Dark-induced and organ-specific expression of two asparagine synthetase genes in *Pisum sativum*

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Nucleotide sequence analysis of cDNAs for asparagine synthetase (AS) of *Pisum sativum* has uncovered two distinct AS mRNAs (AS1 and AS2) encoding polypeptides that are highly homologous to the human AS enzyme. The amino-terminal residues of both AS1 and AS2 polypeptides are identical to the glutamine-binding domain of the human AS enzyme, indicating that the full-length AS1 and AS2 cDNAs encode glutamine-dependent AS enzymes. Analysis of nuclear DNA shows that AS1 and AS2 are each encoded by single genes in *P. sativum*. Gene-specific Northern blot analysis reveals that dark treatment induces high-level accumulation of AS1 mRNA in leaves, while light treatment represses this effect as much as 30-fold. Moreover, the dark-induced accumulation of AS1 mRNA was shown to be a phytochrome-mediated response. Both AS1 and AS2 mRNAs also accumulate to high levels in cotyledons of germinating seedlings and in nitrogen-fixing root nodules. These patterns of AS gene expression correlate well with the physiological role of asparagine as a nitrogen transport amino acid during plant development.

Key words: asparagine synthetase/gene expression/light regulation/nitrogen assimilation/plants

Introduction

In *Escherichia coli* and yeast, the genes for enzymes along amino acid biosynthetic pathways have been well characterized by combined genetic, biochemical and molecular approaches. Until recently studies concerning plant amino acid biosynthetic enzymes have been limited to biochemical approaches. Traditionally, biochemical investigations have been directed at characterizing plant nitrogen metabolic enzymes in terms of their reaction mechanism, number of isozymes, subcellular localizations and roles during plant development (Mifflin, 1980). For several enzymes such as nitrate reductase (Crawford *et al.*, 1986), nitrite reductase (Back *et al.*, 1988) and glutamine synthetase (Cullimore *et al.*, 1984; Tingey *et al.*, 1987), detailed biochemical studies have provided the basis for the isolation and characterization of their corresponding genes. However, for many other important nitrogen metabolic enzymes in higher plants, difficulties encountered in biochemical purification have prevented the characterization of the enzymes and hence their cognate genes.

One amino acid biosynthetic enzyme that has proven to be particularly recalcitrant to biochemical analysis is plant asparagine synthetase (AS; EC6.3.5.4). While asparagine

was the first amino acid to be discovered in plants (Vauquelin and Robiquet, 1896), the enzyme responsible for its synthesis remains poorly understood to date. Asparagine, synthesized from aspartate and glutamine, is the major nitrogen transport amino acid in plants faced with conditions of excess ammonia rather than nitrate. During normal plant growth, conditions of ammonia excess occur under a variety of circumstances which include growth on fertilizers, during nitrogen-fixation, during germination and during seed formation in senescing plants (Lea and Fowden, 1975). In certain species, asparagine can account for up to 86% of transported nitrogen in the above contexts (Lea and Mifflin, 1980). Levels of asparagine transported in a plant are also affected by external factors such as light. Since asparagine has a higher N:C ratio than glutamine, it is a more economical nitrogen transport compound in plants grown in the dark, when carbon skeletons are limiting. Analysis of phloem exudate in *Pisum sativum* reveals that levels of asparagine are higher in phloem exudates from dark-treated versus light-grown plants (Urquhart and Joy, 1981). Consistent with these results is the finding that AS enzyme activity detected in extracts of pea leaves is also higher when plants are grown in the dark (Joy *et al.*, 1983).

Although asparagine plays a crucial role in plant nitrogen transport, the enzyme responsible for its biosynthesis is poorly characterized and has not been purified to homogeneity. The inability to purify the AS enzyme from plants is due, in part, to the fact that the plant AS enzyme is extremely unstable in partially purified extracts (Rognes, 1975; Huber and Streeter, 1984, 1985). In addition, plant extracts contain contaminating asparaginase activity (Streeter, 1977) and specific non-protein inhibitors of AS (Joy *et al.*, 1983) which compound the difficulties in assaying for AS enzyme activity *in vitro*. The inability to purify the plant AS enzyme has made it impossible to precisely characterize the number of AS isozymes in plants, their subcellular localizations and the corresponding gene(s) encoding AS.

To circumvent the problems encountered via biochemical studies on plant AS, we have used a molecular biological approach to directly clone AS cDNAs from *P. sativum*. These plant AS cDNA clones may now be used in a 'reverse-biochemical' approach to characterize the encoded AS enzymes as well as to examine the regulated expression of their corresponding genes in higher plants. Here, we report the isolation and characterization of two classes of plant AS cDNAs (AS1 and AS2) which encode homologous but distinct AS polypeptides. Gene-specific DNA probes were used to monitor the steady-state levels of AS1 and AS2 mRNAs in leaves of light- or dark-grown plants and in various organs during plant development. These studies have shown that the expression of the AS genes in plants are affected by light and developmental conditions of increased nitrogen transport. The regulated expression of the AS genes shows a strong correlation with previous physiological data concerning asparagine transport during plant development.

Results

Isolation and characterization of two distinct AS cDNAs from pea

A cDNA clone encoding human AS (pH131) (Andrulis *et al.*, 1987) was used to isolate cDNAs encoding plant AS from a pea nodule cDNA library. From 50 positive clones identified out of 2×10^5 clones screened, eight clones (λ AS301– λ AS308) were randomly selected for further analysis. Restriction mapping and nucleotide sequence analysis of these clones revealed that all eight contained cDNA inserts which correspond to overlapping portions of a single mRNA species (AS1) (Figure 1A). A cDNA containing the 5' end of the AS1 mRNA (λ AS907) was synthesized *in vitro* using an oligonucleotide primer complementary to the 5' end of λ AS301 (see Materials and methods). The restriction maps of the three overlapping cDNA clones which include the entire AS1 coding region are shown in Figure 1A. The composite full-length AS1 cDNA, pcAS1 (2.2 kb), was constructed by sequential ligation of the restriction fragments (fragments a, b and c) from the three overlapping cDNA clones λ AS907, λ AS301 and λ AS305 respectively as indicated in Figure 1A.

A second type of AS coding sequence (AS2) was detected in peas when a DNA fragment from the coding region of an AS1 cDNA was used to screen a pea genomic library. cDNA clones encoding the AS2 mRNA were subsequently isolated from a pea root cDNA library using an AS2 genomic fragment as a DNA probe. The longest AS2 cDNA clone, λ AS201, which contains a 1.5 kb cDNA insert, was selected for further analysis (Figure 1B). A cDNA containing the 5' end of the AS2 mRNA (pcAS801) was synthesized

in vitro by anchored polymerase chain reaction (A-PCR) using an oligonucleotide primer complementary to the 5' end of λ AS201 as described in Materials and methods. The restriction map of the full length cDNA of AS2 (cAS2) was deduced from the overlapping partial cDNA clones λ AS201 and pcAS801.

The nucleotide sequences of the full-length AS1 and AS2 cDNAs are shown in Figure 2. pcAS1 is 2200 nt long and starting with the first in-frame methionine encodes a protein of 586 amino acids with a predicted mol. wt. of 66.3 kd. The 3' non-coding region of AS1 cDNA is 333 nt long and contains a poly(A) tail (Figure 2A). cAS2 is 2002 nt long and encodes a protein of 583 amino acids with a predicted mol. wt. of 65.6 kd. The 3' non-coding region of cAS2 is 141 nt long and contains a poly(A) tail (Figure 2B). Nucleotide sequence homologies among pea cDNAs of AS1, AS2 and human AS cDNA (pH131) (Andrulis *et al.*, 1987) were compared using the 'fasta' computer program (Pearson and Lipman, 1988). The two pea AS cDNAs (AS1 and AS2) are highly homologous to each other at the nucleotide level within their coding regions (81%) and completely divergent in the 3' non-coding regions. The overall nucleotide homology between either AS cDNA of pea and the AS cDNA of human is ~50–55% within the coding regions. Neither pea AS cDNA shares significant homology to the asparagine synthetase (*asnA*) gene of *E. coli* (Nakamura *et al.*, 1981) (not shown).

The deduced amino acid sequences for the pea AS1, pea AS2 and human AS polypeptides are compared in Figure 3. The polypeptides encoded by AS1 and AS2 cDNAs share an overall homology of 86% at the amino acid level. A

A. AS1 cDNAs

B. AS2 cDNAs

Fig. 1. Restriction maps of AS1 and AS2 cDNA clones. E, *EcoRI*; S, *SstI*; Bm, *BamHI*; Bg, *BglII*; H, *HincII*; Bs, *BstNI*. E⁻ represents an *EcoRI* site in λ AS301 which was destroyed in the process of cloning and selection. Open bars represent the coding regions of each cDNA. (A) pcAS1 (2.2 kb) is a composite full-length AS1 cDNA constructed from restriction fragments (a, b and c) of overlapping cDNA clones λ AS907, λ AS301 and λ AS305. (B) Overlapping cDNA clones pcAS801 and λ AS201 for AS2 mRNA were used to derive the restriction map of full-length cAS2.

A

1	CTA CGT GTT GCT TCT TCC ACA CTC TTT GCT CCT AGT TTT TCG TGT CTT GTT TTC TTT ATC CTC TTC TCA TTC TCT TTG GTT CTT	84
1	M C G I L A V L G C S D D S Q A K R V R I L E L S	25
85	CAA ATC ATA ATG TGT GGC ATA CTT GCT GTA CTT GGT TGC TCT GAT GAT TCA CAA GCT AAA CGA GTT CGC ATA CTC GAG CTT TCT	168
26	R R L K H R G P D W S G L H Q H G D N Y L A H Q R L A I	53
169	CGC AGA TTG AAG CAC CGT GGG CCA GAC TGG AGT GGG CTC CAC CAA CAT GGT GAT AAC TAT TTG GCT CAT CAA AGG TTA GCC ATT	252
54	V D P A S G D Q P L F N E D K S I I V T V N G E I Y N H	81
253	GTT GAT CCT GCT TCT GGT GAT CAA CCT CTC TTC AAT GAA GAC AAA TCA ATT ATT GTC ACG GTG AAT GGA GAA ATC TAC AAT CAT	336
82	E E L R K Q L P N H K F F T Q C D C D V I A H L Y E E H	109
337	GAA GAG CTC AGA AAA CAA TTG CCC AAT CAC AAG TTT TTT ACA CAA TGT GAC TGT GAT GTT ATT GCA CAC CTG TAC GAG GAA CAT	420
110	G E N F V D M L D G I F S F V L L D T R D N S F I V A R	137
421	GGA GAA AAT TTT GTG GAT ATG TTA GAC GGT ATA TTT TCG TTT GTT CTG CTG GAT ACT CGT GAC AAC AGT TTC ATA GTT GCG AGG	504
138	D A I G V T S L Y I G W G L D G S V T W I A S E L K G L N	165
505	GAT GCT ATA GGT GTT ACT TCC TTG TAC ATT GGT TGG GGA CTA GAT GGT TCT TGG ATT GCA TCA GAA TTA AAA GGA CTG AAT	588
166	D E C E H F E V F P P G H L Y S S K E R E F R R W Y N P	193
589	GAT GAA TGT GAA CAT TTC GAA TTT CCG CCC GGT CAC TTA TAC TCG AGC AAA GAA AGA GAG TTT CGT CGA TGG TAT AAT CCT	672
194	P W F N E A I I P S T P Y D P L V L R N A F E K A V I K	221
673	CCA TGG TTC AAT GAG GCT ATT ATT CCG TCA ACA CCT TAT GAT CCT CTA GTT TTG AGG AAC GCG TTT GAG AAG GCT GTG ATA AAG	756
222	R L M T D V P F G V L L S G G L D S S L V A S V T A R Y	249
757	AGG TTG ATG ACC GAT GTG CCT TTC GGG GTT TTA CTA TCG GGA GGT TTG GAT TCA TCG TTG GTC GCG TCT GTC ACT GCT AGA TAC	840
250	L A G T K A A K Q W G A K L P S F C V G L K G A P D L K	277
841	CTT GCT GGT ACA AAA GCT GCT AAG CAG TGG GGA GCA AAA TTG CCC TCT TTC TGT GTA GGC CTT AAG GGC ACA CCA CTA AAG	924
278	A G K E V A D F L G T V H H E F E F T I Q D G I D A I E	305
925	GCT GGA AAG GAG GTA GCA GAT TTC TTA GGA ACT GTC CAT CAT GAA TTT GAG TTT ACT ATC CAG GAC GGT ATA GAT GCA ATT GAA	1008
306	D V I Y H T E T Y D V T T I R A A T P M F L M S R K I K	333
1009	GAT GTC ATC TAT CAC ACA GAA ACA TAT GAT GTT ACT ACG ATA AGG GCT GCA ACA CCT ATG TTT CTG ATG TCT CGT AAG ATC AAA	1092
334	S S G V K W V I S G E G S D E I F G G Y L Y F H K A P N	361
1093	TCA TCC GGA GTC AAA TGG GTG ATT TCT GGA GAA GGA TCT GAT GAG ATC TTT GGA GGG TAT TTG TAT TTC CAT AAG GCG CCA AAC	1176
362	R E E F H Q E T C R K I K A L H R Y D C L R A C A N K S T Y	389
1177	AGG GAA GAG TTT CAC CAA GAA ACA TGC CGC AAG ATC AAA GCT CTT CAT AGA TAT GAT TGT TTG AGA GCC AAT AAA TCA ACA TAT	1260
390	A W G L E A R V P F L D K D F I K V A M D I D P E F K M	417
1261	GCA TGG GGT CTA GAA GCT AGA GTA CCA TTT TTG GAC AAG GAC TTT ACT AAG GGT GCA ATG GAC ATT GAT CCT GAG TTT AAA ATG	1344
418	I K H D E G R I E K W I L R K A F D D E E N P Y L P K H	445
1345	ATA AAA CAT GAT GAA GGA AGA ATT GAG AAA TGG ATT CTA AGA AAG GCC TTT GAT GAT GAA GAG AAT CCA TAT CTG CCT AAG CAC	1428
446	I L Y R Q K E Q F S D G V G Y G W I D G I K D H A A K H	473
1429	ATT TTA TAT AGG CAG AAG GAA CAA TTC AGT GAT GGA GTT GGA TAT GGC TGG ATA GAT GGC ATC AAG GAC CAT GCT GCA AAA CAT	1512
474	V T D R M M F N A S H I F P F N T P N T K E A Y Y Y R M	501
1513	GTC ACT GAC AGA ATG ATG TTC AAT GCT TCT CAC ATC TTT CCT TTC AAC ACT CCA AAT ACC AAA GAA GCA TAT TAC TAT AGA ATG	1596
502	I F E R F F P Q N S A R L T V P G G P S V A C S T E K A	529
1597	ATC TTT GAA AGG TTT TTC CPT CAG AAC TCG ACA AGG CTT ACT AAG GGT GCA GGC CCT AGT GTT GCA TGC AGC ACA GAG AAA GCT	1680
530	I E W D A S W S N N L D P S G R A A L G V H V S A Y E H	557
1681	ATT GAA TGG GAT GCT TCA TGG TCA AAC AAC CTG GAT CCT TCT GGT AGA GCA GCA CTT GGA GTG CAT GTT TCA GCT TAT GAA CAC	1764
558	Q I N P V T K G V E P E K I I P K I G V S P L G V A I Q	585
1765	CAA ATC AAC CCA GTT ACA AAA GGT GTA GAG CCA GAG AAG ATT ATA CCA AAG ATA GGA GTT TCT CCT CTT GGA GTT GCC ATT CAA	1848
586	T *	587
1849	ACC TAG TAT GAG ACA TAG CAA GTA TTA CTT GCT TAA AAA ACC AAG ATA TTA TTA TAC TAT TAG TAT TCA ATA AAA AGA ATA ACA	1932
1933	TAA AGG GAA AAT TTG CCT GTT ATG TAT TTT ATC CAG GTA CAG GTA CAT TTG TAT GTA TAA GCC TTT CTA CTT AGC TGT ATT TAT	2016
2017	GTG TTT TGA TGT TGT GTA ATC CAC ATC TTG TCT TTG CTT TTA ATT GAT GTG GTG ATT TGA ACA CTT TCA GAT TGT AAT TTG GCT	2100
2101	TTT TAA GAA GAG TTG TGT ATT ATG TTA AAT TTG AGT GCA AGT TTC ACT ATT TGA ATA CTA CTT ATA AAT ATA TGT CTT TAC ATT	2184
2185	AAA AAA AAA AAA AAA A	2200

B

1	TT CCA AAG CCA TTA TTA GTA TTA CAA CTA CAT ACA TAT TTT CTT CTT AGT TTA TTC CAA ATT CTG TCT TTG ATT TCA TTA TCG	83
1	M C G I L A V L G C S D P S R A K R V R V L	22
84	TAT AAA ACA TAA ACA ACA ATG TGT GGT ATA CTT GCT GTT CTT GGT TGT TCT GAT CCT TCT CGA GCC AAG AGA GTT CGT GTG TTG	167
23	E L S R R L K H R G P E W S G L H Q H G D C Y L A Q Q R	50
168	GAA CTT TCA CGC AGA TTG AAG CAC CGA GGC CCA TGG AGT GGG CTC CAC CAA CAT GGT GAT TGT TAT TTG GCA CAA CAA CGG	251
51	L A I V D P A S G D Q P L F N E D N P S I V T V N G E I	78
252	TTA GCC ATA GTT GAT CCT GCT TCT GGT GAT CAA CCT CTC TTC AAT GAA GAC AAT CCG TCA ATT GTC ACG GTA AAC GGA GAG ATT	335
79	Y N H E D L R K Q L S N H T F R T G S D C D V I A H L Y	106
336	TAC AAT CAT GAA GAT CTC AGG AAA CAG TTG TCT AAT CAC ACG TTT AGG ACC GGA AGT GAT TGT GAT GTT ATT GCG CAT TTG TAC	419
107	E E Y G E D F V D M L D G I F S F V P L D T R D N S Y I	134
420	GAG GAA TAT GGA GAA GAC TTT GTG GAT ATG TTG GAT GGT ATA TTT TCG TTT GTT CCA TTG GAT ACT CGT GAC AAC AGT TAT ATT	503

135	V	A	R	D	A	I	G	V	T	S	L	Y	I	G	W	G	L	D	G	S	V	W	I	S	S	E	M	K	162	
504	GTG	GCT	AGA	GAT	GCG	ATT	GGT	GTA	ACT	TCT	CTA	TAC	ATT	GGT	TGG	GGA	TTA	GAT	GGT	TCG	GTT	TGG	ATT	TCG	TCG	GAA	ATG	AAA	587	
163	G	L	N	D	D	C	E	H	F	E	C	F	P	P	G	H	L	Y	S	S	K	D	S	G	F	R	R	W	190	
588	GGT	TTG	AAC	GAT	GAT	TGT	GAA	CAT	TTC	GAG	TGT	TTT	CCA	CCT	GGT	CAT	TTG	TAT	TCG	AGC	AAA	GAT	AGT	GGC	TTT	AGA	AGA	TGG	671	
191	Y	N	P	S	W	Y	S	E	A	I	P	S	A	P	Y	D	P	L	A	L	R	H	A	F	E	K	A	V	218	
672	TAT	AAT	CCT	TCT	TGG	TAC	TCT	GAG	GCT	ATT	CCG	TCG	GCT	CCT	TAT	GAT	CCT	CTT	GCT	TTG	AGG	CAC	GCC	TTC	GAG	AAG	GCG	GAT	755	
219	V	K	R	L	M	T	D	V	P	F	G	V	L	L	S	G	G	L	D	S	S	L	V	A	S	I	T	S	246	
756	GTA	AAA	AGG	TTG	ATG	ACA	GAT	GTA	CCT	TTC	GGT	GTT	CTA	CTA	TCC	GGA	GGT	TTG	GAC	TCG	TCA	TTG	GTT	GCA	TCC	ATC	ACT	TCT	839	
247	R	Y	L	A	T	T	K	A	A	E	Q	W	G	S	K	L	H	S	F	C	V	G	L	E	G	S	P	D	274	
840	CGC	TAC	CTA	GCA	ACC	ACG	AAA	GCG	GCT	GAA	CAA	TGG	GGA	TCA	AAA	CTA	CAT	TCA	TTC	TGC	GTT	GGA	CTC	GAG	GGC	TCA	CCT	GAT	923	
275	L	K	A	G	K	E	V	A	D	Y	L	G	T	V	H	H	E	F	T	F	T	V	Q	D	G	I	D	A	302	
924	CTT	AAG	GCT	GGA	AAA	GAA	GTT	GCA	GAT	TAT	CTC	GGA	ACC	GTT	CAT	CAT	GAG	TTT	ACC	TTT	ACT	GTT	CAG	GAT	GGT	ATA	GAT	GCA	1007	
303	I	E	G	D	V	I	Y	H	V	E	T	Y	D	V	T	S	I	R	A	S	T	P	M	F	L	M	S	R	K	330
1008	ATT	GAG	GAT	GTT	ATA	TAC	CAT	GTT	GAA	ACA	TAT	GAT	GTT	ACT	TCA	ATT	AGA	GCA	AGC	ACG	CCT	ATG	TTT	CTC	ATG	TCG	AGG	AAG	1091	
331	I	K	S	L	G	V	K	W	V	I	S	G	E	G	S	D	E	I	F	G	G	Y	L	Y	F	H	K	A	358	
1092	ATT	AAA	TCA	CTT	GGT	GTC	AAA	TGG	GTG	ATC	TCC	GGT	GAA	GGA	TCC	GAT	GAG	ATC	TTT	GGC	GGA	TAT	CTG	TAC	TTT	CAC	AAG	GCA	1175	
359	P	N	K	E	E	F	H	E	E	T	C	R	K	I	K	A	L	H	Q	Y	D	C	Q	R	A	N	K	S	386	
1176	CCG	AAC	AAG	GAA	GAG	TTT	CAC	GAA	GAA	ACT	TGC	CGC	AAG	ATC	AAA	GCA	CTG	CAC	CAA	TAT	GAT	TGC	CAG	AGA	GCT	AAT	AAA	TGC	1259	
387	T	Y	A	W	G	L	E	A	R	V	P	F	L	D	K	A	F	I	N	V	A	M	N	I	D	P	E	N	414	
1260	ACT	TAT	GCT	TGG	GGT	TTA	GAA	GCT	AGA	GTT	CCG	TTT	CTG	GAC	AAG	GCG	TTT	ATC	AAT	GTT	GCG	ATG	AAT	ATT	GAT	CCT	GAG	AAT	1343	
415	K	M	I	K	R	D	E	G	R	I	E	K	Y	I	L	R	K	A	F	D	D	E	E	N	P	Y	L	P	442	
1344	AAA	ATG	ATA	AAA	CGA	GAT	GAA	GGA	CGA	ATT	GAG	AAG	TAT	ATT	TTG	AGG	AAG	GCA	TTT	GAT	GAC	GAA	GAG	AAT	CCT	TAT	CTG	CCA	1427	
443	K	H	I	L	Y	R	Q	K	E	Q	F	S	D	G	V	G	Y	S	W	I	D	G	L	K	A	H	A	A	470	
1428	AAG	CAC	ATT	TTG	TAT	AGG	CAG	AAA	GAA	CAA	TTC	AGT	GAT	GGA	GTT	GGT	TAT	AGC	TGG	ATT	GAT	GGT	CTT	AAA	GCT	CAT	GCT	GCA	1511	
471	K	H	V	T	D	K	M	M	L	N	A	G	N	I	F	P	H	N	T	P	N	T	K	E	A	Y	Y	Y	498	
1512	AAA	CAT	GTG	ACC	GAT	AAA	ATG	ATG	CTT	AAT	GCT	GGT	AAT	ATC	TTC	CCG	CAC	AAC	ACA	CCA	AAC	ACA	AAG	GAA	GCA	TAC	TAC	TAC	1595	
499	R	M	I	F	E	R	F	F	P	Q	N	S	A	R	L	T	V	P	G	G	P	T	V	A	C	S	T	A	526	
1596	AGA	ATG	ATC	TTT	GAG	CGG	TTC	TTC	CCT	CAG	AAC	TCG	GCA	AGA	CTA	ACT	GTT	CCC	GGA	GGA	CCA	ACG	GTT	GCA	TGT	AGC	ACA	GCA	1679	
527	K	A	V	E	W	D	A	A	W	S	N	N	L	D	P	S	G	R	A	A	L	G	V	H	D	S	A	Y	554	
1680	AAA	GCT	GTT	GAG	TGG	GAT	GCT	GCT	TGG	TCA	AAC	AAC	CTC	GAT	CCT	TCT	GGT	AGA	GCA	GCA	CTC	GGA	GTT	CAT	GAT	TCA	GCT	TAT	1763	
555	E	N	H	N	K	V	N	K	T	V	E	F	E	K	I	I	P	L	E	A	A	P	V	E	L	A	I	Q	582	
1764	GAA	AAC	CAT	AAC	AAA	GTC	AAC	AAA	ACT	GTA	GAG	TTT	GAG	AAG	ATT	ATA	CCA	CTG	GAA	GCC	GCT	CCT	GTC	GAG	CTT	GCC	ATC	CAG	1847	
583	G	*																										584		
1848	GGC	TAG	TTT	CAG	CTA	TGG	CAA	GGA	ATG	ACT	GTG	CTA	GAA	GAA	TGA	AGA	TAA	TAA	TTG	AAA	ACT	TAA	CAT	ATA	TGA	AGA	ATT	TGC	1931	
1932	CTT	CTG	TTT	AAT	TTT	ATC	CGG	GGC	GAA	ACA	ATG	CTA	TAT	AAT	ATA	GAT	AAA	GCT	TTA	AAT	AAA	AAA	AAA	AA					2002	

Fig. 2. Nucleotide sequences of cDNAs encoding pea AS1 and AS2. Nucleotide sequences of cDNAs pcAS1 (A) and cAS2 (B) are shown in the mRNA sense. The deduced amino acid sequence is denoted above the nucleotide sequence in the standard one-letter code. Amino acids are numbered starting with the first in-frame methionine as 1. The translation termination codons in each clone are designated as *.

comparison of the pea AS and human AS polypeptides reveals an overall homology of 47% which extends along the entire AS polypeptide. There are several regions of high local homology (>80%) shared between the pea AS and human AS polypeptides (amino acid residues 116–128; 218–243; 340–348; 352–360; 392–401; and 486–500 in the pea AS1 protein). In particular, the first four amino acids of the human AS protein (Met-Cys-Gly-Ile), which have been shown to be the glutamine binding site (Heeke and Schuster, 1989), are perfectly conserved in both the pea AS1 and AS2 proteins. A region of divergence between the pea AS and human AS proteins occurs at amino acid residues 165–234 of the human AS protein. This stretch of amino acids is not found in either pea AS1 or AS2 polypeptide and may be the result of gene modification (deletion or insertion) during evolution of plant versus animal AS.

AS1 and AS2 are encoded by single genes in pea

Southern blot analysis was used to examine the number of genes encoding AS1 and AS2 in *P.sativum*. Pea genomic DNA digested with four restriction enzymes was fractionated on a 0.7% agarose gel and Southern blots were probed with ³²P-labeled cDNA fragments containing the coding

sequences of either AS1 or AS2 cDNAs. The results shown in Figure 4 reveals that in each digestion only a single genomic DNA restriction fragment hybridizes to each probe. In addition, the genomic DNA fragments which hybridize to either AS1 or AS2 cDNA probes are distinct. Similar results were obtained with DNA fragments containing 3' non-coding sequences of pcAS1 or cAS2 (not shown). These results indicate that peas contain a single gene for AS1 and a distinct single gene for AS2.

'Dark-induced' accumulation of AS1 mRNA in leaves

Previous biochemical studies have shown that AS enzyme activity increases when plants are grown in the dark (Joy *et al.*, 1983). To address whether this increase in AS enzyme activity reflects an increase in AS gene expression in the dark, gene-specific probes derived from 3' non-coding regions of AS1 and AS2 cDNAs were used in Northern blot experiments to detect AS mRNAs in leaves of plants grown under different light regimes (Figures 5 and 6). AS1 mRNA (2.2 kb) accumulates to high levels in leaves of mature dark-adapted green plants (Figure 5A, lanes 2 and 3). However, when these plants are transferred to continuous white light, the steady-state levels of AS1 mRNA decrease dramatically

	10	50
AS2	MCGILAVLGCSDFSRKRVVLELSRRLKHRGPE--WSGLHQHGDCYLAQQRLAIVDPA	
AS1	MCGILAVLGCSDDSQAKRVVLELSRRLKHRGPD--WSGLHQHGDNYLAHQRLAIVDPA	
AShuman	MCGIWAIFG-SDDCLS--VQCLS-AMKIAHRGPDARFENVNGYTNCCFGFHLAVVDPL	
	10	50
	100	
AS2	SGDQPLFNEDNPSI-VTVNGEIIYNHEDLRKLSNHTFRGSDCDVIAHLYEEYG-EDFVD	
AS1	SGDQPLFNEDKSII-VTVNGEIIYNHEELRKQLPNHKFFTQCDVIAHLYEEHG-ENFVD	
AShuman	FGMQPIRVKKYPYLWLCYNGEIYNHKKMQQHF-EFEYQTKVDGEIILHLYDKGGIEQTIC	
	100	
	150	
AS2	MLDGIFSFPVPLDTRDNSYIVARDAIGVTSLYIGWLDGSGVWISSEMKGNDCEHFECFP	
AS1	MLDGIFSFPVLLDTRDNSFIVARDAIGVTSLYIGWLDGSGVWIASSELKGLNDECEHFVFP	
AShuman	MLDGVFAFVLLDTANKKVFGRDITYGVRPLFKAMTEDGFLAVCSEAKGLVTLKHSATPFL	
	150	
	200	
AS2	-----PGHLYSSKDSGFRWYNPNWYSEA-IPSAFYDPLALRHAF	
AS1	-----PGHLYSSKEREFRWYNPNWFNEAIIPTFYDPLVLRNAF	
AShuman	EVLDPNGKVASVEMVKYHHCDDVPLHALYDNVEKLFPGFEIETVKNLRLIFN	
	200	
	250	
AS2	KAVVKRIMTDVPFGVLLSGGLDSSLVASITSRYLATTAAEQWGSKLHSCFVGLGSPDL	
AS1	KAVIKRIMTDVPFGVLLSGGLDSSLVASVTARYLACTKAAQWGAQLSFCVGLKGAAPDL	
AShuman	NAVKKRIMTDRIIGCLLSGGLDSSLVA---ATLLKQLKEAQV-QYPLQTFAGMEDSPDL	
	250	
	300	
AS2	KAGKEVADYLGTVHHEFTFTVQDGIDAIEDVIYHVEYDVTISIRASTPMFLMSRKISLG	
AS1	KAGKEVADFLGTVHHEFEFTIQDGIDAIEDVIYHTETDVTIRAAATPMFLMSRKIKSSG	
AShuman	LAARKVADHIGSEHYEVLNFSEEGIQALDEVIFSLITYDITTVRASVGMYLISKYIRKNT	
	300	350
	350	
AS2	VKWWI-SGEGSDEIFGGYLYFHKAPNKEEFHEETCRKIKALHQYDCQRANKSTYAWGLEA	
AS1	VKWWI-SGEGSDEIFGGYLYFHKAPNREEFHQETCRKIKALHRYDCLRANKSTYAWGLEA	
AShuman	DSVVIFSGEGSDELTOGYIYFHKAPSPEKAEESERLLRELYLFDVLRADRTTAHGLEL	
	400	
	400	450
AS2	RVPFLDKAFINVMNIDPENKMIKRDEGRIEKYILRKAFDDEENPYLPKHILYRQKEQFS	
AS1	RVPFLDKFIKAMDIDPEFKMIKHDEGRIEKWILRKAFDDEENPYLPKHILYRQKEQFS	
AShuman	RVPFLDHRFFSYLSLPPMRIPK--NG-IEKHLRETFF--EDSNLIPKEILWRPKEAFA	
	450	
	500	
AS2	DG---VGYSWIDGLKAHAACHVTDKMMNLNAGNIFPHNTPNTKEAYYRMIIFERFFPQNSA	
AS1	DG---VGYGWIDGIKHAACHVTDKMMFNASHIFPHNTPNTKEAYYRMIIFERFFPQNSA	
AShuman	DGITSVKNWFKILQYVEHQVDDAMANAQKFPNTPKTKEGYYYRQVFERHYPGRAD	
	500	
	550	
AS2	RLTVPGGPTVACSTAKAVEWDAASNNLDPSGRAALGVHDSAYENH-NKVNKTVEFEKII	
AS1	RLTVPGGPSVACSTEKAIWDASWSNNLDPSGRAALGVHVSAYEHQINPVTKGVEFEKII	
AShuman	WLSHYWMPKWINATDPSARTLTHYSKAVKA	
	550	
AS2	P-LEAAPVELAIQG	
AS1	PKIGVSPGLGVAIQT	

Fig. 3. Amino acid sequence homology of plant AS and human AS polypeptides. The deduced amino acids sequences encoded by pea pcAS1, pea cAS2 and human AS cDNA (pH131) (Andrulis *et al.*, 1987) are compared. Double dots denote identities between pea AS1 and pea AS2 or pea AS1 and human AS sequences as shown. Dashes in the amino acid sequences represent deletions used to maximize homology of AS proteins. Amino acid alignment is according to 'fasta' computer program (Pearson and Lipman, 1988).

to almost undetectable levels (Figure 5A, lanes 4 and 5). In mature plants, both the dark-induced and light-repressed accumulation of AS1 mRNA can be detected 6 h after changing the light conditions (Figure 5A, lanes 2 and 4

respectively). The steady-state levels of AS1 mRNA in leaves of dark-adapted plants (Figure 5A, lane 3) are 30-fold higher than the AS1 mRNA levels present in leaves of light-grown plants (Figure 5A, lane 5). As a control, mRNA for cytosolic

Fig. 4. Southern blot analysis of AS genes in *P. sativum*. Pea nuclear DNA was digested with the following restriction enzymes: S, *Sst*I (lanes 1 and 5); E, *Eco*RI (lanes 2 and 6); B, *Bam*HI (lanes 3 and 7); H, *Hind*III (lanes 4 and 8), resolved by gel electrophoresis, transferred to nitrocellulose and probed with radioactive probes derived from the coding region of either pcAS1 (A) or cAS2 (B).

GS (1.4 kb) monitored on the same blot revealed no dramatic changes in mRNA levels in response to the light treatments. AS2 mRNA levels were also detected on replicate blots with a DNA probe from the 3' non-coding region of cAS2. These experiments revealed that AS2 mRNA (2.2 kb) is present at much lower levels than AS1 mRNA in leaves of dark-grown plants and is undetectable in leaves of grown plants (not shown).

Northern blots were also performed on RNA isolated from plants at various developmental stages which were grown in continuous white light (Figure 5B, lanes 1, 3, 5 and 7) and then transferred to the dark (Figure 5B, lanes 2, 4, 6 and 8). The results of these experiments reveal that the dark-induced accumulation of AS1 mRNA occurs in plants of all developmental stages but is most dramatic in mature plants. The dark-induced increase of AS1 mRNA varies from 5-fold in 10 day old plants (Figure 5B, compare lanes 1 and 2) to >20-fold in 31 day old plants (Figure 5B, compare lanes 7 and 8). As a control, Northern blots reprobed with a DNA probe encoding a cytosolic form of glutamine synthetase (GS) (Tingey *et al.*, 1988) reveal that the mRNA for cytosolic GS (1.4 kb) is relatively unaffected by the different light treatments (Figure 5B, lower band).

Phytochrome mediates the 'dark-induced' accumulation of AS1 mRNA

AS1 mRNA accumulates to high levels in leaves of etiolated seedlings (Figure 6A, lane 1) and decreases to almost undetectable levels when plants were transferred to continuous white light (Figure 6A, lane 2). The accumulation of AS1 mRNA during dark treatment is in direct contrast to the light-induced accumulation of mRNA for the chloroplast form of GS2 (1.5 kb) (Tingey *et al.*, 1988) in these samples (Figure 6A, lower bands).

Fig. 5. Northern blot analysis of AS1 mRNA levels in leaves of dark-adapted versus light-grown peas. A gene-specific probe from the 3' non-coding region of pcAS1 was used to detect AS1 mRNA (2.2 kb) in RNA from leaves of dark (D) or light (L) treated pea plants. As a control, mRNAs of cytosolic GS (1.4 kb) were also detected on the Northern blots with cDNA probe GS299 (Tingey *et al.*, 1988). (A) Total RNA (20 μ g) from leaves of peas grown in continuous white light for 14 days (lane 1), transferred to the dark for 6 h (lane 2) or 3 days (lane 3) and back to continuous white light for 6 h (lane 4) or 1 day (lane 5). (B) Total RNA (20 μ g) from leaves of peas grown in continuous white light for 10, 17, 24 or 31 days ('L', lanes 1, 3, 5, 7). Dark-treated plants were grown in continuous white light for 7, 14, 21 or 28 days and then transferred to the dark for an additional 3 days ('D', lanes 2, 4, 6, 8) before harvesting.

Fig. 6. Northern blot analysis for AS1 mRNA levels in etiolated pea leaves treated with different light regimes. Detection of AS1 mRNA was as described in Figure 5. mRNA for chloroplast GS (GS2) was detected with cDNA probe GS185 (Tingey *et al.*, 1988). (A) Total RNA (20 μ g) from leaves of peas grown in the dark for 7 days (lane 1) and transferred to the light for 72 h (lane 2). (B) Total RNA (20 μ g) from leaves of peas grown in the dark for 9 days (lane 1) and subsequently treated with a pulse of red light then put back to the dark for 3 h (lane 2), treated with a pulse of red light followed by a pulse of far-red light then put back into the dark for 3 h (lane 3) or transferred to the continuous white light for 8 h (lane 4).

In order to determine whether the plant photoreceptor phytochrome is involved in mediating the dark-induced expression of AS1 gene in peas, AS1 mRNA was examined in etiolated plants treated with light regimes known to activate or inactivate phytochrome (Figure 6B). While AS1 mRNA accumulates to high levels in etiolated plants (Figure 6B, lane 1) the levels of AS1 mRNA decrease dramatically in plants treated with a red-light pulse (Figure 6B, lane 2). The repression of AS1 expression by red light is partially reversed by a subsequent pulse of far-red light (Figure 6B, lane 3). The effects of red and far-red light pulses on accumulation of AS1 mRNA were detected within 3 h after light treatment. These results show that the dark-induced accumulation of AS1 mRNA is mediated, at least in part, through the chromophore phytochrome.

AS1 and AS2 mRNAs accumulate in developmental contexts of increased nitrogen mobilization

To determine whether the level of AS mRNA increases in contexts where large amounts of asparagine are synthesized for nitrogen transport, the steady-state levels of AS1 and AS2 mRNAs were monitored in nitrogen-fixing root nodules of peas and in cotyledons of germinating pea seedlings (Figure 7). Asparagine serves as a major nitrogen transport amino acid during germination (Dilworth and Dure, 1978; Capdevila and Dure, 1977; Kern and Chrispeels, 1978). The results of the gene-specific Northern blots for AS reveals that both AS1 and AS2 mRNAs accumulate to high levels in cotyledons of germinating pea seedlings (Figure 7A). While AS1 mRNA can be detected after 10 days of germination (Figure 7A, lane 5, upper panel), AS2 mRNA is detected earlier (4–6 days of germination) (Figure 7A, lanes 2 and 3, lower panel). There is a >20-fold increase of both AS1 and AS2 mRNAs in cotyledons during a germination time course (Figure 7A, compare lanes 2 and 7). The same Northern blot reprobed with DNA probe for a cytosolic form of glutamine synthetase (GS) (Tingey *et al.*, 1988) reveals that mRNA for cytosolic GS accumulates earlier (2–4 days) than either AS mRNA (not shown).

AS mRNA levels were also examined in nitrogen-fixing root nodules of pea where asparagine serves as a major compound for nitrogen transport from nodules to the rest of the plant (Scott *et al.*, 1976; Reynolds *et al.*, 1982). RNA from nitrogen-fixing root nodules and roots of uninfected plants were probed in Northern blot experiments with gene-specific AS probes (Figure 7B). These experiments reveal that both AS1 and AS2 mRNAs accumulate to very high levels in nitrogen-fixing nodules (Figure 7B, lane 2) compared to uninfected roots (Figure 7B, lane 1). The induction of AS1 mRNA in nodules compared to roots is 20-fold while that of AS2 mRNAs is only 5-fold. The lower fold induction of AS2 mRNA may reflect the higher basal levels present in uninfected roots (Figure 7B, lane 1). As a control, the Northern blot was reprobed with a DNA probe for the β -subunit of the mitochondrial ATPase (Boutry and Chua, 1985) which is expressed at equal levels in roots of uninfected plants and nitrogen-fixing nodules (Figure 7B, lower panel).

Discussion

While asparagine is an important nitrogen transport amino acid in higher plants, the enzyme involved in its synthesis is poorly characterized to date due to enzyme instability *in*

A. cotyledons

B. nodules

Fig. 7. Northern blot analysis of AS1 and AS2 mRNA levels in (A) cotyledons of germinating seedlings and (B) nitrogen-fixing root nodules. Gene-specific DNA fragments from the 3' non-coding region of pcAS1 or cAS2 was used to detect the AS1 mRNA (2.2 kb) or AS2 mRNA (2.2 kb) on Northern blots. As a control, mRNA for the β -subunit of mitochondrial ATPase (2.1 kb) was also detected (Boutry and Chua, 1985). (A) Total RNA (20 μ g) extracted from cotyledons of pea seedlings germinated 2–18 days (lanes 1–8). (B) Poly(A)⁺ RNA (1 μ g) isolated from roots of uninfected peas (lane 1) and nitrogen-fixing root nodules (lane 2).

vitro (Lea and Mifflin, 1980). In this paper, we have directly cloned plant AS cDNAs using a heterologous DNA probe encoding human AS (Andrulis *et al.*, 1987). Two classes of AS cDNAs (AS1 and AS2) that encode homologous but distinct AS proteins were obtained from pea cDNA libraries. The homologies between the pea AS1 and AS2 cDNAs are 81 and 86% at nucleotide and amino acid levels respectively. Full-length cDNAs for AS1 and AS2 of pea are shown to encode proteins whose sizes and amino acid sequences are in excellent agreement with that deduced for the human AS protein. The pea AS1 and AS2 cDNAs and human AS cDNA share an overall nucleotide homology of 50–55% along their entire coding sequence. Regions that are highly conserved between the pea AS and human AS polypeptides (>80% at amino acid level) most likely include important sites for enzyme activity. For example, the first four amino acids in the human AS protein (Met-Cys-Gly-Ile), which have been shown to be the glutamine-binding site and important for enzyme activity (Andrulis *et al.*, 1987; Heeke and Schuster, 1989), are perfectly conserved in both the pea AS1 and AS2 proteins. The high degree of sequence homology between the pea AS and human AS proteins supports the conclusion that the full length AS1 and AS2 cDNAs encode glutamine-dependent AS of peas.

The significance of two homologous but distinct AS polypeptides in plants is intriguing. The AS1 and AS2 cDNAs of pea may encode two distinct subunits of a single AS holoenzyme (heterologous holoenzyme); or each subunit

may assemble into a separate AS holoenzyme (homologous holoenzyme of either AS1 or AS2 subunits). These two possibilities are not mutually exclusive. Partially purified plant AS enzyme preparations have been shown to utilize glutamine as a preferred substrate; however, ammonia can also be used as a substrate in the same preparations albeit with higher K_m values (Scott *et al.*, 1976; Huber and Streeter, 1984, 1985). The existence of glutamine binding sites at the amino terminus of the pea AS1 and AS2 proteins implies that both AS1 and AS2 genes encode glutamine-dependent forms of AS. It is possible, however, that these AS enzymes are able to utilize ammonia as a substrate *in vivo* under conditions of ammonia excess. It is interesting to note that glutamine versus ammonia-dependent forms of AS are encoded by separated genes in *E. coli* (Felton *et al.*, 1980; Humbert and Simoni, 1980) and yeast (Jones, 1978; Ramos and Wiame, 1980). Therefore, we cannot exclude the possibility that plants might contain another distinct AS gene for ammonia-dependent form of AS. Previous biochemical studies have also shown that AS activity can be detected in both soluble and proplastid fractions of nitrogen-fixing nodules of soybean (Boland *et al.*, 1982). The proteins encoded by AS1 and AS2 cDNAs of pea are most likely cytosolic AS since neither of them contains a transit peptide. It is possible that peas contain another distinct gene for plastid AS.

Northern blot analysis has revealed that the steady-state levels of AS1 and AS2 mRNAs parallel asparagine synthesis in various developmental contexts. For example, previous physiological studies have shown that asparagine is the major nitrogen transport amino acid in plants grown in the dark (Urquhart and Joy, 1981), and that AS activity can be enhanced by dark treatment (Joy *et al.*, 1983). Here, experiments have demonstrated that in peas the increase of AS activity in the dark is due, at least in part, to an increase in the steady-state levels of AS1 mRNA. This dark-induced accumulation of AS1 mRNA occurs in leaves of both etiolated seedlings and in mature dark-adapted green plants. Moreover, the magnitude of dark-induced AS1 mRNA accumulation increases significantly during plant development. Kinetic experiments reveal that both dark-induced and light-repressed changes in AS1 mRNA levels can be detected within 3 h in etiolated seedlings (Figure 6B) and mature plants (not shown) after changing the light/dark conditions. Thus the dark-induced accumulation of AS1 mRNA is physiologically significant for plants grown in a short dark period (e.g. at night).

The dark-induced accumulation of the AS1 mRNA classifies the AS1 gene with other genes that are negatively regulated by light such as phytochrome (Otto *et al.*, 1984; Lissemore and Quail, 1988; Kay *et al.*, 1989), protochlorophyllide reductase (Mosinger *et al.*, 1985) and an unidentified mRNA found in *Lemna* (Okubara *et al.*, 1988). As shown for phytochrome (Lissemore and Quail, 1988; Kay *et al.*, 1989) and protochlorophyllide reductase genes (Mosinger *et al.*, 1985), the repression of AS1 mRNA accumulation in the light is a phytochrome-mediated response. However, it remains to be determined whether the dark-induced (or light-repressed) expression of AS1 reflects a transcriptional response as has been shown for phytochrome (Lissemore and Quail, 1988; Kay *et al.*, 1989) and protochlorophyllide reductase (Mosinger *et al.*, 1985)

or whether it represents a post-transcriptional response as has been shown for another dark-induced gene of unknown function (Okubara *et al.*, 1988). In direct contrast to the dark-induced accumulation of AS1 mRNA in leaves, the mRNA for the chloroplast form of glutamine synthetase (GS2) accumulates in the light in a phytochrome-mediated response (Tingey *et al.*, 1988). Parallel molecular studies on the mechanisms for dark-induced accumulation of AS1 mRNA and light-induced accumulation of GS2 mRNA will uncover how two genes encoding nitrogen metabolic enzymes along a common pathway are regulated by light via phytochrome in opposite fashions.

Previous biochemical studies have revealed high levels of AS activity in two developmental contexts where large amounts of asparagine are synthesized for nitrogen transport: in cotyledons of germinating seedlings (Capdevila and Dure, 1977; Dilworth and Dure, 1978; Kern and Chrispeels, 1978) and in nitrogen-fixing root nodules (Scott *et al.*, 1976; Reynolds *et al.*, 1982). Previous studies also showed that actinomycin D treatment abolished the induction of AS activity in cotyledons of germinating cotton seedlings, indicating that AS expression in cotyledons is regulated at the transcriptional level (Capdevila and Dure, 1977; Dilworth and Dure, 1978). Consistent with those findings, we have shown that the accumulation of both AS1 and AS2 mRNAs are induced to high levels in cotyledons of germinating pea seedlings. Comparative studies of AS mRNAs and GS mRNAs in this context show that the steady-state levels of mRNA for cytosolic GS accumulate earlier than those of both AS mRNAs in cotyledons of germinating seedlings (not shown). These results suggest that glutamine synthesized by GS may act as a metabolic signal to induce AS gene expression in this developmental context. The mRNAs of AS1 and AS2 also accumulate to very high levels in root nodules of peas in a parallel fashion with cytosolic GS mRNA. The accumulation of AS1 and AS2 mRNA in nitrogen-fixing nodules may be the result of factors produced by the process of nodulation or/and by metabolic factor(s) such as ammonia or glutamine production in nodules.

Southern blot analysis of genomic DNA reveals that the gene family for AS in peas is composed of at least two genes, AS1 and AS2, which encode homologous but distinct gene products. Expression studies show that AS1 and AS2 genes share some similarities in expression patterns (e.g. induced accumulation of mRNA in cotyledons and nodules); however, they have distinct organ-specific patterns of expression. AS1 mRNA accumulates to higher levels in leaves compared to AS2, while AS2 mRNA accumulates to higher levels in roots than AS1. In this respect, the AS gene family resembles the GS gene family where members of a gene family may be differentially regulated by distinct factors which modulate expression of individual genes in specific contexts during development (Coruzzi *et al.*, 1989). Our continuing studies are aimed at characterizing the AS1 and AS2 genes and gene products as well as defining the *cis*-acting DNA elements responsible for differential regulation of the AS1 and AS2 genes during plant development. A comparative analysis of the AS and GS gene families will elucidate the molecular mechanisms responsible for the co-ordinate induction or repression of genes coding for enzymes along a common nitrogen metabolic pathway in higher plants.

Materials and methods

Growth of plant material

Seeds of *P. sativum* (var. 'Sparkle') obtained from Rogers Brother Seed Co. (Twin Falls, ID) were imbibed and germinated in a Conviron environmental chamber with a day length of 16 h, illumination of 1000 microeinsteins/m²/s [1 einstein (E) = 1 mol of photons], at a day/night cycle of 21/18°C. For etiolated plants, peas were grown for 7–9 days in black lucite boxes contained in a dark environmental chamber. For germination studies, seeds were imbibed in water and germinated in vermiculite. Nodules were isolated from 21 day old pea plants inoculated with *Rhizobium leguminosum* strain 128C53 (Nitragin Co., Milwaukee, WI) as described previously (Tingey *et al.*, 1987).

For phytochrome induction experiments, 9 day old etiolated pea seedlings were irradiated with a 4 min pulse of red light (red fluorescent lamps, General Electric F20T12R) at a fluence of 40 µE/m²/s or were given a 4 min pulse of red light followed by 12 min of far-red light (Westlake, FRF700) at the same fluence and were then returned to the dark for 3 h. For white light treatment, etiolated seedlings were exposed to continuous white light for 8 h.

Isolation of plant AS cDNAs

AS1 cDNA clones were selected from a λgt11 cDNA library previously constructed from *P. sativum* (var. 'Sparkle') nodule mRNA (Tingey *et al.*, 1987) as follows. Nitrocellulose filters containing denatured phage DNA corresponding to 250 000 individual plaques were incubated for 4 h at 45°C in pre-hybridization buffer (6 × SSC, 10 × Denhardt's solution, 0.1% SDS, 1 mM EDTA, 100 µg/ml denatured salmon sperm DNA). Filters were then incubated for 24 h at 45°C in hybridization buffer (6 × SSC, 5 × Denhardt's solution, 0.1% SDS, 1 mM EDTA, 50 µg/ml denatured salmon sperm DNA) plus 0.2 µg of ³²P-labeled cDNA insert (sp. act. 2 × 10⁸ c.p.m./µg) (1.7 kb *Hind*III fragment of pH131) (Andrulis *et al.*, 1987). Filters were washed in 1 × SSC, 0.1% SDS for 15 min at room temperature, followed by 15 min at 45°C and exposed to X-ray film. cDNA clones corresponding to the 5' end of AS1 mRNA were synthesized using a 40 base oligonucleotide primer complementary to the 5' end of cAS301 (809–848 nt, see Figure 2A). This oligonucleotide was annealed with 5 µg pea nodule poly(A)⁺ RNA and cDNA synthesis was performed using a cDNA Synthesis System (Bethesda Research Labs, Gaithersburg, MD). Following second strand synthesis, *Eco*RI linkers were added and the cDNA fragments were ligated into Lambda ZAPII vector (Stratagene, La Jolla, CA). A genomic clone for the AS2 gene was identified when a DNA fragment from the coding region of an AS1 cDNA was used to screen a pea genomic library (Lycett *et al.*, 1985). AS2 cDNAs were subsequently isolated from a pea root cDNA library constructed in λgt11 (Tingey *et al.*, 1987). All cDNA inserts of both λgt11 and Lambda ZAPII clones were initially subcloned into pTZ18U or pTZ19U (GenescribeTM, US Biochemical Corp., Cleveland, OH). Restriction fragments of each cDNA were then subcloned into M13mp18 or M13mp19 and the nucleotide sequence determined by the dideoxy method (Biggin *et al.*, 1983).

The cDNA clones containing the 5' end of AS2 mRNA were amplified from pea nodule poly(A)⁺ RNA by anchored polymerase chain reaction (A-PCR) technique (Loh *et al.*, 1989). First strand cDNA was synthesized in a reaction mix containing 50 mM Tris–HCl pH 8.3, 75 mM KCl, 3 mM MgCl₂, 50 mM dithiothreitol, 0.5 mM dNTP, 5 µg nodule poly(A)⁺ RNA, 200 U M-MLV reverse transcriptase (Bethesda Research Labs, Gaithersburg, MD) and 1 µg oligonucleotide FY13 (5'-GGCCGAATT-CATACAAATGACCAGGTGGAAACAC) which includes an *Eco*RI site plus sequences complementary to the 5' end of cAS201 (617–641 nt, see Figure 2B) at 37°C for 1 h. The reaction was stopped by phenol–chloroform extraction and the supernatant was passed through Linker 6 Quick SpinTM Columns (Boehringer Mannheim Biochemicals, Indianapolis, IN) to remove excess linkers. After ethanol precipitation, the tailing reaction was performed according to manufacturer's instructions in 50 µl of reaction mix containing 20 µM of dGTP, 1 × TdT buffer and 15 U of terminal deoxynucleotidyl transferase (TdT) (Bethesda Research Labs) at 37°C for 30 min. After phenol–chloroform extraction and ethanol precipitation, the tailed cDNAs were redissolved in 20 µl of water and used as templates in an A-PCR reaction. The A-PCR reaction was performed with Taq polymerase (Perkin-Elmer Cetus, Norwalk CT) in 100 µl of a buffer containing 5 µl of the tailed cDNAs, 0.1 mM of dNTP, 2.5 µg of AS2 specific primer (FY13) and 2.5 µg of an anchored primer mix containing a 1:9 ratio of AnC primer (5'-CAGGTCGACTCTAGAGGATCCCCCCCCCCCCCCCC) and An primer (5'-CAGGTCGACTCTAGAGGATCCC). A program of six cycles of low-stringency hybridization and amplification (94°C for 45 s followed by annealing at 37°C for 1 min and elongation at 72°C for 2 min) was followed by 24 cycles of high-stringency hybridization and amplification

(94°C for 45 s followed by annealing at 55°C for 1 min and elongation at 72°C for 2 min). The amplified cDNAs were precipitated by ethanol, digested with *Eco*RI and *Bam*HI, then separated on an agarose gel. A predominant DNA fragment of ~600 bp was recovered from the agarose gel and ligated into the *Eco*RI and *Bam*HI sites of pTZ19U (GenescribeTM). The ligated DNA was introduced into *E. coli* XL1 blue. Clones containing AS2 sequences were isolated and sequenced by the dideoxy method (Biggin *et al.*, 1983).

DNA and RNA analyses

Nuclear DNA from *P. sativum* was analyzed by Southern blot analysis according to the method described in Tingey *et al.* (1987). Briefly, pea nuclear DNA was digested with *Sst*I, *Eco*RI, *Bam*HI or *Hind*III, resolved by gel electrophoresis, transferred to nitrocellulose and probed with a 1373 bp *Sst*I–*Bam*HI ³²P-labeled fragment of pcAS1 (Figure 1A, fragment 'b') or a 872 bp *Bam*HI–*Eco*RI ³²P-labeled fragment of cAS2 (Figure 1A, 3' end of cAS2). The genomic Southern blot was performed at high stringency (hybridized at 70°C and washed at 70°C in 0.1 × SSC and 0.1% SDS) such that cAS1 and cAS2 cannot cross-hybridize to each other. Northern blot analyses of RNA obtained from leaves, roots, nodules or cotyledons of *P. sativum* were performed according to the method described by Tingey *et al.* (1987). Briefly, total RNA or poly(A)⁺ RNA was denatured with glyoxal, resolved by gel electrophoresis, transferred to nitrocellulose and probed with a 423 bp *Bam*HI–*Eco*RI ³²P-labeled fragment of pcAS1 (Figure 1A, fragment 'c') or a 224 bp *Hinc*II–*Eco*RI ³²P-labeled fragment of cAS2 (Figure 1B, 3' end of cAS2) which have sp. act. ~1–2 × 10⁸ c.p.m./µg. The intensities of gene-specific mRNA were determined by densitometer. For Northern blot analysis in which poly(A)⁺ RNA was used, the induction fold was subtracted by the intensities of β-subunit of mitochondrial ATPase mRNA. Sizes of mRNAs were estimated by migration relative to denatured DNA markers.

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