



Rhizobium radiobacter* conjugation and callus-independent shoot regeneration used to introduce the cercosporin export gene *cfp* from *Cercospora* into sugar beet (*Beta vulgaris* L.)*

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Abstract

Leaf spot disease caused by *Cercospora* is responsible for crop and profitability losses in sugar beet crops in the US and worldwide. The *cfp* gene that encodes a protein that exports phytotoxic cercosporins from *Cercospora* was conjugally transferred to sugar beet using *Rhizobium radiobacter* (*Agrobacterium tumefaciens*), to improve *Cercospora*-induced leafspot resistance. Conditions for shoot regeneration were optimized to increase regeneration/transformation efficiencies. Low-light and room-temperature conditions were favorable to sugar beet regeneration without callus when cytokinin had been added to the tissue culture medium. Using this procedure adventitious shoots from leaf pieces were obtained in a simple, one-step regeneration procedure. T7, a *cfp*-transgenic clone verified by PCR with gene-specific primers, is being propagated for leaf spot disease resistance evaluation.

Introduction

Leaf spot disease in sugar beet, caused by *Cercospora beticola* (Imperfecti, Monociliales, Dematiaceae (Barnett & Hunter 1972), is widespread and destructive. Germinating spores on the leaves enter through the stomata to produce small circular spots which coalesce as the disease progresses, producing large necrotic areas or destroying entire leaves (Ruppel 1986). Germplasm screening and selective breeding have yielded only moderately resistant germplasm. All known genetic resistances are multigenic and are thus not easily transferred to agronomically elite germplasm. Smith & Ruppel (1974) quantified the low heritability of *Cercospora* resistance in sugar beet, that resistance was multigenic and intermediate. Bioengineering may safely control *Cercospora* leaf spot disease in sugar beets.

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The lack of an efficient and reproducible method of transformation has slowed the progress of biotechnology applications in sugar beet. Adventitious shoots have been described on the leaves of intact potted plants where the seedling apex was previously treated with the cytokinin *N*⁶-benzyladenine (Saunders 1982, Harms *et al.* 1983); thin layer explants from seedling grown *in vitro* with *N*⁶-benzyladenine (Detrez *et al.* 1988, 1989, Grieve *et al.* 1997), as well as in explants of petioles or leaves of seedlings or shoots from plants cultivated *in vitro* (Detrez *et al.* 1989, Ritchie *et al.* 1989, Sabir & Ford-Lloyd 1991, Grieve *et al.* 1997). None of these regeneration methods has yet been adapted for genetic transformation because of concern about internally preformed initials, the technique not easily being repeated, and/or the low frequency of shoot initiations.

Cercosporin is a red, light-activated, membrane-destabilizing toxin (Daub 1982, Daub & Hangarter 1983, Daub *et al.* 1992) secreted by most phytopathogenic members of the fungal genus, *Cer-*

cospora, including *Cercospora beticola*. The *cfp* gene was enriched and isolated from light-induced cDNAs (Upchurch *et al.* 1991, Callahan *et al.* 1999). Upchurch *et al.* (2002) recently reported that the resistance of *cfp*-carrying transgenic *Cochliobolus heterostrophus* to *Cercospora* toxin was due to production of CFP, a transmembrane facilitator protein that transports cercosporin out of the cell. Recently *cfp*-transgenic tobacco plants were observed to have a 10-fold reduction in the number of lesions and a 50% reduction in the size of leaf lesions induced by *Cercospora* (R.E. Upchurch, personal communication).

Here we present the construction of the first *cfp*-carrying transgenic sugar beet.

Materials and methods

Plant tissue and culture conditions

Greenhouse-grown *Beta vulgaris* cultivar REL-1 plants were used to study the formation of adventitious shoots from leaves, which were either fully expanded, 20% expanded, or 40% expanded. REL-1 was developed for unconventional callus induction, shoot regeneration and somatic embryo systems (Saunders 1982, 1998, Saunders & Doley 1986). This genotype exhibits temperature-dependent, high-frequency, hormone-autonomous regenerative callus from leaf piece explants, and little vitreousness in shoots regenerated from such callus. Leaf tissue was sterilized using two 15 min washes in a solution containing 15% (v/v) commercial hypochlorite and 0.01% SDS (w/v) after which the material was rinsed five times with sterile water. One or two cm² explants were cut from various tissue types and one or two such pieces were placed on the surface of one of the 11 or 13 different media types (see Table 1). All media contained modified Murashige & Skoog (1962) inorganic salts amended with 30 g sucrose per liter, 100 mg myo-inositol per liter, 0.5 mg nicotinic acid per liter, 0.5 mg pyridoxine · HCl per liter, 0.1 mg thiamine · HCl per liter, 0.5 g MES l⁻¹ per liter and 5 g agar per liter (Sigma). The pH was adjusted to 5.8 using KOH before autoclaving and varied concentrations of N⁶-benzyladenine and/or α -naphthaleneacetic acid were added individually or in combination after autoclaving. Medium without hormones was used as a control. After leaf petiole or mid-vein segments were placed on the surface of agar media (35 ml per 100 × 20 mm disposable, sterile petri dish) and the

plates were double-sealed with Parafilm to prevent desiccation. The plates were stored at room temperature (about 23.5 °C) in stacks approximately one meter from Cool White fluorescent bulbs.

Gene transfer

Seeds of REL-1 and C69 were used for gene transfer experiments. C69 seeds were obtained from Dr Bob Lewellen, Salinas, CA, and used for gene insertion experiments since this genotype may be ideal for the transfer of transgenes into elite germplasm. All seeds were surface sterilized in the same manner as described above except an additional 5 min were added to the hypochlorite-SDS washes to accommodate for a rough surface. After 10–14 d of germination in the dark at room temperature on 5% Tryptic Soy Agar (1.5 g Tryptic Soy Broth l⁻¹ and 15 g agar l⁻¹) medium, used to confirm seed sterility, excised seedling cotyledons were aseptically transferred to Murashige & Skoog medium, as described above, with the addition of shoot-inducing 6-benzylaminopurine (BAP) at 1 mg l⁻¹. Since plant leaf discs incubated on tissue culture media containing higher levels of the cytokinin BAP produced both more callus and more vitreous shoots, subsequent transformation experiments involved tissue culture agar medium plates containing 1 mg BAP l⁻¹, which is nevertheless higher than the 0.3 mg BAP l⁻¹ routinely employed for shoot multiplication.

Rhizobium radiobacter strain EHA 105 carrying Plasmid X was maintained on kanamycin-containing agar medium. Freezer stocks (50% glycerol v/v) were used for bacterial storage at -4 °C. Yeast extract/D-mannitol (YEM) medium (Vincent 1970) was routinely used for *Rhizobium* growth. For each culture, 3 ml LB broth were seeded with bacteria from a freezer stock and incubated at room temperature for 1 or 2 d on a rotary shaker.

Axenicly-cultured, sterile cotyledons of sugar beet genotypes REL-1 and C69 were inoculated with bacterial strain EHA105 harboring plasmid X to transfer the *cfp* gene into sugar beet. While on the agar surface, cotyledons were wounded by cutting with a scalpel or puncturing with a medium gauge needle. Then, cotton swabs soaked in fresh broth culture containing about 10⁹ c.f.u. ml⁻¹ of strain EHA105 (plasmid X) were dabbed onto the wounded cotyledons. Plasmid X contained the full length *cfp* cDNA under the control of the 35S promoter in a pBIN19 expression vector derivative (Clonetech Laboratories

Table 1. Number of adventitious shoots formed on plates of tissue culture media (MS + Gamborgs vitamins) containing various hormone concentrations and leaf segments at various stages.

Media names	Exogenous hormone concentration(s) (amount l ⁻¹)	Attached shoots on mature leaf pieces (1/plate)	Attached shoots on 20% expanded leaf pieces (2/plate)	Attached shoots on 40% expanded leaf pieces (2/plate)
MO	None	None	None	None
N.1	100 µg NAA	None	–	–
N.2	200 µg NAA	–	None	None
N.3	300 µg NAA	None	–	–
N1	1 mg NAA	–	None	None
B.3	300 µg BAP	None	None	None
B.3N1	300 µg BAP; 1 mg NAA	None	None	None
B1	1 mg BAP	3 of 4	1 of 5	None
B1N.03	1 mg BAP; 30 µg NAA	None	–	–
B1N.2	1 mg BAP; 200 µg NAA	–	1 of 5	2 of 5
B1N.1	1 mg BAP; 100 µg NAA	None	–	–
B1N.3	1 mg BAP; 300 µg NAA	1 of 2	–	–
B1N1	1 mg BAP; 1 mg NAA	–	None	None
B1N3	1 mg BAP; 3 mg NAA	1 of 3	–	–
B2	2 mg BAP	None	2 of 5	1 of 5
B3	3 mg BAP	–	1 of 5	1 of 5
B3N.2	3 mg BAP; 200 µg NAA	–	1 of 5	2 of 5
B3N1	3 mg BAP; 1 mg NAA	–	None	None
B5	5 mg BAP	–	3 of 5	1 of 5

In one experiment (column 3) data on the number of leaf pieces that developed adventitious shoots from mature leaf pieces of REL-1 greenhouse grown plants on media with varied amounts of hormones (tissue included midvein). In the second experiment the number of leaf pieces forming adventitious shoots from 20% and 40% expanded leaf pieces is illustrated per plate. A dash symbol denotes those media were not used in the experiment. NAA is α -naphthaleneacetic acid and BAP is *N*⁶-benzyladenine.

Inc., Palo Alto, CA) and a kanamycin resistance gene as a selectable marker. The infected cotyledons were incubated in the dark at either 28 or 30 °C for 2–3 d until bacterial growth was evident. Cotyledons were then transferred to selective medium containing 1 mg BAP l⁻¹, 100 mg cefotaxime l⁻¹, and 75 mg kanamycin l⁻¹ in the light at 23.5 °C–25 °C. Leaves that remained green and no longer showed any bacterial growth were transferred to fresh medium of the

same composition. Shoots that developed were then aseptically transferred to fresh medium containing the same BAP and antibiotic concentrations and were allowed to grow until large enough for propagation via shoot multiplication. Shoot cultures were maintained on the same selective medium.

Polymerase chain reaction

Leaf tissue samples were excised for DNA extraction using the Qiagen Plant 'DNA Easy' Kit and some pieces of the leaf tissue samples were incubated in LB liquid for several days to ensure that no donor bacteria had survived antibiotic counter selection. Gene specific primers (given below) were used for PCR reactions in 0.5 ml tubes with a final volume of 50 μ l. PCR reactions were carried out in a PTC-100 by MJ Research, Inc. The program consisted of 35 cycles, and the following conditions: (initial 94° for 2 min) denaturation for 1 min at 94 °C annealing at 67.5 °C for 2 min, primer extension for 3 min at 72 °C (final cycle of 72 °C) followed at the end by a 4 °C hold. PCR products were analyzed by 1% agarose gel electrophoresis. Fragment sizes were estimated with reference to a 1 kb ladder size standard (New England Biolabs, Beverly, MA). Parental REL-1 plant DNA served as a negative control and plasmid X DNA as a positive control.

Sequence of sense primer (5' to 3') was: CCA TCA TCA GCA CAG CAATCC; the sequence of antisense primer (3' to 5') TAC AGC AAC GAC ACG ACC AG.

Results

Adventitious shoots

Low-light and room-temperature conditions were favorable to sugar beet regeneration where cytokinin had been added to the tissue culture medium. Mature leaf pieces including a section of midvein had developed neither callus nor adventitious shoots after six weeks of incubation. By 12 weeks, some plant segments had developed adventitious shoots; shoots issued mostly from the basipetal end. By 15 weeks: (1) attached adventitious shoots had formed on leaf pieces contained in 3 of 4 plates of the 1 mg BAP l⁻¹ media where BAP was the sole source of exogenous growth regulator, and (2) shoots emerge from leaf pieces in the presence of 1 mg BAP l⁻¹ and 1 mg NAA l⁻¹ emerged at a lower frequency than that obtained without the addition of NAA (Table 1).

After 16 weeks, shoot development from 20% and 40% expanded leaves was evaluated, and partially expanded leaf pieces apparently had generated more shoots per leaf piece than mature leaf pieces. Adventitious shoots were observed on increased levels of BAP although with the elevated hormone concentra-

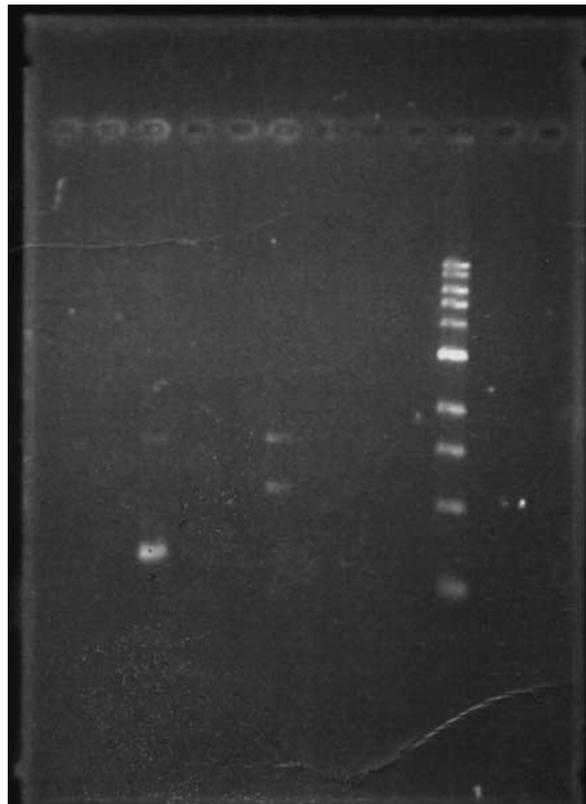


Fig. 1. Agarose gel electrophoresis of PCR products generated using the *cfp*-specific primers and conditions described in Materials and methods. Lane 3 shows the product formed by minute quantities of the DNA of sugar beet transgenic clone #7.

tions shoots were generally smaller, more vitreous, and accompanied with more callus formation evident.

Gene transfer

Shoot regeneration was obtained from 1% of 300 cotyledons of two different genotypes when treated with bacteria bioengineered to transfer desired genes into plant. After co-incubation with *R. radiobacter* and selection, three kanamycin-resistant shoots developed, and these shoots were vegetatively propagated as clones and treated as distinct presumptive *cfp*-carrying transgenics; one from REL-1 and two from C69. The REL-derived *cfp*-transgenic clone initially designated PT7 survived continued propagation on antibiotic-containing medium and consistently tested positive by PCR using gene-specific primers designed to detect the presence of the *cfp* insert, even after many subcultures (Figure 1, lane 3). However, the two genotype C69 clones did not test positive for *cfp* with PCR and did not survive prolonged propagation

on kanamycin-containing medium. Transgenic sugar beet clone T7 is being evaluated by RT-PCR for specific mRNA production. This transgenic clone is being propagated to produce mature plants for leaf spot disease resistance evaluation. Experiments to construct additional transgenic sugar beets are ongoing.

Discussion

This communication reports for the first time the experimental conditions for the regeneration of sugar beet plants from cells by adventitious shoot formation without hormone-independent callus. Improved regeneration of sugar beet will be important for the construction of disease-resistant transgenic plants. The cotyledon infection method we developed is not similar to that of Snyder *et al.* (1999), and this new protocol is simple and repeatable.

That physiological age of tissue influenced regeneration was shown in these experiments since immature tissue regenerated more shoots than mature. We therefore elected to perform *Rhizobium* conjugation and exconjugant selection/shoot regeneration on cotyledons rather than leaf pieces. Leaf pieces are not easily surface-sterilized; losses due to contamination are high; and shoot formation requires prolonged incubation. Early detection of any microbial contamination on seeds made it possible to avoid contaminated cotyledons and therefore we could efficiently test as many as 20 cotyledons per media plate (data not shown). Moreover the physiological age among fully expanded cotyledons may be more uniform than leaves in varied stages of expansion, affording greater uniformity of starting material. Therefore, cotyledons of seedlings from surface-sterilized seeds were used for the selection of antibiotic-resistant, regenerated shoots following bacterial conjugation.

The construction of *cfp*-carrying transgenic clone T7 was successfully performed using the same conditions and medium optimized in leaf disc assays. Thus the new conditions we established for sugar beet shoot regeneration using surface-sterilized leaf pieces incubated at room temperature (about 24 °C) were directly applicable to axenic cotyledons inoculated with *Rhizobium* engineered for the introduction of foreign genes. We envision that the successful introduction of the *cfp* gene into sugar beet via bacterial conjugation will lead to the identification of germplasm, having increased resistance to *Cercospora*-induced leaf spot disease,

which could be used as source of a single dominant leafspot resistance gene in breeding programs.

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