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Detection of the reporter and selection genes in transformed hop (*Humulus lupulus* L.)

Suzana ŠKOF[1], Zlata LUTHAR[2]

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ABSTRACT

Agrobacterium-mediated transformation of hop nodal explants with meristems was used for the introduction of a *gus* reporter gene and *nptII* plant selection gene into Slovenian hop cv. Aurora. Emerging hop regenerants were previously tested for the *gus* gene expression by histochemical analysis of β-glucoronidase (GUS) activity. Approximately six months after the transformation procedure, PCR molecular analysis of shoots originating from previously GUS positive regenerants was performed to check integration of the reporter and selection genes into the hop genome. We also compared whether there were any differences in transgene integration in relation to the intensity of *gus* gene expression (intensive blue coloration on a larger proportion of the leaf surface or just a few blue spots) revealed by GUS-assay. In both cases, the majority of shoots had both transgenes integrated (47.7 or 55.3%) and in smaller number of shoots both transgenes were missing (38.6 or 18.8%). The fewest shoots analyzed showed just *gus* (2.3 or 8.9%) and slightly more *nptII* (11.4 or 17.0%) gene presence.

Key words: hop, transformation, *Agrobacterium tumefaciens*, *gus* gene, *nptII* gene, GUS-assay, PCR

IZVLEČEK

DOLOČANJE TESTNEGA IN SELEKCIJSKEGA GENA V TRANSFORMIRANEM HMELJU (*Humulus lupulus* L.)

Z metodo posredne transformacije z *Agrobacterium tumefaciens* smo vnesli testni *gus* gen in rastlinski selekcijski *nptII* gen v meristeme nodijev hmelja cv. Aurora. V nastalih regenerantih smo predhodno testirali izražanje testnega *gus* gena z metodo histokemičnega testa aktivnosti β-glukuronidaze (GUS). Šest mesecev po transformaciji smo z molekulsko analizo poganjkov, ki so nastali na predhodno GUS pozitivnih regenerantih, s PCR metodo preverili vključenost testnega in selekcijskega gena v rastlinski genom. Primerjali smo tudi, če obstajajo razlike v vključenosti transgenov v poganjke glede na intenzivnost izražanja *gus*

[1] B. Sc., SI-1111 Ljubljana, Jamnikarjeva 101

[2] Associate Prof., Ph. D., SI-1111 Ljubljana, Jamnikarjeva 101

gena (intenzivnejše modro obarvanje na večji površini lista ali le nekaj modrih točk) z GUS testom. V obeh primerih je imela večina poganjkov vključena oba transgena (47.7 oz. 55.3% poganjkov), manj poganjkov ni imelo nobenega transgena (38.6 oz. 18.8%), najmanj pa le *gus* (2.3 oz. 8.9%) ali *nptII* (11.4 or 17.0%) gen.

Ključne besede: hmelj, transformacija, *Agrobacterium tumefaciens*, *gus* gen, *nptII* gen, GUS test, PCR

1 INTRODUCTION

Hop (*Humulus lupulus* L.) is a clonally propagated dioecious perennial plant and commercially important as an essential flavoring in beer. Breeding in hops is a lengthy process and hindered by the lack of male plants. Biotechnological approaches such as genetic transformations are an attractive alternative to conventional breeding methods, since they enable relatively rapid introduction of desirable characteristics into established hop cultivars without altering their quality profiles.

High rate *in vitro* regeneration is a prerequisite for efficient application of gene transfer techniques. Induction of adventitious shoot regeneration is fairly difficult in hop. There are a limited number of reports of efficient hop *in vitro* regeneration, most through callus formation either of some wild varieties (Batista *et al.*, 1996; Batista *et. al.* 2000) or a few commercial cultivars (Motegi, 1979; Connell and Heale, 1986; Heale *et al.*, 1989; Gurriarán *et al*., 1999; Šuštar-Vozlič *et al.*, 1999; Horlemann *et al*., 2003). Rakouský and Matoušek (1994) published direct organogenesis of two commercial Czech hops. Oriniaková *et al.* (1999) reported only transient *gus* (β-glucuronidase) reporter gene expression in transformed hop callus tissue. Two authors achieved stable *gus* reporter gene expression in two genetically closely related hop genotypes (Horlemann *et al.*, 2003; Okada *et al.*, 2003). Since the regeneration ability of hop is highly genotype dependent (Gurriarán *et al.*, 1999), a specific/modified regeneration and subsequently transformation protocol for each variety needs to be established. So far, no successful regeneration and transformation protocol has been published for any Slovenian hop cultivar.

In our study, we tried to establish an efficient *Agrobacterium*-mediated transformation protocol of the most widely grown Slovenian hop cultivar, Aurora. Integration of the *gus* reporter and *nptII* plant selection genes into the genome of hop regenerants, which were previously positive for reporter gene expression by histochemical GUS assay, was analyzed by the PCR method.

2 MATERIAL IN METHODS

2.1 Plant material

Nodal explants with meristems were cut from *in vitro* grown Slovenian hop cv. Aurora and then pre-cultivated in petri dishes on a regeneration medium with MS (Murashige and Skoog, 1962) macro-, microelements and vitamins, supplemented with inositol 100 mg/l, glucose 20 g/l, TDZ (thidiazuron) 1 mg/l, IAA (indole-3-acetic acid) 0.025 mg/l, acetosyringone 100 μM and agar 8 g/l at pH 5.8 for three days. Plant material was grown in a climatic chamber under 16/8 h photoperiod at 24 ± 1 ºC, and illumination of 40 μmol $m^{-2}s^{-1}$.

2.2 *Agrobacterium* cultivation

The *Agrobacterium tumefaciens* strain LBA4404, carrying pCAMBIA2201 plasmid provided with the intron-containing *gus* reporter gene and *nptII* selection gene, both driven by the CaMV 35S promoter, was grown at 28 ºC to log phase on liquid YEB medium (sucrose 5 g/l, beef extract 5 g/l, yeast extract 1 g/l, $MgSO_4 \times 7H_2O$ 1 g/l; pH 7.0) supplemented with bacterial selection antibiotic chloramphenicol 25 mg/l and acetosyringone 100 μM.

2.3 *Agrobacterium*-mediated transformation and regeneration of transformed plants

Hop nodal explants were immersed in liquid MS medium containing bacterial cells and exposed to ultrasound (60 s) and vacuum (10 min) treatment, dried on sterilized filter paper and placed on regeneration medium containing acetosyringone 100 μM. After three days of co-cultivation, explants were rinsed twice with antibiotic timentin [100:1 w/w ticarcillin : clavulanic acid] 200 mg/l solution, dried on sterilized filter paper and plated on regeneration media supplemented with timentin 150 mg/l in order to eliminate *Agrobacterium* growth. Newly formed shoots approximately 2 cm in size were cut from callus tissue and plated on micropropagation medium with MS (Murashige and Skoog, 1962) macro-, microelements and vitamins, supplemented with inositol 100 mg/l, glucose 20 g/l, BAP (6-benzylaminopurine) 1 mg/l and agar 8 g/l at pH 5.8. Plantlets were subcultured every 12 weeks on the same medium.

2.4 Molecular analysis of plant material by PCR method

GUS activity in the leaves of hop regenerants was assayed by histochemical GUS staining (Jefferson *et al.*, 1987; Hiei *et al.*, 1994) 110 days after *Agrobacterium*-mediated transformation. Transformant cells that expressed GUS colored blue. Approximately six months after transformation, total genomic DNA was extracted from the leaves of previously GUS positive shoots and untransformed control plants using the slightly modified protocol including CTAB detergent as described by Kump *et al*. (1992). DNA concentration was estimated by mini DNA fluorometer (Hoefer, TKO 100) and diluted to 20 ng/μl.

GUS expressing shoots were checked by PCR analysis for integration of the reporter and selection genes. The primers used (GUS3for/GUS3rev and NPTIIa/NPTIIb) were designed to amplify a 408 bp fragment in the *gus* gene and a 650 bp fragment in the *nptII* gene, respectively. The PCR reaction mixture contained 1×PCR buffer [10 mM Tris-HCl, 1,5 mM $MgCl_2$, 50 mM KCl pH 8.3], 0.1 mM of each deoxinucleotide (dATP, dGTP, dCTP and dTTP), 0.5 mM of the specific primer (GUS or NPT), 1 unit of *Taq* polymerase enzyme and a corresponding volume of DNA sample. DNA was amplified in a thermal cycler according to slightly modified temperature cycles as described by Lakshmi *et al*. (1998). The samples were initially heated to 94 ºC, then subjected to 35 cycles of 1 min at 94 ºC, 1 min at 58 ºC and 1.5 min at 72 ºC, with a final extension step of 72 ºC for 5 min. The amplified DNA target sequences were analyzed on 1.4% agarose gel in 0.5×TBE buffer and detected by EtBr staining under UV.

3 RESULTS AND DISCUSSION

GUS staining was performed more than three months after explants were subjected to the transformation procedure, so blue staining of tested shoots indicated stable and not only transient reporter gene expression. Of 51 GUS positive shoots, 14 regenerants showed more intensive blue coloration on a larger proportion of leaf surface, while others had just a few blue spots (data not shown). After subcultivation on micropragation medium, the regenerants tended to form clusters of regenerants by formation of callus tissue and, subsequently, new shoots at the base of an original GUS positive shoot. Formation of new shoots was probably caused by the cytokinin BAP content in the micropropagation medium. BAP was later replaced with auxin IBA

(indole-3-butyric acid), which caused elongation and rooting of shoots without the formation of new shoots at the basal end of the regenerants (data not shown).

PCR can be used as a routine analytical tool for quick analysis of plant transformants for the presence of a foreign gene (Hamill *et al.*, 1991). PCR false positives on account of possible agrobacterial contamination persisting in the culture were prevented because our *gus* gene was supplemented with an intron. Fourteen clusters of shoots originating from regenerants with more intensive blue coloration and 33 clusters originating from regenerants with at least one blue spot were checked by PCR analysis for integration of the *gus* reporter and *nptII* selection genes approximately six months after the transformation procedure (Figure 1). Results are shown in Table 1.

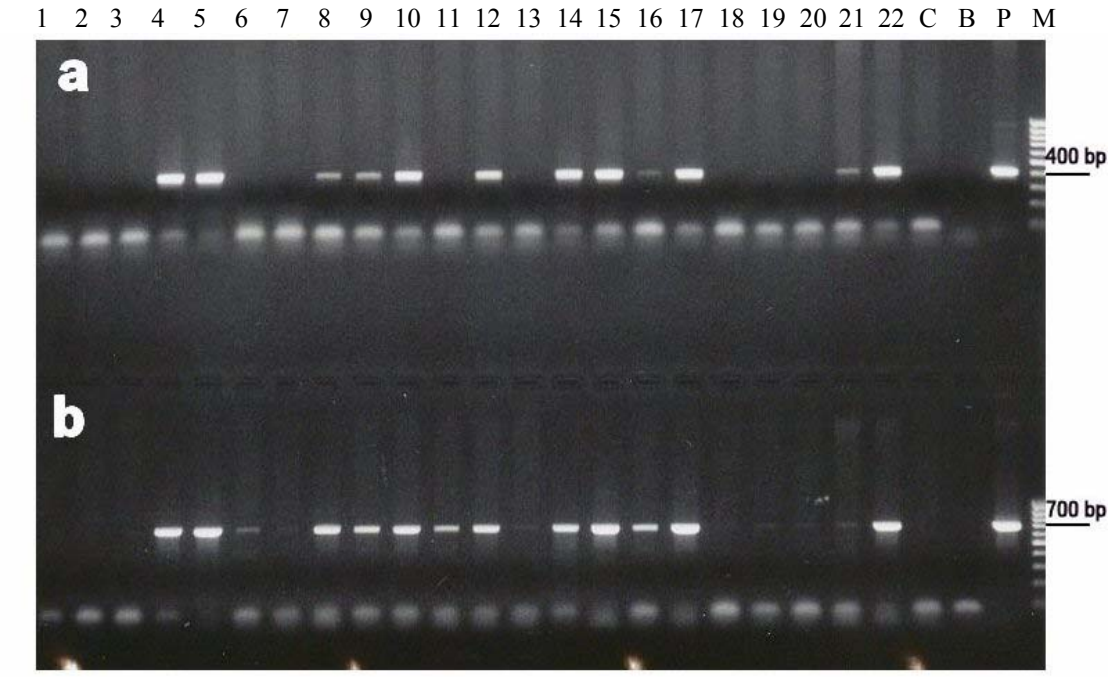

Figure 1: PCR analysis of marker *gus* (a) and selection *nptII* (b) gene integration into the genome of 22 hop shoots originating from GUS-assay positive hop regenerants. 1 to 22 - transformed hops; C - control plant, B - blind sample, P - plasmid pCAMBIA2201; M - marker GeneRuler 100bp DNA Ladder (Fermentas).

Table 1: PCR analysis of 47 clusters of regenerants originating from GUS-assay positive hop regenerants six months after transformation.

Cluster	No. of shoots per cluster	No. of shoots with integrated transgenes in cluster			
		gus and *nptII*	only *gus*	only *nptII*	neither
I/4A*	2	0	1	0	1
I/6B*	5	3	0	1	1
I/1C*	3	1	0	0	2
I/2C*	2	0	0	0	2
I/4B*	2	2	0	0	0
III/3C*	1	1	0	0	0
III/10H*	3	2	0	1	0
IV/3A*	3	1	0	0	2
VI/6A*	5	4	0	0	1
VI/1B*	1	1	0	0	0
VI/12A*	3	0	0	0	3
VI/8C*	4	2	0	1	1
VI/10C*	4	2	0	0	2
VI/5E*	6	2	0	2	2
Total	44	21	1	5	17
I/5C	1	0	0	0	1
I/6C	1	0	0	0	1
I/6D	1	0	0	0	1
I/6F	1	1	0	0	0
II/1C	1	0	0	0	1
II/1D	7	2	0	2	3
II/9D	4	1	0	1	2
II/10B	9	2	0	4	3
II/5D	1	1	0	0	0
II/8F	1	0	0	1	0
II/6F	2	1	0	0	1
III/5B	3	1	2	0	0
III/3B	2	0	0	0	2
III/8B	2	2	0	0	0
IV/2A	1	1	0	0	0
IV/8A	10	1	5	0	4
IV/2B	4	1	2	1	0
VI/9A	3	3	0	0	0
VI/1E	7	7	0	0	0
VI/2C	3	2	0	0	1
VI/2B	4	4	0	0	0
VI/5D	1	1	0	0	0
VI/11D	1	1	0	0	0
VI/2D	3	3	0	0	0
VI/3D	4	4	0	0	0
VI/7F	6	6	0	0	0
VI/8D	6	3	1	1	1
VI/10F	5	3	0	2	0
VI/11E	2	1	0	1	0
VI/11F	5	3	0	2	0
VI/6B	1	1	0	0	0
VI/1D	3	2	0	1	0
VI/12F	6	4	0	1	1
Total	112	62	10	19	21

*clusters of shoots originating from regenerants with more intensive blue coloration

In 44 shoots originating from regenerants with more intensive blue coloration, the majority of shoots showed integration of both marker and selection genes (47.7%), 2.3% of shoots had only the *gus* gene, 11.4% only the *nptII* gene and neither was detected in 38.6% of shoots tested. Similarly, in the majority of 112 shoots originating from regenerants with at least one blue spot, both transgenes were detected in 55.3%, 8.9% of shoots had only the *gus* gene, 17.0% only the *nptII* gene and neither was integrated in 18.8% of shoots tested (Table 2).

Table 2: Percentage of shoots in clusters, originating from GUS-assay positive hop regenerants, with integrated transgenes six months after transformation.

Integrated transgenes	Percentage of shoots in clusters with integrated transgenes (%)	
	Shoots originating from regenerants with intensive blue coloration	Shoots originating from regenerants with a few blue spots
gus and *nptII*	47.7	55.3
only *gus*	2.3	8.9
only *nptII*	11.4	17.0
neither	38.6	18.8

Of the 156 shoots tested, the majority had both reporter and selection genes integrated (53.2%), only in a smaller number was only one transgene (*gus* or *nptII*) detected (7.0% and 15.4%, respectively). No transgenes were identified in 24.4% of shoots assayed. We observed an even better integration rate of both transgenes in shoots originating from regenerants with weaker *gus* gene expression (plants with just a few blue spots) (55.3%) in comparison with shoots originating from regenerants with more intensive reporter gene expression (plants with intensive blue coloration) (47.7%). In the transformation procedures, the whole gene construct (in our case *gus* in *nptII* genes) is randomly integrated into the plant genome (Zupan *et al.*, 2000). In our case, when just one transgene was identified, mutations/deletions or modifications could occur in only one part of the gene cassette. This was more likely in the *nptII* gene, due to the lack of selection antibiotic (kanamycin) in the growth medium. Plant selection antibiotic was not used in the regeneration medium because we observed dying of explants and no regeneration in the preliminary experiments. Another possibility is chimeras (only part of cells successfully transformed) when untransformed tissue could gradually overgrow the transgenic tissue, which was most likely the case when no transgenes were detected in shoots originating from previously GUS positive regenerants. Chimeras are more likely because our starting explants were nodia, in which already preformed axillary buds could be targeted. In only one reported successful transformation protocol of hop Horlemann *et al.* (2003) assayed integration of only the selection *nptII* gene by PCR and confirmed integration of the selection gene in all GUS positive organogenic clusters that grew on the selection medium.

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