Molecular Strategies Towards Anthracnose Resistance in Lupin

by

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Summary

The aim of the project was to develop a strategy towards anthracnose resistance in lupin using molecular techniques. *Colletotrichum* species are considered to be major plant pathogens of cereals and legumes around the world, causing significant crop losses. *Colletotrichum acutatum* causes anthracnose disease on lupin.

Sweet white lupin (*Lupinus albus*) is a high protein grain crop that could alleviate protein shortage in South Africa, since it has the highest protein levels (34-45%) compared to *Lupinus angustifolius*. In an effort to combat the lupin anthracnose threat to the South African lupin industry, which has an annual turnover of approximately 60 million rands, a project was embarked upon to introduce defense genes into a white lupin and a narrow leaf lupin cultivar.

Bean polygalacturonase inhibiting protein (PvPGIP), either extracted from bean or from transgenic tomato expressing the bean *pgip1* gene (*Pvpgip1*), inhibited the *C. acutatum* polygalacturonase (PG) activity (isolate SHK 788) only by 18-25%, compared to apple PGIP (MdPGIP) that inhibited the *C. acutatum* PG activity by 70%. These results led to the *Mdpgip1* gene, rather than the *Pvpgip1* gene, being chosen for genetic engineering of lupin towards anthracnose resistance. However, since plants express more than one PGIP, the protein in the extract prepared from the fruit of apple cv. Granny Smith, could be encoded by any one of at least two closely related copies of *pgip* genes found in apple. Screening of eight putative first generation *Mdpgip1* transformed tobacco plants using PCR, showed that all eight plants contained the *Mdpgip1* gene. Inhibition studies, using the *C. acutatum* PGs, were performed which identified *Mdpgip1* transgenic tobacco plant #8 as being the highest expresser of the MdPGIP1, since the MdPGIP1 extract from this plant exhibited the highest level of *C. acutatum* PG inhibition. The PGIP extract from the non-transgenic tobacco plant, as well as heat denatured MdPGIP1 extracts from the *Mdpgip1* transgenic tobacco plants, resulted in no inhibition of *C. acutatum* PG activity. *Mdpgip1* transgenic tobacco plant #8 was chosen for the purification of MdPGIP1. The protein was purified to apparent homogeneity using anion and cation exchange chromatography. N-terminal sequencing deduced the first 15 amino acids, which aligned 100% to the sequence of a *pgip* gene (called *Mdpgip*) from Golden Delicious apples (Genbank: accession no. MDU 77041), confirming isolation of MdPGIP1. The protein had a molecular mass of approximately 46kDa as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and an isoelectric point of 8.0. Purified MdPGIP1 inhibited the PGs produced by *C. acutatum* and the PGs produced by two apple pathogens, *B. obtusa* and *D. ambigua*. Results indicated that much less MdPGIP1 is required for effective inhibition of the *B. obtusa* and *D. ambigua* PGs, compared to the *C. acutatum* PGs. However, at higher MdPGIP1 concentrations all three fungal PGs were inhibited equally well. A purified *endo*-PG from *Aspergillus niger* was not inhibited by MdPGIP1. This constitutes the first report on the inhibitory activity of MdPGIP1 towards the PGs from *C. acutatum*, and the two apple pathogens *B. obtusa* and *D. ambigua*.

As part of a multigene approach to the production of anthracnose resistant lupin, the use of a yeast exo-β-1,3-glucanase (EXG1) as an antifungal agent towards *C. acutatum* was investigated. The *exo-β-1,3-glucanase* (*exg1*) gene had been isolated from *Saccharomyces cerevisiae*. Yeast cultures transformed with the *exg1* gene, as well as untransformed yeast cultures, were obtained from the Institute for Wine Biotechnology, South Africa. Fungal spore suspensions, from isolate SHK 788, were prepared and used in inhibition studies with spore concentrations ranging from $2.5.10^3$ spores to 80.10^3 spores per flask. Inhibition of *C. acutatum* mycelial growth ranged from 41%, at a fungal spore concentration of $2.5.10^3$ spores, to 20%, at a fungal spore concentration of 80.10^3 spores. Ammonium sulphate concentrated yeast extracts containing the glucanase enzyme did not result in increased inhibition of *C. acutatum* mycelial growth. As an added control, an inhibition study using *Botrytis cinerea* spores yielded similar results to those obtained for the *C. acutatum* inhibition studies. An inhibition of at least 50% for all spore concentrations was set as the criterium to decide that the *exg1* gene is potent enough for genetic engineering of disease resistance. This extent of inhibition was not obtained and the use of the *exg1* gene for protection of lupin against *C. acutatum* was therefore not considered a worthwhile commercial option.

The defense gene plant transformation vectors prepared for lupin transformation, pCAMBIA 3300-virG, pCAMBIA 3301-virG, pCAMBIA 3300-virG-applePGIP and pCAMBIA 0390:applePGIP were successfully transformed into the *A. tumefaciens* strains LBA 4404 and AGL1. Lupin transformation was performed by the transformation group at CSIR Bio/Chemtek using *A. tumefaciens*-mediated transformation of shoot apical meristems. This group showed that the inclusion of the supervirulence *virG* gene enhanced the levels of transient GUS expression in *L. angustifolius* by more than two fold. However, transformation efficiency was

low, and regeneration of the lupin plant proved to be even more difficult. To overcome the difficulties with plant tissue culture-based transformation systems, an *A. tumefaciens* seed vacuum infiltration transformation method was utilised. Extracts obtained from *Mdpgip1* transgenic tobacco plants produced at CSIR Bio/Chemtek (pCAMBIA 3300-virG-applePGIP as well as pCAMBIA 3300-virG/pCAMBIA 0390:applePGIP transformants) inhibited the *C. acutatum* PGs. The *Mdpgip1* gene thus codes for an active protein in the transgenic tobacco plants, and the defense gene constructs prepared for lupin transformation are functional *in planta*.

The *shpx6a* peroxidase gene was isolated from *Stylosanthes humulis*, as the second defense gene to be used in the strategy towards anthracnose resistance in lupin, and substitute for the yeast *exg1* gene. Sequencing data confirmed the successful isolation of the *shpx6a* peroxidase gene, which was subsequently cloned into pCAMBIA 0390:applePGIP upstream from the NOS terminator to produce pCAMBIA 0390:applePGIP:peroxidase.

Seeing that the constitutive CaMV 35S promoter was going to be used upstream from the selection gene (*bar*), the *Mdpgip1* gene and the additional *shpx6a* peroxidase gene, there was a concern that one type of gene silencing could occur. Use of one promoter can block expression of another gene being expressed from the same promoter on account of methylation of the promoter DNA. A 4.2kb fragment containing the inducible *class-III chitinase* (*if3*) promoter was isolated from *L. albus*, using the GenomeWalker™ kit, for use in the pCAMBIA 0390:applePGIP:peroxidase defense gene construct, i.e. upstream from the *shpx6a* peroxidase gene. The 4.2kb fragment was successfully cloned into the pGEM-T Easy vector and sequenced. The sequence was compared to known sequences in the Genbank database but exhibited no significant homology. Using bioinformatic tools, five possible eukaryotic promoter-containing sites, including the TATA boxes, were identified within the isolated 4.2kb fragment. Deletion studies were performed in order to test for the minimal sequence needed for retaining of promoter activity. The 1.818kb, 1.512kb and 1.138kb *if3* promoter-containing fragments were each cloned separately into the pDM327 vector upstream from the *bar-gus* fusion gene to produce pDM327:Prom1.8, pDM327:Prom1.5 and pDM327:Prom1.1 and used in the Biolistic™ transformation of plant tissue. Biolistic™ transformation of *Ornithogalum* and bean callus tissue, as well as maize and lupin immature embryos all demonstrated that the *if3* DNA fragment isolated from *L. albus* contains promoter activity, indicated by the efficient stimulation of the expression of the *gus* reporter gene. Based on these results a provisional patent was filed [Application number: 2003/2405, and entitled "Plant Promoter"].

Bioinformatic analysis indicated the presence of various putative *cis*-acting regulatory elements, that could be important in controlling the expression of the 1.8kb *if3* promoter-containing fragment. A single putative MBS regulatory *cis*-acting element was present in the 1.13kb promoter-containing fragment. It acts as a Myb transcription factor binding site that regulates transcription of several plant genes in response to various environmental factors, including elicitors and wounding. Several CAAT boxes were also identified within the 1.81kb promoter-containing fragment which play an important role in the determination of promoter efficiency. Most of the putative fungal elicitor activated (Box-W1 and ELI-box3) and wound-inducible [WUN-motif and ERE (ethylene responsive element)] *cis*-acting elements were present in the 1.13kb promoter-containing fragment. This supports the hypothesis that all regulatory elements needed for the activation of the *if3* gene promoter are located within the first 1.13kb fragment upstream from the initiation codon of the *if3* gene.

The final evaluation of the main hypothesis that the combinatorial approach, by using two defense genes, will be much more effective than one gene or natural resistance in the suppression of anthracnose in lupin will need to be evaluated once successful transformation and regeneration of lupin has been obtained.

Opsomming

Die doel van die projek was om 'n strategie te ontwikkel wat antraknose weerstandbiedendheid in lupiene sal bewerkstellig d.m.v. die gebruik van molekulêr-genetiese tegnieke. *Colletotrichum* spesies word wêreldwyd bestempel as 'n belangrike plantpatogeen wat op graangewasse en peulgewasse voorkom en wat tot betekenisvolle gewas verliese lei. *Colletotrichum acutatum* is verantwoordelik vir antraknose siekte op lupien.

Soet wit-lupien (*L. albus*) is 'n hoë-proteïen graangewas wat die proteïentekort in Suid Afrika kan verlig en is die mees gesogde spesie omdat dit die hoogste proteïenvlakke bevat (34-45%) in vergelyking met *Lupinus angustifolius*. In 'n poging om die bedreiging van die lupien-antraknose probleem vir die Suid Afrikaanse lupien bedryf (met 'n jaarlikse omset van ongeveer R 60 miljoen) te bekamp, is 'n projek aangepak om verdedigingsgene in 'n wit lupien en 'n nou-blaar lupien kultivar in te plaas.

Boontjie poligalakturonase inhiberende proteïen (PvPGIP), wat geëkstraheer is vanuit boontjie of vanuit transgeniese tamatie wat die boontjie *pgip1* geen (*Pvpgip1*) uitdruk, inhibeer die *C. acutatum* poligalakturonase (PG) aktiwiteit (van isolaat SHK 788) slegs 18-25%, in vergelyking met appel PGIP (MdPGIP) wat die *C. acutatum* PG aktiwiteit met 70% geïnhibeer het. Hierdie resultate het gelei tot die keuse van die *Mdpgip1* geen, in plaas van die *Pvpgip1* geen, vir die genetiese manipulering van antraknose weerstandbiedendheid in lupiene. Omrede plante meer as een PGIP uitdruk, kan die proteïen in die ekstrak wat vanaf appelvrug cv. Granny Smith voorberei is, deur een van ten minste twee naasverwante kopieë van *pgip* gene wat in appel voorkom, uitgedruk word. Evaluering van agt vermeende eerste generasie *Mdpgip1* getransformeerde tabakplante met die gebruik van PCR, het aangetoon dat al agt plante die *Mdpgip1* geen bevat. Inhibisie studies, waarin die *C. acutatum* PGs gebruik is, is uitgevoer en het *Mdpgip1* transgeniese tabakplant #8 geïdentifiseer as die hoogste uitdrukker van MdPGIP1, omdat die MdPGIP1 ekstrak van hierdie plant die hoogste vlakke van *C. acutatum* PG inhibisie aangetoon het. Die PGIP ekstrak van die ongetransformeerde tabakplant, asook die hitte gedenatureerde MdPGIP1 ekstrakte vanaf die *Mdpgip1* transgeniese tabakplante, het geen inhibisie van die *C. acutatum* PG aktiwiteit aangetoon nie. Gevolglik is *Mdpgip1* transgeniese tabakplant #8 gekies vir die suiwering van MdPGIP1. Die proteïen is m.b.v. anioon- en katioonuitruilingschromatografie tot oënskynlike homogeniteit gesuiwer. N-terminale volgorde-bepaling het die eerste 15 aminosure afgelei, wat 100% ooreengestem het met die volgorde van 'n *pgip* geen (genoem *Mdpgip*) vanaf Golden Delicious appels (Genbank: no. MDU 77041), wat die isolasie van MdPGIP1 bevestig het. Die proteïen het 'n molekulêre massa van ongeveer 46kDa getoon en 'n isoelektriese punt van 8.0. Gesuiwerde MdPGIP1 het die PGs wat deur *C. acutatum* uitgedruk word geïnhibeer, asook die PGs wat deur twee appel patogene, *B. obtusa* en *D. ambigua*, gesintetiseer word. Resultate het daarop gedui dat baie minder MdPGIP1 nodig was vir die effektiewe inhibisie van die *B. obtusa* en *D. ambigua* PGs, in vergelyking met die *C. acutatum* PGs; maar teen hoër MdPGIP1 konsentrasies is al drie fungale PGs ewe goed geïnhibeer. 'n Gesuiwerde *endo*-PG vanaf *Aspergillus niger* is nie deur die MdPGIP1 geïnhibeer nie. Hierdie is ook dan die eerste verslag oor die inhiberende aktiwiteit van MdPGIP1 teenoor die PGs vanaf *C. acutatum*, en die twee appel patogene *B. obtusa* and *D. ambigua*.

As deel van 'n multigeen-benadering vir die produksie van antraknose weerstandbiedende lupiene, is die gebruik van 'n gis exo-β-1,3-glukanase (EXG1) as 'n antifungale agent teenoor *C. acutatum* ondersoek. Die *exo-β-1,3-glukanase* (*exg1*) geen is geïsoleer vanuit *Saccharomyces cerevisiae*. Giskulture wat met die *exg1* geen getransformeer is, sowel as ongetransformeerde giskulture, is vanaf die Instituut vir Wyn Biotegnologie, Suid Afrika, verkry. Fungale spoorsuspensies, vanaf isolaat SHK 788, is voorberei, en is in die inhibisie studies gebruik met spoorkonsentrasies wat vanaf $2.5.10^3$ tot 80.10^3 spore per fles gevarieer het. Inhibisie in die groei van *C. acutatum* miselia het gevarieer vanaf 41%, by 'n fungale spoorkonsentrasie van $2.5.10^3$ spore, tot 20%, by 'n fungale spoorkonsentrasie van 80.10^3 spore. Ammonium sulfaat gekonsentreerde gisekstrakte wat die glukanase ensiem bevat het, het nie gelei tot verhoogde inhibisie in die groei van *C. acutatum* miselia nie. As 'n bykomstige kontrole, het 'n inhibisie studie met die gebruik van *Botrytis cinerea* spore soortgelyke resultate gelewer as die wat verkry is in die *C. acutatum* inhibisie studies. 'n Inhibisie van ten minste 50% by alle spoorkonsentrasies was gestel as kriterium in die besluit of die *exg1* geen potent genoeg sou wees vir die genetiese manipulering van siekte weerstandbiedendheid. Hierdie omvang van inhibisie is nie waargeneem nie en die gebruik van die *exg1* geen vir beskerming van lupien teen *C. acutatum* was dus nie beskou as 'n haalbare kommersiële opsie nie.

Die plant transformasie-vektore wat voorberei is vir lupien transformasie met verdedigingsgene, pCAMBIA 3300-virG, pCAMBIA 3301-virG, pCAMBIA 3300-virG-appelPGIP en pCAMBIA 0390:appelPGIP was

suksevol in *A. tumefaciens* lyn LBA 4404 en AGL1 getransformeer. Lupien transformasie deur die gebruik van *A. tumefaciens*-bemiddelde transformasie van loot apikale meristeme is uitgevoer deur die WNNR Bio/Chemtek. Hierdie groep het aangetoon dat die insluiting van die supervirulensie *virG* geen die transformasie-frekwensie verhoog het, en die vlakke van tydelike GUS ekspressie in *L. angustifolius* het meer as twee-voudig verhoog. Transformasie-doeltreffendheid was egter laag, en regenerasie van lupienplante was moeilik. Om die probleme wat met die weefselkultuur-gebaseerde transformasiestelsels ondervind is, te oorkom, is 'n *A. tumefaciens* saad-vakuuminfiltrasie-transformasiemetode gevolg vir die optimisering en transformasie van *L. albus* en *L. Angustifolius*. Daar is egter bevind dat ekstrakte verkry vanaf *Mdpgip1* transgeniese tabakplante wat op WNNR Bio/Chemtek vervaardig is (pCAMBIA 3300-virG-applePGIP sowel as pCAMBIA 3300-virG/pCAMBIA 0390:applePGIP transformante) die *C. acutatum* PGs geïnhibeer het. Die *Mdpgip1* geen kodeer dus vir 'n aktiewe proteïen in die transgeniese tabakplante, en die verdedigingsgeen-konstrukte wat vir lupien transformasie voorberei is, is funksioneel *in planta*.

Die *shpx6a* peroksidase geen vanuit *Stylosanthes humulis*, geïsoleer as tweede verdedigingsgeen vir gebruik in die strategie vir antraknose weerstandbiedende lupiene, en as plaasvervanger vir die gis *exg1* geen. Volgordebepalingsdata het bevestig dat die *shpx6a* peroksidase geen suksesvol geïsoleer is. Daarna was dit suksesvol gekloneer in pCAMBIA 0390:appelPGIP, stroomop vanaf die NOS terminator om pCAMBIA 0390:appelPGIP:peroksidase te produseer.

Omdat die konstitutiewe CaMV 35S promotor stroomop vanaf die seleksie geen (*bar*), die *Mdpgip1* geen en die addisionele *shpx6a* peroksidase geen gebruik sou word, was daar 'n moontlikheid dat daar 'n tiepe van geen 'silencing' sal voorkom. Die gebruik van een promotor kan die ekspressie van 'n ander geen blokkeer wat deur dieselfde promotor beheer word as gevolg van metilering van die promotor DNA. 'n 4.2kb Fragment wat die induseerbare klas-*III chitinase* (*if3*) promotor bevat, is geïsoleer vanuit *L. albus*, met gebruik van die GenomeWalker™ sisteem, vir gebruik in die pCAMBIA 0390:appelPGIP:peroksidase verdedigingsgeen-konstruk; dit is stroomop vanaf die *shpx6a* peroksidase geen. Die 4.2kb fragment is suksesvol gekloneer in die pGEM-T Easy vektor en nukleotiedvolgordebepalings is gedoen. Die volgorde is vergelyk met bekende volgordes in die Genbank databasis maar het geen noemenswaardige homologie aangetoon nie. Bioinformatiese analises het voorspel dat daar vyf moontlike eukariotiese promotor-bevattende setels, wat die TATA elemente insluit, teenwoordig is in die geïsoleerde 4.2kb fragment. 5'-Verkortingstudies is uitgevoer om te toets vir die minimum volgorde wat benodig was om promotoraktiwiteit te behou. Die 1.818kb, 1.512kb en 1.138kb *if3* promotor-bevattende fragmente was elkeen apart gekloneer in die pDM327 vektor stroomop vanaf die *bar-gus* fusiegeen om pDM327:Prom1.8, pDM327:Prom1.5 en pDM327:Prom1.1 te genereer, en was gebruik in die Biolistic™ transformasie van plantweefsel. Biolistic™ transformasie van *Ornithogalum* en boontjie kallusweefsel, asook mielie- en lupien onvolwasse embrios, het bewys dat die *if3* DNA fragment wat vanuit *L. albus* geïsoleer is, promotoraktiwiteit bevat het, soos aangedui deur die doeltreffende stimulasie van die uitdrukking van die *gus* klikkergeen. 'n Voorlopige patent is ingedien [Aansoeknommer: 2003/2405, en getiteld "Plant Promoter"] gebaseer op hierdie resultate.

'n Databasis soektog het die teenwoordigheid van verskillende vermeende *cis*- reguleringselemente geïdentifiseer, wat dalk van belang kan wees in die kontrole van geen ekspressie in die 1.8kb *if3* promotor-bevattende fragment. 'n Enkele vermeende MBS reguleringselement is teenwoordig in die 1.13kb promotor-bevattende fragment. Dit dien as 'n Myb transkripsiefaktor bindingsetel wat transkripsie in verskeie plantgene in respons teenoor verskillende omgewingsfaktore, insluitend elisitore en verwonding, reguleer. Verskeie CAAT-elemente was ook geïdentifiseer binne die 1.81kb promotor-bevattende fragment wat 'n belangrike rol in die bepaling van promotor doeltreffendheid speel. Meeste van die vermeende fungale elisitor-geaktiveerde elemente (W1 en ELI-3) en verwonding-induseerbare [WUN-motief en ERE (etileen responsiewe element)] *cis*-elemente was teenwoordig in die 1.13kb promotor-bevattende fragment. Hierdie ondersteun die hipotese dat al die regulatoriese elemente wat benodig word vir die aktivering van die *if3* geen promotor teenwoordig is binne die eerste 1.13kb fragment stroomop vanaf die inisiasie kodon van die *if3* geen.

Die finale evaluasie van die hoof hipotese dat die kombinatoriese benadering, met die gebruik van twee verdedigingsgene, baie meer effektief sal wees as een geen of natuurlike weerstand in die onderdrukking van antraknose in lupiene sal eers geëvalueer kan word wanneer daar suksesvolle transformasie en regenerasie van lupiene verkry is.