

ARTICLE

Agrobacterium-mediated barley transformation

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ABSTRACT More than ten years have passed since the first successful *Agrobacterium*-mediated barley transformation experiment, however it is still quite challenging to establish a stably functioning agroinfiltration protocol. Efficiency of the method depends mainly on the transformation and co-cultivation conditions, and also the components of the tissue-culture media. With the use of an optimized media we have been able to set up a reliable, properly functioning transformation protocol. The first generation of transgenic barley plants, transformed with a transformation cassette carrying an aldo-keto-reductase gene from *Arabidopsis thaliana* and the hpt marker gene, were analyzed at nucleic acid (both DNA and RNA) and at protein levels. The key factors of success proved to be the use of Silwet L-77 (surfactant) in transformation inoculum, the Cu-content of regenerating media and the continuous visual monitoring of the transformed callus during the somatic embryogenesis.

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KEY WORDS

barley transformation
agroinfiltration
tissue-culture media
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somatic embryogenesis

Barley (*Hordeum vulgare* L.) is the fourth most important crop in the world after maize, wheat and rice. Recently it is becoming a novel cereal model plant, because of its true diploidy and the relative ease of genetic transformation compared to some other monocots. A further advantage is that the barley genome is very similar to the D genome of wheat, so the results with barley should be relevant to transgenic wheat as well.

The genetic transformation of barley has been accomplished in the last twenty years through various methods. Biolistics and agroinfiltration have become the most widely used, because the best transformation efficiency can be reached through these approaches. Agroinfiltration excels with the best transformation efficiencies. Further advantages are that transgene copy number is typically low, there is little genome impairment, unwanted DNA integration is lower and there is a bigger chance for the DNA to integrate into transcriptionally active regions, compared to the biolistic method (Czernilofsky et al. 1986; Konec et al. 1989).

Tingay et al. (1997) reported the very first successful *Agrobacterium*-mediated transformation of barley, using the variety called Golden Promise. Since then, the main object of the methodical studies is to enhance transformation efficiency. This aim is reached via optimizing the conditions of the transformation and co-cultivation, and also the ingredients of the applied tissue-culture media. The method established by Tingay and co-workers (1997) reached a transformation efficiency of 4.2% (independent transgenic lines/number of transformed embryos) without specific Vir-operon activator (such as acetosyringone). Since then e.g. Trifonova et al. (2001) and Shrawat et al. (2007) reported the positive effect

of acetosyringone on transformation efficiency, when applied during the co-cultivation period. Both Trifonova et al. (2001) and Shrawat et al. (2007) have found that a preculturing step of the embryos, for one or two days before transformation, also enhances the transformation efficiency.

Our aim was to establish a functioning barley agroinfiltration protocol in our laboratory. For the sake of the cause we constructed a transformation cassette carrying the uidA reporter gene, and the protocol was further optimized during a new transformation project. Here we also report data on a transgenic barley line carrying an aldo-keto reductase (AKR) gene derived from *Arabidopsis thaliana* for stress-physiology studies.

Materials and Methods

Agrobacterium strain and vector

The transformation experiments were carried out with a gene-construct, that included a 3' His-tagged *Arabidopsis thaliana* akr gene (Gene Bank accession number: DQ837654) between a Nippon-bare derived rice actin promoter followed by the first intron of the rice actin gene, and *A. tumefaciens* nopaline synthase (nos) terminator fragment. The gene-construct was ligated into the multi-cloning site of the binary vector pWB-Vec8 containing the hygromycin-phosphotransferase (hpt) resistance gene, controlled by the cauli-flower mosaic virus 35S promoter and the nos terminator. The transformation cassette was introduced into the AGL1 *A. tumefaciens* strain by heat-shock.

Plant material

Barley plants were potted in a 2:1 mix of peat-containing soil and sand. The plants were grown in a SANYO Fitotron

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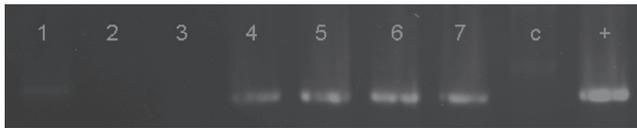


Figure 1. Detection of a 500 bp long fragment, characteristic for the gene of interest, in T0 plants by PCR. Lane 1-7: hpt gene containing regenerated plants; Lane c: untransformed control plant; Lane +: transformation cassette. The PCR-analysis confirmed the presence of the sequence in Lane 1,4,5,6 and 7.

growth chamber at 70% humidity, with a 16 hour photoperiod and light intensity of $700 \mu\text{mol m}^{-2}\text{s}^{-1}$, 15°C day and 12°C night temperature.

Tissue culture media

BCI medium was optimized by Wan and Lemaux (1994); all the other mediums were optimized by Wendy Harwood (personal communication). Both the media and vitamins were filter sterilized.

DBC (for 1 litre): 2,7 g MS salts no NH_4NO_3 (M0238), 165 mg NH_4NO_3 , 750 mg glutamine, 20 g maltose, $5 \mu\text{M}$ CuSO_4 , 2,5 mg 2,4 D, 0,1 mg BAP, 3,5 g Phytigel, 10 ml

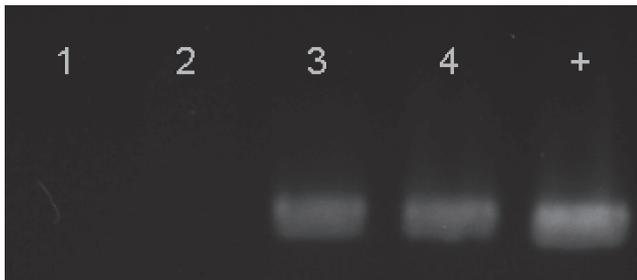


Figure 2A. Detection of a 300 bp sequence at the 3' end of the akr-gene by RT-PCR. 50x diluted (Lane 1) and undiluted (2) control cDNA; 50x diluted (Lane 3) and undiluted (4) cDNA of transgenic barley; transformation cassette (Lane +).

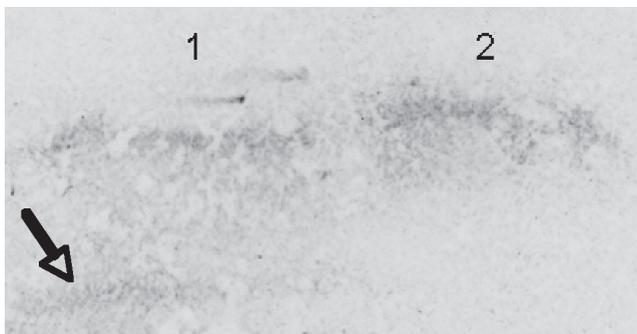


Figure 2B. (right) Detection of the produced *Arabidopsis* AKR protein by Western blot analysis. Transgenic barley plant (Lane 1); control barley plant (Lane c).

100 × FWG vitamins stock (40 mg/l Thiamine-HCl, 10 g/l myo-inositol)

FWG had the same components as DBC, but without the CuSO_4 and hormones.

Barley transformation and tissue culture

Immature barley embryos of 1-2 mm length were isolated from surface-sterilized caryopses. The axis was removed from the scutella. 25 embryos were placed on a BCI medium containing Petri dish and 0.4 ml *Agrobacterium*-suspension ($\text{OD}_{600}=2$) was pipetted onto them. The suspension was prepared by resuspending the *Agrobacterium* cells from an overnight culture in liquid BCI media supplemented with 0,015% Silwet L-77. The excess inoculum was drained off the surface of the medium. The embryos were transferred onto new BCI media without antibiotics for co-cultivation for 3 days. Co-cultivation and all tissue culture steps were carried out at 23°C . On the fourth day, embryos were placed onto BCI-media supplemented with 75 mg/l hygromycin and 150 mg/l timentin for selection, and callus induction. Embryos were kept in dark on this media for 2×3 weeks. When no sign of somatic embryogenesis was detected this period was extended by 1 or 2 weeks.

After the callus induction phase, the explants were placed onto DBC medium supplemented with 75 mg/l hygromycin, and 150 mg/l timentin for 2-6 weeks, until greening was visible. From this step on, the plates were illuminated with $300 \mu\text{mol m}^{-2}\text{s}^{-1}$ of light for 16 hours a day.

Finally, explants were placed onto FWG medium supplemented with 150 mg/l timentin for regeneration. When the shoot length reached 10 cm-s, the plants were potted out.

PCR-reactions

For detection of a 500 bp long region overlapping the 3' end of the rice actin-1-intron and the 5' end of the akr gene, the following primers were used: Prom3'F 5' GTAAC-CACCCCGCCCCCTC 3' and AKR_5'R 5' TGCCATGTTC-CAAGACCCAC 3'. PCR-reactions were carried out in 10 μl volumes containing 0.4 ng plasmids or 20-40 ng genomic plant DNA, 1 × Dynazyme™ enzyme buffer, 0.5-1 μM primers, 1 mM dNTPs, and 1 U Dynazyme™ Taq DNA-polymerase. Samples were predenatured at 95°C for 5 min and then subjected to 36 cycles of amplification in an ICycler™ (BIO-RAD) thermal cycler. Thermocycling conditions were as follows: denaturation at 95°C for 30 s, annealing for 30 s and extension at 72°C for 30 s.

mRNA extraction and reverse transcription

mRNA was isolated from plant leaves with Geno Vision mRNA Isolation kit, according to the manufacturer's instructions. Reverse transcription was conducted by using the iScript kit (BioRed).

Protein extraction and western blot analysis

Leaf total protein was extracted with ice-cold extraction buffer (50 mM Tris HCl, 10 mM EDTA, 50 mM DTT, 0,1 mM PMSF, 0,1 V/V % SDS). Plant leaf tissue was pulverized with a mortar and a pestle at room temperature using equal volume of buffer. The suspension was centrifuged, and then 14 µl of the supernatant was separated by SDS-PAGE on 12% gel. Immuno-blot detection was carried out using a semi dry transblot apparatus (Bio-Rad) as described by Barro et al. (1997).

Results

100 embryos were used in one transformation event. Seven hygromycin B resistant transgenic lines were identified carrying the *akr* gene containing cassette. The *hpt* resistance marker gene was detected in all lines by PCR-analysis (data not shown).

Out of the 7 hygromycin resistant plants, the presence of a 500 bp fragment from our gene-construct was confirmed by PCR in 5 independent lines (Fig. 1) using Prom3'F and AKR5'R primers.

Transgenic plants were analyzed at both RNA and protein levels, (results on transgenic line 4 is presented on Figure 2A and B). A 300 bp long region from the 3' end of the *akr* gene could be detected by RT-PCR (Fig. 2A). The presence of the Arabidopsis AKR protein was also explored in transgenic plants by Western Blot analysis (Fig. 2B).

Discussion

In present study, transgenic barley plants have been produced, as a result of the optimization of our transformation protocol. We have reached the transformation efficiency of 5%. It can be compared to the results of most of the authors (e.g. 6.3%: Trifonova et al. 2001; 6.7%: Shrawat et al. 2007). One of the reasons of the successful transformation could be the use of Silwet-L77 in the inoculation media and the tissue culture media optimized by Harwood (personal communication). Silwet L-77 is a strong surfactant, which helps *Agrobacterium* cells to enter the plant tissue. Hygromycin was used for selection in the highest concentration mentioned in literature for barley (75 mg/l). According to the results of da-Fang et al. (2002) the control embryos cannot grow at all during these conditions. However transformant or transiently expressing embryos could produce callus even at this high level of antibiotics. Most of the non-stable transformants stop growing after two weeks, according to the experiments of Trifonova et al.

(2001) where transient expression was observed in 83% of the explants. On Harwood's pre-regeneration medium the stable transgenic embryogenic calli are able to start greening under these high antibiotic level. This medium contains 5 µM Cu²⁺, which greatly affects plant regeneration (Shrawat 1999). Unlike Harwood, higher concentration of hygromycin was used, but no selection was applied in the last, regeneration media. An important additional advantage of Harwood's media is that plant regeneration occurs during somatic embryogenesis, as in the protocol of Shrawat et al. (2007). During somatic embryogenesis, plants are often developed from a single cell (Ammirato et al. 1983), that's why the frequency of chimeric plants is very low. Also, the visual screening for somatic embryos (as used by Shrawat et al. 2007) supported our work greatly during the selection period.

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