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S. Takumi · K. Murai · N. Mori · C. Nakamura

# *Trans*-activation of a maize *Ds* transposable element in transgenic wheat plants expressing the *Ac* transposase gene

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**Abstract** To develop a transposon tagging system in wheat (*Triticum aestivum* L.), transgenic wheat lines containing a transposase gene of the maize *Activator* (*Ac*) element were produced and characterized. The *Ac* transposase gene under the control of a cauliflower mosaic virus 35S promoter was introduced into cultured wheat embryos by particle bombardment. Several transgenic wheat plants expressing the transposase gene were independently recovered. Southern- and Northern-blot analyses of their progeny showed that the expression of the *Ac* transposase gene was stably inherited, and three fixed Ac lines were established. By RT-PCR analysis, products from fully spliced transcripts of the *Ac* element were confirmed. Cultured embryos isolated from the stable *Ac* lines were further bombarded with plasmids having a maize *Dissociation* (*Ds*) element located between a rice *Act1* promoter and a $\beta$-glucuronidase (*gus*) gene, and transient *gus* expression was observed after the *Ds* excision. These findings suggest that the maize *Ac* transposase gene is precisely processed and an active transposase protein is synthesized in the transgenic *Ac* lines. The *Ds* element is *trans*-activated and excised in wheat cells by the action of the *Ac* transposase gene.

**Key words** *Ac*/*Ds* transposable elements · Transgenic wheat · *Ac* expression · *Ds* excision · Particle bombardment

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S. Takumi (✉) · N. Mori · C. Nakamura
Laboratory of Plant Genetics, Department of Biological and Environmental Science, Faculty of Agriculture, Kobe University, Nada-ku, Kobe 657-8501, Japan
Fax: + 81 78 803 0998
E-mail: takumi@ans.ans.kobe-u.ac.jp

K. Murai[1]
Laboratory of Plant Molecular Genetics, Research Institute of Agricultural Resources, Ishikawa Agricultural College, Nonoichi-machi, Ishikawa 921-8836, Japan

*Present address:*
[1] Department of Bioscience, Fukui Prefectual University, Matsuoka-cho, Yoshida-Gun, Fukui 910-1195, Japan

## Introduction

A genetic transformation system has been developed in wheat using particle bombardment (Vasil et al. 1992; Weeks et al. 1993). The system has been used to manipulate both the quantity and quality of high-molecular-weight glutenin subunits, a class of seed-storage proteins determining the bread-making quality of wheat flour (Altpeter et al. 1996; Blechl and Anderson 1996). A nuclear male-sterility system has also been developed by introducing tapetum-specific expression of the *barnase* gene in transgenic wheat (De Block et al. 1997).

The mutagenic potential of transposable elements has been widely exploited in many organisms to clone genes based on transposon tagging (Walbot 1992; Sundaresan 1996). The maize *Activator* (*Ac*) element is autonomous and has a single open reading frame encoding a transposase protein (Kunze et al. 1987; Finnegan et al. 1988), and the expression of this gene is sufficient to trigger excision of the *Ac*/*Dissociation* (*Ds*) elements in its native host maize genome (Coupland et al. 1988). In maize and heterologous transgenic tobacco, *Ac* transcription results in the formation of a single 3.5-kb transcript which is the mature and correctly spliced mRNA (Kunze et al. 1987; Finnegan et al. 1988). By contrast, in transgenic *Arabidopsis*, which shows a lower activity of *Ac* transposition than tobacco and tomato, the processing of the *Ac* transcript is highly inefficient and inaccurate, thus resulting in premature polyadenylation in the second exon and the splicing of cryptic introns (Jarvis et al. 1997; Martin et al. 1997). Martin et al. (1997) considered that the low frequency of transposition of *Ac* in *Arabidopsis* may be

due in part to the low level of correctly processed transposase transcripts in this species.

The transposition of the *Ds* elements has been demonstrated and applied to the developement of the transposon tagging system in rice (Shimamoto et al. 1993; Izawa et al. 1997). In cultured wheat cells, the introduction and activation of the *Ac*/*Ds* elements have been reported (Laufs et al. 1990; Takumi 1996). However, the mobility of *Ac*/*Ds* has not yet been demonstrated in the genome of transgenic wheat plants. To develop a transposon mutagenesis and tagging system in wheat, transgenic plants with the active *Ac* transposase gene and those with a non-autonomous *Ds* element must first be produced, and *trans*-activation of the non-autonomous element must be demonstrated.

The excision of the maize *Ac*/*Ds* transposable elements from a marker gene can be easily detected by the phenotypic assay developed by Baker et al. (1987). Recently, a simple transient assay for *Ac*/*Ds* activity by particle bombardment has been developed in cells of intact barley tissues based on the $\beta$-glucuronidase (GUS) assay (McElroy et al. 1997). Here, we report the production of several independent transgenic wheat lines expressing the fixed *Ac* transposase gene. Using the McElroy assay, it was demonstrated that the *Ac* transposase gene *trans*-activated *Ds* excision.

## Materials and methods

### Plasmid DNAs for the production of transgenic wheats

The plasmids pUBA and pCKR532 were used to produce transgenic wheat plants. pUBA contains a bialaphos-resistant (*bar*) gene as a marker placed after a maize ubiquitin-1 gene (*Ubi1*) promoter (Toki et al. 1992). pCKR532 contains the *Ac* transposase-coding region controlled by a cauliflower mosaic virus 35S (CaMV35S) promoter (Shimamoto et al. 1993). This *Ac* transposable element was originally derived from the intact *Ac* element of the maize *waxy-m7* allele (Klösgen et al. 1986), and is modified to delete the inverted repeats; thus it cannot transpose by itself. The plasmids were amplified in liquid cultures of *Escherichia coli*, isolated by alkaline lysis, and purified twice by CsCl/ethidium bromide density gradient centrifugation (Sambrook et al. 1989).

### Plant materials, particle bombardment, and selection of transformants

Immature embryos of two common wheat (*Triticum aestivum* L. Thell) cultivars, 'Akadaruma' and 'Norin 12', were used. Immature caryopses from greenhouse-grown plants were sterilized in 70% ethanol and the isolated immature embryos were placed, with scutellar tissues exposed, on Linsmaier-Skoog (LS) medium (Linsmaier and Skoog 1965) containing 2 mg $l^{-1}$ of 2,4-D and 0.25% (w/v) Gelrite (Merck). The conditions of particle bombardment were as described previously (Takumi et al. 1994). The immature embryos were bombarded at 7–10 days after incubation on the LS medium and transferred to selection medium after 1 day. The selection medium comprised LS medium together with 2 mg $l^{-1}$ of 2,4-D and 5 mg $l^{-1}$ of bialaphos. After about 1 month, the bialaphos-resistant tissues were transferred to 2,4-D-free LS medium containing 4 mg $l^{-1}$ of bialaphos for regeneration.

### Molecular analysis of transgenic plants

Genomic DNAs were extracted from 0.5–1 g of leaf tissue by the CTAB method (Murray and Thompson 1980). PCR primers and conditions for detection of the *bar* gene were as previously described (Takumi and Shimada 1996). To detect the integration of the *Ac* element and expression of the *Ac* transposase gene, the following primer set was designed and synthesized based on the sequence of the *Ac* transposase-coding region; 5′-ATTTGATGTTGAGGGATGC-3′ and 5′-TTTGGAGCTGAAGGACTAC-3′. After RT-PCR the primer set was expected to amplify a 542-bp product containing an intron between the second and third exons if not spliced, but if the second intron was correctly spliced a 470-bp product was expected. To further examine the splicing and processing of the *Ac* transposase gene, four primers (INF1-F, INF3-F, INF4-F and INF4-R; see Fig. 4 A) were synthesized according to Jarvis et al. (1997). PCR conditions were as described previously (Takumi 1996). PCR products were cloned in pUC119 and sequenced by DyeDeoxy™ Terminator Cycle Sequencing using an ABI Model 373 DNA Sequencer (Applied Biosystems, USA).

For genomic Southern-blot analysis, total DNA of each *Ac* transformant was digested with *Bam*HI. There were no *Bam*HI sites within the transformed *Ac* transposase gene from pCKR532. The methods of electrophoresis, Southern blotting, hybridization with $^{32}$P-labelled probes, and autoradiography were all according to Liu et al. (1990).

Total RNAs were extracted from 0.5 g of leaves of the *Ac* transformants by ISOGEN (Nippongene). The expression of the *bar* gene and the *Ac* transposase gene was determined by Northern-blot analysis and by RT-PCR analysis. The total RNA (20 µg) was electrophoresed in a 1.2% formaldehyde/agarose gel and transferred to a Hybond $N^+$ nylon membrane (Amersham). The RNA blot was hybridized with the $^{32}$P-labelled probe. The methods of labelling, hybridization, washing and autoradiography were the same as for the genomic Southern-blot analysis. RT-PCR analysis was performed by a first-strand cDNA synthesis kit (Amersham) after removal of DNA contamination by DNase-I treatment. The conditions of RT-PCR for examining the splicing of the *Ac* transposase gene were according to Jarvis et al. (1997) and 25 cycles of PCR were performed.

### Detection of the *Ds* excision

To examine the activity of the introduced transposase gene, the following two plasmids were chosen; pSP-WDV-Act1(DsBar)-GUS.N and pSP-WDV-Act1.GUS.N. These two plasmids contain a rice actin-1 gene (*Act1*) promoter and a GUS gene (*gus*) with and without the *Ds* element, respectively (McElroy et al. 1997). The immature embryos of three Ac lines were bombarded with pSP-WDV-Act1(DsBar)GUS.N and pSP-WDV-Act1.GUS.N, and incubated at 26°C for 2 days prior to the assay for GUS activity. GUS activity was histochemically assessed (Takumi et al. 1994) and the average number of blue spots per embryo was determined. The numbers of blue spots per embryo were counted under the microscope in two separate experiments. In each experiment, more than 20 immature embryos were used.

## Results

### Production of *Ac* lines

Transgenic wheat plants were produced by the same method as that established previously (Takumi and Shimada 1996). In all, 2337 isolated immature embryos were bombarded with two plasmids, pCKR532 and

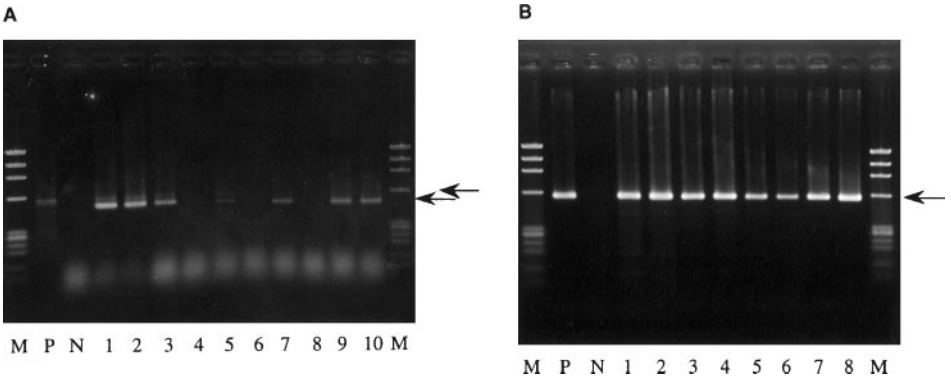
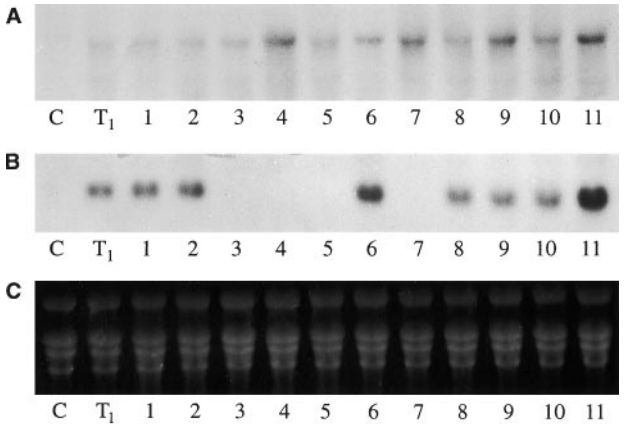



**Fig. 1A, B** Screening of transgenic wheat plants ($T_1$ generation) expressing the *Ac* transposase gene. (**A**) RT-PCR analysis of the *Ac* transposase transcript. *Upper and lower arrows* indicate a 542-bp band of pCKR532 and a 470-bp band of a spliced cDNA, respectively. *M* size marker (*λHin*dIII); *P* pCKR532 as a positive control; *N* non-transformed 'Akadaruma' as a negative control; *lanes 1–10* $T_1$ progeny from five $T_0$ plants. (**B**) PCR amplification of the *Ac* transposase gene. An *arrow* indicates a 542-bp band derived from the integrated *Ac* element. *M*, *P*, *N* same as in (**A**); *lanes 1–8* $T_1$ progeny expressing the *Ac* transposase gene




D

| $T_1$ plant number | No. of analyzed $T_2$ plants | No. of *Ac*-expressing $T_2$ plants | No. of *bar*-expressing $T_2$ plants |
|---|---|---|---|
| Ac1-1 | 8 | 6 | 0 |
| Ac1-2 | 8 | 8 | 0 |
| Ac3-1 | 10 | 10 | 10 |
| Ac3-2 | 11 | 11 | 7 |
| Ac24-1 | 10 | 10 | 0 |
| Ac24-2 | 11 | 6 | 0 |

**Fig. 2** Northern blots of the total RNAs from $T_2$ progeny of Ac3-2 (**A**, **B** and **C**) and a summary of the Northern-blot analyses in the $T_2$ generation (**D**). The PCR-amplified fragments of the *Ac* transposase (**A**) and the *bar* (**B**) genes were used as probes. Ethidium bromide-stained total RNA shown as a loading control (**C**). **C** non-transformed 'Akadaruma' as a negative control; $T_1$ a parental plant (Ac3-2) of the $T_2$ progeny; *lanes 1–11*, $T_2$ progeny from one $T_1$ plant (Ac3-2)

pUBA. After selection, 26 bialaphos-resistant plants were independently regenerated. All the regenerated plants ($T_0$) contained the *bar* gene; confirmed by PCR analysis (data not shown). Twenty one grew into fertile plants. Total RNA was isolated from the leaves of 32 $T_1$ progeny of 16 independent bialaphos-resistant plants (2 $T_1$ plants/$T_0$ plant). The first-strand cDNAs were synthesized from the total RNAs and used for RT-PCR screening of *Ac* transgenic plants. In the transgenic plants expressing the *Ac* transposase gene, a 470-bp band was recognized by RT-PCR amplification, which indicated the correct splicing of the *Ac* transcript at the intron between the second and third exons (Fig. 1 A). In the $T_1$ progeny of six $T_0$ plants (Ac1, Ac3, Ac4, Ac8, Ac20 and Ac24), the inheritance and expression of the transposase gene were confirmed. The co-transformation frequency of the *Ac* transposase gene with the *bar* gene was 37.5%. Total DNA was isolated from leaves of the $T_1$ progeny expressing the transposase gene. The presence of the *Ac* element was confirmed in all $T_1$ progeny expressing the transposase gene by PCR analysis (Fig. 1 B).

Eight to eleven $T_2$ progeny from each of six single $T_1$ plants were grown and total RNAs were isolated to investigate, by Northern-blot analysis, the inheritance of the transgenes into the $T_2$ generation. The *Ac* transposase gene was inherited and expressed in all examined $T_2$ progeny derived from four $T_1$ plants (Ac1-2, Ac3-1, Ac3-2 and Ac24-1), but segregated in the $T_2$ progeny from two other $T_1$ plants (Ac1-1 and Ac24-2) (Fig. 2). In the progeny derived from Ac1 and Ac24, the *bar* gene was not expressed and seemed to be transcriptionally silenced, although integration and inheritance of the *bar* gene were confirmed (data not shown). In the Ac3 progeny, however, the *bar* gene was not silenced and was fixed in the $T_2$ plants from Ac3-1 or else segregated in those from Ac3-2 (Fig. 2).

The *Ac* transposase gene was inherited and expressed in all examined $T_3$ progeny of three $T_2$ plants (Ac1-2, Ac3-1 and Ac24-1) (data not shown). The Southern-blot patterns showed no differences among these progeny (Fig. 3). Ac1-2 and Ac3-1 were derived

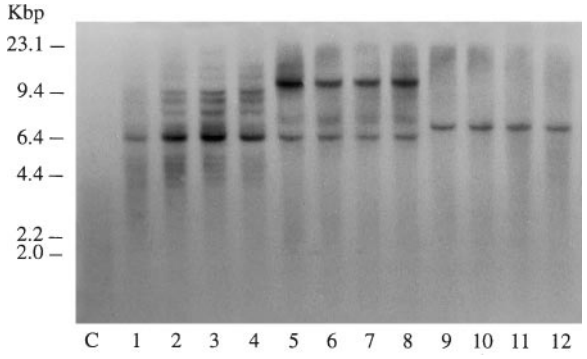



**Fig. 3** Southern blots of the *Bam*HI-digested total DNAs from $T_3$ progeny of Ac1-2, Ac3-1 and Ac24-1. The PCR-amplified fragments of the *Ac* transposase gene were used as probes. *C* non-transformed 'Akadaruma' as a negative control; *lanes 1–4* $T_3$ progeny of Ac1-2; *lanes 5–8* $T_3$ progeny of Ac3-1; *lanes 9–12* $T_3$ progeny of Ac24-1

from 'Akadaruma', and Ac24-1 was from 'Norin12'. From these data we concluded that the *Ac* transposase gene was fixed in the genome of the three transgenic wheat lines, and we used them in the following studies.

## Processing of the *Ac* transcripts

To examine the processing of the maize *Ac* transposase gene in wheat cells, an RT-PCR method was applied. Three forward primers (INT1-F, INT3-F and INT4-F) and one reverse primer (INT4-R), which were based on four of the five exons in the *Ac* element (Fig. 4 A), were designed according to Jarvis et al. (1997). First, we analyzed splicing of the third and fourth introns by using two primer combinations: INT4-F and INT4-R, and INT3-F and INT4-R. In the three Ac lines, the spliced (217-bp for INT4-F and 350-bp for INT3F) transcripts were also correctly produced, but minor bands indicating unspliced (604-bp and 826-bp) transcripts were also present (Fig. 4 B). Next, the fully spliced transcript was generated by the INT1-F and INT4-R primers. The predicted PCR fragment size in this analysis was 3.21 kbp for the primary *Ac* transcript in which all four introns were retained and 2.56 kbp for the fully spliced transcript. The 2.56-kbp fragment was observed in all three Ac lines (Fig. 4 C). Two fragments less than 2.56 kbp were also recognized. They, however, were found to be artifacts because two clones derived from the fragments showed unrelated sequences, and thus were considered not to be the result of the premature termination and polyadenylation of the *Ac* transcripts. These findings indicated that the of *Ac* transposase gene was precisely processed in the three Ac lines.

## *Trans*-activation and excision of the *Ds* element

McElroy et al. (1997) developed a simple transient assay for *Ac*/*Ds* activity in barley cells transformed by particle bombardment with a plasmid containing a rice *Act1* promoter and a *gus* gene interrupted by a *Ds* element. To investigate the activity of the *Ac* transposase gene integrated into the wheat genome,

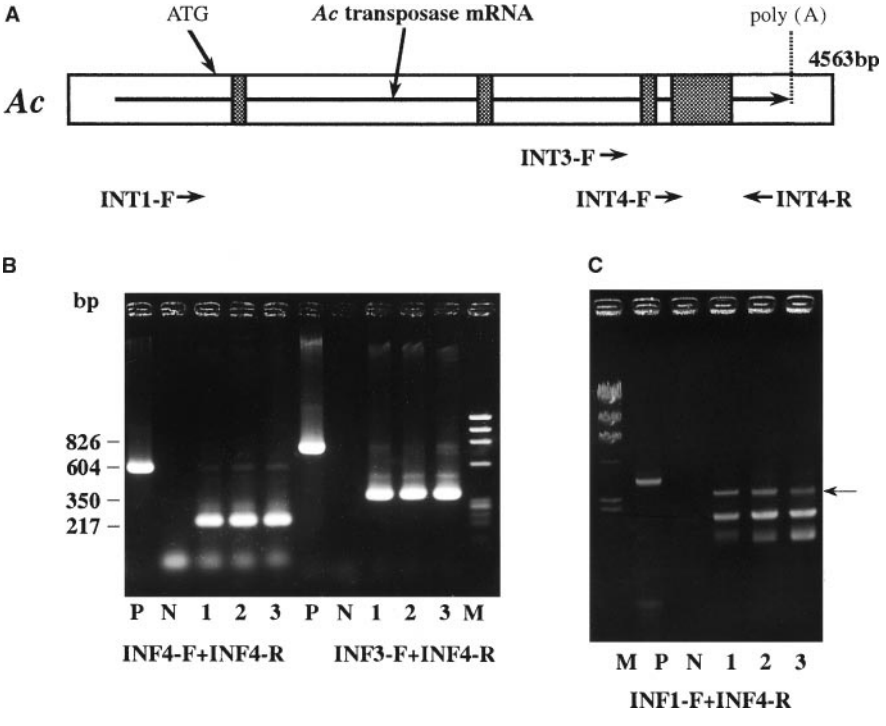



**Fig. 4A–C** RT-PCR analysis of the *Ac* transcripts in the transgenic wheat plants. (**A**) Structure of a coding region of the *Ac* transposase gene and positions of the primers (INF1-F, INF3-F, INF4-F and INF4-R). Four introns are indicated as *dark boxes*. (**B**) Splicing of the third and fourth introns. RT-PCR products corresponding to the spliced (217- and 350-bp) and unspliced (604- and 826-bp) transcripts are indicated. (**C**) Detection of the fully spliced transcript. An *arrow* indicates the fully spliced transcripts (2.56 kbp) of the *Ac* transposase gene. *M*, *P*, *N* see legend to Fig. 1; *lane 1* *Ac*1-2; *lane 2*, Ac3-1; *lane 3* Ac24-1

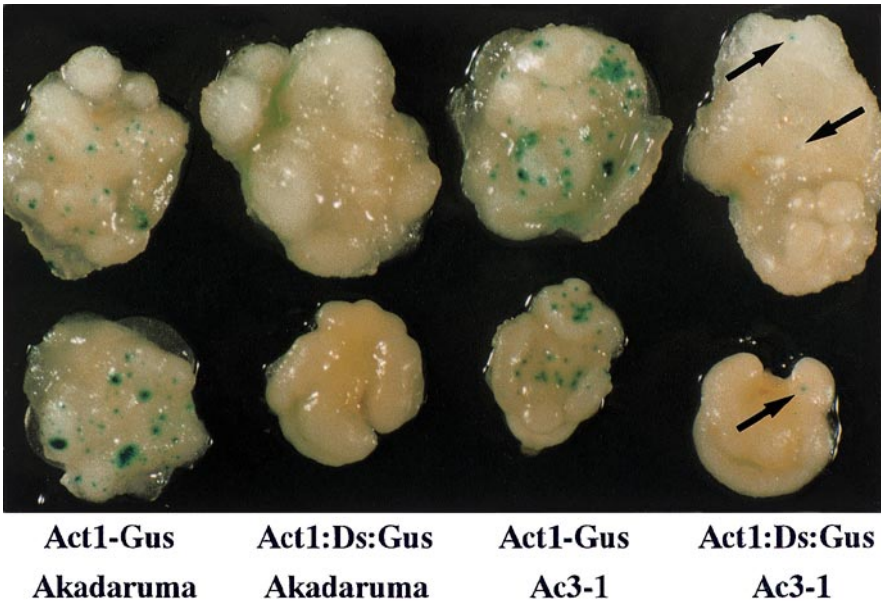



**Fig. 5** Detection of *Ds* excision by transient *gus* expression in cultured immature embryos of 'Akadaruma' (non-transformant) and Ac3-1. The *gus* gene expression (*blue spots*) was assayed 2 days after bombardment with pSP-WDV-Act1.Gus.N (*Act1-Gus*) or pSP-WDV-Act1(DsBar)Gus.N (*Act1 : Ds : Gus*). *Arrows* indicate the blue spots in which the *gus* gene was transiently expressed after excision of the *Ds* element

we performed McElroy's transient assay in the three Ac lines and counted the number of blue spots, which identify cells transiently expressing *gus*. Immature embryos isolated from the self-fertilized seeds of 'Akadaruma' and 'Norin12' and the selfed progenies of the three Ac lines ($T_3$) were cultured and bombarded with pSP-WDV-Act1.GUS.N or pSP-WDV-Act1(DsBar)GUS.N. In the former case 15–45 blue spots per embryo were recognized in 'Akadaruma', 'Norin12' and the three Ac lines. No blue spots were observed in the cultured embryos of the non-transgenic 'Akadaruma' and 'Norin12' bombarded with pSP-WDV-Act1(DsBar)GUS.N. On the other hand, a few blue spots were detected in the cultured embryos of all three Ac lines after bombardment with pSP-WDV-Act1(DsBar)GUS.N (Fig. 5). The number of blue spots per embryo was much less than those bombarded with pSP-WDV-Act1.GUS.N. These observations indicated than that the active transposase protein was translated from the integrated *Ac* transposase gene in the Ac lines and the *trans*-activated *Ds* excision.

The *Ds* excision frequencies in the two Ac lines (Ac3-1 and Ac24-1) were calculated from the number of blue spots per embryo, and compared with their respective parental cultivars. No *gus*-expressing cells were observed in the control cultivars after bombardment with pSP-WDV-Act1(DsBar)GUS.N, and the number of blue spots per embryo bombarded with pSP-WDV-Act1.GUS.N was equivalent in both the control and the *Ac* transgenic lines (Table 1). In the two Ac lines, 0.26 and 0.16 blue spots per embryo were recognized in Ac3-1 and Ac24-1, respectively. Because the number of blue spots was 41 and 16, respectively, when pSP-WDV-Act1.GUS.N was bombarded, the calculated excision frequency was 0.56% and 0.98% in the two Ac lines.

## Discussion

### Production of *Ac* transgenic wheat lines expressing the *Ac* transposase gene

The maize *Ac* transposase gene was successfully introduced into the wheat genome in this study and was co-transformed with a marker gene, *bar*, by particle bombardment. The *Ac* transposase gene was inherited and stably expressed in the $T_1$, $T_2$ and $T_3$ generations, and three Ac lines were established. Our result is in contrast with that of Srivastava et al. (1996) who studied the fate of transgenes in transformed wheat plants, and reported that *gus* expression was lost in $T_2$ plants of all but one line, although the *gus* gene was detected in all $T_0$ plants.

The co-transformation frequency was 37.5% in our transformation experiments to produce the Ac lines. This suggests that a foreign gene co-transformed with *bar* may be integrated and expressed in about one-third of the bialaphos-resistant plants ($T_0$). The co-transformation frequency of unlinked genes in this study was similar to that (45%) reported previously in wheat cultured embryos (Altpeter et al. 1996), but much lower than that (69%) in another report (Blechl and Anderson 1996).

### *Trans*-activation of *Ds* excision by the active *Ac* transposase gene in wheat plants

To examine the processing of the *Ac* transcripts and the activity of the transposase in transgenic wheat plants, we adopted the RT-PCR analysis and the simple transient assay for *Ac* activity developed by McElroy

**Table 1** Excision frequencies (means ± SE) of the *Ds* element in cultured immature embryos of *Ac* transgenic and non-transgenic wheat plants

| Cultivars[a] | No. blue spots per embryo bombarded with pSP-WDV-Act1.GUS.N | No. blue spots per embryo bombarded with pSP-WDV-Act1(DsBar)GUS.N | Excision frequency (%) |
|---|---|---|---|
| Akadaruma | 43.27 ± 1.94 | 0 | 0 |
| Ac3-1 | 41.07 ± 12.57 | 0.264 ± 0.185 | 0.56 ± 0.28 |
| Norin12 | 18.01 ± 3.13 | 0 | 0 |
| Ac24-1 | 15.69 ± 1.27 | 0.155 ± 0.035 | 0.98 ± 0.08 |

[a] Ac3-1 and Ac24-1 were derived from 'Akadaruma' and 'Norin12', respectively

et al. (1997). In the transgenic wheat plants, the *Ac* transposase gene was precisely spliced and most of the transcripts were correctly processed (Figs. 1 A and 4). The results from the RT-PCR analysis in the transgenic wheat plants seem to differ from those in *Arabidopsis* in which splicing efficiencies were 57 and 30% in the third and fourth introns of the *Ac* transposase gene, respectively (Jarvis et al. 1997). In the native host maize and in transgenic tobacco plants, *Ac* transcription results in the formation of a single 3.5-kb transcript which is the mature and correctly spliced mRNA (Kunze et al. 1987; Finnegan et al. 1988). Martin et al. (1997) have also reported that introns of the *Ac* transposase gene were correctly and efficiently spliced in maize and transgenic tobacco with high transposition frequencies, contrary to the low frquency in *Arabidopsis*. Our result confirmed that the processing pattern of the transposase gene in transgenic wheat is similar to that in maize and transgenic tobacco.

Our previous study showed that the *Ds* element was excised in wheat cultured cells by the *Ac* transposase gene and that the mechanism of excision was similar to that in maize (Takumi 1996). The transient *gus* assay now showed that *Ds* excision could be induced by the transposase produced in the transgenic wheat embryos (Table 1, Fig. 5), which is in agreement with the results obtained in cells of intact barley tissue (McElroy et al. 1997). These findings suggest that functionally active transposase polypeptides are produced in the transgenic wheat plants, although the frequency of *Ds* excision is low. We have already established some Ds lines, which carry single or multiple *Ds* copies (unpublished data). Therefore, progeny from the three Ac lines established through this study can be used in the cross with the transgenic wheat plants carrying the *Ds* element to demonstrate the *trans*-activation of the *Ds* element in the wheat genome. The most important requirement is the demonstration of the reintegration of the excised *Ds* element into the genome of wheat.

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