Increased Transgene Expression by Breeding and Selection in White Clover

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ABSTRACT

To determine if standard breeding methodology is applicable to transgenes, phenotypic recurrent selection was used to select for increased transgene expression in white clover, *Trifolium repens* L. Plants were transformed with *npt*II and *gus*A, and selected on 100 mg L^{-1} of kanamycin. Independently transformed plants were intercrossed, and the progeny was germinated on 200, 300, or 400 mg L^{-1} of kanamycin. Those seedlings surviving on 400 mg^{-1} were in turn intercrossed, and the progeny was selected on 300, 400, or 500 mg L^{-1} of kanamycin. NPTII levels were measured in each selected population, and Southern blots were made from individuals in each population. The highest-expressing individual in the T_2 had levels of NPTII that were more than four times higher than those in the highest parent. With selection on increasing levels of kanamycin, average expression across each generation went from 0.033 ng μg^{-1} NPTII in the parents to 0.095 ng μg^{-1} in the selected T_1 plants to 0.539 ng μg^{-1} in the selected T_2 plants. Southern hybridization suggested that plants displaying a heightened level of *npt*II expression in the T_1 and T_2 fell into two categories. The first contained one particular transgenic event, implicating the importance of other genomic factors in modulating gene expression. Alternatively, the plants had an accumulation of various *npt*II loci, suggesting an association between multiple transgene copies and high expression levels. On the basis of these results, selection for transgene expression appears to be a viable option for plant breeding programs.

Transgenic crops commercialized to date (http://www.agbios.com/main.php; verified 22 January 2004) are vegetatively propagated (e.g., potato, *Solanum tuberosum* L.), inbred [e.g., soybean, *Glycine max* (L.) Merr., cotton, *Gossypium* spp.], or hybrids derived from inbred lines (e.g., maize, *Zea mays* L.). For those crops grown from seed, individual transgenes have received regulatory approval for commercialization. These transgenes have then been backcrossed into a variety of different genetic backgrounds. Strategies for deployment of transgenes in cross pollinated, perennial crops, such as forages, are not as obvious or readily evident. Forage cultivars are normally generated as synthetic cultivars, derived by intercrossing tens, if not hundreds, of improved parents. Backcrossing is generally avoided, since backcrossing of the transgene to each individual parent is required. Furthermore, backcrossing leads to inbreeding, and many forage crops are sensitive to inbreeding depression (Jones and Bingham, 1995). To help overcome the limitations associated with traditional backcrossing, a transgene backcross strategy specific for cross-pollinated crops has been designed (Micallef et al., 1995), whereby a different set of parents is used in each backcross generation. Such a strategy is based on the premise that only one transgene should be deployed to avoid gene silencing between transgenes at different loci (Matzke and Matzke, 1990).

If transgenes at different loci do not silence each other, an alternative strategy could consist of simply intercrossing multiple, independently derived transgenics as parents for a synthetic cultivar and selecting for gene expression. Previous work in tobacco evaluated for β-glucuronidase (GUS) levels determined that while some transgenes at different loci can silence each other when crossed into the same plant, other transgenic loci have the ability to act additively and increase the total transgene expression in the plant (Conner et al., 1998; Nap et al., 1997).

Besides the interactions that occur between the same transgene at different loci, the genetic background of the plant can affect transgene expression. Crossing transgenic petunia plants carrying a maize *dfr* gene into elite genotypes bred for stable flower color was sufficient to stabilize a novel orange flower color (Oud et al., 1995). Similarly, GUS expression varied 4-fold between individuals within BC_1, BC_2, and F_2 populations of white clover plants containing the same, single *gusA* locus, although mean *gusA* expression remained the same when averaged between populations as a whole (Scott et al., 1998).

The implications are clear. It should be possible to use plant breeding techniques to enhance transgene expression by selecting for a genetic background that favors transgene expression and/or by the selection or accumulation of transgene loci that have the ability to act additively. Therefore, we performed two cycles of phenotypic recurrent selection on white clover plants transgenic for kanamycin resistance (*npt*II) and GUS (*gus*A).

MATERIALS AND METHODS

Plant Materials

White clover is a perennial, out-crossing forage species with disomic genetics (Taylor, 1985; White et al., 2000), thus facilitating long-term studies for breeding and selection. The seeds used were from the Southern Regional Virus Resistant germplasm (Gibson et al., 1989) or from FL-RL. In this case, a given germplasm represents a mixture of genotypes. Both germplasms are derived from agronomically adapted cultivars; the former being selected for virus resistance, and the latter for the presence of a distinctive red leaf mark, like that described by Pederson (1995).

Bacteria and Transformation

Transformation took place in 1995, by the procedure of Voisey et al. (1994). Briefly, seeds were placed in a tea strainer and sterilized in 70% (v/v) ethanol for 5 min, followed by 15

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min in 25% (v/v) commercial bleach (1.5% NaOCl) and three rinses in sterile water. Seeds were germinated on half-strength Murashige and Skoog (MS) basal medium (Murashige and Skoog, 1962). Explants were obtained from 5-d-old seedlings by cutting the apical meristem and cotyledons off at the constriction found above the hypocotyl. Explants were placed on MS basal medium supplemented with B5 vitamins (Gamborg et al., 1968), 0.1 mg L^{-1} NAA, 1 mg L^{-1} BA, and 100 m*M* acetosyringone. The pH was adjusted to 5.8 and the medium solidified with 3 g L^{-1} GelRite (Merck & Co., Inc., Rahway, NJ).

An overnight culture of *Agrobacterium tumefaciens* strain GV2260 containing pGUSINT (Vancanneyt et al., 1990) was prepared by growing the bacteria overnight in Luria-Bertani broth supplemented with 50 μg mL^{-1} each of rifampicin and kanamycin. The T-DNA of pGUSINT contains *npt*II with the *nos* promoter and terminator, and *gus*A with the 35S promoter and *nos* terminator. *GusA* is interrupted by the second intron of the ST-LS1 gene. The bacteria were centrifuged, and the pellet was resuspended in 1 m*M* $MgSO_4$ and used to inoculate the tissue explants by immersion. Cocultivation was for 5 d, after which tissues were transferred to MS/B5 basal medium supplemented with 0.1 mg L^{-1} NAA, 1 mg L^{-1} BA, 100 mg L^{-1} kanamycin, and 200 mg L^{-1} cefoxitin. After 6 wk with biweekly subcultures, all resulting shoots were transferred to basal MS medium supplemented with 200 mg L^{-1} cefoxitin and allowed to root. All cultures were maintained at 25°C, under a 23-h light, 1-h dark photoperiod provided by cool white fluorescent bulbs, which provided about 50 μmol photons m^{-2} s^{-1}. Rooted plants were placed in sterilized soil in 2.5- by 2.5-cm pots placed inside a GA-7 container (Magenta Corporation, Chicago), given time to recover from the transplantation, and acclimatized by slowly removing the top off the container, then transferred to a greenhouse. Independent transformants were verified through Southern blot analysis. Transgenic plants were maintained in a greenhouse for five years before use, with no special care other than daily watering and periodic cutting-back.

Phenotypic Recurrent Selection

Beginning in 2000, 12 independently derived T_0 plants were intercrossed by hand in a polycross (Fehr, 1987), and 323 T_1 seed harvested. Seeds were scarified by rubbing lightly on sandpaper, surface-sterilized as described before, and placed on MS basal medium supplemented with 0.5 g L^{-1} activated carbon for germination. Upon germination, seeds were transferred to MS basal medium supplemented with 100, 200, 300 or 400 mg L^{-1} kanamycin. Those seedlings that survived on their respective levels of kanamycin were transferred to soil as described before. The T_1 seedlings that survived on 400 mg L^{-1} kanamycin were intercrossed to obtain 120 T_2 seeds, and the process was repeated, this time placing T_2 seedlings on either 300, 400, or 500 mg L^{-1} kanamycin for selection.

Southern Analysis

Genomic DNA was isolated from leaf tissue according to the method of Doyle and Doyle (1990). Ten micrograms of genomic DNA were digested with *Eco*RI, separated on a 0.8% (w/v) agarose gel for 18 h and subsequently transferred to a positively charged nylon membrane (Amersham, Buckinghamshire, UK) using 0.4 *M* NaOH as both a transfer and denaturing agent (Sambrook et al., 1989). *Eco*RI cuts the T-DNA once between the *nos* terminator of *gusA* and the left border; hence the size of the resulting bands in a Southern blot depend on the integration site of the T-DNA. Blots were hybridized in a modified Church's buffer (Church and Gilbert 1984), consisting of 7% (w/v) sodium dodecyl sulfate (SDS), 0.5 *M* $NaPO_4$ pH 7.2 and 0.5 m*M* ethylenediaminetetraacetic acid (EDTA). A probe specific to *npt*II was produced with the Rediprime II random priming labeling kit (Amersham) along with [α–^{32}P]dCTP (PerkinElmer Applied Biosystems, Foster City, CA) and 25 ng of a 633-bp amplicon of *npt*II as a template. Unincorporated nucleotides were removed with a Micro Bio-Spin 6 chromatography column (BioRad Laboratories, Hercules, CA), and the probe denatured 5 min at 100°C and hybridized overnight at 65°C. Posthybridization treatment consisted of three washes in 2× standard saline citrate (SSC; 1× SSC is 0.15 *M* NaCl plus 0.015 *M* sodium citrate)/0.1% (w/v) SDS for 15 min at room temperature, followed by 1-h-long wash at 65°C in 2× SSC/0.1% (w/v) SDS, then a final wash for 1 h at 65°C in 0.2× SSC/0.1% (w/v) SDS. Membranes were exposed to BioMax autoradiography film (Kodak, Rochester, NY) overnight at −80°C.

The hybridization probe was produced by PCR in 25-μL final volume that contained 200 μ*M* each of dATP, dCTP, dTTP, and dGTP, 1 m*M* $MgCl_2$, 1× buffer (PerkinElmer Applied Biosystems), 1μL of 1 U μL^{-1} AmpliTaq Gold polymerase, 10 pg of plasmid DNA, 5 μ*M* of the upstream *npt*II primer corresponding to the region from base 162 to 180 (sequence 5′-CGGTGCCCTGAATGAACT-3′); and 5 μ*M* of the downstream *npt*II primer corresponding to bases 795 to 774 (sequence 5′-TCAGAACAACTCGTCAAGAAGG-3′). PCR cycling parameters were 94°C for 10 min and then 45 cycles of 94°C for 30 s, 55°C for 45 s, 72°C for 1 min, followed by a final extension at 72°C for 7 min. The PCR product was isolated by the Concert Rapid PCR purification system (Life Technologies, Rockville, MD). Bases for *npt*II are numbered according to GenBank accession V00618.

ELISA

The PathoScreen NPTII ELISA assay (Agdia, Elkhart, IN) was performed to quantify the amount of NPTII present. Crude protein extracts were obtained by macerating approximately 0.1 g of fresh leaf tissue, taken from mature plants, in the supplied extraction buffer. The assay was performed according to the manufacturer's instructions with the absorbance read at 450 nm on an EL800 plate reader (BioTek Instruments, Winooski, VT). The amount of NPTII in each sample was calculated and normalized by the total amount of soluble protein in each sample. Total protein was determined with the BioRad Protein Assay solution and reading absorbance at 595 nm. All ELISA readings were taken in triplicate.

β-Glucuronidase Staining

Tissue from each sample was tested for β-glucuronidase (GUS) activity by histochemical staining using 0.1% (w/v) 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (X-gluc) (Biosynth, Naperville, IL) as a substrate (Jefferson et al., 1987). Tissues were cleared after staining by soaking in 95% (v/v) ethanol for 3 h.

RESULTS AND DISCUSSION

All the T_0 clover plants were selected on 100 mg L^{-1} of kanamycin. By the time the breeding process started 5 yr after the initial transformation, the *npt*II gene in all but one of the parents had become silenced, as evidenced by the lack of NPTII detectable by ELISA above background levels. Throughout the selection process, variation in the level of NPTII was present between individual lines and between generations. Accordingly, it was possible to recover four T_1 plants each that survived on the 200, 300, and 400 mg L^{-1} selection levels of kanamycin. The levels of *npt*II expression in the T_1 plants selected

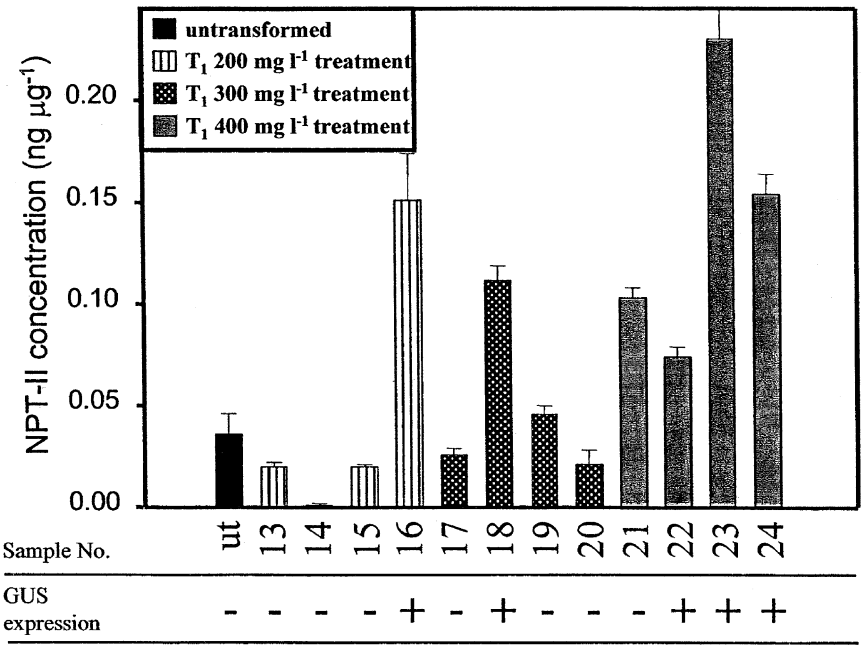

Fig. 1. Transgene expression of T_1 plants. Average NPTII concentrations in relation to total soluble protein are shown for T_1 plants selected on kanamycin at 200 mg L^{-1} (samples 13-16), 300 mg L^{-1} (samples 17-20), and 400 mg L^{-1} kanamycin (samples 21-24). An untransformed plant (ut) was used as a negative control. Histochemical GUS assay results are denoted below the corresponding sample number and were scored as either positive (+) or negative (−). Vertical bars indicate the standard error.

at the different levels of kanamycin are in Fig. 1, and averaged 0.048 ± 0.03, 0.053 ± 0.02, and 0.140 ± 0.03 ng μg^{-1} for 200, 300, and 400 mg L^{-1} kanamycin, respectively. It is plausible that individuals 16 and 18 would have survived on higher levels of kanamycin than 200 mg L^{-1}, while the 400 mg L^{-1} level was effective at selecting against individuals with lower expression.

By the T_2, obtained by intercrossing the only four T_1 individuals that survived selection on 400 mg L^{-1}, it was possible to recover 11 plants on 300, nine on 400, and eight on 500 mg L^{-1} kanamycin, representing 28% of the total seeds that germinated. Average levels of NPTII were 0.191 ± 0.06, 0.854 ± 0.17, and 0.573 ± 0.15 ng μg^{-1}, respectively, for each level of selection (Fig. 2).

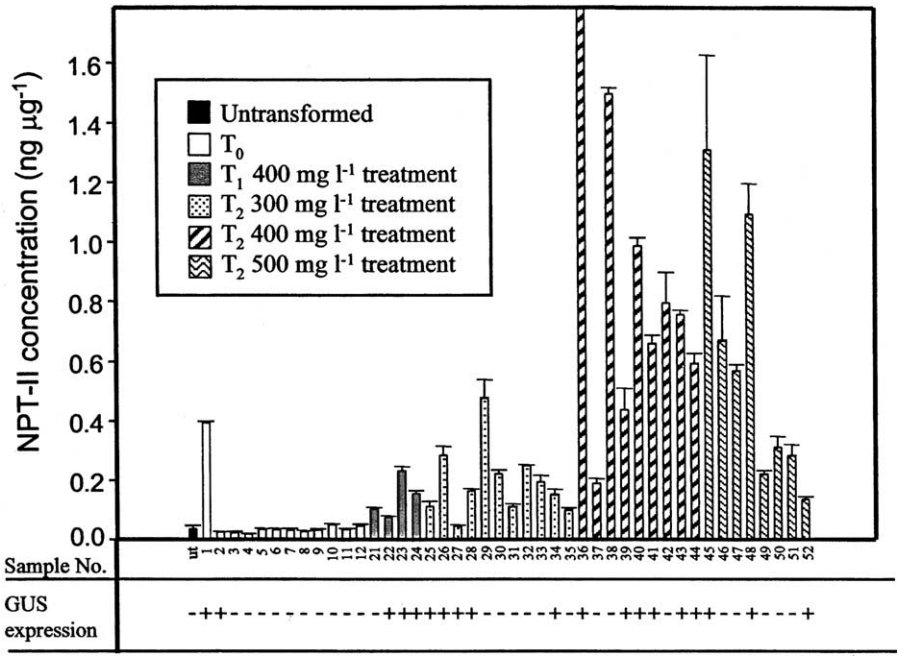

Fig. 2. Transgene expression analysis over three successive generations. Average NPTII protein concentrations in relation to total soluble protein are shown for 12 T_0 plants (samples 1-12); 4 T_1 plants selected on 400 mg L^{-1} kanamycin (samples 21-24); and 28 T_2 plants, 11 of which were selected on kanamycin at 300 mg L^{-1} (samples 25-35), 9 on 400 mg L^{-1} kanamycin (samples 36-44), and 8 selected on 500 mg L^{-1} kanamycin (samples 45-52). An untransformed plant (ut) was used as a negative control. Histochemical GUS assay results are denoted below the corresponding sample number and were scored as either positive (+) or negative (−). Vertical bars indicate the standard error.

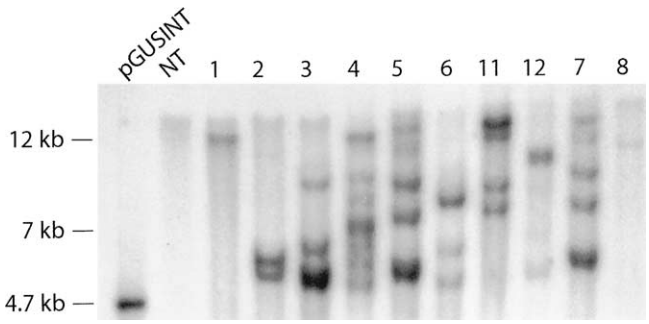

Fig. 3. Southern blot hybridization of total DNA from T_0 plants digested with *Eco*RI, which cuts only once within the plasmid, outside of any gene cassette, and probed with a 633-bp amplicon of the *npt*II gene. Plants 5 and 7 appear to be the same.

The highest-expressing individual in the T_2 had levels of NPTII that were more than four times higher than that of the only expressing T_0 parent. Average expression across each generation went from 0.033 ng μg^{-1} in the parents to 0.095 ng μg^{-1} in the selected T_1 plants to 0.539 ng μg^{-1} in the selected T_2 plants. These results are in contrast to those of Samis et al. (2002), who were unable to obtain enhanced gene expression by crossing two plants containing the transgene for Mn-superoxide dismutase. Perhaps if a greater number of independently derived parents had been used, results would have been different.

On the basis of Southern analysis, the T_0 plants contained multiple transgene loci, ranging from one to five (Fig. 3). The banding number of the T_1 (Fig. 4) and T_2 (Fig. 5–7) suggests that only two of the T_0 parents contributed transgenes that survived the selection process and were passed on to subsequent generations. One was most likely plant 1; the other is more difficult to determine.

Overall, selection for high levels of expression appears to have resulted in either selection of particular transgene inserts, or accumulation of transgene loci. An example of the first phenomenon is evident from the banding pattern of the Southern for the T_1 population selected on 400 mg L^{-1} kanamycin (Fig. 4), in which three out of the four plants appear to only have one transgenic locus, although the presence of multiple copies within the locus might be inferred from the band intensity. In contrast, many T_2 plants show at least two bands, which might indicate the accumulation of transgene loci (Fig. 5–7). Since homozygosity is possible in the T_2, a third phenomenon that could account for higher levels of NPTII is homozygosity of the transgene loci (Stewart et al., 1996).

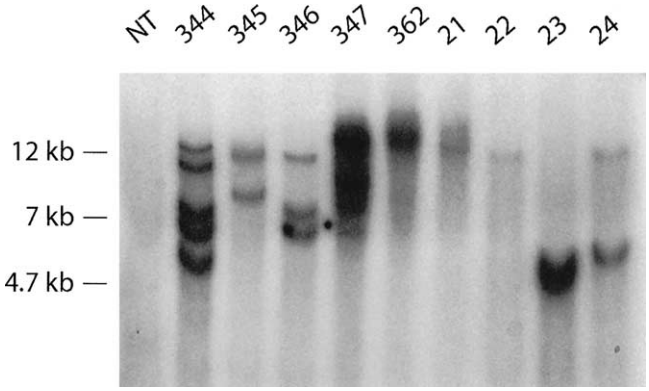

Fig. 4. Southern blot hybridization of total DNA from T_1 plants digested with *Eco*RI, which cuts only once within the plasmid, outside of any gene cassette, and probed with a 633-bp amplicon of the *npt*II gene. Plants numbered 21 through 24 survived 400 mg L^{-1} kanamycin, and were the parents for the T_2 plants.

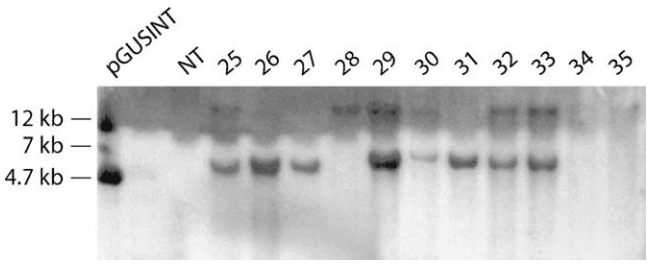

Fig. 5. Southern blot hybridization of total DNA from T_2 plants selected on 300 mg L^{-1} kanamycin, and digested with *Eco*RI, which cuts only once within the plasmid, outside of any gene cassette, and probed with a 633-bp amplicon of the *npt*II gene.

Selection for kanamycin resistance has been attempted previously, but by selection based on cultured plant cells rather than on whole plants (Jones et al., 1994). In that study, cells were obtained after selection that were 8 to 10 times more kanamycin resistant than the original cells, as a result of the amplification of the *npt*II gene. In our example, it is not possible to determine if *npt*II gene amplification has taken place within individual insertion sites. It has long been known that T-DNA can be unstable under certain conditions (Peerbolte et al., 1987). In this case, Southern analysis of the T_2 generation revealed three plants (No. 37, 47, and 48) which appear to have a novel band not found in the original parents, suggesting that selection might be effecting changes in the transgene locus.

GUS expression is indicated for each plant in Fig. 1 and 2. Although physically linked on the same construct used for transformation, *gusA* expression was not correlated with *npt*II expression in any of the generations in the present study. Thus, it appears that selection for expression was limited to the one gene being selected, without affecting the closely linked *gus*A locus. Whether *gus*A expression would have been affected had it had the same promoter as the *npt*II gene is not known.

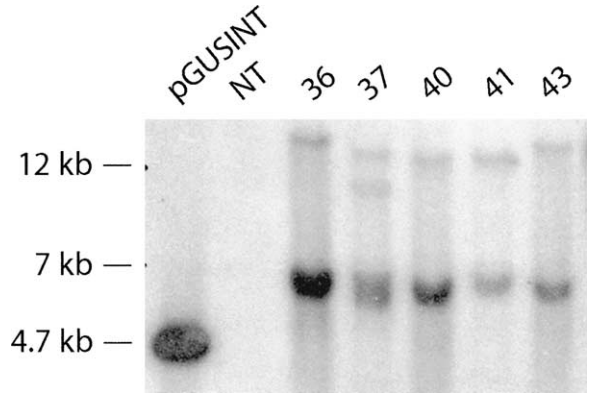

Fig. 6. Southern blot hybridization of total DNA from T_2 plants selected on 400 mg L^{-1} kanamycin, and digested with *Eco*RI, which cuts only once within the plasmid, outside of any gene cassette, and probed with a 633-bp amplicon of the *npt*II gene.

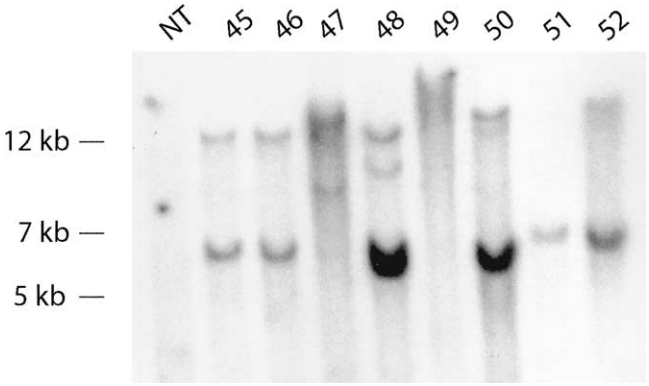

Fig. 7. Southern blot hybridization of total DNA from T_2 plants selected on 500 mg L^{-1} kanamycin, and digested with *Eco*RI, which cuts only once within the plasmid, outside of any gene cassette, and probed with a 633-bp amplicon of the *npt*II gene.

Traditionally, the level of transgene expression in an individual transformant is considered to reflect the level of transcript produced and posttranscriptional control (Ingelbrecht et al., 1994), as influenced by a number of factors, including methylation, copy number (Hobbs et al., 1990), and arrangement of transgenes within a locus (Hobbs et al., 1993). On the basis of the results of selection and on the literature reviewed in this work, the expression of transgenes also is influenced by other genes or factors in the genome that can stabilize and/or increase transgene expression. Therefore, transgenes are amenable to breeding and selection, as is the case for many standard genes selected for and against by plant breeders over the past several decades. Increased transgene expression may be due to the selection for a particular transgenic event with the potential for high expression, homozygosity, the accumulation of transgenes during the breeding process, and perhaps even amplification of transgenes.

This work has implications for crop breeding and transgene deployment. In the case of open-pollinated, out-crossing crops, it is evident that transgenes can be deployed by intercrossing several independently transformed parents, without the need to resort to backcrossing. On the basis of this study and on the studies from the literature reviewed in the introduction, it appears that traditional breeding methods can be used to increase transgene expression for both cross and self-pollinated plants.

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