Fertile transgenic plants obtained from tritordeum inflorescences by tissue electroporation

Abstract
Tissue electroporation was applied to a member of the Triticeae family, namely tritordeum (Hordeum chilense Roem. × Triticum turgidum L. Conv. durum), for the generation of fertile transgenic plants. Two transgenic plants were recovered following the treatment of 361 explants of immature inflorescences (although they were subsequently found to result from the same transformation event). The expression of both inserted marker genes (uidA and bar) was confirmed using standard assays, while transgene integration was confirmed using PCR and Southern hybridization analyses. Integration pattern, segregation ratio and the inheritance of transgene expression in T1 progeny were consistent for the presence of a single transgene locus containing five to ten plasmid insertions. Although this procedure has been applied to other cereal species, stable transformation of the Triticeae using tissue electroporation has not previously been reported.

Keywords
Triticeae · Triticoreum · Inflorescence · Tissue electroporation · Transgene inheritance

Abbreviations
GUS β-Glucuronidase · 2,4-D 2,4-Dichlorophenoxyacetic acid · EB Electroporation buffer · PCR Polymerase chain reaction · PPT Phosphinothricin

Introduction
A number of direct gene transfer methods have been successfully used in plant genetic engineering, providing powerful tools to investigate fundamental and applied problems in plant biology (Lazzeri and Shewry 1993; Jaehne et al. 1998). In cereals, several methods have been found to be suitable for obtaining transgenic plants; these include bombardment of scutellum (Vasil et al. 1992; Weeks et al. 1993; Altpeter et al. 1996; Barro et al. 1997) and inflorescence (Barcelo et al. 1994) cultures, silicon carbide fibre-mediated DNA delivery (Serik et al. 1996) and Agrobacterium tumefaciens transformation (Cheng et al. 1997).

Electroporation of cereal protoplasts has also proved successful (Fromm et al. 1986; Zhang et al. 1988; Huang and Dennis 1989; Tada et al. 1990) but involves prolonged cell treatments and is generally limited by the difficulties of regeneration from cereal protoplast cultures. An alternative approach, involving the electroporation of tissue explants, has been successfully used in rice (Li et al. 1991; Xu and Li 1994) and maize (D’Halluin et al. 1992; Laursen 1994; Pescitelli and Sukhapind 1995), but to date this method has not successfully been applied to stable transformation of the Triticeae. Transient gene expression by tissue electroporation has, however, been reported in leaf base tissues (Dekeyser et al. 1990), immature zygotic embryos (Klöti et al. 1993) and embryogenic callus (Zaghmout and Trolinder 1993; Zaghmout 1994) of wheat. These first studies showed the potential of direct gene delivery into ‘intact’ Triticeae cells via electroporation and, subsequently, He and Lazzeri (1998) described improved conditions for electroporation of the scutellum and inflorescence of wheat and tritordeum and reported reliable transient expression. These results provided the basis for the development of a stable transformation procedure, which was the aim of the investigation reported here.
**Materials and methods**

**Plant material**

Tritordeum [an amphiploid between durum wheat and wild barley (Martin and Sanchez-Monge 1982)] line HT174 ‘donor’ plants were grown under glasshouse conditions under a 16/8 h (light/dark) photoperiod with artificial lighting (fluorescent and tungsten lamps) of 350 mol m⁻² s⁻¹ at 18°C/16°C. The relative humidity was 80%. Immature inflorescences ranging from 0.5 to 1.2 cm in length were harvested 60–65 days after the sowing date, dissected aseptically and cut into pieces of approximately 1 mm in length for in vitro culture following the procedure of Rasco-Gaunt and Barcelo (1999).

**Transgenic plasmid construct**

For this study, plasmid pAHC25 (Christensen and Quail 1996), which contains selectable and recoverable marker genes (uidA and bar), both under the control of the maize ubiquitin promoter, was used.

**Tissue electroporation conditions**

Inflorescences were pre-cultured for 1 day prior to electroporation on L7P4-V medium (Barcelo and Lazzeri 1995) to which 15 g/l sorbitol was added to give an osmolarity of 222 mMOSm. Twenty inflorescence pieces were placed into each electroporation cuvette (BioRad, 4 mm electrode distance) containing 180 μl electroporation buffer [80 mM KCl, 5 mM CaCl₂, 10 mM N-2-hydroxyethylpipperazine-N’-2-ethanesulfonic acid (HEPES), 0.425 M mannitol, 20 μl plasmid DNA (1 μg/μl)] that had been adjusted to pH 7.2 before filter sterilization (D’Halluin et al. 1992). As controls, the same number of pieces were electroporated in 180 μl EB and 20 μl TE. Transformation mixtures were incubated at 24°C for 30–60 min and cooled on ice for 10 min just before electroporation. A single electric pulse of field strength 550 V/cm was discharged from a 960 μF capacitor (Gaunt and Barcelo (1999)). Immediately after electroporation, the cuvettes were incubated for 10 min on ice. Prior to transfer of the explants onto media, 200 μl liquid L7P4-V medium was added to each cuvette to dilute the EB. The explants were then transferred to basal L7P4-V medium at 141 mMOSm (standard osmoticum) and cultured at 23 ± 2°C in darkness for 4 weeks for embryogenic callus induction, following a standard protocol for inflorescence culture (Barcelo and Lazzeri 1995).

**Tissue culture and selection of transformants**

After 4 weeks of culture, electroporated explants were transferred onto plant regeneration medium (RDZ, Barcelo and Lazzeri 1995) supplemented with 0.1 mg/l 2,4-D, 5 mg/l zeatin and 2 mg/l of the herbicide phospinothricin for selection during one passage of 3 weeks. Control cultures were divided randomly and equally into RDZ medium with or without PPT. Subsequently, cultures were transferred onto hormone-free regeneration medium (Barcelo and Lazzeri 1995) containing 3 mg/l PPT for two to three 3-week passages, until all of the control cultures on selection medium had been killed.

**Transgene expression assays**

A histochemical GUS assay was applied to tritordeum inflorescence explants in order to analyse transient GUS expression 4–8 days after electroporation (Barro et al. 1998). The same assay was used on leaf tissue of primary transformants and the progeny to study the inheritance of transgene expression. To examine the expression of the bar gene, we applied BASTA solutions at concentrations of 0.1%, 0.5% or 1.0% (v/v) containing 0.1% (v/v) Tween 20 to leaves of putative transgenic plants by painting with cotton wool buds. The area of leaf necrosis was scored 1–2 weeks after BASTA application.

**Molecular procedures**

**PCR conditions**

PCR was used to confirm the status of the putative transformants and to follow transgene transmission in progeny. Aliquots of 50–200 ng of genomic DNA were added to reaction mixtures containing 50 mM KCl, 10 mM Tris-HCl (pH 8.8), 1.5 mM MgCl₂, 0.1% Triton X-100, 200 μM of each dNTP, 0.3 μM of each primer, and 0.66 U Dynazyme DNA polymerase (Flowgen, Lichfield, UK). The forward primer for the amplification of the uidA gene was 5’-AGTGTACGTACCGTTTGTGTTGAAC-3’, and the reverse primer was 5’-ATCGCCCGTTTGGGACATACTCATCCGTA-3’. The forward primer for the amplification of the bar gene was 5’-GTCGTGACATCGTCAACC-3’, and the reverse primer was 5’-GAAGTCGACGTCGACAAAG-3’. Reactions were carried out in an OmniGene thermal cycler (Hybaid) under the following conditions in four stages. For the uidA gene: PCR stage 1: denaturing at 94°C for 5 min, annealing at 62°C for 30 s and extension at 72°C for 2 min – 1 cycle; stage 2: denaturing at 94°C for 1 min, annealing at 62°C for 30 s and extension at 72°C for 2 min plus 3 s for every cycle – 30 cycles; stage 3: 72°C for 10 min – 1 cycle; stage 4: 30°C for 30 s – 1 cycle. For the bar gene the only difference was an annealing temperature of 57°C for stages 1 and 2. The sizes of the amplification products were 1.05 kb (uidA gene) and 0.46 kb (bar gene).

**Southern hybridization**

Extraction of plant DNA was undertaken using a CTAB method (Barro et al. 1998). For each DNA sample, 10–15 μg was digested with HindIII and 10–15 μg with SacI. Digestion with HindIII releases an approximate 4.2-kb fragment from plasmid pAH25 containing the maize ubiquitin promoter and uidA gene. SacI cuts once within plasmid pAH25. Digested DNA fragments were separated by means of 0.8% agarose gel electrophoresis and capillary blotted to a positively charged nylon membrane. A digoxigenin-labelled probe for part of the uidA coding region was prepared using the PCR-DIG probe synthesis kit from Roche Molecular Biochemicals and the uidA-specific primers described above. Hybridization and the chemiluminescent detection of probe were carried out according to the DIG System User’s Guide for Filter Hybridization (Roche Molecular Biochemicals).

**Results and discussion**

**Transformation by tissue electroporation**

Three transformation experiments were undertaken using plasmid pAH25. In total, 418 explants (including controls) were electroporated. Of these, 84 explants were either used for transient GUS assays or perished due to culture contamination. The frequency of embryogenesis was 40.4% (135 embryogenic calluses from 334). For embryogenic calluses selection pressure was first applied on RDZ regeneration medium containing 2 mg l⁻¹ l-PPT (one 3-week cycle) and then on R medium containing 3 mg l⁻¹ PPT for two to three 3-week cycles. Control explants which were not subjected to selection pressure regenerated at a frequency of 85.7% (18 explants from 21).