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SHORT COMMUNICATION

Glufosinate-tolerant Tobacco Plants Directed by the Promoter of Adenylate Kinase Gene of Rice

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A DNA clone containing the 5′ part of the adenylate kinase (AK) gene was isolated from a rice genomic library, and its nucleotide sequence was determined. This clone consists of 5′ upstream, five exons and four introns of the AK gene. All of the determined donor and receptor sites contained 'GT' and 'AG' consensus splice sequences. Transgenic tobacco plants harbouring a chimeric gene consisting of the 5′ upstream sequence of the AK gene fused with the gene encoding phosphinothricin acetyl transferase were generated. They showed tolerance to glufosinate to a level four times higher than its commercial dose.

Key words: Adenylate kinase, gene promoter, glufosinate resistance, tobacco, rice.

INTRODUCTION

The enzyme adenylate kinase (AK, EC 2·7·4·3) has been reported to occur as a monomeric enzyme to supply ADP in energy-producing systems which are important for growth and maintenance in both prokaryotes and eukaryotes (Noda, 1973). It catalyses the inter-conversion of adenine nucleotides as follows: ATP + AMP $\rightleftharpoons$ 2ADP (Atkinson, 1968). Several isoforms of AK have been purified and characterized from animals (Heil *et al*., 1974), bacteria (Terai, 1974) and yeast (Ito *et al*., 1980). In the case of plants, we reported molecular cloning and characterization of the cDNA encoding functional AK from suspension-cultured rice cells (Kawai *et al*., 1992). The kinetics of rice AK and its cellular localization in vascular tissues of rice plants have been studied (Kawai and Uchimiya, 1995*a*, *b*). Furthermore, our previous studies suggested that salt (Samarajeewa *et al*., 1995) or submergence stress (Kawai *et al*., 1998) stimulates the activity of AK.

In this study, a genomic clone of the 5′ part of AK was isolated from a rice genomic library, and the nucleotide sequence of the 5′-upstream region was determined. To assess the efficacy of this 5′ upstream element as a promoter we constructed a chimeric gene containing the 5′-upstream region of the AK gene fused with the phosphinothricin acetyltransferase (PAT) gene from *Streptomyces hygroscopicus* (Murakami *et al*., 1986; Toki *et al*., 1992). Transgenic tobacco plants bearing this chimeric gene were found to be tolerant to glufosinate treatment.

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MATERIALS AND METHODS

Isolation of the genomic clone containing the AK gene

A genomic library of *Oryza sativa* L. was constructed using λ-GEM12 *Xho*I Half-site Arms Cloning System (Promega Co., Madison, WI, USA). The clone containing the AK gene was identified by plaque hybridization using AK cDNA (Kawai *et al*., 1992) as a gene probe. The clone thus obtained was subcloned into the pBluescript II vector (Stratagene, La Jolla, CA, USA) and sequenced using a Bca BEST Dideoxy Sequencing Kit (Takara Shuzo Co., Tokyo, Japan).

Expression vector and plant transformation

The 5′-flanking region of the AK gene was fused with the upstream end of the GUS (β-glucuronidase) coding sequence of pBI101 (ClonTech Laboratories Inc., Palo Alto, CA, USA) and the resulting plasmid was named pBI/AK-GUS. The GUS gene of pBI/AK-GUS was replaced with the PAT gene and a *bar* gene from *Streptomyces hygroscopicus* (Murakami *et al*., 1986), and the resulting construct was named pBI/AK-PAT. The pBI121 vector (ClonTech) possessing the cauliflower mosaic virus 35S promoter-PAT was used as a positive control vector. All expression vectors contained the neomycin phosphotransferase (NPTII) coding gene as a selectable marker.

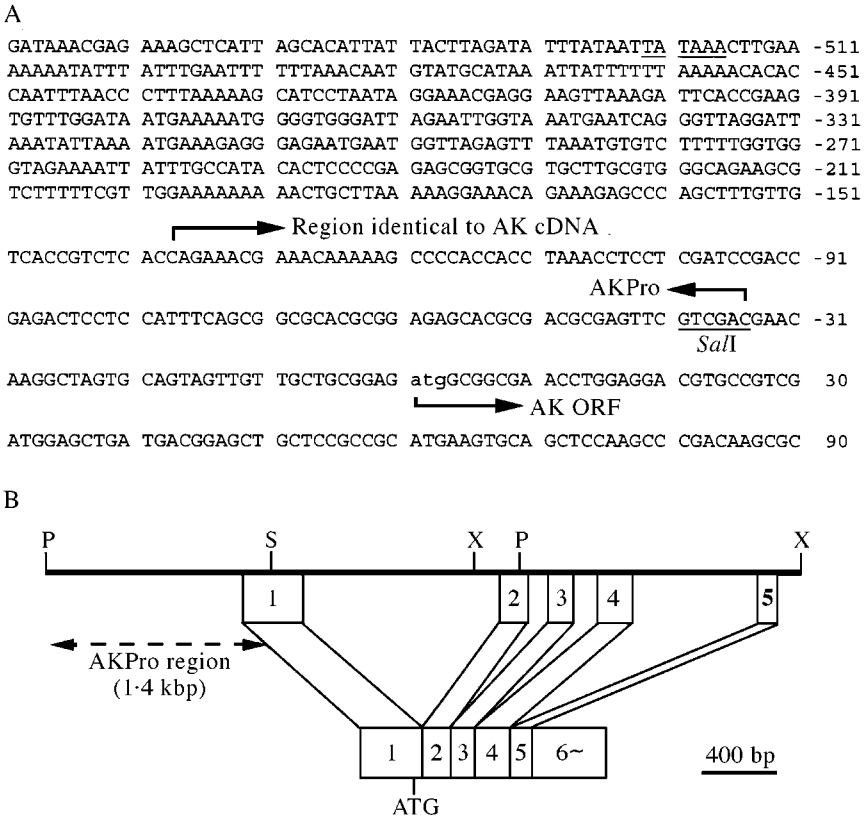

FIG. 1. A, Part of the nucleotide sequence covering the 5′ region of the AK gene: 5′ end of the open reading frame of the AK gene, 3′ end of the AKPro fragment (used in this study) and the regions identical to the AK cDNA sequence are indicated with solid arrows. B, Genomic structure and restriction map of a DNA fragment containing the AK gene (4·2 kbp). The image shows the genomic DNA (top) and mRNA structures (bottom). Boxes indicate exons. The 1·4 kbp fragment (AKPro) at the 5′ end was used as the promoter region. P, *Pst* I; S, *Sal* I; X, *Xba* I.

TABLE 1. *Comparison of nucleotide sequences at the exon–intron junctions of the rice adenylate kinase gene*

Number	Sequence at exon–intron junction: Splice donor . . . splice acceptor	Exon size (bp)	Intron size (bp)	Intron GC content (%)
1	TCG TCG **gt**aacgcc . . . aattttt**ag** GTC CAC	103	1263	38.6
2	AC AAG **gt**agtttt . . . tcttct**ag** GGA GAG	146	87	31.0
3	CAG AAG **gt**gaggtc . . . tggtgc**ag** CTT GAT	120	120	35.0
4	GAT GAT **gt**aagtca . . . tcacaa**ag** GTT ACT	168	644	33.1
5	AAG CCT **gt**atgttt . . . –	90	–	–

The 5′-donor GT and the 3′-acceptor AG of the intron are indicated in bold.

Expression vectors were introduced into *Agrobacterium tumefaciens* strain LBA4404, which was then used to transform leaf discs of *Nicotiana tabacum* 'Bright Yellow' (Horch *et al*., 1985). Transgenic plants regenerated on MS agar medium (Murashige and Skoog, 1962), containing 50 μg ml^{-1} kanamycin were transferred to pots and cultured in a glasshouse under standard conditions.

Detection of inserted genes

Total DNA was extracted from plant tissues with cetyl (hexadecyl) trimethylammonium bromide (CTAB), essentially as described by Wanger *et al*. (1987). Inserted DNA was detected by PCR. In the case of AK-GUS transgenic plants, the sense strand primer 5′-CCCAGCTTTGTT GTCACCGTC-3′ (AAK1) and the antisense strand primer 5′-CCCGGCTTTCTTGTAACGCGCT-3′ (AGUS1) were used. PCR conditions were 30 cycles of 94 °C for 1 min, 55 °C for 1 min, and 72 °C for 1 min using PCR Thermal Cycler MP (Takara). In both AK-PAT and AK-GUS plants, primers AAK1 and 5′-TGAGCGAAACCCTATAAGAA CCC-3′ (F3) were used. PCR conditions were 40 cycles of 96 °C for 1 min, 62 °C for 1 min, and 72 °C for 1 min. PCR products were run on a 1 % agarose gel followed by staining with ethidium bromide.

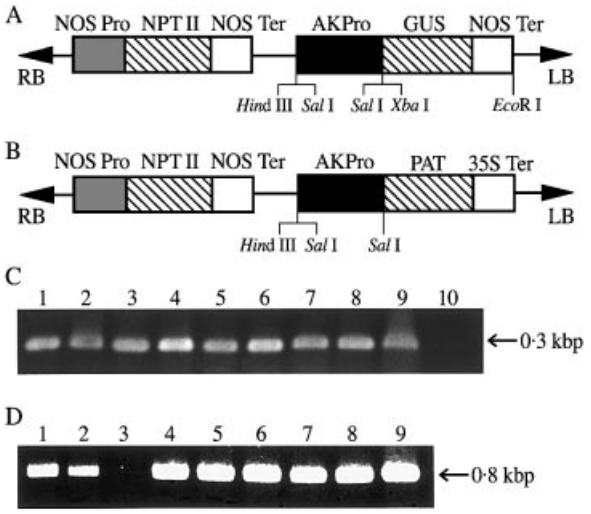

FIG. 2. Transformation vector pBI/AK-GUS (A) and pBI/AK-PAT (B). NPTII, Neomycin phosphotransferase gene; GUS, β-glucuronidase gene; PAT, phosphinothricin acetyltransferase gene; AKPro, promoter sequence of AK; RB and LB, right and left borders of t-DNA, respectively. C, Electrophoretic analyses of PCR products from transgenic tobacco plants. DNA band (0·3 kbp) was amplified using AAK1 and AGUS1 primers. Lanes 1–8, amplified DNA of AK-GUS tobacco plants; lane 9, pBI/AK-GUS positive control; lane 10, wild-type. D, DNA band (0·8 kbp) was amplified using AAK1 and F3 primers. Lanes 1–8, amplified DNA of AK-PAT tobacco plants; lane 9, pBI/AK-PAT positive control.

Histochemical GUS assay

Seeds of AK-GUS tobacco plants (homozygous T_1 generation) were germinated on MS agar plates, and the seedlings were used for histochemical GUS assay (Jefferson, 1987). Plant tissues were incubated in 1 mM 5-bromo-4-chloro-3-indolyl-D-glucuronic acid (X-Gluc), 50 mM potassium phosphate buffer (pH 7·0) and 0·1 % (v/v) Tween-20 at 37 °C overnight. For aerial tissues, pigments were removed by incubation in 70 % ethanol after staining.

Herbicide tolerance test

AK-PAT transgenic tobacco plants were treated with the commercial application rate of phosphinothricin. Scoring for tolerance was carried out 9 d after application of the herbicide. A commercial formulation of glufosinate (L-Basta, Hoechst, Germany) was applied at 40 mg m^{-2}.

RESULTS AND DISCUSSION

Out of 2×10^6 plaques, 11 clones hybridized with the AK cDNA probe. A positive clone containing 20 kbp genomic DNA was found to correspond to the AK sequence. Subclones containing the *Pst*I fragment (2·9 kbp) and the *Xba*I fragment (1·6 kbp) hybridized to the cDNA probe were subjected to entire nucleotide sequencing.

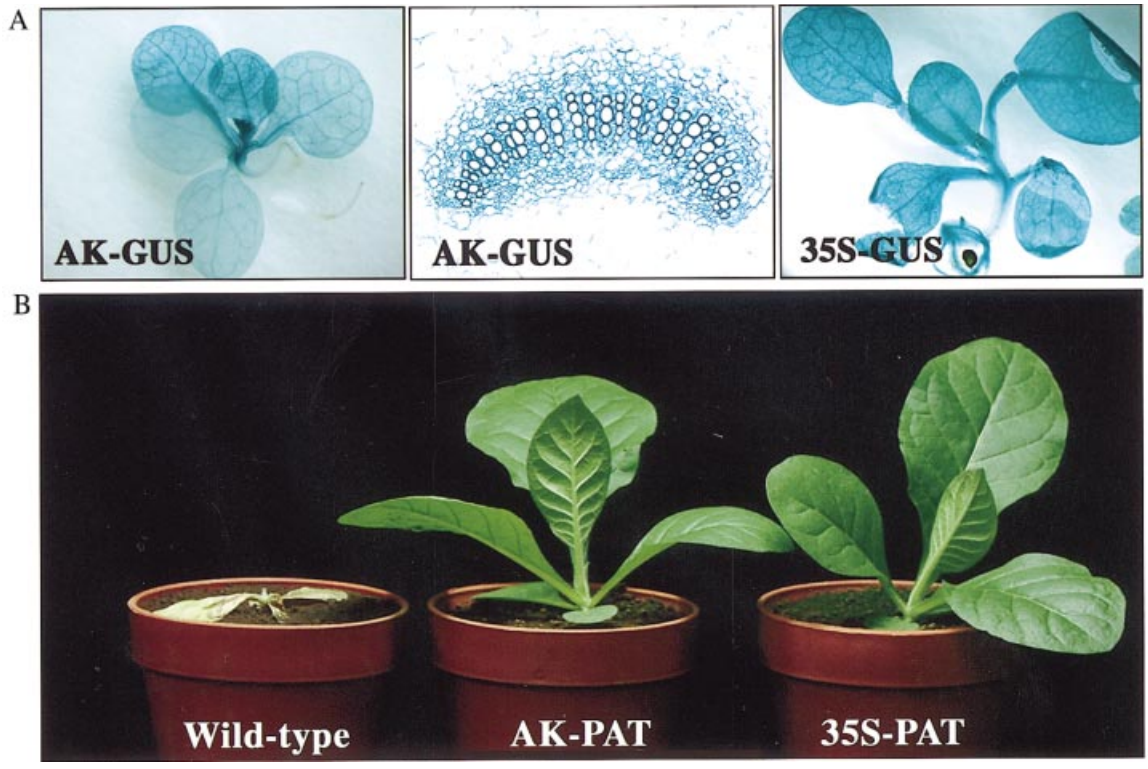

FIG. 3. A, GUS expression in seedlings of transgenic tobacco grown on MS agar medium. Seedlings were stained for GUS activity and decolourised with 70 % ethanol. In the centre is a cross-section of the AK-GUS plant, which shows GUS staining in the region surrounding xylem tissue (horizontal field of the picture approx. 2 mm). B, Glufosinate tolerance of transgenic tobacco plants at commercial application rate (40 mg m^{-2}). Plants were photographed 9 d after herbicide application. AK, Adenylate kinase; GUS, β-glucuronidase; 35S, cauliflower mosaic virus 35S promoter; PAT, phosphinothricin acetyltransferase.

We determined the nucleotide sequence possessing 5′ upstream of AK, namely the 4·3 kbp region between the left *Pst*I site and the right *Xba*I site (Fig. 1B). This sequence has been deposited in the DDBJ database under the accession number AB01773. Figure 1A shows part of the nucleotide sequence of the AK gene. A TATA box is present 0·5 kbp upstream of the first ATG codon of the cDNA. Sequence alignment against cDNA indicates that genomic AK possesses five exons, four introns and a 5′ upstream region of approx. 1·4 kbp (Fig. 1B). The exon/intron boundaries are listed in Table 1. All of the determined donor splice sites and acceptors sites contained 'GT' and 'AG' consensus splice sequences, respectively.

The *Sal*I restriction fragment of the genomic clone (AKPro) was inserted into the *Sal*I site of pBI101 (Fig. 2 A). pBI/AK-GUS digested with *Xba*I–*Eco*RI and filled by T4-DNA polymerase was ligated with the blunt-ended PAT gene (Murakami *et al*., 1986; Fig. 2B).

AK-GUS and AK-PAT transgenic tobacco plants resistant to kanamycin were analysed by PCR to detect the inserted genes. Bands of approx. 0·3 and 0·8 kbp were detected in AK-GUS and AK-PAT tobacco plants, respectively (Fig. 2C and D).

In AK-GUS tobacco plants, GUS staining was seen in the vascular tissues. In contrast to 35S-GUS, GUS staining in AK-GUS plants was very weak in the lower part of the plants including the roots (Fig. 3A).

We previously reported an intense expression of rice AK protein in vascular tissues by a tissue-print immunoblot method (Kawai and Uchimiya, 1995*b*), but the precise localization of this protein was not investigated thoroughly. In the present study we isolated a genomic clone of AK and demonstrated its promoter activity in tobacco plants. Using a histochemical GUS assay, expression of the AK promoter was observed mainly in the upper part of the plant, in the growing tip and the vascular system.

Hayashi and Chino (1990) reported the presence of high levels of adenine nucleotides in the phloem sap of rice plants. In phloem sap, ATP is the direct source of energy for phloem loading of sucrose (Spanswick, 1986). Thus, localization of AK in sieve elements or companion cells suggests a possible role of this enzyme in the synthesis or loading of sucrose (Fig. 3A). This hypothesis is supported by the high AK promoter activity observed in young leaves and growing tips, but weak activity in old leaves.

Germination tests in the presence of bialaphos (10–100 mg l^{-1}) showed that only seedlings harbouring AK-PAT were able to grow; control plants did not grow (data not shown). At the whole plant level, the resistance of AK-PAT plants against glufosinate treatment was comparable with that of 35S-PAT plants. However, the tolerance was found to be weak in the lower part of AK-PAT plants. AK-PAT plants showed tolerance to glufosinate to a level four times higher than the commercial application rate (40 mg m^{-2}). This indicates that the promoter activity of AKPro was sufficient to drive tolerance to exogenously supplied herbicide in tobacco plants. Further application of this promoter for ectopic expression of other useful genes will be the subject of future studies.

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