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A novel system for *Agrobacterium*-mediated transformation of wheat(*Triticum aestivum* L.) cells

XU YAO[1*], BAOJIAN LI*, JINGFEN JIA**
**Biotechnology Research Centre, Zhongshan University, Guangzhou 510275. **Department of Biology, Lanzhou University, Lanzhou 730000, China.*

ABSTRACT

A new approach for transforming the cultured cells of wheat(*Triticum aestivum* L. cv. Ganmai 8) was developed using *Agrobacterium tumefaciens.* The features of the optimum procedure were: (a) both combined synthetic signal molecules and multiple natural extracts from susceptible plants were used to pretreat the primary vigorous *Agrobacterium*(PVA) cells for approximately 16 h; (b) the gyratory magnetic field condition was used during cocultivation; (c) the cocultivating period and selecting condition were modified; (d) the recipient cells were at exuberant metabolism and active division while infected with *Agrobacterium.* Both neomycin phosphotransferase and nopaline synthase assays demonstrated the expression of NPT II and NOS genes, located on the T-DNA segment of chimaeric plasmid pGV3850::1103neo, in transformed wheat cell colonies by adopting the techniques of dot blot, ndPAGE or high voltage paper electrophoresis. Integration of the foreign genes into wheat genome was confirmed by Southern blot hybridization. Moreover, a relatively rational method was described for the estimation of transformation frequencies from cultured cell levels.

Key words: *genetic engineering, gyratory magnetic field, natural susceptible extracts, synthetic signals, transformation system, wheat cells.*

1. To whom correspondence should be addressed

INTRODUCTION

It is well known that *Agrobacterium*-mediated transformation of monocotyledonous plants, especially important cereal crops, is one of the most difficult vital problems in the research on plant gene engineering. Up to now there have been only a few reports [2, 4 -7, 12, 14, 16, 18] to indicate that exceptional monocots or their varieties could be transformed by *Agrobacterium tumefaciens*. However, in this regard progeny testing for foreign genes has not yet been reported so far. It is generally accepted that the transformation system mediated by *Agrobacterium* is one of the most efficient and reliable methods among the several current techniques for gene transfer in higher plants. Therefore, an efficacious system for *Agrobacterium*-mediated transformation of the cereal crop cells is urgently needed. In this report, we described a novel procedure for *Agrobacterium*-mediated transformation of cultured wheat cells by using some unique means.

MATERIALS AND METHODS

Establishment of wheat cell lines

The initial cultures used to establish suspension cell lines were those derived from young ears of *Triticum aestivum* L. var. 'Ganmai 8'. When the wheat suspension cells were initially cultured, the loose callus cultures were inoculated to 250 ml flasks containing 50 ml N_6 medium[3] supplemented with 2.0 mg /l 2,4-D(2,4-dichlorophenoxyacetic acid), 200 ml YE(yeast extracts), 250 mg /l CH(casein hydrolysate), and 2% sucrose, pH 5.8. This medium was designated as N6D. Every 7-10 d, the suspension cultures were subcultured by adding fresh N_6D solution. Simultaneously, they were filtered through 900 μm stainless steel sieves to remove larger cell clusters. At each time, the filtrated cells were incubated on a gyratory shaker (100 r/min) at 26°C and under diffuse light. After about 6-8 cycles of subculture, the suspension cells, following their retransfer to fresh medium and incubation for another 24-48 h, were used in cocultivation with *Agrobacterium.*

Bacterial strains and plasmids

The bacterial strains and plasmids used in this experiment were A. *tumefaciens* strain C58C1 Rif^r (pGV3850::1103neo) and *E.coli* strain HB101 (pLGV2103), respectively. Their relevant characteristics and growth conditions were as previously described[8].

Synthetic signal molecules (SSM)

The substances utilized to induce the activation and expression of virulence-region genes on the *Agrobacterium* Ti plasmid were acetosyringone(AS), catechol, gallic acid, pyrogallic acid, ρ-hydroxybenzoic acid, protocatechuic acid, β-resorcyhc acid and vanillin. These 8 compounds, designated as 8SSM, were totally combined or separately dissolved each at 5 mg/ml in 50% dimethyl sulfoxide to make a stock solution. It was sterilized by filtration through a 0.45 μm filter and used at a concentration of 10 mg/l each in the treatment.

Preparation of natural susceptible extracts (NSE)

Several different kinds of growing plantlets or seedlings at length of 4-7 cm, matured fruits, and cultured cells were independently pestled and extracted with MS medium. These included: (a) 30 sterile tobacco plantlets; (b) 100 mung bean seedlings; (c) 200 cucumber

seedlings; (d) 200 tomato seedlings; (e) 3 matured tomato fruits; and (f) 5 g of cultured carrot cells. Prior to grinding, the leaves and stems of all the seedlings and plantlets were wounded to varying degrees and then incubated overnight at 26°C. Following extraction, all the crude homogenates were mixed together and then filtrated and/or centrifuged to discard the pellets. The supernatant was adjusted to 500 ml with MS culture solution. Finally, 2% of sucrose and half components of LB medium were added to the mixed solution, pH 5.5. This combined extract was designated as NSE, which was sterilized by filtration.

Pretreatment of agrobacteria and modification of cocultivation

Prior to the interaction of donor and receptor cells, first of all, a single agrobacterial colony was transferred into 20 ml YEP medium (10 g/l peptone, 5 g/l NaCl, and 10 g/l yeast extract) containing the approriate antibiotic and incubated for 16 h at 28°C on a rotary shaker. The cultures were used as the primary vigorous agrobacteria, designated as PVA cells. Secondly, about 2 ml aliquots of PVA cells were taken and loaded into 5 volumes of different treating solutions containing the appropriate antibiotic. Finally, the PVA cultures were incubated for a further 12 h and then utilized as secondary activating *Agrobacterium,* marked as SAA cells. These treating solutions included a series of different YEP culture solutions with or without acetosyringone(AS), combined 8 synthetic signals (8SSM), 1/3 volume of natural susceptible extracts(NSE), or 1/3 volume of NSE containing 8SSM (marked as N8S). At the time of cocultivation, the specially pretreated agrobacteria were separately inoculated into different flasks containing wheat suspension cells which were at exuberant metabolism and active division as mentioned above. The average amount of bacteria inoculated was 1 ml per 30 ml wheat cells. The solutions of cocultivation, each adjusted to pH 5.8, included three kinds: N_6 medium solution containing 2,4-D(N_6D), natural susceptible extract(NSE) solution and high osmotic solution(HOS). The HOS was an N_6D solution supplemented with 10% polyethylene glycol (MW 6,000) and 50 mmol $CaCl_2 \cdot 2H_2O$. Besides the ordinary conditions (at 28°C and 120

Tab 1. Treatment combinations for cocultivation of *Agrobacterium* with wheat cells*

Name of treatment	Treating solution for PVA cells	Cocultivating solution	Condition of cocultivation
YEP	YEP	N_6D	28° C and 120 r/min
AS	YEP+10 mg/1 AS	N_6D	28° C and 120 r/ min
8SSM	YEP+SSSM(10 mg /l, each)	N_6D	28° C and 120 r /min
NSE	2/3YEP+1/3NSE	N_6D	28° C and 120 r/min
N8S-1	2/3YEP+1/3NSE+8SSM	N_6D	28° C and 120 r/min
N8S-2	2/3YEP+1/3NSE+8SSM	N_6D	28° C,120 r/min, and GMF
N8S-3	2/3YEP+1/3NSE+8SSM	HOS	28° C and 120 r/min
CK	Control without *Agrobacterium*, followed the same operation as YEP treatment		

*Abbreviations:
AS=acetosyringone; GMF=gyratory magnetic field; HOS=high osmotic solution; N_6D=N_6 medium containing 2.0 mg/l 2,4-D; N8S=YEP culture solution containing both 1/3 volume of NSE and 8SSM (10 μg/ml, each); NSE=combined natural susceptible extracts; PVA=primary vigorous agrobacteria; 8SSM=combined 8 synthetic signal molecules; YEP=YEP medium solution.

r/min), a condition of gyratory magnetic field(GMF) was also adopted in one treatment during cocultivation, as shown in Fig 1. In this way, the total combinations of cocultivating system used in the present experiment were listed in Tab 1. After 24 h of coincubation of cultured wheat cells with specially pretreated agrobacteria, upper bacterial suspensions were gently poured out of the flasks. Additionally, fresh corresponding solutions were added into flasks containing the remaining cultures which were then cocultivated for a further 36 h. At the specified intervals of cocultivation, samples for the scanning electron microscope observation were prepared using routine protocols.

Isolation and selection of transformed cell colonies

Following the cocultivation of wheat cells with *A*. tumefaciens, the mixed suspensions were sieved through a 280 μm stainless steel screen, and the cell clusters and aggregates were discarded. The cell filtrates were alternately washed more than eight times in sterilized water or N_6D solution containing 600 μg /ml cefotaxime(Cef) and 200 μg/ml ampicillin(Amp). After removing free bacteria, the cocultivated cells of wheat were resuspended in the latter solution (N_6D+Cef+Amp) and incubated for another 3 d at 26°C and 100 r/min. The culture solution was decanted and the cells were adjusted in the same fresh solution to an appropriate density. An equal volume of cell suspensions was drawn and then plated on solidified (0.7% agar) cefotaxime-ampicillin-containing N_6D medium with or without 120 mg/l kanamycin, respectively. After drawing superfluous solution, the culture plates were further incubated for isolation and selection of transformed cells. Each treatment was conducted with at least 3-5 replications. After being cultured for 4-6 wk, the proliferating microcolonies with kanamycin resistant growth were scored for further selecting culture, expanding reproduction, and subsequent regeneration. In the case of agar-containing medium, 0.5% activated carbon was also supplemented.

Detection of NPT II and NOS enzyme activities

The presence of neomycin phosphotransferase II(NPT II) or nopaline synthase activity in transformed cell lines was assayed by dot blot method[10], ndPAGE method[13], and high voltage paper electrophoresis [15]. The sample for NPT II analysis was extracted as described elsewhere[19].

Estimation of transformation frequencies

According to the method described by Xu et al.[17], the transformation frequencies of wheat ceils were modified as follows:

$$\text{Formation frequency of kanamycin resistance}(\%) = \frac{\text{Number of colonies formed on kanamycin-containing medium from the } \textit{Agrobacterium}\text{-cocultivated wheat cells}}{\text{Number of colonies formed on kanamycin-free medium from an equal amount of the same cell population}} \times 100$$

$$\text{Maintenance frequency of kanamycin resistance}(\%) = \frac{\text{Number of colonies capable of keeping proliferation in the presence of kanamycin}}{\text{Number of formed kanamycin-resistant colonies reinoculated on kanamycin-selecting medium}} \times 100$$

Transformation frequency

= formation frequency of kanamycin resistance(%) x maintenance frequency of kanamycin resistance(%) x frequency of NPT II activity from kanamycin-resistance-maintaining colonies(%)

DNA isolation and Southern blot analysis

Wheat DNA was prepared from callus cultures of both transformed and non-transformed cell lines using the procedures of Bevan[1]. The DNA precipitates were dissolved in TE buffer (pH 7.5) and treated with 20 μg/ml RNase A for 30 min at 37℃, then extracted with phenol/chloroform and precipitated with ethanol. After resolving in TE buffer, the DNA concentration was estimated in comparison with a DNA standard by electrophoresis in an agarose gel and staining with ethidium bromide. About 20 μg DNA of each sample was thoroughly digested overnight at 37℃ with both EcoR I and Hind III restriction endonucleases. The digested DNA fragments were separated by electrophoresis in 0.8% agarose gel, and Southern blot analysis was conducted with the method of Southern as described by Maniatis et al.[9], ^{32}P-labeled radioactive probe was a nick-translated EcoR I/Hind III fragment of pLGV2103 plasmid containing NPT II specific sequence. The hybridized filters were exposed to X-ray films with an intensifying screen at -20℃.

RESULTS AND DISCUSSION

Role of different treatment in Agrobacterium-mediated transformation of wheat cells

Even in the transformation experiment for dicotyledonous plants, a successful transformation is associated not only with the physiological and metabolic state of recipient cells but also with the activation of donor cells. The callus lines derived from wheat young ear had been rapidly growing and proliferating in the course of subculture. They were rather loose and suitable for liquid suspension culture. After 6–8 cycles of suspension culture and filtration, cell lines became highly steady in growth and relatively uniform in state. When the suspension cells were retransferred and incubated for up to 1–2 d, a major proportion of the cells was exuberantly metabolic and actively dividing at this stage, which was in a beneficial recipient state for *Agrobacterium*-mediated transformation.

In order to obtain effectively state-activated *Agrobacterium* capable of transforming wheat cells, a certain amount of primary vigorous agrobacteria was treated for 12 h with shaking incubation using the following factors prior to *in vitro* infection, including common culture solution; single synthetic signals or combined 8 different compounds that were capable of triggering the activation and expression of vir genes on Ti plasmid; multiple natural extracts from the cells, tissues and organs of different plants susceptible to *Agrobacterium*; or both the combined synthetic signal molecules and the natural susceptible extracts. Besides ordinary cocultivating conditions, the special condition of gyratory magnetic field (Fig 1) or high osmotic solution were also used during cocultivation, which might impel agrobacterial attachment to the recipient cell surfaces, the cell division, and even the foreign DNA integration into wheat genome.

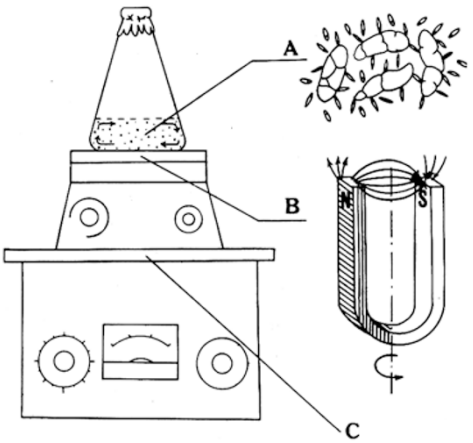

Fig 1. ▷ Diagrammatic representation of the cocultivating system with gyratory magnetic field used in this experiment. (A) The cocultivating plant cells and agrobacteria. (B) A rotating U-shaped magnet. (C) A shaking incubator (at 26℃, 120 rpm).

Following 24 h of cocultivation, the upper bacterial suspension in the cocultivating flasks was removed and fresh corresponding solution was added again, and then cocultivated for another 36 h in order to allow a through interaction between the donor and recipient cells, to complete at least one cell cycle for most recipient cells during this process, and not to be detrimental to the division and growth of wheat cells by excessive *Agrobacterium* at the same time. After cocultivation, the bacterium-free wheat cells were first incubated for 3 d so as to minimize the adverse effects of selective stress on expression of the foreign DNA just integrated into the recipient cells. On the other hand, removing the larger cell clusters by filtration was to decreases the number of chimaeric transformants and the proportion of nontransformed cells.

As shown in Tab 2, although the wheat cells plated on kanamycin-selecting media in both YEP and CK treatment were able to form a few celt colonies, these colonies retransferred onto kanamycin-selecting media were incapable of proliferating and growing continuously (Fig 2c). In the treatment N8S-3, the sharp drop of the formation number of cell colonies might be imputable to the impropriety of high osmotic solution used, i.e. a poor effect on the growth of recipient cells. Nevertheless, both the formation frequency and maintenance frequency of kanamycin resistance were obtained to a different extent from other treatments (Tab 2; Fig 2a, 2b, 2d and 2e), among which the optimum one was the treatment N8S-2. Its characteristics was that not only both combined synthetic signal molecules and natural susceptible extracts capable of inducing effective activity of *Agrobacterium* was used, but also the cocultivating condition of gyratory magnetic field (Fig 1) was supplemented. The role of this treatment might be related to the more efficient stimulation of the activation and expression of vir genes on Ti plasmid or other genetic loci of *Agrobacterium*, which were essential and suitable to the transformation of wheat cells. The gyratory magnetic field might also stimulate cell division, DNA replication, and even transfer and integration of foreign genes. Yet, there has been

no direct evidence for this hypothesis.

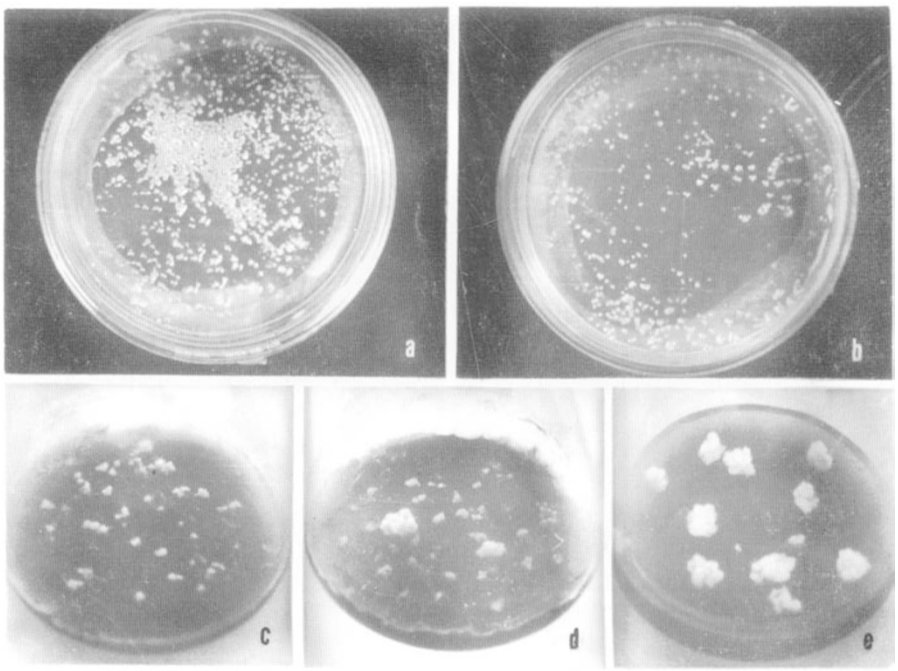

Fig 2. △ Selection and subculture of transformed kanamycin-resistant wheat cells. Cell colonies were formed on plates without kanamycin (a) or with 120 mg/l kanamycin (b) after 1 m of culture from *Agrobacterium*-cocultivated wheat cells using the transforming treatment N8S-2. When cell colonies produced on kanamycin-containing media were retransferred onto the same fresh media and cultured for further 6 wk, some of these derived from the transformating treatment continued to grow (d) whilst those from the control treatment failed to show further growth (c). Transformed kanamycin-resistant colonies kept on growing when subcultured on selection medium (e).

Tab 2. Agrobacterium-mediated transformation of wheat cells in different treatments

Treatment*	Number of cell colonies formed (Mean±S.E.M.)		Frequency of kanamycin resistance (%)		Ratio of kanamycin resistance-sustained colonies exhibiting NPT II activity	Transformation frequency
	On kanamycin-free midium	On medium with 120 mg/l kanamycin	Percentage of formation	Percentage of maintenance.		
YEP	144±32	3±2	2.1	0	—	—
AS	207±42	6±4	2.9	8.9	1/3 (33.3)	8.6×10^{-4}
8SSM	191±18	7±3	3.7	10.4	0/2 (0)	0
NSE	317±58	18±5	5.7	7.8	3/11 (27.3)	1.2×10^{-3}
N8S-1	291±64	37±7	12.7	11.2	6/12 (50)	7.1×10^{-3}
N8S-2	234±37	58±13	24.8	18.6	7/12 (58.3)	2.7×10^{-2}
N8S-3	86±23	0	0	—	—	—
CK	287±38	5±3	1.7	0	—	—

* As described in Tab 1

Agrobacterium-mediated transformation of wheat cells

Under the most optimum treatment, a lot of agrobacterial cells were observed to attach to the surface of wheat cells and produce fibrils (Fig 3a and b). In addition, the attachment of agrobacteria to the wheat cell wall seemed to be related to the physiological state of host cells because more bacteria were found to adhere to younger cells than to older ones.

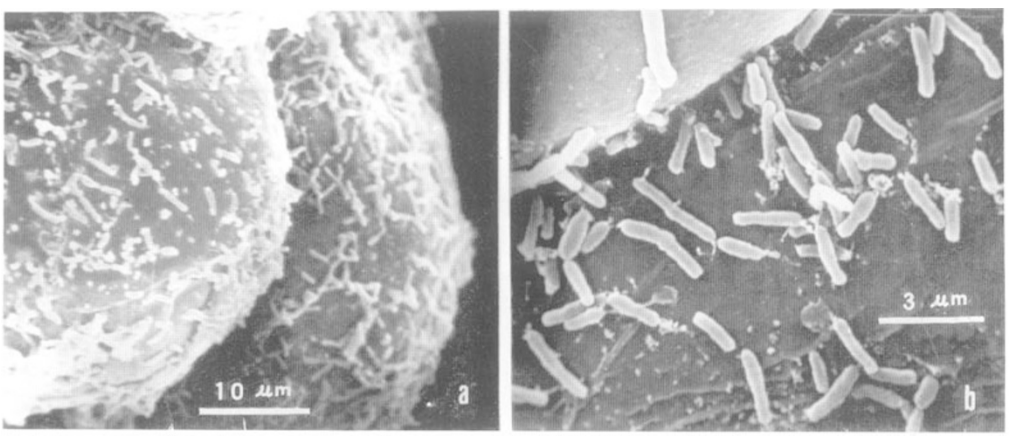

Fig 3. △ Dense attachment of agrobacteria to the surface of wheat cells (a) and formation of fibrils from specified bacteria (b) during cocultivation of the treatment N8S-2.

Enzyme assays of NPT II and NOS in transformed and nontransformed cell lines

The kanamycin-resistent cell colonies formed from wheat cells that had been treated by different transforming proceduces were separately transferred onto the corresponding selective media for sustaining isolation, selection and culture. After expanding reproduction of each kanamycin-resistant cell colonies, a certain amount of samples was drawn at random from each treatment. At first, the kanamycin-resistant cell lines exhibiting NPT II enzyme activities and their proportions (Tab 2) were determined by the dot blot method[10] and further verified by the ndPAGE method[13]. Fig 4 showed an ndPAGE-NPT II analysis of 3 typical kanamycin-resistant cell lines. Then, the NOS activities in the 3 cell lines were detected using high voltage paper electrophoresis method[15], as shown in Fig 5. Neither NPT II nor NOS enzyme activity was detected in the nontransformed normal callus cultures of wheat. Therefore, it was demonstrated that both foreign genes were expressed in wheat cell colones transformed by *Agrobacterium tumefaciens.*

Transformation frequency of wheat cells

In order to rationally calculate the frequency of transformation from cultured cell

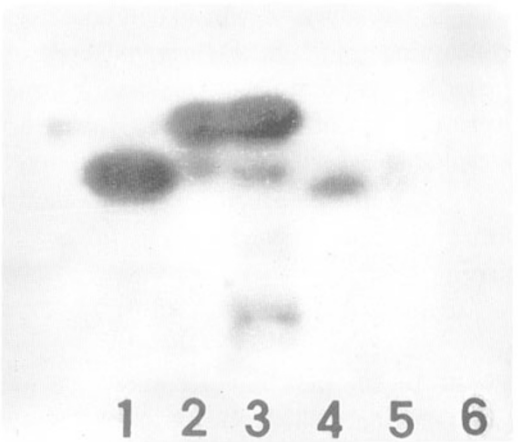

Fig 4. ▷ The ndPAGE analysis for neomycin phosphotransferase II activity in wheat callus cultures from transformed cells. Lanes 1–6 correspond to assays of various extracts as follows: lane 1, *E. Coli* strain HB101 containing pLGV2103; lanes 2–4, 3 typical transformed wheat cell lines resistant to kanamycin (previously shown to express NPT II activity by the dot blot method); lanes 5 and 6, nontransformed normal wheat callus cultures, respectively.

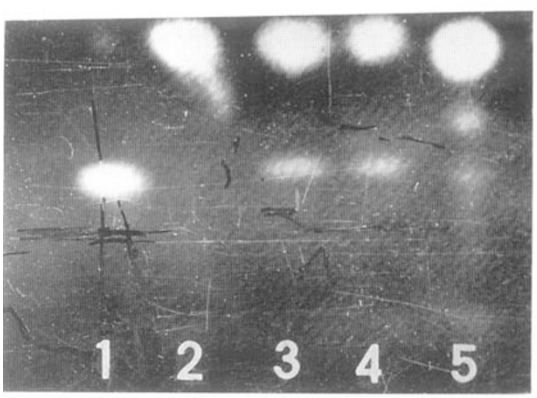

Fig 5. ▷ Detection of nopaline, in different wheat callus tissues. Lane 1, nopaline standard; lane 2, nontransformed normal wheat callus cultures; lanes 3–5, 3 independent transformed cell lines not only resistant to kanamycin but also exhibiting NPT II activity.

level, we have developed a new method instead of using a routine one whose calculation was based on estimating the cell density initially plated. In this method, the cell suspensions of several flasks from same treatment were mixed together after cocultivation and removal of free bacteria, and fairly well-shaken whenever they were drawn. Then, the formation number of cell colonies was compared after culturing an equal amount of the same cell population on media in the presence or absence of selection pressure, and the formation frequency of kanamycin resistance was calculated. On the basis of this, the transformation frequencies of wheat cells of each treatment by *Agrobacterium* were further estimated according to comprehensive consideration of both maintenance frequency of kanamycin resistance and frequency possessing NPT II activity in kanamycin-resistance-maintaining colonies(Tab 2).

Regeneration experiment of transformed cell lines

The transformed wheat cell colonies obtained from several cycles of kanamycin-containing selection were transferred onto kanamycin-free differentiating media in order to regenerate the transgenic plants. The nontransformed calli of normal wheat were capable of differentiating into whole plants on the agar-solidified N_6 medium supplemented with 3% sucrose, 100 mg/l CH, 0.2% activated carbon, 0.1 mg/l 2, 4D,

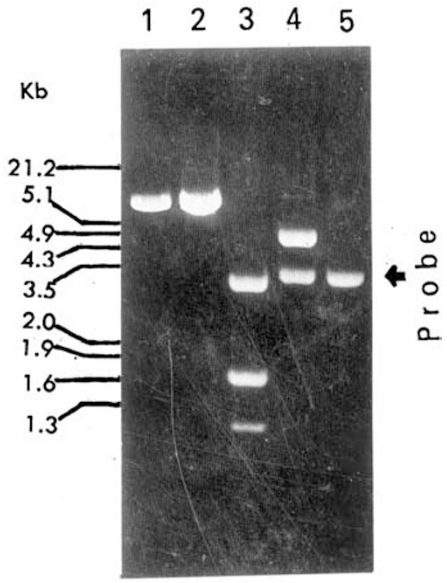

Fig 6. ▷ Digestion assay of pLGV2103 plasmid and electrophoresis identification of isolated probe fragment. Lanes 1–4, pLGV2103 plasmid digested with EcoR I (1), Hind III (2), Pst I (3) and EcoR I + Hind III (4), respectively; Lane 5, isolated Ec0R I-Hind III fragment of pLGV2103 plasmid containing the NPT II gene using the method of low melting agarose gels.

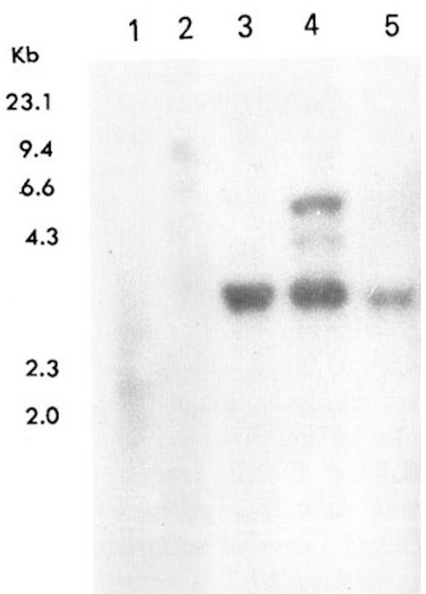

Fig 7. ▷ Southern blot analysis of the EcoR I-Hind III-digested DNA isolated from transformed and nontransformed wheat tissue cultures. About 20 μg DNA from wheat callus cultures was completely digested with EcoR I and Hind III restriction enzymes and electrophoresed on 0.8 % agrose gel. The separated fragments were then blotted to nitrocellulose membrane and hybridized with a ^{32}P-labeled DNA probe as shown in Fig 5. Phage lambda Hind III marker was displayed in Kb. Lane 1, nontransformed normal wheat callus lines; lanes 2–5, 4 independent transformed wheat cell lines with both kanamycin resistance and NPT II activity.

0.5 mg/l BA (benzyl-adenine) and 1.0 mg/l KT (kinetin). On the basis of this regenerating medium, approximately 20 kinds of different medium treatments for regeneration were designed using the following factors: MS medium, B_5 medium, various strength of inorganic salts, concentration and kind of sugar (e.g. sucrose and glucose), level and ratio of various plant hormones, hormone-free condition, amino acids (e.g. proline and glutamine), $AgNO_3$, etc.. Unfortunately, there has been no sign of differentiation in the transformed wheat cell lines cultured for more than one and half year up to date, which restricted the further analysis of the transformed cell lines. The loss of regeneration ability from the cell lines might be concerned with the long-term subculture and several selections for kanamycin resistance.

DNA analysis of foreign gene integration

To confirm that the sustained growth of cell lines on media containing kanamycin was the result of chromosomal integration of the chimaeric NPT II gene from *Agrobacterium* to *T. aestivum*, we carried out a Southern analysis of DNA from both control wheat calli and putative transformants. These DNAs were completely digested with Hind III and EcoR I restriction enzymes. The ^{32}P-labeled probe was about a 3.3 Kb fragment of pLGV2103 plasmid containing the coding sequence of NPT II gene(Fig 6). As shown in Fig 7, DNA from nontransformed wheat calli did not show any hybridized signal, while DNAs from those of 4 typical transformed cell lines analyzed, which possessed both kanamycin resistance and NPT II activity, showed apparent hybridized bands except one. It could be of interest to note that there were 3 bands with different molecular sizes in one of the 4 transformed cell lines, thus suggesting that there would be several possibilities for T-DNA transformation of this cell line, including partial integration, molecular rearrangements, or multiple insertions. Southern blot analysis further confirmed the presence of DNA fragments complementary to the introduced foreign gene in the *Agrobacterium*-transformed wheat cell lines. It was concluded, therefore, that the relatively novel transformation system developed here was efficacious to *Agrobacterium*-mediated transformation of cultured cells of wheat material used. At present we are aiming at approaching and identifying whether this system will be also suitable for the *Agrobacterium*-mediated transformation of other important cereal crop cells.

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