cooling (or winter cooling) in the northern Arabian Sea is expected to be the regions of convective formation of Arabian Sea High Salinity water mass[3,15]. The formation of water mass must involve downward transport of heat and salt from the high salinity surface layer leading to the formation of salt fingers. A recent study by Prasanna Kumar and Prasad[15] elucidated the physical forcing that led to the formation of Arabian Sea High Salinity Water mass and Madhupradap *et al.*[16] have shown the mechanism of the biological response to winter cooling in the northern Arabian Sea.

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ACKNOWLEDGEMENTS. I thank the India Meteorological Department for providing the INSAT precipitation data. I acknowledge financial support from DST, New Delhi, as part of TOGA-I program. I am also thankful to the anonymous referee for valuable suggestions.

Received 18 February 1997; revised accepted 12 September 1997.

Particle bombardment: A simple and efficient method of alfalfa (*Medicago sativa* L.) pollen transformation

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Direct delivery of DNA into pollen was used to obtain transgenic alfalfa (*Medicago sativa* L.). Plasmid pBI121 bearing the β-glucuronidase (GUS) reporter gene was introduced into the pollen by microprojectile bombardment. The bombarded pollen expressed GUS activity. Pollinating flowers of male-sterile plants with the bombarded pollen produced fertile seeds. Thirty per cent of plants derived from the fertile seeds showed integration of GUS plasmid, which was confirmed by PCR and Southern analysis. However, after ten vegetative generations, some transgenic plants (T_0) apparently lost the integrated GUS plasmid, while in few others, GUS gene copy number decreased. Presently, we are not able to establish the possible causes for the loss of integrated DNA after several vegetative generations. However, it would be a worthwhile avenue for future work to establish the reasons for the loss of integrated DNA since this method of transformation employs simple techniques to transform pollen and produce transgenic plants through the natural plant reproductive process.

ALFALFA (*Medicago sativa* L.) is a highly valued forage legume crop cultivated worldwide on 32 million hectares in warm and cool subtropical regions[1,2]. Improvements of alfalfa, earlier was restricted largely to conventional breeding methods. Recently, attempts to genetically engineer alfalfa by plant transformation have relied on gene introduction techniques demanding tissue culture methods[3–6]. Plant transformation methods involving tissue culture are time-consuming, require special techniques, and the efficiency of obtaining stable

regenerants is low[7]. Particle bombardment might be an alternative approach to negate some problems of tissue culture techniques depending on the target tissue used for transformation. Transforming pollen via particle gun would be advantageous since pollen is easily amenable as a free cell in large numbers, and naturally produce fertile plants through normal plant reproductive process.

In this paper we describe a pollen transformation protocol via particle bombardment, which precludes the necessity of tissue culture techniques using β-glucuronidase (GUS) reporter plasmid pBI121. Pollinating *in situ* with the transformed pollen generated fertile seeds. Molecular analysis done on plants derived from the fertile seeds confirmed the integration of the reporter GUS gene in transgenic F_1 (T_0) plants, until ten vegetative generations.

Materials and methods

Vector

Plasmid pBI121 (Figure 1) (Clonetech Laboratories, USA) containing the (GUS)[8] reporter gene, was used for pollen transformation. The GUS gene has only one restriction site for *Sna*BI in the coding region. This gene is driven by a 5′ up-stream cauliflower mosaic virus (CaMV) 35S promoter and terminated by nopaline synthase (NOS).

Pollen collection and preparation for microprojectile bombardment

Mature alfalfa flowers were collected randomly from greenhouse-grown plants. Flowers in each raceme were tripped open by gently rubbing the sepals between fingers to expose anthers enclosed in the fused keel petals[9]. Pollen grains were suctioned from the anthers into a narrow glass tube attached to a cyclone spore collector[10], then were transferred into a sterile microfuge tube and stored dry on ice until future use.

Before microprojectile bombardment, pollen grains were washed in sterilized 13% sucrose solution by vortexing at setting 4 on an orbital shaker (Bell Biotechnology, USA) for 10 min, and sedimented in a bench-top microfuge with 15-second short pulses of centrifugation. The sedimented pollen was resuspended in 1.5 ml fresh 13% sterilized sucrose solution and vortexed briefly to disperse the pollen uniformly in the liquid medium. Approximately 100 µl of the pollen liquid suspension was pipetted onto sterile nylon membrane (Boehringer Mannheim, Germany), which was anchored in a 9 cm petri plate containing 1.5% bacto agar (Difco Laboratories, USA) overlaid with two layers of sterilized Whatman No. 1 filter paper. The pollen suspension on the nylon membrane was spread evenly over a 2 cm^2 area with the conical end of 1.5 ml sterile eppendorf tube before microprojectile bombardment. Pipetting operations involving pollen liquid suspensions were always carried out with wide bore pipet tips.

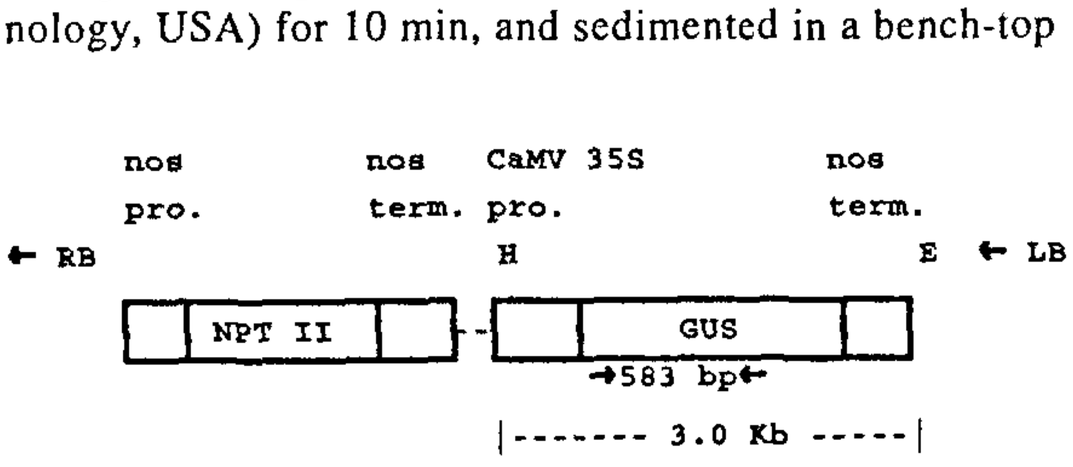

Figure 1. The diagram of plasmid pBI121: pro, promoter; term, terminator; RB, right border; LB, left border; Primers,→ ←; GUS gene, |-----|; H, *Hin*dIII; E; *Eco*RI.

Preparation of plasmid DNA and adsorption to microprojectiles for particle bombardment

Plasmid DNA was isolated by alkaline lysis and further purified by equilibrium centrifugation in a $CsCl_2$-ethidium bromide gradient[11].

Approximately 60 mg of M17 or M25 tungsten microparticles (Bio-Rad Laboratories, USA) for every ml of 50% glycerol was prepared for adsorption following the procedure described by Sanford *et al.*[12]. For adsorption of plasmid onto tungsten particles, the broad steps outlined by Sanford *et al.*[12] were followed with certain modifications. Nonlinearized $CsCl_2$-plasmid DNA (21 µg) was ethanol precipitated, and the air-dried pellet was resuspended in 10 µl of sterile water. Tungsten particles (50 µl) suspended in 50% glycerol were vortexed for 30 min on a bench top vortexer at a low speed (setting 4), and then added to the plasmid DNA suspended in 10 µl of sterile water. To this suspension containing tungsten particles and plasmid DNA, 75 µl of coating adjuvants, $CaCl_2$ (2.5 M) (ref. 13) or PEG (8 %) (ref. 14) was added and vortexed briefly, after which, 25 µl of 0.1 M spermidine (free base) was added. The final suspension mixture was vortexed vigorously for a total of 15 min, incubating on ice for 3 min after every 5 min intervals of vortexing.

The vortexed suspension was incubated on ice for 30 min and then centrifuged for 30 s in a bench top centrifuge. The resultant supernatant was discarded and the DNA-coated tungsten pellet was gently washed, first in 300 µl of cold 70% ethanol, and then with 300 µl of absolute ethanol. The DNA-coated tungsten particles were resuspended in 200 µl cold ethanol and stored on ice until applied onto the surface of macrocarriers.

The final DNA-tungsten suspension contained 3 mg of tungsten and 21 µg of plasmid DNA, assuming that there was no loss of either tungsten particles or plasmid DNA. The DNA-coated particles were briefly vortexed and then 20 µl (2.1 µg of plasmid DNA and 300 µg tungsten

particles) was applied onto macrocarriers. The macrocarriers anchoring the DNA–tungsten particles were air dried inside a laminar flow hood. Several preliminary experiments detailed in the result section were performed to optimize the particle gun parameters for transforming pollen, which eventually could germinate following bombardment. Pollen contained in petri plates were bombarded only once.

In situ pollination of female parent to derive F_1 plants

Cytoplasmic male-sterile alfalfa (*Medicago sativa* L.) was used as a female parent. Plants were maintained in greenhouse conditions at 24°C supplemented to a photoperiod of 14 h light to achieve a high degree of flowering[15]. Racemes having many unopened matured flowers were chosen for artificial pollination. In the tripped raceme, immature flower buds were pinched off by tweezers and mature flowers were tripped open with a toothpick to expose the pollen-recipient stigma.

For collecting pollen from the top of nylon membranes, microprojectile-bombarded petri plates incubated in a refrigerator (4°C) were retrieved and placed in a laminar flow hood. The top lid of the petri plate was removed and placed face down (inverted). The nylon membrane anchoring microprojectile-bombarded pollen was placed inside the inverted top lid of the petri plate. Several aliquots of sterile, 10% sucrose was added on the nylon membrane and the bombarded pollen slurry was gently brushed to the middle using a dry camel's hair paintbrush. The bombarded pollen was absorbed onto the paintbrush and resuspended into 1.5 ml sterile eppendorf tubes. The final volume of the pollen slurry collected from each petri plate was approximately 200 µl. Tungsten particles were sedimented from the pollen slurry by spinning for 20 to 30 seconds in a bench top centrifuge. The supernatant was pipetted into another sterile eppendorf tube and stored on ice until pollinating the female recipient flowers, which usually took less than 30 min.

Wide bore pipet tips were used to collect 200 µl aliquots of the bombarded pollen suspension stored on ice. Gently, 10 to 20 µl of the pollen suspension were pipetted onto the initially tagged and tripped female recipient flowers, such that the whole hollow groove formed by the petals was filled with the microprojectile-bombarded liquid-pollen-suspension.

The pollinated flowers completed seed set in about a month. The seeds derived from the harvested pods were mechanically scarified by viewing underneath a dissecting microscope and making a small incision with a sterile razor blade. The scarified seeds were imbibed in sterile water to ensure the seeds were scarified and then germinated in sterile sand. After a month in growth chambers (24°C), the seedlings were transplanted to pots and maintained in greenhouse conditions.

GUS assay

Histochemical GUS assay of the microprojectile-bombarded pollen was carried out according to the method of Jefferson[16]. To the microprojectile-bombarded pollen 500 µl of GUS histochemical buffer (3 mM, 5-bromo-4-chloro-3-indolyl-β-glucuronide in 0.1 M sodium phosphate buffer, pH 7.0) was added and the petri plates were incubated in dark for 4 h at 37°C. GUS expression was scored based upon the number of pollen grains showing blue spots by randomly selecting with the ocular micrometer 0.01 mm^2 focal areas at 40X magnification.

PCR analysis

Putative transgenic plants derived from pollinating male sterile plant flowers were screened by PCR analysis. Plant DNA was extracted by maize miniprep method[17]. The GUS primers (Genosys Biotechnologies, TX, USA) were deprotected, desalted and dried. A typical 50 µl PCR contained 100 ng genomic DNA; 200 µM dNTPs; 15 pmols of each primer; 1.5 mM $MgCl_2$; 0.5 U Tfl thermostable DNA polymerase (Epicenter Technologies, USA); and the manufacture supplied 20X reaction buffer. Initially, the PCR mixture without the reverse GUS primer and Tfl thermostable polymerase was heated for 7 min at 95°C on the thermocycler, and then a mixture of the reverse GUS primer and the polymerase enzyme was added to each well. The PCR amplification reaction was performed for 42 cycles of: 15 s at 94°C, 1 min at 63°C, and 2 min at 72°C. PCR products were separated by 0.8% agarose gel electrophoresis and visualized with ethidium bromide. Primers were designed for GUS coding regions at positions 535 (Forw) and 1118 (Rev); the forward sequence is 5'GGG CAG GCC AGC GTA TCG TGC TGC3' and the reverse sequence is 5'GAC CGG ATG CCG ACG CGA AGC GGG3'. For restriction digestion analysis of PCR product, amplified fragment was extracted from the gel and purified in a Gene Capsule Kit (Geno Technology, USA).

Southern blot analysis

Approximately 15 µg of genomic DNA was digested with *Eco*RI and *Hin*dIII and separated electrophoretically in 0.8% agarose (FMC Bioproducts, USA) gel. The DNA fragments were depurinated and transferred overnight onto positively-charged nylon membranes (Boehringer Mannheim, Germany) under alkaline con-

ditions[11]. The membranes were prewashed in 2X SSPE (pH 7.5) for 1 h and fixed by exposing to UV light for 45 s. The hybridization probe was prepared by random primer labelling (Stratagene, USA) according to manufacture instruction using [α-^{32}P] dCTP (Dupont, USA) and purified on a sephadex G-50 column. Blots were prehybridized for 3 h at 42°C in hybridization buffer (50% formamide, 6X SSPE (pH 7.5), 0.5% SDS, 0.5% nonfat milk, 5X Denhardt's solution). After prehybridization, the membranes were washed twice at 42°C for 5 min in fresh prehybridization buffer. The purified radiolabelled probe was added to 20 ml prehybridization buffer, and the membranes were hybridized overnight. After hybridization the membranes were washed twice with 0.5X SSPE, 0.1% SDS for 15 min at room temperature, followed by two 15 min washes with 0.1X SSPE, 0.1% SDS at 65°C. Finally the probe-target hybrid was detected by exposing to Kodak X-omat LS X-ray film (Eastman Kodak Company, Rochester, NY, USA) at –70°C for 12 to 24 h. A similar procedure was used for Southern analysis of PCR-amplified product.

Results and discussion

It is thought that the supercoiled plasmid DNA and the target genome are nicked during particle gun bombardment. These nicks increase the chance of integration of the plasmid into the target tissue genome[18,19]. However, in particle gun bombarded pollen, stable integration and expression of the reporter gene, including the ability to produce transgenic seeds, depends largely on the severity of harsh conditions imposed on pollen, during and after bombardment[20]. The microprojectile-bombarded pollen has more likelihood of losing its ability to germinate because of mechanical aberrations endured on the membranes and other cell organelles during high velocity penetration of tungsten particles[7]. Thus a study was conducted to find an optimum combination of particle gun parameters such that the tungsten particles penetrate deep into the pollen and yet maintain the pollen viability.

The particle gun chamber vacuum pressure and velocity of the microprojectiles are inversely related[13]. Higher chamber vacuum presents less air resistance to the microprojectiles and causes deeper penetration into the cell and their organelles. Using maximum chamber vacuum (28 inch of Hg), tobacco (*Nicotiana tabacum*) pollen was transformed with GUS encoding plasmid pBI121 by Twell *et al.*[21] via particle gun. The transformed tobacco pollen expressed GUS activity efficiently, and were capable of germinating. Thus, we attempted to introduce the same reporter plasmid into alfalfa pollen at the highest chamber vacuum pressure (24 inch of Hg) that could be achieved in our particle gun.

Table 1. Effects of tungsten particle size, helium pressure, and target distance on pollen germination and transformation efficiency

Tungsten particles	Helium pressure[a]	Target distance[b]	GUS score[c] Expt 1	2	Pollen germination (%) Expt 1	2
M17 (1.1 µM)	1100	6	++++	++++	63	72
		9	++	+++	70	65
		12	++	++	76	84
	900					
		6	++	++	70	64
		9	++	++	74	78
		12	+	+	86	90
M25 (1.7 µM)	1100	6	+++	+++	34	47
		9	++	++	40	48
		12	+	+	42	47
	900	6	+++	+++	48	45
		9	++	++	51	54
		12	+	+	55	56

[a]Burst pressure of rupture disk in psi.
[b]Approximate distance (cm) from the stopping screen.
[c]The score is an arbitrary estimate of the number of pollen expressing GUS activity at 100X magnification: very low (+), low (++), intermediate (+++), and high (++++).
Tungsten particles were adsorbed with GUS plasmid pBI121 using $CaCl_2$ (2.5 M) as the coating adjuvant. The stopping screen was in the middle level (8 mm) and the chamber vacuum was 24 inch of Hg in all of the experiments. The bombarded petri plates were incubated for 30 min at room temperature (24°C) before assaying for GUS or pollen germination. From each bombarded petri plate, one half the pollen anchored on the nylon membrane was used for GUS histochemical assay, and the other half for conducting pollen germination tests. Each experiment consisted of three bombarded petri plates.

Tungsten particles of sizes 1.1 and 1.7 μM coated with pBI121 plasmid were accelerated at 900 and 1100 psi helium burst pressures (Table 1). The pollen grains were bombarded at flight distances of 6 cm (level 2), 9 cm (level 3), and 12 cm (level 4). Bombarded pollen grains viability was tested by observing pollen tube growth in 10% sterile sucrose solution with 40X magnification. Pollen transformation efficiency was confirmed by GUS histochemical assay.

Irrespective of tungsten particle size used for particle bombardment, increasing the target distance or decreasing the acceleration of tungsten particles (helium burst pressure), generally enhanced germination of bombarded pollen, but at the expense of lower amounts of pollen being transformed. Most pollen bombarded with small size (1.1 μM) tungsten particles at a target distance of 6 cm expressed high GUS activities that could germinate (63 to 72%). Bombarding with larger size tungsten particles (1.7 μM) decreased not only the number of pollen transformed but also their ability to germinate, without regard to the burst pressure of tungsten particles or the target distance used.

To generate the maximum number of transformed pollen that could germinate, in all future pollen bombardment experiments we followed the optimum gun conditions defined in Table 2, determined based on the preliminary experiments conducted earlier (Table 1).

Several critical factors were identified that influenced the ability of flowers receiving microprojectile-bombarded pollen to set transgenic seeds: 1) tungsten particles should efficiently adsorb and adhere DNA strongly on their surface until delivered into the pollen; 2) the microprojectile-bombarded pollen must be arrested from premature germination and extensive pollen tube growth until pollinating male sterile flowers; 3) the bombarded pollen should be rejuvenated to germinate faster once applied to the stigma of the male sterile flowers to evade nuclease activity. To reduce all the above-mentioned deleterious effects on bombarded pollen, several experiments were conducted.

The adsorption procedure is important since DNA that is bound strongly to tungsten particle ensures greater amounts of delivery into pollen[13], and would be less susceptible to pollen nuclease attack after entering the pollen, because fewer amounts of free DNA become available as a substrate. Tungsten particle coating adjuvants, (2.5 M) $CaCl_2$ (ref. 13) or (8%) PEG (ref. 14) was added during adsorption process for greater adherence of pBI121 to the surface of tungsten particles.

Only 1 to 5% of bombarded pollen indicated GUS activity when both PEG and $CaCl_2$ were eliminated during the adsorption process (data not shown). Addition of PEG yielded 30% increase in the number of pollen expressing GUS. However, the highest amount (90%) of GUS positive pollen was observed with tungsten particles adsorbed in the presence of $CaCl_2$. Since $CaCl_2$ treatment was significantly effective, we did not attempt to combine $CaCl_2$ and PEG during the adsorption process. A photograph of pollen expressing GUS activity after bombarding with (2.5 M) $CaCl_2$ adsorbed tungsten particles is shown in Figure 2.

Table 2. The optimum conditions of particle gun used for pollen transformation

Parameter	Conditions
Microcarrier	M17 Tungsten (1.1 μM)
Macrocarrier travel distance	8 mm
Chamber vacuum	24 in Hg
Helium pressure	1100 psi
Flight distance	6 cm (level 2)

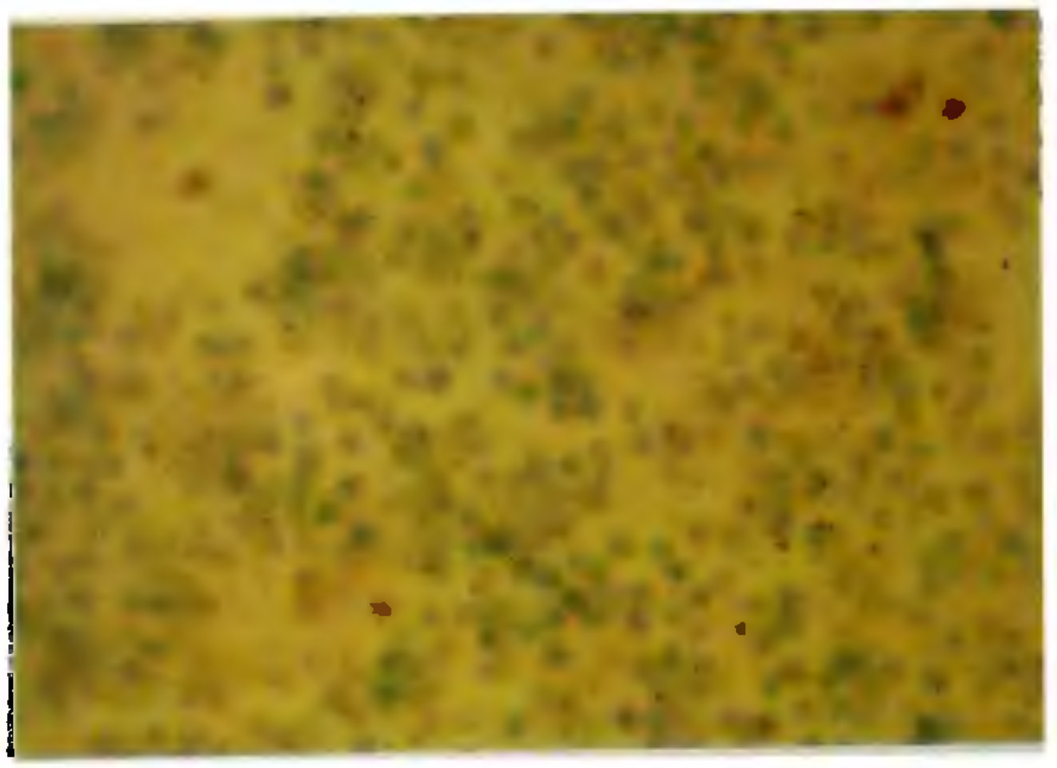

Figure 2. Histochemical GUS assay of pollen bombarded with pBI121 adsorbed tungsten particle (M17, 1.1 μM) using $CaCl_2$ (2.5 M) coating adjuvant.

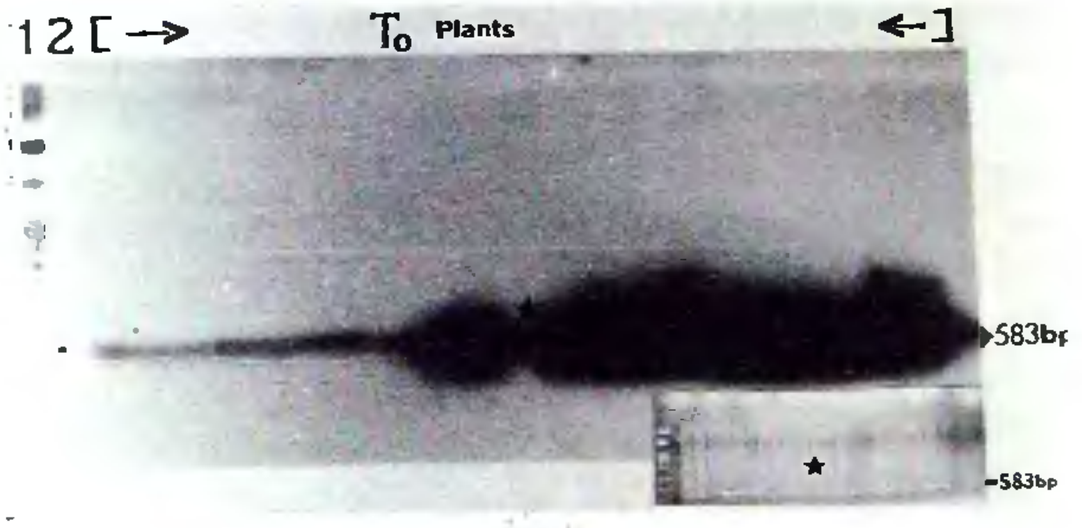

Figure 3. Southern analysis of PCR product using GUS primers and F_1-derived plant DNAs. Figure in the inset depicts the ethidium stained gel of PCR amplified product. Lane 1, *Hin*dIII digested λ. Lane 2, PCR product without template DNA. Lane marked with a star designates PCR product of male sterile plant DNA. All other lanes designated T_0 are PCR products of transgenic F_1 plants.

The next task was to decrease the number of transformed pollen from germinating prematurely before pollinating male-sterile flowers since extensively germinated alfalfa pollen are often less effective in fertilization[22]. Li *et al.*[20] have shown that the number of GUS positive pollen increased significantly by incubating microprojectile-bombarded white spruce (*Picea glauca (Moench.) voss*) pollen at 25°C for 18 h in dark before assaying for GUS. In contrast to Li *et al.*[20] procedure,

Table 3. Percentage of pollen showing transient GUS expression

Duration of cold incubation after microprojectile bombardment in hours →.								
	0	1	2	3	4	6	12	24
Pollen grains/0.01 mm^2	60	65	70	59	66	51	54	61
Pollen showing GUS[a] activity	52	47	60	38	30	26	18	15
Percent	87	85	86	64	45	51	33	25
S.E ±	3	5	4	8	6	7	9	5

[a]Histochemical GUS assay was conducted after thawing the cold incubated petri plates for 15 min at room temperature (24°C).

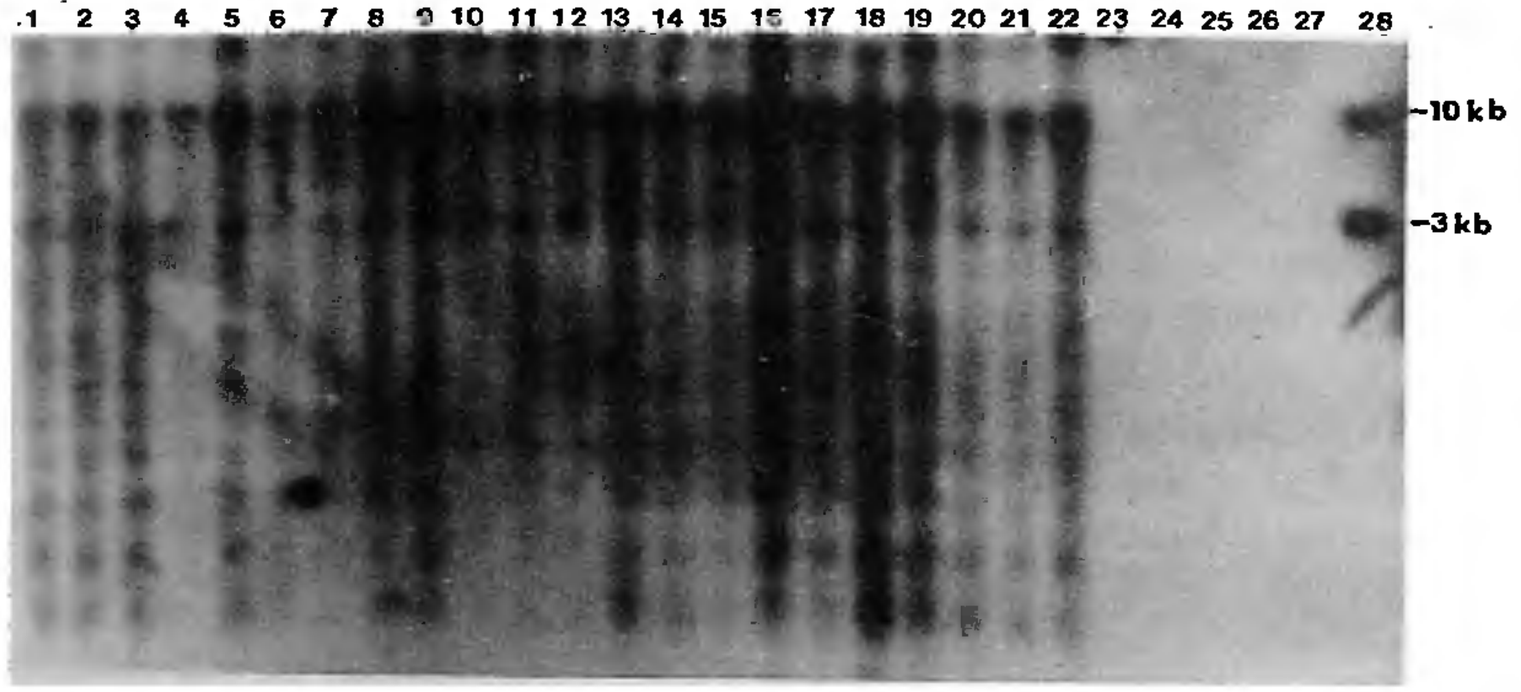

Figure 4. Southern analysis of *Hin*dIII and *Eco*RI digested genomic DNA of T_0 plants probed with *Eco*RI digested pBI121 plasmid. Lanes 1–22, DNA of transgenic plants; Lanes 23 and 24, DNA of pollen recipient male sterile plant; Lanes 26 and 27, DNA of nontransformed plant; Lane 28, plasmid pBI121 that has released the 3 kb GUS insert after restriction digestion with *Hin*dIII and *Eco*RI.

instead of incubating the bombarded pollen at 25°C, we incubated the bombarded pollen at 4°C with the presumption that pollen germination would be delayed at lower temperatures[22] and the nicks in the pollen DNA caused by microprojectile bombardment may slowly trigger a repair mechanism to religate, during which process the plasmid DNA gets integrated into the pollen genome.

Cold incubated microprojectile-bombarded petri plates were retrieved from the refrigerator at one-hour intervals and checked for GUS activity in the bombarded pollen to determine how long the GUS gene existed in the pollen without being degraded (Table 3). The mean number of pollen grains showing GUS activity was 86% until 2 h of cold incubation, and then gradually declined, with severe loss occurring after 24 h. This suggests that the nuclease activity in the pollen grains was slowed down at lower temperatures and therefore, may have taken a longer time to degrade plasmid DNA or perhaps, the DNA was physically lost by slow diffusion through the micropores created in the bombarded pollen cell wall due to microprojectile entry.

The pollen germination process in alfalfa begins within a few minutes after suspending in any liquid suspension. However, incipient germinated pollen can be maintained in liquid suspension containing sucrose without loss of ability to fertilize, until 2 h (ref. 22). It has been reported that alfalfa pollen producing long pollen-tubes promote growth of neighbouring pollen by an unknown mechanism, sustains longer periods of growth and fertilizes more ovules[9]. Further, pollen suspended in 10% sucrose always produce long pollen tubes[23]. Thus to increase the chances of seed-set, we resuspended the bombarded pollen after 2 h cold incubations in 10% sucrose before pollinating. The stable integration of the GUS gene in F_1 plants was first confirmed by PCR after establishing the seedlings for six weeks in the greenhouse, later by Southern analysis following two vegetative generations (cuttings).

A 583 bp fragment of the GUS coding region was PCR amplified. The amplified GUS fragment contains a SnabI restriction site located 150 bp beyond the GUS 535 forward primer. PCR analysis indicated a clear band of the predicted size (583) in twenty of the seventy-five F_1 plants screened, suggesting a transformation efficiency of 26% (inset of Figure 3). The identity of the amplified fragment was confirmed by Southern analysis of PCR products, probing with the 3 kb GUS insert in plasmid pBI121 (Figure 3).

The probe hybridized to all the lanes that contained the PCR amplified product confirming the amplified region was related to GUS. The possibility of plasmid contamination in our PCR mixture was ruled out, since there was no amplified product detected either in the agarose gel or in Southern analysis, when PCR reaction was conducted without genomic DNA (template) (lane 2 of Figure 3, and lane 2 in inset of Figure 3).

A Southern analysis was conducted using DNA of PCR-screened GUS positive T_0 plants after two vegetative generations (cuttings). Probing *Hin*dIII and *Eco*RI digested genomic DNAs of T_0 plants with *Eco*RI digested plasmid pBI121 showed several hybridization bands (Figure 4). There were two additional T_0 plants showing the presence of GUS that were not included in the PCR analysis because of failure to obtain healthy leaves (Figure 4, lanes 21 and 22). Hybridization with the probe was not detected in the DNA of pollen recipient male sterile plant (Figure 4, lanes 23 to 26). All T_0 plants showed a prominent hybridization band at 10 kb plasmid unit length and at 3 kb GUS insert unit length (Figure 4, lanes 1–22). The hybridization patterns of all T_0 plants were similar. The intensities, though, especially that of 3 kb GUS unit length differed in a few (lanes 4, 6, 7, 20, and 21). Though the exact copy number could not be authenticated from our studies, we presume the differing 3 kb hybridization banding intensities are related to the number of copies of the GUS gene in T_0 plants. There was one hybridization band, larger than 10 kb, invariably showing up in all the transgenic plants probably due to incomplete digestion of genomic DNA. The Southern analysis also revealed several hybridization bands smaller than the 3 kb GUS unit length, evidently indicating partial deletion of the GUS insert from the T_0 plant genome, which is a common feature during gene delivery via microprojectile bombardment[24].

There are two critical barriers associated with pollen transformation: 1) the nuclease activity[25,26] and 2) lack of stable integration of foreign DNA in the transformed pollen-derived plant[14,27]. In this investigation we have shown the integration of GUS plasmid pBI121 at least in F_1 generation at least after two vegetative cuttings. A brief outline of the procedure by which we accomplished transgenic alfalfa is shown in Figure 5.

Regarding inhibiting nuclease activity, salts such as EDTA[27,28], Na_3PO_4 (ref. 29), KNO_3 and $MgSO_4$ (ref. 30), NaCl and/or $ZnCl_2$ (ref. 31) were avoided, in case these salts, when used in combination with sucrose, would change the osmotic potential of the pollen liquid suspension causing large-scale rupture of pollen grains[32]. Additionally, salts EDTA, Na_3PO_4, NaCl, and $ZnCl_2$ inhibit pollen germination[29,31]. Instead, we tried to decrease the amount of nucleases by introducing certain critical steps in our pollen transformation protocol.

Proteins are mobile and diffuse out freely from moistened pollen, including from the plasmalemma membrane of incipient germinated pollen[33]. Most of the nucleases localized in the subcellular fractions: the mitochondrial fraction (including lysosomes and peroxisomes), the nuclear fraction, and microsomal fraction are released within a few minutes into the supernatant after very low speed vortexing or centrifugation[28]. Accordingly, Booy *et al.*[31] showed, washing maize (*Zea mays*) pollen for 10 min reduced the nuclease activity. Negrutiu *et al.*[27], while attempting to transform mature tobacco (*Nicotiana tabacum*) pollen for kanamycin-resistances washed the pollen twice for 5 min to protect the plasmid from pollen nuclease degradation. In our studies, washing pollen in 13% sucrose for 10 min before microprojectile-bombardment should have decreased the nuclease content, and the activities of whatever residual nuclease remained may have reduced during the 2 h cold incubation treatments rendered to pollen after microprojectile bombardment.

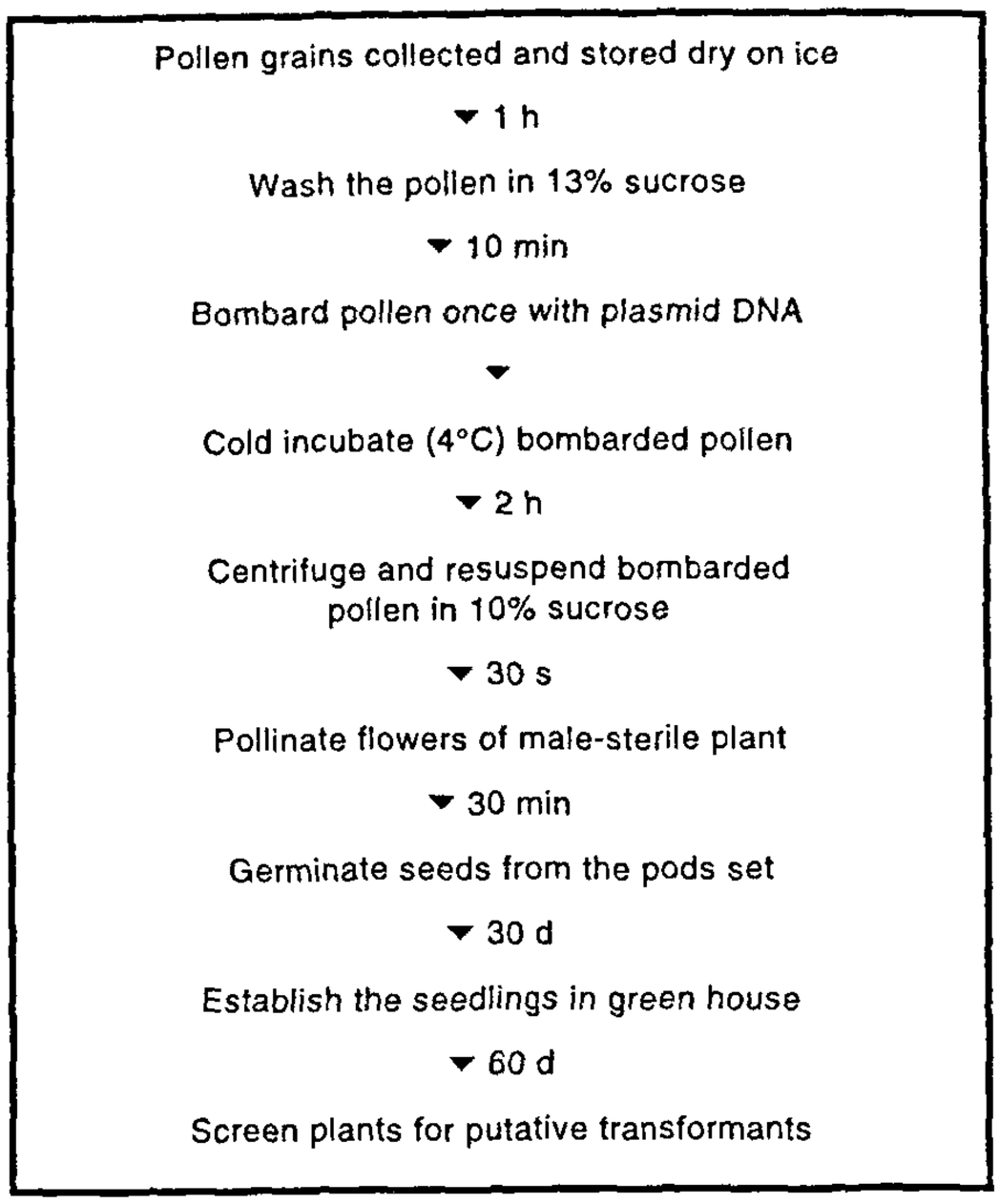

Figure 5. Outline of the pollen transformation procedure to obtain transgenic alfalfa plants.

The foreign gene integrated in T_0 plants is occasionally lost[27] or the level of the alien gene expression is significantly reduced[34,35]. However, this phenomenon is observed only in sexual segregating generations. A Southern analysis was conducted on T_0 plant DNAs after ten successive vegetative generations to investigate whether the loss and/or reduction of copy number of integrated foreign DNA occurs in asexual vegetative generations (Figure 6). Indeed, there was no hybridization signal detected in 5 of the 22 plants, which earlier showed strong hybridization (Figure 4, lanes, 4, 6, 7, 20, and 21) to the probe, *Eco*RI digested pBI121 plasmid (data not shown). In the remaining sixteen T_0 plants, the prominent hybridization bands less than 3 kb, detected at second vegetative generations were either faint or completely lost (Figure 6, lanes 1–14, 16, 17). In addition, the 10 and 3 kb fragments exhibited weak hybridi-

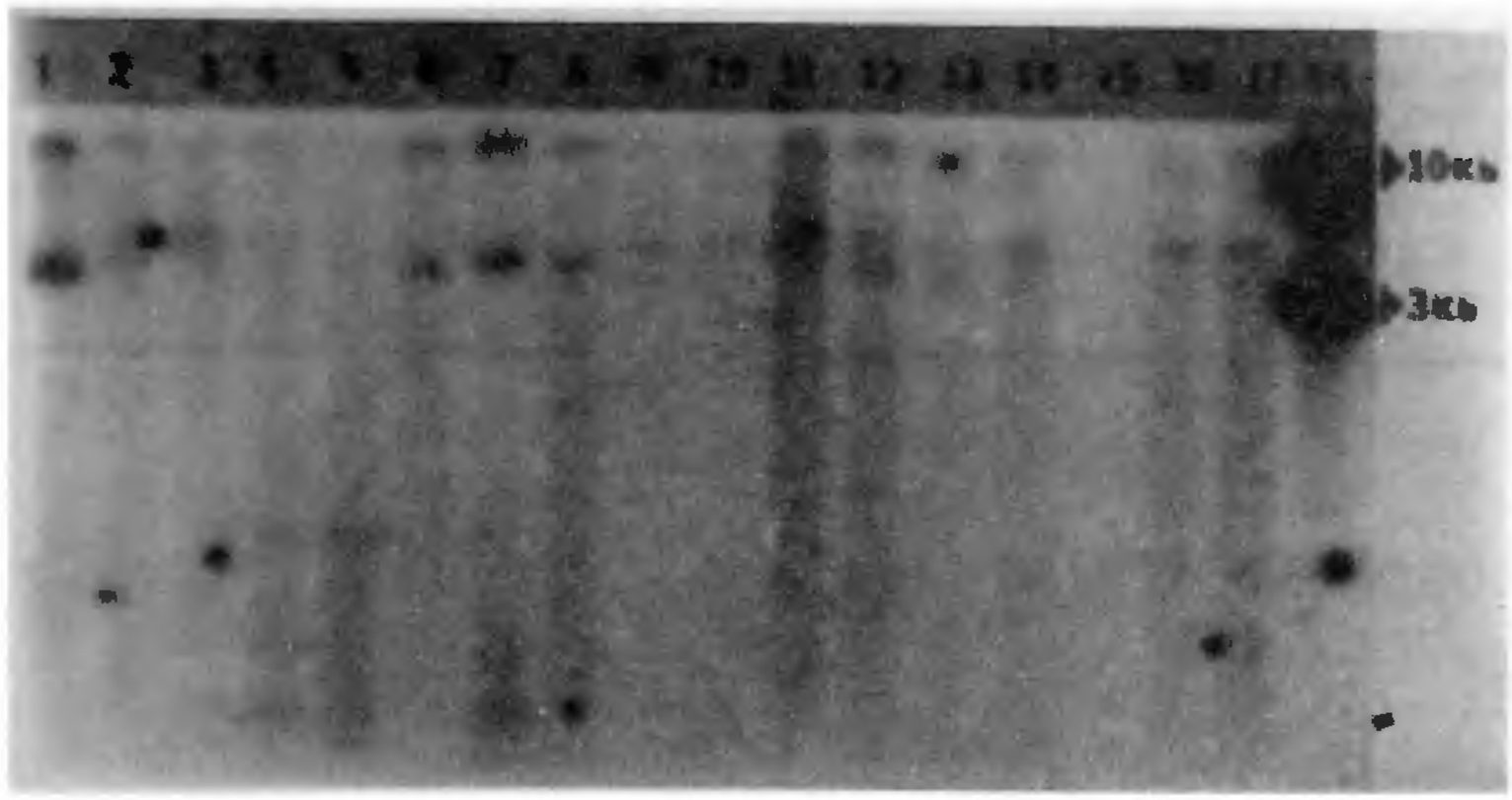

Figure 6. Southern analysis of *Hin*dIII and *Eco*RI digested genomic DNA of T_0 plants extracted after ten vegetative generations and probed with *Eco*RI digested pBI121 plasmid. Lanes 1–14, 16, 17, DNA of T_0 plants; Lane 15, DNA of pollen recipient male sterile plant; Lane 18, plasmid pBI121 that has released the 3 kb GUS insert following restriction digestion with *Hin*dIII and *Eco*RI.

zation signals, suggesting a possible reduction in the copy number of integrated plasmid DNA in some T_0 plants (lanes 2–5, 9, 10, 13, and 14). Ultimately, all transformed plants appeared to lose the integrated foreign DNA. We do not have an explanation for the apparent loss and reduction of the integrated plasmid DNA copy number, after ten asexual vegetative generations.

This work describes for the first time, the use of the particle gun to transform alfalfa pollen and obtain T_0 plants through natural plant reproductive process. As a next step, we plan to reevaluate this pollen transformation protocol by using a plasmid (pMON410) coding for kanamycin resistance and investigate the plausible causes for the loss of integrated plasmid after several vegetative generations in T_0 alfalfa plants.

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ACKNOWLEDGEMENTS. We thank T. K. Fish, L. L. Klocke, M. S. Schabel, D. Obert, and P. C. St. Amand for technical help. This investigation was supported by a research grant provided to D. Z. Skinner from USDA-ARS.

RESEARCH COMMUNICATIONS

Strain determination from three known stretches – A trigonometric solution

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The determination of strain from three coplanar, non-parallel stretches has been a classical problem in structural geology. A simple trigonometric solution to this problem is presented here.

THE problem of determination of the shape of strain ellipse from three known extensions was first recognized by Ramsay[1]. He proposed a Mohr circle solution which was also adopted by Ramsay and Huber[2]. Another graphical method using an alternate Mohr circle construction was proposed by Lisle and Ragan[3]. The graphical Mohr circle method tends to be rather lengthy and less accurate than an equivalent mathematical method. A few workers have therefore attempted alternate mathematical solutions to this problem. Ragan[4] has used matrix inversion and eigenvector determination while Sanderson[5] and De Paor[6] have used other algebraic methods. The simplest of them is by De Paor[6]. In the present paper, a simple equation is derived for the direction of the longest principal strain axis, which has further been used for determination of magnitudes of both the principal stretches. Whereas the '... amount of calculation involved is time-consuming without the aid of a computer...'[6], the present simple expression can easily be evaluated using a hand calculator.

The derivation is based on the appreciation of the fact that to define a strain ellipse, three variables are required, viz. λ_1 (long axis), λ_2 (short axis) and θ (direction of λ_1 with respect to any direction A along which the strain is known). If we know three coplanar non-parallel strain vectors (A, B, and C) with quadratic elongations λ_A, λ_B and λ_C respectively and their angular interrelationship (Figure 1), the shape and orientation of the strain ellipse can be determined.

The inverse quadratic strain along any direction A (λ_1^A = θ) is:

$$\lambda'_A = \lambda'_1 \cos^2\theta + \lambda'_2 \sin^2\theta \text{ [From eqs. (3)–(31) of ref. 1]}$$

Therefore inverse quadratic strain along A, B and C (Figure 1) can be represented by:

$$\lambda'_A = \lambda'_1 \cos^2\theta + \lambda'_2 \sin^2\theta\,, \tag{1}$$

$$\lambda'_B = \lambda'_1 \cos^2(\theta+\theta_1) + \lambda'_2 \sin^2(\theta+\theta_1)\,, \tag{2}$$

$$\lambda'_C = \lambda'_1 \cos^2(\theta+\theta_2) + \lambda'_2 \sin^2(\theta+\theta_2)\,, \tag{3}$$

where θ_1 and θ_2 are known.

By means of trigonometric relationships:

$$2\cos^2\alpha = 1 + \cos 2\alpha,$$

$$2\sin^2\alpha = 1 - \cos 2\alpha,$$

we get:

$$2\lambda'_A = \lambda'_1 + \lambda'_2 + (\lambda'_1 - \lambda'_2)\cos 2\theta\,, \tag{4}$$

$$2\lambda'_B = \lambda'_1 + \lambda'_2 + (\lambda'_1 - \lambda'_2)\cos 2(\theta+\theta_1)\,, \tag{5}$$

$$2\lambda'_C = \lambda'_1 + \lambda'_2 + (\lambda'_1 - \lambda'_2)\cos 2(\theta+\theta_2)\,. \tag{6}$$

Subtracting eq. (5) from (4) and eq. (6) from (4) we get:

$$2(\lambda'_A - \lambda'_B) = (\lambda'_1 - \lambda'_2)[\cos 2\theta - \cos(2\theta + 2\theta_1)]\,, \tag{7}$$

$$2(\lambda'_A - \lambda'_C) = (\lambda'_1 - \lambda'_2)[\cos 2\theta - \cos(2\theta + 2\theta_2)]\,. \tag{8}$$

Because for any value of α and β:

$$\cos\alpha - \cos\beta = 2\sin\frac{\alpha+\beta}{2}\sin\frac{\beta-\alpha}{2}\,,$$