Theor Appl Genet (1998) 97:810–815 

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# Characterization of transgenic rice plants that express *rgp1*, the gene for a small GTP-binding protein from rice

Received: 21 September 1997/Accepted: 31 March 1998

**Abstract** The *rgp1* gene, which encodes a small GTP-binding protein from rice, was introduced into rice protoplasts by electroporation. Transformed protoplasts were cultured on liquid protoplast-culture medium for 1 month, and then cells that had proliferated were transferred to a selection medium that contained 50 mg/l hygromycin B. Among 50 colonies that were selected and transferred to regeneration medium, 3 colonies generated shoots. However, two of the three shoots failed to form roots and ceased growing. A single regenerated shoot that formed roots was planted in soil and transferred to a greenhouse. Southern hybridization showed that the regenerated plant harbored a single copy of the introduced gene. The transformant ($T_0$) plant was shorter than the controls, it developed three times as many tillers as controls, it developed three times as many tillers as control plants but it produced mostly sterile seeds. In a test of hygromycin resistances, viable seeds segregated into resistant and sensitive seedings at a ratio of approximately 1:3. The progeny ($T_1$) plants were short with many tillers, and some produced seeds normally. The $T_2$ seedlings grew more rapidly than control seedlings for the first 28 days after germination, but control plants subsequently outgrew the $T_2$ plants. Northern blotting analysis revealed that the *rgp1* gene in $T_2$ plants was expressed consitutively throughout all developmental stages. The results suggest that the observed phenotypic changes were due to expression of the exogenous *rgp1* gene.

Communicated by Yu. Gleba

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**Key words** Protoplast · Electroporation · *rgp1* · Tillering · Transgenic rice

## Introduction

Since the discovery of the *ras* proto-oncogene in 1981 (Gruenbaum et al. 1981), more than 100 genes from eukaryotes that encode small GTP-binding proteins have been described (Matsui et al. 1989; Anai et al. 1991; Anuntalabhochai et al. 1991; Sano and Youssefinan 1991; Palme et al. 1992; Drew et al. 1993; Yang and Watson 1993; Youssefian et al. 1993; Merkle et al. 1994; Dombrowski and Raikhel 1995). Detailed analysis has revealed that small GTP-binding proteins with molecular masses of 20–30 kDa constitute a superfamily which can be divided into three major subfamilies according to the functions of the various proteins (Chardin et al. 1989b). The protein in the *ras* subfamily are essential for signal transduction (Barbacid 1987), the proteins in the *rho* subfamily are required for cytoskeletal organization (Chardin et al. 1989a) and those in the *rab* subfamily are involved in intercellular transportation (Balch 1989, 1990).

More than 40 genes that encode small GTP-binding proteins have been isolated from higher plants, and most of these genes belong to the *rab* subfamily (Ma 1994; Redhead and Palme 1996). Their physiological functions remain, however, to be characterized. Complementation analysis of mutant strains of yeast has revealed that a number of plant *rab*-related proteins do indeed function in intracellular trafficking (Redhead and Palme 1996). A genetic approach using transgenic plants has also provided some clues to the biological functions of small GTP-binding proteins. In transgenic soybean plants that expressed sRAB1 or vRAB7, both the growth of nodules and nitrogenase activity were severely reduced, an indication that these genes might be involved in membrane synthesis (Cheon et al. 1993).

When tobacco plants were transformed with *rgp1*, a distinct morphological change was observed, with plants exhibiting a reduction in apical dominance and dwarfism (Kamada et al. 1992). Further analysis revealed that these plants constitutively produced high levels of endogenous cytokinins, with resultant abnormal responses to mechanical wounding, such as the accumulation of salicylic acid (Sano et al. 1994). It was, therefore, suggested that *rgp1* might be involved in the biosynthesis of cytokinins and/or in related metabolic pathways (Sano et al. 1994).

In order to obtain more information about the function of *rgp1* and, also, to determine whether or not the phenotypic changes observed in transgenic tobacco plants might reflect a general effect of *rgp1*, we attempted to introduce the gene into rice plants. The present paper describes the construction of stable rice transformants that harbored an exogenous *rgp1* gene. The phenotypic characteristics of these transformants resembled those of the tobacco counterparts.

## Materials and methods

### Plant materials and culture of protoplasts

Anthers of rice (*Oryza sativa* L. var 'Yamahoushi') containing uninucleate pollen grains were platted on B5 medium (Gamborg et al. 1968) supplemented with 2 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D) and 0.8% (w/v) agar to induce the formation of callus. The resultant calli (wet wt., approx 2 g) were transferred to 35 ml of AA medium (Toriyama and Hinata 1985) and cultured, with reciprocal shaking at 120 rev/min, in a 100-ml flask to establish a suspension culture of cells. Cells were grown in darkness at 25°C and subcultured at 2-week intervals.

Crude protoplasts were prepared by incubating suspension-cultured cells in a solution of enzymes [1.6% (w/v) Cellulase Onozuka R10 (Yakult Biochemicals, Japan), 0.3% (w/v) Macerozyme R10 (Yakult Biochemicals), 8% (w/v) mannitol nd 0.1% (w/v) $CaCl_2$-$2H_2O$ (pH 5.5)] for 3 h at 25°C. The mixture was filtered over a nylon net (50-μm mesh), and protoplasts were washed twice with MES buffer [0.5 m*M* 2-(morpholino) ethanesulfonic acid (MES), 7 m*M* KCl, 4 m*M* $CaCl_2$-$2H_2O$ and 0.36 *M* mannitol] by centrifugation at 80 *g* for 5 min. Protoplasts were loaded on a 25% (w/v) solution of sucrose, subjected to centrifugation at 80 *g* for 5 min and washed once with MES buffer. The population density of the protoplasts was adjusted to $10^7$/ml, and plasmid DNA was added to a final concentration of 100 μg/ml. After the mixture had been subjected to an electrical pulse (alignment, 40 Vpp for 30 s; electroporation, 400 V in a 20 pulse width) with a BTX Electro Cell Manipulator 200 (model ECM-200; Biotechnologies and Experimental Research, San Diego, Calif.), protoplasts were washed once with washing solution [8% (w/v) manitol and 0.1% $CaCl_2$-$2H_2O$] and diluted with an equal volume of Gelrite solution [0.3% (w/v) Gelrite (Wako Pure Chemical Industries, Japan), 3% (w/v) glucose]. The electroporated protoplasts were then suspended in protoplast culture medium [MS medium (Murashige and Skoog 1962) plus 3% sucrose, 5% glucose, 1 mg/l 2,4-D and 0.5 mg/l kinetin] in plastic petridishes. Non-treated protoplasts were cultured under the same conditions.

During the first month of culture, cells were incubated in darkness at 25°C. Then they were transferred, for selection, to medium supplemented with 50 mg/l hygromycin B and grown under continuous fluorescent light (4 W/m$^2$) at 25°C. Selected microcalli were subsequently cultured in shooting medium (MS medium plus 3% sucrose, 1 mg/l N6-benzylaminopurine and 0.1 mg/l naphthaleneacetic acid). After 2 months, regenerated shoots were transferred to rooting medium (MS medium without hormones), and the single regenerant that formed roots was further cultured in a greenhouse.

### Construction of the vector

A sense-oriented 1.3-kb cDNA derived from *rgp1*, which had originally been subcloned at the *Eco*RI site (position 872) of pUC119, was inserted into the binary vector pBI121 (Clonetech) at the *Sma*I and *Sac*I sites. The resultant vector was digested with *Eco*RI and *Hind*III to excise the fragment that included *rgp1*, the CaMV 35S promotor and the Nos terminator, which was then inserted into pUC119. A hygromycin-resistance gene (kindly provided by Dr. K. Shimamoto, Nara Institute of Science and Technology, Japan) was inserted into the pUC119-based vector. The resulting recombinant plasmid was designated pRGP119A and used for transformation (Fig. 1).

### Cytological analysis

Pollen obtained from transgenic ($T_0$) and control rice plants was stained with a 1% (w/v) solution of acetocarmine. The frequency of stained pollen grains was determined for an estimation of the fertility rate.

### Hygromycin-resistance test

Seeds of transgenic ($T_1$) and control rice plants were surface-sterilized for 15 min in a 2% (w/v) solution of sodium hypochlorite, washed twice in distilled water, placed on solid MS medium supplemented with 3% (w/v) sucrose plus 0.8% (w/v) agar and cultured under continuous fluorescent light (4 W/m$^2$) at 25°C. After days, seedlings at the same developmental stage were transferred to solid MS medium supplemented with 30 mg/l hygromycin B, 3% (w/v) sucrose and 0.8% (w/v) agar, and cultured under the same conditions for 2 weeks. Growth rates were evaluated by measuring lengths of shoots.

### Isolation and analysis of DNA

Total DNA was prepared from entire transgenic rice ($T_2$) and control rice seedlings by the method described by Honda and Hirai (1990). After digestion of the DNA with appropriate endonucleases (*Pst*I, *Eco*RV, *Bam*HI, *Hind*II, *Eco*RI, *Xho*I, *Bgl*II, *Sal*I and *Sac*I), Southern hybridization was performed with a non-radioactive DNA

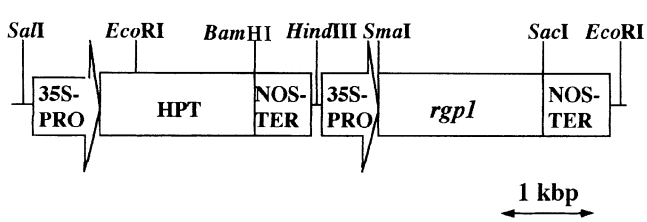


**Fig. 1** Construction of plasmid pRGP119A. *HPT* gene for hygromycin phosphotransferase, *NOS-TER* terminator of a gene for nopaline synthase, *35S-PRO* CaMV 35S promoter, *rgp1* rice cDNA encoding a GTP-binding protein. This fragment was inserted in pUC119 to generate pRGP119A

labeling and detection kit (Boehringer Mannheim, Germany). A cDNA of the hygromycin-resistance gene (*HPT*) was used as the probe.

### Isolation and analysis of RNA

Entire young plants were homogenized and homogenates were placed in a centrifuge tube with 5 ml of extraction buffer [100 m*M* LiCl, 100 m*M* Tris-HCl (pH 8.0), 10 m*M* ethylenediaminetetraacetic acid (EDTA) and 1% (w/v) sodium dodecyl sulfate (SDS)] and 5 ml phenol at 65°C. Each extract was mixed with 5 ml of a 24:1 (v/v) mixture of chloroform and isoamyl alcohol and then centrifuged at 9,250 *g* for 15 min at 4°C. The upper layer was supplemented with 5 ml of 4 *M* $LiCl_2$ and incubated for 1 h at −70°C. After centrifugation at 9,250 *g* for 15 min at 4°C, the pellet was dissolved in 1 ml of water. Northern hybridization analysis was performed with a non-radioactive DNA labeling and detection kit (Boehringer Manheim). A cDNA of the *rgp1* gene was used as the probe.

## Results

### Transformation of protoplats

Rice cells were maintained in suspension medium by routine sub-culture at 2-week intervals. For the isolation of protoplasts, cells from a 2-month-old suspension culture were harvested 4–6 days after the last sub-culture (Fig. 2a). Protoplasts were adjusted to a final population density of $10^7$/ml and mixed with vector plasmid DNA (pRGP119A; Fig. 1) at a final concentration of 100 μg/ml. After electroporation, cells were cultured for 1 month, at which time the colonies became visible to the naked eye (Fig. 2b). These colonies were transferred to the selection medium, which contained 50 mg/l hygromycin B. Among 50 colonies obtained, only 3 colonies regenerated shoots (Fig. 2c). Two these regenerants failed to form roots, but a single regenerant successfully formed roots and was grown to maturity in the greenhouse (Fig. 2d).

### Characterization of the $T_0$ transgenic plant

The original transgenic plant ($T_0$) had distinct phenotypic characteristics (Fig. 2d). It produced about three times as many tillers as the control plants (Table 1). In addition, the transgenic plant was at least 20 cm shorter than the control plants (Table 1). The transgenic plant produced spikes and flowering organs in the normal manner. However, while pollen grains from control plants were strongly stained with acetocarmine, most of the pollen grains from the trasgenic plant were stained (Fig. 2e), an indication that they were sterile. The transgenic plant produced a total of 146 viable seeds.

### Characterization of $T_1$ progeny plants

Seeds obtained from the original $T_0$ transgenic plant were tested for resistance to hygromycin B (30 mg/l).

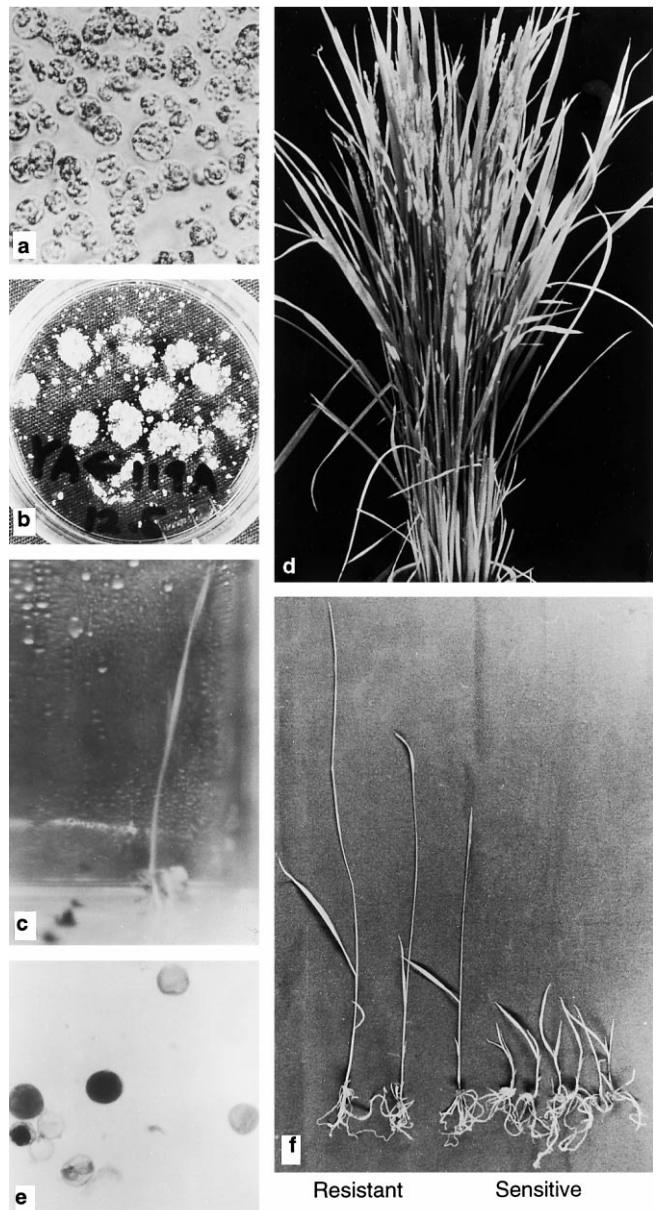


**Fig. 2** **a** Protoplasts isolated from rice cells in suspension culture, **b** colonies after 1 month in culture, **c** regeneration of a shoot from a selected callus, **d** transgenic rice plant, **e** pollen from the transgenic plant ($T_0$) after staining with 1% acetocarmine, **f** test for resistance to hygromycin

We found that 20 seedlings were resistant and 47 seedlings were sensitive to the drug (Fig. 2f). The ratio of resistant to sensitive seedlings was approximately 1:3. The $T_1$ progeny selected for resistance to hygromycin were all fertile. The mature $T_1$ plants were again shorter but produced three times more tillers than the control plants. The number of seeds per ear of $T_1$ plants was slightly lower than that of the control plants. However, because of the increased number of tillers, the total number of seeds of each $T_1$ plant was 2.6 times higher, on average, than that of the control plants (Table 2).

**Table 1** Characterization of the transgenic rice plant ($T_0$)

| Plant | Number of tillers | Fertility (%) | | Plant height (cm) | Number of seeds per ear |
|---|---|---|---|---|---|
| | | Seeds | Pollen | | |
| Yamahoushi | 11.5 (± 1.6)[a] | 99.8 (± 10.1) | 97.4 (± 6.5) | 107.3 (± 8.4) | 142 (± 27.2) |
| Transgenic rice ($T_0$) | 32.0 | 6.1 | 30.6 (± 1.3) | 87.0 | 4.2 (± 1.3) |

[a]Values in parentheses are standard errors ($n \geq 10$)

**Table 2** Characterization of the transgenic rice plants ($T_1$)

| Plant | Number of tillers | Fertility of seeds (%) | Plant height (cm) | Number of seeds per ear |
|---|---|---|---|---|
| Yamahoushi | 3.8 (± 1.2)[a] | 93.9 (± 1.8) | 111 (± 2.7) | 154 (± 27) |
| Transgenic rice ($T_1$) | 11.2 (± 2.9) | 95.3 (± 1.6) | 99.8 (± 2.3) | 138 (± 23) |

[a]Values in parentheses are standard errors ($n \geq 10$)

## Growth of $T_2$ plants

We examined the effect of *rgp1* on plant growth by monitoring the height of $T_2$ plants after germination. Seeds were incubated at 37°C for 2 days in darkness to allow germination, and then seedlings were grown in the greenhouse. During the first week after germination, the heights of $T_2$ and control plants were similar. (From 10 and 25 days after germination $T_2$ plants) grew more rapidly than control plants. However, the growth of $T_2$ plants was slower than that of control plants for the next 4 weeks. Finally, the height of mature $T_2$ plants was approximately 80% of that of control plants (Fig. 3). Flowering occurred 118 days after germination in $T_2$ plants, whereas it occurred 124 days after germination in wild-type plants.

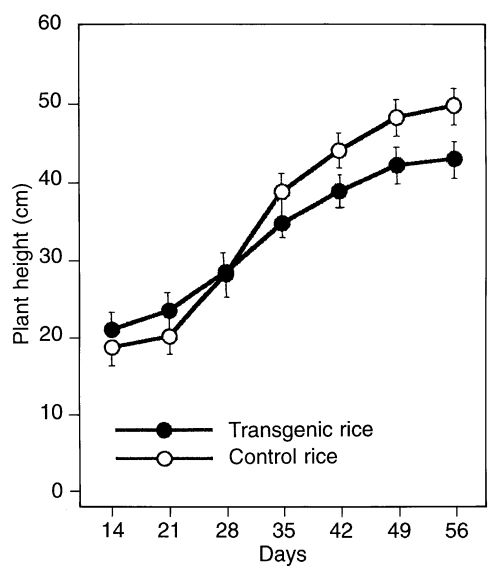


**Fig. 3** Increases in plant height during the growth of transgenic and control seedlings. Results are means from eight or more plants in each case. *Bars* show standard errors ($n \geq 10$)

## Southern and northern hybridization

The number of copies of the *rgp1* transgene was estimated by Southern hybridization. To avoid cross-hybridization of the probe for the transgene with endogenous *rgp1*, we used the *HPT* gene as the probe. Total DNA was isolated from seedlings of a $T_2$ plant, digested with appropriate endonucleases (*Pst*I, *Eco*RV, *Bam*HI, *Hind*III, *Eco*RI, *Xho*I, *Bgl*I, *Sal*I and *Sac*I) and subjected to hybridization. One or two specific bands were obtained with most of the restriction endonucleases, indicating that *rgp1* had been introduced as a single-copy gene into the genomic DNA of the $T_2$ plants (Fig. 4).

To investigate the expression of the *rgp1* transgene, we performed northern hybridization with total RNA that had been extracted from entire young plants 14, 28, 42 or 56 days after germination. After electrophoresis, bands of RNA were allowed to hybridize with *rgp1* cDNA as the probe. In $T_2$ transgenic plants that contained an exogenous *rgp1* gene, as confirmed by Southern analysis, *rgp1* appeared to be expressed constitutively at all stages of development (Fig. 5). In control plants, the level of transcripts of the endogenous *rgp1* gene was generally low and expression was transient, with the transcript detectable only from 28 to 42 days after germination (Fig. 5).

## Discussion

This paper describes the production of transgenic rice plants that expressed an exogenous *rgp1* gene. These plants had distinct characteristic, namely, dwarfism, early flowering and high grain yield, and these characteristics were stable and heritable.

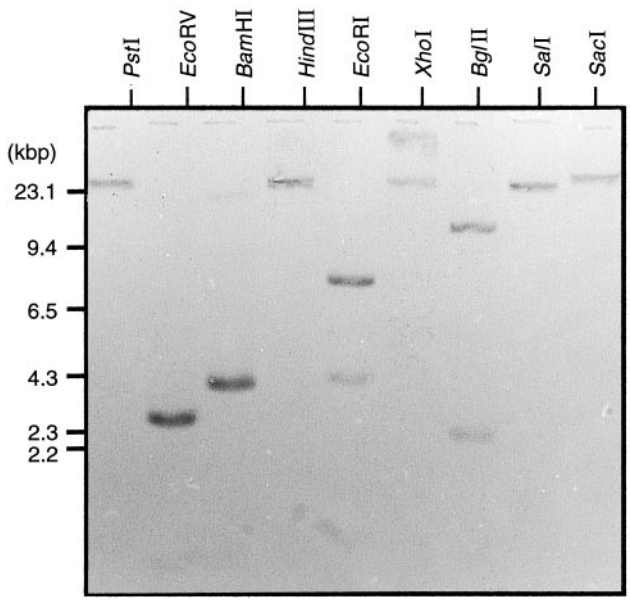


**Fig. 4** Results of Sourthern hybridization of genomic DNA. Aliquots of 20 μg of total DNA were digested with *Pst*I (*lane 1*), *Eco*RV (*lane 2*), *Bam*HI (*lane 3*), *Hind*III (*lane 4*), *Eco*RI (*lane 5*), *Xho*I (*lane 6*), *Bgl*II (*lane 7*), *Sal*I (*lane 8*) and *Sac*I (*lane 9*), and digests were fractionated by electrophoresis on a 0.8% agarose gel. Bands of DNA were transferred to a nylon membrane and allowed to hybridize with nonradioactive digoxigenin-labeled the *HPT* gene as probe

We previously isolated a gene for a small GTP-binding protein from rice, which we designated *rgp1* (Sano and Yousssefian 1991). The molecular structure of the encoded protein was similar to that of the *rab* group of small GTP-binding proteins, which are involved in intracellular transportation via the activation of individual vesicles around the endoplasmic reticulum and Golgi apparatus (Balch 1989). The similarity between the product of *rgp1* and *rab* proteins suggests that the protein might function in vesicle transport, but no clear evidence has so far been presented. In an attempt to determine the physiological functions of *rgp1*, we introduced this gene into tobacco plants. The resultant transgenic plants showed clear morphological and physiological differences from the wild type, with a reduction in apical dominance, dwarfism, abnormal flower structure and enhanced resistance to viral and bacterial infection (Kamada et al. 1992; Sano et al. 1994). The levels of endogenous cytokinins in the transgenic plants were six fold higher than in wild-type tobacco plants, and it was suggested that the specific characteristics of the transgenic plants were primarily a consequence of abnormally high levels of cytokinins (Sano et al. 1994). However, the relationship between elevated levels of cytokinins and *rgp1* remains to be explained.

In order to obtain more precise insight into the physiological functions of *rgp1*, we attempted to construct transgenic rice plants in which the level of the product was either enhanced by the introduction of a sense gene or reduced by an antisense gene. The introduced gene originated from rice itself and, therefore, we would expect the effects to be more direct and "natural" than in the case of the transgenic tobacco plants. Transgenic rice protoplasts were obtained by electroporation of sense-oriented *rgp1*, with the *HPT*

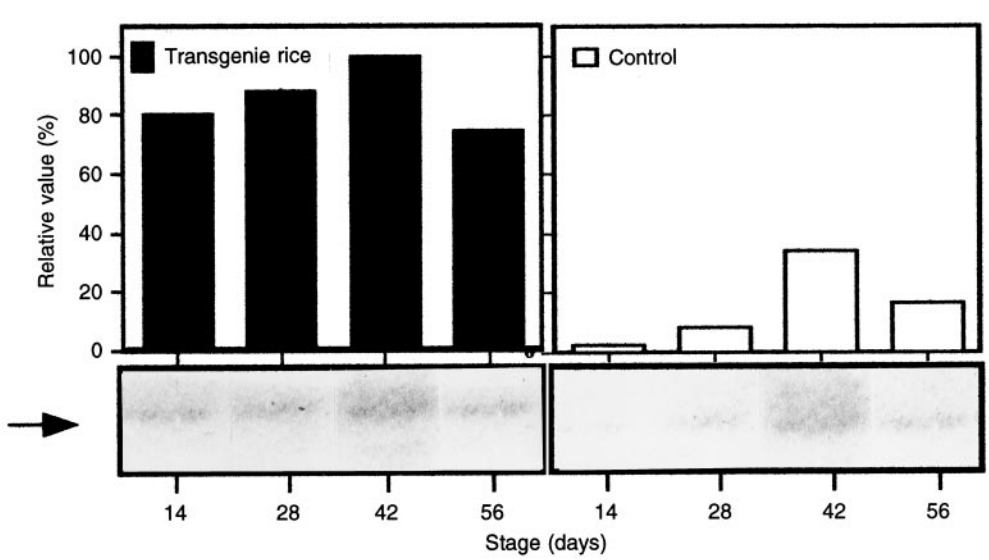


**Fig. 5** Northern hybridization analysis of the expression of the *rgp1* gene in transgenic ($T_2$) plants and control plants. Aliquots of 50 μg of total RNA were frationated by eletrophoresis on a 1.5% formaldehyde/agarose gel. Bands of RNA were transferred to a nylon membrane and allowed to hybridize with digoxigenin-labelled cDNA of the *rgp1* gene as probe

gene as the selective marker. Although many calli were formed on MS medium supplemented with 50 mg/l hygromycin B, only 1 of these regenerated shoots, suggesting that the balance of endogenous might have been changed by introduction of the gene. The transgenic $T_0$ plant grew to maturity and had distinct morphological characteristics. It was short in stature, with early flowering and tillering, and all these features were stably inherited by the $T_1$ and $T_2$ progeny. In the $T_2$ progeny, although the number of seeds per ear was slightly lower than in wild-type plants, the number of tillers was much greater so that the total number of seeds was 2.6-fold higher than in wild-type plants.

Accumulation of *rgp1* transcripts in transgenic plants was constitutive at all stages of growth. In wild-type plants, transcripts were first detected 14 days after germination, reaching a maximum level at 42 days and then gradually decreasing. It seems very likely that the consitutive accumulation of *rgp1* transcripts was the cause of the observed morphological alterations. These alterations were similar to those seen in transgenic tobacco plants. Thus, it seems that *rgp1* functions generally at a particular stage of plant growth, perhaps by regulating levels of hormones, for example, cytokinins. Moreover, since the effect of the *rgp1* transgene is similar in rice and tobacco plants, this gene may be useful in the creation of crop plants with new traits, such as improved grain yield.

**Acknowledgements** This study was carried out under the Joint Research Program of the Institute of Genetic Ecology, Tohoku University.

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