Brief communication

Heat-inducible Cre-*lox* system for marker excision in transgenic rice

ABHILASHA KHATTRI[1], SOUMEN NANDY[1] and VIBHA SRIVASTAVA[1,2,*]

[1]*Department of Crop, Soil and Environmental Sciences,* [2]*Department of Horticulture, University of Arkansas, Fayetteville, AR 72701, USA*

**Corresponding author (Fax, 479-575-7465; Email, vibhas@uark.edu)*

The present study assessed the efficacy of a heat-inducible *cre* gene for conditional removal of the marker gene from a rice genome via Cre-*lox* recombination. A *cre* gene controlled by the soybean heat-shock promoter was introduced into the rice genome along with the recombination target (*lox*) construct. Cre-mediated recombination was expected to remove the marker gene and activate the promoter-less *GUS* gene. Six transgenic lines displayed well-regulated heat-inducible Cre activity in the callus. However, only one line that contained a single copy of the *cre* gene maintained this property in the regenerated plants and their progeny. Marker-free progeny were obtained from the plant that was heat-treated at the seedling stage, indicating the inheritance of the recombination 'footprint'. The presence of the 'footprint' was verified by polymerase chain reaction and Southern analysis. Therefore, the *cre* gene controlled by the soybean heat-shock promoter is an effective tool for conditional removal of the marker gene in rice.

[Khattri A, Nandy S and Srivastava V 2011 Heat-inducible Cre-*lox* system for marker excision in transgenic rice. *J. Biosci.* **36** 37–42] **DOI** 10.1007/s12038-011-9010-8

The presence of selectable marker genes in commercially applicable transgenic plants is undesirable; therefore, many regulatory agencies recommend its removal from field-grown transgenic lines. Selection marker genes such as antibiotic resistance or herbicide resistance genes perform a critical function in identifying a transformed cell, but serve no purpose upon isolation of the transformed clones. Several molecular tools for the removal of marker genes from transgenic clones have been described (Puchta 2003), including the use of site-specific recombination systems such as Cre-*lox* (Ow 2002; Gilbertson 2003; Gidoni *et al.* 2008). The Cre-*lox*-based technique is particularly attractive as it involves precise DNA excision. The principle of Cre-*lox*-mediated marker removal is based on the recombination of two *lox* sites catalysed by Cre recombinase, resulting in the deletion of the intervening DNA fragment (Hamilton and Abremski 1984). Initially, marker removal techniques involved the development of two separate plants consisting of either the *cre* gene or the *lox*-flanked marker gene. Marker removal was accomplished by crossing the two plants or by re-transformation of the marker-containing plant with the *cre* gene (Dale and Ow 1991; Russell *et al.* 1992). These initial demonstrations of Cre-mediated marker removal established the efficacy of the Cre-*lox* system in a number of plant species (Ow 2002; Gilbertson 2003). Subsequently, inducible or tissue-specific Cre-*lox* systems were developed for marker removal applications (reviewed by Gidoni *et al.* 2008). The inducible Cre-*lox* systems mainly consist of chemical- or heat-induced systems (Hoff *et al.* 2001; Zuo *et al.* 2001). The heat-inducible system is particularly attractive as it only requires exposure to high-temperature environment (42°C) to initiate recombination. Additionally, induction can be performed with the callus or seedlings. The heat-inducible Cre-*lox* system has been shown to work in *Arabidopsis*, tobacco, potato and maize (Hoff *et al.* 2001; Zhang *et al.* 2003; Liu *et al.* 2005; Wang *et al.* 2005). The present study describes the use of the heat-inducible Cre-*lox* system for marker removal from rice genome.

Two plasmid constructs were developed for rice transformation: (a) pSM24, containing a *cre* gene controlled by the promoter of soybean heat-shock gene, *HSP17.5E* (Czarnecka *et al.* 1992) and (b) pAK5, containing a *loxP*-

Keywords. Cre-*lox*; inducible promoter; marker removal; site-specific recombination; transgenic rice

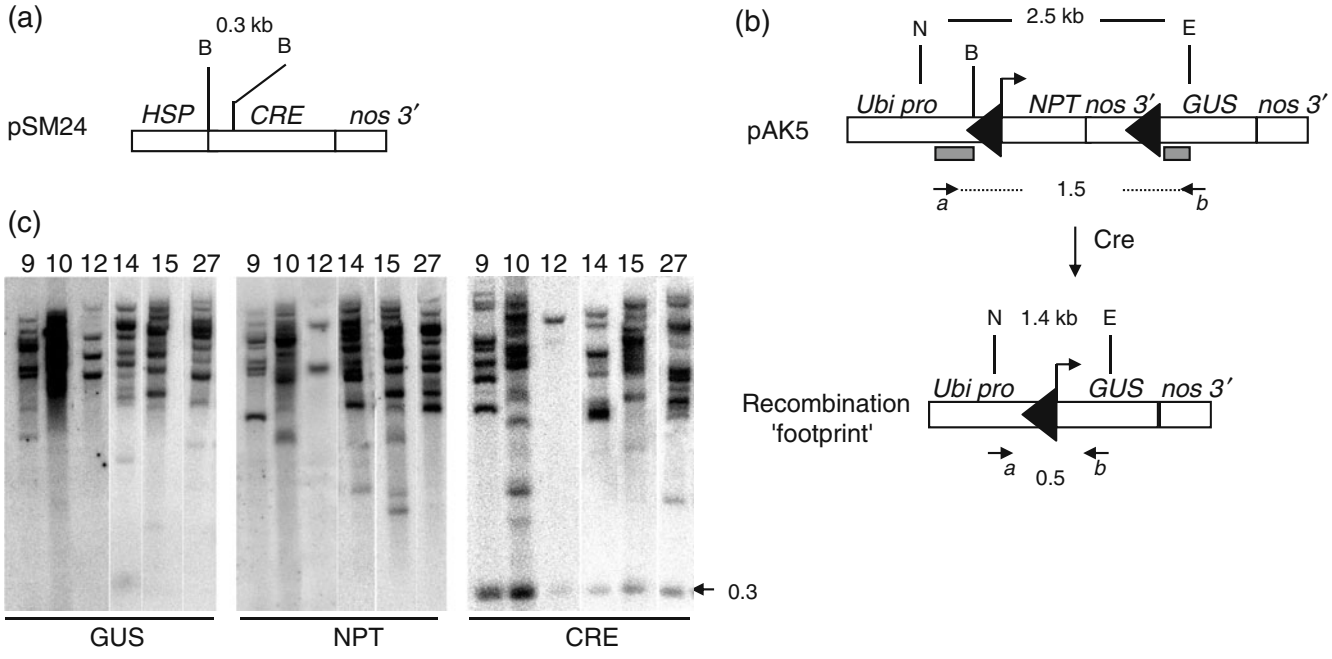

Figure 1. Plasmid constructs and Southern analysis. (**a**, **b**) Schematic diagrams of pSM24 and pAK5, and production of the recombination 'footprint' as a result of Cre-*lox* recombination, induced by HS treatment. Restriction sites, *Bam*HI (B), *Nhe*I (N), *Eco*RV (E) are shown along with the expected fragment sizes. The arrows below the construct (*a* to *b*) represent PCR primers. The expected sizes of the PCR fragments are indicated. The filled blocks below the construct represent the Ubi and GUS probes used in the Southern hybridization displayed in figure 3c. (**c**) Southern hybridization patterns of *Bam*HI-digested genomic DNA of six transgenic lines. *HSP*, soybean HS promoter; *nos 3′*, transcription terminator of nopaline synthase gene; *Ubi pro*, maize ubiquitin-1 promoter; *NPT*, neomycin phosphotransfease gene; *GUS*, promoter-less β-glucuronidase gene.

flanked neomycin phosphotransferase II (*NPT*) gene under the control of maize ubiquitin promoter (Ubi pro), and a promoter-less β-glucuronidase (*GUS*) gene (figure 1a, b). Removal of the *NPT* fragment by Cre-*lox* recombination, induced by heat-shock (HS) treatment, would place the *GUS* gene under the transcriptional control of Ubi pro (figure 1b). Thus, the *GUS* gene serves as a convenient marker for Cre-*lox* recombination. These plasmids were introduced in rice (Nipponbare cultivar) by particle bombardment of scutellar calluses. The bombarded calluses were selected on geneticin (100 mg/l) at room temperature (RT). Protocols of rice tissue culture and regeneration were derived from Nishimura *et al.* (2006). Several geneticin-resistant lines were obtained, six of which (C9, C10, C12, C14, C15 and C27) were regenerated into transgenic plants. Southern analysis confirmed the presence of both constructs in each line; however, only one line, C12, was found to contain a single copy of *cre* gene with two to three copies of pAK5 construct (figure 1c; table 1). To assess the heat-inducible Cre activity, calluses and leaf cuttings of these lines were stained for GUS activity before heat treatment (at RT) or after heat treatment by placing them at 42°C for 4 h followed by recovery at RT for 48 h (heat-shocked, HS). While each line displayed heat-inducible GUS activity in the callus cells, leaf cuttings of only line C12 showed strong staining in HS leaf samples, with some background activity in RT samples (figure 2). Thus, heat-inducible Cre activity was present in the callus cells of all six lines but not necessarily in their regenerated plants. Only C12 plants contained the heat-inducible *cre* gene, while other lines possibly contained a silenced *cre* or *GUS* gene, a charac-

Table 1. Transmittance of recombination 'footprint' to progeny

T1 plants	[a]PCR	Number of T2 progeny analysed	GUS positive	[a]PCR positives	[b]Efficiency
HS-1	+	12	0	ND	0
HS-2	+	31	22	22	94
HS-3	+	12	0	1	11
NHS-1	−	12	0	0	0
NHS-2	ND	12	0	ND	0

[a] Detection of recombination 'footprint'.

[b] Number of GUS positives/(Total × 0.75).

ND not detected.

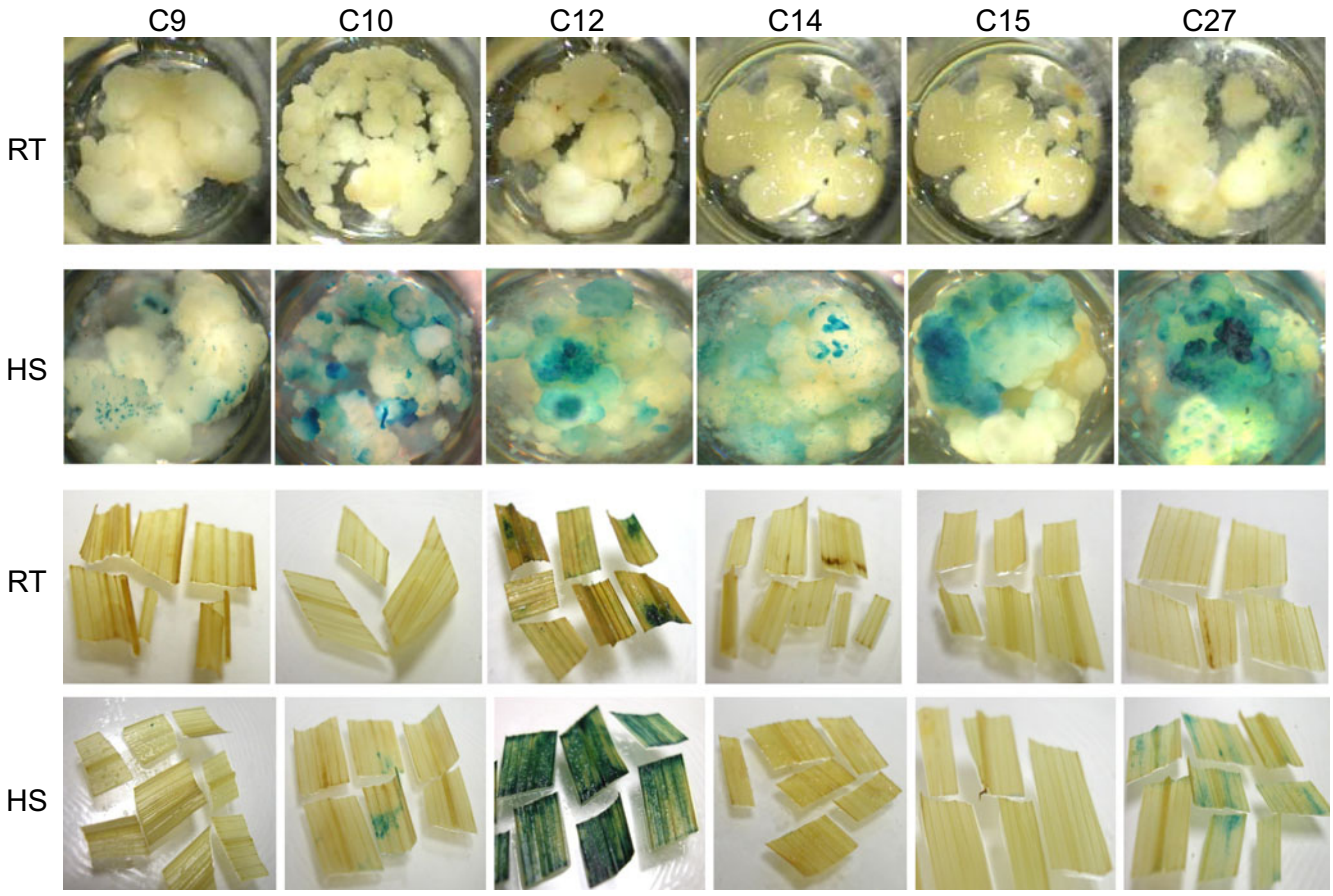

Figure 2. Histochemical staining of callus and leaves of six different transgenic lines in X-gluc solution after heat-shock (HS) treatment or at room temperature (RT), i.e. without heat-shock treatment.

teristic often associated with complex integration locus (Kumpatla and Hall 1998). Next, T1 seedlings of each line, except C9 (which did not set seeds), were assessed for heat-inducible Cre activity. Again, only C12 seedlings displayed heat-inducible Cre activity (figure 3a). For the molecular confirmation of the Cre-*lox* recombination, a few NHS (non-heat-shocked, i.e. kept at RT) and HS seedlings (kept at 42°C for 4 h) of C12 were grown in the greenhouse and analysed by PCR and Southern hybridization. The greenhouse was maintained at 25–30°C to prevent ectopic induction of the *cre* gene. After 4–5 weeks of growth, leaf cuttings of these plants were stained for GUS activity. While samples of NHS plants did not show any GUS activity, that of HS plants displayed uniform GUS activity, indicating successful Cre-*lox* recombination in the seedling stage (data not shown). PCR on genomic DNA with primers *a* (5′-TCTAACCTTGAG TACCTATCTATT-3′) and *b* (5′-AATTACGAATATCTC GATCGG-3′) (figure 1b) amplified the 0.5 kb fragment from HS T1 plants, indicating the presence of the recombination 'footprint'. The same PCR reaction on NHS plants amplified the 1.5 kb band, indicating the presence of the original locus (figure 3b). For Southern analysis, genomic DNA was digested with *Nhe*I and *Eco*RV, and hybridized with Ubi and GUS probes (figure 1b). Southern hybridization showed the expected 2.5 kb band in NHS plant, and a 1.4 kb band in HS plants as predicted for the recombination 'footprint' (figure 3c). However, C12 locus contains three copies of pAK5 constructs as per GUS hybridization (figure 1c), two of which appear to be recombination-incompetent. Therefore, the recombination 'footprint' (Ubi-*loxP*-GUS) is derived from only one of the copies, while the remaining two were rearranged upon Cre-*lox* recombination (figure 3c).

Finally, to study the inheritance of the recombination 'footprint', three different T1 seedlings were heat-shocked (HS) recurrently for 3 h each day for 3 consecutive days and planted in soil in the greenhouse. Two control seedlings (NHS), which were kept at 25°C throughout, were also transferred to the greenhouse. Each of the HS (1–3) and NHS (1–2) plants was confirmed to contain both *cre* and *GUS* genes by PCR (data not shown). The presence of the recombination 'footprint' in the HS plants was also

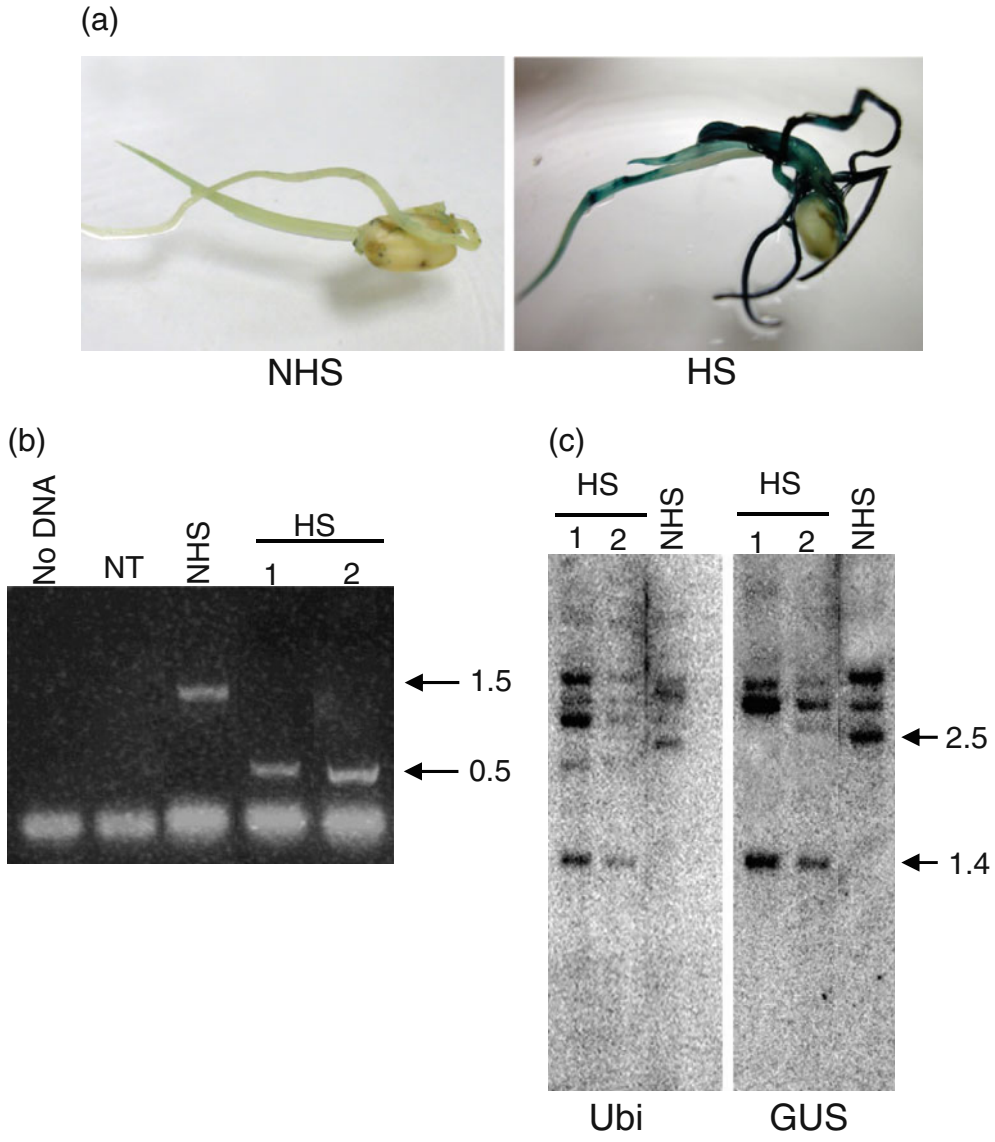

Figure 3. Heat-inducible Cre activity in T1 plants. (**a**) Heat-shocked (HS) and non-heat-shocked (NHS) T1 seedlings of line C12 displaying GUS activity as a result of Cre-mediated recombination. (**b**) PCR on genomic DNA isolated from HS and NHS T1 plants using primers *a* and *b* (figure 1a). Non-transgenic (NT) and 'no DNA' controls are also shown. (**c**) Southern hybridization on *Nhe*I and *Eco*RV double-digested genomic DNA from HS and NHS plants with Ubi and GUS probes (figure 1b). NHS plants contain the expected 2.5 kb fragment representing the pAK5 locus, and HS plants display 1.4 kb fragment representing the recombination 'footprint'.

confirmed by PCR (figure 4a; table 1). T2 progeny of these plants were tested for the presence of GUS activity and the recombination 'footprint' was tested by PCR. At least 12 T2 seedlings derived from each HS and NHS plants were analysed. Of the three HS plants, only one (HS-2) produced progeny that displayed GUS activity, indicating the inheritance of the recombination 'footprint' (table 1). The progeny of the remaining two HS plants (HS-1 and HS-3) did not display any GUS activity, indicating the lack of inheritance. As expected, T2 seedlings derived from the NHS plants were GUS-negative. The absence of GUS activity but the presence of the transgene among T2 seedlings of the two HS plants suggests that the Cre-*lox* recombination was inefficient in the gametophytic tissue of their parents. GUS-positive HS-2 T2 seedlings amplified the recombination 'footprint' in a PCR reaction (figure 4b; table 1). As the *cre* gene cannot be segregated from the target *lox* sites, it is difficult to confirm whether the GUS activity in T2 seedlings was induced *de novo* or inherited from the parent plant. However, the presence of uniform

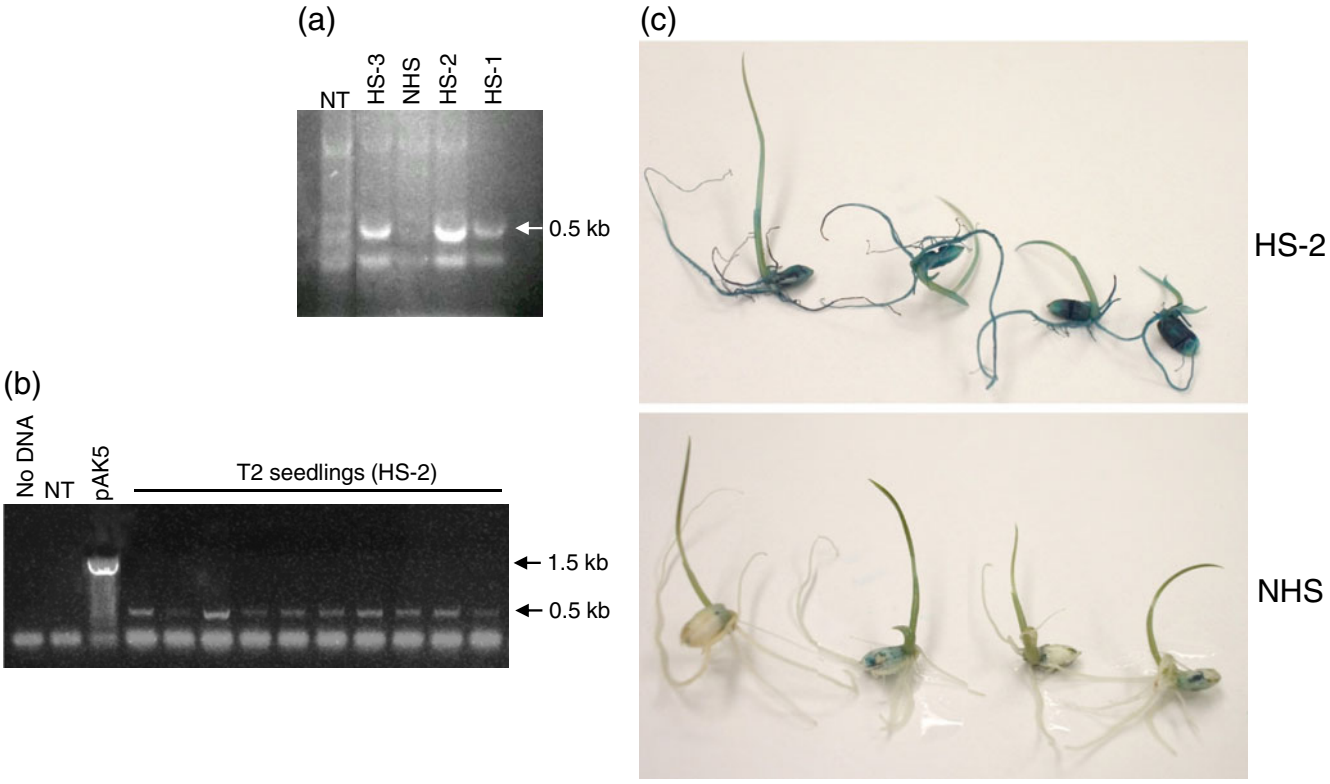

Figure 4. Transmittance of the recombination 'footprint' to T2 progeny of line C12. (**a**) PCR with primers *a* and *b* on T1 plants. HS plants display the 0.5 kb recombination 'footprint', while NHS and non-transformed (NT) do not. This PCR was carried out with 30 s extension time, which precludes the amplification of 1.5 kb fragment from the original pAK5 locus. (**b**) PCR with primers *a* and *b* on genomic DNA isolated from T2 seedlings derived from HS-2 T1 plant. Presence of the 0.5 kb fragment indicates the presence of recombination 'footprint'. (**c**) GUS staining in T2 seedlings derived from HS-2 or NHS T1 plants.

GUS activity in T2 seedlings, and the absence of GUS activity in the control NHS plants and also in the two HS plants, suggests that GUS activity originated from an inherited locus rather than from *de novo* somatic recombination (figure 4c). As HS-2 plant was hemizygous for transgene locus, only 75% of the progeny would inherit the transgene; therefore, the overall efficiency of the marker removal in HS-2 plant was calculated to be ~94% (table 1).

Thus, the present study confirmed the effectiveness of the soybean *HSP17.5E* promoter in rice. Successful marker excision and inheritance of the excised locus was observed in a single line that contained a single copy of the *cre* gene. By virtue of the simple locus structure, this single-copy C12 line displayed a well-regulated *cre* expression, while complex lines succumbed to gene silencing. A previous study validated the use of *HSP17.5E* promoter in maize (Zhang *et al.* 2003) by demonstrating marker excision mediated by the heat-shock *cre* gene. Zhang *et al.* reported high-efficiency marker excision in maize callus lines subjected to heat treatment. The regenerated plants and their progeny were also marker-free, indicating the efficacy of the heat-shock promoter and stability of the recombination 'footprint' in the germline. The soybean-*HSP17.5E*-controlled Cre-*lox* system was also successfully used for removing the marker gene from tobacco plants, which transmitted the recombination product to progeny with high efficiency (Wang *et al.* 2005). Similarly, *Arabidopsis* heat-shock promoter, *HSP81-1*, was successfully utilized to excise marker genes from *Arabidopsis* and tobacco (Hoff *et al.* 2001; Liu *et al.* 2005). While heritability of the recombinant locus in these studies was not analysed, excision efficiency based on activation of *GUS* gene was found to be high in a few selected lines. In another study carried out on potato, the heat-inducible Cre-*lox* system was based on the use of a promoter derived from *Drosophila* heat-shock gene, *HSP70* (Cuellar *et al.* 2006). As *HSP70* promoter activity is weak in potato, excision efficiency was relatively lower; however, fully excised, marker-free clones were recovered at efficiencies ranging from 0% to 14%. Thus, inducible Cre-*lox* systems can serve as a valuable tool for plant biotechnology. The functionality of Cre-*lox* for marker

excision has previously been validated in rice (Moore and Srivastava 2005; Hoa *et al.* 2002), including that of a chemical (β-estradiol)-inducible Cre-*lox* (Sreekala *et al.* 2005). Each of these systems was effective in generating marker-free transgenic line. The heat-inducible system described here serves as an alternative Cre-*lox* system that would be useful for rice biotechnology.

Acknowledgements

This work was supported by the USDA-CSREES grant (BRAG 2006-33120-17718), and the ABI-Arkansas Division of Agriculture grant to VS. We thank Dr Yinong Yang and Dr David Ow for the gift of *HSP* promoter and *cre* gene, respectively.

References

Cuellar W, Gaudin A, Solorzano D, Casa A, Nopo L, Chudalayandi P, Medrano G, Kreuze J and Ghislain M 2006 Self-excision of the antibiotic resistance gene *nptII* using a heat inducible Cre-*loxP* system from transgenic potato. *Plant Mol. Biol*. **62** 71–82

Czarnecka E, Ingersoll JC and Gurley WB 1992 AT-rich promoter elements of soybean heat shock gene Gmhsp17.5E bind two distinct sets of nuclear proteins *in vitro*. *Plant Mol. Biol*. **19** 985–1000

Dale EC and Ow DW 1991 Gene transfer with subsequent removal of the selection gene from the host genome. *Proc. Natl. Acad. Sci. USA* **88** 10558–10562

Gidoni D, Srivastava V and Carmi N 2008 Site-specific excisional recombination strategies for elimination of undesirable transgenes from crop plants. *In Vitro Cell. Dev. Biol. Plant* **44** 457–467

Gilbertson L 2003 Cre-*lox* recombination: Creative tools for plant biotechnology. *Trends Biotechnol.* **21** 550–555

Hamilton D and Abremski K 1984 Site-specific recombination by the bacteriophage P1 *lox*-Cre system: Cre-mediated synapsis of two *lox* sites. *J. Mol. Biol.* **178** 481–486

Hoa TTC, Bong BB, Huq E and Hodges TK 2002 Cre/*lox* site-specific recombination controls the excision of a transgene from the rice genome. *Theor. Appl. Genet*. **104** 518–525

Hoff T, Schnorr KM and Mundy J 2001 A recombinase-mediated transcriptional induction system in transgenic plants. *Plant Mol. Biol.* **45** 41–49

Kumpatla SP and Hall TC 1998 Recurrent onset of epigenetic silencing in rice harboring a multi-copy transgene. *Plant J.* **14** 129–135

Liu H-K, Yang C and Wei Z-H 2005 Heat shock-regulated site-specific excision of extraneous DNA in transgenic plants. *Plant Sci.* **168** 997–1003

Moore SK and Srivastava V 2005 Efficient deletion of transgenic DNA from complex integration locus of rice mediated by Cre/*lox* recombination system. *Crop Sci.* **46** 700–705

Nishimura A, Aichi I and Matsuoka M 2006 A protocol for *Agrobacterium*-mediated transformation in rice. *Nat. Protocol* **1** 2796–2802

Ow DW 2002 Recombinase-directed plant transformation for the post-genomic era. *Plant Mol. Biol.* **48** 183–200

Puchta H 2003 Towards the ideal GMP: homologous recombination and marker gene excision. *J. Plant Physiol.* **160** 743–754

Russell SH, Hoopes JL and Odell JT 1992 Directed excision of a transgene from the plant genome. *Mol. Gen. Genet*. **234** 49–59

Sreekala C, Wu L, Gu K, Wang D, Tian D and Yin Z 2005 Excision of a selectable marker in transgenic rice (*Oryza sativa* L.) using a chemically regulated Cre/*loxP* system. *Plant Cell Rep.* **24** 86–94

Wang Y, Chen B, Hu Y, Li J and Lin Z 2005 Inducible excision of selectable marker gene from transgenic plants by the Cre/*lox* site-specific recombination system. *Transgenic Res.* **14** 605–614

Zhang W, Subbarao S, Addae P, Shen A, Armstrong C, Peschke V and Gilbertson L 2003 Cre/*lox*-mediated marker gene excision in transgenic maize (*Zea mays* L.) plants. *Theor. Appl. Genet.* **107** 1157–1168

Zuo J, Niu QW, Moller SG and Chua NH 2001 Chemical-regulated, site-specific DNA excision in transgenic plants. *Nat. Biotechnol.* **19** 157–161

MS received 03 September 2010; accepted 06 December 2010

*e*Publication: 14 March 2011

Corresponding editor: IMRAN SIDDIQI