Biotechnology Letters **26:** 723–727, 2004.

Expression in sugar beet of the introduced cercosporin toxin export (CFP) gene from *Cercospora kikuchii*, the causative organism of purple seed stain in soybean

L. David Kuykendall[1,*] & Robert G. Upchurch[2]
[1]*Agricultural Research Service, U.S. Department of Agriculture, Molecular Plant Pathology Lab, Plant Sciences Institute, Beltsville Agricultural Research Center, Beltsville, MD 20705, USA*
[2]*Agricultural Research Service, U.S. Department of Agriculture, Soybean and Nitrogen Fixation Unit, North Carolina State University, Raleigh, NC 27695, USA*
[*]*Author for correspondence (Fax: +1 (301) 504-5449; E-mail: kuykendl@ba.ars.usda.gov)*

Received 7 January 2004; Revisions requested 23 January 2004; Revisions received 1 March 2004; Accepted 2 March 2004

Key words: Beta vulgaris, *Cercospora beticola*, leafspot disease, major facilitator, transgenic plant

Abstract

The *Cercospora kikuchii* cercosporin export gene, CFP, introduced into *Beta vulgaris* L. by conjugation with *Rhizobium radiobacter*, was stably maintained during vegetative propagation as verified by PCR using primers specific for the CFP gene. Transcriptional expression of the CFP gene in leaves was determined by RT-PCR using CFP-specific primers. CFP protein was detected using Western analysis with an affinity-purified polypeptide-specifc antibody. Analysis of the relative susceptibility of CFP-transgenic and non-transgenic sugar beet plants is planned but will probably take several years to complete.

Introduction

Plants have numerous mechanisms for defense against pathogenic microorganisms including structural barriers against infection, production of antimicrobial metabolites and either constitutive or inducible expression of enzymatic proteins (PR proteins) with antimicrobial function. Plants also possess ABC-type multidrug resistance proteins with roles ranging from development to defense and Major Facilitator Superfamily transporters, not so well characterized, but with known functions such as nutrient transport (Lemoine 2000, Quirino *et al.* 2001). Thus far, however, neither multidrug proteins nor major facilitators of plant origin have been shown to specify any degree of resistance to cercosporin or other singlet oxygen photosensitizers. Fungi of the genus *Cercospora* are pathogens of a variety of economically important crops such as sugar beet, tobacco and soybean (*C. beticola*, *C. nicotianae* and *C. kikuchii*, respectively). The non-host specific, phytotoxic polyketide, cercosporin is a lipid-soluble perylenequinone that, upon photoactivation, catalyzes the production of highly reactive oxygen species, principally singlet oxygen (Daub 1982). Singlet oxygen-catalyzed peroxidation of membrane lipids results in loss of membrane integrity, cytoplasmic leakage, and cell death (Daub & Ehrenshaft 2000). *Cercospora* hyphae enter the host plant passively through open stomata and grow intercellularly, and toxin-mediated disruption of the cellular membranes of host cells probably provides the pathogen with nutrients for *in situ* growth and sporulation.

Cercosporin-deficient mutants of *C. kikuchii* did not produce lesions on soybean, suggesting that cercosporin is an essential virulence factor (Upchurch *et al.* 1991). Plant cellular resistance to cercosporin has been reported in Louisiana red rice which exhibits resistance to a high concentration of cercosporin in illuminated seedling assays (Batchvarova *et al.* 1992).

Recent studies have focused on identifying genes for resistance to cercosporin in *Cercospora* fungi themselves (Daub & Ehrenshaft 2000). One such

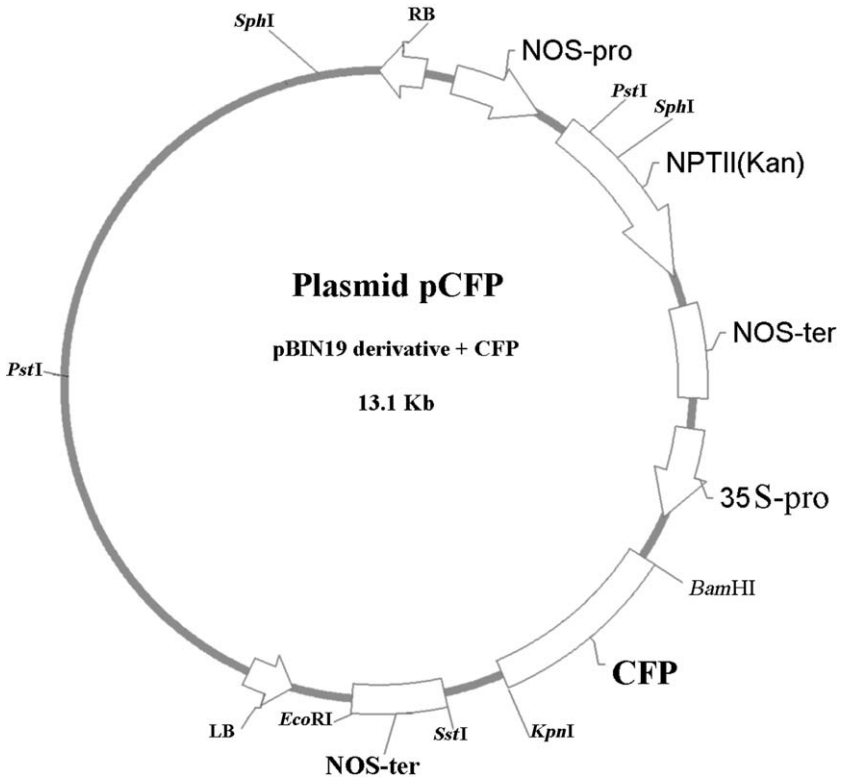

Fig. 1. Representation of the relevant features of the constructed vector plasmid pCFP.

resistance mechanism apparently involves the export action of the Major Facilitator (MF)-like protein gene, CFP, which was isolated from *C. kikuchii* (Callahan *et al.* 1999). Targeted disruption of the CFP gene resulted in mutants that lacked virulence on soybean and were inhibited by cercosporin. Cercosporin export was substantially elevated in CFP multi-copy strains of *C. kikuchii* that expressed elevated levels of CFP protein (Upchurch *et al.* 2001); and transgenic expression of CFP, in the cercosporin sensitive fungus *Cochliobolus heterostrophus*, resulted in significantly increased resistance to the toxin (Upchurch *et al.* 2002).

To assess the potential for bioengineering plant resistance to *Cercospora* infection we (R.G. Upchurch, unpublished work) have expressed CFP in the plant model *Xanthi* and transgenic tobacco had enhanced resistance to frog-eye leaf spot as compared with the parental line. This report presents molecular data on the expression of CFP in transgenic sugar beet. This research is aimed at enhancing resistance to *Cercospora* leafspot disease.

Materials and methods

Construction of the transformation vector

The 13 kb pBIN19-based (Bevan 1984) binary plant expression vector, pBI121, was modified by removal of the promoterless 1.87 kb GUS cassette. Digestion of the vector with *Bam*HI and *Kpn*I and ligation with 1.9 kb *Bam*H1-*Kpn*1 fragment containing the entire cDNA sequence of CFP produced a recombinant plasmid, pBCFP, into which the CFP gene had been placed into a unique, directional cloning site behind the CaMV 35S promoter in proper orientation (Figure 1). The GenBank accession number for CFP is AF091042. pBCFP was introduced into *Rhizobium radiobacter* (Young *et al.* 2001) EHA105 by electroporation (Weaver 1993).

Plant transformation, regeneration and growth

Leaf explants of *Beta vulgaris* L. genotype REL1 were inoculated with *R. radiobacter* strain EHA105 carrying pBCFP. Selection of kanamycin-resistant exconjugants and the regeneraton of transgenic plants

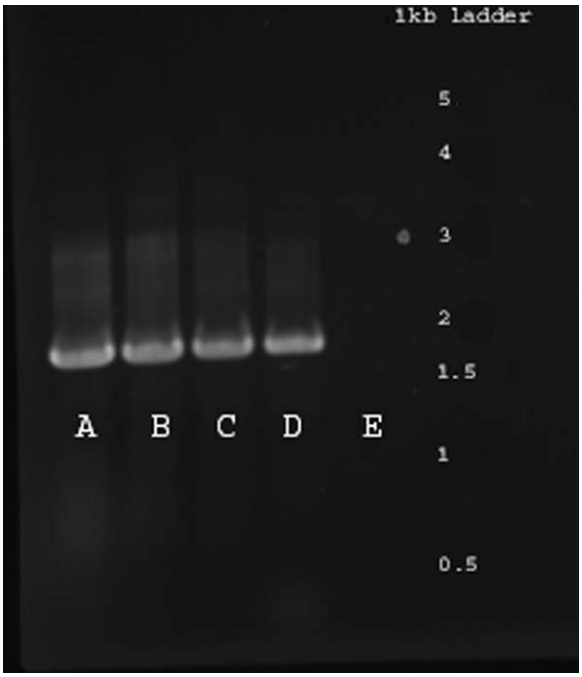

Fig. 2. RT-PCR analysis of total RNA with primer pair CFPF1-CFPR1. Lanes A–D are Rel-1 plants transformed with CFP showing a 1.7 kb band (thereafter PT7). Lane E represents a non-transformed Rel-1 tissue. The primers used were 5′-GCGGCTGGCCTCTGGTATTTCTC- and 5′-CGCGTCGTCCCATTAGTTTTCTTG-.

were as previously described by Kuykendall *et al.* (2003). Plants regenerated from kanamycin-resistant shoots (R_o) were transferred to potting soil and grown at room temperature (about 24 °C) under fluorescent lights with a 14 h photoperiod.

Analysis of genomic plant DNA and RNA

DNA was isolated from sugar beet leaf tissue with the DNeasy Plant Mini Kit and protocol supplied by Qiagen (Santa Clarita, CA). *Beta vulgaris* genomic DNA was analyzed for the presence of CFP by PCR using a CFP coding sequence-specific primer pair as previously described (Kuykendall *et al.* 2003). The Qiagen RNeasy Plant Mini Kit and protocol was used to extract total RNA from leaf tissue. RT-PCR reactions were performed using the directions and materials supplied in the RT-PCR kit (Epicentre MasterAmp, Madison, WI). Thermal Cycler program: (MJ Research, Inc. PTC 100 Thermocyler). Using the following primers, CFPF1-5′-GCGGCTGGCCTCTGGTATTTCTC-3′ and CFPR1-5′-CGCGTCGTCCCATTAGTTTTCTTG-3′, the first step was synthesis of cDNA from the RNA and then amplification of the target cDNA as follows: 20 min 60 °C, 50 cycles [(94 °C, 1 min) (62 °C, 1 min) (72 °C, 3 min)], and stabilization at 72 °C, 4 °C hold. The 1.9 kb CFP amplicon was visualized by staining agarose electrophoresis gels with ethidium bromide and examination under u.v. light at 310 nm.

Sequencing of the RT-PCR product was performed with an automated DNA sequencer (ABI Prism model 377) at the Center for Agricultural Biotechnology, University of Maryland, College Park, MD.

Preparation of total cellular proteins and Western blot analysis

Total proteins were extracted from fresh leaf material from mature, one year old plants using an extraction buffer containing 1% SDS, 50 mM Tris/HCl, pH 8, 10 mM KCl, 2.5 mM DTT, and 1 mM EDTA. Liquid N_2 was added to 0.1 g leaf sample which was then ground in microtubes with a pestle and then 200 ul sample buffer was added. Debris was removed by centrifugation. About 1/3 volume of 3× sample buffer (30% v/v glycerol, 9% v/v SDS, 15% v/v 2-mercaptoethanol, 0.188 M Tris/HCl, pH 6.8, Bromophenol Blue) was added. Samples were frozen then boiled for 10 min prior to loading on the gel. Twenty ul of sample was separated by SDS-PAGE (12% separating gel). The proteins were then transferred to an Immobilon-P membrane (Millipore Corp., Bedford, MA) using the LKB 2117-250 Nova blot Electrophoretic transfer apparatus and protocols (LKB, Bromma, Sweden). The electrodes and power supply were attached and protein was transferred at 40 mA for 1 h. After protein transfer, the blot was allowed to air dry. The blot was then incubated for 1 h with the primary antibody diluted (1:400) in blocking buffer containing 0.05% Tween 20 (blocking buffer consists of 1% BSA in phosphate-buffered saline, pH 7.2). The antibody was prepared by Quality Controlled Biochemicals Inc. (Hopkinton, MA). The antibody used was an affinity-purified, polyclonal antibody specific to a hydrophilic and putatively highly antigenic region of the amino terminus (ACREIEDPEKGQSAEIVC-Amide) of CFP (Callahan *et al.* 1999). The blot was then washed in PBS twice for 10 s. The blot was then incubated for 30 min with goat, anti-rabbit IgG (whole molecule) (Promega, St.Louis, MO) in blocking buffer containing 0.05% Tween 20. The blot was washed in PBS two times for 10 s after which Western blue-stabilized substrate for alkaline phosphatase (Promega, St Louis, MO) was added.

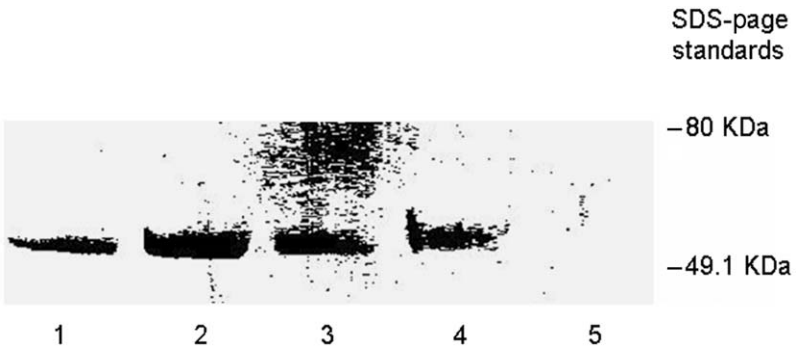

Fig. 3. Western blot analysis for CFP protein detection in the total cellular proteins sugar beet using affinity-purified, peptide-specific rabbit polyclonal antibody. Lanes 1–4 are CFP carrying plants showing the presence of a reactive polypeptide of approx. 65 kDa; while lane 5 is an Rel-1 parent.

Results

Generation and analysis of sugar beet CFP$^+$ transformants

Kanamycin-resistance clones were regenerated *in vitro* following conjugal mating of wounded REL-1 leaf pieces with *Rhizobium radiobacter* carrying pB-CFP. Transgenic plants were confirmed by PCR of leaf DNA using CFP-specific primers (Kuykendall *et al.* 2003). Moreover, vegetatively propagated kanamycin-resistant plants and seed-grown transgenic REL-1 plants stably maintained the ability to produce a DNA product of the approximate size predicted for PCR using the CFP-specifc primers. In this present study, RT-PCR was performed using RNA isolated from CFP-transgenic plants and primers suitable for amplifying the entire CFP gene to produce the predicted 1.9 kb CFP fungal amplicon from *C. kikuchii*. Figure 2 shows that by RT-PCR of total RNA, the four transgenic plants contained a transcript for CFP. DNA sequence analysis of the RT-PCR amplicon confirmed the CFP sequence. The presence of the intact CFP gene as a product of RT-PCR of total cellular RNA clearly indicates active transcriptional expression of CFP in transgenic sugar beet plants.

CFP protein detection in cfp$^+$ transgenic sugar beet

Western blot analysis (Figure 3) of total cellular protein, using an affinity-purified polypeptide-specific antibody from rabbit serum, showed the presence of a reactive polypeptide of approx. 65 kDa. This corresponds to the size predicted for the CFP protein. This protein was not observed in parental control plants. Although there may not be an accumulation, the presence of detectable CFP protein in the sugar beet transgenics means that the gene was successfully expressed both transcriptionally and translationally.

Discussion

Significant resistance to cercosporin is provided by the fungal MF-like protein, CFP, in *Cercospora fungi*. Disruption of the CFP gene in *C. kikuchii* results in an approximate 50% reduction in fungal growth on medium containing 10 μM cercosporin (Callahan *et al.* 1999). CFP was hypothesized as conferring toxin resistance by lowering cellular and/or membrane concentrations of the toxin via toxin export. Cercosporin export and resistance were both substantially elevated in engineered CFP multi-copy strains of *C. kikuchii* (Upchurch *et al.* 2001). Membrane stabilization by CFP is another possible explanation for these observed phenomena but this hypothesis has not yet been tested.

We have hypothesized that the CFP gene, carried in the pBCFP vector in *Rhizobium radiobacter*, given successful transfer and regeneration of transgenic plants, may provide both cercosporin resistance and pathogen resistance in crop plant pathosystems where cercosporin is critically involved in pathogen virulence. Although the testing of this hypothesis in transgenic plants may take several years, we were able to dicern the expression of the introduced CFP in sugar beet using RT-PCR and Western blot analysis with and affinity purified peptide specific antibody. Transgenic expression of the CFP gene in the cercosporin-sensitive fungus *Cochliobolus heterostrophus* resulted in significantly increased resistance to cercosporin due to either toxin export (Upchurch *et al.* 2002) or membrane stabilization.

Introduction of candidate genes into crop plants is a general approach for both phenotypic analysis and for possibly obtaining transgenic plants with enhanced disease resistance against particular pathogens. In this study, the expression of CFP was placed under the control of a constitutive promoter (CaMV 35S) rather than a pathogen-inducible or stress-inducible promoter. The reasoning was that CFP would need to be present and functioning within the plant cellular membranes prior to *Cercospora* infection and toxin elaboration in order to contribute to plant defense. Although not specifically localized, CFP protein was detected immunochemically (Figure 3) in Western blot analysis of gel electrophoretically separated total proteins of CFP transgenic sugar beet plants.

Acknowlegements

We are indebted to Rose Hammond who generously performed protein electrophoresis and transfer to membranes. RT-PCR and DNA sequence analyses were professionally performed by Mrs Tammy Stockett Murphy. Ms Kristi Bottner and Mr Joe Kelly also offered significant, skilled assistance. LDK also thanks the Beet Sugar Development Foundation, Denver, Colorado, for financial support under projects 830 and 831; and, specifically, Tom Schwartz, Executive Vice President, consistently provided both useful encouragement and kind support.

References

Batchvarova RB, Reddy VS, Bennett J (1992) Cellular resistance in rice to cercosporin, a toxin of *Cercospora*. *Phytopathology* **82**: 642–646.

Bevan M (1984) Binary *Agrobacterium* vectors for plant transformation. *Nucl. Acids Res.* **12**: 8711–8721.

Callahan TM, Rose MS, Meade MJ, EhrenshaftM, Upchurch RG (1999) *CFP*, the putative cercosporin transporter of *Cercospora kikuchii*, is required for wild type cercosporin producion, resistance, and virulence on soybean. *Mol. Plant-Microbe Interact.* **10**: 901–910.

Daub ME (1982) Cercosporin, a photosensing toxin from *Cercospora* spp. *Phytopathology* **72**: 370–374.

Daub ME, Ehrenshaft M (2000) The photoactivated *Cercospora* toxin cercosporin: contributions to plant disease and fundamental biology. *Annu. Rev. Phytpathol.* **38**: 461–490.

Kuykendall LD, Stockett TM, Saunders JW (2003) *Rhizobium radiobacter* conjugation and callus-independent shoot regeneration used to introduce the cercosporin export gene *cfp* from Cercospora into sugar beet (*Beta vulgaris* L.). *Biotechnol. Lett.* **25**: 739–744.

Lemoine R (2000) Sucrose transporters in plants: update on function and structure. *Biochim. Biophys. Acta* **1465**: 246–262.

Quirino BF, Reiter WD, Amasino RD (2001) One of two tandem *Arabidopsis* genes homologous to monosaccharide transporters is senescence-associated. *Plant Mol. Biol.* **46**: 447–457.

Upchurch RG, Rose MS, Eweida M (2001) Over-expression of the cercosporin facilitator protein, *CFP*, in *Cercospora kikuchii* up-regulates production and secretion of cercosporin. *FEMS Microbiol. Lett.* **204**: 89–93.

Upchurch RG, Rose MS, Eweida M, Callahan TM (2002) Transgenic assessment of *CFP*-mediated cercosporin export and resistance in a cercosporin sensitive fungus. *Curr. Genet.* **89**: 179–183.

Upchurch RG, Walker DC, Rollins JA, Ehrenshaft M, Daub ME (1991) Mutants of *Cercospora kikuchii* altered in cercosporin synthesis and pathogenicity. *Appl. Environ. Micrbiol.* **57**: 2940–2945.

Weaver JC (1993) Electroporation: a general phenomenon for manipulation cells and tissues. *J. Cell. Biochem.* **51**: 426–435.

Young JM, Kuykendall LD, Martínez-Romero E, Kerr A, Sawada H (2001) A revision of *Rhizobium* Frank 1889, with an emended description of the genus, and the inclusion of all species of *Agrobacterium* Conn 1942 and *Allorhizobium undicola* de Lejudie *et al.* 1998 as new combinations: *Rhizobium radiobacter, R. rhizogenes, R. rubi, R. undicola* and *R. vitis*. *Intern. J. Syst. Evol. Microb.* **51**: 89–103.