Evaluation of polygalacturonase-inhibiting protein (PGIP)-mediated resistance against *Verticillium dahliae*, a fungal pathogen of potato

by

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SUMMARY

Polygalacturonase-inhibiting proteins (PGIPs) are plant proteins believed to play a role in the defence against pathogenic fungi. In this study, it was hypothesized that apple PGIP1 could be used to confer enhanced resistance against *Verticillium*-wilt, a major disease of potato caused by the fungus *Verticillium dahliae*. Transgenic lines containing the apple *pgip1* gene under control of the enhanced CaMV 35S (e35S) promoter had been generated previously. Stable integration of the transgene into the potato genome was shown by the polymerase chain reaction (PCR) and Southern blot with a DIG-labelled apple *pgip1* fragment as probe.

Polygalacturonase (PG)-inhibiting assays (the agarose diffusion assay and reducing sugar assays) were employed to investigate the inhibiting activity of apple PGIP1 extracts, prepared from the transgenic potato lines, on the PGs secreted by *V. dahliae* grown on pectin medium. Inhibition was successful for all but one of the transgenic lines. Active PGIP1 was expressed in the leaves of *in vitro*- and glasshouse grown plants, as well as in roots of *in vitro*-grown plants. Due to the success of the *in vitro* inhibition results, it was anticipated that the apple *pgip1* transgene would protect the transgenic lines against *Verticillium*-wilt in a subsequent glasshouse trial.

The transgenic lines and untransformed BP1 potato control were planted in soil inoculated with *V. dahliae* microsclerotia and control soil. Assessments of the visual symptoms of yellowing and wilt were made on a scale of 1-5. Colonisation of stem sections was determined by plating onto potato dextrose agar plates. Disease index values were calculated from the symptom and colonisation data. Analysis of variance indicated six lines to be significantly different from the rest when grown in the inoculated soil, but five of them also showed significantly slower senescence symptoms when grown in the control soil. It is proposed that the physiological effect of an extended juvenile phase resulted in the apparent increased disease resistance. This could be caused by transformation or tissue culture-induced somaclonal variation of the potato plants. The hypothesis that transformation of the apple *pgip1* gene into potato would confer enhanced resistance against *Verticillium*-wilt was not supported by the data that was obtained.

Expression of antifungal genes by pathogen-inducible promoters is a valuable strategy in the development of disease resistant crops of importance. A construct containing the apple *pgip1* gene under control of the pathogen-inducible *gst1* promoter from *Arabidopsis thaliana* (L.) Heynh was generated. *Agrobacterium tumefaciens* GV3101(pMP90RK) was transformed with the plant transformation vector pCAMBIA2300 containing the *gst1* and e35S promoter-*pgip1* inserts. *A. thaliana* was transformed using the floral-dip method, and putative transgenic progeny were selected

by kanamycin selection of the seeds. PCR verified the insertion of the transgene into the genomes of T2 and T3 lines. Gene expression from the two promoters was compared by performing PGIP extractions and the agarose diffusion assay. The *gst1* promoter was active even without induction by methyl-salicylate. Both constructs led to the expression of active apple PGIP1 against *V. dahliae* PG in the heterologous plant *A. thaliana*.

OPSOMMING

Poligalakturonase-inhiberende proteïene is plant proteïene wat 'n rol speel in die beskerming teen patogeniese fungi. In hierdie studie word dit voorgestel dat appel PGIP1 gebruik kan word om verhoogde weerstand teen *Verticillium*-verwelk, 'n belangrike siekte van aartappel veroorsaak deur die fungus *V. dahliae*, te verleen. Transgeniese lyne wat die appel *pgip1* geen onder beheer van die verbeterde CaMV 35S (e35S) promoter bevat is voorheen gegenereer. Stabiele integrasie van die transgeen in die aartappel genoom is bewys deur die polimerase ketting reaksie (PKR) en Southern klad met 'n DIG-gemerkte appel *pgip1* fragment as 'n peiler.

Poligalakturonase (PG)-inhiberende analises (die agarose diffusie en reduserende suiker analises) is aangewend om die inhiberende aktiwiteit van appel PGIP1, berei van die transgeniese aartappel lyne, op die PG ensieme afgeskei deur *V. dahliae* in pektien medium te ondersoek. Inhibisie was suksesvol vir al die potensiële transgeniese lyne behalwe een. Aktiewe PGIP1 was uitgedruk in die blare van *in vitro*- en glashuis gekultiveerde plante, asook die wortels van *in vitro* plante. As gevolg van die sukses van die *in vitro* inhibisie resultate, was dit voorspel dat die appel *pgip1* transgeen die transgeniese lyne teen *Verticillium*-verwelk sal beskerm in die daaropvolgende glashuisproef.

Die transgeniese lyne en ongetransformeerde BP1 aartappel kontrole is geplant in *V. dahliae* mikrosklerotia-geïnokuleerde grond en kontrole grond. Evaluasies van die visuele simptome van vergeling en verwelk is gemaak op 'n skaal van 1-5. Kolonisasie van die stingels is bepaal deur dit uit te plaat op aartappel dekstrose agar plate. Siekte-indekse is bereken van die simptome en kolonisasie data. Analise van variansie het aangedui dat ses lyne betekenisvol verskil het van die res toe dit in die geïnokuleerde grond gegroei is, maar vyf van hulle het ook betekenisvol stadiger veroudering simptome getoon toe dit in die kontrole grond gegroei is. Dit word voorgestel dat die fisiologiese effek van vertraagde volwassenheid verantwoordelik is vir die skynbare verhoogde siekte-weerstand. Hierdie kon veroorsaak gewees het deur transformasie of somaklonale variase geinduseer deur weefselkultuur van die aartappelplante. Die hipotese dat die transformasie met die appel *pgip1* geen in aartappel verhoogde weerstandbiedendheid teen *Verticillium*-verwelk kon verleen was nie ondersteun deur die verkrygde data nie.

Uitdrukking van fungi-werende gene deur patogeen-stimuleerbare promoters is 'n waardevolle strategie in die ontwikkeling van siekte-bestande belangrike gewasse. 'n Konstruk wat die appel *pgip1* geen onder beheer van die patogeen-stimuleerbare *gst1* promoter van *Arabidopsis thaliana* (L.) Heynh bevat is gegenereer. *Agrobacterium tumefaciens* GV3101(pMP90RK) is getransformeer met die plant transformasie vektor pCAMBIA2300 wat die *gst1* en e35S promoter-*pgip1* insetsels bevat.

A. thaliana is getransformeer met die blom-doop metode, en potensiële transgeniese nageslag is geselekteer met kanamycin seleksie van die sade. Integrasie van die transgeen in die genome van T2 en T3 lyne is geverifieer met PKR. Geen-ekspressie van die twee promoters is vergelyk deur PGIP ekstraksies te toets met die agarose diffusie analise. Die *gst1* promoter was aktief selfs sonder induksie deur metiel-salisilaat. Beide konstrukte het gelei tot die ekspressie van aktiewe appel PGIP1 teen *V. dahliae* PG in die heteroloë plant *A. thaliana*.

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LIST OF ABBREVIATIONS

ADA	Agarose diffusion assay
ARC	Agricultural Research Council
AS	Ammonium sulphate
ATP	Adenosine triphosphate
Avr	Avirulence
bp	Basepair
BSA	Bovine serum albumin
CaMV	Cauliflower mosaic virus
CSPD	Disodium 3-(4-methoxyspiro{1,2-dioxetane-3,2'-(5'-chloro)tricyclo[3.3.1.1^{3,7}]decan}-4-yl) phenyl phosphate
CTAB	Hexadecyl trimethyl ammonium bromide
dATP	Deoxyadenosine triphosphate
dCTP	Deoxycytosine triphosphate
dGTP	Deoxyguanidine triphosphate
dH_2O	Distilled water
DIG	Digoxygenin
DIG-11-dUTP	Digoxygenin-11-deoxyuridine triphosphate
DMF	Dimethylformamide
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
DPE	Diphenyl ether
dTTP	Deoxythymidine triphosphate
EDTA	Ethylenediamine tetraacetic acid
endoPG	Endopolygalacturonase
ERE	Ethylene-responsive element
exoPG	Exopolygalacturonase
gDNA	Genomic DNA
GST	Glutathione *S*-transferase
GUS	β-glucuronidase
kb	Kilobasepair
lsd	Least significant difference
LB	Luria Bertani
LRR	Leucine-rich repeat

MCS	Multiple cloning site
Me-Sa	Methyl-salicylate
MS	Murashige and Skoog
NaAc	Sodium acetate
nptII	Neomycin phosphotransferase II
PAHBAH	*p*-hydroxybenzoic acid hydrazide
PCR	Polymerase chain reaction
PDA	Potato dextrose agar
PG	Polygalacturonase
PGA	Polygalacturonic acid
PGIP	Polygalacturonase-inhibiting protein
PVP	Polyvinylpyrrolidone
RNA	Ribonucleic acid
RNase	Ribonuclease
rpm	Revolutions per minute
TAE	Tris-acetate ethylenediamine tetraacetic acid
TE	Tris ethylenediamine tetraacetic acid
TEV	Tobacco etch virus
Tm	Melting temperature
TNE	Tris-sodium chloride ethylenediamine tetraacetic acid
Tris	Tris hydroxy methyl aminoethane
X-gal	5-bromo-4-chloro-3-indolyl-β-galactoside

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