CONSTRUCTION OF VECTORS FOR THE INTRODUCTION AND REGULATION OF NOVEL TRANSGENES IN WALNUT PLANTS.

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ABSTRACT:

This project is aimed specifically to a) develop vectors that will permit the introduction of novel transgenes, b) identify regulatory sequences that will permit enhanced expression of transgenes in walnut plants, and c) analyze transgenic walnut plants and tissues that express the various transgenes. To this end we report construction of 4 vectors for the enhanced expression of genes encoding insecticidal crystal protein (ICP) of Bacillus thuringiensis (Bt), 3 vectors for resistance to fungal infection and 2 vectors for the modification of plant form and architecture. In addition we have completed the qualitative analysis of 61 embryo lines transformed with a vector containing a second generation gene encoding ICP of Bt. We observed that in 34.4% of these lines (21/61) that we have designated "class A", expression was high enough to kill a vast majority of the codling moth larvae (98% mortality). Twelve clones (19.7%) were designated "class B" and these showed a marked retardation of larval development and a mortality between 50-94%. The remaining 28 lines (45.9%) although transformed were indistinguishable from the control. Our qualitative analysis of ICP and quantitative analysis of GUS enzymatic activity has revealed the strong position effects in walnut for the inserted gene and allowed the identification of lines that highly express resistance to codling moth larvae. These highly resistance lines will undergo further testing.

OBJECTIVES:

1. Construction of vectors for the testing of horticulturally useful transgenes in walnut.

2. Development of vectors for tissue-specific and cell-type specific expression of transgenes in walnut.

3. Analysis and characterization of transgenic plants and tissues expressing horticulturally useful transgenes.

PROCEDURES:

1. Construction of vectors for the testing of horticulturally useful transgenes in walnut.

Pest tolerance through expression of second generation synthetic constructs encoding Cry1Ac of Bt:
Synthetic full length cry1Ac gene of Bt encoding ICP: A binary vector pMON1058 was obtained from David Fischoff at Monsanto that contained a full length synthetic construct of the cry1Ac gene of Bt regulated by an enhanced Cauliflower Mosaic Virus 35S promoter (ECaMV35S). In addition, we obtained pMON755 a plasmid that contained a chimeric GUS gene also regulated by the ECaMV35S promoter. The vector pMON10518 was cut with the restriction endonuclease NotI, dephosphorylated and then ligated to a 2.8 kb NotI fragment (containing the chimeric GUS gene) of pMON755 after separation on agarose and subsequent purification with glass milk. The ligated mixture was transformed into E.coli DH5α. Plasmid DNA from Str25 and Spc25 (media containing the antibiotic streptomycin and spectinomycin at 25 μg/ml each) resistant colonies was analyzed by restriction analysis with BglII to identify the desired construct and then confirmed using other restriction enzymes. The plasmid designated pDU91.3 (Fig. 1) represents one of the two possible orientations of the NotI fragment and was introduced into Agrobacterium strain ABI. Agrobacterium colonies resistant to Km50 (media containing the antibiotic kanamycin at 50 μg/ml) and Spc25 were analyzed for plasmid DNA to confirm the presence of pDU91.3.

A derivative of pDU91.3 was constructed to provide a control for the above construct for walnut transformation experiments. This construct lacks the synthetic cry1Ac coding sequences and otherwise is essentially the same as the above vector. This vector was constructed by digesting pDU91.3 with ScaI that releases the cry1Ac coding region. An 11 kb fragment that contained the rest of the plasmid was isolated from a gel after separation and then allowed to ligate. After ligation the DNA was digested with XhoI to linearize any contaminating uncut plasmid and then transformed into E.coli DH5α. This vector was designated pDU91.6 (Fig. 1). Further analysis and subsequent transformation into Agrobacterium was essentially identical to that described for pDU91.3.

Synthetic truncated Cry1Ac gene of Bt encoding ICPF: Two additional vectors became available to us from Kevin McBride at Calgene as part of an ongoing apple transformation program. These were modified as part of this program by introducing a gene encoding GUS (β-glucuronidase). These constructs are described below. The two Calgene constructs were pCGN4070 and pCGN4072 that contain a chemically synthesised truncated cry1Ac gene. In pCGN4070 this version of the chemically synthesised truncated cry1Ac gene was further modified at the N-terminal with the addition of the 5′-noncoding region and translational leader context and the first 6 amino acids from the gene encoding the small subunit of RUBP carboxylase from tobacco. This modification should further improve the translational efficiency of this gene thus further improving its expression. The vector pCGN4070 was modified by introducing the gene encoding GUS thus creating two new vectors pDU92.67 and pDU92.63 containing the both possible orientations of GUS (Fig. 1). In a similar manner the vector pCGN4072 was modified creating pDU92.710 and pDU92.15 (Fig. 1).
We have now introduced these vectors into three different strains of Agrobacterium, C58C1, EHA101 and AGL1

**Disease resistance:**

The SAR8.2 gene is a gene encoding resistance to Phytophthora in tomato. A construct expressing the cDNA of this gene designated pCGN1790C was obtained from Danny Alexander at Calgene. The vector pCGN1790C was modified by us by the insertion of a chimeric gene encoding GUS thus creating the vector pDU92.213 (Fig. 1). This vector has been introduced into the Agrobacterium strain AGL1.

Genes encoding chitin binding lectins with anti fungal properties have also been used for constructing vectors. Three genes have been targeted from different sources, barley, rubber tree (hevein) and stinging nettle. Constructs containing these genes were obtained from Thea Wilkins at UCD. Two vectors have been completed; one is still under construction. The vector pDU92.1710 contains the gene encoding the barley lectin along with GUS and a selectable marker gene APH (Fig. 1). The second vector pDU92.164 contains the gene encoding the lectin hevein along with GUS and APH (Fig. 1). The two vectors have been introduced into the ABI strain of Agrobacterium. Initially they were introduced into the AGL1 but were found to be unstable.

**Alteration of plant form and architecture:**

Two vectors were constructed that contained the rolABC and rolB genes from Agrobacterium rhizogenes strain A4. Subclones of the TR-DNA of pRiA4 were used. The rolABC region was obtained as a 4.3 kb EcoRI fragment, whereas the rolB gene was obtained as a 1.8 kb HindIII fragment. These two fragments were purified through gel electrophoresis and subcloned into pBluescript creating two plasmids designated pDN152 (containing rolABC) and pDN113 (containing rolB). In addition we subcloned the gene cassette containing an intron containing GUS gene (355GUS-INT) into pBluescript creating plasmid pDN352 (containing 355GUS-INT). The GUS gene from pDN352 and the rolABC and rolB fragments from pDN152 and pDN113, respectively, were introduced into the binary vector pBIN19 creating two new vectors, pDN3521 (containing rolB; Fig. 2) and pDN3514 (containing rolABC; Fig. 3). The details of this construction are quite involved and have been described elsewhere (Negri, 1992).

2. **Development of vectors for tissue-specific and cell-type specific expression of transgenes in walnut.**

**Construction of cDNA libraries:** Standard recombinant DNA methodologies have been used to construct a cDNA library from walnut somatic embryos. Total RNA was extracted by a modification of earlier procedures (Cathala et al., 1983; Chirgwin et al., 1979; Verwoerd et al., 1989) and the mRNA separated (poly A+ RNA; Aviv and Leder, 1972). Synthesis and cloning of cDNA was
accomplished using a kit from Stratagene. The double stranded cDNA (ds-cDNA) was cloned directionally into the lambda vector uni-Zap, packaged in vitro (Gigapak Gold, Stratagene) and transfected into E.coli XL1-Blue cells.

Evaluation of cDNA libraries: The primary libraries were amplified to produce high titre phage lysates (>2x10^{10}/ml). We have approximately 100 ml of lysate. Recombinant phagemids containing the cDNA were recovered after purification from single plaque isolates through excision from the uni-Zap lambda phage in XL1-Blue cells by infecting with the helper phage R408 according to procedures provided by Stratagene. Plasmid DNA was isolated from individual colonies and characterized by restriction analysis.

3. Analysis and characterization of transgenic plants expressing horticulturally useful transgenes.

Second generation construct expressing full length synthetic cryIA(c) gene of Bt:

Phase IA (Qualitative insect bioassay): In the phase 1A trial 61 transformed embryo lines and one nontransformed (control) line was tested for efficacy against the target insect species codling moth (CM). The embryo lines were bulked up in the laboratory of Gale McGranahan and then transported to Fresno where the tests were carried out. Transformed E_2 lines were chosen that showed a blue reaction with the GUS substrate X-Gluc. Our purpose was to categorize the embryo lines using an arbitrary scheme based on mortality of first-instar CM or retardation of larval rate of development. Embryos were removed from DKW basal medium, rinsed in sterile, distilled water, and then placed into 2.5 ml plastic vials (about 2 to 3 embryos per vial). The embryos were infested with one newly hatched (first-instar) CM and the vial closed with a snap-cap. The test was evaluated after seven (7) days at 27°C to determine larval mortality and development. We recorded the instar of each live or dead larva. A replication of the feeding trial consisted of ten larvae (ten vials) per clone. Controls were included with each experimental set and consisted of untransformed embryos belonging to the SU2 line. Feeding trials were replicated two or three times (20 to 30 larvae per clone).

Phase IB (Quantitative insect bioassay): The phase 1B trial is currently in progress. Serial dilutions of freeze-dried embryos were assayed against first-instar CM and navel orange worm (NOW) by exposing the larvae to surface contaminated insect diet. Trials were evaluated after seven (7) and 14 days.

RESULTS AND CONCLUSIONS:

1. Construction of vectors for the testing of horticulturally useful transgenes in walnut.

Pest tolerance: The production of transgenic plants expressing
the insecticidal crystal protein(s) (ICPs) of *Bacillus thuringiensis* (Bt) provide a useful approach toward a non-insecticide approach to combat insect pests. The ICPs of Bt have been used for many years and are highly toxic to many Lepidopteran insect pests. One of the objectives of our program is to achieve resistance to *Cydia pomonella* (coding moth, CM), the key pest in walnut. We have previously demonstrated that purified insecticidal crystal protein fragments (ICPFs) of *Bacillus thuringiensis* encoded by cryIA(c) and cryIA(b) of this organism were shown to be lethal to coding moth. A binary vector (pWB139) was used to introduce one of these genes, cryIA(c) with limited success against CM mainly due to the low expression of cry1Ac. This particular vector construct contained the coding sequences from the bacteria Bt. Examination of the natural ICP coding regions from *Bacillus thuringiensis*, especially those present in the construct pWB139 that we have used, reveal many features that could give problems for expression in plants, in general, and walnuts in particular. There is a strong codon bias preferring A/T in the third position (Table 1). The preference in plants is for codons, XXC/G rather than XXA/T (Murray et. al., 1989; Campbell and Gowri, 1990). In addition, we find that many of the codons in the sequence have CG or AT in the second and third position (Table 1). In most eukaryotes there is an absence of XCG and XAT codons (Beutler et. al., 1989). Apart from the incompatibility observed at the codon usage level we can identify at least 28 instances where "killer sequences" are present (Fig. 4). These are sequences that cause instability in mRNA and have been shown to be caused by many types of sequences; killer sequence – ATTAA (Shaw and Camen, 1986), polyA like signals – AATAA (Dean et. al., 1986), T-rich regions, e.g., TTTPTR or >TTTT and A-rich regions, e.g., >AAAA (Goodall and Filipowicz, et. al., 1989). A strategy to circumvent these problems was reported by Perlak et. al., (1991) and involved making alterations of this gene through chemical synthesis. The gene product has the identical amino acid sequence with the major differences being at the nucleic acid level. This altered gene functioned very well in plants having levels of expression >500 fold higher than those expressed using the coding region from the bacteria (Perlak et. al., 1991). We refer to the genes containing the bacterial coding regions as "first generation" and the synthetic genes with altered coding regions as "second generation".

Last year we described the transformation experiments with the second generation vector pDU91.3, but did not give the details of the construction. Preliminary results on efficacy with respect to CM were very positive: a more detailed analysis is presented below. The second generation vectors can be divided into two classes one that encode the ICP (protoxin) and the other that codes for ICPF (active fragment of the β-endotoxin of Bt). The vector pDU91.3 belongs to the former class. We have now constructed 4 new vectors that should express the latter class (ICPF). These vectors are pDU92.67, pDU92.63, pDU92.710 and pDU92.15. In the vectors pDU92.67 and pDU92.63 the second
generation cry1Ac gene encoding ICPF was further modified at the N-terminal to include the 5’ nontranslated region and first 6 aminoacids from the mRNA encoding the small subunit of RUBP carboxylase of tobacco. This has been previously shown to enhance the translational efficiency of the transcripts.

Disease resistance: Vectors have been constructed to investigate two different modes of resistance to fungi, one involves the gene encoding SAR8.2 and the other genes encoding chitin binding lectins.

It was shown some years ago that plants, like animals, have an immune response that acts as a defense like the inducible antigen-antibody response in animals. Although plants do not contain the antibody-antigen mechanism like animals they can be immunized against both disease causing microorganisms and insect pests (Kuc, 1982). This increase in resistance to subsequent pathogen attack was observed in parts of the plant that were not infected by the pathogen and the term systemic aquired resistance or "SAR" was used to describe this phenomenon (Ross, 1961). A variety of genes are induced during SAR and these have been characterized (Bol et. al., 1990; White and Antoniw, 1991). Some of the proteins encoded by these genes have been shown to be inhibitory towards Phytophthora infestans (Woloshuk et. al., 1991). We have obtained one of these genes designated SAR8.2 from Danny Alexander and encodes resistance to the ‘Black Shank’ fungus Phytophthora. The vector pDU92.213 was constructed that contains the SAR 8.2 gene for transformation into walnut.

Plant lectins like wheat germ agglutinin have been shown to interact with chitin and inhibit fungal growth (Mirelman, 1975) and are proposed to have a role in plant defense (Chrispeels and Raikhel, 1991). The hypothesis here is that fungal hyphal expension and septa formation involve the synthesis of chitin in hyphal tips and septa (Cabib, 19; Wessels, 1988). Correspondingly growth of fungi that contain chitin in their cell walls has been shown to be sensitive to proteins that bind (chitin binding lectins) or degrade chitin (chitinase) (Broekaert, 1988; 1989; Parijs, 1991 and Schlubaum, 1986). In order to test this hypothesis in walnut three candidate genes have been chosen. These all three encode lectins from three different plant species that have strong chitin binding properties, and these are barley (Lerner and Raikhel, 1989, Peumans et. al., 1982), rubber-tree (Hevein; Parijs et. al., 1991) and stinging nettle (Broekaert et. al., 1989). Constructs containing genes encoding these three candidates were obtained from Thea Wilkins. Two vectors have been completed; one is still in the process of construction. The vector pDU92.1710 contains the gene encoding the barley lectin and pDU92.164 contains the gene encoding Hevein, the lectin from the rubber tree (Hevea brasiliensis). The vector containing the lectin from stinging nettle (Urtica dioica L.) is presently under construction.

Alteration of plant form and architecture: Although similarities
exist between Agrobacterium rhizogenes and Agrobacterium tumefaciens in the mechanism of virulence and T-DNA transfer the physiology of the disease process is distinctly different. Mutational analysis revealed 6 loci designated rolA-F that affect the disease phenotype and are present on the T-DNA of the Ri plasmid, rolB mutants were avirulent (White et al., 1985). Further characterization of these loci revealed that the hairy root phenotype could be reproduced in some plants using a DNA fragment of the Ri plasmid that contained rolABC and that the presence and expression of either rolB or rolC was sufficient to induce roots in tobacco leaves and stems (Cardarelli et al., 1987; Spena et al., 1987; Vilaine et al., 1987). It has also been shown that when investigated individually rolA, B and C were shown to induce developmental alterations of plant architecture in the transgenic plants with rolB playing a central role in root formation and hairy root syndrome (Capone et al., 1989; Oono et al., 1987; Schmulling et al., 1988; Sinkar et al., 1988). Recent studies indicate that the rolB gene encodes a β-glucosidase able to hydrolyse indole β-glucosides (Estruch et al., 1991a) and rolC encodes similarly a cytokinin-β-glucosidase (Estruch et al., 1991b). The action of these two enzymes alter the levels of auxin and cytokinin in the transformed plant tissues. However, this is a very simplified explanation of the complex and as yet poorly understood physiology of the hairy root cells and the role played by the expression of these two genes. An example of this is the high auxin sensitivity that has been observed to be a common feature of hairy roots and plants regenerated from hairy roots (Shen et al., 1988). The rolB gene product has been implicated for this phenotype and has also been proposed to encode an auxin receptor or a protein that may be closely associated with the functioning of the auxin receptor (Barbier-Brygoo et al., 1990; Maurel et al., 1990; Shen et al., 1988; Spano et al., 1988. The inescapable conclusion of all these studies is that the expression of rolB and/or rolC modulate plant metabolism and development in profound ways and this may have a useful application in the modification of plant architecture. In order to investigate this we have used two regions from the T-DNA of the A4 strain of Agrobacterium rhizogenes, a segment that contains rolABC and another that contains rolB, to construct two vectors, pDN3514 and pDN3521 respectively (Fig. 2 and 3).

2. Development of vectors for tissue-specific and cell-type specific expression of transgenes in walnut.

Construction of cDNA libraries: One cDNA library has been constructed using mRNA from proliferating somatic embryos of the SU2 line that has been used in transformation experiments. This library of cDNA is contained in a bacteriophage vector. The primary library contained about 1.5 million phage particles. This primary library was amplified through infection of E.coli on plates to produce a high titre lysate containing 9.5 $10^9$ phage particles per ml. We have confirmed that over 98% of these phage particles are recombinant in that they contain inserted DNA, presumably cDNA. One of the experiences in constructing this
library has been that we get a variability both in the amount and quality of mRNA from walnut tissues. This is due to many physiological and developmental factors of the tissue used but also on the concentration of phenolic and interfering substances found in these tissues.

Evaluation of cDNA libraries: We have evaluated this library in two ways, one by screening using plaque hybridization procedures and the other by restriction digest of plasmid excised from the phage particles. For the plaque hybridization we have used heterologous DNA probes corresponding to cDNA encoding the proline biosynthetic gene P5C reductase, a gene that we expect to be globally expressed in walnut, i.e., in all tissues. We have some isolates that need to be confirmed. Using the excision approach we have isolated some cDNA clones and would like to identify those that are highly expressed in walnut embryo tissues. We can conclude from the initial results that we have a good cDNA library that represents the mRNA populations in the walnut embryo. The only negative observation has been that the size of most of the random cDNA inserts is small, ~600-800 bp. It is still possible that full length clones are present but may be at a lower concentration. This can only be confirmed when we are able to isolate a known cDNA.

3. Analysis and characterization of transgenic plants expressing horticulturally useful transgenes.

Second generation constructs expressing cryIA(c) gene of Bt:

Phase IA: Classification of transgenic walnut embryos based on a qualitative insect bioassay: Feeding trials with first-instar CM larvae included 61 clonal lines of transformed embryos, one control clone (SU2), and a total of 1,556 larvae (Table 2). We identified 21 Class A clones. The clones were producing ICP at levels sufficient to kill 98% (452 out of 454) of CM larvae feeding on them. These 21 clones will be the ones included in future tests. Phase II assays of shoots and plants, and Phase III assays of greenhouse or field planted material. We observed a decreased rate of larval development and a relatively low mortality response in 12 clones. The embryos were producing low-levels of ICP and were placed in Class B. All but a few of the class B embryos were discarded. Class C clones were those that had little or no effect on the rate of larval development nor produced significant mortality, compared to controls. These embryos had significant expression of ICP and were discarded.

In the future, we may be able to make preliminary screening based on the MUG assay. Table 3 shows the mean reaction rates from MUG assays for each of the three classes of embryos as determined by bioassay. Class A embryos had a mean 238 nmol/mg/min, Class B and C embryos had a mean values of 129 and 42, respectively. At this point, values of 100 to 150 nmol/mg/min may be considered a reasonable threshold for preliminary screening of putative embryos. However, results obtained from this assay vary widely as
seen by the high standard deviations (Table 3). Eventually, we hope to correlate results from chemical analysis of the transgenic plant material with results from bioassay procedures. This would greatly simplify and expedite early screening of putative embryos.

Phase IB: Quantitative analysis of class A embryo lines: To date we have conducted range-finding bioassays using first-instar CM to define the range of doses that mortality responses are obtained. We have just completed the first series of trials designed to determine the range of doses producing mortality responses in the two species. results of trials designed to determine dose-response curves of the two species to Class A embryos will not be available until later.

REFERENCES:


Table 1. Codon distribution of the crylAc gene sequences encoding the ICPF in the vector pWB139.

<table>
<thead>
<tr>
<th>Codon</th>
<th>Frequency</th>
<th>Amino Acid</th>
<th>Frequency</th>
<th>Codon</th>
<th>Frequency</th>
<th>Amino Acid</th>
<th>Frequency</th>
</tr>
</thead>
<tbody>
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<td>TTT Phe</td>
<td>30 (4.9)</td>
<td>Ser 11 (1.8)</td>
<td>TAT Tyr 23 (3.8)</td>
<td>TGG Cys 1 (0.2)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TTC Phe</td>
<td>6 (1.0)</td>
<td>Ser 7 (1.1)</td>
<td>TAC Tyr 4 (0.7)</td>
<td>TGC Cys 1 (0.2)</td>
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<td></td>
<td></td>
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<td>TTA Leu</td>
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<td>Pro 15 (2.5)</td>
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The codons represent 610 amino acids:
Table 2. Phase 1A feeding trial: classification of 61 line of transgenic walnut somatic embryos.

<table>
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<tr>
<th>Class Designat.</th>
<th>Class Description</th>
<th>No. of Clones</th>
<th>No. of Larvae</th>
<th>% Mortality</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>95 to 100% mortality no development (high expression)</td>
<td>21</td>
<td>454</td>
<td>98%</td>
</tr>
<tr>
<td>B</td>
<td>50 to 94% mortality retarded development (Low-level expression)</td>
<td>12</td>
<td>284</td>
<td>48%</td>
</tr>
<tr>
<td>C</td>
<td>0 to 49% mortality normal development (no signific. expression)</td>
<td>28</td>
<td>601</td>
<td>12%</td>
</tr>
<tr>
<td>Control</td>
<td>0 to 10% mortality normal development (no expression)</td>
<td>1</td>
<td>217</td>
<td>9%</td>
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<tr>
<td>Total</td>
<td></td>
<td>62</td>
<td>1556</td>
<td>N/A</td>
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Table 2. Results of fluorimetric MUG assays (Mean, SD, Range) of transgenic walnut somatic embryos by bioassay class (Table 2).

<table>
<thead>
<tr>
<th>Class</th>
<th>MUG (nmol/min/mgFW)</th>
<th>Notes</th>
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<td></td>
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<td>SD</td>
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Fig. 1. Maps of the T-DNA regions of different vectors constructed.

Vectors derived from Monsanto vectors pMON10518 and pMON755

pDU91.3

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RB|7S-3'|CAVIAAY|CRP|mac-5'|35S-3'| GUS|...|35S-5'|E35S-5'|...|nos 3'
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pDU91.6

```
RB|7S-3'|E35S-5'|spc/str|nos 3'| KAN|...|35S-5'|E35S-5'|...|GUS|...|nos 3'
```

Vectors derived from Calgene vector pCGN4072 and our GUS construct

pDU92.710

```
RB|mas 3'|CAVIAAY|CRP|mac-5'|35S-3'|...|GUS|...|35S-5'|tm1 3'|...|KAN|...|35S-5'|LB
```

pDU92.15

```
RB|mas 3'|CAVIAAY|CRP|mac-5'|35S-5'|...|GUS|...|35S-3'|tm1 3'|...|KAN|...|35S-5'|LB
```

pDU92.67

```
RB|35S-3'|...|GUS|...|35S-5'|nos 3'|CAVIAAY|CRP|D35S-5'|tm1 3'|...|KAN|...|35S-5'|LB
```

pDU92.63

```
RB|35S-5'|...|GUS|...|35S-3'|nos 3'|CAVIAAY|CRP|D35S-5'|tm1 3'|...|KAN|...|35S-5'|LB
```

Vectors for disease resistance

pDU92.213

```
LB|mas 5'|...|KAN|mas 3'|D35S-5'|SAR 8.2|tm1 3'|35S-5'|...|GUS|...|35S-3'|RB
```

pDU92.1710

```
RB|7S-3'|Barley Lectin|E35S-5'|35S-3'|...|GUS|...|35S-5'|spc/str|nos 3'|...|KAN|...|35S-5'
```

pDU92.164

```
RB|7S-3'|HEVEN|E35S-5'|35S-3'|...|GUS|...|35S-5'|spc/str|nos 3'|...|KAN|...|35S-5'
```
Fig. 2. Map of the vector pDN3514 that contains the rolABC genes of Agrobacterium rhizogenes.
Fig. 3. Map of the vector pDN3521 that contains the rolB genes of Agrobacterium rhizogenes.
Fig. 4. Killer sequences within the natural coding region of the Bt encoded CRYIA(c) ICPF in pWB139.