

Isolation of cDNAs Encoding Asparagine Synthetase from *Coronilla rostrata*

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Abstract

Asparagine synthetase (AS) is one of the enzyme involves in ammonium metabolism in plant, it catalyzes the transfer of the amino group of glutamine to aspartate giving asparagine. Asparagine serves as a major nitrogen transport and storage molecule in many higher plants. This research used legume plant *Coronilla rostrata* callus culture for primary and secondary metabolism study. On primary metabolism, the culture were used to study the phenomenon of asparagine accumulation. Because of the responsiveness of the culture, we investigated the use of this culture for model system to conduct molecular biology study of the nitrogen metabolism. The aim of this study was to isolate...cDNAs encoding Asparagine synthetase from *C. rostrata* because of the asparagine accumulate in this culture. Several PCR based approaches were conducted such as RT-PCR, LM-PCR and RACE. Using these methods, two AS cDNAs was isolated from *C. rostrata*. AS1 (GenBank no AY081945) which a complete cDNA sequence and AS2 which a partial cDNA sequence. (GenBank no AF488726). These two cDNAs had high homology with the legume AS.

Keywords: cDNA, nitrogen metabolism, asparagine synthetase, *Coronilla rostrata*

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Introduction

For most plants, nitrogen is one of the major limiting nutrients required for growth. Many important processes in plant growth, such as seed germination and the production of protein-rich food, depend on the availability of sufficient nitrogen. Because of the crucial role that nitrogen plays in plant growth, a more detailed knowledge about biochemical and molecular events regulating nitrogen metabolism is required (Vance & Griffith, 1990).

The common sources of nitrogen for plants are nitrate from the soil (fertiliser, manure, and mineralisation of organic matter) and from the atmosphere (symbiotic N₂ fixation). Nitrate and N₂ have to be reduced to ammonium before further metabolism. Assimilation of inorganic nitrogen into organic matter is an important process for both plant growth and development (Oliveira *et al.*, 1997). Nitrogen has a great impact in determining the quality of plant products. It is an indispensable elementary constituent of numerous organic compounds including

nucleic acids, amino acids and proteins which are important for plant growth and crop production (Mengel & Kirkby, 1987; Mengel, 1992).

Essential studies include the characterization of the genes encoding the enzymes involved in nitrogen assimilation, the mechanism on how these genes are regulated, the pathways of nitrogen assimilation and the identification of the rate-limiting enzyme(s) in the pathways. Overall, by means of genetic manipulation the amount of nitrogen can be regulated. Since nitrogen is usually the rate-limiting element in plant growth, increasing its availability should improve crop quality (Oliveira *et al.*, 1997).

Asparagine synthetase (AS) is one of the enzyme involve in ammonium metabolism, it catalyzes the transfer of the amino group of glutamine to aspartate giving asparagine. Asparagine serves as a major nitrogen transport and storage molecule in many higher plants (Lam *et al.*, 1995; Oliveira *et al.*, 1997; Lea *et al.*, 2007; Gaufichon *et al.*, 2010).

It is known that many enzymes involved in plant metabolism occur as multiple

isoenzymes, asparagine synthetase can result from either the ASN1, ASN2 or ASN3 genes (Oliveira *et al.*, 1997). While the ASN2 gene product is active under light conditions, the ASN1 gene product works in the dark (Lam *et al.*, 1995). In sun flower (HAS1, HAS1.1 and HAS2) (Herrera-Rodríguez *et al.*, 2004) and tobacco (Beato *et al.*, 2010), light, carbon and nitrogen availability control asparagine synthesis by regulating their AS genes. It is also found that in sun flower expression of the AS genes also affected by environmental stressed such as osmotic stress, salt stress and heavy (Herrera-Rodríguez *et al.*, 2007).

Many studies of asparagine synthetase have been carried out in nodulated legumes including lupin (Shelp & Atkins, 1984), soybean (Huber & Streeter, 1985), and alfalfa (Ta *et al.*, 1988). Others have reported asparagine synthetase activity from soybean seedlings (Streeter, 1973), various plant parts of lupin cotyledons (Rognes, 1975; 1980), maize and soybean cotyledons (Oaks & Ross, 1983), and pea leaves (Joy *et al.*, 1983).

Tsai & Coruzzi (1990, 1991) have successfully used a molecular biological approach to study this enzyme. From a pea cDNA they have sequenced two glutamine-dependent asparagine synthetase genes (AS1 and AS2). The sequence showed high homology with the human asparagine synthetase enzyme (Tsai & Coruzzi, 1990). In their work they observed that high levels of AS1 mRNAs was induced by a dark treatment. They also observed high levels of AS1 and AS2 mRNAs in cotyledons of germinating seeds and nitrogen fixing root nodules. This correlates well with asparagine's role in nitrogen transport during plant development (Tsai & Coruzzi, 1990). They have suggested that the negative regulation of AS1 and AS2 genes by light is a general phenomenon in plants (Tsai & Coruzzi, 1991). Since then many other AS genes has been reported from other plants such as *Arabidopsis thaliana* (Lam *et al.*, 1995), *Lotus japonicus* (Waterhouse *et al.*, 1996), *Glycine max* (Hughes *et al.*, 1997; Yamagata *et al.* 1998), *Phaseolus vulgaris* (Osuna *et al.*, 1999), *Hordeum vulgare* (Moler *et al.*, 2003), *Zea mays* (Todd *et al.*, 2008).

In our lab we have been using *C. rostrata* callus culture for primary and secondary metabolism study. On primary metabolism the culture were used for study the phenomenon of

asparagine accumulation (Temple, 1985; Artanti, 1993). Whereas for secondary metabolism the culture, were used to study the nitro propanoic acid production. Because of the responsiveness of the culture we aimed to use this culture for model system to conducted molecular biology study of the nitrogen metabolism. The first step to pursue this objective was by isolating and sequences the genes that involve in ammonium assimilation. The aim of this research was to isolate the gene/cDNA encoding asparagines synthetase from *Coronilla rostrata*, as starting point for further molecular studies of this nitrogen metabolism enzyme using this culture system. Asparagine synthetase was chosen to be the first gene/cDNA to isolate, because of the asparagine accumulate in this culture, and literature stated that assay of this enzyme is difficult to be conducted because of the present of inhibitor in the extraction. Therefore, molecular approach is another alternative to study this enzyme.

Materials and Methods

Plant Materials. *Coronilla rostrata* Boiss. Sprun seeds were initially obtained from Kew Botanical gardens. For the work reported here with *C. rostrata*, *ex hortico* F1 seeds derived from the Kew seeds were used. A voucher specimen of a mature *C. rostrata* plant producing F1 seeds has been lodged with the UNSW Herbarium (22325). *C. rostrata* callus was initiated from leaf explants of about 5 cm tall aseptic seedling. The medium used for callus intiation and maintenance was modified of Murashige & Skoog (1962) medium supplemented with 10 μ M NAA and 1 μ M kinetin. Cultures were grown at 25°C constant temperature room with continuous cool white fluorescent light. Callus of *Acacia holocericca*, *A. melanoxydon*, *A. podalyriifolia*, and *A. salicina*, were a gift from other fellow lab member of this laboratory (Plant Cultures Lab., School of Biochemistry and Molecular Genetics, UNSW).

Oligonucleotides. Oligo dT₍₁₂₋₁₈₎ was obtained from Life Technologies (Melbourne, VIC). Other primers, unless otherwise stated, were custom-synthesised (desalted) by Life Technologies (Melbourne, VIC). Asparagine synthetase primers were as described by

Dransfield (1994). The primers for LM-PCR as described by Siebert *et al.* (1995) were custom-synthesised by Operon Technologies Inc. (USA). The cDNA specific primers were used for the RACE-PCR protocol based on Frohman (1995). The RACE adaptor primers were also based on Frohman (1995). The cDNA specific primers were used for the RACE-PCR protocol using GeneRacer™ kit. Primers of pUC/M13 for plasmid sequencing were purchased from Promega (USA) or custom-synthesised (desalted) by Life Technology (Melbourne, VIC). The following primers were *C. rostrata* cDNA specific primers for sequencing clone of PCR products using the primer pair CRAS3 and 2: NACAS32C1F (5'-ACTTCCCTCTACATTGGTT GG-3') and NACAS32C1R (5'-CATGGTG GACAGTCCCAAGAT-3'). Oligo nucleotide primers used in these studies are designed based on conserved region of published AS cDNA sequences from plants determined using ClustalW in ANGIS. Some are based on specific *C. rostrata* sequences obtained from sequencing results and analysed using Amplify v.1.2.

DNA and RNA extraction. DNA was extracted using either the procedure of Doyle and Doyle (1991), Graham *et al.* (1994) or the Qiagen DNeasy kit. Total RNA was extracted using TRI Reagent™.

General Polymerase Chain Reaction (PCR). Typically PCR was carried out in a total volume of 50 µl containing 1× PCR buffer, 3 mM MgCl₂, 0.8 mM dNTPs, 0.2 U *Taq* polymerase, 10 pmoles of each primer and 1 µl of genomic DNA (*ca.* 25-100ng) as template. For some experiments various MgCl₂ and template concentrations were tested to improve the yield of product. The PCR reaction was performed using either a Corbett Research FTS 960 Thermal Sequencer with hot bonnet or a STRATAGENE RoboCycler Gradient 96 Temperature Cycler. Generally the following PCR conditions were used: 1 cycle of denaturation at 94°C for 1 min 30 sec, annealing at 45°C for 1 min and extension at 72°C for 1 min. This was followed by 30 cycles of denaturation at 94°C for 45 sec, annealing at 45°C for 45 sec, extension at 72°C for 45 sec, with a final extension at 72°C for 4 min. For some experiments various annealing

temperatures were tested to improve the yield of product.

Reverse Transcriptase -PCR (RT-PCR). Approximately 5 µg total RNA and 0.25 µg oligo dT₍₁₂₋₁₈₎ were incubated for 15 min at 70°C and cooled to 42°C. The following were added (final concentration) dNTPs (0.8 mM), DTT (10 mM), 1× PCR buffer, MgCl₂ (3 mM) in a total volume of 20 µl. The reaction was incubated for 5 min at 42°C, 25 U (0.5 µl) of MuLV reverse transcriptase was added and the incubation continued for another 50 mins at 42°C. The reaction was stopped by heating at 70°C for 15 min.

PCR of cDNA. PCR component and conditions for amplifying from cDNA were as described in General PCR method above, except that 1 µl of the RT reaction mixture was used as the source of template DNA.

Ligation mediated PCR (LMPCR). Ligation-mediated PCR was conducted using a modification method by Siebert *et al.* (1995). *C. rostrata* genomic DNA were digested using *Dra*I, *Eco*RV, or *Pvu*II and the adaptor ligated to the fragments at 37°C overnight. LMPCR was carried out in a total volume of 20 µl containing 1× PCR buffer, 1.5 mM MgCl₂, 0.8 mM dNTPs, 35 mM KCl, 1 U *Taq* polymerase and 0.1 U Pfu-Turbo *Taq* polymerase, 10 pmoles of each primer and 40 ng of digested and adaptor ligated DNA as template. The LMPCR reactions were performed using either a Corbett Research FTS 960 Thermal Sequencer with hot bonnet or a STRATAGENE RoboCycler Gradient 96 Temperature Cycler. PCR conditions used were: 1 cycle of denaturation at 95°C for 3 min followed by 40 cycles of denaturation at 95°C for 30 sec, annealing and extension at 63°C for 5 min with a final extension at 63°C for 10 min.

Rapid amplification of cDNA ends by PCR (RACE PCR). The method used for rapid amplification of cDNA ends was modified from the method for 5' and 3' RACE described by Frohman (1995). For 3' RACE, the RT reaction was as described in RT-PCR method above, except that oligo dT was replaced by QT PCR conditions were as described in General PCR method above using the primers Q0 and a RACE specific primer

for the appropriate target gene from *C. rostrata*. If the yield of PCR product was low (faint band on agarose gel electrophoresis), a second round of PCR using primers Q1 and the RACE specific primers was performed. For 5' RACE, RT reaction was as described in General PCR method above, except that oligo dT was replaced by RACE specific primers for the appropriate target gene from *C. rostrata*. The RT reaction product was purified using the GeneClean kit. The recovered volume was adjusted to 10 µl, and the following reagents added 5× TdT tailing buffer (4 µl), 25 mM CoCl₂ (1.2 µl), 1 mM dATP (4 µl) and 10 units of TdT. The reaction mixture was incubated for 5 min at 37°C followed by 5 min at 65°C. The tailed cDNA was purified using the GeneClean kit and the recovered volume was adjusted to 50 µl. PCR was carried out in a total volume of 50 µl containing 1× PCR buffer, 3 mM MgCl₂, 0.8 mM dNTPs, 1 U Taq polymerase, 10 pmoles of RACE primer, 10 pmoles of Q0, 2 pmoles of QT and 1 µl of tailed cDNA. The following PCR conditions were used: 1 cycle of denaturation at 94°C for 5 min, annealing at 50°C for 2 min and extension at 72°C for 40 min. This was followed by 30 cycles of denaturation at 94°C for 1min, annealing at 50°C for 1min, extension at 72°C for 1min, with a final extension at 72°C for 10 min.

GeneRacer Kit. The method used for 5' and 3' rapid amplification of cDNA ends using Gene Racer Kit was according to manufacturer procedure Version D.

Purification of PCR Products from Agarose Gels. Under low intensity UV illumination, PCR product fragments were excised from EtBr stained agarose gels using a sterile surgical blade. The PCR product was recovered from the gel using either the QiaQuick Gel Extraction kit or GeneClean® kit following the manufacturer procedure.

Cloning and Transformation. Purified PCR products were cloned using the pGEM®-T Vector System II kit (PROMEGA) according to the manufacturer's procedure. *E. coli* strain JM109 competent cells were provided in the kit and transfections were carried out according to the manufacturer's procedure using SOC medium. Blue and white colonies selection for transformants was conducted on

LB agar plates containing ampicillin, IPTG, and X-Gal After overnight incubation at 37°C, putative transformants carrying the recombinant vector were identified as white colonies. Putative clones were verified by colony lift PCR using appropriate primers.

Plasmid Preparation. Putative clones were grown over night in LB medium. Plasmids were isolated using QIAprep® Spin Miniprep kit (QIAGEN) according to the manufacturer's procedure.

Sequencing. Purified PCR products and plasmid from positive clones were sequenced using the automated fluorescent sequencing facility in the School of Biochemistry and Molecular Genetics at the University of New South Wales. Sequencing reactions were performed using the ABI Prism™ Big Dye™ Terminator Cycling Sequencing Ready reaction Kit with one of the appropriate primers for purified PCR products or M13 universal primer for plasmid on clones. When PCR reactions were performed using either a Corbett Research FTS 960 Thermal Sequencer with hot bonnet or Perkin Elmer, the following PCR conditions were used: 1 cycle of denaturation at 96°C for 5sec, follow by 25 cycles denaturation at 96°C for 10 sec, annealing 50°C for 5 sec and extension at 60°C for 4 min. Otherwise when PCR reactions were performed using a STRATAGENE RoboCycler Gradient 96 Temperature Cyclers, the following PCR conditions were used: 1 cycle of denaturation at 96°C for 10 sec, follow by 30 cycles denaturation at 96°C for 20 sec, annealing 50°C for 20 sec and extension at 60°C for 4 min. Sequencing products were separated on the ABI PRISM 377 DNA sequencer machine (Applied Biosystems).

Computational Analysis. Sequencing result were analysed using ABI Prism™ Sequencing 2.1.1, DNA Strider 1.2 and programs provided on The Australian National Genomic Information Service (ANGIS) server (CLUSTALW, BLAST N, BLAST P and FASTA) (<http://www.angis.org.au/WebANGIS/>).

Results

Primer design

Primers for AS used in this project were previously designed by Ngan (1992), Dransfield (1994) and Schibrowski (1998), new design primers for RACE PCR. The position of the primers based on published *Pisum sativum* (X 52179) AS sequence and simulation of the expected PCR product sizes analyses using Amplify ver 1.2 is shown in Figure 1.

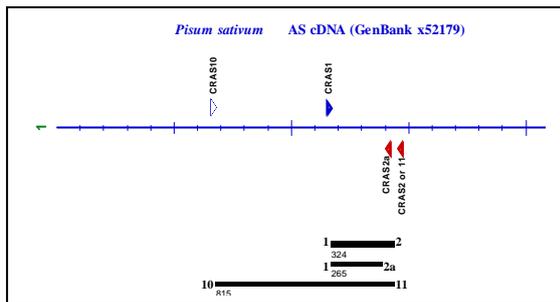


Figure 1. Position and expected size of PCR product for AS cDNA.

PCR and RT-PCR Approach

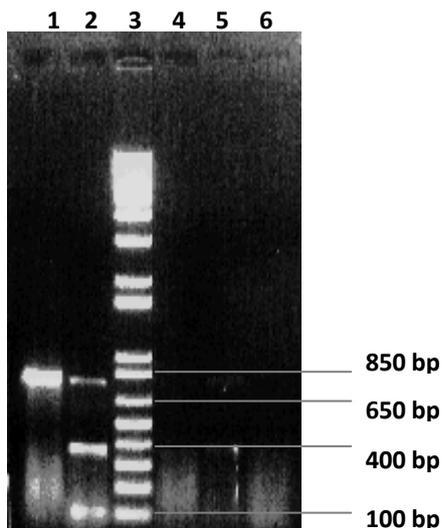
Using *C. rostrata* total RNA as template for RT step for the RT-PCR approach with primers previously designed, desired PCR products were obtained from primer pairs that not gave any products when genomic DNA

was used as template. Whereas primer pairs that gave product using genomic DNA also gave expected size of cDNA. These primers were also used for RT-PCR using RNA from several Acacias species to show whether this primer could be used for amplification of AS from other legume or only specific for *C. rostrata*. Figures 2 and 3 and Table 1 show typical result of the RT-PCR for AS.

The 3' region of AS1 cDNA were obtained using home made RACE PCR reagent, whereas further 5' region of AS1 and AS2 cDNA were obtained using the commercial kit for RACE PCR from Invitrogen. The results is shown in Figures 4 and 5.

Sequencing and Sequence Analysis

Direct sequencing was conducted to check whether or not the PCR product obtained is the desired product. For AS cDNA, direct sequencing on PCR product in this study from primer pair CRAS10 and 11 only gave very short good quality sequence as shown in Table 2. Although only short sequences obtained, BLASTN analysis of that sequences showed that those sequences is part of AS cDNA sequences. Therefore, it is necessary to clone the PCR product for sequencing AS cDNA.



- Lane 1 : CRAS10 & 11; *C. rostrata*
- Lane 2 : CRAS10 & 11; *A. holosericea*
- Lane 3 : 1 kb Plus Ladder
- Lane 4 : CRAS10 & 11; *A. melanoxylon*
- Lane 5 : CRAS10 & 11; *A. podalyriifolia*
- Lane 6 : CRAS10 & 11; *A. salicina*

Note: expected PCR product band is ~ 800 bp, band present ~ 400 bp is a false positive

Figure 2. RT-PCR results for AS primer pair CRAS 10 & 11 using total RNA from callus of *C. rostrata* and Acacia species.

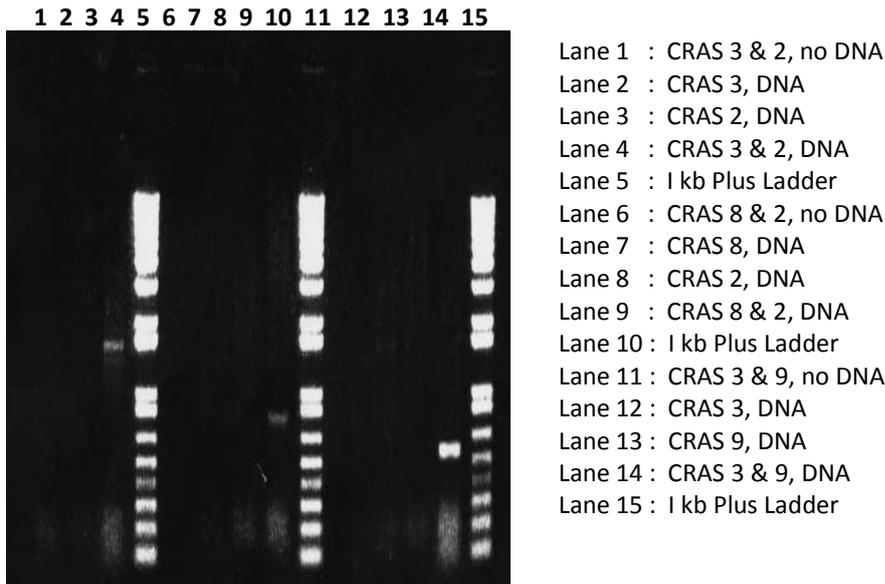


Figure 3. RT-PCR results for AS primer pair CRAS 3 & 2, 8 & 2 and 3 & 9 using total RNA from callus of *C. rostrata*

Table 1. Results on different primer pairs for AS using RT product as PCR template.

Species	Tissue	Primer pairs	Amplicon and description
<i>C. rostrata</i>	callus	CRAS 1 and 2	yes; bright
		CRAS 1 and 2a	yes; bright
		CRAS 10 and 11	yes; bright
<i>A. holocericea</i>	callus	CRAS 10 and 11	yes; bright
<i>A. melanoxylo</i>	callus	CRAS 10 and 11	No
<i>A. podalyriifolia</i>	callus	CRAS 10 and 11	yes; dim
<i>A. salicina</i>	callus	CRAS 10 and 11	No

Note: bright means band clearly show on the gel, dim means band only faintly showed on the gel

Table 2. Direct sequencing results of primer pair CRAS 10 and 11 (815bp)

Species	Partial sequenced size (bp)	Best BLASTN search result
<i>C. rostrata</i>	87	<i>Lotus japonicus</i> AS (X89409); cDNA
<i>A. podalinifolia</i>	51	<i>Pisum sativum</i> AS (X52180); cDNA

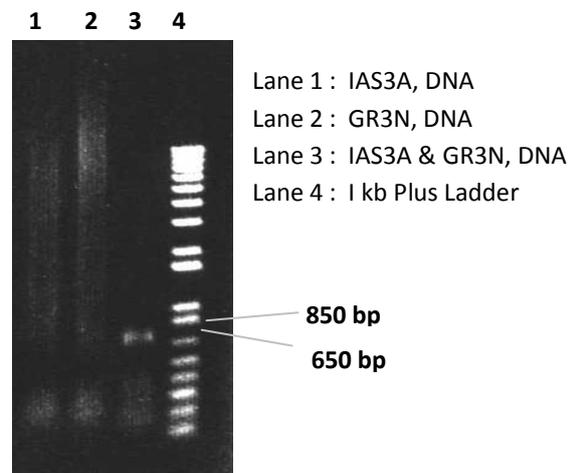


Figure 4. 3'RACE PCR result for primer RCAS1 using total RNA from callus of *C. rostrata*

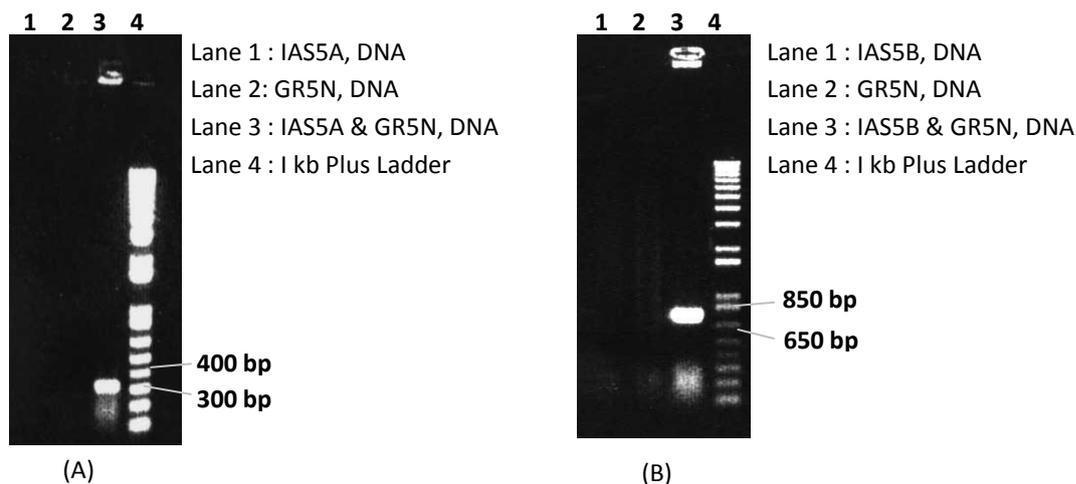


Figure 5. 5'RACE PCR result using total RNA from *C. rostrata* callus for AS1 (A) and AS2 (B)

Discussion

The RT PCR results obtained showed that the primers designed could be used for *C. rostrata* and *A. holocericea* and *A. podalyrifolia* to give the desired products but no products obtained from *A. melanoxyton* and *A. salicia*. Although further sequencing was not conducted for *Acacia* species, this result suggests that *C. rostrata* AS cDNA may have

relatively high homology with *A. holocericea* and *A. podalyriifolia* than with *A. melanoxyton* and *A. salicia*. Comparison of *C. rostrata* AS1 and AS2 cDNA sequences with other AS using BLASTN analysis is shown in Figures 6 and 7. The deduced amino acid sequences analysed using BLASTP is shown in Figures 8 and 9.

Sequences producing significant alignments:	Score	E
	(bits)	Value
emb X89409.1 LJAS1GENE <i>L. japonicus</i> mRNA for asparagine synthetas	2498	0.0
emb AJ009952.1 PVU9952 <i>Phaseolus vulgaris</i> mRNA for asparagine sy	1911	0.0
gb U77678.1 GMU77678 <i>Glycine max</i> asparagine synthetase 2 (AS2) m	1907	0.0

Figure 6. Best three sequences match of BLASTN result for *C. rostrata* AS1

Sequences producing significant alignments:	Score	E
	(bits)	Value
emb X89410.1 LJAS2GENE <i>L. japonicus</i> mRNA for asparagine synthetas	1639	0.0
gb U55874.1 GMU55874 <i>Glycine max</i> asparagine synthetase mRNA, com	1298	0.0
emb AJ133522.1 PVU133522 <i>Phaseolus vulgaris</i> mRNA for asparagines	1275	0.0

Figure 7. Best three sequences match of BLASTN result for *C. rostrata* AS2

Sequences producing significant alignments:	Score	E
	(Bits)	Value
sp P49092.2 ASNS1 LOTJA RecName: Full=Asparagine synthetase [...	1149	0.0
gb ABU95104.1 asparagine synthetase [<i>Phaseolus vulgaris</i>]	1148	0.0
ref NP_001235721.1 asparagine synthetase 2 [<i>Glycine max</i>] >gb...	1126	0.0

Figure 8. Best three sequences match of BLASTP result for *C. rostrata* AS1

Sequences producing significant alignments:	Score	E
	(bits)	Value
emb X89410.1 LJAS2GENE <i>L. japonicus</i> mRNA for asparagine synthetas	1639	0.0
gb U55874.1 GMU55874 <i>Glycine max</i> asparagine synthetase mRNA, com	1298	0.0
emb AJ133522.1 PVU133522 <i>Phaseolus vulgaris</i> mRNA for asparagines	1275	0.0

Figure 9. Best three sequences match of BLASTP result for *C. rostrata* AS2

Sequences obtained from this study were *C. rostrata* of AS1 (AY081945) complete cDNA sequence and AS2 (AF488726) cDNA partial CDNA sequence, Nucleotide sequences were translated into protein sequences using STRIDER version 1.2 than identity of the sequences were based on analysis using BLASTN and BLASTP in ANGIS.

Although many sequences available on GeneBank database (www.ncbi.nlm.nih.gov) from other legume species especially for GS and AS, only 8 other sequences reported for the genera of *Securigera* or *Coronilla* beside the ones submitted from our research group, and none of them is from *Securigera parviflora* which is the accepted name for *C. rostrata* according to International Legume Database and Information Service (www.ildis.org/LegumeWeb) and none of them are ammonium assimilation genes.

C. rostrata AS1 complete cDNA sequence has 93% identity with AS1 cDNA of *Lotus japonicus* (X89409) and protein sequence shows 94% identity with *L. japonicus* AS1 protein. This partial sequence of *C. rostrata* AS cDNA has been lodge into Genbank database (AY081945) which contain 5' untranslated region, complete coding region, stop codon (TGA), 3' untraslated region, and polyA tail.

C. rostrata AS2 partial cDNA sequence has 90% identity with AS2 cDNA of *Lotus japonicus* (X89410) and protein sequence shows 93% identity with *L. japonicus* AS2 protein. This partial sequence of *C. rostrata* AS2 cDNA has been lodge into Genbank database (AF488726) which contain partial coding region.

The nucleotide sequence of *C. rostrata* AS1 and AS2 has high homology with the other plant AS with the best three nucleotides sequences according to BLASTN analysis are legume AS. The deduced amino acid sequence of both *C. rostrata* AS contained the purF-type Gln binding domain, consisting of three triad forming residues (Cys2, Asp 34, His 104) (Mei & Zalkin, 1989). Other residues important for the glutamine binding and positioning identified from the *E. coli* Asn B enzyme are Arg50, Leu1, Ile53, assn 75, Gly76, Glu77 and Asp 98 (Larsen *et al.*, 1999) and essential residues for binding of aspartate and ATP, Thr316, Thr317, Arg318 and Cys 523 (Boehlein *et al.*, 1997a,b) are also present in both *C. rostrata* AS sequences. Based on

sequence similarity and characteristic, it is assume that both isoform use glutamine as their primary substrate.

As conclusion, in this study, two different AS cDNA sequences were obtained from *C. rostrata* and designated as AS1 and AS2. Complete cDNA AS1 *C. rostrata* (GenBank AY081945) was obtained whereas for only partial AS2 cDNA (GenBank AF488726) sequence was obtained. This finding is important to be use for further studies of plant nitrogen metabolism in general and better understanding of the asparagine synthetase role. The present of many patents and current patents application on development of transgenic plants which include addition of asparagine synthetase genes show the potential commercial value of these genes (US 6846969; US20110293815; US20110321197).

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