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Research note.

Detection of single nucleotide polymorphisms (SNPs) and transgenes in DNA from small individual seeds and half-cotyledons in *Lotus japonicus*

Qunyi Jiang and Peter M. Gresshoff*

Australian Research Council Centre of Excellence for Integrative Legume Research, The University of Queensland, St Lucia, Qld 4072, Australia, Tel. +61-7-3365-3550, Fax. +61-7-3365-3556, Emails. q.jiang@uq.edu.au and p.gresshoff@uq.edu.au

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Abstract

Single nucleotide polymorphisms (SNPs) and transgene sequences were detected by allele-specific PCR in DNA isolated from small (1 mg mass) individual seeds and half-cotyledons of the model legume *Lotus japonicus*, allowing fast determination of a seedling's genomic status. This permitted a shortening of the breeding cycle for multi-trait seed lines.

Keywords: DNA diagnostics, symbiosis, legume, genomics, nodulation

Abbreviations: *etr1*, ethylene insensitive; *har1*, hypernodulation and aberrant root formation; SNPs, single nucleotide polymorphisms

1. Introduction

Genetic analysis of plant development is based on either phenotypic or genotypic variation. Classical procedures relied on the determination of the phenotype to imply the genotype which at times involves time delays due to organism development (e.g., flowering), by plasticity of the phenotype (e.g., yield), or variable environmental conditions (e.g., escapes in resistance screening). In contrast, DNA analysis provides insights into the genotype. The ultimate genetic marker is the gene itself; thus defining a SNP ultimately defines a locus and its allelic status. Knowledge of that status in either F1 hybrids or F2 segregants saves time as plants lacking the appropriate genetic complement can be eliminated.

To analyse mechanisms of plant development, the description of epistatic and pleiotropic gene interactions is progressively moving into the research foreground, as these determine the functional, spatial and temporal sequence of gene action. This analysis often is facilitated through the construction of double or triple mutants utilising either recessive or dominant alleles, as well as dominant transgenes.

*The author to whom correspondence should be sent.

We have long focused on the genetic dissection of the control of nodulation in legumes and have defined several pathways controlling the number of nodules (for summary see Gresshoff, 2004). In short, ethylene control of nodulation (ECN) as well as systemic autoregulation of nodulation (AON) regulate the number of nodules after initiation (Lohar et al., 2005; Penmetsa et al., 2003). Critical genes such as *LjHAR1* in *Lotus japonicus* (Wopereis et al., 2000; Krusell et al., 2002; Nishimura et al., 2002), *GmNARK* in soybean (Searle et al., 2003; Men et al., 2002) as well as ethylene insensitive 2 (*MtEIN2*) in *Medicago truncatula* (Penmetsa et al., 2003) were defined. The dominant *AtETR1-1* was transferred into *Lotus japonicus* to produce ethylene insensitive nodulation. Promoter-trapped, beta-glucuronidase (GUS) expressing lines of *L. japonicus* such as Fata Morgana (Martirani et al., 1999; Buzas et al., 2005) were produced to follow expression domains. Interaction of such genes and pathways now promised to reveal the physiological complexity of nodulation control. For that purpose, multi-mutant legume lines are being produced. The confirmation of their genetic status and subsequent stability becomes paramount.

Here we utilised allele-specific or transgene-specific PCR and coupled it with micro-DNA isolation utilising individual seeds/half-cotyledons of *Lotus japonicus*. As

only a portion of the seed was used, the diagnosis allowed quick and reliable genetic analysis. The procedure quickens the development of multiple mutant/transgene lines and serves to verify phenotypic assignments.

2. Materials and Methods

Plant material

Lotus japonicus (Regel) Larsen seeds were germinated as previously described (Jiang and Gresshoff, 1997). The hypernodulation and aberrant root mutant *har1-1* was described previously (Wopereis et al., 2000; Krusell et al., 2002; Nishimura et al., 2002; Jiang and Gresshoff 2002). The *GUS* trap line Fata Morgana contains a single T-DNA/ GUS insertion that is expressed in the pericycle and resultant nodule tissue after inoculation (Buzas et al., 2005). The ethylene insensitive transgenic line *Lj129-3*, containing a single homozygous insert of the *Arabidopsis ETR1-1* ethylene insensitivity gene, was described (Lohar et al., 2005). All plants were grown in a PC2 controlled glasshouse with an 18/6 h day/night cycle and 24°C/18°C day/night temperature regimes. The growth substrate was Vermiculite Grade 3 (Chillagoe Perlite, Kewarra Beach, Qld 4879, Australia) and half strength B&D medium (see Lohar et al., 2001). Leaf tissues were collected 35–40 d post-germination.

DNA extraction of single dry seed or half-cotyledons

The whole single dry seed DNA was extracted according to Hee Wan Kang et al. (1998). DNA from a single pre-germinated individual seed (18 h after scarification and imbibition on moistened filter paper) was obtained by removing a loosened seed coat and extracting it as described for dry seeds. Alternatively, the pre-germinated individual seeds were permitted to germinate on 1.4% agar plates containing half strength B5 medium (Lohar et al., 2001) in a growth cabinet with an 18/6 h day/night cycle and 24°C/18°C day/night temperature regime. The single distal half of a cotyledon 2 days after incubation was cut for DNA extraction. Plantlets after cotyledon excision were used for phenotypic analysis (nodulation, GUS, and triple response tests as required). Half-cotyledon DNA extraction was described by Klimyuk et al (1993).

Primers and PCR

PCR primers were designed using PRIMER3, and synthesised by PROLIGO (Australia; Table 1). Allele-specific primers were designed from *HAR1-1* mRNA sequence (NCBI: AJ495844, Krusell et al., 2002), *Arabidopsis thaliana Etr1* mRNA sequence (NCBI: NM_105305, Town et al., 2002), and plasmid pΔgusBin19 vector (GenBank accession number U12638, Jefferson et al., 1986).

Gradient PCR was used to optimise the detection of the SNP in *har1-1*. PCR thermal cycling consisted of denaturation for 3 min at 94°C, then 35 cycles of 45 s at 94°C, 30 s at specific annealing temperature (66–74.9°C), and 1.5 min at 72°C. The final extension was for 6 min at 72°C. The final, optimal annealing temperature was set at 69.5°C for *har1-1* SNPs detection.

PCR for T-DNA and *GUS* was for 3 min at 94°C, then 35 cycles of 45 s at 94°C, 30 s at 60°C, and 1.5 min at 72°C. The final extension was for 6 min at 72°C. The PCR condition for *ETR1-1* detection was for 3 min at 94°C, then 40 cycles of 15 s at 95°C, 60 s at 58°C, and 1.5 min at 72°C. The final extension was 6 min at 72°C. The PCR mixture (20 µl) was composed of 1 unit of *Taq* DNA polymerase (INVITROGEN), 1 x PCR buffer, 0.2 mM dNTPs, 1.5 mM $MgCl_2$, 0.5 µM combined primers and 100 ng template DNA. PCR were performed in a HYBAID thermal cycler. Amplification products were separated on a 1% agarose gel with ethidium bromide staining.

Table 1. Primer pairs used for allele-specific PCR

Primer pair	Name		Sequence (5'-3')	Description of primer
PP1	WT	(F)	ctg cac cga gcg cag gcc tg	2107–2126 bp (AJ495844)
	Reverse	(R)	gtt cga aac gta ccc cag aa	2382–2411 bp (AJ495844)
PP2	*Har1-1*	(F)	ctg cac cga gcg cag gcc ta	2107–2126 bp (AJ495844)
	Reverse	(R)	gtt cga aac gta ccc cag aa	2382–2411 bp (AJ495844)
PP3	WT	(R)	ctg gaa cgc ggt gag ctt cc	2145–2126 bp (AJ495844)
	Forward	(F)	gaa aaa cct cag agc gtt gc	1536–1555 bp (AJ495844)
PP4	*Har1-1*	(R)	ctg gaa cgc ggt gag ctt ct	2145–2126 bp (AJ495844)
	Forward	(F)	gaa aaa cct cag agc gtt gc	1536–1555 bp (AJ495844)
PP5	GUS1		tag cgg gac ttt gca agt g	1373–1391 bp (pΔgusBin19)
	GUS2		gtt ttt gca gca gaa aag cc	2439–2420 bp (pΔgusBin19)
PP6	TDNA1		cga tag cag cac cgt aat ca	3261–3280 bp (pΔgusBin19)
	TDNA2		gac tct tgg ggt tcg aaa tga	4163–4142 bp (pΔgusBin19)
PP7	ETRP1		ccg att tct tca ttg cga tt	236–255 bp (NM_105305)
	ETRP2		tga tac ggg ttt gag caa ca	2316–2297 bp (NM_105305)

3. Results and Discussion

SNP detection in the F2 population of HAR1-1

One hundred and eighty three F2 seeds from the cross between B-159-S9 Gifu *har1-1* and ecotype B-581 Funakura (Jiang and Gresshoff, 1997, 2002) segregated for the hypernodulation and aberrant root phenotype at a classical Mendelian 3:1 ratio. No evidence of co-dominance was detected. The mutant allele leads to hypernodulation when inoculated, and an aberrant root phenotype when grown in the absence of *Mesorhizobium loti* inoculant. The nodulation phenotype is controlled by the shoot, suggesting long distance signalling involving the mutated LRR receptor kinase (Jiang and Gresshoff, 2002; Krusell et al., 2002).

Sequencing of *har1-1* DNA revealed a single nonsense mutation (TGG–>TAG) located in codon 676 (2,125–2,127 bp; Krusell et al., 2002; Nishimura et al., 2002). We designed two groups of *har1-1* primer pairs (PPs). Forward primers of PP1 and PP2 carried a selective 'G' nucleotide (wild-type) or 'A' (mutant). A common reverse primer was used. For the second group, the diagnostic primer sequence (terminating in C-3' and T-3'; detecting the other strand) was in the reverse primer, sharing the same forward primer. The estimated sizes of the PCR products from the two groups of PPs were 305 bp and 610 bp, respectively. Both groups of PPs showed the appropriate products after amplification (data not shown; verified by sequencing). We used PP3 and PP4 for further analysis because the 610 bp product was easier to detect.

To confirm the genotypic make-up of the F2 population, we designed allele-specific primers from the *HAR1-1* mRNA sequence (NCBI: AJ495844). Eight randomly selected F2 lines with wild-type nodulation and 12 F2 lines with hypernodulation were used for SNP detection. With PP3, designed to detect the wild-type allele, the control Gifu (Fig. 1, upper lane, 22) and the wild-type subpopulation (Fig. 1, upper lanes, 1, 3, 5–10) showed a single, anticipated amplification product of 610 bp. The control *har1-1* (Fig. 1 upper lane, 21) and the hypernodulation subpopulation (Fig. 1 upper lanes, 2, 4, 11–20) were negative (null). With PP4, designed to detect the mutant allele, the control *har1-1* (Fig. 1, lower lane, 21) and all hypernodulation subpopulation (Fig. 1, lower lanes, 2, 4, 11–20) had the anticipated 610 bp band; five phenotypically wild-type lines (Fig. 1, lower lanes, 6–10) also showed the 610 bp band with the *har1-1* allele-specific primer pair. These five lines also showed the wild-type allele indicating that they were heterozygotes. PP1 and PP2, designed by a different paradigm detecting the same heterozygotes, confirmed the assignment in all cases, though with a weaker band due to its smaller size.

Gradient PCR determined the appropriate annealing temperature for PCR. For PP3 and PP4, the predicted optimal annealing temperature was 65°C. At temperatures in the 66–68°C range with primer pairs PP3 and PP4, both wild-type and *har1-1* lines showed 610 bp bands without selectivity. The SNP was only detected in the 69.3–73.4°C range. There were no products above 73.4°C. Thus, 69.3°C was used for optimal annealing, because it gave the strongest signal and a clear polymorphism.

Confirmation of the F1 status at the individual seed level

To develop an integrated nodule developmental model based on gene interactions, we developed multiple mutants involving autoregulation, non-nodulation, hormone-insensitive, and promoter-trapped lines by sexual crosses. The F1 nature from crosses *har1-1* x *Lj129-3 (etr1-1)* and *har1-1* x Fata Morgana was confirmed using DNA extracted from individual dry seed or pre-germinated seedling cotyledon.

Triplicate single dry seeds from either the *har1-1* x *Lj129-3 (etr1-1)* and *har1-1* x Fata Morgana cross were used for DNA extraction. Seed DNA from *har1-1* x *Lj129-3 (etr1-1)* and *har1-1* x Fata Morgana was amplified at 69.3°C (Fig. 2A). With allele-specific PP3, all F1 single seed DNA (lanes 1–6) and wild-type Gifu (lane 9) showed a band at 610 bp. The negative control *har1-1* (single seed DNA, lane 7) and *har1-1* (leaf tissue DNA, lane 8) missed the diagnostic band. With PP4, all F1 seed DNA (lanes 10–15), positive control *har1-1* (single seed DNA, lane 16), and *har1-1* (leaf tissue DNA, lane 17) showed the 610 bp band. The negative control Gifu (lane 18) missed the band.

PP7 was derived from *Arabidopsis thaliana Etr1-1* cDNA sequence (Lohar et al., 2005) and used to confirm contribution of the male parent *Lj129-3*, without phenotypic characterisation, in the *har1-1* x *Lj129-3 (etr1-1)* cross. Three individual seed DNA samples of putative F1 status from the *har1-1* x *Lj129-3 (etr1-1)* (lanes 1–3) cross and *Lj129-3 (etr1-1)* (leaf tissue DNA, lane 5) control showed a 2,081 bp (the anticipated band). The negative control *har1-1* (single seed DNA, lane 4), *har1-1* (leaf tissue DNA, lane 6), and wild-type Gifu (leaf tissue DNA, lane 18) missed the band (Fig. 2B).

Fata Morgana, a *GUS* trap line expressing the reporter gene in pericycle and nodule tissue in response to *Mesorhizobium loti* inoculation (Buzas et al., 2005), was crossed with *har1-1* to follow early inoculation events during the absence of autoregulation of nodulation. As the female parent *har1-1*, its F1 nature was confirmed by SNP. We further confirmed the genetic contribution of the male parent with T-DNA and *GUS* primers from vector pΔgusBin19. In Fig. 2C, three F1 single seed DNA samples of *har1-1* x Fata Morgana (lanes 1–3) and Fata Morgana (leaf tissue DNA, lane 4) presented a unique band at 903 bp with T-DNA primers PP6. Meanwhile, the same samples, amplified with *GUS* primers PP5, showed a consistent result. Three individual F1 seed DNA samples of *har1-1* x Fata Morgana (lanes 6–8) and Fata Morgana (leaf tissue DNA, lane 9) presented a unique band at 1,067 bp

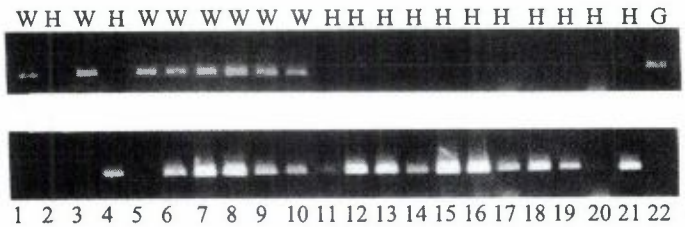

Figure 1. DNA amplification profile with allele-specific primer pairs PP3 and PP4 in the F2 population of *har1-1* x Funakura. Upper bands were amplified with primer pair PP3 and lower one was amplified with primer pair PP4. No 1–20. F2 individual lines, W means wild type, H means *har1-1*. Sample No 2 is weak positive. No 21. *Har1-1* control, No. 22. Wild type control Gifu. *All samples were extracted from leaf tissue.

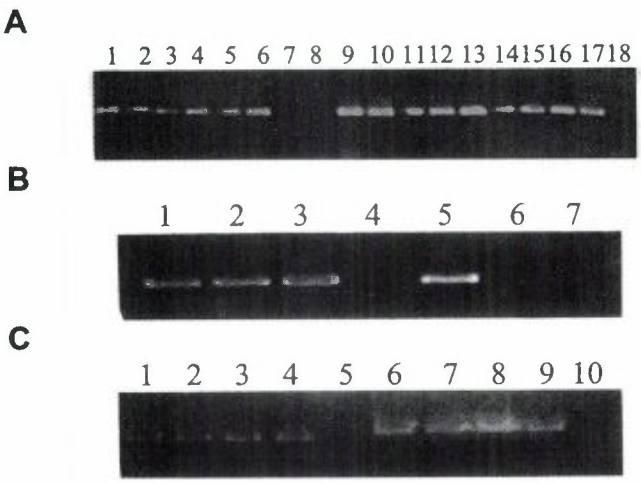

Figure 2. PCR with specific primers in F1 heterozygous seed of *L. japonicus*. A, with *har1-1* allele-specific primers. 1–3. *har1-1* x Fata Morgana (F1, seed DNA), 4–6. *har1-1* x *Lj129-3 (ETR1-1)* (F1, seed DNA), 7. *Har1-1* (seed DNA), 8. *Har1-1* (leaf tissue DNA), 9. Gifu (seed DNA), 10–12. *har1-1* x Fata Morgana F1, seed DNA), 13–15. *har1-1* x *Lj129-3 (ETR1-1)* (F1, seed DNA), 16. *HAR1-1* (seed DNA), 17. *HAR1-1* (leaf tissue DNA), 18. Gifu (seed DNA). *1–9, amplified with PP3; 10–18 amplified with PP4. B, with *ETR1-1* specific primers. 1–3. *har1-1* x *Lj129-3 (ETR1-1)* (F1, seed DNA), 4. *Har1-1* (seed DNA), 5. *Lj129-3 (ETR1-1)* (seed DNA), 6. *Har1-1* (leaf tissue DNA), 7. Gifu (seed DNA). C, with T-DNA and GUS primers. 1–3. *har1-1* x Fata Morgana (F1, seed DNA), 4, Fata Morgana (seed DNA), 5. *Har1-1* (seed DNA), 6–8. *har1-1* x Fata Morgana (F1 seed DNA), 9. Fata Morgana (seed DNA), 10. *Har1-1* (seed DNA). *1–5, amplified with PP6; 6–10, amplified with PP5.

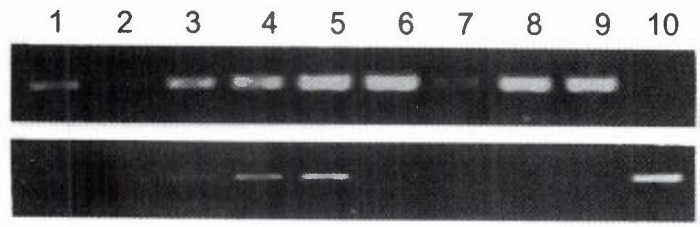

Figure 3. DNA amplification profile with allele-specific primer pairs PP3 and PP4 in the F2 population of *har1-1* x Funakura. Upper bands were amplified with primer pair PP3 and lower one was amplified with primer pair PP4. No 1–8. F2 individual lines; No 9. Wild type control Gifu; No. 10. *Har1-1* control. *All F2 samples were extracted from the distal half of cotyledon.

with *GUS* primer pair, while *har1-1* as a negative control (single seed DNA, lanes 5, 10) gave no PCR products with both T-DNA and *GUS* primers.

The F1 nature of *har1-1* x *LjETR1* and *har1-1* x Fata Morgana was confirmed by SNP and primers from *Arabidopsis thaliana Etr1-1* cDNA sequence, T-DNA and *GUS* derived from pΔgusBin19 at the single dry seed level. Three individual F1 seeds from above crosses showed the same F1 nature.

Confirmation of SNP using half-cotyledons of single seedlings

According to the seed classification, *Lotus japonicus* forms exalbuminous seed, which consist mainly of testa and cotyledons. Instead of the endosperm in albuminous seed, the cotyledon stores abundant nutrients material. Based on this fact, we have tested the half-cotyledon PCR assay in comparison to the dry seed DNA extraction method.

Like seeds of tomato and sesame, the seeds of *Lotus japonicus* are difficult to cut into half, and are too small (800 seeds/gram, average diameter = 1 mm) to permit the maintenance of DNA-profiled plantlets after a half dry seed approach. To overcome this, we germinated the seeds from the F2 population of *har1-1* x Funakura and cut the distal half of each cotyledon for DNA extraction, leaving the remainder of the plant for growth and phenotype determination. Eight half-cotyledon DNA samples from randomly selected seedlings were used for SNP detection with primers PP3 and PP4 (anticipated amplification product of 610 bp). Eight F2 lines were WT phenotype with primer pair PP3, designed to detect the wild-type allele

(Fig. 3). Three lines with the 610 bp band were detected as heterozygotes with the *har1-1* allele-specific primer pair PP4. Like Fig. 1, we observed the same SNP pattern and segregation from half-cotyledon DNA as those from single dry seed DNA and leaf tissue DNA. The remaining seedlings grew on the 1/2 strength B5 medium agar plates for 7 days after capping of the cotyledon, and could be transplanted into soil.

To study gene-gene interactions or tissue-gene interplay among *GUS* expression, ethylene insensitive, hypernodulation, and non-nodulation, we were generating multi-trait double or triple mutants/transgenics. Use of single seed/half-cotyledon DNA and SNP for genetic analysis helped to accelerate the selection of such multi-trait mutants.

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