P L A N T B R E E D I N G A N D S E E D S C I E N C E
Volume 50 2004

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TRANSFORMATION OF WILD *SOLANUM* SPECIES RESISTANT TO LATE BLIGHT BY USING REPORTER GENE *GFP* AND *MSH2* GENES

ABSTRACT

Green fluorescent protein (*gfp*) reporter gene and *nptII* marker gene were used to optimize *Agrobacterium tumefaciens* (agro) mediated transformation of wild *Solanum* genotypes resistant to late blight. Different genotypes of *Solanum bulbocastanum*, *S. chacoense*, *S. microdontum* and *S. verrucosum* were assessed for their regeneration ability on MS based media and for agro–mediated transformation. As the first step reporter genes were used to optimize transformation protocol for each species and then the transfer of genes involved in mismatch repair of DNA were attempted in *Solanum chacoense*. For transformation, either leaf or stem fragments were used. It was shown that *gfp* is a valuable and elegant tool for monitoring the efficiency of transformation or the occurrence of chimera in all genotypes. Transformation efficiency was dependent on a plant genotype. A number of genotypes have been successfully transformed and they expressed constitutively the bright green fluorescence of *gfp* without any side effects. The most recalcitrant species proved to be *S. microdontum*, which did not regenerate plants although different media and phytohormones had been used. The best protocol for *S. chacoense* transformation was also found to work in the transfer of *msh2* genes. *Msh2* isolated from *Arabidopsis* was used and transferred either as mutated (*Apa*) or antisense (*As*) gene. The integration of *msh2*–mutated gene into *S. chacoense* genome was demonstrated by PCR amplification and confirmed by RT–PCR for some of the putative transgenic clones. The implications of mismatch repair in homologous recombination and its importance for potato improvement are discussed.

Key words: *Agrobacterium tumefaciens* – mediated transformation, DNA mismatch repair, *gfp*, *nptII* marker gene

INTRODUCTION

Although wild *Solanum* tuber–bearing species related to the cultivated potato represent an important reservoir of genetic diversity (Hawkes 1990), interspecific incompatibility between cultivated potato and the majority of wild species has been a limiting factor in the exploi–

Communicated by Ewa Zimnoch–Guzowska

tation of this gene pool in potato breeding (Chen *et al.* 2003). Moreover, a great diversity and heterogeneity of resistance genes in wild populations make the exploitation even more difficult (Chen *et al.* 2003). Somatic hybridization between wild and cultivated potato has already produced some interesting pre–breeding material (Thieme *et al.* 1997), and novel breeding tools such as the use of bridge species or embryo rescue have been developed. Nevertheless, new techniques and knowledge aiming to improve multiple gene transfer from resistant wild genotypes to cultivated potato are still needed.

Late blight caused by oomycete *Phytophthora infestans* (Mont.) de Bary is considered to be the most important and devastating disease of potato worldwide. To control late blight, two main strategies: breeding for resistance based on race specific *R*–genes derived from *S*. *demissum* Lindl. and/or the use of systemic or residual fungicides are applied (Ross 1986). The recent appearance of new, more aggressive genotypes of *P. infestans* in North America and Europe has raised the interest in novel ways to produce stable resistant potato varieties by the transfer of multiple genes.

Many biological processes have evolved to prevent or repair mutation that can occur during DNA replication or genetic recombination. DNA mismatch repair (MMR) contributes to genome stability by recognizing and correcting mispaired bases. As mismatches arise during recombination between homologous sequences, MMR reduces recombination between the diverged sequences, thereby hindering the introgression of useful genes from wild relatives (Pelletier *et al.* 1990). Manipulating MMR genes was therefore thought to facilitate the transfer of genes from wild to cultivated species. The eukaryotic MMR system is more complex than bacterial one. Yeast has six different *mutS* homologous genes (*msh1–msh6*) and four *mutL* homologous genes (*mlh1–mlh3* and *pms1*), which have different functions. The maize and *Arabidopsis* genomes encode a seventh MUTS homologous protein, MSH7. From the proteins involved in MMR, only MUTS interact with the heteroduplex DNA molecule. Another enzyme, MUTL, interacts with MUTS to fix DNA mismatch, and different other proteins are involved in processing the mismatched base pairs. In the interspecies mating, MMR activity resulted in the breakdown of heteroduplex DNA containing multiple mismatches. It appeared that large genomic fragments from the donor could functionally replace the corresponding genes from the recipient. In other words, MMR deficiency allowed the creation of a heteroduplex intermediate, containing multiple mismatches, and the exchange of genetic information through recombination (Pelletier *et al.* 1990). The currently low meiotic DNA recombination in interspecific crosses in plants can be improved through the controlled expression or inhibition of the MMR system. This new strategy for the enhanced meiotic recombination during interspecific hybridization is taken here into account to be evaluated in potato breeding.

In this paper we report an attempt to transform of wild *Solanum* species by using DNA vector, *Agrobacterium tumefaciens*. Different genotypes of *S. bulbocastanum, S. chacoense, S. microdontum* and *S. verrucosum* were assayed for transformation. *S. chacoense* and *S. verrucosum* transgenic plants carrying *gfp* and *nptII* genes were regenerated and analysed. Two genotypes of *S. chacoense* (2095 and PI 458310) were successfully transformed either by using a construct carrying both reporter gene *gfp* and marker gene *nptII* or gene contructs carrying *msh2* genes. Either antisense or complementation strategies were applied for *msh2* transformation and kanamycin was used for putative transgenic clone selection. The integration of *msh2*–mutated gene into *S. chacoense* genome was demonstrated by PCR amplification and confirmed by RT–PCR for some putative transgenic clones.

MATERIAL AND METHODS

Plant material and transformation

Different genotypes of wild species: *S. bulbocastanum* – *S. b* GLKS 1741; *S. chacoense* – *S. c* GLKS 138; *S. microdontum* – *S. m* GLKS 850 and *S. verrucosum* – *S. v* GLKS 777 – all characterized for high resistance to *Phytophthora infestans* (IPK Genebank Gatersleben, Germany); *S. chacoense* 2095; *S. microdontum* 2035; *S. verrucosum* 2032 – the latter three from the Institute of Potato Production (ICPCC) Brasov, Romania; and *S. chacoense* PI 458310 from the NPGS Sturgeon Bay, USA, have been assessed for their regeneration ability on MS based media (Murashige and Skoog 1962) and for agro–mediated transformation. *Agrobacterium*–mediated transformation of stem or leaf fragments was performed according to the protocol by Kumar (1995). Selection and regeneration media were either LSR1 and LSR2 (Kumar 1995) or MSt medium alone (a modified MS medium containing: MS salts, 16 g/l glucose, 0.5 mg/l folic acid, 0.05 mg/l biotine, 40 mg/l adenine, 2 mg/l glycine, 100 mg/l meso–inositol, 0.5 mg/l nicotinic acid, 0.5 mg/l B6 vitamin, 0.1 mg/l B1 vitamin, agar 7 g/l, pH 5.8) (R. Thieme – personal communication).

Bacterial strains

Agrobacterium tumefaciens LBA 4404 carrying different plasmids was used for transformation:

— plasmid pHB2892 (*gfp*), carrying reporter gene *gfp* driven by CaMV 35S promoter and two CaMV enhancers and *npt II* driven by *nos* promoter (Molinier *et al.* 2000);

— binary vector FRG–MSH2–As (*As*), containing the sequence for *msh2* antisense inhibition, with the 3' conserved region of *msh2* from *Arabidopsis thaliana* in antisense orientation, and a selectable marker gene *nptII* driven by *nos* promoter (G. Ispas – unpublished);

— binary vector FRG–MSH2–Apa (*Apa*), containing the sequence for *msh2* mutant in sense orientation and a selectable marker gene *nptII* driven by *nos* promoter (G. Ispas – unpublished).

Selection and analysis of transgenic plants

Putative transgenic clones obtained by applying agro–mediated transformation plasmid pHB2892 were selected primarily on the media (LSR or MSt) with kanamycin (50 mg/l), and then by analyzing green fluorescence of GFP under UV by epifluorescence microscopy (Olympus BX60). Moreover, molecular analysis based on PCR amplification with specific primers for *nptII* gene and electrophoretic profiles were performed, as described by Davey *et al.* (1995). Transgenic clones obtained with the use of *msh2* genes were selected on kanamycin (50 mg/l) containing medium (LSR or MSt) further assessed for root development on kanamycin containing MS media (50 mg/l) and analyzed by PCR ampfication of *msh2* gene and RT–PCR.

PCR analyses: DNA was extracted using DNAeasy Kit (Qiagen). The *msh2* specific primers (*msh2*–A: atggagaatacatgatatcttcaagc and *msh2*–D: ctttcagtgtcaatgtgagcgctgac) were used in the PCR reaction. Amplified DNA fragment was visualized on 1% agarose gel.

RT–PCR analyses: total RNA was extracted from young leaves of putative transgenic lines using RNAeasy Kit (Qiagen). Total RNA was reverse transcribed with Superscript RT–PCR one–step kit with Platinum Taq (Invitrogen). The amount of template for RT–PCR analyses was equilibrated according to the spectrophotometric measurements.

RESULTS AND DISCUSSIONS

Transformation efficiency was found to depend on a plant genotype. Nevertheless, some genotypes have successfully been transformed and expressed constitutively the bright green fluorescence of *gfp* without any side effects (Table 1, Fig. 1). The most recalcitrant species proved to be *S. microdontum*, which did not regenerate plants on the variations of media and phytohormones tested. With this species only callus was regenerated, and *gfp* expressing callus was observed mainly in genotype *S. m* GLKS 850 (Fig. 2). Similarly, *S. b* GLKS 1741 regenerated only callus, part of which expressed green fluorescence of *gfp* after transformation with the construct pHB2892 (Fig. 2). The untransformed tissue exhibits the native red fluorescence generated by chlorophyll, in contrast to green fluorescence of GFP – a fluorescence differentiation that allows very good screening of transgenic tissues and plants. So far, the best results have been obtained with some genotypes of *S. chacoense* and *S. verrucosum* (Table 1, Fig. 1). For all the genotypes, visualizing *gfp* expression allowed good screening of transformation efficiency and chimerical plant tissues (Fig. 1). The expression of *gfp* monitored

Table 1

Efficiency of organogenesis and genetic transformation of *Solanum chacoense* and *Solanum verrucosum* genotypes transformed with *Agrobacterium tumefaciens* LBA4404 carrying the construct pHB2892

Species / genotype	Culture medium[A]	Type of explants	Regeneration [%][B]	Transformation [%][C]
S. chacoense 2095	LSR	stem	21.5	0.0
		leaf	32.0	0.0
	LSR+C+K	stem	18.0	38.9
		leaf	5.0	40.0
S. chacoense PI 458310	LSR	stem	7.5	0.0
		leaf	5.7	0.0
	LSR+C+K	stem	2.2	67.0
		leaf	0.9	0.0
S. verrucosum 2032	LSR	stem	12.0	0.0
		leaf	0.0	0.0
	LSR+C+K	stem	12.0	33.3
		leaf	0.0	0.0

[A] LSR – control; C – cefotaxime; K – kanamycin
[B] Proportion of regenerated shoots in a total number of inoculated explants
[C] Transformation efficiency as the percentage of regenerated shoots expressing constitutively the *gfp* gene

microscopically in relation with selection on kanamycin containing media proved to be a very good strategy for transgenic clone selection, as previously reported for potato (Aurori and Rakosy–Tican 2000, Rakosy–Tican *et al.* 2000, 2003). Once callus tissue formed, the green fluorescence of *gfp* was seen under the microscope very early, at first as green fluorescent spots, then as green fluorescent roots and shoots. For genotype *S. chacoense* 2095 the highest percentage of transformation was obtained with stem and leaf fragments, although the number of regenerated shoots was lower than that in the controls (Table 1). It is noteworthy that, apart from the transgenic shoots selected on kanamycin and expressing *gfp* (39%), also chimeric shoots (17%) as well as shoots which did not express *gfp* (44%) could be visualized in this genotype (Fig. 1b). These data suggest that selection on kanamycin media alone is not a sufficient way to select a putative transgenic line. They raise the question of the origin of chimera and non–*gfp* plants. Molecular analysis showed that all plants expressing *gfp* were also integrating the *nptII* gene (one example is presented in Fig. 3). Genotype *S. chacoense* PI 458310, compared to *S. chacoense* 2095, showed a lower ability to regenerate from both explants, and only stem fragments regenerated shoots on kanamycin containing LSR media. Sixty–seven percent of regenerated shoots expressed *gfp*, whereas the remaining shoots (33%), although able to grow on the media with kanamycin, have not expressed till now green fluorescence of GFP (Table 1, Fig. 1). In genotype *S. verrucosum* 2032 only stem fragments were able to regenerate plants on LSR media. With this explant, the putative transgenic shoots

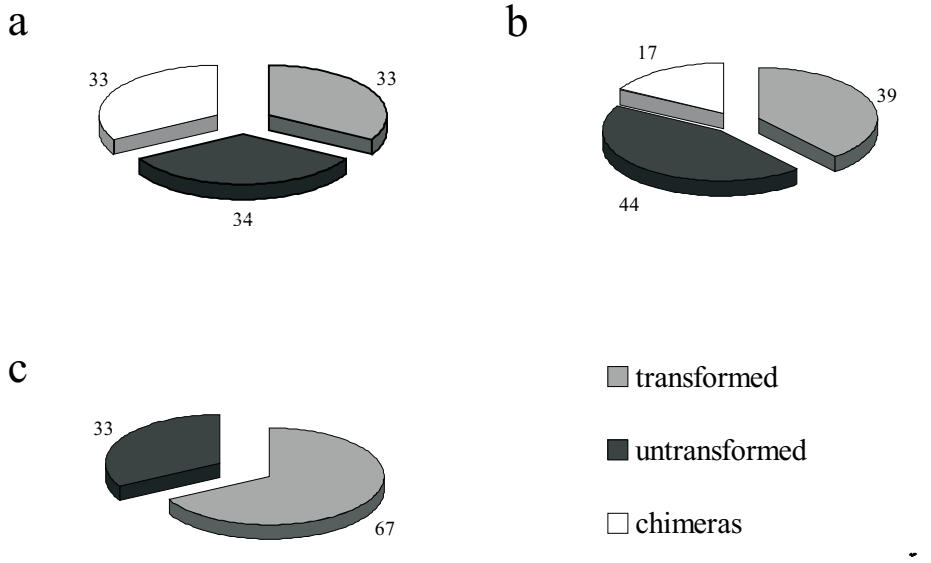

Fig. 1. Proportion [%] of *gfp* expressing shoots (transformed) compared to shoots, which did not express *gfp*, and chimeric regenerated shoots: a – *S. verrucosum* (GLKS 777 and 2032); b – *S. chacoense* 2095; c – *S. chacoense* PI 458310

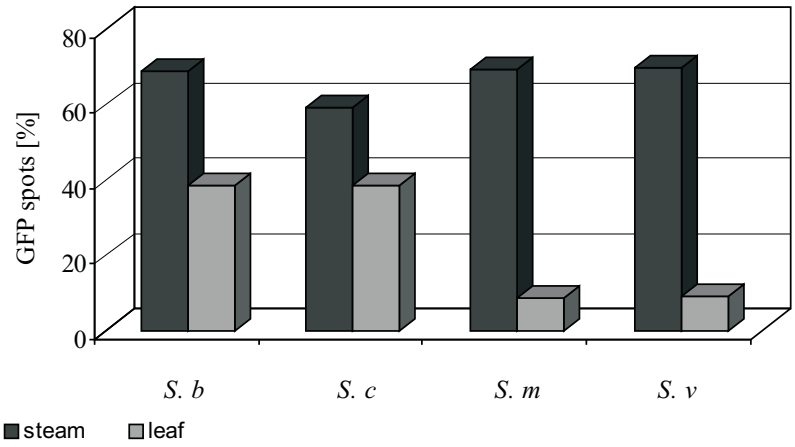

Fig. 2. *Agrobacterium*–mediated transformation of stem and leaf explants (plasmid pHB2892) as percentage of callus with visible *gfp* fluorescent spots for wild *Solanum* genotypes highly resistant to late blight: *S. b* – *S. bulbocastanum* GLKS 1741; *S. c* – *S. chacoense* GLKS 138; *S. m* – *S. microdontum* GLKS 850; *S. v* – *S. verrucosum* GLKS 777

were selected on media with kanamycin. Out of these, only 33% also expressed *gfp*, another 33% were chimeras with both green and red fluo–

rescent tissues, whereas the remaining 34% did not express *gfp* at all (Table 1, Fig. 1). These results suggest that selection with the use of kanamycin alone may lead to overestimation of the transformation efficiency. The expression of the *gfp* transgene was stable but, sometimes, the reduction of expression due to a physiological condition or rarely transgene inactivation, shown by disappearance of green fluorescence during *in vitro* cloning, was also observed. So far, genotypes *S. b* GLKS 1741, *S. c* GLKS 138, *S. m* GLKS 850 and *S. v* GLKS 777 have only regenerated callus, and efficiency of transformation was estimated as the percentage of callus with green fluorescent spots (Fig. 2). Stem and leaf fragments formed callus in all genotypes, but the stem samples showed more green fluorescent spots than did the leaf samples. These observations have confirmed that this type of explant gives better results in agro–mediated transformation of *Solanum* species (Rakosy *et al.* 2003).

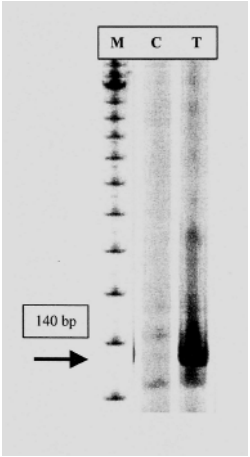

Fig. 3. PCR amplification products of *nptII* gene in transgenic *Solanum chacoense* 2095 shoots expressing *gfp* – example for one transgenic clone (T) as compared to the control (C); M = molecular marker 100 bp

The best results were obtained with *S. chacoense* 2095 and PI 458310, first on LSR media and improved by culture on MSt medium, the latter being also used for the transfer of *msh2* genes (Table 2). The number of regenerated shoots after agro–infection and kanamycin selection was higher with *msh2* in genotype 2095 for both contructs, *Apa* and *As*, as compared to genotype PI 458310. The percentage of putative transgenic clones able to form a good root system on MS medium with 50 mg/l kanamycin differed for the genotypes and constructs, ranging from 14% to 60%. PCR analysis was only performed for the plants transformed with *Apa* construct in both genotypes, and three plants in *S. chacoense* PI 458310 and one plant in genotype 2095 were proved to be transgenic integrating the gene (Table 2). PCR amplification revealed the expected 1 Kb amplified DNA fragment. RT–PCR analysis confirmed the expression of a mutated *msh2* gene in three transgenic clones (Fig. 4). The PCR

controls applied in parallel confirmed the absence of DNA contamination in the RT–PCR reaction.

Table 2

The efficiency of *msh2* genes (*Apa* and *As*) transfer to genotypes *S. chacoense* 2095 and PI 458310 as revealed by kanamycin selection during *Agrobacterium*–mediated transformation (1), root development on kanamycin containing MS media (2) and molecular analysis (3)

	S. chacoense 2095		*S. chacoense* PI 458310	
	Apa	*As*	*Apa*	*As*
(1) Number of putative transgenic clones	30.00	76.00	14.00	15.00
(2) % of plants with very well developed roots	41.37	52.94	14.28	60.00
(3) Number of transgenic clones confirmed by PCR analysis of mutated *msh2* gene	1.00	n.d.	3.00	n.d.

n.d. – not determined yet

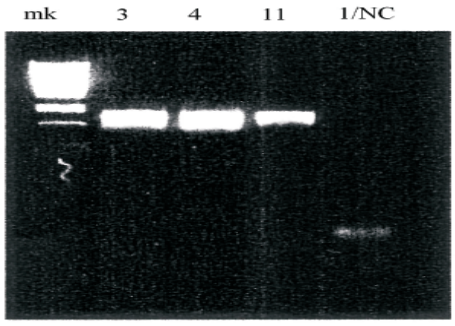

Fig. 4. Confirmation by RT–PCR of *msh2* gene – *Apa* – integration in transgenic clones (mk – marker; lanes 3, 4, 11 – transgenic clones, 1/NC – negative control)

The transgenic plants carrying *msh2* gene either as mutated or antisense gene are further analyzed, cloned *in vitro* and transferred to a greenhouse. They will be used for somatic or sexual hybridization to examine their utility in increasing interspecific recombination between wild species and cultivated potato and further genetic analyses.

CONCLUSIONS

- *Gfp* reporter gene is a valuable tool for monitoring genotype–dependent transformation; it allows optimization of transformation procedure and its application for the transfer of other genes, as exemplified by *msh2* genes in this study.
- Transgenic clones carrying *msh2* genes are to be further used for molecular analysis and sexual and somatic hybridization in order to

prove mismatch repair involvement in heterologous interspecific re–combination and its possible genetic manipulation**.**

ACKNOWLEDGEMENTS

Romanian Ministry of Education (grant BIOTECH 02–2–PED–500) is gratefully acknowledged for financing part of these studies and a bi–lateral project BIL01/53 from the Ministry of Flemish Community.

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