TRANSFORMATION OF SUGAR BEET WITH *CFP* TO IMPROVE *CERCOSPORA* LEAFSPOT RESISTANCE

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ABSTRACT

Sugar beet leaf spot disease, caused by *Cercospora beticola*, results in sucrose percentage and tonnage both being diminished. The *cfp* gene from *Cercospora* may stabilize eukaryotic membranes when the light-activated form of the potent phytotoxin cercosporin is present. Greg Upchurch, ARS/USDA at North Carolina State University, discovered that transgenic tobacco carrying the *cfp* gene has increased resistance to infection by *Cercospora*. The construction of a *cfp*-carrying sugar beet in our laboratory has now been successfully performed. This advance was largely due the late Joe Saunders' recent improvement in sugar beet regeneration.

INTRODUCTION

Our laboratory is concerned with the construction of transgenic sugar beets that can safely control crop losses due to fungal disease. Bioengineering is needed for the control of leaf spot disease in sugar beets since germplasm improvement via screening and selective breeding has yielded only moderate resistance that is multigenic and thus not readily incorporated into commercial germplasm. Sugar beet plants survive fungal infection but both tonnage and sucrose percentages are significantly diminished. Development of *Cercospora* leaf spot disease-resistant sugar beet germplasm would potentially increase crop profitability an overall 25% or more. Although fungicides are still used to combat predicted epidemics of foliar disease, this practice unfortunately selects for mutants of the virulent pathogen with newly acquired fungicide resistance. Fungicide-tolerant strains of *Cercospora* have long been hypothesized to diminish the effectiveness of spraying with chemical fungicides.

In 1999, Snyder, Ingersol, Smigocki & Owens reported the development of transgenic sugar beets carrying genes encoding bacterial cytokinin and plant pathogen defense-related proteins under transcriptional control of stress or wound inducible promoters. These novel plant genotypes were examined for their ability to inhibit *Cercospora* (Kuykendall and Smigocki, 1999). Two promising transgenic sugar beet genotypes, OOT and *osm*PrS, with antimicrobials under the control of the strong osmotin promoter, were examined in the growth chamber for *Cercospora* leafspot resistance (Kuykendall, 2001). Both transgenics were significantly more susceptible to leaf spot disease than their Rel-1 parental germplasm from which they had earlier been derived.

The concept that a pathogen's gene encoding a toxin pump can confer resistance in the host is new. The *cfp* gene, which specifies a cercosporin export protein, had been found to produce transgenic tobacco resistant to *Cercospora* infection (R.G. Upchurch, personal communication). In this report we describe preliminary success in experimentally constructing *cfp*-carrying transgenic sugar beet plants. The success of this particular project has been largely possible due to the recent development of improved sugar beet regeneration (Saunders et al., 2001). Adventitious shoots were obtained without a callus intermediate. This method was herein applied to genetic transformation.

MATERIALS AND METHODS

Seeds of the C69 breeding line, developed by Dr. Bob Lewellen at Salinas, CA, and of the Rel-1 biotechnology clone, developed by Dr. Joe Saunders, ARS/ MSU, were used as starting material. The *cfp* gene from *Cercospora* was supplied to us by Dr. Greg Upchurch, ARS/NCSU, Raleigh, North Carolina. Plasmid X contains the full length cDNA clone of *cfp* under the transcriptional control of the S35 promoter contained in pBIN19 (Clontek). Plasmid DNA was electroporated into *Rhizobium radiobacter* EHA105, and transformants were selected on kanamycin-containing LB agar medium.

Sugar beet seeds were surface-sterilized using a solution containing 15% commercial bleach (5.25% sodium hypochlorite) and 0.01% SDS. Two twenty minute washes were performed, then the seeds were rinsed with sterile water 5 times and they were allowed to dry in a laminar flow hood. Rel1 and C69 seeds were individually germinated on petite 1/20 TSA-containing plates in the dark. After 14 days, approximately 70% germination was typically obtained and contamination about 25%. Cotyledons were excised from seedlings 2-3 days post germination and were aseptically transferred to a modified MS medium (Murashige and Skoog base with the Gamborg's vitamins; 0.5 g/l of MES buffer; 30.0 g/l of sucrose; pH 5.8, adjusted with KOH and solidified with 5.0g/l of Agargel) with 1.0 mg/l of 6-benzylaminopurine (BAP). Cotyledons were wounded either by cutting with a scalpel or piercing and then were infected with strain EHA105 carrying pX grown from freezer stocks as 3 ml liquid cultures grown at 25°C for 1-2 days on a rotary shaker to high viable cell densities, greater than 10^9 CFU/ml. Cotton swabs dipped in strain EHA105 (pX) were used to transfer the plant-conjugative bacteria onto the surface of the freshly wounded cotyledons. Inoculated cotyledons were incubated in 30°C dark conditions for about 3 days, to allow time for multiplication and interkingdom conjugation. Green, still viable cotyledons were then transferred to selective medium.

Selective plates were placed in low light and room temperature conditions earlier determined (Saunders et al., 2001) to produce adventitious shoot regeneration without an intermediate of hormone-independent callus.

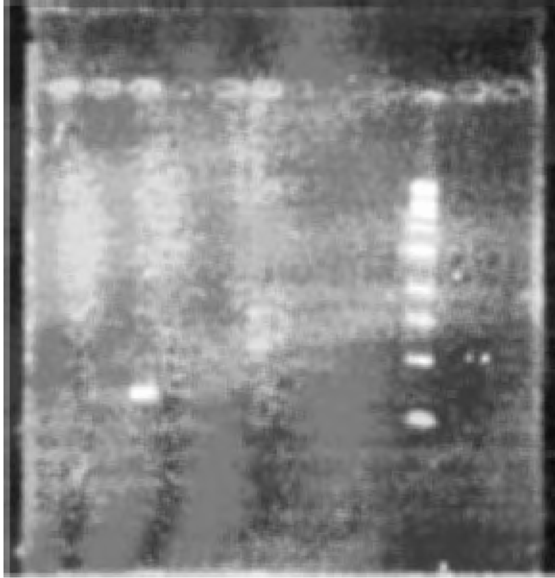
Cotyledons were transferred to medium containing 0.3 mg/l of BAP, 100 mg/l cefotaxime and 75 mg/l of kanamycin sulphate and then exposed to light (3200 Lux) and room temperature, about 24°C. Those with shoots forming without bacterial growth evident were transferred to fresh medium containing the same BAP and antibiotic concentrations, and then allowed sufficient incubation time to

grow large enough to be propagated *in vitro*. Such shoot cultures were maintained, and after at least 4 or 5 transfers, leaf tissue was excised for DNA extraction. Plant leaf tissue was also placed in LB broth and incubated at 37•C to test for growth of any surviving bacteria on the leaf surface. Qiagen plant DNAeasy™ kits were used to extract DNA for PCR analysis. Mention of a trademark is not intended to imply endorsement of a particular commercial product. Gel electrophoresis of PCR products obtained using *cfp*-specific primers was performed (Sequence of sense primer (5' to 3') CCA TCA TCA GCA CAG CAATCC; Sequence of antisense primer (3' to 5') TAC AGC AAC GAC ACG ACC AG). PCR products were analyzed by 1% agarose gel electrophoresis. Fragment sizes were estimated with reference to a 1kb 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.

RESULTS AND CONCLUSION

In order to obtain *cfp*-carrying transgenic sugar beets with resistance to *Cercospora* leaf spot disease, we treated hundreds of cotyledons of different genotypes with bacteria bioengineered to transfer desired genes into plants. About 1% regeneration was obtained, resulting in 3 distinct, putative *cfp*–carrying transgenics, one from Rel-1 and two from C69 germplasm. Gel electrophoresis of PCR products revealed that the Rel1 transformant was verified since it's genomic DNA produced a fragment of the expected size (lane 3) whereas a putative C69 transformant could not be confirmed since it's genomic DNA gave two fragments of anomalous size (lane 6) (Figure 2). The PCR product amplified from the genomic DNA of the Rel-1 transformant is being sequenced. Experiments on infecting new cotyledons are underway. The transgenic clone, termed T7, is being vegetatively propagated to test mature plants for *Cercospora* leaf spot resistance. The successful introduction of the *cfp* gene into sugar beet via transformation could lead to the identification of germplasm with resistance to *Cercospora* leaf spot infection. If ideally successful, the resultant germplasm could be used in commercial breeding programs as a source of a single dominant leaf spot resistance allele.

FIGURE 1. *Gel electrophoresis of PCR amplification products. The gel was stained with ethidium bromide and examined under uv. A fragment of the predicted size (lane 3) was obtained with genomic DNA of a Rel-1 transformant. Anomalous fragments (lane 6) were obtained with a putative C69 transformant. A 1 kb ladder was used (lane10).*



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