomiyama *et al.*, 2003), respectively. Following treatment of rice protoplasts with the elicitor, induction of *PBZ1* and *chitinaseIII* was detectable by RT-PCR as early as 2 h and reached a high level at 4 h after treatment (Fig. 3A). A Western blot was also performed with anti-PBZ1 antibody on the *M. grisea* elicitor-treated protoplasts. The result showed that the accumulation of PBZ1 protein was induced as early as 4 h after treatment, and enhanced significantly with increased treatment time (Fig. 3B).

The promoter fragments of the *PBZ1* and *chitinasIII* genes were then isolated from rice genome DNA by PCR and fused to both the firefly luciferase reporter gene (*luc*) and the *gfp* gene. The derived constructs were tested transiently in the transfected rice protoplasts. For the *luc* constructs assay, the construct containing the β-glucuronidase reporter gene (*gus*) (Jefferson *et al.*, 1987) driven by the constitutive maize ubiquitin promoter (Christensen and Quail, 1996) was co-transfected into the protoplasts as an internal control. Consistent with the RT-PCR results, the relative LUC/GUS activities in the *M. grisea* elicitor-treated protoplasts were six- and five-fold, respectively, of that of

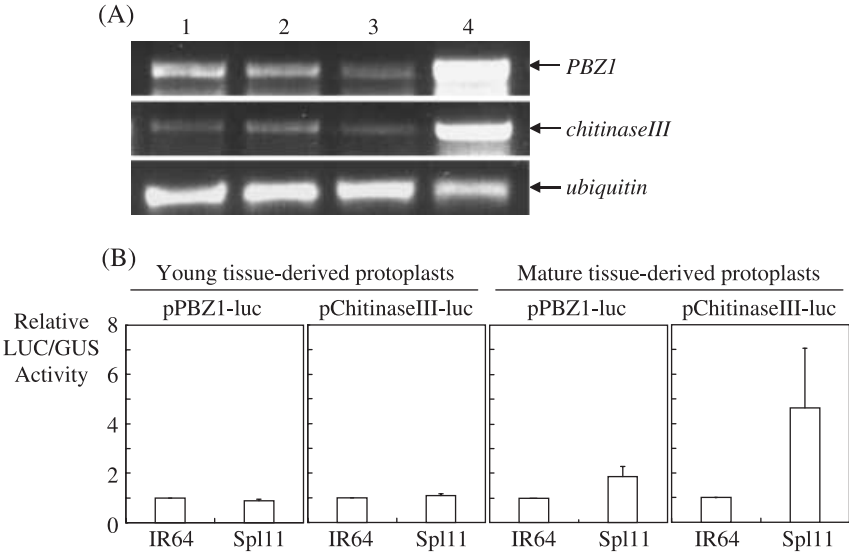

Fig. 4 Transient reporter system of defence-related genes in mature tissue-derived protoplasts. (A) RT-PCR analysis of *PBZ1* and *chitinaseIII* expression. 1, young IR64 protoplasts; 2, young *spl11* protoplasts; 3, mature IR64 protoplasts; 4, mature *spl11* protoplasts. (B) Transient expression assay of activities of *PBZ1* and *chitinaseIII* promoters using the reporter *luc* gene. Protoplasts were detected 10 and 12 h after transfetction with pPBZ1-luc or pChitinaseIII-luc, respectively. Promoter activities are represented as relative LUC/GUS activity, and the value of *spl11* protoplasts samples was normalized to the value of IR64 protoplast samples.

non-elicitor-treated samples 10 h after the transfection with pPBZ1-luc and pChitinaseIII-luc constructs (Fig. 3C). Similar results were observed in rice protoplasts when the pPBZ1-gfp and pChitinaseIII-gfp constructs were tested. Although no or only very weak fluorescence could be observed in the non-elicitor-treated protoplasts, bright GFP fluorescence was observed in both elicitor-treated pPBZ1-gfp- and pChitinaseIII-gfp-transfected protoplasts (Fig. 3D).

Mature plant protoplast system for developmentally regulated PR gene expression analysis

Previous studies have shown that the expression of defence-related genes in many rice mutant plants was often developmentally regulated (Fitzgerald *et al.*, 2004; Takahashi *et al.*, 1999; Yin *et al.*, 2000). Therefore, the protoplasts isolated from young seedling tissues are not appropriate for analysing those developmentally regulated gene expression. To exploit the utilization of the rice protoplast system for studying developmentally regulated gene expression, we isolated protoplasts from mature rice plants. Similar to the results observed in protoplasts isolated from aetiolated young seedling tissues, a large number of protoplasts could be isolated from the stem and sheath tissues of mature plants, whereas very few protoplasts were obtained from the leaves of the same plants (data not shown). An average yield of $1-2 \times 10^6$ protoplasts per gram of tissue was routinely obtained from mature rice plants, and the transformation efficiency of the mature tissue protoplasts was consistently in range 50–70% (data not shown), similar to that of the protoplasts from young tissues.

To determine whether the protoplasts isolated from mature tissues retain the developmentally regulated defence-related gene expression patterns, the rice lesion mimic mutant *spl11*, in which the defence-related gene expression correlates with lesion development in mature plants (Yin *et al.*, 2000), was selected to test the expression of two defence-related genes, *PBZ1* and *chitinaseIII*. Protoplasts were isolated from both aetiolated young seedlings and mature plants of IR64 and *spl11*. RT-PCR analysis indicated that *PBZ1* and *chitinaseIII* were constitutively activated in the mature *spl11* protoplasts, but not in young *spl11*, IR64 or mature IR64 protoplasts (Fig. 4A). Consistent with the RT-PCR data, transient expression assays showed that *PBZ1* promoter- and *chitinaseIII* promoter-controlled *luc* was induced in mature *spl11* protoplasts (Fig. 4B), demonstrating the feasibility of the mature tissue protoplast system for analysis of developmentally regulated gene expression.

Protoplast-based BiFC technique for detecting protein associations in living rice cells

To test the feasibility of the protoplast transient assay system for the detection of protein–protein associations in rice cells, we used a pair of split-YFP constructs, pA735SGUS-NYFP and pA735SGUS-CYFP (Fig. 5A, provided by Dr H. Q. Yang, Institute of Plant Physiology and Ecology, Chinese Academy of Sciences, Shanghai, China). The two constructs have been tested in bombarded onion tissues, and the complementation of YFP fluorescence was achieved by homodimerization of the GUS protein (H. Q. Yang, personal communication). In addition, we generated another pair of split-YFP constructs, pA7spin1-NYFP and pA7spin1-CYFP (Fig. 5A). The *SPIN1* gene encoding a putative RNA binding protein was identified from a yeast two-hybrid screen when *Spl11* was used as the bait (Zeng *et al.*, 2004, and unpublished data). Transfection of pA735SGUS-NYFP, pA735SGUS-CYFP

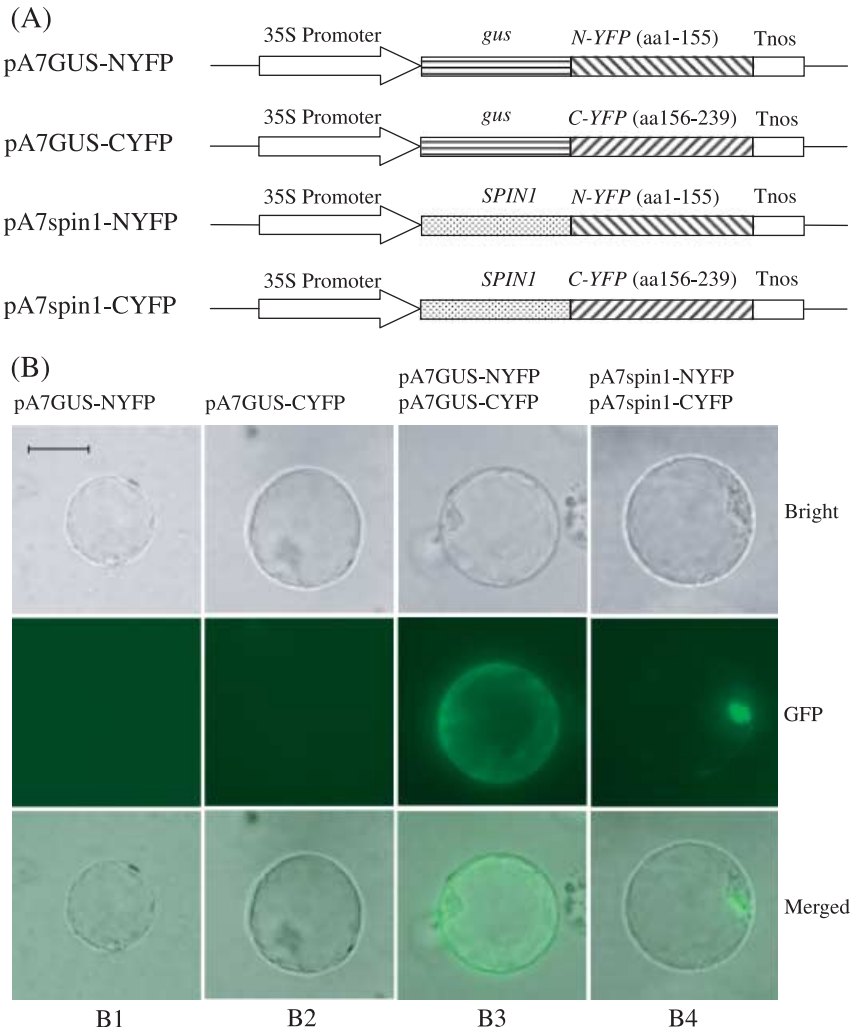

Fig. 5 BiFC visualization of protein homodimerization in rice protoplast cells. (A) Diagrams of split-YFP constructs used in the transfections. (B) Microscopy detection of transfected rice protoplast cells. Scale bar, 20 μm.

(Fig. 5B1, B2), pA7spin1-NYFP or pA7spin1-CYFP alone, or co-transfection of pA735SGUS-NYFP and pA7spin1-CYFP or pA7spin1-NYFP and pA735SGUS-CYFP (data not shown) did not yield any YFP fluorescence. By contrast, obvious YFP fluorescence mediated by homodimerization of GUS or SPIN1 was observed when pA735SGUS-NYFP and pA735SGUS-CYFP (Fig. 5B3) or pA7spin1-NYFP and pA7spin1-CYFP (Fig. 5B4) were co-transfected into the rice protoplasts. The GUS homodimerization-induced YFP fluorescence was observed in the whole cell, and the SPIN1 homodimerization-induced YFP fluorescence was observed exclusively inside the nucleus region. These results demonstrate that our protoplast-based BiFC method is a useful tool for *in-situ* protein–protein interaction assays.

DISCUSSION

In rice, protoplasts isolated from suspension culture cells have been used for transformation studies (Datta *et al.*, 1990; Shimamoto *et al.*, 1989) and transient gene expression assays (Isshiki *et al.*, 2006; McElroy *et al.*, 1990) for many years. However, the protoplast system based on rice suspension culture cells has some limitations. For example, the protoplasts isolated from suspension culture cells lose their differentiated state; and the isolation of large-scale protoplasts is not easy for many rice varieties owing to the difficulties in the induction of suspension culture cells. Plant tissue-derived mesophyll protoplasts retaining their cell identity and differentiated state have been proven to be versatile systems for studying gene activity and signal transduction in several plant species, such as maize, Arabidopsis (Sheen, 2001) and tobacco (Tao *et al.*, 2002). However, the isolation of mesophyll protoplasts from rice is difficult because of its leaf structure and size. With our improved protoplast isolation method, we have established a highly efficient protoplast-based transient system for gene functional expression and protein–protein interaction studies in rice. Unlike other protocols used in many plant species such as maize, Arabidopsis and tobacco that

used leaf tissues for protoplast isolation (Sheen, 2001; Tao *et al.*, 2002), stem and sheath tissues were used as the source material. Based on our protocol, more than 10×10^6 protoplast cells could be obtained easily from 105 2-week-old rice seedlings, sufficient for more than 50 transfection experiments (2×10^5 cells can be used per transfection). Our results also indicated that the protoplast system is ideal for RNA and protein assays, such as RT-PCR and Western blotting. Moreover, the stem and sheath tissue-derived protoplasts showed very high transformation efficiency, similar to Arabidopsis mesophyll protoplasts (Sheen, 2001). Compared with the protoplast system based on suspension culture cells or other developed transient assay methods that are usually less effective in rice, such as the bombardment transient assay (Bruce *et al.*, 1989) and tissue electroporation (Dekeyser *et al.*, 1990), the easy isolation of large numbers of protoplasts, the highly efficient gene expression or suppression combined with convenient assays such as reporter gene assays, RNA- and protein-level analyses, enable the stem- and sheath-derived protoplast system to be used more extensively for gene and function studies in rice in the future.

Protoplast transient assay systems were usually considered to be inappropriate for the study of developmentally regulated gene expression (Kapila *et al.*, 1997), mainly because of the use of young seedling tissues or suspension culture cells as sources of protoplasts. In this study, as an attempt to study developmentally regulated gene expression, we tested the isolation of protoplasts from mature plants grown in the greenhouse or growth chamber. As a previous report suggested that high concentrations of disinfectant, such as ethanol or Clorox, used for tissue sterilization might damage tissues and result in a low yield of protoplasts (Liang *et al.*, 2002), the stem and sheath tissues were not treated with any disinfectant in our protocol. By adding ampicillin in the enzyme solution, a high yield of viable protoplasts was obtained from the stem and sheath tissues and no bacterial contamination of the protoplasts was observed even after 36 h of incubation (data not shown). Based on this protocol, we selected the rice lesion mimic mutant *spl11* to test the expression of defence-related genes in mature tissue-derived protoplasts. We have shown previously that the activation of defence-related genes was found only in mature *spl11* plants with lesion mimic phenotype, but not in young seedlings without this phenotype (Yin *et al.*, 2000). As expected, the constitutive induction of two defence-related genes, *PBZ1* and *chitinaseIII*, in the mature *spl11* mutant-derived protoplasts was detected by both RT-PCR (Fig. 4A) and transient reporter gene assays. We noted that the induced LUC activity of the *PBZ1* promoter-controlled *luc*-transfected sample was not as high as that of the *chitinaseIII* promoter-controlled *luc*-transfected sample (Fig. 4B). The lower induced LUC activity of the *PBZ1-luc* sample might be because the the *PBZ1* promoter fragment used in this study was too short. When the transient expression assays were performed in protoplasts treated with *M. grisea* elicitor, we also found that the *PBZ1* promoter–reporter had a lower basal expression level and a lower induced expression level compared with the expression level of *chitinaseIII* promoter–reporter (Fig. 4B). Nethertheless, the RT-PCR results of *PBZ1* and *chitinaseIII* and the transient expression result of *chitinaseIII* promoter-*luc* indicated that the protoplasts isolated from mature tissues retain similar gene expression profiles to that of intact plant tissues. Therefore, our system will be suitable for analysis of developmentally regulated gene expression.

Protoplast transient assay systems have proven very useful for dissecting a broad range of plant signal transduction pathways and transcriptional regulatory networks (De Sutter *et al.*, 2005). One successful example is the dissection of plant defence signalling pathways. Based on the protoplast assay approach, Asai *et al.* (2002) developed an inducible defence gene expression system, and identified a complete MAP kinase cascade in Arabidopsis innate immunity. In this study, we developed a rice protoplast transient expression system in which the defence genes *PBZ1* and *chitinaseIII* are induced in protoplasts by *M. grisea* elicitor treatment, and spontaneously activated in mature tissue protoplasts of the lesion mimic mutant *spl11*. Defence genes have been widely used as markers for characterization of genes related to disease resistance in rice (Chern *et al.*, 2005; Takahashi *et al.*, 1999; Yin *et al.*, 2000). The method established here can thus be very useful for large-scale screening of candidate defence genes from microarray or expression studies when their over-expression or RNAi constructs are co-transfected with an important defence gene reporter construct in rice protoplasts.

The functions of many genes are known to be mediated through the association of their coding proteins with other proteins. Several methods have been developed to test protein interactions and protein complexes. Traditionally, the yeast two-hybrid is the general choice for screening interacting proteins. However, this method has limitations for screening for plant interacting proteins using the heterologous yeast system. FRET is a direct read-out technique to detect protein–protein interactions in plant cells and has been used in Arabidopsis (Seidel *et al.*, 2005). However, FRET detection has not been widely used owing to its complicated procedure and low detection efficiency. Recently, alternative approaches based on the complementation of split reporter proteins, such as BiFC (Bracha-Drori *et al.*, 2004; Hu *et al.*, 2002; Walter *et al.*, 2004) and LCI (Luker *et al.*, 2004), have been developed. In this study, we tested the BiFC method using the protoplast transient expression assay system. The YFP complementation mediated by homodimerization of GUS and SPIN1 was observed in transfected rice protoplasts with different subcellular localizations, suggesting that the protoplast-based BiFC is a convenient tool to detect protein–protein interactions in rice cells. With the improvements made to assay techniques, for

example with the automated and standardized assay (De Sutter *et al.*, 2005), the protoplast transient expression system can now be used as an alternative to the yeast two-hybrid system for large-scale detection of protein–protein interactions in living plant cells.

In conclusion, we have established a rice transient assay system based on stem- and sheath-derived protoplasts. Although the procedure might be not suitable for studies of complex organisms, its versatility for assays of gene over-expression or gene suppression, dissection of signalling pathways and detection of protein–protein interactions will facilitate large-scale functional analysis of defence-related genes in rice, in combination with genetic, genomic and proteomic approaches.

EXPERIMENTAL PROCEDURES

Protoplast isolation

For isolating protoplasts from young seedling tissues, rice seeds were germinated on half-strength MS medium under light for 3 days. Seedlings were then cultured on half-strength MS medium in the dark at 26 °C for 10–12 days. Tissues of aetiolated young seedlings were cut into approximately 0.5-mm strips and placed in a dish containing K3 medium (Kao and Michayluk, 1975) supplemented with 0.4 M sucrose, 1.5% cellulase R-10 (Yakult Honsa) and 0.3% macerozyme R-10 (Yakult Honsha). The chopped tissue was vacuum-infiltrated for 1 h at 20 mmHg and digested at 25 °C with gentle shaking at 40 r.p.m. After incubation, the K3 enzyme medium was replaced by the same volume of W5 solution (154 mM NaCl, 125 mM $CaCl_2$, 5 mM KCl and 2 mM MES, adjusted to pH 5.8 with KOH). Protoplasts were released by shaking at 80 r.p.m. for 1 h, followed by filtering through a 35-μm nylon mesh. Protoplasts were collected by centrifuging at 300 ***g*** for 4 min at 4 °C. Pellets were resuspended in W5 solution. The leaves and the stems including sheaths were used to compare protoplast yields with a 12-h digestion time. To optimize the digestion time, the tissues were digested for 1, 2, 4, 6, 8 or 10 h. Protoplasts were counted in a haematocytometer, and viability was determined by the Evans blue staining method (Asai *et al.*, 2000).

For isolating protoplasts from mature plants, rice plants were grown in a growth chamber, with cycles of 12 h light at 26 °C, 80% relative humidity, and of 12 h dark at 20 °C, 60% relative humidity, and under a light intensity (photosynthetically active radiation value) of ~180 μmol/m^2/s. Stems including sheaths of 2-month-old rice plants grown in a growth chamber were rinsed with sterile water and briefly dried and cut into approximately 0.5-mm strips. The chopped tissue was digested for 4 h with K3 enzyme medium plus 100 μg/mL ampicillin. Other procedures followed the protocol for isolation of young tissue protoplasts.

DNA constructs

Plasmids pGDG and pGDR, which contain a CaMV 35S promoter-driven *gfp* gene and *DsRed* gene, respectively, were described by Goodin *et al.* (2002).

Plasmids pGFPi and pSPL11i, which contain inverted repeats of *gfp* and *Spl11* (Zeng *et al.*, 2004), respectively, were made using the Gateway RNAi vector pANDAmini described by Miki and Shimamoto (2004). The oligonucleotide primers 5′-CACCGACCATCTTCTTCAAGGA-3′ and 5′-TTGTATAGTTCATCCATGCCATG-3′, and 5′-CACCCAGCAGCTCTCTTCAACTTGTG-3′ and 5′-CTAAATGCACAAGATGGTGCTC-3′ were designed to amplify fragments of *gfp* and *Spl11*, respectively. The amplified 426-bp *gfp* and 300-bp *Spl11* fragments were used to make the RNAi constructs following the procedure described by Miki and Shimamoto (2004).

Plasmids pPBZ1-luc and pPBZ1-gfp contain a *luc* and a *gfp* driven by the promoter of the *PBZ1* gene (Midoh and Iwata, 1996), respectively. To make the two constructs, an intermediate plasmid, pPBZ221, was derived from pBI221 (Clontech, CA) by replacing the *Hin*dIII/*Bam*HI 35S CaMV promoter fragment with a 1032-bp fragment 45 pb upstream from the *PBZ1* gene (provided by Dr Yinong Yang, Arkansas State University). pPBZ1-luc was derived from pPBZ221 by replacing the *gus* with the *luc* from a 35S-LUC construct (Tao *et al.*, 2002). PPBZ1-gfp was derived from pPBZ221 by replacing the *gus* with the *gfp* amplified from plasmid pGDG using primers 5′-AGTGGATCCATGGGTAAAGGAGAAG-3′ and 5′-TGCGAGCTCATTTGTATAGTTCATCC-3′. Plasmids pChitinaseIII-luc and pChitinaseIII-gfp contain a *luc* and a *gfp* driven by the promoter of the *chitinaseIII* gene, respectively. The 2044-bp promoter fragment 10 bp upstream from the *chitinaseIII* gene was amplified by PCR from rice cv. Nipponbare genomic DNA with primers 5′-GATTATTGAGCAGTTCCACTCCG-3′ and 5′-CTGTGATGGAGAAATTGCTTGC-3′. pChitinaseIII-luc and pChitinaseIII-gfp were derived from pPBZ1-luc and pPBZ1-gfp, respectively, by replacing the *PBZ1* promoter with the *chitinaseIII* promoter.

Split-YFP constructs pA7-NYFP, pA7-CYFP, pA7GUS-NYFP and pA7GUS-CYFP were kindly provided by Dr H. Q. Yang (Institute of Plant Physiology and Ecology, Chinese Academy of Sciences). pA7-NYFP and pA7-CYFP contain an N-terminal YFP (amino acids 1–155) coding sequence and a C-terminal YFP (amino acids 156–239) coding sequence driven by CaMV 35S promoter, respectively. pA7GUS-NYFP and pA7GUS-CYFP contain in-frame fusions of *gus* with N-terminal *YFP* and C-terminal *YFP*, respectively. The cDNA region encoding the SPIN1 protein was amplified by RT-PCR using rice cv. Nipponbare RNA with primers 5′-AGTGTCGACATGTCGGGGCTGTAC-3′ and 5′-GAGGATCCCTGGCCCGTTTTCGC-3′. The *SPIN1* cDNA was then digested with *Sal*I/*Bam*HI, and inserted into the *Sal*I/*Bam*HI sites of pA7-NYFP and pA7-CYFP to generate pA7spin1-NYFP and pA7spin1-CYFP, respectively.

All plasmid DNAs were prepared by using Qiagen Plasmid Maxi or Midi Kits (Qiagen, Valencia, CA), and dissolved in double-distilled water. DNA concentration was determined by UV spectrometry.

Protoplast transfection and fluorescence microscopy

Protoplast transfection was performed essentially as described (Tao *et al.*, 2002). The collected protoplasts were resuspended in an appropriate volume of suspension medium (0.4 M mannitol, 20 mM $CaCl_2$ and 5 mM MES, adjusted to pH 5.7 with KOH). Plasmid DNAs (about 10 μg DNA of each construct) were mixed with 200 μL of suspended protoplasts (usually 1.5–2.5 $\times 10^6$ cells/mL). The DNA and protoplasts mixture was added to 40% PEG solution [40% PEG 4000, 0.4 M mannitol and 100 mM $Ca(NO_3)_2$, adjusted to pH 7.0 with 1 M KOH] and mixed immediately by gently shaking, and then incubated for 20 min at room temperature. After incubation, 1.0 mL W5 medium was added to the tube to dilute PEG. For the experiments designed for fluorescence microscopy, the K3 medium was used instead of the W5 medium.

Fluorescence microscopy was performed under a Nikon Eclipse E600 fluorescence microscope (Nikon, Tokyo, Japan). Excitation and emission filters Ex450–490/DM510/BA520-560, and Ex540–580/DM595/BA600-660 were used for GFP and DsRed (Qi *et al.*, 2004), respectively. Images were captured with a SPOT 2 Slider charge-coupled device camera.

M. grisea elicitor extraction and treatment for protoplasts

Rice blast fungus (race 70-15) was cultured in a liquid minimum medium (0.2% yeast extract, and 1% sucrose) at 28 °C at 200 r.p.m. for 3 days. *M. grisea* cell-wall elicitor was then extracted according to the method described by Matsumura *et al.* (2003). Elicitor was added to rice protoplasts at a final concentration of either 0.5 or 1.0 mg/mL.

RT-PCR

Total RNA was isolated from the protoplasts by using the Qiagen RNeasy Plant Mini Kit (Qiagen) and treated with RNase-free DNase I (Ambion, Austin, TX) for removal of DNA contamination. One microgram of total RNA was subjected to reverse transcription using the Promega Reverse Transcription System (Promega, Madison, WI). RT-PCR was carried out under standard conditions with specific primers for *PBZ1*, *chitinaseIII*, *Spl11* and the internal control *actin1* or *ubiquitin* genes. Primer sequences were 5′-TGATGGCTCCGGCCTGCGTC-3′ and 5′-AGGGTGAGCGACGAGGTAGTC-3′ for *PBZ1*; 5′-GCAAGCAATTTCTCCATCACAG-3′ and 5′-TGCTCGTAGTCGATGTCGATGC-3′ for *chitinaseIII*; 5′-GATGCTTGCCTTATTGTCCTCA-3′ and 5′-ACGGATTGATATGCCTGACGAT-3′ for *Spl11* cDNA; 5′-CACCCAGCAGCTCTCTTCAACTTGTG-3′ and 5′-CTAAATGCACAAGATGGTGCTC-3′ for the *Spl11* RNAi fragment; 5′-CGTCTGCGATAATGGAACTGG-3′ and 5′-CTGCTGGAATGTGCTGAGAGAT-3′ for *actin1*; and 5′-AAGAAGCTGAAGCATCCAGC-3′ and 5′-CCAGGACAAGATGATCTGCC-3′ for *ubiquitin*.

Western blot assay

Protoplasts were harvested by centrifugation at 300 ***g*** for 5 min and resuspended with 2× sodium dodecyl sulphate loading dye (New England Biolabs, Ipswich MA). About 40 μg of total protein per lane was subjected to SDS-PAGE. Western blot analysis was performed using standard protocols described by Sambrook and Russell (2001). Anti-PBZ1 antibody was raised by the Umemura lab (Meiji Seika Kaisha Ltd, Health & Bioscience Laboratories, Japan) and used for detection of the PBZ1 protein level in protoplasts.

LUC and GUS assays

LUC and GUS assays essentially followed previously described methods (Tao *et al.*, 2002). Transfected protoplasts were harvested by centrifuging at 300 ***g*** for 4 min. LUC assays were performed by using the Promega Luciferase Assay System (Promega). The LUC activity in each sample was expressed relative to GUS activity to normalize data for variation in transformation efficiency and cell viability.

NOTE ADDED IN PROOF

The establishment of a novel rice protoplast system for gene silencing is also presented in the paper by Bart R., Chern, M., Park, C.J., Bartley, L. and Ronald, P.C. (2006) A novel system for gene silencing using siRNAs in rice leaf and stem-derived protoplasts, *Plant Meth.* **2**, 13.

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