Vacuum infiltration of lupin seed for genetic engineering lupin in resistance to anthracnose

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1. Executive summary

The aim of this project was the transformation of *Lupinus albus* cv. Esta with a pgip construct, via *Agrobacterium tumefaciens*, to produce transgenic lupin plants resistant to anthracnose. Great success had been achieved with the vacuum transformation procedure currently used to produce transgenic soybean (patent PCT/IB99/01676). Thus it was applied on lupin with the necessary changes as it may be possible that the same procedure can be effective in lupin as both are legumes. Much developmental work were however needed to gain a reproducible transformation system for lupin, as transformation of lupin is not yet a highly successful and routine based procedure.

The transformation technique was optimized to provide the best transformation efficiency. Various criteria that could influence the transformation efficiency were looked at eg: 1) final concentration of culture, 2) media for resuspension of *Agrobacterium* cultures, 3) length of seed radicle, 4) length of vacuum applied 5) time of planting of seeds 6) type of *Agrobacterium* strain 7) inclusion or exclusion of *Vir G* gene in pCambia plasmid. The best procedure proved to be: Surface sterilization of Lupin seed, cultivar Esta, and germination for 2 days on 1% agar at 29°C. *Agrobacterium tumefaciens* containing a gene construct of interest, e.g. pCambia 3301, were inoculated into LB containing specific antibiotic. This culture was allowed to grow for 2 days at 27°C while shaking, after which the cultures was concentrated by centrifugation at 10 000 rpm at 4°C for 10 minutes. The concentrated culture was resuspended in 50% 0.2M phosphate buffer containing 0.01% Breakthru®. The degree of germination of the seeds played no role as long as the radicle was visible. After resuspension the germinated seeds were subjected to vacuum pressures of up to –88kPa in the grown culture. Seeds were not left in media overnight, as the seed survival decreases dramatically with this step included in the procedure. After vacuum infiltration the seeds were well washed, ultimately rinsed in 1% Benlate solution, before planted out in the greenhouse. Two different races were used, AGL 1 en LBA 4404. The two races were compared regarding transformation efficiency. The highest transformation efficiency was found with the *Agrobacterium* race AGL1.

The construct pCambia 3301 plasmid that was used for optimizing the procedure, contained a *gus* and *bar* gene to use as selector markers. The first experiments indicated that GUS expression was found in T0 generation (leaves) and GUS colouring of the seed showed that the *gus* gene was transferred to the offspring. GUS positive plants were tested for herbicide resistance by spraying the plants with Ignite. The putative transgenics showed considerably less damage than the control plants. Root growth on various concentrations of Ignite was tested. It was found that based on the amount of plant leaves that colour blue after a XGLUC colouring, a transformation efficiency of 11% was reached with the vacuum infiltration transformation method.

As preliminary results indicated that the technique has merits for *Lupinus albus* transformation and it was decided to transfer a construct containing the *Pgip* gene, pCambia 0390, to lupin in order to combat anthracnose. A co-transformation of pCambia 3301 and pCambia 039 were performed with the rational that the marker genes (*gus* and *bar* in pCambia 3301) will be out cross later. After a series of transformation experiments, 200 putative transformant seed were transferred to the greenhouse to produce seed. The seed (5000) were germinated via a tissue culture step, in order to pre-select the plants tolerant to the herbicide. A very low survival rate was experienced on tissue culture as a result of low germination rate due to damage obtained by the transformation process and fungal infection. An additional washing step with Benlate in subsequent experiments solved this problem. The 1200 plantlets that survived the tissue culture screening were transplanted to the greenhouse. Some seedlings did not survive the transplanting into the greenhouse due to various reasons: i.e. some of the seedlings' roots were damaged by the herbicide in the tissue culture medium, some of the seedlings had to be sterilized during the tissue culture growth period with sodium hypo-chloride due to fungal infiltration and the roots were damaged. The 600 plants that survived all the pre selection were sprayed with 0.1% ignite to screen for *bar* expression, where after it were test for the presence of the bar and *pgip* gene with PCR. At first 125 plants looked resistant, but in the end all the plants but 4 died. All 125 plants were however evaluated for the presence of the *bar* and *pgip* gene with PCR, but no gene was amplified. This negative result could have been due to a number of reasons:

- the co-transformation – percentage transfer of combined genes are smaller than for a single construct
- the gene was not transferred from T0 generation to T1
- as the selection was for the bar gene, pgip transgenics could have been lost
- the transformation process do not work

Subsequently transformation experiments were performed with only the pCambia 0390 construct to investigate the possibility of the "lost" of transgenics via bar selection. Forty plants survived the vacuum procedure and were transplanted into the greenhouse. DNA was extracted from these plants (T0) to perform PCR analysis to determine the existence of pgip gene. Amplification of the Pgip gene was observed in at least 4 DNA samples. *Agrobacterium* primers confirmed that the gene is inserted into the genome and the *Pgip* amplification was not due to *agrobacterium* still present in the plant. It however doesn't confirm that the gene is expressed or that it will be transferred to the off spring.

Another series of experiments were performed to test different co-transformation ratios of Esta with pCambia 0390 and pCambia 3301. Various ratios of the gene constructs were used in the co-transformation process i.e. pCambia 0390:pCambia 3301; 90:10; 10:90; 85:15 and 15:85. A hundred seeds were used in each ratio for transformation. All the seeds that survived the transformation process were grown in the glasshouse. XGLUC colouring indicated that the 10:90 ratio was the best, with 8 plants that coloured blue. These plants were subjected to a PCR analysis of *gus* and *pgip*. The S35 –gus primer proved that the *gus* gene was inserted into the T0 plants genome and the pgip primer proved that the pgip gene was inserted into the T0 plants genome.

The T1 seed of the 4 pCambia 0390 plants as well as the 8 pCambia 0390 : pCambia 3301 10:90 plants that proved to contain the *gus* and/or *pgip* genes were planted the following season in pots in the greenhouse for testing. DNA extracts of the plant leaf material were performed and tested for the presence of the gus and *pgip* gene by means of PCR. Unfortunately, none of the seeds (T1) tested positive by PCR analysis for the presence of *pgip*. This indicated that the gene was not transferred to the next generation.

It might be that the gene was incorporated into the T0 plants genome, but only temporary, as it doesn't transfer towards the next generation. It can thus be concluded that the transformation technique is not stable yet, as it efficiency is fickle and the gene tend to been lost towards the next generation.

Since successful vacuum transformation has been achieved with soybean it was with great excitement that transformation of lupin was launched, but the lupin seed was not so receptive to foreign DNA as in the case of soybean and the transformation procedure couldn't be adopted with constant success.