Expression of the *Arabidopsis thaliana* Histone Gene *AtHTA1* Enhances Rice Transformation Efficiency

Ye Zheng[a,2], Xiao-Wei He[a,2], Ying-Hui Ying[a], Jiang-Feng Lu[a], Stanton B. Gelvin[b] and **Hui-Xia Shou[a,1]**

a State Key Laboratory of Plant Physiology and Biochemistry, College of Life Sciences, Zhejiang University, Yuhangtanghe Road 368, Hangzhou 310058, China
b Department of Biological Sciences, Purdue University, West Lafayette, IN 47907-1392, USA

ABSTRACT We expressed the *Arabidopsis thaliana* histone *AtHTA1* in rice under the control of the maize ubiquitin promoter. Transformation efficiencies of rice plants that constitutively expressed *AtHTA1* were 28–44% higher than calli containing an empty vector control. Furthermore, co-infection of rice calli with a vector containing *AtHTA1* and another vector with the target gene increased transformation by 27–50%. Thus, expression of *AtHTA1* either transiently or in stably transformed cells improved rice transformation efficiency.

Key words: *Arabidopsis*; histone H2A; *Agrobacterium*; rice; transformation.

INTRODUCTION

Plant genetic transformation is an important tool for both fundamental research and agricultural biotechnology. The advantages of *Agrobacterium*-mediated transformation over other methodologies include convenience of manipulation, high transformation efficiency, low copy number of integrated transgenes and a relatively simple integration pattern. The efficiency of *Agrobacterium*-mediated plant transformation depends on the success of the plant–*Agrobacterium* interaction, which involves numerous genes from both *Agrobacterium* and plants (Gelvin, 2000). Increasing the copy number of *virG* or using a mutant version of the gene in *Agrobacterium* increased transformation efficiency (Hansen et al., 1994; Liu et al., 1992). Super-binary vectors that carry extra copies of a portion of *virB*, *virC*, and *virG* have been used to transform recalcitrant genotypes of rice and maize with high efficiency (Hiei et al., 1994; Ishida et al., 1996). An alternative strategy is to target plant genes involved in the transformation process (Gelvin, 2000, 2003a, 2003b; Hwang and Gelvin, 2004). Using a forward genetic screen in *Arabidopsis*, mutants resistant to *Agrobacterium*-mediated transformation (*rat* mutants) were identified (Zhu et al., 2003). Genes identified in this screen were involved in the processes of bacterial attachment, T-DNA and virulence protein transfer, cytoplasmic trafficking, nuclear targeting of the T-complex and T-DNA integration (Mysore et al., 2000; Zhu et al., 2003). The *Arabidopsis* histone H2A gene *HTA1* (*RAT5*) is one of those genes that are involved in T-DNA integration into the plant genome (Mysore et al., 2000; Zhu et al., 2003). Overexpression of *AtHTA1* gene increased *Agrobacterium* transformation efficiency in wild-type *Arabidopsis* plants, and also restored transformation competence to the *rat5* mutant (Mysore et al., 2000; Yi et al., 2006). Besides overexpression of other histone H2A and H4 genes, overexpression of the VirB2 interacting protein BTI1 (Hwang and Gelvin, 2004), and VIP1 (Gaspar et al., 2004; Hwang and Gelvin, 2004; Tzfira et al., 2002) resulted in increased transformation susceptibility of *Arabidopsis* and tobacco.

Despite intensive efforts, many agronomically important plant species or specialized genotypes of these species remain recalcitrant to this method of transformation. In this study, we show that both stable and transient overexpression of the *Arabidopsis HTA1* gene in rice improve rice transformation frequency. The approach of co-infection by two *Agrobacterium* strains is applicable to improve the transformation of recalcitrant plant species or genotypes.

[1] To whom correspondence should be addressed. E-mail: huixia@zju.edu.cn, fax 86-0571-88206133, tel. 86-0571-88206146.

[2] These authors contributed equally to this work.

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RESULTS

Generation of *HTA1* Overexpressing Transgenic Rice and Calli

To investigate the effect of *HTA1* on rice transformation, the *Arabidopsis HTA1* gene (At5g54640) (Yi et al., 2006) was amplified by RT–PCR and ligated to the 3′ end of a maize ubiquitin promoter (Christensen et al., 1992). The *HTA1* expression cassette was inserted into the T-DNA binary vector pTF101.1 (Paz et al., 2004), resulting in the vector pUbi–*HTA1* (Figure 1A). pTF101.1 was used as the vector control. Both pUbi–*HTA1* and pTF101.1 were introduced into the rice *Japonica* variety Nibbonbare using *A. tumefaciens* EHA101. Transgenic plants ectopically expressing *AtHTA1* or its empty vector control were obtained by *Agrobacterium*-mediated rice transformation (Chen et al., 2003; Nishimura et al., 2007). The putative transgenic plants were confirmed by a leaf-spray of 100 m L^{-1} Liberty® (Aventis, Strasbourg, France; Figure 1B).

A DNA blot assay was performed on T_1 seedlings of eight transgenic events, TR1–TR8, to confirm the integration of the transgene (Figure 1C). Five micrograms of genomic DNA of events TR1–TR8 were digested with the restriction endonuclease *Eco*RI and hybridized with a *bar* gene probe (Figure 1A). The results showed that all eight events are independent and contain the transgene. Semi-quantitative RT–PCR analysis revealed that the *AtHTA1* gene was highly expressed in all eight transgenic lines (Figure 1C). Compared to wild-type and pTF101.1 transgenic plants, T_1 plants from all eight pUbi–*HTA1* lines showed no notable differences in plant growth and development, suggesting that *HTA1* has no significant negative effect on plant growth and development (Supplemental Figure 1).

To generate *HTA1* transgenic calli, callus initiation medium containing 2 mg L^{-1} bialaphos was used to induce calli from mature T_1 seeds of two of both the pTF101.1 and pUbi–*HTA1* (TR1 and TR3) independent events. Using this concentration of bialaphos, seeds not expressing the *bar* gene cannot initiate calli, whereas ones containing the *bar* gene expression cassette can initiate calli (Figure 1D). The segregation ratio of T_1 seeds forming bialaphos-resistant calli to ones not able to form resistant calli followed Mendelian segregation ratios in all tested lines (data not shown). DNA samples from the induced calli grown on bialaphos-containing medium were extracted and PCR analysis was conducted to ensure the presence of the *bar* and *HTA1* genes. All induced calli contain the *bar* and *HTA1* genes.

Effect of Stable Expression of *HTA1* on Rice Transformation Efficiency

To evaluate the effect of expression of *HTA1* on the efficiency of *Agrobaterium* re-transformation, 20 calli (in duplicate) from each of the re-transformed lines were stained for GUS activity. The level of GUS expression was divided into four groups (Figure 2). The results showed that overall GUS expression in the pUbi–*HTA1* calli was stronger than that in pTF101.1 calli

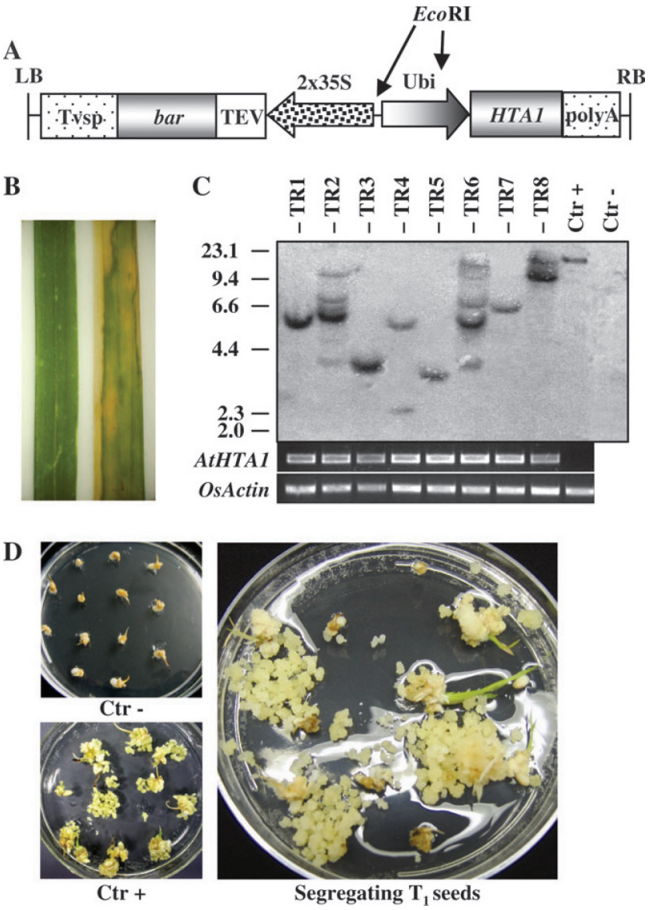

Figure 1. Expression of the *Arabidopsis HTA1* Gene in Rice.

(A) T-DNA region of the binary vector pUbi–*HTA1*. LB, left border; RB, right border; *bar*, phosphinothricin acetyltransferase gene; 2 × 35S, CaMV double 35S promoter; TEV, tobacco etch virus translational enhancer; Tvsp, soybean vegetative storage protein terminator; Ubi, maize ubiquitin promoter; *HTA1*, *Arabidopsis HTA1* gene; polyA, T-DNA polyadenylation site.

(B) Leaves after spraying with the herbicide glufosinate. Leaf from the *bar*-expressing plantlet on the left (resistant) remains alive, whereas a leaf from the non-expressing plant on the right (sensitive) turned yellow.

(C) DNA blot and reverse transcription PCR analyses of the pUbi–*HTA1* transgenic events. Five micrograms of genomic DNA were digested with *Eco*R I and hybridized with a *bar* gene probe. Ctr–, negative control, genomic DNA from non-transformed rice variety Nipponbare; Ctr+, one copy of positive control, non-transgenic rice genomic DNA spiked with 2 pg of pUbi–*HTA1* plasmid DNA digested with *Eco*R I. TR1–TR8 are eight independent transgenic lines overexpressing *AtHTA1*. The bottom of the figure shows the RT–PCR results of *AtHTA1* in pUbi–*HTA1* transgenic events. Samples, from left to right, are TR1–TR8, and the WT cDNA control. *OsActin* was used as the internal standard.

(D) Calli initiated on callus induction medium containing 2 mg L^{-1} bialaphos. Left top, callus induction of the negative seeds; left bottom, callus induction of homogenous transgenic seeds with *bar* gene; right, segregation of *bar* gene among T_1 seeds derived from T_0 plants.

(Figure 2). The percentage of the moderate and high expressers was 53.8% in pUbi–*HTA1* transgenic calli, which was 10% higher than that of pTF101.1 transgenic calli (Figure 2). About a quarter of the assessed pTF101.1 calli were non-expressers, which was about 8% higher than that of the pUbi–*HTA1* calli. Due to the small sample size for the assay of transient expression, the experimental error was relatively high. Thus, we conducted a stable transformation experiment to verify these conclusions.

To test the effect of *HTA1* on stable transformation, calli induced from pUbi–*HTA1* and pTF101.1 transgenic seeds were used for a standard rice transformation procedure. Beginning 4 weeks after infection, hygromycin-resistant callus events were identified and histochemically analyzed for stable expression of the *gusA* gene (Supplemental Figure 2A and 2B). The number of hygromycin-resistant calli generated from re-transformation of the two pUbi–*HTA1* independent transgenic lines (Table 1 and Supplemental Figure 2C, right) was significantly higher than that from pTF101.1-containing lines (Supplemental Figure 2C, left). When using a 'standard' density of *Agrobacterium* cells (10^7 cfu ml^{-1}; A_{600} = 0.01) for inoculation, the average re-transformation efficiency for pUbi–*HTA1* calli was 73.5%, which is significantly higher than that of the pTF101.1 calli (57.0%, Table 1). When the density of *Agrobacterium* cells was diluted to 10^6 cfu ml^{-1} (A_{600} = 0.001), the transformation efficiency of pTF101.1 calli was reduced to 49.5% (Table 1 and Supplemental Figure 2D), which is 7.5% lower than that using the standard inoculum density. This dilution of *Agrobacterium* had little impact on the transformation efficiency of pUbi–*HTA1* calli (Table 1 and Supplemental Figure 2D, right). The transformation efficiency we calculated refers to the number of co-cultured calli that can yield hygromycin-resistant calli over the total number of co-cultured callus pieces.

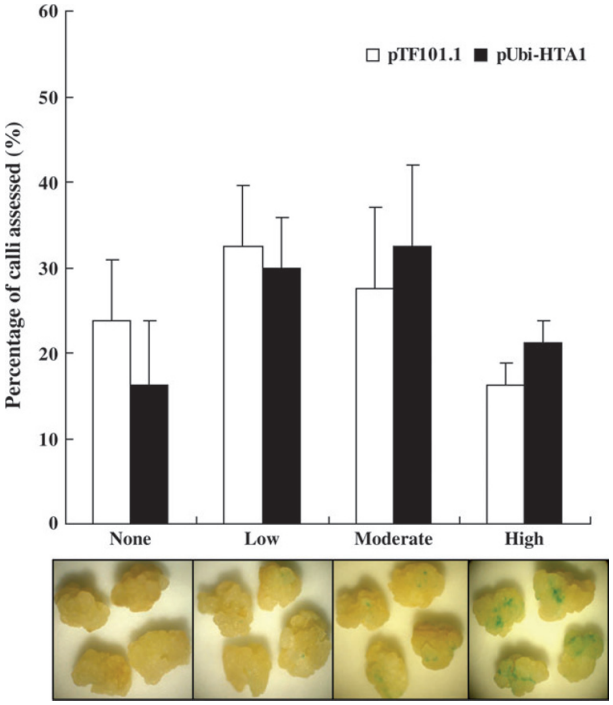

Figure 2. Transient Expression of the *gusA* Gene Following Re-Transformation of pTF101.1 and pUbi–*HTA1* Transgenic Plants with pC1301.

GUS staining of calli categorized into different groups. None, no blue foci in the assayed calli; low expresser, 1–10 blue foci in the assayed calli; moderate expresser, 10–25 blue foci in the assayed calli; high expresser, >25 foci in the assayed calli.

Effect of Transient *HTA1* Expression on the Transformation Efficiency of Rice

To test whether transient expression of the *HTA1* gene can facilitate *Agrobacterium* infection, transformation experiments were conduced by co-inoculation of wild-type rice calli with two different *Agrobacterium* strains. One strain carried the target vector pC1301 with the *gusA* gene, and the other contained either the pUbi–*HTA1* vector or pTF101.1 as a 'helper'. One hundred calli were used for each treatment, and the experiment was duplicated. The two *Agrobacterium* strains were mixed at a 1:1 ratio with each strain at a cell density of 10^7 cfu ml^{-1}. As shown in Figure 3, infection by *A. tumefaciens* carrying pC1301 alone resulted in 14.5% of the calli classified as high expressers, whereas co-infection with pTF101.1 reduced the percentage of high expressers to 10.0%. Co-infection with pUbi–*HTA1* significantly increased the proportion of high expressers of transient GUS activity to 24.5%. Meanwhile, the percentage of non-expressers was decreased from 42.5 to 29.0% by co-transformation with the pUbi–*HTA1* helper vector.

Table 1. Effect of *HTA1* Expression on Rice Transformation Efficiency.

Bacterial density*	Calli initiated from	No. of calli infected	No. of events expressing the *gusA* gene	Hygromycin-resistant calli produced from re-transformation(%)**
10^7 cfu ml^{-1}	pTF101.1 transformants	200	114	57.0 ± 2.12%[a]
	pUbi–*HTA1* transformants	200	147	73.5 ± 4.60%[b]
10^6 cfu ml^{-1}	pTF101.1 transformants	400	198	50.0 ± 7.33%[a]
	pUbi–*HTA1* transformants	400	288	72.0 ± 7.02%[b]

* Cell density of *A. tumefaciens* used for infection. 10^7 cfu ml^{-1} is equivalent to A_{600} = 0.01; 10^6 cfu ml^{-1} is equivalent to A_{600} = 0.001.
** Mean ± STD; different letters of superscripts indicate significant differences between two transgenic constructs ($p < 0.01$) according to the student's *t*-test.

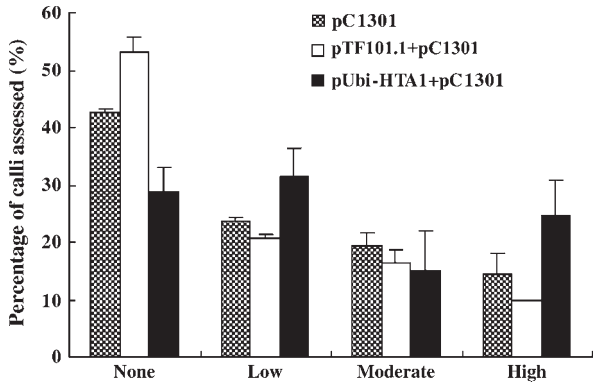

Figure 3. Co-Inoculation of Rice Calli with Two *A. tumefaciens* Strains.

Transient expression of the *gusA* gene after co-infection with two *A. tumefaciens* strains containing pC1301 plus pTF101.1, and pC1301 plus pUbi–*HTA1*.

To confirm these results, stable transformation experiments were carried out by co-infection of wild-type rice calli with either pC1301 plus pUbi–*HTA1* or with pC1301 plus pTF101.1. When each bacterial strain was set at 10^7 cfu ml^{-1}, the stable transformation efficiencies of pC1301 and pC1301 + pTF101.1 were 56 and 51.5%, respectively (Table 2 and Supplemental Figure 3), which were significantly lower than that using pC1301 + pUbi–*HTA1* (71.3%, Table 2). Dilution of *Agrobacterium* cells to 10^6 and 5×10^5 cfu ml^{-1} reduced the transformation efficiency of pC1301 alone to 48.9 and 38.7%, respectively. The corresponding transformation efficiencies using co-infection with the empty vector pTF101.1 were 43.5 and 38.7%, respectively. When pUbi–*HTA1* was used as the helper vector, the transformation efficiencies increased to 72.0 and 52.7%, respectively (Table 2 and Supplemental Figure 3).

DISCUSSION

Manipulation of expression of genes involved in the transformation process could improve *Agrobacterium*-mediated plant transformation efficiency by increasing the virulence of the *Agrobacterium* strain or the susceptibility of the plants' cells (Gelvin, 2000, 2003b; Hwang and Gelvin, 2004). We show that ectopic expression of the *Arabidopsis HTA1* gene in rice significantly increases the re-transformation frequency. When using low inoculum concentrations of *A. tumefaciens*, the effect of *HTA1* expression is greater (Table 1). Furthermore, expression of *HTA1* in transgenic calli improved transient GUS expression following re-transformation by an *A. tumefaciens* strain containing a *gusA*-intron gene within the T-DNA, confirming the role for *HTA1* on the improvement of re-transformation by increasing the competence of plant cells to *Agrobacterium*-mediated transformation. Thus, *AtHTA1* is effective in increasing the frequency of transformation in *Arabidopsis* (Yi et al., 2002) and rice.

Table 2. Effect of *HTA1* on Rice Co-Transformation Efficiency.

Bacterial density*	Vectors for co-transformation	No. of calli infected	No. of callus events expressing the *gusA* gene**
10^7 cfu ml^{-1}	pC1301	200	56.0 ± 3.2^a
	pC1301 + pTF101.1	200	51.5 ± 2.8^a
	pC1301 + Ubi–HTA1	200	71.3 ± 2.7^b
10^6 cfu ml^{-1}	pC1301	200	48.0 ± 2.8^a
	pC1301 + pTF101.1	200	43.5 ± 2.1^a
	pC1301 + Ubi–HTA1	200	72.0 ± 7.1^b
5×10^5 cfu ml^{-1}	pC1301	300	38.7 ± 5.0^a
	pC1301 + pTF101.1	300	38.7 ± 4.2^a
	pC1301 + Ubi–HTA1	300	52.7 ± 5.1^b

* Cell density of *A. tumefaciens* used for infection. 10^7 cfu ml^{-1} is equivalent to A_{600} = 0.01; 10^6 cfu ml^{-1} is equivalent to A_{600} = 0.001; 5×10^5 cfu ml^{-1} is equivalent to A_{600} = 0.0005.
** Mean ± STD; different letters of superscripts indicate significant differences between two transgenic constructs ($p < 0.01$) according to the student's *t*-test.

It can be difficult to generate transgenic plants from many species or genotypes that are recalcitrant to *Agrobacterium*-mediated transformation. Thus, even if ectopic overexpression of the *HTA1* gene could improve re-transformation efficiency, the application of this methodology may be difficult. In this study, we showed that using a *HTA1* overexpression vector as a 'helper' to co-infect plant cells with the target vector could improve rice transformation both transiently and stably. This technique has great potential for establishment and development of transformation systems in many recalcitrant plant species or genotypes.

METHODS

Construction of Binary Vectors and Rice Transformation

The *Arabidopsis* histone *HTA1* cDNA was amplified by RT–PCR as previously described (Yi et al., 2006). It was then excised from the cloning plasmid using *Pst*I and *Xba*I and ligated into the multiple cloning site of the binary vector pTF101.1 (Paz et al., 2004). A maize ubiquitin promoter (Christensen et al., 1992) and a polyadenylation signal sequence were fused onto the 5′ and 3′ ends of the *AtHTA1* cDNA, respectively. The resulting construct was designated as pUbi–*HTA1* (Figure 1A) and introduced into *A. tumefaciens* EHA101 (Hood et al., 1986) for rice transformation. Rice transformation was conducted as described (Chen et al., 2003; Nishimura et al., 2007). Bialaphos (Meiji Seika Kaisha Ltd, Tokyo, JP) was used at a concentration of 2 mg L^{-1} for selection of transformed calli.

For the re-transformation experiment, T_1 seeds harvested from T_0 pUbi–*HTA1* or pTF101.1 transgenic plants were placed onto callus-inducing medium containing 2 mg L^{-1} bialaphos to select transgenic calli. Bialaphos-resistant calli were re-transformed by *A. tumefaciens* EHA105 (Hood et al., 1993)

carrying the T-DNA binary vector pCAMBIA1301 (pC1301, CAMBIA, Canberra, Australia), which contains a *gusA* gene with an intron in its coding region (Vancanneyt et al., 1990) to prevent GUS activity in *A. tumefaciens* cells. The A_{600} of the *Agrobacterium* suspension was adjusted to 0.01 (10^7 cfu ml^{-1}) for the standard transformation. Ten-fold dilutions of the *Agrobacterium* suspension were made to evaluate the transformation efficiency. Re-transformed resistant calli were generated from hygromycin selection, 50 and 80 mg L^{-1}, each for 2 weeks.

In co-infection experiments, the binary vector pC1301 was used as the plant selection vector for transformation. pUbi–*HTA1* served as the 'helper' vector and pTF101.1 as an empty vector control. Three treatments were set for *Agrobacterium* inoculation, consisting of: (1) 100% EHA105/pC1301, (2) a mixture of 50% EHA105/pC1301 and 50% EHA101/pUbi–*HTA1*, and (3) a mixture of 50% EHA105/pC1301 and 50% EHA101/pTF101.1.

A glufosinate leaf-spray test was used to screen for putative transgenic plants. The herbicide Liberty® (Aventis, Strasbourg, France) at a final concentration of 100 mg L^{-1} was sprayed on T_0 regenerated rice plants. Three days later, plants expressing the *bar* gene cassette remained alive whereas plants lacking the *bar* gene turned yellow.

RT–PCR Analysis

Total RNA was extracted from leaves using TRIzol Reagent (Invitrogen, CA, USA) according to the manufacturer's recommendations. First-strand cDNA was synthesized from total RNA using SuperScript II reverse transcriptase and served as a template for RT–PCR. The rice actin gene, *OsActin*, was used as an internal standard. The primer sequences were as follows: *HTA1* forward, 5′-TGGTCGTGGAAAAACTCTTG-3′; *HTA1* reverse, 5′-TTGCTTAGCTCCTCATCGTT-3′; *OsActin* forward, 5′-GCGATAATGGAACTGGTATG-3′; *OsActin* reverse, 5′-CTCCATTTCCTGGTCATAGTC-3′. Amplification was performed as follows: one cycle of denaturation at 94°C for 5 min; 28 cycles for *OsActin* and 30 cycles for *HTA1* of denaturation at 94°C for 30 s, annealing at 58°C for 30 s, and extension at 72°C for 30 s; one cycle of elongation at 72°C for 10 min.

DNA-Blot Analysis of Transgenic Plants

Genomic DNA was isolated using the CTAB method (Murray and Thompson, 1980) and was digested with the restriction endonuclease *Eco*RI. Five micrograms of total genomic DNA was separated electrophoresis through 0.8% agarose gels. After that, the digested DNA was transferred to Hybond-N1 nylon membrane (Amersham Pharmacia) and hybridized with a [^{32}P]dCTP-labeled *bar* gene probe. The blots were washed three times at 65°C. The three washing buffers contain 0.1% SDS and 2, 1, or 0.1 SSC, respectively. Then the blot was exposed to a phosphorimager for 48 h and subsequently scanned with a variable mode imager (Typhoon 8600, Molecular Dynamics, Sunnyvale, CA, USA).

Histochemical Analysis of GUS Activity

Histochemical GUS assays (Jefferson, 1987) were used to assess transient (3 days after infection) and stable expression of the *gusA* gene in calli or plants. The level of transient GUS expression was assessed on a per callus basis according to Frame et al. (2002) with different grouping criteria. Infected calli were categorized into groups as shown in Figure 2: non-expresser (no blue foci), low expresser (1–10 blue foci), moderate expresser (10–25 blue foci), or high expresser (>25 blue foci). The number of calli in each of these groups was compared with the total number of calli assessed to determine the percent of total calli in each of the expression categories.

Histochemical GUS assays were also used to assess stable expression of the *gusA* gene in bialaphos-resistant calli and in leaf tissue of transgenic plants. Samples were submerged in the substrate and incubated at 37°C overnight.

SUPPLEMENTARY DATA

Supplementary Data are available at *Molecular Plant Online.*

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