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A protocol for sonication-assisted *Agrobacterium rhizogenes*-mediated transformation of haploid and diploid sugar beet (*Beta vulgaris* L.) explants*

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Hairy root cultures obtained after *Agrobacterium rhizogenes*-mediated genetic transformation can serve as a model system for studying plant metabolism and physiology, or can be utilized for the production of secondary metabolites. So far no efficient protocol of hairy root development in sugar beet has been publically released. In this work, two *A. rhizogenes* strains (A4T and LBA1334) carrying a binary vector pBIN-m-gfp5-ER or pCAMBIA1301 possessing *gfp* and *uidA* reporter genes were used to transform petiole explants of haploid and diploid sugar beet genotypes. Five treatment combinations of sonicated-assisted *Agrobacterium*-mediated transformation were compared. Hairy roots appeared on 0% to 54% of explants depending on the treatment combination used. The highest frequency was achieved when explants of a diploid genotype were sonicated for 15 s in the inoculum containing *A. rhizogenes* of OD_{600}=0.5 and then co-cultured for three days. Using the same treatment combinations the explants of haploid genotypes developed hairy roots with the frequency ranging from 10% to 36%. Transformation efficiency was independent on the bacterial strain used. The results indicate that haploid sugar beet explants are amenable to transformation using *A. rhizogenes,* and that the efficiency of that process can be increased by applying short ultrasound treatment.

Key words: genetic modification, hairy root, SAAT, ultrasound

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INTRODUCTION

Sugar beet is an important agricultural crop cultivated in 2012 on a total area of 4.9 million hectares, predominantly in Europe (FAOSTAT). It is mainly used for sugar production, but a number of by-products, such as leaves, molasses, sugar beet pulp are successfully used as fertilizers, animal feed or in food industry (Smigocki *et al.,* 2008). For several years, sugar beet has been also used for ethanol and biofuel production (Bessou *et al.,* 2011).

Breeding new sugar beet varieties aims both at increase yield (polyploid varieties) and development of varieties with desirable traits such as resistance to pests and diseases and increased tolerance to abiotic stress (Biancardi *et al.,* 2005). Sugar beet breeding process is difficult because of biennial life cycle of the species, high inbreeding depression and, additionally, by a limited range of available genetic variation. In recent decades, biotechnological techniques, including tissue cultures, transgenesis and molecular assisted selection have been successfully incorporated in breeding programs, for example by the production of doubled haploid (DH) lines useful for creation of hybrid varieties (Bosemark, 2007). Genetically modified (GM) H7-1 variety characterized by herbicide resistance was successfully commercialized in USA in 2008 and dominated sugar beet production. It is grown on 95% sugar beet plantation area in USA (Nehls *et al.,* 2010).

H7-1 variety was developed in a process of *Agrobacterium tumefaciens*-mediated transformation (Dillen *et al.,* 2013). *A. tumefaciens* is the most frequent vector used to deliver heterologous genes into a host plants. However, sugar beet is considered as one of the highly recalcitrant species to *Agrobacterium.* Thus, there is only a limited number of reports showing successful transformation and development of GM sugar beet plants (Smigocki *et al.,* 2008). Several factors affect the efficiency of sugar beet transformation with the host plant genotype being one of the most critical one, what is explained by a high sugar beet heterozygosity. In comparison to other species, publicly released protocols of sugar beet transformation are inefficient and time consuming, and the GM plant production requires at least two years (Gurel *et al.,* 2008).

A. rhizogenes is another bacteria capable of plasmid T-DNA transfer to higher plants and causing hairy root syndrome that can be utilized for the development of GM plant tissue. The appearance of hairy roots at the infection site of a plant explant occurs usually within two to four weeks after the inoculation. The excised hairy roots can be maintained on hormone free media where they grow extensively (Britton *et al.,* 2008). An evident drawback of the use of *A. rhizogenes* is the need to force hairy root tissue to redirect its morphogenesis towards shoot development that is often difficult because of the presence of a high level of endogenous auxins (Giri & Narasu, 2000, Ninković *et al.,* 2010). In sugar beet there has been no reports showing shoot morphogenesis from hairy roots so far. Nevertheless,

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Abbreviations: BAP, 6-benzylaminopurine; DH, doubled haploid; GFP, green fluorescent protein; GM, genetically modified; GUS, β-glucuronidase; NAA, 1-naphthaleneacetic acid; OD, optical density; SAAT, sonication-assisted *Agrobacterium*-mediated transformation

sugar beet hairy root cultures were used in research to answer questions related to agricultural problems like plant-pathogen and plant-pest interactions (Cai *et al.,* 2003). Additionally, hairy roots can be used for the production of secondary metabolites as demonstrated in red beet (Taya *et al.,* 1992, Rudrappa *et al.,* 2005). The efficiency of transgenic hairy roots is dependent on a number of factors, such as bacterial strain, genotype, type of explant, condition and duration of co-culture with bacteria. Most of those factors have not been studied extensively in *A. rhizogenes*-mediated sugar beet genetic transformation. Also, there are no reports available on the use of ultrasound treatment to increase efficiency of sugar beet *Agrobacterium*-mediated transformation. Sonication-assisted *Agrobacterium*-mediated transformation (SAAT) is a procedure utilizing ultrasounds to help bacteria interfere with plant cells. It is postulated that brief ultrasonic treatment results in the formation of micro wounds on the surface of explants and the secreted phenolic compounds enhance transformation. Wounds make explant penetration by bacteria also more feasible. SAAT was successfully used for genetic transformation in plant species particularly considered as resistant to *Agrobacterium* (Liu *et al.* 2006, Pathak & Hamzah, 2008).

In the present paper we compare several SAAT procedures using *A. rhizogenes* for the development of sugar beet hairy roots. We indicate the most efficient protocol for genetic transformation of haploid and doubled haploid genotypes.

MATERIALS AND METHODS

Plant material and culture conditions. Three haploid (Nos. 168, 169, 170) and one DH (No. 24) sugar beet (*Beta vulgaris* L.) genotypes were provided by Kutnowska Hodowla Buraka Cukrowego w Straszkowie (KHBC) breeding company, Straszkow, Poland. Haploids were produced via gynogenesis from unpollinated ovules and the DH genotype was obtained by diploidization of another haploid shoot as described before (Klimek-Chodacka & Baranski, 2013).

All shoots were micropropagated by dividing into separate rosettes with intact meristems every three weeks. The shoots were placed on 0.7% agar-solidified Murashige and Skoog (1962) salts and vitamins medium (MS; Duchefa Biochemie) supplemented with 0.3 mg l^{-1} 6-benzylaminopurine (BAP; Sigma), 0.1 mg l^{-1} 1-naphthaleneacetic acid (NAA; Sigma), 0.3 mg l^{-1} thiamine (Sigma) and 30 g l^{-1} sucrose; pH 5.8. To prevent bacterial contamination 200 mg l^{-1} cefotaxime (Polfa, Tarchomin) was added to the medium. Micropropagated shoots were cultured in 500 ml containers at 25 ± 1^{o}C under a 16 hrs photoperiod of 1:1 Daylight (Philips) and Fluora (Osram) fluorescent tubes (55 μmol m^{-2} s^{-1}).

***Agrobacterium* strains and plasmids**. *Agrobacterium rhizogenes* strains A4T (McInnes *et al.,* 1989) and LBA1334 (Offringa *et al.,* 1986) both harboring pBIN-mgfp5-ER plasmid (thereafter called A4T-gfp and LBA1334-gfp, respectively) and strain A4T with plasmid pCAMBIA1301 (thereafter called A4T-gus) were used. The T-DNA of pBIN-mgfp5-ER plasmid contained m-*gfp5*-ER gene coding for a green fluorescence protein and *npt*II gene conferring plant resistance to kanamycin. The T-DNA of pCAMBIA1301 plasmid contained β-glucuronidase (*uidA*) gene and *hpt*II gene conferring plant resistance to hygromycin. Both plasmids possessed kanamycin resistance gene expressed in bacteria.

Bacteria were grown overnight at 25°C in liquid Luria–Bertani medium supplemented with 50 mg l^{-1} rifampicin and 50 mg l^{-1} kanamycin with shaking (200 rpm). Bacteria were harvested by centrifugation at room temperature for 10 min and then resuspended in MS salts and vitamins inoculation medium supplemented with 0.4 mg l^{-1} NAA, and 30 g l^{-1} sucrose, 100 μM acetosyringone and the inoculum was adjusted to OD_{600} = 0.5. After additional 1 h shaking incubation the inoculum was diluted to the final bacteria concentration (OD_{600} = 0.05 or 0.5).

Transformation procedure. Petiole and midrib explants were obtained from two-week-old shoots micropropagated *in vitro* and immersed in the inoculation medium containing one of the bacterial strain, or in the inoculation medium free of *Agrobacterium*. After sonication (15–240 s) explants were gently shaken for 15 min. When sonication was carried out in a solution without *Agrobacterium*, the inoculum was added after ultrasonic treatment followed by gentle shaking for 15 min. The explants were then blotted on sterile filter paper and transferred to Petri dishes containing 0.7% agar-solidified MS medium with 30 g l^{-1} sucrose and 100 μM acetosyringone and were co-cultured for 3 or 4 days in the dark at 25 ± 2°C. Next, explants were transferred to a fresh medium supplemented additionally with 400 mg l^{-1} cefotaxime and 100 mg l^{-1} timentin for *Agrobacterium* elimination. Ten explants were placed in each Petri dish and experiments were set up in 5–14 replications. Cultures were kept in the dark at 25 ± 2°C. Emerging hairy roots were excised out of explants and cultured using the same medium and conditions, and were subcultured every three weeks.

GUS staining and GFP detection. β-glucuronidase activity in hairy roots obtained after transformation with strain A4T-gus was detected by histochemical staining essentially according to Jefferson *et al.* (1987). The transformed hairy roots were incubated overnight at 37°C in 50 mM sodium phosphate buffer (pH 7.0) with 1% Triton X-100 (Sigma-Aldrich) and 2mM 5-bromo-4-chloro-3-indolyl glucuronide (Duchefa) as a substrate.

The presence of GFP protein in hairy roots obtained after transformation with strains A4T-gfp and LBA1334-gfp was detected by observation of green fluorescence of hairy roots illuminated with UV lamp (UVP-100BP).

DNA isolation and molecular analysis of transformants. Genomic DNA was isolated from transgenic hairy roots using a CTAB method described by Rogers and Bendich (1988). The presence of transgenes was confirmed by PCR reaction using *uidA* and *gfp* gene specific primers that amplified 1202 kb and 500 kb products, respectively (Hamill *et al.,* 1991, Higgins *et al.,* 2006). 10 μl PCR reactions contained 1 μl 10x buffer, 250 μM of each dNTP, 0.5 μM of each primer and 0.5 U DreamTaq Green DNA Polymerase (ThermoScientific). PCR was performed in the Eppendorf Master Gradient thermocycler by applying denaturation at 94°C for 4 min followed by 35 cycles (45s denaturation at 94°C; 30 s primer annealing at 56°C for *gfp* primers or 65°C for *uidA* primers; 60s elongation at 72°C) and then final 5 min elongation at 72°C. A potential bacterial contamination was verified using the same PCR parameters as for *uidA* gene and applying primers specific to virD2 sequence (Haas *et al.,* 1995). Amplified products were separated by electrophoresis in 1 % agarose gel in 1xTBE buffer. For Southern blot hybridization, 5μg DNA was digested with restriction enzymes (*Hind*III or *Bam*HI for hairy roots obtained with 102 and 105 or 131 *Agrobacterium* strain, respectively), separated in 1 % agarose gel and transferred onto nylon membrane (Roche). Probes

were obtained using *uid*A and *gfp* gene specific primers (Hamill *et al.,* 1991 and Lipp *et al.,* 2001, respectively) and were labeled with DIG using PCR DIG Probe Synthesis kit (Roche). Plasmid DNA, non-restricted DNA from genetically modified plants and control DNA from sugar beet were used for reference. After hybridization with labeled probes at 65°C, detection of signals was performed using a chemiluminescent substrate according to the manufacturer instructions.

Statistical analysis. Treatment means were calculated and accompanied by their standard errors. Data were subjected to one-way ANOVA available in the Statsoft Statistica v.8.0 package. Percentage data were transformed using Bliss transformation arcsin $\sqrt{x}$ before the analysis.

RESULTS AND DISCUSSION

In the process of genetic transformation wounding of plant tissue plays a crucial role as the released plant metabolites may activate a cascade of bacterial virulence genes (Gaba *et al.,* 2006). In plants that are susceptible to *Agrobacterium* tissue wounding done during explants preparation is thus usually sufficient. However, in recalcitrant species like sugar beet, it is necessary to use additional mechanical injuries using needles or by tissue scratching (Hoshi *et al.,* 2004). Alternatively, explants are treated with ultrasounds, which disrupt tissue. Based on our preliminary experiments, genetic transformation without ultrasound treatment of sugar beet explants was inefficient. In control, explants were inoculated with bacteria, but the sonication step was omitted. Hairy roots usually did not occur at all or in some replications they occurred on individual explants only. After SAAT the development of hairy roots was abundant. They started to emerge usually 10 days after the inoculation mainly at the explant wounding site (Fig. 1). The number of hairy roots ranged from 1 to 12 per explant. Hairy roots were characterized by a typical phenotype described in other species (Giri & Narasu, 2000). They grew rapidly, formed lateral branches and showed lack of geotropism. After transfer to a hormone free medium they continue fast growth and proliferation.

Comparison of SAAT treatment variants using diploid sugar beet revealed that explants developed hairy roots with various frequency ranging, on average, from 6% to 54% (Table 1). In general, short ultrasound treatments lasting for 15s and 30s better stimulated hairy root development than 1–4 min treatments. Also, high optical density of inoculum promoted hairy root formation but at short ultrasonic treatments only. Ultrasounds cause tissue disruption leading finally to its damage as shown in other plant species (Liu *et al.*, 2006). Thus, short sonication is recommended, although its time must be adjusted to explant type. Trick & Finer (1997) indicated that the efficiency of transformation process is independent on the presence of *Agrobacterium* during sonication. Hence most of SAATs is usually carried out in the inoculation medium already containing bacteria (Pathak & Hamzah, 2008). In contrast, our results indicate that higher percentage of explants form almost two times more hairy roots when sonication is applied to explants immersed in the inoculum than when *Agrobacterium* is added after sonication ($P < 0.05$). Such reaction indicates that ultrasounds promote explant penetration by bacteria.

Time of explant co-culture with bacteria is essential for proper T-DNA transfer to a host cell. One to two day co-culture is usually allowed before *Agrobacterium* is eliminated from the culture. Extended time may be beneficial for species recalcitrant to *Agrobacterium* as shown by Mishutkina *et al.* (2010) who got higher efficiency after a 4-day co-culture. In our experiments a 3-day co-culture was found more advantageous than a 4-day co-culture.

Table 1 Percent of diploid explants (genotype No. 24) developing hairy roots after SAAT using inoculum with LBA1334-gfp strain depending on duration of ultrasonic treatment.
The bacterial inoculum of OD_{600}=0.05 or 0.5 was added before sonication, and co-culture lasted for 3 days. Means ±standard error, number of explants per treatment *n*=100

Duration of ultrasonic treatment [s]	OD_{600} of bacterial inoculum			
	0.05		0.5	
15	19.0	±3.79	54.0	±3.40
30	9.0	±2.77	52.0	±5.33
60	7.0	±2.60	33.0	±4.73
120	6.0	±2.67	8.0	±2.49
240	7.0	±2.60	7.0	±2.13
Mean	9.6	±1.43	30.8	±3.34

Table 2. SAAT efficiency of three haploid explants immersed in three bacterial inocula of OD_{600}=0.5 and after a 3-day co-culture.
Means ±standard error

Haploid genotype	*Agrobacterium* strain	No. of explants	Percentage of explants forming hairy roots		Total number of hairy roots	Percentage of *gfp/gus* + hairy roots
168	A4T-gfp	80	28.8	±9.72	117	53.8
168	LBA1334-gfp	130	30.8	±5.00	187	64.2
168	A4T-gus	130	30.0	±4.39	212	35.9
169	A4T-gfp	100	17.0	±5.78	38	36.8
169	LBA1334-gfp	50	36.0	±5.10	28	64.3
169	A4T-gus	50	10.0	±0	13	0
170	A4T-gfp	90	11.1	±3.89	20	50.0
170	LBA1334-gfp	140	10.0	±2.96	29	62.1
170	A4T-gus	140	16.4	±3.87	57	36.4
Total/Mean		2270	20.8	±1.87	701	54.8

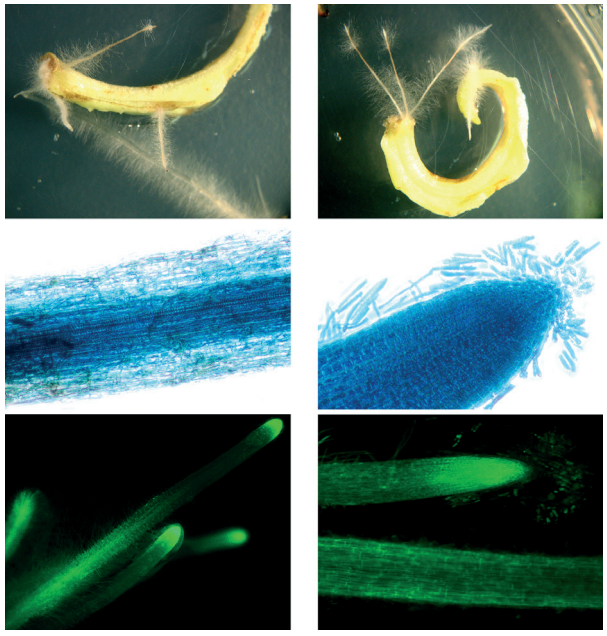

Figure 1. Hairy roots developing at wounding sites of haploid genotype No. 170 explants after SAAT (upper). Transgenic hairy roots expressing *uid*A gene and stained in blue after β-glucuronidase assay (middle) and expressing *gfp* gene visible by green fluorescence in UV light (bottom).

This later resulted in a reduced number of hairy roots by 30% and additionally caused tissue colonization by bacteria and decay similarly as observed by Jacq *et al.* (1993).

In total, 226 hairy roots were obtained from 500 explants and 107 out of them (47%) showed green fluorescence in UV light that confirmed transfer of the *gfp* gene from *Agrobacterium* and its expression in plant tissue. When short sonication times (15 s and 30 s) were applied, the expression of GFP was observed in 54.3% and 47.5% of hairy roots, respectively.

SAAT conditions found the most effective for diploid explants (15 s sonication in the presence of *A. rhizogenes* inoculum of OD_{600} = 0.5, 3-day co-culture) were subsequently verified using explants of three haploid genotypes. Other two bacterial strains were also additionally used. The mean transformation efficiency of haploids was 20.8% and significantly depended on the genotype ($P<0.005$), but not on the bacterial strain used ($P=0.23$) (Table 2). On average, hairy roots were formed most effectively on explants of 168 genotype (30.0%). The other two genotypes 169 and 170 responded with lower efficiency 20% and 12.7%, respectively, and the difference between them was significant. Both haploid genotypes were derived from two ovules of the same donor plant, which was not closely related to a donor of 168 genotype. Thus, the observed variation was due to genetic background of explants, a common factor identified in most research (Gurel *et al.*, 2008; Smigocki *et al.*, 2008).

In total, 701 hairy roots were obtained from haploid explants (Table 2). The expression of transgenes (*gfp* or *gus*) was observed in 54.8% of the examined roots (Fig. 1), that a similar rate as for the diploid explants No. 24. The highest percentage of roots exhibiting transgene expression was obtained using A4T-gfp strain, independently on the explant genotype, and for which green fluorescence in UV light was observed in over 60% of hairy roots.

Results of PCR reactions carried out using primers specific to the transgenes confirmed T-DNA transfer in 26 out 44 hairy roots (59%), as amplified fragments had expected sizes (Fig. 2). For 13 hairy root clones no PCR products of the expected size were obtained. In further five clones PCR results remained ambiguous as virulence genes were detected indicating contamination of the plant material with bacterial DNA. Southern hybridization confirmed integration of the transgenes, which copy

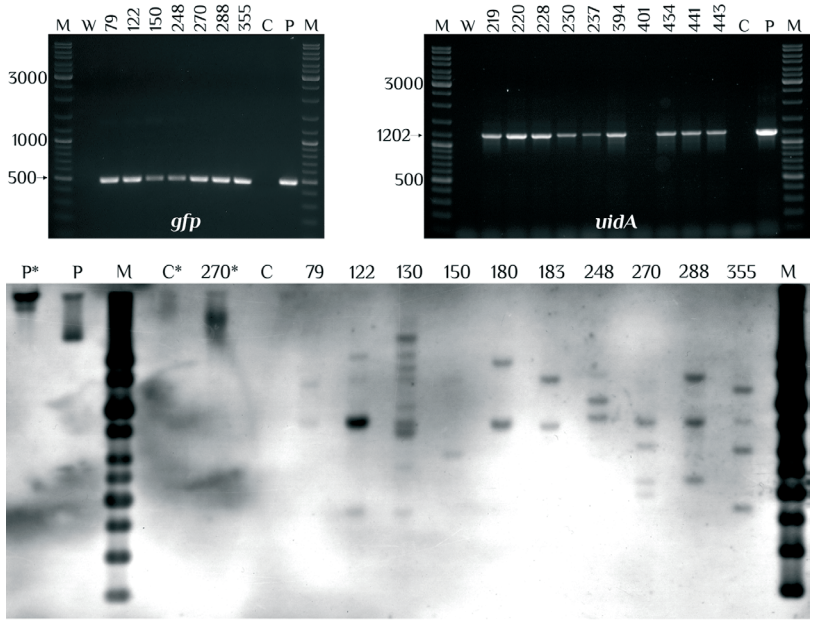

Figure 2. Products of PCR amplification of *gfp* and *uidA* fragments using gene specific primers (upper) and Southern blot of hairy root DNA obtained after transformation of haploid genotypes with A4T-gfp and LBA1334-gfp strains and hybridized with gfp probe (bottom).
M — DNA Ladder Mix, W — water, C — DNA of non-transformed plant, P — pBIN-mgfp5-ER plasmid, 79–443 — DNA of independent transgenic hairy roots, *undigested DNA.

number varied from one to nine depending on the transformation event (Fig. 2).

So far, haploid explants have never been used in sugar beet genetic transformation in contrast to the use of diploid tissues (Kishchenko *et al*., 2005, Jafari *et al*., 2009). Haploids may be valuable material for genetic transformation as diploidization induced after a gene transfer would lead to the development of a homozygous tissue, including a transgene locus. Thus, the locus becomes fixed (Dunwell, 2010). Haploids are routinely produced and utilized by sugar beet breeding companies for the development of DH lines. Our results show for the first time that sugar beet haploid tissue is amenable to genetic transformation using *A. rhizogenes* and that its efficiency can be enhanced using SAAT method. This opens new perspectives for a fast development of homozygous GM sugar beet tissue useful primarily in basic research. Its further application can be possible once a protocol for shoot morphogenesis from sugar beet hairy roots is released.

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