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Genetic engineering of Brassicas for pest and disease control and improved storage

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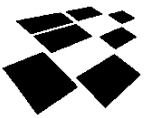

Horticulture Australia

Genetic enginering of brassicas for pest and disease control and improved storage

Final report for project VG98085

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Purpose of the report:
This project (VG98085) was directed towards:

1. the development of methods to genetically modify vegetable brassicas

2. the preliminary assessment of genetically modified vegetable brassicas with agronomically useful genes

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Text by:
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1.0 Introduction

During the last decade, genetic modification has become a reality, with the first crops being commercialised. World-wide in excess of 44 million hectares of genetically modified plants worth about US $ 3 billion were grown in 2000. This is anticipated to increase to 50 million hectares in 2001 (www.isaaa.org). While vegetables represent, only a small portion of these figures (< 100,000 h), potato cultivars with resistance to Colorado beetle and Potato Leaf Roll Virus (PLRV), tomatoes with delayed ripening and squash with virus resistance have been commercialised in the USA.

This report summarises research to develop and use genetic modification to transfer agronomically useful genes to vegetable brassicas and describes four aspects of our research:

- the development of tissue culture regeneration systems
- the development of appropriate gene transfer systems
- the preparation of gene constructs and
- the preliminary assessment of genetically modified plants for there response to clubroot and diamondback moth

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1.1 Media summary

Gene technology has been used and evaluated with vegetable brassicas to increase their resistance to insect pests and diseases and improve shelf-life.

In Australia and world-wide, the insect pest diamondback moth and the fungal disease clubroot are major problems, reducing both the yield and quality of produce. The vegetable industry in partnership with the Institute for Horticultural Development - Knoxfield and Horticulture Australia Ltd are developing integrated crop management (ICM) programmes for diamondback moth and clubroot. For the past three years, researchers at IHD – Knoxfield have been developing ways to genetically modify vegetable brassicas with genes that may be useful to control insects and diseases to complement the ICM programmes.

Methods have been developed to transfer genes into a number of brassica crops. The necessary tissue culture systems for about 40 cultivars have been developed and the important factors that influence the regeneration of adventitious shoots identified. Gene transfer systems using *Agrobacterium* have been developed for the broccoli cultivar Marathon, the cauliflower cultivars Atlantis and Plana and the Chinese cabbage Pak Choi.

A population of genetically modified plants has been produced with different types of genes for insect and disease control and to increase shelf life. These plants are being assessed in glasshouse trials to find suitable lines for more detailed study. To date genetically modified lines with an insect control gene and a disease control gene have been screened and some promising lines identified.

1.2 Technical summary

The insect pest diamondback moth and fungal disease clubroot are major problems world-wide with vegetable brassicas, resulting in reduced yield and produce quality. Integrated crop management systems for both organisms are being developed to reduce reliance on chemical control. For crops such as broccoli, improved shelf life is also a desirable attribute.

Breeding for resistance to diamondback moth and clubroot and improved shelf life are difficult, as known sources of resistance are either not available or difficult to incorporate into cultivated lines using sexual hybridisation. Gene technology is able to overcome these barriers and provides a new, novel and powerful tool to study these problems.

Tissue culture methods have been developed to reliably and reproducibly regenerate adventitious shoots from broccoli (8 cultivars), Brussels sprout (3 cultivars), cabbage (2 cultivars) and cauliflower (15 cultivars). There is considerable variation in the way individual cultivars regenerate with an order of magnitude difference between the worst and best cultivars.

Gene transfer systems have been developed for a number of cultivars. Research using a construct with the *gusA* gene has not been particularly useful to develop a transformation system. Despite this difficulty, a population of genetically modified vegetable brassicas has been produced with potentially useful genes. In excess of 21,000 explants were processed resulting in 105 transgenic lines. This transformation frequency is very low and requires improvement.

A number of gene constructs have been produced, with different anti-microbial genes, a proteinase inhibitor gene and a gene associated with cytokinin biosynthesis. These have been transferred to a number of cultivars, including Marathon (broccoli), Atlantis and Plana (cauliflower) and Pak Choi (Chinese cabbage). These transgenic lines are in various stages of assessment in glasshouse trials.

Transgenic material has been screened in the glasshouse for their response to clubroot and diamondback moth and a number of lines with promise identified.

Future work will characterise these further and screen the remaining transgenic lines.

2.0 Tissue culture regeneration

2.1 Introduction

Brassica oleracea is a highly polymorphic species and includes a number of cultivated forms of vegetables such as broccoli (var. *italica*), Brussels sprout (var. *gemmifera*), cauliflower (var. *botrytis*) and cabbage (var. *capitata*).

Reliable adventitious shoot regeneration systems are an essential requirement if gene technology is to be applied to crop improvement. Adventitious shoots have been regenerated from various explants for important *Brassica* species. These include cotyledons, hypocotyls and roots (Bhalla and Smith 1998), hypocotyls (Pua *et al.* 1999), and peduncles (Eapen and George 1997).

With *Brassica* species, incorporation of silver, which inhibits ethylene action, has been shown to enhance shoot regeneration (Chi & Pua 1989, Palmer 1992) and improve androgenesis (Dias and Martins 1999). Similarly De Block *et al.* (1989) reported that silver was essential for shoot recovery of transformed *B. napus* explants.

Results presented here evaluate the influence and form of silver, seedling age and the influence of cultivar on adventitious shoot regeneration from hypocotyls of *Brassica oleracea*. Our long-term objective is to use the regeneration system for *Agrobacterium*-mediated gene transfer. For meaningful studies with transgenic plants it will be necessary to evaluate clonal lines and for this reason, factors were studied that influence shoot proliferation from adventitious shoots and shoot tips.

2.2 Methods and materials

2.2.1 Seed germination

Seeds were rinsed in 70% ethanol for five minutes, surface sterilised in 2% available chlorine with 0.1% Tween 20 for 15 minutes and rinsed three times in sterile water. Seeds were germinated in 250 mL polycarbonate tubs containing MS (Murashige and Skoog 1962) basal medium with 3% sucrose and solidified with 0.8% agar (Difco). The pH was adjusted to 5.7 prior to autoclaving at 100 kPa for 20 minutes. Cultures were incubated at 22 ^{0}C under a 16 hour : 8 hour (light : dark) photoperiod. The light source was cool-white fluorescent tubes providing 50 µmol m^{-1} s^{-1} at the culture level.

3.0 Gene transfer system

3.1 Introduction

The production of genetically modified plants is a time consuming and complex process. When using *Agrobacterium* as a gene transfer system, a cascade of events occur. Briefly, phenolic compounds produced by wounded plant cells initiate the transfer of T-DNA from the *Agrobacterium* through the induction of the bacterial *vir* genes. The T-DNA, as a single stranded molecule, moves through the bacterial membrane and is transferred to the plant cell where it moves to the nucleus and is integrated at random (Galun and Galun 2001). Once this has occurred it is then necessary for the transgenic cells to regenerate to shoots thence plants. From the original inoculation of bacterial and plant cells to the eventual development and growth of a transgenic plant can take from four to six months.

From a practical point, the transfer of genes, when using *Agrobacterium*, involves three distinct stages, (1) the manner in which the bacterial cells are inoculated with an explant, (2) the duration of the co-cultivation period of the bacterial cells with the plant tissue and (3) the selection and regeneration of transgenic cells. During the last 10 years, the *gusA* gene has become a valuable tool to study factors that influence gene transfer (Martin *et al.* 1992). It is easy to detect; a substrate, x-gluc (5-bromo-4-chloro-3-indolyl-β-D-glucuronide) is reacted with plant tissue and GUS positive tissue turns blue (Jefferson 1987). The main problems with using GUS is the assay is destructive and results are obtained some 24 hrs after setting up. Recently, a new reporter gene system based on the green fluorescent protein (GFP), isolated from the jellyfish, *Aequorea victoria* has been developed (Chalfie and Kain 1998). The main advantage of GFP are that no substrate is required, expression can be monitored with fluorescence microscopy and it is non-destructive.

Some of the data presented in this section results from a series of experiments to evaluate factors that could influence gene transfer, using the *gusA* gene. The *gusA* gene used has been modified to prevent expression in *Agrobacterium* through a portable plant intron, so that the gene expression can be attributed to the treatment imposed (Vancanneyt *et al.* 1990). Information is also provided about the other gene constructs used in the project (see section 4.0 Preparation of gene constructs for further details).

~~**3.2 Methods and materials**~~

~~**3.2.1 Agrobacterium growth**~~

~~The *gusA* gene construct (pBECKS$_{400}$ GUSintron. nptII. hph) (M^{c} Cormac *et al.* 1997) (Fig. 4.1) used to develop the gene transfer system was mobilised to~~

5.0 Screening transgenic plants against clubroot

5.1 Introduction

Clubroot (*Plamodiophora brassicae*) is an important soil-borne pathogen of vegetable brassicas world-wide with a complex life cycle (Ingram and Tommerup 1972). Primary infection occurs in root hairs resulting in the development of uninucleate plasmodia. Secondary infection of cortical cells results in multinucleate plasmodia and the development of the characteristic galls.

Breeding for clubroot resistance is difficult, as it appears to be a recessive character controlled by more than one gene (Chiang and Crête 1970).

Plants are able to defend themselves against invading pathogens by a number of mechanisms. Specific host-pathogen responses involve the interaction between pathogen *avr* (avirulence) genes and the corresponding *R* (plant resistance) genes (Dangl and Jones 2001). Cysteine-rich and basic polypeptides have also been recognised as being involved with plant defense against pathogens. Such compounds include thionins (Bohlmann 1994), lipid transfer proteins (Garcia-Olmedo *et al.* 1995) and plant defensins (Terras *et al.* 1995).

Glucose oxidase (GO) has been found effective against fungal and bacterial pathogens *in vitro*, which is attributed to the production of hydrogen peroxide (H_2O_2) as a result of the oxidation of GO (Peng and Kuc 1992). Expression of a *glucose oxidase* gene in transgenic potato plants has resulted in enhanced disease resistance (Wu *et al.*1995).

Results presented here describe the preliminary analysis resulting from the challenge of 10 transgenic lines of the cauliflower cultivar Atlantis containing the *glucose oxidase* gene under the control of the 35S constitutive promoter.

5.2 Methods and materials

5.2.1 Production of transgenic plants

Transgenic plants were produced using methods described (see section 3.2.2 Transformation) with the gene construct p35S glucose oxidase (Table 3.1, Fig. 4.3). Ten transgenic lines of Atlantis (cauliflower) were clonally propagated *in vitro*, acclimatised and grown in the containment glasshouse at IHD - Knoxfield for three weeks prior to inoculation. Non-transgenic control plants were regenerated from hypocotyl explants.

5.2.2 Clubroot challenge

Transgenic lines were inoculated with 200 µL of spores (10^4 or 10^8 spores mL^{-1}) collected from an infected site at Trentham (Victoria) or water (zero inoculum) (Fig. 5.1). Control plants were treated as above. Each treatment was replicated five times.

Plants were grown for eight weeks prior to analysis. Soil was washed off the plants (Fig. 5.2) and the root system evaluated using the 'root galling assessment scale'. The shoot and root system of each plant was oven dried and weighed (Appendix 2).

5.3 Results and discussion

As expected the zero inoculum (water) treated plants, irrespective of whether transgenic lines or control plants, resulted in no galls. Plants inoculated with the lowest spore density (10^4 spores mL^{-1}), all had low ratings, with one or less galls per plant. This result indicates the level of inoculum is too low to induce symptoms. There were variable results with the highest inoculum density (10^8 spores mL^{-1}), ranging from 3.6 (lines 1152 and 1153) to five and above (lines 1213, 1214, 1217 and control (Table 5.1). This type of result is common with transgenic plants and demonstrates the importance of screening numerous plants before selecting those for more detailed analysis. It is encouraging that some of the tested lines (1152, 1153, 1154, 1215 and 1218) have lower gall ratings than the control. There is no apparent relationship between the plant dry weight data and the gall rating, which indicates that the interactions between transgenic lines, clubroot and plant response are likely to be complex. The response of plants from the clubroot challenge can be found in Fig. 5.3.

Table 5.1 Ratings of transgenic Atlantis lines inoculated with clubroot spores at two densities [1]

Line	Inoculum density (spores mL^{-1})		
	0	10^4	10^8
1152	1.0	1.4	3.8
1153	1.0	1.0	3.6
1154	1.0	1.0	4.4
1213	1.0	1.0	5.6
1214	1.0	1.0	5.6
1215	1.0	1.0	4.0
1216	1.0	1.2	4.8
1217	1.0	1.0	5.2
1218	1.0	1.0	4.0
1219	1.0	1.0	4.6
Control	1.0	1.2	5.0

[1] Plants were rated using the 'root galling assessment scale'.

1= no galls
2= single gall
3= several small galls
4= mild tap root infection
5= moderate tap root infection
6= severe tap root infection, many galls

5.4 Conclusions

A preliminary screen of transgenic plants with the *glucose oxidase* gene has identified some lines with reduced gall numbers. These transgenic lines need to be tested again, using only one inoculum density. In addition, the glucose oxidase activity needs to be determined, both before and after inoculation.

Additional transgenic lines with glucose oxidase (Tables 3.5 and 3.6) and a truncated thionin gene (Table 3.7) need to be assessed.

6.0 Screening transgenic plants against diamondback moth

6.1 Introduction

Diamondback moth (*Plutella xylostella*) is the most important insect pest of vegetable brassicas world-wide. Control of this pest has been an issue for the Australian vegetable industry since the 1980's, when resistance to pyrethroid insecticides was identified. Consequently, integrated crop management (ICM) systems were developed to reduce the reliance on conventional insecticides. A desirable part of the solution for ICM programmes is the availability of cultivars with a level of tolerance (or ideally resistance) to the target.

Resistant cultivars can be produced by either conventional breeding or gene technology. Unfortunately, breeding for diamondback moth resistance is difficult due to the lack of appropriate germplasm. Gene technology has been proven to be effective, and two compounds, the proteinase inhibitors and the insecticidal proteins from *Bacillus thuringiensis*, have been identified and demonstrated to have wide application in controlling insect pests of plant species (Gatehouse *et al.* 1992, Peferoen 1992).

Proteinase inhibitors are proteins produced naturally in certain plants as a defence mechanism (Ryan 1990). The gene encoding for a proteinase inhibitor from the giant taro (*Alocasia macrorrhiza*) has been cloned and characterised and its effectiveness tested against cotton bollworm (*Helicoverpa armigera*) in transgenic tobacco lines (Wu *et al.* 1997). The giant taro proteinase inhibitor (GTPI) has two reactive sites, which are active against trypsin and chymotrypsin, the two major proteinases present in the insect larval midgut. Therefore, GTPI should have an impact on the digestive capacity of insects following consumption.

This section provides preliminary data on the growth and development of diamondback moth larvae fed leaf tissue from eight transgenic lines of the cauliflower cultivar Plana.

6.2 Methods and materials

6.2.1 Production of transgenic plants

Transgenic plants were produced using the methods described (see section 3.2.2 Transformation) with the gene construct p35S AM8 (Table 3.1, Fig. 4.6) and screened for the presence of transgenes with PCR. Lines were cloned *in vitro*, acclimatised and grown in the containment glasshouse at IHD - Knoxfield. Non-transgenic control plants were grown from seeds.

6.2.1 Diamondback moth challenge

A diamondback moth colony derived from insects collected at Berwick (Victoria) was reared in the laboratory at 25 °C on cabbage seedling leaves. Eggs were collected on a Parafilm® sheet and first instar larvae (within 24 hr of hatching) were used in the feeding trial. One-cm leaf discs from glasshouse grown plants were placed in small clear plastic cups (Solo® plastic portion cups, 28 mL) and used for the feeding trials (Fig 6.1). For the first three days of the feeding trial, a single leaf disc was used and subsequently two (or more) discs were used. Leaf discs were replaced every second day. Larvae were weighed after seven days and maintained until the adults emerged. Mortality was recorded at seven day intervals.

The experimental unit was an individual cup containing a larva. Four cups of each transgenic line and control material were placed in a closed 18 litre plastic container (a replication) lined with moistened paper towel (Fig 6.2). Three plastic containers were maintained at 25 °C and 80% relative humidity.

6.3 Results and discussion

Larvae fed on seven of the eight transgenic lines had a lower body weight than control fed larvae (Table 6.1). The greatest reduction in larval weight was found with line 1122, which was 12.5 times less than the control fed larvae. In contrast, larvae fed on line 1129 were heavier than control fed larvae. Lines 1122 and 1127 resulted in a higher larval mortality than other transgenic lines and the control. With lines 1123, 1127 and 1129, the percentage emergence of moths from pupae was reduced.

The observed slower growth, increased larval mortality and the failure of moths to emerge from pupae could be due the levels of GPTI expressed in transgenic lines. The mechanism of GTPI on growth retardation has not been studied, however it may have been due to a decrease or increase of gut proteiolytic activity, as observed by Broadway and Duffy (1986).

Table 6.1 Response of diamondback moth larvae after feeding on transgenic lines

Plant number	Larval body weight (mg) [1]	Laval mortality (%) [2]	Adults emerged from larvae (%)
1122	0.2	33.3	100
1123	1.1	8.0	82
1124	1.3	0.0	100
1125	1.2	8.3	100
1127	1.5	33.3	88
1128	1.0	0.0	100
1129	3.5	8.3	90
1130	1.4	8.3	100
Control	2.5	16.6	100

[1] at seven days
[2] at 21 days

6.4 Conclusions

This preliminary screen of transgenic lines with the GTPI gene has identified some lines with potential, due to reduced larval weight and increased mortality. Some of the transgenic lines need to be tested again in a more detailed study.

6.5 References

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Peferoen M (1992) Engineering of insect-resistant plants with *Bacillus thuringiensis* crystal protein genes. In: Plant Genetic Modification for Crop Protection, 135-53. (Eds. AMR Gatehouse, VA Hilder, D Boulter). CABI International.

7.0 Technology transfer

7.1 Publications/Conferences/Seminars

Members of the project team participated in the clubroot research team meeting and industry day at IHD-Knoxfield in October 1998.

Genetic engineering – will it help? Plutella Update (No. 37, May 1999)

Genetic engineering – can it help? Galls and All (No.8, July1999)

Department of Natural Resources and Environment, Horticultural Conference (6 and 7 September 2000)

Kaul V, Maheswaren G, Hutchinson JF. Genetic engineering of brassicas for pest and disease control and improved storage. 'Growing for Profit' field day, Gympie, Queensland. Queensland Fruit and Vegetable Growers and HRDC.

Genetic engineering for improved vegetable brassicas. Institute for Horticultural Development Annual Report 2000/01

Seminars at Crop and Food Research (New Zealand), University of Melbourne (School of Forestry and Institute of Land and Food Resources) on 'Biotechnology research with vegetable brassicas and potatoes at IHD'

7.2 Laboratory visits

Meeting organised by Patrick Ulloa, Industry Development Officer, Victoria on improving communication skills between growers and researchers (Melbourne Market, April 1999)

Werribee-Expo and discussed with the Young Growers Group their R&D priorities (May 1999)

Some members of the HRDC Brassica Commodity Group visited to see how the project was progressing. This laboratory visit was associated with the clubroot meeting organised by Caroline Donald and Ian Porter (23 and 24 August 2000)

Ontario Processing Vegetable Growers Association. Members included the Directors and six growers. (www.ovgmb.org) January 2001

Two delegations from the Biotechnology Inovation Centre, Democratic Peoples Republic of Korea. As part of ACIAR visit. March 20001

Patrick Ulloa, the Vegetable Industry Development Officer for Victoria, led a group of vegetable growers to IHD - Knoxfield in December 1999 to learn about the project. Growers were shown tissue culture and gene transfer systems and given a guided tour of the laboratory facilities. Much of the discussion was about safety issues associated with gene technology. This was a most beneficial exercise and additional visits from any sectors of the vegetable industry are welcome

This visit was reported in Vege Link (Issue 4, March 2000) Is genetic engineering the way to go?

Vegetable growers visit IHD - Knoxfield

8.0 Recommendations

1. Gene technology has considerable potential for the improvement of vegetable brassica germplasm and it is highly recommended that this research continue.

2. It is recommended that the technologies developed during the project be capatilised on. Genetic modification is an ideal way to conduct targeted breeding. We have developed an enabling technology that can be used to transfer any available gene to vegetable brassicas.

3. The transgenic lines generated from this project need to be fully evaluated in glasshouse trials. To date only some transgenic lines with glucose oxidase and GTPI have been screened in preliminary glasshouse trials. Further transgenic lines to be assessed include additional lines with *glucose oxidase* and all those with a truncated *thionin* and the *ipt* genes.

4. Due to the low number of transgenic plants produced, it is important that ways to improve vegetable brassica transformation continue. Research using the green fluorescent protein maybe a better alternative than the frequently used *gusA* gene.

5. During this research project it was announced (December 2000) that the genome of *Arabidopsis thaliana* had been sequenced. *Arabidopsis* has become the 'model species' for plant research as it has a small genome, well-known genetics, a short life cycle (eight to 10 weeks) and can be easily transformed with *Agrobacterium*. Importantly for the vegetable brassica industry is that *Arabidopsis* is susceptible to clubroot and diamondback moth. This makes *Arabidopsis* an ideal experimental plant to study problems associated with vegetable brassicas. It is recommended that a small research programme with *Arabidopsis* proceed in parallel with the main brassica project.

6. The vegetable industry needs to be aware of the advantages and disadvantages of gene technology, so that meaningful decisions can be made about future investments. The internet is a powerful resource with a wealth of information. A number of URLs are suggested.

AgBio View (www.agbioworld.org) and
AgNet (www.plant.uoguelph.ca/safefood)

These sites provide daily updates on biotechnology and discuss the advantages and disadvantages.

Horticulture Australia GMO (Guiding Meaningful Opinions) newsletter (www.horticulture.com.au/)

Provides an overview of activities, with a horticultural slant.

Tomorrow's Bounty (www.tomorrowsbounty.org)

Put together by a number agricultural commodity organisations in the USA with the specific purpose of providing information to the agricultural community.