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ORIGINAL ARTICLE

Sinorhizobium meliloti nifA mutant induces different gene expression profile from wild type in Alfalfa nodules

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Several studies have demonstrated that the *Rhizobium nifA* gene is an activator of nitrogen fixation acting in nodule bacteria. To understand the effects of the *Sinorhizobium meliloti nifA* gene on Alfalfa, the cDNA-AFLP technique was employed to study the changes in gene expression in *nifA* mutant nodules. Among the approximately 3,000 transcript-derived fragments, 37 had differential expression levels. These expression levels were subsequently confirmed by reverse Northern blot and RT-polymerase chain reaction. Sequence analyses revealed that 21 cDNA fragments corresponded to genes involved in signal communication, protein degradation, nutrient metabolism, cell growth and development.

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Keywords: cDNA-AFLP, gene expression, nodule, *Sinorhizobium meliloti*, *nifA*

Introduction

Nitrogen fixation in legumes takes place in highly specialized root nodules that result from the association between *Rhizobia* and legume plants [1-3]. The bacteria enter the nodule via an infection thread, and then differentiate into nitrogen-fixation bacteroids, which are capable of fixing atmospheric nitrogen into ammonia that provided to the plant. In return, the host plant provides carbon and energy to the bacteroids in the form of C_4-dicarboxylic acids [4, 5]. Nodules are unique among plant organs as their development arises as a result of interactions among many cooperating plant and bacterial genes. However, at the molecular level, only a few events in the communication between bacteria and plants are understood. In plants, a set of early nodulin genes encode products that are involved in infection and nodule development. The products of late nodulin genes participate in the contribution of the endosymbiont to the specialized metabolic activity of the nodule [6, 7]. Within bacteroids, nitrogenase synthesis (*nif*) and microoxic respiration (*fix*) genes and an oxygen-limited condition are required for nitrogen fixation [2, 8].

In *Sinorhizobium meliloti*, the *nifA* gene as well as *nifHDKE*, *fixABCX* and *fixLJ* are located in a large plasmid, pSymA [9]. The *fixL* and *fixJ* genes encode a two-component regulatory system in which the oxygen sensor FixL transfers phosphate to the response regulator FixJ. Phosphorylated FixJ positively controls transcription of *fixK* and *nifA* [10, 11]. FixK induces expression of *fixNOQP* and negatively affects expression of *nifA*, whereas NifA is required for transcription of *fixABCX*, *nifN* and *nifB*, as well as for transcription of the *nifHDK* operon that encodes the subunits of the nitrogenase [12]. However, the FixL/FixJ system is not required for *nifA* gene expression in *Bradyrhizobium japonicum* [13]. In addition to *nif* and *fix* genes, NifA controls some other genes not directly involved in nitrogen fixation, such as genes related to nodulation competitiveness, rhizopine synthesis, nodule development and bacteroid persistence in *S. meliloti* [14].

Previous studies inferred that *nifA* mutants not only failed to fix nitrogen but also elicited numerous small nodules whose necrotic interior was reminiscent of a hypersensitive response characteristic of non-compatible host-pathogen interactions [3]. The nodules induced by the *S. meliloti nifA* mutant differed from those induced by wild type in that the fixation zone was less extensive and many of the cells

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interspersed within the fixation zone contained senescent bacteroids. In addition, a significantly larger senescent zone was observed in *S. meliloti nifA* mutant nodules [15]. We previously found that small, white and ineffective nodules were also formed in plants infected with *S. meliloti* that had been transformed with a multi-copy plasmid carrying *nifHDK* genes driven by a P1 promoter [16]. This indicated that the redundant *nifHDK* promoter brought about a shortage of NifA protein necessary for normal nodulation in the host plant. Thus, it is speculated that *nifA* not only regulates *nif/fix* genes but also genes involved in nodule formation and maintenance. Recently, our unpublished data indicated that *nifA* affects the nodulation on other lateral roots in split root system. However, little is known about how host genes act in response to the expression of *S. meliloti nifA*. In the present study, we compared gene expression profiles in an *S. meliloti nifA* mutant nodules with wild-type. We aimed to characterize the molecular events taking place during symbiotic association, and to provide new clues concerning the relationship between signaling molecules and the *S. meliloti nifA* gene.

Materials and Methods

Bacterial strains

The wild-type *S. meliloti* strain Rm1021 and its *nifA* mutant Sm1354 (*nifA*::Tn5) strain were used in this study [15]. *Escherichia coli* DH5α was used for plasmid DNA transformation.

Plant cultivation

Seeds from *Medicago sativa* were surface sterilized, germinated and then grown in plastic barrels on nitrogen-free vermiculite. Wild-type Rm1021 and *nifA* mutant Sm1354 were inoculated on plants as described previously [17]. The nodules induced by Rm1021 or Sm1354 were harvested 15 and 30 days after inoculation and stored at -70 ºC.

cDNA-AFLP analysis

cDNA-AFLP was carried out according to standard procedures with little modification [18]. Briefly, total RNA was extracted from the nodules 30 days after inoculation (Total RNA isolation Kit IV, Watson, China) according to the manufacturer's instructions. mRNA was purified using an mRNA isolation system (Watson, China). Double-strand cDNA was synthesized using an anchored oligo-$(dT)_{18}$ primer and Superscript II RNase H reverse transcriptase (Invitrogen)

Table 1 Primers used in RT-PCR experiments for 24 TDFs

AFLP fragment No.	Primers
G3	F: 5′ ATTTATAAAGGGAGGAAG3′; R: 5′ACCATTTGTAGCAGTAGAGC3′
G5	F: 5′ACCTAACAAAACCCTAGCGAAGT3′; R: 5′AAGCAGAACAAAGCCAAATAAAA3′
G6	F: 5′GTTGGATATGACTGAACGCCTCTA3′; R: 5′GGCCACTCTGCCACTTCAATAC3′
G7	F: 5′CAACAGCCTCAGCAGAACAA3′; R: 5′GCTGGGTTTAGATACAGA3′ATCATAG3′
G8	F: 5′ACTGTCTTACATTGCGGGTTTG3′; R: 5′TCAATTTGACCAGGGTGATGCT3′
G9	F: 5′GACACCTTTGTGAACTTCCCCA3′; R: 5′AGATGATCTGTTTTAGCCTCCG3′
G10	F: 5′CAAATCCATAACCGTAACAAGAAC3′; R: 5′CCAGATCCAGGAAAGAAGAGCC3′
G11	F: 5′ACCCAGAAAAGCCGCCAAGTGT3′; R: 5′AAATAGGAGCATTCCCCAACAT3′
G12	F: 5′GATCGAGATCCATGCGGG3′; R: 5′GCTCTGAATCGAAGCCCCA3′
G13	F: 5′GGGCACCAGGGACTATACATAC3′; R: 5′GAAACCGACAGATCCGAAAGA3′
G15	F: 5′GCTGCAGGTCGACGATTGAT3′; R: 5′GGGGAGTCTTCTTATGAGGGTTT3′
G16	F: 5′TTTAGTAGTCGGGCTGTTGA3′; R: 5′ACTGCGTACCAATTCCCCTG3′
G17	F: 5′CAGGTCGACGATTGATGAGT3′; R: 5′CTGCGTACCAATTCCCATAT3′
G19	F: 5′GATATGGGAGGAAGGGCAAAGGG3′; R: 5′GGGGGAATTTCATTACATCACCATCTTA3′
G20	F: 5′TGCTGCGGTCGACGATTGAT3′; R: 5′CGTTTTTTATATTCTCCCAA3′
G22	F: 5′ATTGCAGTTGCCAGTGCATCAT3′; R: 5′CTGCAGCAAAAGGCTTGTTCTC3′
G23	F: 5′CAGGCTAGAAAGAAAATGAAGATAC3′; R: 5′CTAAGATGAGGGTACTGGAGAA3′
G24	F: 5′TAGTGCCCAGAAATGAAA3′; R: 5′CAAAGATTGACGATGTTCCA3′
G26	F: 5′TCGCAAAGTCAAGCAGAAGA 3′; R: 5′GCGCAGGATTACTATAACCAT3′
G29	F: 5′TCTCCTCACCAGGTAACCACATC3′; R: 5′ATCACCTGAAGAAAGAAAATCCC3′
G30	F: 5′CAATGATAGGAAGAGCCGACAT3′; R: 5′TGGAAACAGCGAAAGTGAAATA3′
G31	F: 5′CCCTATTGCGTCCTTTCTTGT3′; R: 5′ATTCGCCGTCCCAGCCTTGTC3′
G34	F: 5′GGCGATAGGGTGAAACATT3′; R: 5′GCATCAGCAGCATAAAAGGA3′
G35	F: 5′ATCAAAGACCACAAAATCTGAAAT3′; R: 5′AAAATATGGCTAAAGTCGCTAAGT3′

according to the manufacturer's protocol. The pre-amplification step for 16 cycles of polymerase chain reaction (PCR) was performed with an *Eco*RI+0/*Mse*I+0 adaptor primer combination. Pre-amplified productions were diluted 50-fold and 5 μl was used as template for final amplifications using γ^{33}-labeled *Eco*RI primer with two selective nucleotides and an *Mse*I primer with two selective nucleotides. The obtained radioactive amplification products were separated on 5% polyacrylamide gels and run at 90 Watts in Sequi-Gen GT nucleic acid sequencing cell (Bio-Rad) until the xylene-cyanol dye reached the 1/3 position from the bottom. Gels were covered with plastic film and positionally marked before being exposed to Kodak film for 3 days.

Fragment characterization

The differentially expressed bands were individually collected as template for re-amplification with the same primers used in AFLP analysis. Re-amplified products were analyzed by agarose electrophoresis, cloned into pMD18-T vector (Takara) and sequenced. The obtained sequences were compared to nucleotide and protein sequences in publicly available databases (http://www.medicago.org/genome/blast.php).

Reverse Northern hybridization

Reverse Northern hybridization was used to confirm the differential gene expression results obtained from cDNA-AFLP. To prepare hybridization templates more conveniently, a pair of specific primers were designed according to pMD18-T vector sequence at cloning site (F: 5′GCG GAT AAC AAT TTC ACA CAG3′; R: 5′CCA GGG TTT TCC CAG TCAC3′). Equal volumes of PCR products (about 1 μg) were loaded into 1.5% agarose gels. DNA samples were then transferred to nylon membranes (Amersham Biosciences). Total RNA was extracted from 15- and 30-day-old nodules. Radiolabeled cDNA probes were synthesized by reverse transcription of 10 μg of total RNA for 1 h in the presence of 100 μCi [^{32}P]dCTP with the Superscript reverse transcriptase. Probes derived from each RNA sample were hybridized against one set of transcript-derived fragments (TDFs) obtained above. Hybridization was performed in 0.5 M Na-phosphate (pH 7.2), 1 mM EDTA, 7% SDS buffer for 16 h at 65 ºC, washed twice for 5 min with 40 mM Na-phosphate/1 mM EDTA/5% SDS and then washed four times for 10 min with 40 mM Na-phosphate/1 mM EDTA/1% SDS. Hybridization signals were observed first on Kodak X-ray film and then quantified by Dots hybridization analysis system (Tanon 2.20, China).

RT-PCR

To control for equal amounts and quality of RNA template, an additional RT-PCR was performed under the same conditions as PCR with specific primers based on the sequence of Ribosome large subunit gene (F: 5′ACC AGA GCC GCT AAG GTT3′; R: 5′CAG CTT ACA TAA CCG GTC3′). The primers used for 24 cDNA TDFs are listed in Table 1.

Results

Characterization of differentially expressed TDFs

cDNA-AFLP reactions were performed with 64 combinations of *Eco*RI and *Mse*I primers having two selective 3′ terminal nucleotides. This reduced the number of fragments per fingerprint to ~50. Although the majority of

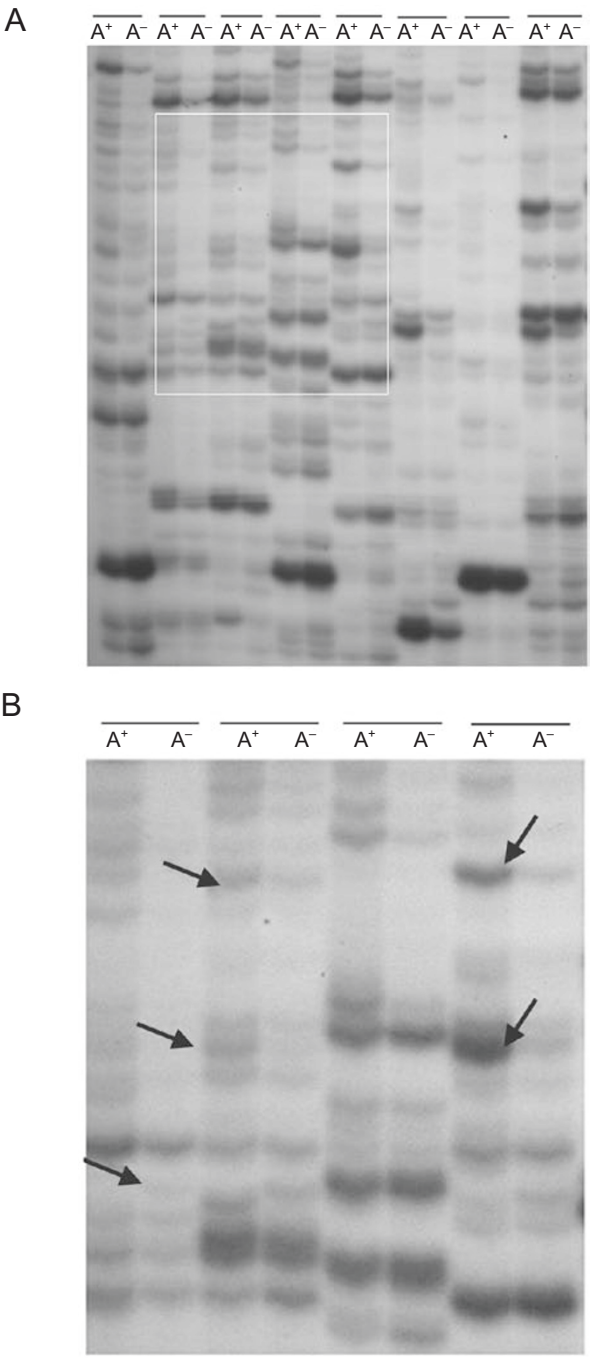

Figure 1 cDNA-AFLP display of transcripts in nodules elicited by *S. meliloti* wild-type strain Rm1021 and *nifA* mutant Sm1354. RNA was extracted from 30-day-old nodules induced by wild-type Rm1021 (A^+) and *nifA* mutant Sm1354 (A^-). Lanes are in groups of two, and each group was amplified using one combination of *Eco*RI and *Mse*I primers having two selective 3′ terminal nucleotides. **(A)** A selection of cDNA-AFLP display. **(B)** Enlarged view of the box in **(A)**. The arrow shows the differentially expressed TDFs.

Table 2 The EST database in GenBank showing homology with differentially expressed TDFs

AFLP fragments	Size (bp)	GenBank Accession No.	Homology			Score/e-value
			Accession No.	Species	Tissue type	
G3	162	DR025667	CB827529.1	*Lotus japonicus*	Nodule library 5 and 7 weeks old	32/1e-07
G4*	412	DR159684	BQ136242.1	*Medicago truncatula*	Elicited cell culture	210/2e-113
G5*	530	DR159685	CO513060.1	*Medicago sativa*	Glandular trichomes	454/0.0
G6	244	DR159686	CO5132971	*Medicago sativa*	Glandular trichomes	229/5e-125
G7	245	DR159687	CO515538.1	*Medicago sativa*	Glandular trichomes	224/5e-122
G8*	654	DR159688	CX550739.1	*Medicago truncatula*	Roots	459/0.0
G9*	593	DR159689	CB892042.1	*Medicago truncatula*	Roots	537/0.0
G10*	465	DR159662	CX549961.1	*Medicago truncatula*	Roots	433/0.0
G11*	417	DR159663	CX550724.1	*Medicago truncatula*	Roots	377/0.0
G12	200	DR025668	CK550724.1	*Heterodera glycines*	Gland cell	191/4e-102
G13*	388	DR159664	CX530322.1	*Medicago truncatula*	Root cell suspension culture	229/2e-119
G15*	643	DR159665	CB893436.1	*Medicago truncatula*	3-day-old seedling roots	570/0.0
G16*	477	DR159666	BE324025.2	*Medicago truncatula*	Phosphate-starved leaf	419/0.0
G17*	526	DR159667	BG454790.1	*Medicago truncatula*	Developing leaf	448/0.0
G18	118	DR159668	CX526702.1	*Medicago truncatula*	Aphid-infected shoots	70/2e-30
G19*	403	DR159669	AW287853.2	*Medicago truncatula*	Phosphate-starved root	328/0.0
G20*	511	DR159670	BF650554	*Medicago truncatula*	Cell cultures derived from root tissues	377/0.0
G21*	560	DR159671	CO513447.1	*Medicago sativa*	Glandular trichomes	316/1e-176
G22*	472	DR159672	CF069452.1	*Medicago truncatula*	Various stages mixed tissues	388/0.0
G23	276	DR159673	CA920444.1	*Medicago truncatula*	Various stages mixed tissues	247/1e-135
G24	253	DR025670	AJ847674	*Medicago truncatula*	Whole roots	61.9/3.8e-12
G25*	489	DR159674	CB891349.1	*Medicago truncatula*	Seedling roots	316/1e-176
G26	148	DR025671	CX539620.1	*Medicago truncatula*	Germinating seed	27/3e-05
G27	278	DR159675	CO514116.1	*Medicago sativa*	Glandular trichomes	153/1e-79
G29*	537	DR159676	CA920444.1	*Medicago truncatula*	Various stages mixed tissues	329/0.0
G30*	690	DR159677	BG646644.1	*Medicago truncatula*	3-day-old seedling roots	681/0.0
G31	300	DR159678	DR107844.1	*Canis familiaris*	Mixed	332/0.0
G33	83	DR025673	CB861201.1	*Hordeum vulgare*	1-day-old coleoptile	66/3e-28
G34*	352	DR159680	CB893273.1	*Medicago truncatula*	3-day-old seedling roots	301/8e-168
G35*	533	DR159681	AL380616.1	*Medicago truncatula*	Symbiotic root nodules	263/6e-145
G36*	314	DR159682	CB892026.1	*Medicago truncatula*	Seedling roots	247/1e-135
G37*	523	DR159683	BI310237.1	*Medicago truncatula*	Immature seeds	493/0.0
G1	66	DR159661				
G2	75	DR025666				
G14	162	DR025669				
G28	155	DR025672				
G32	68	DR159679				

*The fragments were extended on the basis of GenBank data.

the cDNA-AFLP fragments did not show any significant change, 44 differentially expressed TDFs were detected. Thirty-seven differentially expressed fragments were excised from the gels, re-amplified by PCR and sequenced. There were technical problems with sequencing the other seven fragments and thus they were not studied. Figure 1

Table 3 AFLP, Northern blot and RT-PCR analyses of the differentially expressed TDFs in *nifA* mutant Sm1354 nodules at 30 days after inoculation

AFLP fragments	Size (bp)	GenBank Accession No.	Homology	BLAST score/e-value	AFLP	Northern blot	RT-PCR
Signal transduction							
G7	245	DR159687	Adhesive/proline-rich protein (Q7F1U6)	126.9/5.0e-30	U	U	U
G10	465	DR159662	Ethylene response factor ERF3a (Q6TKQ4)	106.1/2.2e-32	D	D	D
G16	477	DR159666	G-protein a-subunit (Q9C516)	233.2/5.2e-62	D	D	D
G19	403	DR159669	Cytokine IK (Q13123)	72.6/9.5e-13	U	D	D
G25	489	DR159674	Receptor-like kinase RHG4 (Q8LKR3)	178.6/8.9e-45	D	D	N
G31	300	DR159678	Calmodulin-binding protein (Q8GEG1)	183.9/3.5e-47	D	D	D
Degradation							
G9	593	DR159689	Ubiquitin carboxyl terminal hydro lase (Q8LMT7)	119.8/9.1e-27	D	D	D
G11	417	DR159663	Ubiquitin-conjugating enzyme (Q9SJ44)	168.4/1.6e-42	D	D	D
Cell growth and death							
G6	244	DR159686	Senescence-associated protein (Q9AVH3)	27.2/9.5e-06	D	U	U
G21	560	DR159671	Retinoic-interferon-induced mortality 19 (O49313)	188.5/1.5e-48	D	D	N
G30	690	DR159677	Senescence-associated protein (Q9AVH2)	239.5/6.3e-78	U	U	U
Development							
G22	472	DR159672	Leghemoglobin 1 (Q43789)	203.9/3.2e-53	D	D	D
G28	155	DR025672	Cellulose synthase-like protein D3 (Q8GV00)	57.2/4.9e-09	D	D	N
G35	533	DR159681	Late nodulin (Q9LEF6)	44.5/3.2e-05	D	D	D
Primary and secondary metabolism							
G5	530	DR159684	Arabinogalactan protein (Q9LYF6)	51.9/1.9e-07	D	U	U
G8	223	DR159688	Phenylalanine ammonia-lyase class II (P19142)	373.6/2.7e-104	D	U	U
G15	643	DR159665	Beta 1,4*N*-acetylglucosaminyltransfe rase (Q599J1)	201.1/2.2e-52	D	D	D
G20	511	DR159670	UDP-glycosyltransferase 89B2e (Q6VAA5)	112.8/1.4e-25	D	D	D
G34	352	DR159680	Cyanogenic beta-glucosidase precursor (P26205)	201.8/1.4e-52	D	D	D
G36	314	DR159682	PhospholipaseA2 (Q9LYH2)	152.9/7.4e-38	D	D	N
G37	218	DR159683	Amino acid permease like (Q6H6H8)	254.3/2.2e-68	D	D	N

Table 3 AFLP, Northern blot and RT-PCR analyses of the differentially expressed TDFs in *nifA* mutant Sm1354 nodules at 30 days after inoculation (continued)

AFLP fragments	Size (bp)	GenBank Accession No.	Homology	BLAST score/e-value	AFLP	Northern blot	RTPCR
Unknown function protein							
G12	392	DR025668	Hypothetical protein (Q877Q8)	103.6/7.1e-26	D	U	D
G17	526	DR159667	Hypothetical protein (Q9SU16)	391.7/1.2e-111	U	D	D
G23	276	DR159673	Hypothetical protein (Q8LEF8)	247/1e-135	U	D	D
G29	537	DR159676	Hypothetical protein (Q8LEF8)	131.4/2.1e-31	U	D	D
No match							
G1	66	DR159661			D	D	N
G2	75	DR025666			D	D	N
G3	162	DR025667			D	D	D
G4	412	DR159684			D	D	N
G13	388	DR159664			D	D	D
G14	162	DR025669			D	D	N
G18	118	DR159668			D	D	N
G24	253	DR025670			D	D	D
G26	148	DR025671			U	D	D
G27	278	DR159675			D	D	N
G32	68	DR159679			D	D	N
G33	83	DR025673			D	D	N

U, up regulated; D, down regulated; N, untested.

shows an example of a typical cDNA-AFLP banding pattern. *Medicago sativa* is a tetrasomic plant and its sequence information was limited. However, the database of the diploid plant *Medicago truncatula* provided reliable and sufficient information for sequence comparison. TDF fragments that were only 50–300 bp long were subsequently extended to ~500 bp for Northern and RT-PCR experiments, if homologous sequences were available in public databases (Table 2). The extended cDNA fragments were obtained by RT-PCR, and then cloned into pMD18-T for sequence analysis. Through BLAST analysis, TDFs were organized into several categories according to their putative functions, including primary and secondary metabolism, signal communication, protein degradation, cell growth and development (Table 3).

Reverse Northern and RT-PCR verification

To assess the results of cDNA-AFLP analysis, gene expression patterns were first verified by reverse Northern blot. The results from hybridization-based methods were not consistent with cDNA-AFLP, as only 28 TDFs (75.6%) showed coincident expression patterns with those in AFLP experiments (Figure 2, Table 3). In order to confirm our results, RT-PCR was carried out on 24 TDFs, including all of the nine TDFs showing conflicting expression patterns between cDNA-AFLP and Northern blot results. All of the RT-PCR products were sequenced directly for verification. The results of 23 TDFs (95.8%) in RT-PCR experiments were consistent with those from Northern blot analyses, whereas the remaining G12 was consistent with the result from cDNA-AFLP. This indicated that reverse Northern blot is more reliable than cDNA-AFLP (Figure 3, Table 3).

Analysis of gene expression time series in nifA mutation nodules

The expression patterns of TDFs are dynamic in nodules. In Northern blots of the nodules 15 days after inoculation, the expression patterns of some TDFs differed from those 30 days after inoculation. Compared with wild-type nodules, the phospholipaseA2 (G36), receptor-like kinase (G25) and three unknown functional cDNA fragments (G3, G17, G18) showed no distinct difference in expression level in 15-day-old *nifA* mutant nodules. A senescence-related protein (G30) was down-regulated in 15-day *nifA* mutant nodules but up-regulated in 30-day nodules. In contrast, 13 TDFs (35%) including one nodulin (G22) and the G-protein α-subunit (G16) were up-regulated in 15-day nodules, and

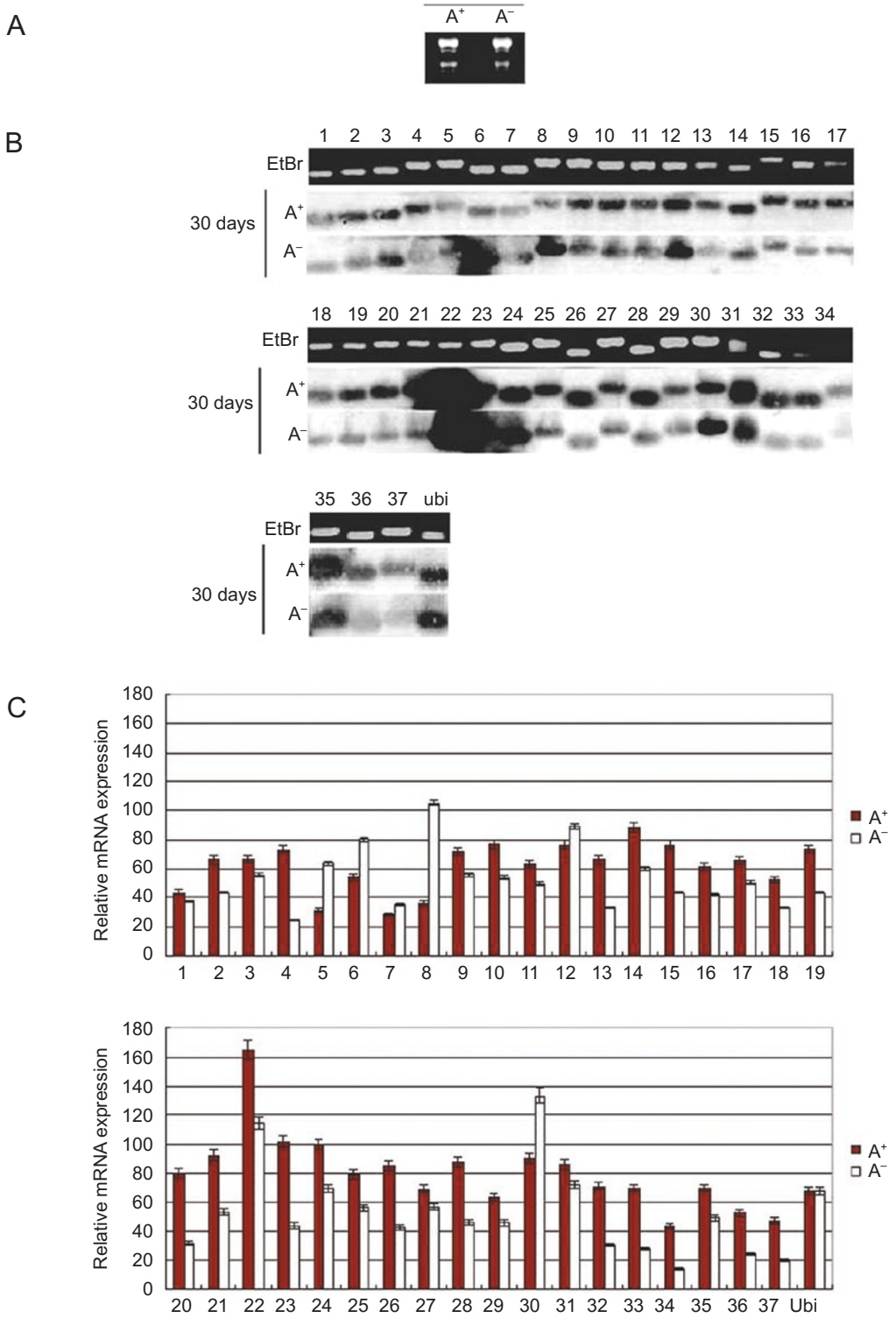

Figure 2 Reverse Northern blot analysis of TDFs. **(A)** Electropherogram of methanol denaturing gel electrophoresis of total RNA from 30-day-old nodules induced by wild-type Rm1021 (A^+) and *nifA* mutant Sm1354 (A^-). **(B)** Autoradiograms of reverse Northern hybridization. The duplicate membranes were hybridized against total cDNA probes resulting from wild-type Rm1021 and Sm1354 nodules, separately. Upper part show agarose gel electrophoresis of the 37 amplified cDNA fragments stained with EtBr. **(C)** Quantification of mRNA expression levels. Bands in their intensities were quantified by scanning and normalized with ubiquitin control. The differences in expressions were determined by Student's *t*-test. N=3, p<0.01.

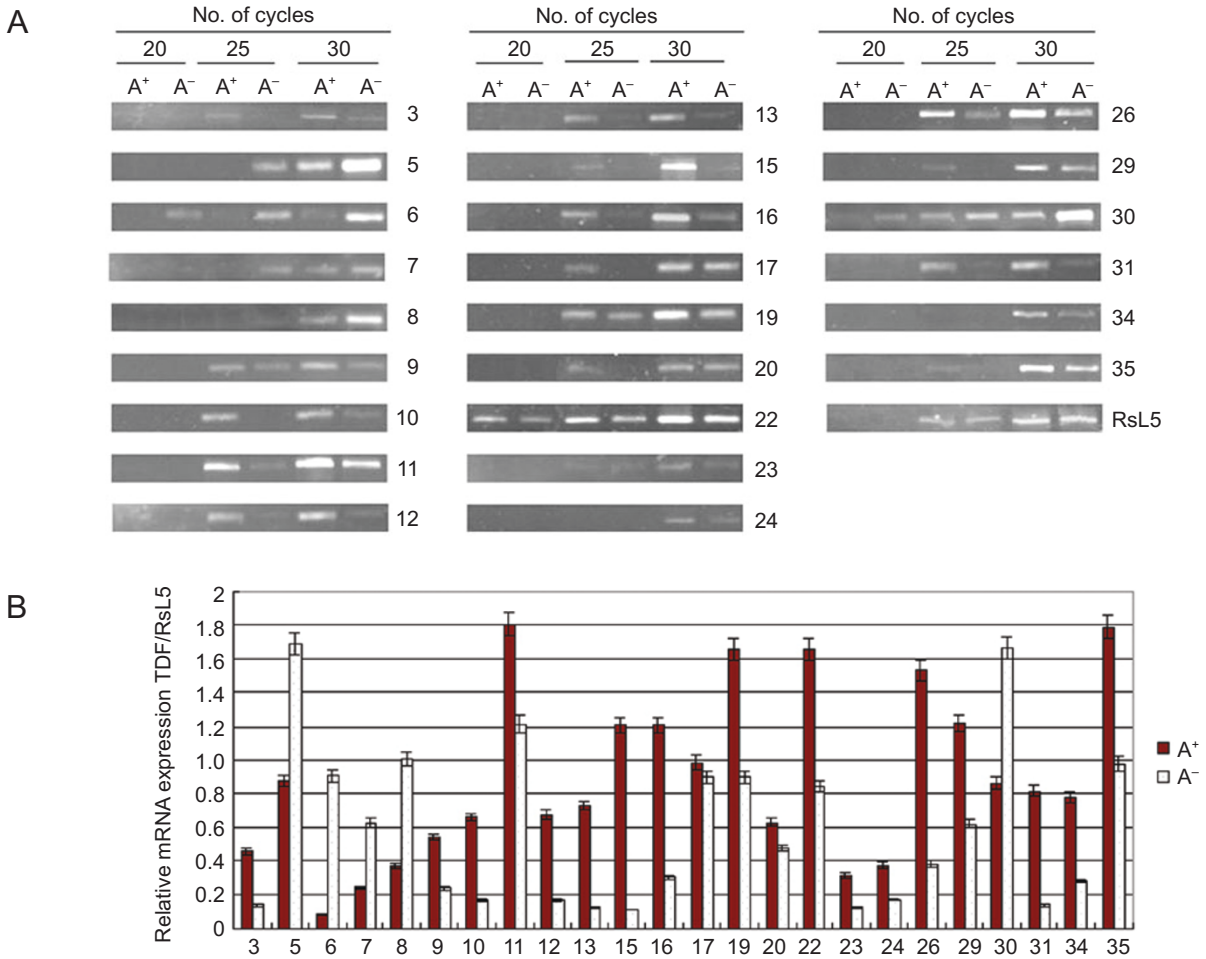

Figure 3 RT-PCR analysis of differentially expressed TDFs in 30-day-old nodules induced by wild-type Rm1021 (A^+) and *nifA* mutant Sm1354 (A-). **(A)** Agarose gel electrophoresis of PCR productions. The expressions of 24 TDFs were checked at 20, 25 and 30 cycles. RsL5, ribosome large subunit gene, was used as control. **(B)** Quantification mRNA levels. Bands in their intensities were quantified by scanning. The differences in expression rates were determined by Student's *t*-test. N=3, p<0.01.

yet they were down-regulated at 30 days (Figure 4).

Discussion

The cDNA-AFLP technique is a reliable method for revealing small differences between close individuals, especially in non-sequenced organisms. Since its first application on potato tuber development, several modifications of this technique have improved its validity and advantages over other fingerprinting techniques and DNA chip-based approaches [18-20]. In most cases, it has been employed for surveying transcriptional changes in host plants during interactions with fungi, bacteria, nematode or environmental stimuli [21-29]. In this study, cDNA-AFLP was successfully used to identify 37 differentially expressed host plant genes in Alfalfa nodules.

Among the 37 TDFs obtained in this study, six were found to be related to signal transduction. The ethylene response factor contains a core sequence of GCC box, which is recognized by ethylene-responsive element-binding proteins to regulate ethylene responses via regulating gene transcription and expression [30-32]. Calmodulin is recognized as a major calcium sensor and orchestrator of regulatory events through its interaction with a diverse group of cellular proteins, including cytoskeletal elements, ion channels, kinases and phosphatases. [33-35]. In *Arabidopsis* and rice plants, a wide range of processes including seed germination, shoot and root growth, and stomatal regulation are altered by mutations in G-protein components [36-41]. In Alfalfa, G proteins mediate the *nod*

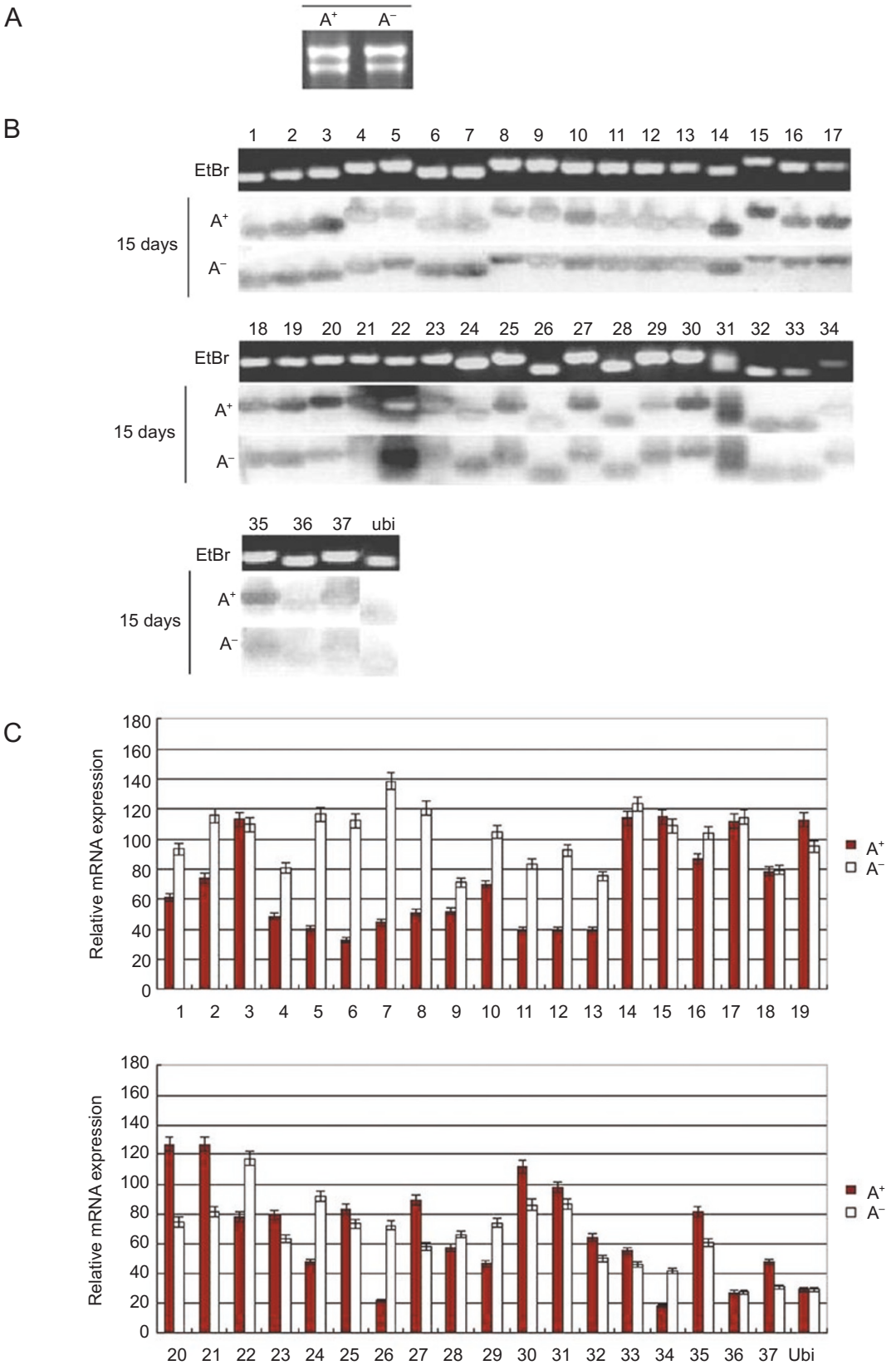

Figure 4 Same as **Figure 2**, except for 15-day-old nodules.

factor signaling pathway during symbiosis [42]. However, expression patterns of up-regulated G-protein-coupled receptors (G7) and down-regulated G-protein α-subunit (G16) indicate a complex regulation pattern of G proteins in nodules. The retinoid-interferon-induced mortality-19 gene (*GRIM*-19) and cytokine IK are novel cell death regulators characterized only in animals [43-45]. Further studies on their homologous genes in plants may provide new insights into apoptotic plant cell death.

The ubiquitin/26S proteasome pathway is a major route for the selective degradation of cytoplasmic and nuclear proteins in eucaryotes [46, 47]. At least three enzymes are involved in this action – a ubiquitin-activating enzyme (E1), a ubiquitin-conjugating enzyme (E2) and a ubiquitin ligase (E3) – which work sequentially in a cascade [48, 49]. In the present study, one ubiquitin carboxyl terminal hydrolase and one ubiquitin-conjugating enzyme (E2) were down-regulated in 30-day *nifA* mutant nodules. This indicates that the elaborate regulation of protein is damaged to some degree in *nifA* mutant nodules. The effects of protein degradation are not well understood in legume nodules and further study is needed.

The nutrient exchange between *Rhizobia* and legume plants is executed by a number of genes including those involved in primary and secondary metabolism. In *nifA* mutant bacteroids, a number of genes related to central intermediary metabolism are down-regulated in bacteroids 30 days after inoculation (unpublished data). In our study, seven host plant genes related to central intermediary metabolism were identified in *S. meliloti nifA* mutant nodules. Five genes were down-regulated in 30-day *nifA* mutant nodules, whereas the phenylalanine ammonia-lyase class II (G8) gene and arabinogalactan (G5) were up-regulated. PAL catalyses the non-oxidative deamination of L-phenylalanine to *trans*-cinnamic acid. This enzyme is ubiquitous in plants and is involved in the production of phenylpropanoids such as lignin and phytoalexins [50, 51]. The amino-acid permease (G37) is an integral membrane protein involved in the transport of amino acids into the cell. A study of *Saccharomyces cerevisiae* found that the amino acid permease AGP1 is dependent on an ubiquitin ligase complex, suggesting that an ubiquitination step is required for amino acid signal transduction [52].

The *nifA* gene exerts its pleiotropic nature in controlling further nodulation by eliciting host plant defense reactions at early stages in development [3]. In addition to the *nif/fix* genes, four nodulation-specific genes (*nodH*, *nodL*, *nolF* and *noeB*) were down-regulated in 30-day nodules induced by the *S. meliloti nifA* mutant (unpublished data). In our study, two late nodulin genes (G22, G35) were found to be down-regulated in *nifA* mutant nodules. In addition, the cellulose synthase-like protein D3 was also down-regulated. Its homologous gene in *Arabidopsis* is involved in biosynthesis of polysaccharides required during root hair elongation [53-55]. As root hair deformation is a defined procedure for nodule initiation, cellulose synthase-like protein D3 is likely to influence nodulation development. Considering our results together with previous findings, the data suggest that nodule development is affected by mutations of the *S. meliloti nifA* gene.

Although several molecular events taking place in *nifA* mutants are illustrated in the present study, there are not enough data to fully understand the relationship between these molecular events and the *nifA* gene. Even though time-dependent gene expression was deduced from the expression patterns of 15- and 30-day nodules, more studies are needed to elucidate the roles of these genes in nodule development.

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