Investigation of alternative approaches to narrow-leafed lupin (Lupinus angustifolius) genetic transformation

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by

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Declaration

I declare that this thesis is my own account of my research and contains as its main content work which has not previously been submitted for a degree at any tertiary education institution.

........................................

(Kanokwan Ratanasanobon)
Abstract

Narrow-leafed lupin (*Lupinus angustifolius*) is one of the top six crops that contribute value to the Australian economy. Gene transfer technology has been studied as a strategy to improve lupin varieties against diseases to improve yield, production and seed quality. However, the established method used for transformation of lupin is based on *Agrobacterium* and embryonic axes as explants is a method of low efficiency. The aim of this project was to investigate the alternative genetic transformation methods for genetic manipulation of narrow-leafed lupin (*L. angustifolius*) to improve the transformation efficiency. Two potential genetic transformation methods were investigated: particle bombardment (direct gene transfer), and *in planta* transformation (*Agrobacterium*-based transformation). In addition, lupin-*Agrobacterium* interactions were studied to provide information of the factors limiting transformation, and whether the involvement of an additional *virG* using construct carrying *virGN54D* (constitutive *virG* mutant carrying Asn-54 to Asp amino acid substitution) improved lupin transformation efficiency.

In this project, a genetic transformation protocol using particle bombardment for narrow-leafed lupin was accomplished. The following conditions were identified as being optimal for transformation via particle bombardment using a helium inflow particle gun with lupin embryonic axes as target explants:

a) Embryonic axes used as explants were pre-treated in MS media supplemented with 5 mg/L BAP and 0.5 mg/L NAA, 3% sucrose and 0.7% agar for 3 days in the dark at 25°C.

b) Pre-treated explants were placed onto MS media with 0.3 M mannitol as osmoticum 4 h prior to bombardment.

c) Bombardment was carried out by:
   - A precipitation protocol using plasmid DNA prepared at 2 ng DNA per μg tungsten particles.
   - Bombardment was carried out twice at 400 psi with a 7 cm target distance with 10 μL coated particles.

d) Bombarded explants were kept on osmoticum media (MS media with 0.3 M mannitol) for 4 h then transferred to pre-/post-treatment media for post-treatment for another 3 days and kept in the dark at 25°C.
e) After post-treatment, explants were transferred to selection media (MS medium with 1 mg/L BAP and 0.1 mg/L NAA, 3% sucrose, 0.7% agar and 10 mg/L PPT) for 8 weeks with subculture every two weeks. Surviving shoots were transferred to rooting media and analysed for presence of the transgene by PCR. A transformation efficiency of 0.4% for T0 production was achieved as confirmed by amplification of a gus gene by PCR. However those transformed explants did not form roots.

In planta transformation of seedlings and flowers of narrow-leaved lupin was investigated. For seedling transformation, factors essential for delivering A. tumefaciens to the target tissues (L2 layer of apical meristem of seedlings) and to enhance the ability of A. tumefaciens cells to transform plant cells were studied and optimised. Sonication and vacuum infiltration facilitated penetration of A. tumefaciens cells to the target tissue, sonicating seedlings 15 min before 10 min infiltration with A. tumefaciens cells gave the best overall balance of both gene transfer determined by GUS staining and seedling survival rate. The Agrobacterium induction condition and infiltration medium was developed after testing and optimising of media and Agrobacterium growth. Modified LB medium with glucose 30 g/L was the best medium that gave the highest percentage of shoots showing GUS expression, at 35±5% which was significantly higher than the control infiltration media used for Medicago and A. thaliana in planta transformation at the 0.05 level Tukey HSD. The combined optimised conditions were further tested. Some shoots, picked at random, were positive for GUS expression, including the whole apical area and parts of leaves of new shoots, indicating gene transfer and stable transformation although chimeric. However, transformants were not obtained. Further investigations suggested that there may not have been enough viable A. tumefaciens on seedling shoots for successful transformation. Survival of A. tumefaciens cells on the plant tissues was about 10^3 times less than routinely used for transformation of lupins in the established in vitro lupin transformation method.

In in planta transformation of lupin flowers, experiments were designed to deliver Agrobacterium cells to lupin ovules as target tissues. Factors reported to contribute to success in this type of transformation, such as using surfactant, infiltration period and times under vacuum and composition of infiltration medium were tested and optimised
for lupins. Thirty plants with floral inflorescences having flowers ranging from the
dome to open stages were infiltrated twice for 3 min each with MS liquid medium
containing 10 mM glucose, 0.01% Silwet L-77 and *A. tumefaciens* cells in early
exponential stage at concentration of OD 1.87. Pod set was 10.82%. No transformant
was obtained. The same infiltration media and conditions were used with *Arabidopsis
thaliana* cv Columbia as control plants and transformants obtained at 0.255%.
Histology studies of lupin flower structure by SEM and wax-embedded sectioning
revealed that there did not appear to be a physical channel for *A. tumefaciens*
cells to gain access to the ovule via the stigma or style before anthesis. Furthermore,
*Agrobacterium* cells could not gain access to the ovule through the immature carpel of
young flowers as the developing carpel closed while the ovule developed inside.

Interactions between *Agrobacterium* and lupin were studied to determine which stages
limit transfer of genes from *Agrobacterium* to lupins, and which might be modified to
achieve and/or improve transformation efficiency by *A. tumefaciens* in a genotype-
independent fashion. The stages studied were: the attachment of *Agrobacterium* to the
lupin explants, T-DNA transport across the cell wall and through the cell membrane. In
addition, the effect of an extra *virG* was examined to find out if it would increase T-
DNA transport. The interaction studies were done by comparing reactions to gene
transfer in lupin cultivars Merrit and Quillinock which have significant difference in
transformation efficiency (6.5% for cv Merrit and ~1% for cv Quillinock). The results
of the attachment of *Agrobacterium* cells experiment showed no significance
statistically in the number of bacterial cells attached to the explants (half embryonic
axes) of six cultivars of narrow-leafed lupin (cvs Merrit, Quillinock, Belara, Illyarrie,
Yorrel and Danja), indicative that the attachment stage was not the factor limiting gene
transfer. T-DNA transport through the cell wall and cell membrane was evaluated
through *gus* expression in experiment using cell suspensions (cells with cell walls) and
protoplast (cells without cell walls) with T-DNA transfer determined by the relative
intensities of the RT-PCR amplicons. The results showed, unexpectedly, that cv Merrit
had less T-DNA transferred by *A. tumefaciens* than cv Quillinock in cell suspensions
but not in protoplasts. The results were supported with MUG assays of transient
expression of the *gus* reporter gene in cell suspensions of both cultivars. This indicated
that the differences in cell wall composition between these two cultivars played an
important role in gene transfer, but the factors limiting transformation success in
Quillinock were downstream from T-DNA transfer to the cytoplasm of the host cell, possibly involving in T-DNA integration and/or expression, or selection and recovery of whole plants. The effect of an extra \textit{virG} was examined with lupin, \textit{virG}N54D increased transient expression of \textit{gus} only in cv Quillinock cells, not in cv Merrit cells. Constructs carrying \textit{virG}N54D may, therefore, be of some use as a component of a transformation protocol for cv Quillinock, and possibly other recalcitrant lupin cultivars.

This work has confirmed the relative difficulty of transforming narrow-leafed lupins, and it is concluded that despite investigating a series of alternative approaches, the method based on ‘stab inoculation’ of apical meristems, followed by selection of chimeric tissues to generate transgenic inflorescences, appears to be the most reliable approach. However, it is strongly genotype dependant, and improvements in efficiency and reduced genotype dependence are still desirable.
Table of contents

Chapter 1. Introduction and Literature Review ..........................................................15
  1.1 Lupins: usage and importance ............................................................................. 15
  1.2 Plant genetic transformation ............................................................................... 16
    1.2.1 Indirect gene transfer system ................................................................. 17
      i) Agrobacterium-mediated gene transfer system ........................................... 17
      ii) Vector systems used in Agrobacterium-mediated transformation ............. 23
          a) Cointegrative Vectors ........................................................................... 23
          b) Binary Vectors ...................................................................................... 23
      iii) Factors affecting the efficiency of Agrobacterium-mediated plant
           transformation ......................................................................................... 26
          a) Agrobacterium strains .......................................................................... 26
          b) Wounding methods ............................................................................... 28
    1.2.2 Direct gene transfer systems ....................................................................... 29
      i) Macroinjection ........................................................................................... 30
      ii) Microinjection ........................................................................................... 30
      iii) Laser microbeam ..................................................................................... 30
      iv) Electrophoresis .......................................................................................... 31
      v) Electroporation .......................................................................................... 31
      vi) Silicon carbide fiber (whisker)-mediated transformation .......................... 31
      vii) Particle bombardment transformation (Biolistics) .................................... 31
      viii) Poly (amidoamine) dendrimer for direct DNA delivery to plant cells ...... 32
  1.3 Legume transformation ......................................................................................... 32
  1.4 Current status of genetic modified crops ............................................................ 36
  1.5 Aims of the project .............................................................................................. 37
  1.6 Future of genetically modified crops .................................................................... 38
  1.7 Aims of project .................................................................................................... 42

Chapter 2: General Materials and Methods ............................................................... 43
  2.1 Plant materials ..................................................................................................... 43
    2.1.1 In vitro cultures ......................................................................................... 43
    2.1.2 Lupins in the glasshouse .............................................................................. 43
2.1.3 Surface-sterilisation of seeds......................................................... 43
2.1.4 Embryonic axes isolation.............................................................. 43
2.2 Agrobacterium tumefaciens............................................................. 44
  2.2.1 Storage ....................................................................................... 44
  2.2.2 Inoculum preparation................................................................... 44
  2.2.3 Preparation of electro-competent cells......................................... 44
  2.2.4 Electroporation of electro-competent A. tumefaciens cells .......... 45
  2.2.5 A. tumefaciens culture preparation for plant transformation......... 45
2.3 Plant expression vectors.................................................................... 46
2.4 Plant DNA extraction........................................................................ 47
  2.4.1 Miniprep method ....................................................................... 47
  2.4.2 Easy DNA High Speed Extraction method (for plants)............... 48
2.5 Analysis of transgenic plant material................................................ 48
  2.5.1 Spraying with phosphinothricin ............................................... 48
  2.5.2 Polymerase chain reaction......................................................... 49
  2.5.3 Histochemical GUS assay......................................................... 50
2.6 Histology .......................................................................................... 50
  2.6.1 Paraffin wax embedded plant tissues with microtome................. 50
  2.6.2 Dissection of plant tissues by hand.............................................. 51

Chapter 3. Lupin Transformation via Particle Bombardment............... 52
3.1 Introduction ..................................................................................... 53
  3.1.1 The significance of particle bombardment transformation........... 53
  3.1.2 Particle bombardment devices................................................... 56
    a) Helium-modified bombardment device ......................................... 56
    b) Particle accelerator or Accel particle gun .................................... 56
    c) Microtargeting device ................................................................ 56
    d) Particle inflow gun ...................................................................... 56
    e) Helios gene gun ......................................................................... 57
  3.1.3 Parameters influencing transformation success........................... 57
  3.1.4 Transgene integration................................................................. 59
3.2 Materials and Methods................................................................. 60
  3.2.1 Plant material and culture conditions......................................... 60
  3.2.2 Plasmid preparation ................................................................. 61
3.2.3 Microparticle bombardment ................................................................. 62
3.2.4 Selection procedure of putative transformants ................................. 63
3.2.5 Rooting .................................................................................................. 63
3.2.6 Data analysis ......................................................................................... 64

3.3 Results .................................................................................................... 64
3.3.1 Optimisation ........................................................................................ 64
   i) Plasmid preparation method ................................................................. 64
   ii) Helium pressure .................................................................................. 64
   iii) Effect of helium pressure and target Distance .................................. 68
   iv) Mannitol concentration .................................................................... 69
   v) Shoot regeneration versus bombardment pressure ............................ 70
   vi) Concentration of plasmid DNA ......................................................... 70
   vii) Particle load ...................................................................................... 71
   viii) Combining parameters to effect stable transformation .................. 72
   ix) Pre- and post-treatment of explants .................................................. 73
   x) Number of bombardments ................................................................. 75
   xi) DNA fragment length ...................................................................... 76
3.3.2 Using optimised protocol to generate transformants ....................... 77

3.4 Discussion ............................................................................................... 79
3.4.1 Optimisation ........................................................................................ 79
   a) Plasmid preparation method ................................................................. 80
   b) Helium pressure and target distance .................................................. 80
   c) Concentration of mannitol in osmoticum medium ............................ 80
   d) Concentration of DNA ........................................................................ 81
   e) Particle volume loaded ........................................................................ 81
   f) Combining optimised parameters ....................................................... 81
   g) Pre- and post-treatment of explants .................................................... 82
   h) The number of bombardments ............................................................ 83
   i) DNA fragment size ............................................................................. 83
3.4.2 Developing and optimised protocol .................................................... 84

3.5 Conclusion ............................................................................................... 86

Chapter 4 In planta Transformation of Narrow-leafed Lupin .................... 87
4.1 Introduction ........................................................................................................................................ 87

4.1.1 Types of \textit{in planta} transformation .................................................................................. 87
  i) Pollen transformation .................................................................................................................. 87
  ii) “Clip’n Squirt” .......................................................................................................................... 88
  iii) Seed transformation .................................................................................................................. 88
  iv) Vacuum infiltration .................................................................................................................... 88
  v) Floral dip or spray ...................................................................................................................... 88

4.1.2 Mechanism of \textit{in planta} transformation ............................................................................. 89

4.1.3 Factors influencing successful \textit{in planta} transformation .................................................. 89
  i) Explants: stages and genotypes .................................................................................................. 89
  ii) Components of infiltration and inoculation media ................................................................. 90
  iii) Conditions of treatment ......................................................................................................... 90

4.2 Materials and Methods ............................................................................................................. 91

4.2.1 Plant materials ....................................................................................................................... 91
  a) Narrow-leafed lupin .................................................................................................................. 91
  b) \textit{A. thaliana} .......................................................................................................................... 91

4.2.2 Transformation of lupins using vacuum infiltration ............................................................ 91

4.2.3 Wounding explants with sonication .................................................................................... 92

4.2.4 Isolation of \textit{A. tumefaciens} cells from infiltrated lupin seedlings and 3-ketolactose test for \textit{Agrobacterium} cells ........................................................................................................ 92

4.2.5 Sample preparation for scanning electron microscopy (SEM) ............................................ 93

4.2.6 \textit{In planta} transformation of \textit{A. thaliana} ................................................................................. 94

4.3 Results ........................................................................................................................................... 94

4.3.1 Vacuum infiltration transformation of lupin seedlings ....................................................... 94
  i) Infiltration time and sonication time ....................................................................................... 94
  ii) \textit{A. tumefaciens} growth phase ............................................................................................. 98
  iii) Composition of infiltrated medium and \textit{gus}-intron transient expression ......................... 99
  iv) The transformation of lupin seedlings by sonication and infiltration .................................... 101
  v) Viability of \textit{A. tumefaciens} after infiltration versus gene transfer rate (\textit{in vitro}) .............. 102

4.3.2 Vacuum infiltration transformation of \textit{L. angustifolius} flowers .......................................... 104
  i) Effect of wetting agent and BAP in infiltration medium ......................................................... 104
  ii) Effect of infiltration time ........................................................................................................ 106
  iii) Flower infiltration with optimum media and conditions .................................................... 107
iv) Mechanism of *A. tumefaciens* delivery to flowers................................. 112
4.3.3 *A. thaliana* in planta transformation.............................................. 116
4.4 Discussion .......................................................................................... 117
4.4.1 Vacuum infiltration of lupin seedlings .............................................. 117
4.4.2 Vacuum infiltration of lupin flowers ............................................... 123
4.4.3 *A. thaliana* in planta transformation.............................................. 125
4.5 Conclusion......................................................................................... 126

Chapter 5: Studies on *Agrobacterium* and Lupin Interactions .................. 127
5.1 Introduction ........................................................................................ 127
5.1.1 Plant and *Agrobacterium* interactions.......................................... 127
5.1.2 Lupin genotype-dependent response to *Agrobacterium tumefaciens* ...... 129
5.2 Materials and methods .................................................................... 129
5.2.1 Plant materials, *Agrobacterium* and plant expression vector.............. 129
5.2.2 Determination of the number of *Agrobacterium* cells attached to lupin explants ................................................................. 130
5.2.3 Lupin protoplast culture .................................................................. 130
5.2.4 Cell suspension preparation of narrow-leafed lupin ......................... 132
5.2.5 RNA extraction for plant tissue ..................................................... 132
5.2.6 Analysis of GUS expression by RT-PCR ......................................... 132
5.2.7 Fluorimetric assay for β-glucuronidase (MUG assay)....................... 133
5.3 Results ............................................................................................... 134
5.3.1 Determination of the number of *Agrobacterium* attached to lupin explants ................................................................. 134
5.3.2 Determination of T-DNA transfer efficiency to cell suspensions and protoplasts by RT-PCR ......................................................... 134
5.3.3 Determination of T-DNA transfer to lupin cell suspensions by a fluorimetric assay for β-glucuronidase (MUG assay)................................. 137
5.4 Discussion ........................................................................................ 140
5.4.1 Binding of *A. tumefaciens* to plant cell walls .................................. 140
5.4.2 Transient gene expression ............................................................. 142
5.5 Conclusion......................................................................................... 143

Chapter 6 General Discussion ................................................................. 144
6.1 Investigations using particle bombardment........................................ 144
6.2 Investigations using *in planta* transformation ................................. 146
6.3 Studies on *Agrobacterium* and lupin interactions .......................... 150
6.4 Conclusion.......................................................................................... 151

References............................................................................................... 152

List of Abbreviations............................................................................... 183

Publications and Presentations.............................................................. 186
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Dedication

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