A Chemically Inducible Gene Expression System and Its Application to Inducible Gene Suppression in Rice

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Abstract: The viability of the estrogen-receptor (ER)-based chemically inducible gene expression system LexA-VP16-ER (XVE) in combination with the site-specific DNA recombination Cre/loxP system in rice was examined using transgenic plants introduced with a plasmid vector pUH-GFP2 that controls the expression of a green fluorescent protein (GFP) gene. \(\beta\)-estradiol applied to the germinating seeds of the transgenic plants, successfully induced the mRNA expression of the GFP gene. Inducible gene suppression was also tested by replacing the GFP gene by an RNAi cassette; this cassette targeted \(OsSPS1\), a gene encoding sucrose phosphate synthase. When the RNAi plants were treated with the inducer, the transcript levels of \(OsSPS1\) decreased. Concomitantly, the plant length became shorter or the sucrose/starch molar ratio in the leaf blades decreased, suggesting the successful suppression of the target gene. Finally, the utility and remaining problems of this inducible expression system are discussed.

Key words: Chemically inducible expression, Gene suppression, Green fluorescent protein, \textit{Oryza sativa} L., Sucrose phosphate synthase, Transgenic plants.

Since completion of whole genome sequencing of some model plants, including rice, the primary concern of physiologists is to understand the function of each gene. Currently, technologies such as the overexpression and suppression of target genes are common practice for the elucidation of plant gene function. However, the constitutive expression of a transgene is sometimes detrimental or even lethal to the host plant. This often occurs when the gene of interest is involved in essential steps of the plant’s life. This problem may be overcome by expressing the transgenes under precise temporal and/or spatial control; this strategy is increasingly required to study gene function. Recently developed inducible gene expression systems may provide a solution to this problem (see Moore et al., 2006 for review).

The chemically inducible XVE system utilizes estrogen as the inducer, and gene expression is tightly regulated and highly inducible in tobacco and \textit{Arabidopsis} (Zuo et al., 2000). Zuo et al. (2001) subsequently developed a chemically induced site-specific DNA excision system by combining the XVE system with the site-specific DNA recombination Cre/loxP system, and they showed its validity in transgenic \textit{Arabidopsis}. Sreekala et al. (2005) modified this system for use in monocot species by replacing both the G10-90 synthetic promoter and the kanamycin resistant gene with the maize ubiquitin promoter and the hygromycin resistant gene, respectively. In these systems, estrogen can activate the transcription of the Cre DNA recombinase gene, which initiates a site-specific recombination of the transgene to induce the strong constitutive expression of the target gene (see Fig. 1 and Materials and Methods for details). The resultant plasmid pUH-GFP2 was introduced into rice calli, and the results showed for the first time that the XVE system is effective in rice. Very recently, Okuzaki et al. (2011) introduced a plasmid construct containing a gene for green fluorescent protein (GFP) regulated by the XVE system into rice calli and conducted a detailed study on the correlation between the estradiol concentration and GFP expression. However, the studies by Sreekala et al. (2005) and Okuzaki et al. (2011) both primarily dealt with induction in rice calli, and we therefore have a very limited understanding regarding gene induction in intact rice plants. In addition, the plasmid construct used by Okuzaki et al. (2011) lacks the Cre/loxP recombination component.
that confers an advantage on pUH-GFP2; this Cre/loxP component allows for successful induction to be discerned by screening for the structural change of the transgene using the polymerase chain reaction (PCR). After the induction and subsequent DNA recombination is completed, there is no need to reapply the inducer to maintain the induction in the transgenic plants. Thus, it is important to examine the inducer treatment conditions in intact rice plants transformed with the XVE system containing Cre/loxP.

Another important question regarding inducible expression systems is whether they are applicable for conditional gene suppression. Guo et al. (2003) first reported an XVE-Cre/loxP-inducible RNA interference (RNAi) system in Arabidopsis. Xu et al. (2009) recently performed a successful functional analysis of glycolate oxidase by XVE-mediated antisense suppression in rice, although they employed a plasmid that contained an XVE system but lacked the Cre/loxP component. When successful induction and recombination occur in pUH-GFP2, the target \( \text{GFP} \) gene is located downstream of the maize ubiquitin promoter; this ensures the strong constitutive expression of the target gene in monocot species (McElroy and Brettell, 1994). It is therefore important to test whether pUH-GFP2 can be utilized for inducible gene suppression by replacing the \( \text{GFP} \) gene with any RNAi cassette. In this study, we first checked if the XVE-Cre/loxP system is effective in the intact transgenic rice seedlings harboring pUH-GFP2. We next sought to induce the RNAi suppression of \( \text{OsSPS1} \), a gene encoding sucrose phosphate synthase, a key enzyme in the biosynthesis of sucrose in higher plants. Finally, we discuss the validity and remaining problems of this inducible expression system.

**Materials and Methods**

1. **Plant material and transformation**
   The japonica rice cultivar Nipponbare (Oryza sativa L. cv Nipponbare) was used throughout this study. The transformation of the rice plants was carried out following the Agrobacterium-mediated method described by Hiei et al. (1994). The transgenic plants were grown in a temperature-controlled natural-light greenhouse at the Hokuriku Research Center, Joetsu, Niigata, Japan (37°06′ N, 138°16′ E).

2. **Plasmid construction**
   A plasmid vector pUH-GFP2 developed by Sreekala et al. (2005) was kindly provided by Dr. Nam-Hai Chua, Rockefeller University. pUH-GFP2 is essentially a derivative of pX6-GFP that contains a chemical induction component called the XVE system (Zuo et al., 2001). XVE is a chimeric transcriptional activator comprised of the DNA binding domain of the bacterial repressor \( \text{LexA} \) (X), a viral transactivating domain \( \text{VP16} \) (V) and the regulatory region of the human estrogen receptor (E). In pUH-GFP2, the expression of \( \text{XVE} \) is controlled by a constitutive maize ubiquitin promoter, and the transactivating activity of the XVE protein is strictly regulated by estrogen, i.e., it is inactive without estrogen (Fig. 1). Once it is activated by estrogen, XVE can stimulate the target promoter \( \text{Olex-46} \) that consists of eight copies of the \( \text{LexA} \) binding sequence fused upstream of the ~46 CaMV 35S minimal promoter. \( \text{Olex-46} \) drives the expression of the \( \text{Cre} \) DNA recombinase derived from the P1 bacteriophage before it excises the sequence between the two \( \text{loxP} \) recognition sites. As a result, the \( \text{GFP} \) gene is located just downstream of the maize ubiquitin promoter and its expression is strongly induced. We also examined whether this inducible

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**Fig. 1.** Schematic diagram showing the structure of the T-DNA region of pUH-GFP2. Before induction, a constitutive ubiquitin promoter \( \text{Pubi} \) drives the expression of the chimeric transcriptional activator \( \text{XVE} \). When \( \text{XVE} \) is activated by the estrogen inducer, it stimulates the target promoter \( \text{Olex-46} \) to express the \( \text{Cre} \) DNA recombinase. Cre recombinase excises the region between the two \( \text{loxP} \) sites, and as a result, \( \text{Pubi} \) becomes juxtaposed to \( \text{GFP} \) to drive its constitutive expression. This DNA recombination can be monitored by genomic PCR using the primers \( \text{P1}, \text{P2}, \text{P3} \) and \( \text{P4}’ \). \( \text{KpnI} \) and \( \text{SpeI} \) restriction sites for replacement of \( \text{GFP} \) are also indicated. \( \text{hpt} \), a gene for hygromycin phosphotransferase; \( \text{Tnos} \), NOS terminator; \( \text{TrbcS} \), Arabidopsis \( \text{rbcS} \) terminator.
expression system could be applied to conditional gene suppression through RNAi. For this purpose, a 297-bp cDNA fragment of the OsSPS1 gene (Os01g0919400), which encodes the rice sucrose phosphate synthase, was amplified by PCR using the first strand cDNA from young green leaves as a template; the primer pair used is listed in Table 1. The amplified fragment was cloned into the plasmid vector pENTR D/TOPO (Life Technologies, Carlsbad, CA, USA), and sequenced to confirm that it encoded the intended sequence. The cDNA fragment was subsequently transferred into the RNAi vector pANDA (Miki and Shimamoto 2004) by the Gateway LR reaction (Life Technologies, Carlsbad, CA, USA), and the resultant RNAi cassette was excised and ligated into pUH-GFP2 to replace the GFP gene using Kpn I/Spe I restriction sites (Fig. 1).

3. Induction of the transgene by β-estradiol

β-estradiol (Sigma E8875), an inducer for the XVE system, dissolved in dimethylsulfoxide at a concentration of 100 mM was used as the stock solution that was diluted with distilled water to 10, 30, 50 or 100 μM before use.

4. Analyses of DNA and RNA

Genomic DNA was extracted from leaf segments (ca. 1.0 cm²) according to the method of Wang et al. (1993), and the DNA was used as template for the polymerase chain reaction (PCR). Multiplex PCR was performed using four PCR primers, P1, P2, P3 and P4' (Table 1). The reaction mixture (10 μL) consisted of 1 μL of template DNA, 1 μL of 10x PCR buffer, 0.2 mM each of dNTPs, 0.25 μM of each of the four primers and 0.5 units of ExTaq DNA polymerase (Takara Bio Inc., Shiga, Japan). Thermal cycling was done at 98°C for 30 s followed by 30 cycles of 98°C for 10 s, 59°C for 30 s and 72°C for 60 s.

Total RNA was extracted from leaf samples using the RNeasy Plant Mini Kit (QIAGEN K.K., Tokyo, Japan) following the manufacturer’s instructions. Ten nanograms of total RNA per reaction was subjected to real-time quantitative reverse transcription PCR using the One Step SYBR PrimeScript PLUS RT-PCR Kit (Takara Bio Inc., Shiga, Japan) and Smart Cycler (Cepheid Co., Sunnyvale, CA, USA) with the PCR primers listed in Table 1. The transcript levels of each sample were standardized against those of constitutive poly-ubiquitin gene (RUBIQ1, Wang et al., 2000; accession number U37687).

5. Determination of sugar and starch

The samples were ground with a mixer mill (MM-300, QIAGEN K.K., Tokyo, Japan) under a cryogenic condition, immediately weighed and then extracted twice with 80% ethanol at 80°C. After centrifugation at 10,000 × g for 5 min, the supernatant was dried in vacuo, dissolved in distilled water and used to assay for sucrose, glucose and fructose by the enzymatic method (Nakamura and Miyachi, 1982). The starch content was determined by resuspending the pellet in distilled water, boiling for 8 h and digesting with amyloglucosidase (Roche Diagnostics Co., Mannheim, Germany) for 20 min at 55°C; the amount of resultant glucose was determined enzymatically (Nakamura and Miyachi, 1982). The starch content was calculated as the molar concentration of glucose unit.

Results

To characterize the inducible expression of the transgene, we examined the site-specific DNA recombination and mRNA accumulation of GFP gene using the young green seedlings of the transgenic rice plants introduced with pUH-GFP2. The seeds of the transgenic plants were germinated on MS medium containing 0, 10, 30 or 50 μM of β-estradiol and grown for one week. PCR analysis was then conducted to detect the specific recombination.

| Primer   | Purpose                      | Nucleotide sequence (5’ to 3’) |
|----------|------------------------------|--------------------------------|
| P1       | Detection of DNA recombination | GGTTGGGGCGGTGGTCTGATTGTC      |
| P2       |                              | CTGTCAATTCACAGGGCCATCAGT      |
| P3       |                              | CTGGACACAGTTGGGCTGTTGCGA      |
| P4'      |                              | AGTGAAGAATGTCTTCTCCCTTACGG    |
| SPS1-L1  | RNAi suppression of OsSPS1    | ACCAGGATAGGCGCATGCG          |
| SPS1-R1  |                              | GTCATGGCCCGGGAATGG           |
| GFP-162F | Real-time PCR of GFP transcript| TGTTCCTTGCCACACCTGG         |
| GFP-422R |                              | AACCTGTGGCCGGAGGTT           |
| Rubi-L1  | Real-time PCR of Ubiquitin transcript | GGAGCTGCTGCTGTTCTTGG       |
| Rubi-R1  |                              | CACATGAAACGGCCACAGA          |
| OsSPS1-L2| Real-time PCR of OsSPS1 transcript | AGGTCAACAGCCTCAATGGTAC      |
| OsSPS1-R1|                              | GCACGCATGATCTCATCGG          |

Table 1. List of PCR primers used.
events caused by the excision of a DNA fragment flanked by two loxP sites. Before the site-specific recombination, the priming sites of the two PCR primers P1 and P4' locate about 6.0 kbp apart, which makes it difficult to amplify the corresponding fragment with the primer pair under the PCR conditions employed. Once successfully induced, the excision of the DNA fragment helps in juxtaposing the ubiquitin promoter and GFP gene, and thus the priming sites become closer to easily amplify a band of approximately 500 bp with the P1/P4' primer pair (Fig. 1). In the leaves of the transgenic plants, the P1/P4' primer pair generated a PCR product of the expected size only when the leaves were treated with β-estradiol (Fig. 2A). The P1/P2 and P3/P4' PCR products that were indicative of the non-recombinant construct were evident in all samples (Fig. 2A). Concomitantly, the transcript levels of GFP in the induced seedlings were four to eight times higher than in the uninduced seedlings. However, the transcript levels did not differ significantly among the seedlings treated with β-estradiol at any concentration examined (Fig. 2B).

To explore the applicability of the chemically inducible pUH system to conditional gene suppression, we replaced the GFP gene of pUH-GFP2 with an RNAi cassette harboring a 297-bp cDNA fragment of OsSPS1 gene; this construct was subsequently introduced into rice plants. The cDNA fragment corresponds to the linker region located between the two conserved domains within the SPS proteins, the glucosyltransferase domain and the SPP-like domain (Castleden et al., 2004). The nucleotide sequence of this linker region is not well-conserved in the members of the OsSPS gene family. The seeds of the transgenic plants were sown on the MS agar plates containing 30 μM of β-estradiol and grown for a week. In two of the three independent transgenic lines that were examined, the transcript levels of OsSPS1 in the leaf blades of the β-estradiol-treated plants were significantly less than the levels in the control plants (Fig. 3A). Concomitantly, the shoot length of the β-estradiol-treated plants was shorter than that of the control plants (Fig. 3B). In another experiment, the induced seedlings were transplanted into plastic pots filled with soil and were further grown for four weeks. During this period, 100 μM of β-estradiol containing 0.02% Tween-20 was sprayed onto the terrestrial portions of the plant parts every week (three times in total). Again, the OsSPS1 mRNA levels in the leaf blades decreased significantly in two of the three transgenic lines (Fig. 4A). In the leaf blades of the induced plants, the molar ratio of sucrose to starch decreased significantly when compared to the uninduced control plants (Fig. 4B).

Discussion

An advantage of pUH-GFP2, the chemically inducible vector used in this study, is that its successful induction can be easily verified using PCR. We confirmed this in the seedlings of the transgenic plants germinated on inducer-containing agar plates (Fig. 2A). In another trial, successful induction was confirmed by PCR also in the leaf segments incubated with the inducer (data not shown). These are the first observations that XVE-Cre/lox induction system works in intact tissues of rice plants because in the first application of this system to rice by Sreekalra et al. (1995) inducer was treated to calli before regeneration. The DNA recombination was induced by β-estradiol at concentrations as low as 10 μM, and mRNA levels of GFP did not vary with the inducer concentration, implying that the effect of inducer was saturated (Fig. 2B). In rice, Sreekala et al.
applicability of the XVE-Cre/loxP system to inducible gene suppression in rice, we replaced the GFP gene of pUH-GFP2 by an RNAi cassette to suppress the OsSPS1 gene. OsSPS1 encodes sucrose phosphate synthase (SPS; EC 3.1.3.24), a key enzyme of sucrose biosynthesis in higher plants, and has been shown to be an isoform that is preferentially expressed in source leaves among five members of the gene family (Okamura et al., 2011). When the OsSPS1-RNAi transgenic plants were treated with the inducer, some of them showed decreased transcript levels of OsSPS1 (Figs. 3 and 4). Concomitantly, either the length of the terrestrial portion of the seedling or the sucrose/starch molar ratio of the leaf blades significantly decreased (Figs. 3 and 4). Seneweera et al. (1995) reported that elongation rate of rice leaf blades positively correlates with SPS activity. In addition, Ishimaru et al. (2004) demonstrated that a qualitative trait locus for the longer plant length of arabidopsis and Tobacco. The PCR analysis indicated that the uninduced transgenes indicated by the P1/P2 or P3/ P4’ PCR product consistently existed in all samples; this suggests that the tissues were not uniformly induced and that they are a chimera of induced and uninduced cells. Zuo et al. (2000) observed a patchy-type expression of GFP in the induced transgenic Arabidopsis seedlings. It can be speculated that the accessibility of the inducer may vary from cell to cell, resulting in a patchy induction.

Chemically inducible gene suppression would be a powerful and fascinating tool that crop physiologists could use to elucidate a gene’s function. To address the applicability of the XVE-Cre/loxP system to inducible gene suppression in rice, we replaced the GFP gene of pUH-GFP2 by an RNAi cassette to suppress the OsSPS1 gene. OsSPS1 encodes sucrose phosphate synthase (SPS; EC 3.1.3.24), a key enzyme of sucrose biosynthesis in higher plants, and has been shown to be an isoform that is preferentially expressed in source leaves among five members of the gene family (Okamura et al., 2011). When the OsSPS1-RNAi transgenic plants were treated with the inducer, some of them showed decreased transcript levels of OsSPS1 (Figs. 3 and 4). Concomitantly, either the length of the terrestrial portion of the seedling or the sucrose/starch molar ratio of the leaf blades significantly decreased (Figs. 3 and 4). Seneweera et al. (1995) reported that elongation rate of rice leaf blades positively correlates with SPS activity. In addition, Ishimaru et al. (2004) demonstrated that a qualitative trait locus for the longer plant length of

Fig. 3. Effects of β-estradiol on the transcript levels of OsSPS1 in the leaf blades (A) and plant lengths (B) of the inducible RNAi transgenic plants.

The seeds of the transgenic plants were sown and cultured on MS medium containing 30 μM of β-estradiol for one week. The transcript levels of OsSPS1 are expressed relative to those of a poly-ubiquitin gene (RUBIQ1, Wang et al., 2000). Error bars indicate standard error (n=3−4). Asterisks represent a significant difference between the induced and uninduced plants (p<0.05, t-test).

Fig. 4. Effect of β-estradiol on transcript levels of OsSPS1 (A) and the molar ratio of sucrose to starch (B) in the leaf blades of the inducible RNAi transgenic plants.

The induced seedlings were grown in plastic pots for four weeks. During this period, 100 μM of β-estradiol was sprayed onto the terrestrial portions of the plants every week. The transcript levels of OsSPS1 are expressed relative to those of a poly-ubiquitin gene (RUBIQ1, Wang et al., 2000). Error bars indicate standard error (n=3−4). * and ** represent a significant difference between the induced and uninduced plants at p<0.05 and p<0.01, respectively (t-test).
rice corresponds to \textit{OsSPS1}. A change in sucrose/starch molar ratio in the leaves has been repeatedly observed to be accompanied by the alteration of SPS activity in many plant species such as tomato (Galtier et al., 1993), potato (Tobias et al., 1999) and rice (Ono et al., 1999; Takahashi et al., 2000). Therefore, our observations in the \textit{OsSPS1}-RNAi plants can be reasonably attributed to the decreased level of the \textit{OsSPS1} transcript through induced gene suppression. This study has demonstrated for the first time that these phenotypic changes were caused by altered SPS gene expression levels because the plants have an identical genetic background.

Despite the potential utilities discussed above, there remain several problems regarding the practical use of pUH-GFP2 or its derivative inducible RNAi system. First, due to its complicated structure, pUH-GFP2 composes a T-DNA of a relatively large size (9.2 kbp). The large size of a T-DNA often causes technical difficulties in various steps of the experimental process such as plasmid construction. During the course of this study, we encountered relatively poor regeneration of transgenic plants, which may also be related to the large size of the T-DNA. The former problem could be settled by employing \textit{in vitro} site-specific recombination technologies for cloning. A second problem is that induction can occur non-uniformly in a plant. This problem is important because non-uniform patchy induction may dilute the effect of the induced transgene on the whole tissue level. This may be concerned with the fact that our preliminary trial of a single shot application of inducer to uninduced seedlings resulted in limited success due to unstable induction (data not shown). To address this problem, we need to explore how the inducer reaches the target cells, of which we are hardly informed so far. More quantitative assessments of the induction efficiency are also necessary. In summary, our results suggest that the XVE-Gre/loxP chemically inducible system is effective in rice, and although some structural modifications and/or the refinement of the administration of the inducer are necessary to better improve its accessibility, this technology combined with RNAi is a promising technique for stage- or tissue-specific gene suppression.

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