MOLECULAR BIOLOGY AND BIOCHEMICAL ASSESSMENT OF ACETOLACTATE SYNTHASE (ALS)-MEDIATED PROTEIN-PROTEIN INTERACTIONS IN *ARABIDOPSIS THALIANA* (L.) HEYNH.

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ABSTRACT

The biosynthesis of the branched-chain amino acids (BCAAs) isoleucine, leucine and valine, in microorganisms and in plants, is accomplished by a series of enzymatic reactions where acetolactate synthase (ALS) is the first enzyme common to three pathways. This study was aimed at molecular and cell biological analyses of protein-protein interactions in *Arabidopsis thaliana* involving the ALS catalytic subunit and putative ALS-interacting proteins, and characterization of genes encoding ALS-interacting proteins. The study was conducted primarily to biochemically validate the persistence of *in vivo* protein-protein interactions between the *A. thaliana* ALS catalytic subunit and two ALS-interacting proteins, AIP1p and AIP3p, which were selected on the basis of their structural similarity to ALS regulatory subunits from prokaryotic and eukaryotic species.

Characterization of *AIP1* and *AIP3* genomic DNAs, cDNAs and their deduced gene products revealed both similarities and differences between these two entities with respect to size, chromosomal location, sub-cellular localization, and primary sequence similarity to prokaryotic and eukaryotic orthologues. The observed similarity between AIP1p and AIP3p and their respective prokaryotic counterparts suggests that domains of prokaryotic origin may serve as the common ancestral cores for both polypeptides.

The fusion of His_6 tags to either the N- or C-terminus of the chlorsulfuron-resistant At*ALS-csr1-1* transgene for generation of transgenic *A. thaliana* lines enabled the capture and purification of His_6-tagged AtALS-CSR1-1 using an immobilized metal-

ion affinity chromatography (IMAC) system, and the identification of co-enriched proteins that interacted with ALS. The use of the herbicide-resistant At*ALS-csr1-1* allele allowed a prior functional assessment of the genetically-modified ALS enzyme. Specifically, an IMAC Co^{2+} batch chromatography system was used successfully to determine the persistence of protein-protein interactions between ALS and AIP1p and/or AIP3p. The results constitute direct biochemical evidence in support of the existence of ALS:AIPx protein-protein interactions in plant extracts.

In bacteria, the ALS holoenzyme has been proposed to assume a tetrameric conformation consisting of two identical catalytic subunits and two identical regulatory subunits forming an $\alpha_2\beta_2$ structure. In plants, based on evidence obtained in this study and elsewhere, the ALS holoenzyme appears to exhibit a different conformation involving $\alpha\beta\beta'$ (i.e. a single catalytic subunit plus a heterodimer consisting of two regulatory subunits), $\alpha_2\beta\beta'$, $\alpha\beta$, $\alpha\beta'$, $\alpha_2\beta$ and/or $\alpha_2\beta'$ heteromers.

The postulate that AIP1p and AIP3p are ALS regulatory subunits was supported by the study of transgenic lines ectopically-expressing *AIP1* and *AIP3* transgenes. Transgenic lines carrying *AIP1* or *AIP3* displayed altered contents of BCAA and twelve other amino acids. The introduction of *AIP1* and *AIP3* transgenes somehow caused disruption of regulation of the BCAA and other amino acid biosynthetic pathways with a total net increase of the fifteen free amino acids analyzed, regardless of whether the endo- and trans-genes were down- or up-regulated via ectopic expression or co-supression. These results suggest that the disruption of regulation of BCAA metabolism may be perceived as a direct consequence of AIP1p and AIP3p being regulatory subunits of ALS that mediate negative allosteric regulation of BCAA biosynthesis.

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DEDICATION

I dedicate this thesis to...

...my mother, mother-in-law, and
my wife, Aditia, and our children,
Arvin, Adriel, Aselia...

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LIST OF ABBREVIATIONS

a.a.	amino acid(s)
ATP	adenosine triphosphate
β-ME	β-mercaptoethanol
bp	base pair(s)
BSA	bovine serum albumin
CaMV	cauliflower mosaic virus
CoA	coenzyme A
cpm	counts per minute
d	day(s)
dATP	deoxyadenosine triphosphate
dCTP	deoxycytidine triphosphate
ddH_2O	distilled, deionized water
dGTP	deoxyguanosine triphosphate
dH_2O	distilled water
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
dpm	disintegrations per minute
DTT	dithiothreitol
dTTP	deoxythymidine triphosphate

EDTA	ethylenediaminetetraacetic acid
FAD	flavin adenine dinucleotide
g	gram(s)
h	hour(s)
HRP	horse radish peroxidase
kan	kanamycin
kb	kilobase(s)
kbp	kilobase pairs
kDa	kilodalton(s)
min	minute(s)
MOPS	3-(*N*-morpholino)propanesulfonic acid
mRNA	messenger RNA
NAD^+	nicotinamide adenine dinucleotide (oxidized form)
NADP(H)	nicotinamide adenine dinucleotide phosphate (reduced form)
nt	nucleotide(s)
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PEG	polyethylene glycol
Pfu	*Pyrococcus furiosus*
PLP	pyridoxal phosphate
PMP	pyridoxamine-5'-phosphate
PMSF	phenymethylsulphonyl fluoride

Pol	DNA polymerase
PVDF	polyvinylidene difluoride
PVP	polyvinylpyrrolidone
RNA	ribonucleic acid
RT-PCR	reverse transcriptase-polymerase chain reaction
s	second(s)
SDM	site-directed mutagenesis
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SOR	shikimate oxidoreductase
SSC	sodium saline citrate
ssDNA	single-stranded DNA
Taq	*Thermus aquaticus*
TBE	tris-borate-EDTA buffer
TBS	tris-buffered saline
TCA	trichloroacetic acid
T-DNA	transfer DNA
TEMED	N,N,N',N'-tetramethylethylenediamine
temp	temperatures(s)
Ti	tumor inducing plasmid
TPP	thiamine pyrophosphate
Tris	tris(hydroxymethyl)aminomethane
U	enzyme units
UV	ultraviolet

V	volts
v	volume
w	weight

1. INTRODUCTION

The branched-chain amino acid (BCAA) biosynthetic pathway catalyzes the biosynthesis of branched-chain amino acids, and has been widely studied in bacteria, fungi and plants yielding abundant information pertaining to the enzyme constituents of the pathway and the structural genes which encode them (Wittenbach *et al.*, 1992). As shown in Figure 2.1, the BCAA pathway consists of four enzymatic steps catalyzing the conversion of pyruvate and 2-oxobutyrate to isoleucine and valine, and four additional enzymatic steps catalyzing the conversion of 2-ketoisovalerate (an intermediate of valine biosynthesis) to leucine (Umbarger, 1978; Bryan, 1980; Barak *et al.*, 1990).

The BCAA or isoleucine, leucine and valine (*ilv*) biosynthetic pathway is post-translationally regulated in response to branched-chain amino acid pools (Rathinasabapathi *et al.*, 1990; Wu *et al.*, 1994). Regulation of the pathway is thought to be mediated, at least in part, by direct feed back inhibition of the ALS enzyme by isoleucine, valine and leucine. While partially purified ALS activity was directly inhibited by these amino acids *in vitro*, the purification state of the enzyme was variable and millimolar concentrations of amino acid were required (Miflin, 1971).

Acetolactate (acetohydroxyacid) synthase (ALS, AHAS; EC 4.1.3.18), as the first enzyme in the BCAA biosynthetic pathway, has justifiably been the subject of the most intense study. In eukaryotes, ALS activity, together with that of other enzymes in the pathway, is found in the mitochondrion in yeast and fungi (Ryan and Kohlhaw, 1974) or in the chloroplast in algae and higher plants (Miflin, 1974; Reith and

Munhulland, 1993). In *Arabidopsis thaliana*, genetic studies suggest that a single locus (CSR) on chromosome 3 encodes the ALS catalytic subunit; these studies have also implied that ALS enzymatic activity in *A. thaliana* is the product of a single structural gene which is active throughout development (Haughn and Somerville, 1986).

To date, conventional biochemical studies have been problematic for defining protein-protein interactions relevant to *in vivo* ALS activity (W.L. Crosby, personal communication). Several laboratories have attempted conventional biochemical purification of ALS from diverse plant sources. In most cases, the enzyme was reported to be very labile during purification, with activity half-lives of 4-6 h at 0°C (Bekkaoui *et al.*, 1993). Enzymatically-active ALS has been partially purified using various protocols, which has led to reports of molecular masses ranging from 55 kDa to over 400 kDa. The lability of ALS *in vitro* has hampered efforts to purify the enzyme and to determine its quaternary structure. In *Brassica napus*, the ALS holoenzyme has been reported to be a dimer of two 65 and/or 66 kDa subunits (Bekkaoui *et al.*, 1993). Parallel studies using stromal extracts from *A. thaliana* indicated an identical apparent Stoke's radius and other chromatographic properties of the enzyme from this species (Bekkaoui *et al.*, unpublished). In contrast to the regulatory subunit reported to co-purify with ALS in bacteria (Umbarger, 1978), the *B. napus* enzyme did not co-purify with other polypeptides, and extensive purification of the *B. napus* ALS enzyme failed to enrich other interacting proteins (Bekkaoui *et al.*, 1993).

More recent findings suggest that the ALS catalytic subunit may require other proteins in order to function. Southan and Copeland (1996) reported that ALS in crude extracts of wheat leaves was strongly inhibited by end-products of the pathway, but after partial purification the enzyme was almost insensitive to feedback inhibition. This

suggested, among other things, that the enzyme might require other (protein?) factors in order to exhibit feedback inhibition.

Another approach to the analysis of protein-protein interactions involving ALS was carried out by W.L. Crosby and his team at the Plant Biotechnology Institute (PBI) in Saskatoon, Canada. Using the yeast two-hybrid system, they identified ten classes of ALS-interacting proteins. Two classes (AIP1p and AIP3p) of gene products were determined to be similar to bacterial ALS regulatory subunits, and interacted with the ALS catalytic subunit of *A. thaliana* in the yeast two-hybrid system (Kohalmi and Crosby, unpublished). Although these findings were suggestive of protein-protein interactions involving the ALS catalytic subunit, they were insufficient to confidently conclude the persistence of the protein-protein interactions *in planta*. For example, the results from the heterologous yeast two-hybrid system may not be readily applied to plants since correct post-translational modifications that take place in yeast may not occur in the plant species of interest, while proper protein folding and stability, and maintenance of fusion protein activity, may also differ in yeast and plants (Phizicky and Fields, 1995). Therefore, it was important to independently verify and confirm the protein-protein interactions in plants or in plant-derived fractions. To do this, *AIP1* and *AIP3* were selected from among the ten cDNA classes previously identified, and these were subjected to further study. Since both proteins are homologous to multiple ALS regulatory subunits from prokaryotic as well as from eukaryotic organisms, it was postulated that these were independent subunits of the plant ALS enzyme.

Arabidopsis thaliana offers technical advantages as a model species for molecular and genetic studies of plant processes (Pruitt *et al.*, 1987; Somerville, 1993; Schmidt, 1998) and information obtained will likely be relevant to other members of the

Brassicaceae, including *Brassica* species. Its small size, short generation time and prolific seed production have resulted in the identification of numerous genetic loci controlling diverse developmental and metabolic processes (Meyerowitz and Somerville, 1994). A somewhat low content of repeated DNA sequences typifies the genome of *A. thaliana* (Leutwiler *et al.*, 1984; Pruitt and Meyerowitz, 1986) which has an estimated c-value of approximately 125 Mbp (*The Arabidopsis Genome Initiative*, 2000), thus its genome is much smaller than the genomes of almost all other widely-investigated plants (Schmidt, 1998).

The overall objective of this study was to investigate the molecular biology and the biochemical basis of protein-protein interactions involving the catalytic subunit of ALS and putative ALS-interacting proteins from *A. thaliana*. Specific objectives included investigation of the genomic structure, expression and characteristics of the *AIP1* and *AIP3* cDNAs, as well as investigation of the AIP1p and AIP3p proteins that have been shown to interact with the catalytic subunit of ALS using the yeast two-hybrid system, which included assessment of their expression, subcellular localization and *in vivo* protein-protein interaction potential.

2. LITERATURE REVIEW

2.1 *Arabidopsis thaliana* as a model plant system for molecular biology studies

Arabidopsis thaliana (L.) Heynh. (thale cress; mouse-eared cress) is a small annual plant species belonging to the *Brassicaceae* family (Bowman, 1994). *Arabidopsis thaliana* is naturally found in forests of the subtropical region of the world growing as a winter annual species (Rédei, 1992), although its range extends from the high mountains of equatorial Africa and the highlands of the Himalayas to most of temperate Europe (Bowman, 1994). Berger (1965), as cited in Rédei (1992), suggested the Central Asian highlands of the Western Himalayas as the origin of the plant, and not likely North America, since its dispersion was localized to the transportation routes of early (European) settlers in North America (Rédei, 1992).

Botanists have been familiar with this plant for at least four hundred years and have used it in experimental research for nearly one hundred years (Bowman, 1994), beginning with Friedrich Laibach who published, in 1907, a significant paper concerning the individuality and continuity of the chromosome (Rédei, 1992). Since then, the species has become increasingly popular as an experimental object of study in the field of natural science. As mentioned in the INTRODUCTION section, *A. thaliana* has become an ideal species for molecular and genetic analysis of plant processes, and accordingly has become an important model plant system for molecular biology studies (Schmidt, 1998).

As described by Zambryski (1997), two general conclusions can be drawn from initial efforts on cloning single genes from *A. thaliana* that stress its use for gene organization studies. Firstly, genes recovered from *A. thaliana* generally cross-hybridize with homologous genes from diverse plant species. Secondly, multiple genes or families of genes in various plants that encode particular proteins are likely represented by a single gene or a relatively few genes in this model species. Several topics from various authors and edited by Anderson and Roberts (1998) summarized the use of *A. thaliana* as an experimental model in numerous research areas including genomic analysis, biochemical genetic analysis of metabolic pathways, growth regulation, secretory systems and machinery for protein targeting, sexual reproduction, embryogenesis, vegetative development, gene regulation, light regulation, and biological clocks and programmed cell death, among others.

Arabidopsis thaliana is an innocuous weed of no special economic value (Zambryski, 1997). Thus, as Rédei (1992) describes, the value of *A. thaliana* for mankind is justified by the depth of scientific information that can be collected through its study. Although its usefulness parallels its development as a research tool, the fundamental value of the scientific information provided eventually determines its merits.

2.2 An overview of studies on metabolic pathways in *A. thaliana*

In general, metabolic pathways consist of a series of successive or matrix enzymatic reactions, which yield a specific product or family of products. As described by Voet and Voet (1995), there are at least four general characteristics of metabolic pathways based on their function in cells: 1) metabolic pathways are not reversible, 2)

each metabolic pathway has a first executed step, 3) all metabolic pathways are controlled, and 4) metabolic pathways in eukaryotic cells often take place in particular cellular compartments.

Experimentally, a metabolic pathway can be studied at various levels. Firstly, it can be studied in terms of a series of sequential enzymatic reactions where an input compound is converted to one or more end products. Secondly, one can study mechanisms whereby each transitional compound is changed to its subsequent compound - to achieve this, one may have to isolate and characterize particular enzymes catalyzing every reaction step. Thirdly, studies can be done which address control mechanisms that govern the flux of metabolites through the pathway. To accomplish all of these, techniques are required to disturb the system and detect the effect of the disruption on growth or on the yield of metabolic intermediates. For example, metabolic inhibitors can be used to inhibit a metabolic pathway at a particular enzymatic step, or the study of genetic variants that abolish or modify particular steps in the metabolic pathway of interest can be undertaken (Voet and Voet, 1995).

Studies of *A. thaliana* as a model plant system have contributed considerably to the advancement of our understanding of plant metabolic pathways (Cobbett, 1998). Studies to confirm molecular and genetic aspects of biochemical pathways have benefited from the isolation and characterization of mutants lacking or modified in particular enzymes. Moreover, such mutants have been a useful source of experimental objects for further studies on pathway roles or specific metabolic compounds in numerous segments of plant physiology (Cobbett, 1998). Cobbet (1998) compiled and tabulated the metabolic pathways in *A. thaliana* that have been studied via the generation and analysis of mutants. In this compilation, the author showed that several

metabolic pathways had been more comprehensively dealt with than others, including the metabolism of nitrogen and amino acids involving twenty-four loci (including *csr1-1*, the locus for the chlorsulfuron resistant variant of ALS), carbohydrates with thirteen loci, fatty acids and waxes with twenty-three loci, and metabolic pathways responding to environmental stresses with twenty-two loci.

2.3 The branched-chain amino acid (BCAA) biosynthetic pathway in plants

2.3.1 Biochemistry and molecular biology of the pathway

Amino acids are essential for the function of all organisms. In the mammalian kingdom, amino acids can be divided into two groups - essential and non-essential. Non-essential amino acids are those that can be intrinsically synthesized, whereas essential amino acids are those that must be obtained from the diet and are indirectly derived from microorganisms and/or plants (Voet and Voet, 1995). The essential amino acid group is further divided into several families based on their metabolic precursors and their chemical properties (e.g. aromatic or aliphatic). These are the aspartate family (lysine, methionine, threonine), the pyruvate family (leucine, isoleucine, valine), the aromatic amino acids (phenylalanine, tryptophan), histidine and arginine, which is essential for child growth and is grouped as a member of the glutamate family along with non-essential proline and ornithine (Voet and Voet, 1995).

This thesis is principally concerned with the so-called *ilv*, pyruvate-derived, biosynthetic pathway or BCAA pathway leading to the formation of the branched-chain amino acids (BCAAs) isoleucine, leucine and valine.

In the past, isoleucine was grouped as a member of the aspartate family, although biosynthetically it may be better grouped with the pyruvate family (Umbarger, 1978). The biosynthetic pathways leading to isoleucine, leucine and valine of the pyruvate family are best considered together (as shown in Figure 2.1), not so much because of their common aliphatic properties but because their pathways and regulation are interrelated (Umbarger, 1978).

As shown in Figure 2.1, the biosynthesis of the five-carbon valine and the six-carbon isoleucine takes place in a parallel series of reactions, as summarized by Umbarger (1978), Bryan (1980), and Barak *et al.* (1990). The first reaction in valine biosynthesis involves the condensation of pyruvate with an activated acetaldehyde derived from a second molecule of pyruvate, yielding 2-acetolactate. A similar reaction takes place in isoleucine biosynthesis where instead of pyruvate, 2-ketobutyrate (derived from deamination of threonine by threonine deaminase) is the acetaldehyde acceptor, forming 2-aceto-2-hydroxybutyrate. Since this parallel step involves acetolactate synthase (ALS), the enzyme of interest in this research, it will be more fully described in the next section.

The first step in the pathway yields two acetohydroxy acids, which undergo an NADPH-dependent reduction and alkyl group migration catalyzed by ketol-acid reductoisomerase (KARI, EC1.1.1.86) to yield the dihydroxy acid precursors 2,3-dihydroxyisovalerate and 2,3-dihydroxy-3-methylvalerate in the isoleucine and valine pathways, respectively. These precursors undergo subsequent dehydration reactions, which are catalyzed by dihydroxyacid dehydratase (DH, EC4.2.1.19) to produce 2-keto-3-methylvalerate and 2-ketoisovalerate, respectively. These intermediates, in turn, undergo transamination reactions with glutamate as the $-NH^{3+}$ donor, and catalyzed by

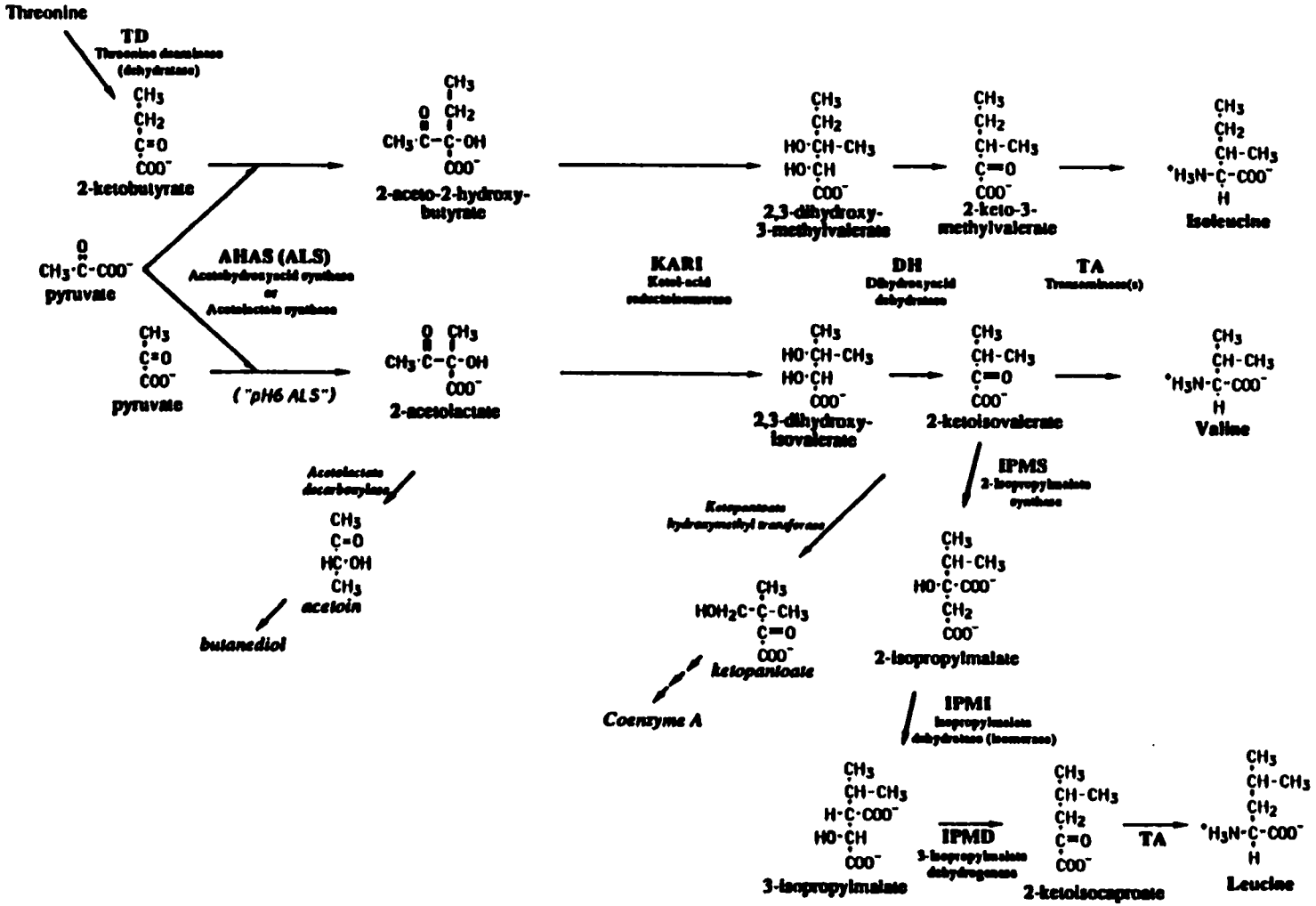


Figure 2.1. A flow chart of branched-chain amino acid (BCAA) biosynthetic pathways adapted from Barak *et al.* (1990).

branched-chain amino acid transaminase (BCAA-TA, EC2.6.1.42), yielding isoleucine and valine, respectively.

An intermediate in valine biosynthesis, 2-ketoisovalerate, is a branch point compound that serves as the substrate for the first enzyme in leucine biosynthesis, 2-isopropylmalate synthase (IPMS, EC4.1.3.12), which catalyzes the condensation reaction of this substrate with acetyl CoA to yield 2-isopropylmalate, which in turn undergoes a rearrangement reaction catalyzed by isopropylmalate isomerase (dehydratase; IPMI, EC4.2.1.33) to yield 3-isopropylmalate. This isomer undergoes dehydrogenation with NAD^+ as the proton acceptor, catalyzed by 3-isopropylmalate dehydrogenase (IPMD, EC1.1.1.85), to yield 2-ketoisocaproate. With the catalyzing action of BCAA-TA, leucine is produced from 2-ketoisocaproate.

Table 2.1 summarizes some of the cognate genes and biochemical properties of the enzymes involved in BCAA biosynthesis as compiled from various sources (Barak *et al.*, 1990; Schomburg, 2000-http://www.brenda.uni-koeln.de/brenda/brenda_copyright.html; Enzyme Nomenclature Database-http://www.expasy.ch/enzyme/). In prokaryotes, detailed reviews of the biochemical characteristics of all enzymes involved, including aspects of their molecular biology and gene regulation, have been previously summarized (Umbarger, 1978; Umbarger, 1996). Subsequent sections of this thesis focus on ALS as the first common enzyme in the BCAA biosynthetic pathway.

2.3.2 Acetolactate synthase (ALS), the first common enzyme in the pathway

Acetolactate synthases (EC4.1.3.18) can be grouped into two classes based on their distinct physiological roles, substrate specificities, and cofactor requirements (Chipman *et al.*, 1998). The first class of enzymes is involved in the first common

Table 2.1. Selected biochemical and genetic properties of enzymes involved in the BCAA biosynthetic pathway as compiled from various sources[1].

Pathway and enzymes	Gene		Molecular mass (kDa)		Cofactor/ prosthetic group
	Prokaryote[2]	Eukaryote[3]	Prokaryote	Eukaryote	
Isoleucine, leucine, valine pathway:					
ALS (EC4.1.3.18)	*ilvBN* *ilvGM* *ilvIH*	*ILV1* *ILV2* [4]*CSR1*	140-600	55-440	TPP, FAD, Mg
KARI (EC1.1.1.86)	*ilvC*	*ILV5*	220	70-235	NADPH
DH (EC4.2.1.9)	*ilvD*	*ILV3*	130-155	63-110	Cysteine
TA (EC2.6.1.42)	*ilvE* *avtA* *tyrB*	*BAT1* *BAT2*	80-197	34-96	PLP, PMP
Leucine pathway:					
IPMS (EC4.1.3.12)	*LeuA*	*LEU4*	114-175	68-137	CoA
IPMI (EC4.2.1.33)	*LeuC*	*LEU1*	88	70-90	None
IPMD (EC1.1.1.85)	*LeuB*	*LEU2*	50-90	39-72	NAD

[1]Data were compiled from Barak *et al.* (1990), Schomburg (2000)-http://www.brenda.uni-koeln.de/brenda/brenda_copyright.html and Enzyme Nomenclature Database -http://www.expasy.ch/enzyme/.

[2/3]Gene designations are based on bacterial and yeast genetic nomenclatures, respectively (Mol. Cell. Biol. - Instructions to authors, Jan 2001).

[4]Gene designation is based on the *A. thaliana* genetic nomenclature (Meinke and Koornneef, 1997).

enzymatic step in the biosynthetic pathway leading to the formation of the branched-chain amino acids isoleucine, leucine and valine (Umbarger, 1987). This enzyme is found in bacteria (Stormer and Umbarger, 1964; Schloss *et al.*, 1985; Barak *et al.*, 1988; Schloss *et al.*, 1991), fungi (Poulsen and Stougaard, 1989), algae (Hartnett *et al.*, 1987) and higher plants (Miflin, 1971; Durner and Böger, 1988; Durner and Böger, 1990), but not in animals. The enzymes are flavoproteins with a dependence on flavin adenine diphosphate (FAD) as co-factor (Schloss, 1992). Most are sensitive to feedback inhibition by one or more BCAAs as end products of the pathway. They are also sensitive to at least three classes of herbicides, namely the sulfonylureas (Chaleff and Mauvis, 1984; LaRossa and Schloss, 1984; Ray, 1984), the imidazolinones (Shaner *et al.*, 1984; Muhitch *et al.*, 1987) and the triazolopyrimidines (Kleschick, 1994; Hawkes *et al.*, 1989). The second class of enzymes is typified from *Aerobacter (Enterobacter) aerogenes* strain 1033 (Johansen *et al.*, 1975) where ALS is committed solely to the post exponential growth phase production of acetoin in bacteria (Halpern and Even-Shoshan, 1967; Holtzclaw and Chapman, 1975). This enzyme does not require FAD, is not sensitive to inhibition by BCAAs (Stormer, 1968) and has a relatively low capacity for acetohydroxybutyrate production (Huseby and Stormer, 1971; Gollop *et al.*, 1990). This enzyme is often called 'pH 6' acetolactate synthase, since it tends to exhibit a somewhat low pH optimum (Stormer, 1967). The FAD-independent, acetolactate synthase holoenzyme from *Aerobacter aerogenes* has a molecular mass of 232 kDa and is comprised of a tetramer of identical 58-kDa subunits (Huseby *et al.*, 1971). Enzymes from other microorganisms with no requirement for FAD have been studied and appear to be similar (Holtzclaw and Chapman, 1975; Malthe-Sorenssen and Stormer, 1970; Peng *et al.*, 1992).

2.3.2.1 Biochemistry and molecular genetics of ALSs from prokaryotes and eukaryotes

There is little difference in the biochemical function of FAD-dependent ALS enzymes in prokaryotes and eukaryotes. They all catalyze the non-oxidative, thiamine-pyrophosphate (TPP)-dependent decarboxylation of pyruvate and transfer of the active acetaldehyde group to either pyruvate or 2-ketobutyrate (Umbarger, 1996), although they may be distinct in enzymatic characteristics including substrate preference, feedback inhibition and kinetics (Chipman *et al.*, 1998). These two parallel reactions yield 2-acetolactate and 2-aceto-2-hydroxybutyrate, respectively, which in turn undergo the subsequent enzyme-catalyzed reactions (Umbarger, 1978) leading to the formation of valine (and leucine) and isoleucine, respectively (Schloss, 1992). As a flavoprotein, ALS has an absolute requirement for FAD, TPP and divalent metal cations (Mg^{2+} or Mn^{2+}) for activity (Schloss *et al.*, 1985; Schloss, 1992). Singh *et al.* (1988a) suggested that the essential requirement for the divalent cation Mg^{2+} or Mn^{2+} was presumably for the binding of TPP to the enzyme. The FAD requirement of ALS may reflect a structural role of the flavin in the stabilization of the quaternary structure of the polypeptide, as was first suggested by Durner and Böger (1990).

In wild type *Escherichia coli* and *Salmonella typhimurium*, three different ALS isozymes that catalyze the formation of acetohydroxyacids have been identified (Blatt *et al.*, 1972; O'Neill and Freundlich, 1972; De Felice *et al.*, 1974; Shaw *et al.*, 1980). All three isozymes exhibit a quaternary structure consisting of two large catalytic subunits and two smaller regulatory subunits (Umbarger, 1996).

As summarized by Schloss (1992), isozyme I from *E. coli* (ALS-I) is a tetramer composed of two identical large subunits of 60.4 kDa encoded by *ilvB* and two identical

small subunits of 11.1 kDa encoded by *ilvN*. *Escherichia coli* isozyme III (ALS-III) is also a tetramer, but differs in the size of its subunits *vis-à-vis* those of ALS-I where the large subunit is of 61.8 kDa and encoded by *ilvI* and the small subunit is of 17.5 kDa and encoded by *ilvH*. Isozyme II from *S. typhimurium* (ALS-II) contains two identical 59.3-kDa α (large) subunits encoded by the *ilvG* locus, plus two identical 9.7-kDa β (small) subunits encoded by *ilvM*. Acetolactate synthase-II is the-best studied prokaryotic enzyme thus far, and exhibits a quaternary structure of $\alpha_2\beta_2$, resulting in a native molecular mass of 138 kDa. In the presence of pyruvate, ALS-II persists as native $\alpha_2\beta_2$ and $\alpha_4\beta_4$ oligomers. One mole of FAD is bound per mole of αβ protomers, regardless of enzyme activity losses upon storage or purification. On the contrary, TPP is bound by the purified enzyme in less than unit stoichiometry i.e. 0.35 mol/mol of αβ protomers, and the capacity of the enzyme to bind TPP decreases as the enzyme loses activity upon storage (Schloss and Aulabaugh, 1990).

Most prokaryotes other than *E. coli* (Blattner *et al.*, 1997), and for which complete genome sequences are now publicly accessible, appear to encode only a single ALS enzyme. Examples include *Haemophilus influenzae* (Fleischmann *et al.*, 1995), *Bacillus subtilis* (Kunst *et al.*, 1997), the cyanobacterium *Synechocystis sp.* Strain PCC6803 (Kaneko *et al.*, 1996) and the two archaea, *Methanococcus jannaschii* (Bult *et al.*, 1996) and *Archaeoglobus fulgidus* (Klenk *et al.*, 1997) as compiled by Chipman *et al.* (1998), plus the archaean *Methanobacterium thermoautotrophicum* deltaH (Smith *et al.*, 1997), *Campylobacter jejuni* (Parkhill *et al.*, 2000a), *Neisseria meningitidis* Z2491 (Parkhill *et al.*, 2000b), and possibly other prokaryotes with completely-sequenced

genomes. Each organism presents only a single gene that can be characterized as encoding an ALS catalytic subunit based on sequence similarity (Chipman *et al.*, 1998).

In eukaryotes, ALS activity, together with other enzymes of the BCAA biosynthetic pathway, is compartmentalized in the mitochondrion in yeast and fungi (Ryan and Kohlhaw, 1974) or the chloroplast in algae and higher plants (Miflin, 1974; Reith and Munholland, 1993). Unlike their prokaryotic counterparts, eukaryotic ALS holoenzymes exhibit a wide range of molecular masses, often with conflicting accounts of the holoenzyme/quaternary content of the enzyme. Partially-purified ALS from *Neurospora crassa* exhibited four peaks having ALS activity, at molecular masses of 51, 68, 140 and 500 kDa, following chromatographic analysis of crude extracts (Tanaka and Kuwana, 1984). These different molecular-mass species were not considered isozymes, but rather different aggregates of a single ALS subunit. This was inferred since *N. crassa* has been shown by genetic analysis to have only one functional *ALS* gene. Furthermore, the 68 kDa subunit was indicated to be the basic subunit of valine-sensitive ALS, since a 51 kDa species was observed that lacked valine sensitivity (Tanaka and Kuwana, 1984).

Purified ALS isolated from barley was reported to be a homo-octamer of 58 kDa subunits in the presence of FAD, that dissociated to a tetramer in the presence of pyruvate or in the absence of FAD (Durner and Böger, 1988). On the other hand, a more recent isolation of the barley enzyme, using a different enrichment procedure, resulted in an enzyme complex assumed to be a tetramer of 65 kDa polypeptides (Chong *et al.*, 1997). On this basis, it was suggested that the 58 kDa polypeptide was a degradation product (Chipman *et al.*, 1998). Acetolactate synthase purified from maize embryogenic cell suspension cultures exhibited a native molecular mass of 440 kDa

(Muhitch *et al.*, 1987). In contrast, the existence of two active ALS peaks corresponding to molecular masses of 193 and 55 kDa was reported upon size exclusion chromatography of extracts from Black Mexican sweet corn cell cultures, suggesting this species might exhibit different oligomeric forms of the same polypeptide (Singh *et al.*, 1989). In wheat, purified ALS was reported to contain 58, 57 and 15 kDa polypeptides, with a native molecular mass of ca. 128 kDa. The smallest subunit was implied to be a partial degradation product of a larger subunit polypeptide, or simply an impurity that was co-enriched in the preparations. On the other hand, it has not been ruled out that the plant enzyme may contain a small regulatory subunit analogous to that in bacteria (Southan and Copeland, 1996). The native molecular mass of the wheat ALS was similar to that of the *Brassica napus* ALS described by Bekkaoui *et al.* (1993), who found that the *B. napus* ALS enzyme was comprised of two subunits of 65 and 66 kDa molecular mass. In some other reports, a single 65-kDa polypeptide has been identified in immunoblots of crude extracts from a wide range of plant tissues using monoclonal antibodies raised against synthetic peptides as immunogen, or polyclonal antibodies raised against an *A. thaliana* ALS fusion protein expressed in *E. coli* (Singh *et al.*, 1991a and 1991b).

Taken together, the quaternary structure of eukaryotic ALS enzymes remains unclear as reports in the literature are often contradictory, making it difficult to establish a consistent pattern.

2.3.2.1.1 Regulatory subunits of ALS in eukaryotes

In prokaryotes, the quaternary structure of the ALS holoenzyme has been well established. The large "catalytic" and small "regulatory" subunits have been identified,

investigated and characterized by many researchers. The $\alpha_2\beta_2$ structure is generally accepted to be the common quaternary structure for most prokaryotic ALSs. Their physicochemical and molecular genetic properties have also been summarized (see Umbarger, 1987; Umbarger, 1996; Chipman *et al.*, 1998 for detailed reviews).

As for eukaryotic ALS, much of the work to date has focused on the large "catalytic" subunit of ALS. Several investigators have suggested alternative quaternary structures for ALS even though, in some cases, the ALS enzyme was isolated from the same organism. Most investigators have inferred the existence of ALS as a homo-oligomer i.e. the enzyme contains identical subunits in multiple stoichiometry. The question of the existence of a low molecular weight "regulatory" subunit of ALS was first raised by Singh *et al.* (1992). They found that ALS from *A. thaliana* seedlings was sensitive to feedback inhibition by pathway end-products, namely valine and leucine. However, ALS activity extracted from *E. coli* expressing recombinant *A. thaliana* ALS was insensitive to inhibition by valine and leucine in both *in vitro* assays and *in vivo* complementation studies. One of several explanations for the observed insensitivity of ALS to valine and leucine was that a second subunit normally present in the plant and required for the feedback regulation sensitivity of the enzyme was absent in the recombinant bacterial expression system. This argument was supported by a previous finding that the prokaryotic ALS isozyme I (ALS-I) required a second "small" subunit for sensitivity to valine inhibition (Eoyang and Silverman, 1986).

More recently, it has been reported that ALS in crude extracts of wheat leaves was strongly inhibited by end-products of the pathway. However, after partial purification the enzyme was less sensitive to feedback inhibition. This finding suggests

that other factors (e.g. a second subunit) are required for the feedback inhibition response, factors that might have been lost by consecutive purification procedures (Southan and Copeland, 1996). In agreement with a previous report by Singh *et al.* (1992), Chang and Duggleby (1997) found that *A. thaliana* ALS expressed in *E. coli* was insensitive to feedback inhibition by branched-chain amino acids. Possible reasons included the improper folding of the enzyme in its native form, incorrect processing of a transit peptide and the absence of a small subunit (Singh *et al.*, 1992). They argued that the requirement for the small subunit for feedback inhibition is feasible since such a role has been demonstrated for the small subunit in isozyme III of *E. coli* ALS (Vyazmensky *et al.*, 1996). In the same year, Duggleby (1997) reported a putative ALS small subunit from two eukaryotes, namely yeast, *Saccharromyces cerevisiae*, and red algae, *Phorphyra purpurea*, based on alignment similarity to the bacterial ALS small subunit from *Bacillus flavum*.

A recent study (Hershey *et al.*, 1999) identified a cDNA clone that encoded a putative small subunit of plant ALS based upon homology with various bacterial ALS small subunits. *In vitro* studies involving mixing of a partially-purified *E. coli* recombinant small subunit with the ALS catalytic subunit from tobacco or *A. thaliana* resulted in both increased specific activity and increased stability of the enzyme activity. However, no feedback inhibition of activity by branched-chain amino acids was observed using *in vitro* assays.

Evidence for the possible existence of a small subunit that mediates post-translational feedback inhibition of ALS is accumulating in the eukaryotic taxa. In addition to that described above, it has been reported that a disruption of the yeast gene mentioned by Duggleby (1997) affects the sensitivity of yeast ALS to feedback response

in crude extracts (Cullin *et al.*, 1996). Duggleby (unpublished), as noted by Chang and Duggleby (1997), has identified expressed sequence tags (EST) that may represent portions of a putative ALS regulatory small subunit in rice and *A. thaliana.*

Kohalmi *et al.* (unpublished) used the yeast two-hybrid system to survey for protein-protein interactions using the *A. thaliana* ALS as the 'bait' against an *A. thaliana* cDNA expression library, and found ten classes of proteins encoded by forty-five cDNA clones that interacted with the ALS catalytic subunit. Two of the observed protein classes exhibited significant similarity to multiple prokaryotic small subunits and putative eukaryotic small subunits. Although an intriguing finding, an independent biochemical assessment is required to verify the observed protein-protein interactions *in planta.*

One of the principal goals of this study has been to carry out a biochemical assessment of protein-protein interactions involving the ALS catalytic subunit from *A. thaliana.*

2.3.2.1.2 An overview of ALS kinetic properties

Most kinetic studies involving ALS have been carried out using prokaryotic ALS as the model enzyme. Thus, the kinetic scheme highlighted here is based on the kinetics of the prokaryotic enzyme from *E. coli* or *S. typhimurium.* As summarized by Chipman *et al.* (1998), nearly all ALS enzymes display noticeable specificity for 2-ketobutyrate over pyruvate as the second subtrate. Chipman *et al.* (1990) provided a definitive kinetic analysis by developing analytical procedures for determining the second substrate specificity of ALS by analyzing the biosynthesis of acetolactate as the valine

precursor, and acetohydroxybutyrate as the isoleucine precursor. They defined a parameter, R, using the formula shown below:

$$R = \frac{V_{AHB}/V_{AL}}{[\text{2-ketobutyrate}]/[\text{pyruvate}]}$$

where AHB is acetohydroxybutyrate, AL is acetolactate and V is the rate of formation. This formula was then used to calculate and compare the R values for the three *E. coli* isozymes, ALS-I, ALS-II and ALS-III. The formula was first developed based on experiments carried out with ALS-III. In one experiment, analysis was performed at a constant pyruvate concentration (1 mM) and several different concentrations of 2-ketobutyrate, where the amount of AHB formed increased as the 2-ketobutyrate concentration increased, while the amount of AL formed decreased. The ratio of the concentrations of the two products stayed proportionally constant to the ratio of the concentrations of the substrates (R= 41 ± 3) over an extended range of 2-ketobutyrate concentrations. In a second experiment with the same enzyme, pyruvate was prepared over a series of known concentrations while maintaining the 2-ketobutyrate concentration constant (0.1 mM). The proportion (R) was constant (40 ± 3) in this experiment over a broad range of pyruvate concentrations and was comparable to the value from the first experiment with ALS-III. In the absence of, or at a constant concentration of, 2-ketobutyrate, the dependence of acetolactate formation by ALS on the pyruvate concentration obeyed Michaelis-Menten kinetics, although two molecules of pyruvate are involved in the reaction. Schloss *et al.* (1985) suggested that this kind of

response indicated that an irreversible step may take place between the addition of the first and second pyruvate to the enzyme.

2.3.3 Metabolic regulation of ALS

In bacteria, genetic regulatory mechanisms for ALS include induction/repression, activation/deactivation and attenuation, as reviewed by several authors (Umbarger, 1987; Tsui and Freundlich, 1990; Umbarger, 1996). In plants, work has been carried out to demonstrate end-product repression of the BCAA biosynthetic enzymes. However, no repression was found when ALS from barley was examined (Miflin and Cave, 1972). Therefore, as summarized by Wallsgrove (1990), there was no proof for metabolic control by induction/repression, activation/deactivation or attenuation of plant BCAA enzymes.

In terms of metabolic (biochemical) regulation, BCAA pathways in both prokaryotic (Umbarger, 1987) and eukaryotic (Wallsgrove, 1990) organisms display similar responses to metabolic regulation by end-product feedback inhibition. This section highlights the biochemical regulation of ALS from higher plants.

As reviewed by Azevedo *et al.* (1997), studies have been performed to investigate the metabolic regulation of ALS by feedback inhibition. Acetolactate synthase of plant origin was found to be inhibited by each of the three BCAAs or co-operatively by leucine plus valine (Miflin, 1971; Miflin and Cave, 1972). In maize, end-product inhibition occurred when the enzyme assumed at least the dimeric form, as pointed out by Singh *et al.* (1988a). In etiolated barley shoots and *B. napus* cotyledons, two forms of ALS were detected by ion exchange chromatography, where inhibition by isoleucine, leucine and valine was reported to exhibit only minor differences in

sensitivity for the two enzyme forms (Durner and Böger, 1990; Bekkaoui *et al.*, 1993). It has been reported that inhibition by the three BCAAs displayed mixed competition with regard to pyruvate (Hawkes *et al.*, 1989; Rathinasabapathi and King, 1991; Mourad *et al.*, 1995). In *A. thaliana*, the enzyme was 60% inhibited by 1 mM valine or leucine, but was no longer inhibited (less than 10%) when the corresponding gene was expressed in recombinant *E. coli*. The results suggested that a second subunit was required to mediate the response of the enzyme to feedback inhibition by BCAAs (Singh *et al.*, 1992).

In another study, it was revealed that exogenous branched-chain amino acids inhibited plant growth. In particular, the growth of barley embryos was inhibited by valine, which was restored by isoleucine but increased by the addition of leucine. For the inhibition of growth caused by leucine, however, both valine and isoleucine were needed for reversal (Miflin, 1969). Borstlap (1981) reported similar results with *Spirodela polyrhiza*, where the co-operative action of valine and leucine inhibited ALS activity and blocked the formation of isoleucine. Isoleucine also inhibited growth, but its effect increased the soluble pool of valine, suggesting that inhibition was not due to action on ALS activity but instead was due to the blockage of the conversion of valine to leucine. Hagelstein *et al.* (1993) obtained corroborative data on leucine biosynthesis that confirmed this proposed explanation.

Wu *et al.* (1994) reported that a mutant of *A. thaliana* had been identified that showed resistance to growth inhibition by valine but not to isoleucine or leucine, and where ALS activity from the mutant exhibited reduced sensitivity to all three BCAAs. The results indicated that the growth inhibitory mechanism of isoleucine and leucine was not due to a direct effect on ALS activity. In a different study, seven valine-

resistant tobacco protoplast mutants were regenerated to whole plants, and four were found to exhibit defects in amino acid uptake (Bourgin *et al.*, 1985). When ALS was isolated from the remaining three mutants, the regulatory properties of the enzyme were found to be modified, in that the activity was less sensitive to inhibition by leucine or valine (Relton *et al.*, 1986). Hervieu and Vaucheret (1996) cloned the ALS catalytic subunit gene of valine-resistant tobacco, and upon sequencing the mutant allele found a missense mutation at position 214, changing serine to leucine.

Pang and Duggleby (1999) were the first to report an ALS small subunit from yeast that functions in *in vitro* feedback inhibition of the ALS catalytic subunit by valine. The putative small subunit protein, designated ILV6, and the catalyitc subunit of yeast ALS (ILV2), were each overexpressed in *E. coli* and purified to near homogeneity. Reconstitution studies revealed that the ILV6 protein stimulated the catalytic activity of the ILV2 protein by up to sevenfold and conferred sensitivity to valine inhibition. Without direct evidence, they speculated that the two proteins interacted through hydrophobic interactions, since the subunit association could only be observed in a high ionic strength buffer (i.e. approximately 1 M potassium phosphate), which favours such interactions. Unfortunately, such ionic strength of the buffer does not reflect a physiologically-relevant intracellular ionic strength of yeast.

2.3.4 Molecular characterization of plant ALSs

Acetolactate synthase has been identified, cloned and characterized from several eukaryotes, including *Saccharomyces cerevisiae* (Falco *et al.*, 1985), *Schizosaccharomyces pombe* (Bekkaoui *et al.*, 1993a), *A. thaliana* (Mazur *et al.*, 1987), *Nicotiana tabacum* (Mazur *et al.*, 1987; Lee *et al.*, 1988), *B. napus* (Wiersma *et al.*,

1989), *Gossypium hirsutum* (Grula *et al.*, 1995) and *Zea mays* (Fang *et al.*, 1992). In *S. cerevisiae*, on the basis of its ability to complement a deletion mutant, a single gene was identified and cloned from a plasmid genomic library. The gene, termed *ILV2*, encoded a deduced polypeptide of 687 amino acids and was mapped to the right arm of chromosome XIII (Falco and Dumas, 1985). The gene was deduced to be required and sufficient for ALS function in yeast, since inactivating the wild type $ILV2^{+}$ caused *ilv* auxotrophy (Falco and Dumas, 1985).

Using the yeast gene as a heterologous probe, Mazur *et al.* (1987) isolated genomic clones encoding the corresponding ALS protein in *A. thaliana* and *N. tabacum*, where they found a single gene in *A. thaliana* and two genes in the allotetraploid *N. tabacum*. Molecular characterization revealed that both genes lacked introns and exhibited homologies of 73% and 84% at the nucleotide level and the amino acid level, respectively to those of yeast and *E. coli*. Unmodified polypeptides of 670 and 667 amino acids of predicted molecular mass of 72.6 and 72.9 kDa were deduced from the *A. thaliana* and tobacco *ALS* genes, repectively. Keeler *et al.* (1993) named the two *ALS*-like tobacco genes as *SurA* and *SurB*. Both genes were expressed in all organs and developmental stages analyzed, and their relative expression was co-ordinately regulated. Although the transcript of *SurB* was always present at higher levels than that of *SurA*, maintenance of gene expression at a constant proportion was observed, suggesting a common regulatory mechanism (Keeler *et al.*, 1993). An *ALS* gene family was cloned and characterized in cotton, *G. hirsutum*, where four of the six genes were functional and structured as tandem pairs in which the genes were separated by only 2-3 kb (Grula *et al.*, 1995). At least two of the four genes were in the two tandem pairs.

These genes exerted either constitutive, low level expression or specific high level expression in reproductive tissues.

Brassica napus is an allotetraploid originating from a fusion between *Brassica campestris* and *Brassica oleracea*. Rutledge *et al.* (1991) identified five *ALS* genes which were subsequently cloned and sequenced. Genes of *ALS1* and *ALS2* were constitutively expressed in a wide range of somatic and reproductive tissues. The strong sequence homologies of both the coding and non-coding regions of *ALS1* and *ALS3* suggested a common origin and function (Rutledge *et al.*, 1991; Ouellet *et al.*, 1992). The *ALS2* was shown to be structurally distinct from the other genes and was only expressed in mature ovules and extra-embryonic tissues of immature seeds. The *ALS4* was not expressed in any tissue studied, and was probably a pseudogene (Ouellet *et al.*, 1992). In separate studies, genomic and cDNA *ALS* clones were isolated from *B. napus* (Wiersma *et al.*, 1989; Bekkaoui *et al.*, 1991b). Using the *A. thaliana ALS* gene as a heterologous hybridization probe, a genomic *ALS* clone, designated *ALS*-1, was isolated from a *B. napus* genomic library (Wiersma *et al.*, 1989). The *ALS*-1 genomic clone exhibited an open reading frame (ORF) that appeared to lack introns and encoded a predicted polypeptide of 637 amino acids. A cDNA clone designated *ALS*-2 was isolated from a *B. napus* cDNA library derived from five-day-old seedlings (Bekkaoui *et al.*, 1991b). This clone differed from *ALS*-1 in that it encoded a polypeptide of 599 amino acids. Based on Southern blot analysis, both genes appeared to be members of an *ALS* multiple gene family consisting at least four genes, in agreement with the results of Rutledge *et al.* (1991).

The N-terminal regions of ALS-1 and ALS-2 exhibited a putative transit peptide sequence that may be required for import into the chloroplast, although the predicted

domains showed little sequence similarity to each other or to the *A. thaliana ALS* gene. Wiersma *et al.* (1989) used the unique transit peptide sequence of *ALS*-1 as a hybridization probe against genomic DNA of *B. campestris* and *B. oleracea.* Based on Southern blot data, they concluded that the *B. napus ALS*-1 gene originated in the *B. campestris* progenitor. The *ALS*-1 transit peptide was also used as a unique probe to examine the developmental and tissue specific expression of the gene. Based on Northern blot analysis, low levels of expression were detected and only in relatively young flower buds and zygotic embryos (P. Wiersma, unpublished, cited in Babic, 1991). The expression of *ALS*-2 was analyzed by the then-novel approach of quantitative PCR (Qt-PCR), which revealed that *ALS*-2 transcripts were detected at various levels in all tissues examined, with the lowest levels of the *ALS*-2 transcript found in roots and the highest levels in flower buds of three-month-old plants (Bekkaoui *et al.*, 1991a). However, Qt-PCR failed to detect *ALS*-1 transcripts in any of the tissues investigated. The *ALS*-1 transcript appears to be identical to the *ALS2* gene that was identified and described by Rutledge *et al.* (1991).

2.3.5 Acetolactate synthase as a herbicide target

For more than a decade, ALS has attracted the attention of biotechnologists since it was found to be the metabolic inhibitory target of several structurally diverse classes of herbicides (Bekkaoui *et al.*, 1993). At least three major classes of compounds have been shown to be potent inhibitors of ALS, including the sulphonylureas, the imidazolinones and the triazolopyrimidines. The detailed chemistry of these substances has been described by several authors (Schloss *et al.*, 1994; McGee and Hay, 1994;

Ladner, 1994; Kleschick, 1994). In general, all compounds have a strong herbicidal activity at very low application rates i.e. 2-75 g ha^{-1}.

In particular, chlorsulfuron (2-chloro-N[[(4-methoxy-6-methyl-1,3,5-triazin-2-yl)amino]carbonyl]benzenesulfonamide) is a herbicide compound of the sulfonylurea class along with sulfometuron methyl, metsulfuron methyl and nicosulfuron, among others. Sulfonylurea class compounds consist of three moieties i.e. an aryl group where a phenyl group is a common ortho substitution to a sulfonylurea link, a heterocycle moiety with a symmetrical pyrimidine or triazine as the common moiety, and a sulfonylurea bridge joining the former two moieties. Slight substitution changes in any part of the molecule can cause large changes in biological activity and selectivity (Devine *et al.*, 1993). These substances are immediately taken up by roots or leaves and are transported to phloem and xylem. Field application rates as low as 2 g ha^{-1} of these highly-active substances were reported (Tachibana, 1987). A chlorsulfuron-resistant mutant of *A. thaliana* was developed more than a decade ago (Haughn and Somerville, 1986) where a single mutation (C to T) in the wild type *ALS* changed its residue 197 from proline to serine (Haughn *et al.*, 1988).

The kinetics of enzyme inhibition have been studied in depth in various organisms by several investigators, including Hawkes *et al.* (1989), Subramanian *et al.* (1990), Schloss (1992), Ott *et al.* (1996) and Southan and Copeland (1996). Avezedo *et al.* (1997) summarized the inhibition constant (K_i) values of these classes of herbicides; K_i values in the region of 5-20 nM and 2-60 μM were observed for sulphonylurea and imidazolinone herbicides, respectively. Although the K_i values are different, both classes of herbicides have similar field-application rates. The discrepancy between *in*

vivo and *in vitro* effectiveness may explain differences in the abilities of the herbicides to induce irreversible loss of enzyme activity (Durner *et al.*, 1991; Shaner and Singh, 1991).

La Rossa *et al.* (1990) pointed out a possible mechanism of herbicide action since these ALS inhibitors induced the accumulation of 2-ketobutyrate and the transamination product 2-aminobutyrate, causing the death of microorganisms and plants. However, a study by Shaner and Singh (1993) showed that in maize, using imazaquin herbicide, there was no correlation between the pool sizes of 2-ketobutyrate and 2-aminobutyrate and the growth inhibition effects. They argued instead that the herbicidal action was due to starvation of branched-chain amino acids, particularly valine and leucine.

Hofgen *et al.* (1995) reported that a transformed potato with a recombinant antisense *ALS* gene under the control of the *CaMV 35S* promoter exhibited a 14% reduction in ALS activity compared to the wild type, and was accompanied by the typical symptoms of herbicide treatment. Although low levels of 2-aminobutyrate were detected in the antisense-transformed plants, there was no accumulation of 2-ketobutyrate, although soluble amino acid pools were increased (Hofgen *et al.*, 1995). A similar observation, suggesting an increase in soluble amino acids due to protein breakdown, has been reported following treatment with the sulfonylurea herbicide, chlorsulfuron (Rhodes *et al.*, 1987). The growth inhibitory effects observed in the antisense plants could not be reversed by the addition of BCAA, although some reversal was obtained with casamino acids, suggesting that an imbalance in the soluble amino acids, particularly lysine and threonine, may be responsible for the growth inhibition (Rhodes *et al.*, 1987).

As summarized by Avezedo *et al.* (1997), there has been considerable interest in obtaining plants that are tolerant to the effects of ALS specific herbicides. Mutant lines of *A. thaliana* (Haughn and Somerville, 1986), soybean (Shaner and Singh, 1993), *B. napus* (Swanson *et al.*, 1989), *G. hirsutum* (Subramanian *et al.*, 1990), *Datura innoxia* (Rathinasabapathi and King, 1991), maize (Anderson *et al.*, 1989; Bright, 1992) and flax (McSheffrey *et al.*, 1992) have been isolated that display various degrees of resistance to the herbicides.

2.4 Protein-protein interactions: selected methodology for detection and analysis

2.4.1 General overview

Many authors have expressed the essential requirement for, and study of, protein-protein interactions in biological systems (Phizicky and Fields, 1995; Jones and Thorton, 1996; Kohalmi *et al.*, 1998; Vergnon and Chu, 1999). Kohalmi *et al.* (1998) stated: "the mechanistic basis of fundamental life processes as diverse as gene expression, metabolism, morphogenesis and development, signal transduction, and cell division are increasingly being understood in terms of quaternary protein complexes and the constituent protein-protein interactions they contain" (Kohalmi *et al.*, 1998). In a recent editorial, Srere (1999) further described that, for approximately four decades, a growing body of proof had accumulated that implied that almost all proteins in the cell exist in multiprotein complexes. The description of heterologous protein-protein interactions that form quaternary structures had grown in the past three decades from a "cottage industry" to a main "industrial complex." He noted that some researchers had dubbed

protein-protein interactions "the new biochemistry" in spite of the extensive historical perspective of this aspect of protein function.

Protein complexes which are comprised of more than one subunit are evident in diverse and well-studied proteins such as the multi-component complexes of photosystem I (PSI) and photosystem II (PSII) of the thylakoid membrane, the pyruvate dehydrogenase multienzyme complex and the widely-known G-protein complex, among others. Purification of these proteins revealed multimeric complexes, thus confirming their involvement in protein-protein interactions (Phizicky and Fields, 1995). Other examples of multimeric proteins include the supramolecular protein complexes ("the metabolon") involved in the TCA cycle (Velot *et al.*, 1997), cyclins and cyclin-dependent kinases (CDKs) involved in regulating cell cycle proliferation (Germino *et al.*, 1993) and the widely-known acetyl-CoA carboxylase, the first enzyme in fatty acid biosynthesis.

Temporal protein-protein interactions that, subsequently, control diverse cellular processes are also found in many biological systems. Post-translation modifications of proteins, including phosphorylation, glycosylation, prenylation, myristoylation, palmitoylation and compartmentation, often mitigate such interactions, mediated by corresponding protein-modification enzymes. It is well known that these protein modifications, in turn, play a role in the diversity of protein-protein interactions in the cell and govern many essential processes including cell growth, the cell cycle, metabolic pathways and signal transduction (Phizicky and Fields, 1995), gene expression, morphogenesis and development (Kohalmi *et al.*, 1998).

Klotz *et al.* (1975) summarized the conceptual advantages of multi-subunit proteins over individual proteins as functional units as follows:

1. A single, simpler subunit as a building block for an oligomeric protein is much more metabolically economical to synthesize than a single protein equivalent in size to that of an oligomeric protein.
2. An increase in errors in translation may be apparent if larger proteins are to be translated as compared to translation of several small subunits which in turn self-assemble to form a large protein complex. In the case of errors, only the defective smaller subunit is lost instead of the larger oligomeric complex.
3. Multiple-protein complexes characteristically exhibit association-dissociation properties that allow assembly and disassembly of oligomeric proteins under different conditions, and a subunit can be synthesized in one place and transported easily, due to its smaller size, to a different compartment for final assembly. These properties are generally lacking in a single large protein.
4. Oligomeric proteins are evolutionarily preferable to a single large protein, when each subunit of the oligomer self-associates in an antiparallel orientation, since a single mutation in a smaller subunit will be more effective in modifying the functionality of the oligomeric protein than that in a single large molecule which, in turn, is more amenable to natural selection resulting in more efficient protein structures and interaction potential.

Phizicky and Fields (1995) pointed out an additional feature of multimeric assemblies over a single large subunit, namely the capability of exchanging monomeric partners to impart a different complex and function. For example, adult hemoglobin ($\alpha 2\beta 2$) and fetal hemoglobin ($\alpha 2\gamma 2$) are each comprised of hetero-oligomers with an identical α subunit, but they differ in their second subunit; consequently, they display distinct

oxygen binding properties that are important in enabling oxygen to be supplied to the unborn baby from the mother. Other examples include the interaction of *Jun* with *Fos*, or with itself, resulting in distinct transcription activities due to the different transcription activating properties of the different complexes (Kerppola and Curran, 1991). Likewise, the TATA-binding protein binds with the transcription machinery of RNA polymerase I, II or III where it imparts a different effect on each RNA polymerase in transcription initiation (White and Jackson, 1992).

In order for a protein-protein interaction to take place, a specific complementary recognition of two protein subunits is required to form a stable complex (Duquerroy *et al.*, 1991) where a hydrophobic interaction is essentially required for protein-protein complex stabilization (Chothia and Janin, 1975). The hydrophobic interaction is defined as a gain in free energy that takes place as non-polar residues of proteins form a complex in aqueous surroundings (Kauzmann, [1959] as cited by Jones and Thornton, [1995]). Assembly of subunit-subunit proteins conceals the hydrophobic residues inside the complex which in turn reduces the chance of thermodynamically-unfavourable solute-solvent interactions and instead results in favourable subunit-subunit interactions (Jones and Thornton, 1995).

Complementarity of shape and group charges on the surfaces is another essential aspect in facilitating subunit-subunit assembly. A hydrogen bond between donor and acceptor electrogenative atoms is an integral part of a protein-protein interaction in which hydrogen bonds and salt bridges provide specificity of association while hydrophobic forces mediate protein-protein interactions (Fersht, 1984). Subunit-subunit assembly can also be achieved via complementarity of the structural shape of their interfaces (Jones and Thornton, 1995).

In protein-protein interactions, it is known that both small and large binding sites can facilitate the association of one subunit to another. Examples of well-characterized motifs or domains responsible for protein-protein interactions include leucine zippers, which were first described by Landschulz *et al.* (1988), and SH2 (Src homology 2) and SH3 (Src homology 3) domains as reviewed by Koch *et al.* (1991). Landschulz *et al.* (1988) identified an enhancer binding protein as a segment of thirty amino acid residues of the C/EBP transcription factor. They found this segment to contain leucine on every seventh residue; upon dimerization, the segments integrate in a way that results in a zipper-like motif, hence the term "leucine zipper." Leucine zippers are an example of interactions between two surfaces over a large area, where a stretch of α-helix forms a surface that spatially interacts with another α-helix from another subunit (Ellenberger *et al.*, 1992; O'Shea *et al.*, 1991). The SH2 domain was first characterized to be a non-catalytic conserved domain of cytosolic tyrosine kinases of a non-receptor type (Sadowski *et al.*, 1986) and was later identified as a domain of approximately one hundred amino acids involved in protein-protein interactions of various cytosolic signaling proteins e.g. cytosolic protein-tyrosine kinases, phospholipase C_γ, $p21^{ras}$ GTPase-activating protein (GAP), among others (Moran *et al.*, 1990). In interactions involving the Src homology 2 (SH2) domain, a domain of one protein interacts tightly with a small peptide, where the SH2 domain binds to specific, small, phosphotyrosyl-containing peptide domains resulting in a very strong interaction (dissociation constant of approximately 0.3-3 nM) due to a particular interacting cleft different from a classical substrate-interacting cleft (Felder *et al.*, 1993). Mayer *et al.* (1988) identified two domains in the $p47^{gag\text{-}crk}$ protein encoded by an avian sarcoma virus, CT10 oncogene *crk*,

the previously-identified SH2 domain (Sadowski *et al.*, 1986), and the other domain of approximately 50-60 amino acids that was later designated as the SH3 domain (Koch *et al.*, 1991). It is widely known that the SH3 domain, which is present in various proteins participating in tyrosine kinase signal transduction, cytoskeletal organization, and enzyme complexes, is also involved in protein-protein interactions in a manner similar to the SH2 domain (Buday, 1999). Detailed information and examples of other domains responsible for protein-protein interactions (e.g. phosphotyrosine binding [PTB] domains, pleckstrin homology domain and LIM domains, among others) can be found in any of several reviews edited by Pawson (1998).

2.4.2 Selected methods for protein-protein interaction detection and analysis

Protein-protein interactions can manifest a number of different biological effects. First, modifications of kinetic properties of proteins as reflected in substrate binding, catalysis or allosteric-property modifications of the complex are examples of protein-protein interaction roles (Phizicky and Fields, 1995). Examples include the binding of proliferating-cell nuclear antigen with DNA polymerase δ which modifies the processivity of DNA polymerase (Prelich *et al.*, 1989), the binding of succinate thiokinase and α-ketoglutarate dehydrogenase that reduces the K_m for succinyl coenzymeA by 30-fold (Porpaczy *et al.*, 1983), and the synergetic interaction of oxygen to hemoglobin and the allosteric control of aspartate transcarbamylase are mediated by the association of subunits. Second, the assembly of multi-enzyme quaternary complexes offers a mechanism for substrate channeling between different subunits of a complex (Srere, 1987). Third, protein-protein interactions may cause formation of a

novel binding entity, e.g. the formation of an ADP site at the interface of the α and β subunit of *E. coli* F1-ATPase (Weber *et al.*, 1993). Fourth, protein-protein interactions can render a protein non-active, as in the widely-known interaction of trypsin with its inhibitor proteins e.g. Bowman-Birk and Kunitz protein inhibitors. Lastly, substrate preference of a protein may be altered due to protein-protein interactions. For example, the binding of lactalbumin with lactose synthase reduces the K_m for glucose by 1000-fold (Hill and Brew, 1975); likewise, transcription factors which bind to RNA polymerase result in the polymerase selectively binding to different promoters (White and Jackson, 1992).

Subsequent sections will highlight selected methods for the identification and characterization of protein-protein interactions.

2.4.2.1 The yeast two-hybrid system

Since its introduction over a decade ago (Fields and Song, 1989; Chien *et al.*, 1991), the heterologous yeast two-hybrid system has proven to be a robust approach for the identification and analysis of protein-protein interactions *in vivo* (McAlister-Henn *et al.*, 1999). Several authors have reviewed the success and widespread application of this procedure (Mendelsohn and Brent, 1994; Young, 1998) including several reviews of current practical approaches (Bai and Elledge, 1996; Kohalmi *et al.*, 1997; Golemis and Khazak, 1997; Gietz *et al.*, 1997; Kohalmi *et al.*, 1998). According to McAlister-Henn *et al.* (1999), applications, assessment and improvement of these systems are widely presented in the published literature sufficiently often so as to render previously-

published articles quickly out-of-date. This section focuses on the basic methodology of the yeast two-hybrid system.

This novel genetic system for detecting protein-protein interactions was first described by Fields and Song (1989). In designing the system, they exploited the properties of the GAL4 protein of the yeast *S. cerevisiae*. The GAL4 protein acts as a transcriptional activator involved in the expression of genes encoding galactose-utilization enzymes, namely galactokinase (GAL1), permease (GAL2), galactose-1-phosphate uridylyltransferase (GAL7), uridine diphosphoglucose 4-epimerase (GAL10) and α-galactosidase (MEL1) (Johnston, 1987). Two separable and functionally essential domains are found in this protein, where an amino-terminal domain acts as the DNA-binding domain (Keegan *et al.*, 1986) and a carboxy-terminal domain containing acidic regions as the transcriptional region (Ma and Ptashne, 1987).

As shown in Figure 2.2 (adopted from Fields and Song, 1989), the native GAL4 protein is comprised of DNA binding (DB) and trans-activating (TA) domains where the DB domain binds to the upstream activating sequence (UAS) of the *GAL1-lacZ* marker gene. Upon binding, the protein induces *GAL1-lacZ* transcription when yeast is grown in a medium containing galactose (Figure 2.2a). When the DB domain is fused to a protein (X) or the TA domain to a protein (Y) to form individual fusion hybrids and expressed independently in different cells, there is no transcription of *GAL1-lacZ* gene observed in either cell. On the other hand, when the two hybrid fusion proteins are expressed in the same cell, and if X and Y interact thus bringing the DB and TA domains in relatively close proximity, then transcription of the marker genes is observed. Based on this design principle, Fields and Song (1989) examined two yeast

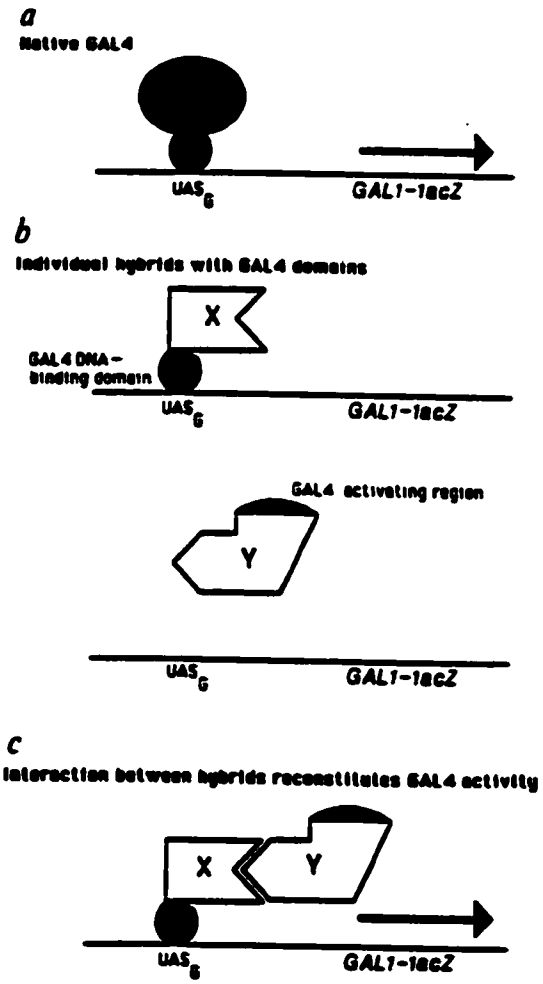


Figure 2.2. The principle of the yeast two-hybrid system (Fields and Song, 1989). It was used a GAL4 transcriptional factor and *GAL1-lacZ* reporter gene system: a) the native GAL4 protein containing DNA-binding domain (DB, small filled circle) and transactivating domain (TA, large filled circle) induces transcription of *GAL1-lacZ*, b) protein hybrids of either DB and X protein (upper) or TA and Y protein (lower) with no induction in transcription, c) an X-Y protein interaction resulting in the GAL4 domains into close proximity, hence, inducing *GAL1-lacZ* transcription.

proteins, SNF1 and SNF4, that are known to interact. They obtained a high transcriptional activity only when both hybrids were present in the cell, confirming that the DB and TA domains were reconstituted to a common transcriptional activating complex via the SNF1-SNF4 interaction. Fields and Sternglanz (1994) reviewed the two-hybrid system as an assay for protein-protein interactions and provided a universal graphical outline of the principle of the yeast two-hybrid system.

Since it was first introduced, many investigators have taken advantage of the two-hybrid method (Chien *et al.*, 1991; Fritz and Green, 1992; Chevray and Nathans, 1992; Bartel *et al.*, 1993; Kohalmi *et al.*, 1995; Kohalmi *et al.*, 1998) for the discovery of novel protein-protein interactions. Kohalmi *et al.* (1995) developed a laboratory guide for cDNA library screening with the yeast two-hybrid system, where known gene(s)/gene product(s) were used as "bait" which was fused to the GAL4 DB domain and screened using a cDNA-fusion library (e.g. from *A. thaliana)* fused to the GAL4 TA domain as "prey" (target) for the discovery of unknown, potentially biologically relevant interactors.

Ito *et al.* (2000) generated a comprehensive system to examine two-hybrid interactions in all possible combinations between proteins of *S. cerevisiae*, with the goal of defining a comprehensive protein-protein interaction map of this budding yeast. To do this, all 6,300 yeast open reading frames (ORFs) were cloned as a DB domain fusion "bait" construct in a *MATa* strain, and as a TA domain fusion "prey" library resident in a corresponding *MATα* strain. Their findings, after initial examination of $4x10^6$ different combinations (i.e. 10% of the total to be tested), revealed 183 independent two-hybrid interactions of which more than half were unknown before. They argued that the system

will provide leads for integration of various cellular functions and will serve as a major driving force in unraveling the whole circuitry of protein-protein interactions within a cell, thus allowing the construction of a comprehensive protein-protein interaction map of an organism.

Although there are many beneficial features of the yeast two-hybrid system, some limitations are also apparent, as reviewed by Fields and Sternglanz (1994) and Phizicky and Fields (1995). For example, the technique is confined to binary protein interactions that can be consolidated to the yeast nucleus, thus preventing its application with particular extracellular proteins. Post-translational modification schemes of a particular protein may take place differently (or not at all) in yeast cells than in the species of interest, and observed protein-protein interactions in yeast may not actually exist *in vivo* in the species in question (Fields and Sternglanz, 1994). Other limitations are mentioned elsewhere in the INTRODUCTION section.

2.4.2.2 Co-immunoprecipitation

Immunoprecipitation methods are employed in many areas of life science research to identify the molecular mass of an antigen, to characterize the specificity of an antibody, to identify molecules associated with antigens (co-immunoprecipitation), to quantify antigens with radio-immunoassays, and to determine binding constants (Otto and Lee, 1993). Practically speaking, immunoprecipitation is a technique involved in the precipitation of an antigen (e.g. a protein) from a crude blend, often a cell or tissue homogenate containing other proteins and/or biological molecules. The technique commonly employs an antibody against the protein of interest to precipitate the complex, allowing its fractionation and enrichment from the initial mixture (Anderson,

1998). Co-immunoprecipitation, which is an extended application of immunoprecipitation, is employed to co-precipitate a protein associated with the protein of interest that is recognized by the antibody (Anderson, 1998). This technique has been employed widely and is a powerful biochemical tool in assessing protein-protein interaction potential (McNabb and Guarente, 1996).

There are two primary approaches in using such co-immunoprecipitations (Anderson, 1998). Firstly, it is used to test the hypothesis that two or more known proteins interact, or to confirm interactions that have been revealed by other methods (e.g. the yeast two-hybrid system). In this approach, immunological probes against each protein must be available for detection purposes. A second application is to search for unknown proteins potentially able to interact with a known protein, where the unknown protein is *a priori* labeled. The interacting proteins are resolved by gel electrophoresis or some other technique, and detected using a detection procedure specific for the label used (Anderson, 1998).

In practice, Otto and Lee (1993) described the principal steps for immunoprecipitation as follows:

1. Solubilization of the antigen preparation.
2. Depleting the preparation of any insoluble materials and molecules that potentially interact with protein A, a protein that binds specifically to IgG antibodies.
3. Primary antibody incubation.
4. Primary antibody immobilization with protein A or protein G that is attached to a matrix (e.g. agarose resin).
5. Immunoprecipitate washes to reduce non-binding contaminants.

6. Immunoprecipitate resolution via gel electrophoresis.

In this method, if multiple proteins are present in the co-immunoprecipitate complex, they can be simultaneously detected using their corresponding antibodies as probes in Western blot analysis (Anderson, 1998).

2.4.2.3 *In vitro* transcription/translation and binding assays

It has been shown that abundant quantities of active mRNA can be produced *in vitro* and the *in-vitro*-transcribed RNA, in turn, used for *in vitro* translation (Turner and Foster, 1998). This *in vitro* synthesis of proteins was first described by Herrlich and Schweiger (1974) using an *in vitro* system isolated from *E. coli*. Likewise, Anderson *et al.* (1983) used a eukaryotic wheat germ system and Woodward *et al.* (1974) developed the rabbit-reticulocyte-based system. The transcription and translation can be carried out separately; however, it is also possible to perform the transcription and translation simultaneously in a single coupled-reaction using the transcription/translation system introduced by Gold and Schweiger (1971) and Zubay (1973). The coupled transcription/translation system is now commercially available as a kit (e.g. from Promega, Madison, WI).

In vitro transcription is accomplished by cloning a full-length cDNA of interest into a plasmid containing an upstream T7, T3 or SP6 bacteriophage promoter; subsequently, the construct is used for transcription by RNA polymerase (Chan *et al.*, 1997) or by PCR amplification of a recombinant ORF using terminal primers containing one of these promoter regions; subsequently, the linear PCR-amplified fragment is purified and used for transcription (Crosby *et al.*, unpublished). *In vitro* translation of mRNA is achieved by carrying out the reaction in extracts that contain all the required

components for protein synthesis, including ribosomes, translation factors, tRNA and amino acids (Turner and Foster, 1998). A detailed procedure for standard *in vitro* transcription and translation was presented by Turner and Foster (1998) and Ranganathan and Kern (1999) for the synthesis of lipoprotein lipase.

In vitro protein synthesis may provide advantages over *in vivo* methods for the production of labeled protein using recombinant approaches, where some serious limitations can be encountered *in vivo*, such as toxicity of recombinant proteins to host cells and the overall yield of the desired protein and other proteins necessary for growth, resulting in the need for complicated enrichment procedures (Stiege and Erdmann, 1995).

The potential application of proteins synthesized by *in vitro* transcription/ translation systems have been reviewed in detail by Stiege and Erdmann (1995). In particular, translated proteins radioisotopically labeled *in vitro* can be used for the study of protein-protein interactions. Several researchers have used this approach in protein-protein interaction experiments (Hennessey *et al.*, 1991; Liu *et al.*, 1992; Kao *et al.*, 1992). For example, using the *Drosophila* Act88F actin gene transcribed and translated *in vitro* from a plasmid construct that contained a bacteriophage T7 RNA polymerase promoter sequence, Hennessey *et al.* (1991) studied the significance of N-terminal processing for normal actin function. They found that processed and unprocessed actins translated *in vitro* interacted with DNase I equivalently. However, uncleaved actins were less able to co-polymerize with bulk actins than were processed actins, suggesting that the regular actin function asks for proper post-translational modification of the N-terminal region.

In another study, Liu *et al.* (1992) investigated two main core proteins of bluetongue virus, VP3 and VP7, to assess whether they could interact *in vitro* to form a morphological structure. In this study, the two proteins were transcribed *in vitro* using SP6 RNA polymerase, and subsequently translated using a rabbit reticulocyte lysate system. They reported that the structures recovered by sedimentation through a sucrose gradient were found to simulate VP3-VP7 core-like particles (CLPs) expressed *in vivo*, indicating that the *in-vitro*-translated proteins did interact. These experiments also showed that *in-vitro*-translated outer capsid proteins (VP2 or VP5) retained the capacity to interact with a preformed CLP when *in-vivo*-synthesized CLPs (VP3 and VP7) were incubated with either of these two outer capsid proteins.

Kao *et al.* (1992) reported that helicase 1a and polymerase-like 2a, both RNA replication proteins of Brome mosaic virus (BMV), were co-purified through various purification steps during purification of RNA-dependent RNA polymerase from plants infected by BMV. To determine whether these proteins also interacted, the two proteins were synthesized *in vitro* using a rabbit reticulocyte lysate system and were found to form a specific complex *in vitro*, suggesting the existence of a helicase 1a and polymerase-like 2a protein-protein interaction necessary for BMV RNA replication.

2.4.2.4 Immobilized metal-ion affinity chromatography (IMAC)

Immobilized metal-ion affinity chromatography or IMAC (also called metal chelate chromatography, metal ion interaction chromatography or ligand-exchange chromatography [Yip and Hutchens, 1994]) was first introduced as a new approach to protein fractionation by Porath *et al.* (1975). This affinity chromatography technique has been regarded as halfway between highly specific, high- and bio-affinity

bioseparation methods, and broader range, low-specificity adsorption methods such as ion exchange chromatography (Yip and Hutchens, 1994).

Sulkowski (1989) summarized the mechanism of IMAC-based purification based on previous reports, most notably those of Porath (1985) and Porath *et al.* (1975, 1988a and 1988b). As a protein molecule containing amino acid residues with electron donor groups (e.g. histidine, tryptophan and cysteine) on its surface, at neutral pH, is exposed to a chromatographic column containing chelated and immobilized metal ions (e.g. Co^{2+}, Ni^{2+}, Cu^{2+} and Zn^{2+}), a coordination bond or bonds is/are formed, causing retention of that protein. The protein is subsequently eluted from the column by applying low pH or imidazole-containing buffer to disrupt the coordinated bond. As a result, both the column matrix containing immobilized metal ion (e.g. IDA-Me^{2+} [iminodiacetate-$Metal^{2+}$]) and the protein are re-established to their previous state.

Mechanistically, one can visualize the following course of events during IMAC fractionation in which a histidine residue and the IDA-Me^{2+} matrix are used as an example, as shown in Figure 2.3 (Sulkowski, 1989). Firstly, a target protein with a single histidine residue on its surface, as shown in the left panel, makes contact with the IDA-Me^{2+} ligand and establishes a coordination bond. Upon applying a washing buffer, the flow of the buffer washes proteins from the column, particularly those that lack a single histidine residue on their surfaces. The histidine-containing protein is then eluted by applying an elution buffer. Secondly, as shown in the middle panel, a protein having two histidine residues coordinates to the IDA-Me^{2+} ligand more often during its travel through the column, since the chance of prolific collisions is proportionally intensified. Lastly, when a protein presenting two adjacent histidines on its surface makes contact with the IDA-Me^{2+}, as shown in the right panel, the resultant two coordination bonds

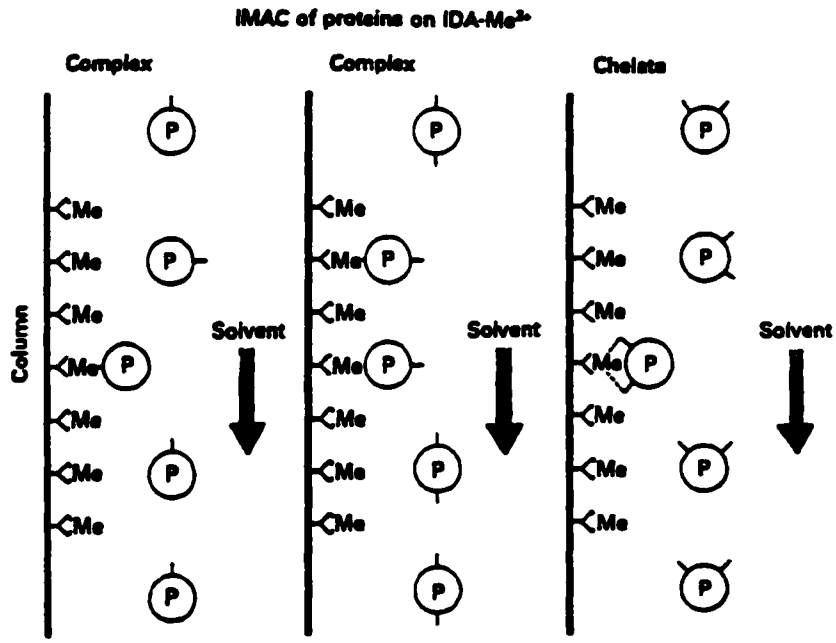


Figure 2.3. A Diagram depicting the retention of protein (P) on an IDA-Me^{2+} column based on the multiplicity of the histidyl residues of a protein (Sulkowski, 1989).

form a chelate rather than a complex, as previously described. The chelating effect is adequate in magnitude to impart retention of the protein, even on IDA-Co^{2+} and IDA-Zn^{2+} columns, the weakest in the series of this metal group (Co, Ni, Cu and Zn).

Schmid *et al.* (1997) covalently bound a chelator, nitrilacetic acid (NTA), to a hydrophilic quartz surface and subsequently loaded it with a divalent metal cation (Ni^{2+}). The matrix was used to bind green fluorescent protein from jellyfish, *Aequorea victoria*, fused to a hexahistidine domain. The intrinsic chromophoric properties of this protein allowed the progress and efficiency of purification to be readily monitored.

In another application, the IMAC technique was used to purify, in one step, a recombinant human papillomavirus type 16 E7 oncoprotein (Kasher *et al.*, 1993). They found that the efficiency of purification correlated with the number of histidine and tryptophan residues in the chelator which was oligopeptides instead of IDA or NTA. Several metal(II) cations were assessed for their affinity to proteins; it was found that each metal(II) exhibited a different degree of affinity to the analyzed protein.

Porath (1992) pointed out advantages and disadvantages of IMAC techniques. Several select advantages include: 1) specific sites on the molecular surface of proteins can be targeted for affinity; 2) Denatured, native or refolded proteins can be resolved; 3) quantitative recovery of dilute protein solutions can be achieved through multiple-cycle purification steps; and 4) pH changes (decreasing pH for soft and intermediate types of metal ions and increasing pH for hard metal ions) can be used for elution. On the other hand, possible disadvantages include: 1) metal ion transfer (MIT) may take place from the ligand to the protein and *vice versa*; 2) incidental elution (metal leakage) of the metal

ions together with the protein may take place; and 3) oxidation and catalysis can potentially take place, although this phenomenon has not been reported.

3. MATERIALS AND METHODS

3.1 Plant materials

Arabidopsis thaliana ecotypes 'Columbia' and 'C24' (*Arabidopsis* Biological Resource Center, ABRC, Colombus, OH) were used in extract preparation and genetic transformation experiments. The chlorsulfuron resistant (*csr1-1*) mutant line was provided by ABRC and *CaMV 35S::GUS* and *CaMV 35S::ASK1* lines by Drs. R. Datla and E.P. Risseeuw (Plant Biotechnology Institute, Saskatoon, SK), respectively. Plants were grown in pots or flats containing horticultural grade soil (Terra-Lite Redi-Earth,® W.R. Grace & Co, Ajax, ON) and placed in growth chambers at 22°C under a 16/8-h light/dark cycle.

3.2 Strains of bacteria and culturing procedures

Escherichia coli and *Agrobacterium tumefaciens* strains and growth media used in this research are listed in Tables 3.1 and 3.2, respectively. From a frozen stock, *E. coli* strains were initiated by inoculating 2, 5 or 50 mL of 2x YT media, depending on the purpose of the experiment; if required, appropriate antibiotics were added at the required concentrations. For liquid cultures, incubations were carried out at 37°C for 16-18 h in a shaking incubator (New Brunswick Scientific Co. Inc., Edison, NJ) set at ~250 rpm. For solid media, incubation was carried out at the same temperature and duration in an incubator oven.

Table 3.1 Strains of bacteria used in this research.

Strain	Genotype	Comments	Reference
Escherichia coli			
DH5α	*F⁻ ϕ80dlacZΔM15 Δ(lacZYA-argF)U169 endA1 recA1 hsdR17*($r_K^- m_K^+$) *deoR thi-1 supE44 λ⁻ gyrA96 relA1*	- host for pUC and other α-complementation vectors; pBR322 - useful for cDNA cloning	Bethesda Research Laboratories (Bethesda, MD)
DH10B	*F⁻ mcrA Δ(mrr-hsdRMS-mcrBC) ϕ80dlacZΔM15 ΔlacX74 endA1 recA1 deoR Δ(ara, leu)7697 araD139 galU galK nupG rpsL λ⁻*	- host for pUC and other α-complementation vectors; pBR322 - useful for generating genomic libraries containing methylated cytosine or adenine residues - useful for plasmid rescue procedures	Grant *et al.* (1990)
NM522	*F' lacI^q Δ(lacZ)M15 proA⁺B⁺ Δ(hsdMS-mcrB)5* ($r_K^- m_K^+$) *Δ(lac-proAB) supE thi-1*	- used as transformation host - as *lacZ* host	ATCC[1] (Manassas, VA)
NM538	*supF hsdR trpR lacY*	- used for assay and propagation of bacteriophage λ vectors	Frischauf *et al.* (1983)
Agrobacterium tumefaciens			
LBA1115		- Ti helper plasmid: pMOG410, Km^r	Hood *et al.* (1993)
GV3101		- plasmid: pMP90, Gm^r	Koncz and Schell (1986)

[1]American Type Culture Collection

Table 3.2 Growth media and their composition.

Growth Medium	Ingredients (amount/L of solution)	Reference
LB	Bacto-tryptone, 10 g; yeast extract, 5 g; NaCl, 10 g. Adjust pH to 7.0 with 5 N NaOH	
2x YT	Bacto-tryptone, 16 g; yeast extract, 10 g; NaCl, 5 g. Adjust pH to 7.0 with 5 N NaOH	Miller (1972)
SOC	Bacto-tryptone, 20 g; yeast extract, 5 g; NaCl, 0.5 g; KCl, 2.5 mM; $MgSO_4$, 10 mM; $MgCl_2$, 10 mM; glucose, 20 mM. Adjust to pH 7.0 with 5 N NaOH	
NZY	Yeast extract, 5 g; casamino acids, 10 g; NaCl, 5 g; $MgCl_2$, 2 g. Adjust to pH 7.0 with 5 N NaOH	
MS (commercially-prepared)	NH_4NO_3, 1.6 g; KNO_3, 1.9 g; $CaCl_2.2H_2O$, 0.44 g; $MgSO_4.7H_2O$, 0.37 g; KH_2PO_4, 0.17 g; KI, 0.0008 g; H_3BO_3, 0.0062 g; $MnSO_4.4H_2O$, 0.0223 g; $ZnSO_4.7H_2O$, 0.0086 g; $CuSO_4.5H_2O$, 0.000025 g; $CoCl_2.6H_2O$, 0.000025 g; Sequestrene 300 Fe (EDTA-ferric salt), 0.043 g and 3% sucrose. Adjust pH to 5.6	Murashige and Skoog (1962)

Agrobacterium tumefaciens strains were grown in liquid or solid LB medium at 29°C for 18-20 h or 72 h, respectively.

For long-term storage, 930 µL of the bacterial culture was mixed with 70 µL DMSO in a pre-sterilized cryo vial (Fisher Scientific Co., Edmonton, AB) and immediately frozen in liquid nitrogen prior to storage at -80°C. For working stocks, master stocks were picked with a toothpick and streaked on an agar plate or inoculated to liquid media for culturing.

3.3 Solutions, buffers and growth media

All chemicals used in this study were of reagent grade and stored as suggested by the supplier. Solutions and buffers were prepared in distilled de-ionized water (ddH_2O) and sterilized by autoclaving for 20 min at 121°C. Heat-labile solutions (e.g. antibiotics) were filter-sterilized using pre-sterilized 0.22 µm filter units (Millipore, Etobicoke, ON). Buffer pH was measured using a pH meter calibrated against standard buffers (BDH Chemicals, Toronto, ON). If necessary, buffer pH was adjusted with 5 N NaOH or concentrated HCl.

All media were prepared in ddH_2O using commercially-available ingredients and autoclaved at 121°C for 20-30 min depending on volume. Yeast extract, tryptone, agar and phytagar were purchased from Life Technologies (Burlington, ON). MS media along with vitamins, various inorganic salts and antibiotics were purchased from Sigma Chemical Co. (Mississauga, ON) with the exception of carbenicillin, which was purchased from Ayerst (Montreal, PQ). Types of growth media used and their ingredients are listed in Table 3.2, with the exception of those required for

Agrobacterium-mediated root transformation of *A. thaliana* and which have already been described in detail (Vergunst *et al.*, 1998). One-half strength MS medium with 0.8% phytagar was used for germination and early growth phase of plants.

3.4 Methods for DNA estimation, manipulation and characterization

3.4.1 Determination of DNA concentrations

DNA concentrations were determined using a spectrophotometric method. In particular, the measurement was carried out using an UltraSpec II UV/Visible spectrophotometer (Pharmacia Biotech, Baie d'Urfe, PQ) equipped with a 100 μL-capacity cuvette. DNA samples were diluted accordingly (10 – 100x dilution factor) and measured at 260 nm. The use of a single wavelength at 260 nm for measurement was sufficient providing that DNA samples were quite free from impurities (Boyer, 1993). The OD 260/280 nm ratio is required when one wishes to determine protein contamination in DNA solution or *vice versa*. When an accurate measurement was not critical, which was the case in most preparations, a visual inspection was done by comparison of known concentrations of DNA standards and the DNA sample for their fluorescence intensity induced by EtBr. A mixture of λ DNA digested with *Hind*III and ΦX174 DNA digested with *Hae*III was used as DNA standards that displayed a DNA ladder profile of 23130, 9416, 6557, 4361, 2322, 2027, 1353, 1078, 872, 603, 310, 281 and 271 bp.

3.4.2 Isolation and purification of plasmid DNA (small and mid-scale) and phage DNA

3.4.2.1 Plasmid DNA

Recombinant plasmid extraction and purification were carried out using different methods depending on the purpose of the experiment. Alkaline lysis and phenol-chloroform-isoamyl alcohol (25:24:1/v:v:v) methods modified from Sambrook *et al.* (1990) were used primarily for the purposes of preliminary screening of positive clones. Commercial small- and mid-scale preparation kits (Promega, Madison, WI) were used in preparation for sequencing and other purposes. Bacteria harbouring the recombinant plasmid of interest were grown and selected at 37°C for 16-18 h in the presence of the appropriate antibiotic. The culture was centrifuged for 5 min at 10,000 x *g* in a microfuge or centrifuge and the pelleted cells were collected. For alkaline lysis plasmid preparation, cells were resuspended in 15 volumes of ice-cold Solution I (50 mM glucose, 25 mM Tris-HCl pH 8.0, 10 mM EDTA pH 8.0). Freshly prepared lysis Solution II (0.2 N NaOH, 1% SDS) was added at a 7.5:1 volume ratio to the resuspended cells, mixed by inverting the tube five times, and incubated on ice for 10 min. To precipitate cell debris, chromosomal DNA and proteins, and to neutralize the suspension, Solution III (5 M potassium acetate, 115 mL/L glacial acetic acid) was added at a 10:1 volume ratio and the suspension was incubated further for an additional 3-5 min on ice. The supernatant was collected after centrifugation at 12,000 x *g* for 10 min at 4°C and transferred to a fresh tube. An approximately equal volume of phenol-chloroform-isoamyl alcohol (25:24:1, v:v:v) was added to the supernatant to extract proteins and other organic-solvent-soluble substances with vigorous shaking for 10 min at ~200 rpm. To separate the aqueous and organic layers, the mixture was then

centrifuged at 12,000 x *g* for 2 min, and the aqueous phase was transferred to a fresh tube. To precipitate the plasmid DNA, two volumes of ethanol and one-tenth volume of 3M Na-acetate were added and the mixture was allowed to stand for 12-15 min at -80°C, or overnight at -20°C. The DNA precipitate was centrifuged at 12,000 x *g* for 10-15 min at 4°C, the supernatant aspirated away and the pellet washed twice with ice-cold 70% ethanol. The washed pellet was vacuum dried and then dissolved in the desired volume of TE buffer (10 mM Tris-HCl pH 7.4, 1 mM EDTA pH 8.0) or ddH_2O.

For mini- and mid-scale purification, Wizard Plus™ minipreps and midipreps DNA purification systems were used (Promega, Madison, WI).

3.4.2.2 Phage DNA

Escherichia coli strain NM538 was used as the host for propagating and screening the λ bacteriophage genomic library of *A. thaliana*. Following screening by *in situ* phage hybridization (Sambrook *et al.*, 1990), a plaque derived from infection of a single bacterial colony by a plaque-purified phage containing the DNA segment of interest was extracted and purified according to the protocol described (Sambrook *et al.*, 1990). As an alternative to NZCYM or LB medium, NZY medium (Table 3.2) was used for solid and liquid cultures. The purified phage DNA was cut with various restriction enzymes and subjected to Southern blotting and hybridization to determine the exact fragment size that contained the DNA of interest. Once the positive fragment was identified, further restriction cuts and electrophoresis gel purification were carried out in order to clone the DNA of interest.

3.4.3 Oligonucleotide design and synthesis

Several approaches were used in the design of synthetic oligonucleotides used for sequencing, PCR amplification and site-directed mutagenesis (SDM). For sequencing purposes, a "prime-as-you-go" approach was taken. Recombinant plasmid inserts were first end-sequenced by the DNA Technologies Unit at the National Research Council of Canada-Plant Biotechnology Institute (NRCC-PBI), Saskatoon, SK using commercially-available universal (UP, for forward direction) and reverse primers (RP, for reverse direction). Subsequently, the sequence data obtained from the first round of sequencing was used to design primers to further the sequence. The cycle of primer design and sequencing was continued until the overlapping UP and RP contiguous sequences (contigs) were identified.

For purposes of PCR amplification where sequence data were already available, primer design was done using a commercial computer application, Vector NTI v.4.0 (Informax, Bethesda, MD).

For SDM, mutagenic primer design for N-terminal tagging experiments was carried out using Vector NTI v.4.0 (Informax, Bethesda, MD). The primers were designed as 36-mers containing 18-mers of 5'CAT CAT CAT CAT CAT CAT encoding six histidine molecules plus 9-mer segments flanking both 5' and 3' ends of the 18-mers. The terminal 9-nucleotide segments were complementary to either strand of the DNA region at the N-terminal end of the mature coding region sequence. By this design, the *His*$_6$-encoding segment was inserted in a region immediately downstream of the encoding transit peptide sequence. In similar fashion, C-terminal tagging was achieved by insertion of a *His*$_6$-encoding domain immediately upstream of the termination codon.

Synthesis of all oligonucleotides was carried out using a Beckman Oligo1000 M (Beckman Instruments Inc., Fullerton, CA) and standard phosphoramidite chemistry. Oligos were purified using an HPLC system equipped with a Delta Pack 15 m C18 300A column (Waters Canada Ltd., Mississauga, ON).

3.4.4 DNA electrophoresis in agarose and polyacrylamide gels

3.4.4.1 Agarose gels

Agarose gel electrophoresis was employed for preparation and analysis of DNA. Restriction-cut DNA fragments were electrophoresed through 0.8%-agarose gels prepared in 1x TBE buffer (10.8 g/L Tris base, 5.5 g/L boric acid, 4 mL/L of 0.5 M EDTA, pH 8.0). The agarose-containing solution in 1x TBE was either autoclaved or microwaved to melt the agarose and allowed to cool to approximately 55-60°C before being poured into a gel tray. The gel tray was then placed into the gel box containing 1x TBE as running buffer. The DNA sample to be resolved was mixed with 0.1x reaction volume of 10x gel loading/stop buffer (50% glycerol, 0.7% SDS, 1 mM EDTA and 0.1% Bromophenol blue) prior to loading into gel wells. Typically, the gel was subjected to a voltage of 1-9 V/cm (measured as the distance between electrodes). In order to estimate the size of the resolved fragments, DNA standards of known fragment size were co-electrophoresed with the target DNA sample. After completion of electrophoresis, the gel was stained in 0.5 μg/mL ethidium bromide in 1x TBE or water for 30-45 min at room temperature and subsequently visualized using a short wave UV light transilluminator (Foto/Prep I, Fotodyne, Bio/Can Scientific, Mississauga, ON) and photographed using either a Polaroid MP-4 Land Camera or a Gel Doc 2000 system

(BioRad, Hercules, CA). To determine fragment sizes of the DNA sample, the migration distance of each fragment was interpolated against that of the DNA standards. A regression curve of migration (mm) vs. log10 of molecular size (bp) of each standard fragment was generated and, subsequently, the molecular sizes of the unknowns were estimated.

3.4.4.2 Non-denaturing polyacrylamide gels

In order to resolve smaller DNA fragments, non-denaturing polyacrylamide gels were employed using a vertical electrophoresis apparatus typically used for protein separations. Twelve-percent polyacrylamide gels were prepared by mixing 4.0 mL aqueous solution containing 29% acrylamide and 1% bis-acrylamide, 2.0 mL 5x TBE, 0.07 mL of 10% ammonium persulfate, 3.93 mL dH_2O and 0.02 mL TEMED. The separation was carried out as described by Sambrook *et al.* (1990). Gel loading buffer, DNA standards, staining solution, visualization and photographing were as described in the preceding section for agarose gels.

3.4.4.3 Isolation and elution of DNA from an agarose gel

Several methods for isolation and elution of DNA from agarose gels were used including electroelution, GENECLEAN, Nucleospin and DEAE membrane procedures.

3.4.4.3.1 Electroelution

Gel slices approximately 10x5x5 mm in size were excised from agarose gels and placed in the horseshoe shaped slot of an electroeluter (Analytical Unidirectional

Electroeluter Model UEA-3615, International Biotechnologies Inc., New Haven, CT) containing 0.5x TBE buffer. A 400 µL salt block (7.5 M NH_4OAc, 0.1% Bromophenol blue in dH_2O) was loaded into the V-channel to trap the eluted DNA. An electrical potential was then applied at ~100 V for 60 min. When completed, the high salt cushion containing the eluted DNA was withdrawn using a Pasteur pipette and placed in a microfuge tube. To recover the DNA sample, ethanol precipitation was carried out as described in Section 3.4.2.1, but omitting the Na-acetate addition.

3.4.4.3.2 GENECLEAN procedure

A GENECLEAN II Kit was used to purify DNA from excised agarose gels according to the manufacturer's protocols (BIO 101 Inc., Vista, CA). The DNA was eluted from the complex with an appropriate volume of ddH_2O or TE buffer and stored at 4 or -20°C for short- or long-term storage, respectively.

3.4.4.3.3 NucleoSpin® procedure

NucleoSpin® Nucleic Acid Purification Kits were used to purify DNA from excised agarose gels or PCR products according to the manufacturer's protocols (Clontech, Palo Alto, CA). The DNA was eluted with an appropriate volume of 10 mM Tris-HCl pH 8.5 and stored at 4 or -20°C for short- or long-term storage, respectively.

3.4.4.3.4 Band intercept method using S&S NA-45 DEAE membranes

An NA-45 DEAE anion exchange membrane is a cellulose support containing diethylaminoethyl functional groups. It is used for basic transfer techniques including

the band intercept method for eluting DNA bands out of agarose gels. With minor modification, a protocol from Schleicher & Schuell (Keene, NH) was used. To increase binding capacity, membranes were pre-washed for 10 min with 10 mM EDTA pH 7.6, followed by 0.5 N NaOH and several rapid rinses in ddH_2O and then stored in ddH_2O for up to several weeks. To purify DNA from agarose gels, after electrophoresis in a horizontal slab gel apparatus followed by EtBr staining, a strip of NA-45 DEAE membrane was inserted into an incision made in the gel just ahead of the DNA band of interest. The gel was then allowed to electrophorese further for ~15 min at 4 V/cm or until the DNA band was completely intercepted as judged by EtBr fluorescence from the membrane seen under long-wave UV light. The low-salt washing step to remove any residual agarose was omitted; instead, the residual agarose was removed by scraping it using a clean scalpel or by placing the strip into a centrifuge tube and centrifuging it for 2-3 min at maximum speed after incubation in sufficient high-salt (HS) NET buffer (1.0 M NaCl, 0.1 mM EDTA, 20 mM Tris-HCl pH 8.0) to cover the membrane strip. For elution of the DNA, the tube was incubated at 55-68°C for 30-60 min with occasional swirling, and then centrifuged as mentioned above to remove any residual agarose. The HS-NET buffer containing DNA was transferred to a clean microfuge tube and the DNA was precipitated with ethanol as described in Section 3.4.2.1, but omitting the Na-acetate addition.

3.4.5 Enzymatic manipulations of DNA

3.4.5.1 Restriction endonuclease digestion

All digestion reactions were carried out using enzymes purchased from Life Technologies (Burlington, ON), Pharmacia Biotech (Baie d'Urfé, PQ) or New England BioLabs (Mississauga, ON) and stored under the conditions recommended by the suppliers. Restriction enzyme digestions were used to isolate specific DNA fragments, linearize plasmid vectors for subcloning or modification, analyze (screen) for positive recombinants and map recombinant clones. Generally, the digestion reactions were performed in a 20 μL reaction volume containing 1 unit of the required enzyme per 1 μg DNA and buffer according to the recommendations of the vendor. The reaction was stopped by inactivation of the enzyme by either addition of 0.1x reaction volume of 10x "stop" buffer (50% glycerol, 0.7% SDS, 0.1 M EDTA and 0.1% Bromophenol blue) or heat treatment at the recommended time and temperature for a particular enzyme.

3.4.5.2 DNA ligation

For DNA ligation, typical reactions were done in a 10 μL volume consisting of 0.1x reaction volume of 10x ligase buffer (500 mM Tris-HCl pH 7.6, 100 mM $MgCl_2$, 100 mM DTT, 26 μM NAD^+, 500 μg/mL BSA), the DNA and the linearized plasmid vector at a DNA insert-to-vector molar ratio of 2:1. The reaction mixture was incubated either overnight at 16°C or for 1-2 h at ambient temperature and stopped by heat inactivation at 65°C for 15 min.

3.4.5.3 Synthesis of radio-labeled DNA probes

Radio-labeled DNA probes were synthesized using a commercial Oligolabelling Kit (Pharmacia Biotech, Baie d'Urfé, PQ). With the exception of radio-labeled deoxycytidine-5'-triphosphate ([α-^{32}P]dCTP) which was purchased from Dupont (Mississauga, ON), all required components were provided in the Oligolabelling Kit. The template DNA to be labeled was isolated and purified according to methods described in Section 3.4.4.3. Template DNA (25-50 ng) in a maximum of 34 µL TE or ddH_2O was denatured by heating for 2-3 min at 95-100°C. The denatured DNA was immediately placed on ice for 2 min, centrifuged briefly, and processed according to the protocol recommended by the vendor. When the reaction was completed, the unincorporated (oligo)nucleotides were removed using a ProbeQuant™G-50 Micro column (Pharmacia Biotech, Baie d'Urfé, PQ) according to the protocol recommended by the manufacturer. A 5-µL aliquot of the probe was counted in a Dupont BC2000 beta counter (Dupont, Mississauga, ON). Conversion of counts from cpm to dpm was based on 1% counting efficiency of the beta counter. For the purpose of nucleic acid hybridizations, approximately 500,000 dpm of probe was used for a membrane size of 8x10 cm.

3.4.6 DNA sequencing

Nucleotide sequence determinations were carried out by the DNA Technologies Unit at NRCC-PBI (Saskatoon, SK) using a Perkin Elmer Applied Biosystems 373 or 377 DNA Sequencer STRETCH™ in conjuction with the ABI PRISM™ Dye Terminator Cycle Sequencing Kit in combination with sequencing primers designed for particular

DNA templates. As mentioned elsewhere, sequencing and PCR primers used were also synthesized by the DNA Technologies Unit. Sequence analyses and editing were carried out using Lasergene software (DNASTAR* Inc., Madison, WI).

3.4.7 Southern blotting and hybridization

Southern blotting and hybridization were carried out as described by Sambrook *et al.* (1990), with some modifications. After electrophoresis and photographing, DNA fragments in the gel were depurinated by soaking in 0.25 N HCl for 15 min followed by denaturation with 0.5 N NaOH for 30 min. Gels were then neutralized with 1.0 M Tris-HCl pH 7.5 for 30 min followed by further neutralization with a solution of 0.5 M Tris-HCl pH 7.5, 1.5 M NaCl for 30 min. Gels were blotted to a nitrocellulose membrane (MicroSeparations Inc., Westborough, MA) by the Southern blotting method (Southern, 1975; Sambrook *et al.*, 1990). In this method, a glass plate was placed in a reservoir such that it was supported by four plastic columns of equal height (1.5 cm) in order to allow free movement of buffer. The glass plate was covered with a sheet of 3MM Whatman paper and the reservoir was filled to just below the glass plate with 10x SSC blotting buffer (1.5 M NaCl and 0.15M Sodium Citrate pH 7.0). The treated gel was placed onto the 3MM-covered glass plate and covered with the nitrocellulose membrane that had been pre-soaked in 6x SSC for 2 min, followed by a piece of 3MM Whatman paper. Layers of absorbent paper (e.g. paper towel) were placed on top of the paper-covered membrane followed by another glass plate and a weight of ~500 g. Transfers were allowed to take place overnight, after which the membrane was rinsed briefly in 2x SSC and vacuum-dried in an oven at approximately 70°C for 2 h.

For hybridization, the membrane was placed in a hybridization bottle (Bio/Can Scientific, Mississauga, ON) and pre-hybridized for 2 h at 65°C in 5 mL of pre-hybridization solution containing 5x SSPE (43.83 g/L NaCl, 22.05 g/L sodium citrate, 1.85 g/L EDTA, pH 7.4), 5x Denhardt's reagent (1 g/L each of Ficoll type 400, polyvinylpyrrolidone and BSA), 0.1% SDS and 5 μg/mL denatured herring sperm DNA. After pre-hybridization, a 100-μL aliquot of boiled probe with radioactivity of 500,000 dpm/membrane plus 0.25 μg/μL herring sperm DNA in ddH_2O was added and hybridization was carried out overnight. The membrane was then washed twice at 62°C with ~10 mL 2x SSPE/0.1% SDS, each for 20 min, and once with ~10 mL 0.5x SSPE/0.1% SDS for 20 min. After washing, the membrane was exposed to X-ray film at -80°C for at least overnight, after which the X-ray film was developed and inspected.

3.4.8 Northern transfer and hybridization

For analysis of mature transcripts (polyA$^+$ mRNA fractions) by Northern transfer and hybridization, total RNA was isolated and purified from roots, stems, leaves and flowers of approximately 40-43 day-old *A. thaliana* plants. TRIZOL® Reagent and recommended protocols (Life Technologies Inc., Burlinton, ON) were used for the isolation and purification. The purified total RNA was electrophoresed through formaldehyde gels using a standard procedure (Sambrook *et al.*, 1990) with some modifications. A 5-μL aliquot containing 5-20 μg of RNA was mixed with 2 μL of 10x MOPS buffer (0.2 M MOPS pH 7.0, 50 mM NaCl, 10 mM EDTA pH 8.0 in diethyl pyrocarbonate [DEPC]-treated ddH_2O), 3 μL deionized 37% formaldehyde (pH must be more than 4.0) and 10 μL formamide. This mixture was heated at 65°C for 10 min, after

which 2 μL of 10x RNA loading dye (50% glycerol, 10 mM $NaHPO_4$ pH 7.0, 0.4% Bromophenol blue) was added. The sample mixture was subsequently loaded to a gel, prepared by solidifying a mixture of 1.2 g melted agarose, 85 mL ddH_2O, 10 mL 10x MOPS and 5 mL deionized 37% formaldehyde (pH > 4.0), and placed in a GNA 100 horizontal slab gel box (Pharmacia Biotech, Baie d'Urfé, PQ). The gel apparatus components were pre-treated to remove contaminating RNase by soaking in 0.1 N NaOH/0.5% SDS solution and rinsed thoroughly with DEPC-treated ddH_2O. The gel was subjected to electrical current at a maximum setting of 5 V/cm, after which it was stained with EtBr (0.5 μg/mL) for 30 min and subsequently photographed. For blotting purposes, the gel was destained and pretreated (3x10 min washes in DEPC-treated ddH_2O) to remove formaldehyde. After pretreatment, the resolved RNA sample in the gel was transferred to a nitrocellulose membrane in the fashion described for Southern blotting (Section 3.4.7). The transferred RNA was cross-linked to the membrane by UV treatment while still moist. Pre-hybridization and hybridization were performed as for Southern hybridization as described in Section 3.4.7 (but in this case, it was DNA-RNA hybridization) using corresponding radio-labeled DNA probes for the transcripts of interest. After washing, membranes were exposed to X-ray film for at least overnight.

3.4.8.1 Quantitative RT-PCR

When the detection of mature transcripts ($polyA^+$ mRNA) by Northern blotting and hybridization was not successful due to low abundance of target transcripts, a quantitative reverse transcriptase (RT)-PCR technique was employed. This technique was used to determine not only the presence but also the relative abundance of the

transcripts quantitatively. Total RNA samples prepared by the TRIZOL® method (Section 3.4.8) were used as the starting material for the synthesis of single strand cDNA. Approximately 5 μg of total RNA from each plant organ was used for the first strand cDNA synthesis using oligo(dT) primer and SUPERSCRIPT II RT (Life Technologies Inc., Burlington, ON). The cDNA synthesis was carried out according to the manual provided by the manufacturer (SUPERSCRIPT™ First-Strand Synthesis System for RT-PCR). An average of 5-10 ng/μL of cDNA was obtained from each reaction as determined by counts of incorporated radiolabeled dCTP. Approximately 5 ng of cDNA was used as input for quantitative PCR reactions. The PCR amplification was carried out in a 50 μL total reaction volume according to a method previously described (Bekkaoui *et al.*, unpublished). A 0.5 pg aliquot of cDNA previously synthesized from rabbit β-globin mRNA was included in all PCR reactions as internal standard with a pair of specific primers defining a 404-bp region of the β-globin structural gene. In the same fashion, PCR amplifications using a DNA template of recombinant plasmid DNA corresponding to each of the cDNAs in a series of known concentrations were performed. This PCR amplification served as external standard, which was used for determination of the molar concentration (or number of molecules) of the unknown by regression interpolation.

A 20-μL aliquot of the completed RT-PCR reactions was resolved in 12% polyacrylamide gels, which were subsequently dried and exposed for at least 1 h to a phosphorscreen containing fluors. The phosphorscreen was then scanned in a PhosphorImager™ SI scanner (Molecular Dynamics Inc., Sunnyvale, CA), after which the signal intensity of each band, corresponding to the molar concentration/number of

molecules of the amplified DNA, was analyzed using ImageQuant v.4.1 software (Molecular Dynamics Inc., Sunnyvale, CA).

3.4.9 Polymerase chain reaction (PCR) and purification of PCR products

The polymerase chain reaction (PCR, Mullis *et al.*, 1986) was performed for a variety of purposes such as *in situ* colony screening and amplification of genomic or recombinant DNA for recovery of specific amplicons. Other purposes included incorporation of new terminal endonuclease sites into DNA amplicons, site-directed mutagenesis (SDM) and quantitative RT-PCR, as described elsewhere. In general, PCR reactions were set up in a mixture containing 1-200 ng of template DNA, 50 pmoles of each of the primers, 0.1x total reaction volume of 10x PCR buffer (500 mM KCl, 100 mM Tris-HCl pH 8.3, 15 mM $MgCl_2$ and 0.1% BSA), 200 μM dNTPs with a final addition of either two units of *Taq* polymerase (Pharmacia Biotech, Baie d'Urfé, PQ) or five units of *Pfu* polymerase (New England Biolabs, Mississauga, ON).

The reaction was carried out in a programmable thermocycler (PTC-100™; MJ Research Inc., San Fransisco, CA) or Genius (Techne Inc., Princeton, NJ). A typical combination of time-temperature profiles is as follows: an initial denaturation at 94-96°C for 0.75-1.15 min, a second denaturation taking place in every cycle at 94-96°C for 0.5-0.75 min, primer annealing at 52-55°C for 0.5-0.75 min, primer extension at 72-75°C for 0.5-1.0 min/kb of amplicon template when using *Taq* Pol or 1.0-2.0 min/kb when using *Pfu* Pol. The steps from the second denaturation to extension were cycled 25-30 times followed by a final extension at 72°C for 10 min, and then stopped by chilling at 4°C. In

this time-temperature profile, the temperature setting at maximum range was selected when *Pfu* Pol was usually used.

In some cases, the template DNA was diluted to a series of known concentrations to determine what concentration would yield a reasonable result. In addition, a hot start approach (Birch *et al.*, 1996) was used when a single copy of a genomic DNA target was desired.

PCR products were resolved in agarose gels or isolated/purified using the phenol-chloroform extraction method followed by ethanol precipitation as described in Section 3.4.2.1. NucleoSpin® Nucleid Acid Purification Kits (Clontech, Palo Alto, CA) as described in Section 3.4.4.3.3 were also used but buffer NT2 instead of NT1 was used.

3.4.10 Site-directed mutagenesis (SDM)

Site-directed mutagenesis (SDM) was employed in one specific application of this project. In this approach, eighteen nucleotides encoding six histidine molecules were inserted at the N- or C-terminal ends of the *A. thaliana* chlorsulfuron resistant mutant of the *ALS* gene (*csr1-1*) using the SDM primers described in Section 3.4.3. The gene, including its native promoter region, defined as an *Xba*I-*Xba*I fragment of ~5.7 kb in pTZ18R (pBI144), was subjected to the QuickChange™ Site-Directed Mutagenesis Kit protocol (Stratagene, La Jolla, CA). The PCR program and reaction were also carried out as described in the protocol.

The SDM reaction products were electroporated into *E.coli* DH10B and screened for positives. Successful SDM mutants carrying N- and C-terminal insertions were

predicted to lose *BslI* and *DdeI* restriction sites, respectively and this fact was exploited for screening mutants. Of twenty mutants screened for N- and C-terminal insertions, six and four, respectively, were observed to have lost the corresponding restriction sites. To confirm that these mutations were correct, direct sequencing was undertaken. The data showed that two out of six recombinant DNAs analyzed had the desired N-terminal 18-nucleotide insertion, and all four had the correct insertion at the C-terminus.

Based on these results, recombinant plasmids of two positive mutants from each N- and C-terminal insertion were isolated, purified and cut with *XbaI* to yield the SDM-mutated At*ALS-csr1-1* gene. These in turn were subcloned into the binary T-DNA plasmid pRD400, which was used for plant transformation. Ligations of the mutant gene into the pRD400 vector were carried out, and the recombinant DNAs were subsequently electroporated into *E. coli* DH5α. A screening of positives was carried out by *in situ* colony hybridization techniques (Sambrook *et al.*, 1990) using the At*ALS-csr1-1* mutant gene as probe. Positive *E. coli* recombinants were identified and their plasmids transformed into *A. tumefaciens* strain LBA1115. Recombinant *A. tumefaciens* strains were used to generate transgenic *A. thaliana* plants carrying the modified *ALS* catalytic subunit gene as described in Section 3.5.1.1.

3.4.11 Transformation of recombinant DNA to bacteria

3.4.11.1 Electro-competent cell preparation

Escherichia coli strain DH5α or DH10B was grown and prepared for electro-competent cells. A single *E. coli* colony or a small aliquot of frozen stock cells was used to inoculate 3 mL of 2x YT medium and incubated overnight at 37°C with vigorous

shaking. An aliquot of 1-2 mL of the overnight culture was used to inoculate 500 mL of 2x YT liquid medium and the culture was grown to an OD_{600} of 0.6-0.8. The cell culture was transferred to sterile centrifuge tubes, kept on ice for 30 min and centrifuged at 5,000 rpm for 10 min at 4°C. The supernatant was discarded and the pellet resuspended in 100 mL of ice-cold sterile water. The cells were centrifuged at 2,400, 2,800 and 3,000 rpm for 10 min each time, with a resuspension in 100 mL of ice-cold sterile water between centrifugations. After the final centrifugation, the pellet was washed twice with 100 mL of ice-cold sterile water and resuspended in 2.5 mL of ice-cold sterile water. The cells were kept on ice for 1-3 h before proceeding to the electroporation procedure. For the purpose of long-term storage (6 mo. to 1 yr.), the resuspended cells were stored in 30% glycerol, quickly frozen in liquid nitrogen and kept at -80°C.

3.4.11.2 Electroporation

The electro-competent cells prepared as described above were used for transformation with recombinant DNA by the electroporation method, which is a technique that utilizes a high voltage of direct electric current (DC) to break open bacterial membranes so as to make them amenable to transformation. In this procedure, a 2-μL aliquot of a ligation product (Section 3.4.5.2) was transferred to a sterile 0.2 cm-gapped electroporation cuvette (BioRad, Hercules, CA) placed on ice. Into the cuvette, 100 μL of cells (or 20 μL of commercially-available cells) were added. The suspension was mixed and immediately electroporated using "The Porator" (Invitrogen, Carlsbad, CA) set up as follows: 40 μF, 1,500 V (DC), 250 Ω, 50 W, 50 mA, with 2-3 s of exposure time. The electroporated cells were transferred to 1 mL 2x YT containing 1%

glucose, incubated at 37°C for 1 h and subsequently plated at the desired dilutions on 2x YT medium containing appropriate antibiotics. The plates were incubated overnight at 37°C and the following day observed for the presence of, and counted for, positive colonies. In the event that putative positives were present, a further confirmation was undertaken using either colony PCR or hybridization, or a restriction endonuclease cut of the purified, putatively-positive recombinant DNA.

3.4.12 Computer-aided editing and analysis of DNA sequences

All DNA sequence data obtained were subjected to computer-aided editing and analysis using Lasergene software (DNASTAR* Inc., Madison, WI), including, but not limited to, EditSeq2, SeqMan II and MegAlign. Sequence data were provided by the DNA Technologies Unit as a contig for each sequencing primer used. Thus, a gene or DNA segment would have several contigs after sequencing. These primary sequence data in floppy discs were imported and edited using EditSeq2. Approximately 700-1000 bp that would make up a raw contig and which would contain some ambiguous nucleotides, usually at the 5' and 3' ends, were edited and truncated to a 400-500 bp contig. Several contigs from the sequencing results of a gene were then assembled in a single large contig spanning the entire gene using SeqMan II application. The SeqMan II analyzed and matched one contig to others by comparing nucleotide homology at both the 5' and 3' ends. Since a primer was designed based on a previous contig, each contig would have overlapping sequences at each end.

Using the MegAlign application, the edited and assembled large contig representing the entire gene was aligned against an existing sequence database of diverse genes from various organism to determine whether sequence homology existed.

MegAlign was also used to determine exon/intron position by aligning a genomic sequence against its corresponding cDNA sequence.

Another software package, Vector NTI v.4.0 (Informax, Bethesda, MD), was also used for editing, restriction mapping, PCR and sequencing primer selection and design, and graphical construction of a gene.

With the availability of the bioinformatics system through the Internet, edited gene or protein sequences were analyzed against worldwide DNA or protein databases to determine whether a gene or a protein had homologue(s), or whether the same gene had been discovered previously, using *BLAST (Basic Local Alignment Search Tool,* Altschul *et al.*, 1997*)* available at the *National Centre for Biotechnology Information, NCBI* (Bethesda, MD) with its website at http://www.ncbi.nlm.nih.gov/BLAST/. To determine nucleotide and protein homology between AIP1 and AIP3, the "*BLAST* 2 Sequences" algorithm (Tatusova and Madden, 1999) was employed. For comparison with cDNAs of *AIP1* and *AIP3*, gene prediction tools i.e. Grail (Genomix, Oak Ridge, TN), GenScan (C. Burge, http://worf.molbiol.ox.ac.uk/documentation/gene_predict/genscan.htm) and MZEF (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY) applications were used to determine exons of *AIP1* and *AIP3* available in the existing *A. thaliana* database. After retrieval of AIP1p and AIP3p orthologous sequences using local alignment (*BLAST*), AIP1p and AIP3p sequences were divided into N- and C-domains and subsequently compiled together with the orthologous and ALS sequences as *FASTA* files. The *FASTA* file containing either the N- or C-domain was subjected to the multiple sequence alignment algorithm Clustal W (Thompson *et al.*, 1994) provided by EMBOSS (The European Molecular Biology Open Software Suite, EMBnet, Belgium) and available at a website (http://bioinfo.pbi.nrc.ca) of the PBI Bioinformatics

Centre (Saskatoon, SK). Genomic and cDNA sequences of AIP1p and AIP3p were also subjected to *BLASTN* and *BLASTX* analyses, which search for local alignments of nucleotide and amino acid sequences in six frames, respectively (using an e value of 10 and a gap penalty of 10) against the *A. thaliana* genome database, to search for gene duplication in other *A. thaliana* chromosomes using the *PANDA BLAST* application of the PBI Bioinformatics Centre (Saskatoon, SK).

3.5 Genetic transformation of *A. thaliana* by *A. tumefaciens*

3.5.1 Root transformation

3.5.1.1 Generation of transgenic *A. thaliana* lines containing His_6-tagged derivatives of chlorsulfuron resistant ALS

Following SDM (Section 3.4.10), *Agrobacterium* LB1115 containing recombinant pRD400::His_6-At*ALS-csr1-1* plasmids was used to transform *A. thaliana* by infecting root explants, as described by Vergunst *et al.* (1998). An independent *Agrobacterium* colony containing a construct of either the SDM-mutated N- or C-terminal end of the gene was grown accordingly and used to infect root explants harvested from hydroponically-grown seedlings. Root explants from each flask were divided into two clusters, where each cluster was chosen in such a way as to avoid using root clusters from the same flask to be transformed by a particular construct. Two independent colonies from N- or C-terminal constructs were grown as separate cultures, after which each independent culture was divided into duplicates. Each duplicate culture was then used to infect two different clusters of root explants. Thus, for each construct, a total of four root-explant clusters were infected by each independent culture.

From callus propagation to subsequent steps, 100 nM chlorsulfuron (CS) was used as the selection agent. Small plants arising from each independent callus were transferred to soil and grown to maturity. Since there were two constructs used in the transgenic propagation, a proper designation was assigned to each independent line. For example, in the case of DPC712-3, DP and C would represent the investigator's initials and the C-terminal end as the SDM insertion target, respectively, 7 the number assigned to the recombinant plasmid construct in the *E. coli* host, 1 the replication number of the *E. coli* colony used to transform *Agrobacterium*, 2 the replication number of the root explant cluster, and 3 the serial number of the independent line harvested.

A segregration test was done to determine whether a line was homo- or hetero-zygous by growing ~100 seeds under 100-nM CS selection. The homo- or hetero-zygocity was determined by counting plants (after one week of germination) that survived under the selection. In the case of a 100% survival rate, the line was declared homozygous.

3.5.2 Flower infiltration method

3.5.2.1 Promoterless-*GUS* constructs

For the purpose of determining promoter region validity, *cis*-regulatory segments of *AIP1* and *AIP3* were subcloned to a promoterless-*GUS* (expressing β-glucuronidase) cassette of pBI101.1 vectors (Jefferson *et al.*, 1986). The *AIP1* promoter segment of approximately 0.7 kb was *Pfu*-amplified using a pair of primers containing an added *Sal*I site at both 5' sense- and antisense-primer ends, in order to have a restriction-cut compatibility with the vector. The *AIP3* promoter segment was also *Pfu*-amplified using

a pair of primers containing an added *Xba*I site at both the 5' sense- and antisense-primer ends. It was previously concluded by examining the existing *AIP3* cDNA and exon/intron positions that the promoter segment was approximately 2.1 kb in length. However, with the recent availability through the Internet of gene (exon) prediction tools (e.g. GenScan, Grail and MZEF), which analyze existing sequences to construct a putative ORF, later analyses using these prediction tools revealed that the *AIP3* structural gene was approximately one third longer than previously thought. The additional segment, unfortunately, was found upstream of its cDNA, resulting in the promoter segment previously thought to be 2.1 kb in length being only ~0.5 kb in length (i.e. ~1.6 kb shorter). Nevertheless, the plant transformation and transgenic plant analyses were carried out to completion as planned since time did not permit modification of the construct, as this would have required, among other things, screening a genomic library for the extra sequence upstream of the genomic sequences, which has not been available publicly.

An *Agrobacterium* culture containing *AIP1*- or *AIP3-GUS* constructs was used to transform *A. thaliana* flowers using the flower infiltration method (Clough and Bert, 1998). Seeds of putative transformants were grown under a selection agent, kanamycin, at 100 μg/mL in order to identify true transformant lines. The seeds harvested from the plants that survived the first selection were grown on kan^{100} plates and transferred to soil after 7-10 d. Each independent line at several growth stages was stained for GUS activity according to the method of Jefferson (1987) and photographed.

3.5.2.2 Cauliflower Mosaic Virus (CaMV) *35S*::target gene constructs

A pBI832 vector containing *CaMV 35S* strong promoter was used to fuse and cDNAs of *AIP1* (E73 clone) and *AIP3* (E84 clone) respectively, immediately downstream of the strong promoter in the sense orientation. The fused constructs were subsequently subcloned to pRD400, which in turn was used to transform *A. thaliana* flowers by *Agrobacterium* infection, also using the flower infiltration method (Clough and Bert, 1998). Seeds of putative transformants were grown under a selection agent, kanamycin, at 100 μg/mL in order to identify true transformant lines. The seeds harvested from the plants that survived the first selection were grown on kan^{100} plates and transferred to soil after 7-10 d. Each independent line at 22-25 day-old was extracted once as described in Section 3.6.1 for determination of protein levels by Western blotting technique.

3.6 Methods for protein preparation, quantitation and analysis

3.6.1 Plant protein extractions

Approximately 2 g of aerial tissue was used to prepare stromal protein extracts (the soluble fraction) and thylakoid membranes (the insoluble fraction) from *A. thaliana*. The preparation was based on protocols previously described (Gualberto *et al.*, 1995) and was carried out at 4°C with the following modifications. Instead of 0.33 M sorbitol, 0.45 M sucrose was used, and for steps requiring Hepes-KOH buffer, KPO_4 buffer was used instead (Bekkaoui *et al.*, 1993b). Chloroplasts were resuspended and fractionated into stromal and thylakoid fractions by lysing using hypotonic shock. After 10 min lysis, 1 volume of 2x stromal protein buffer pH 8.0 (Bekkaoui *et al.*, 1993b) was added

to the suspension and the resulting stromal protein extract was used for further experiments (e.g. protein-protein interaction experiments, enzymatic assays). Thylakoid fractions were purified as described by Gualberto *et al.* (1995) and subsequently used for the detection and localization of the proteins of interest.

A one-step extraction from aerial tissues of 22-25 d-old *A. thaliana* plants was used to prepare protein extracts in preparation for Western blot analysis of proteins of interest from wild type and mutant lines generated as described in Section 3.5.2.2. Approximately 0.2 g of plant material was ground (~2 min) to fine powders under liquid nitrogen, after which 0.5 mL of plant lysis buffer (200 mM Tris-HCl pH 7.5, 20 mM $MgCl_2$, 2 mM EDTA, 1 mM PSMF, 1 μg/mL leupeptin, 1 μg/mL peptatin, 40% glycerol) was added and grinding continued for 1 min. The ground suspension was transferred to a microfuge tube and centrifuged at approximately 12,000 x *g* for 12 min at 4°C. The supernatant was then transferred to a clean microfuge tube and the total protein concentration determined using the Bradford procedure (Section 3.6.2.2). From each sample, aliquots containing equal amounts of total protein were used for SDS-PAGE followed by Western analysis.

3.6.2 Protein quantitation

3.6.2.1 Direct UV spectrophotometric measurement

For purposes of antibody concentration determination, a non-destructive, direct protein quantitation by UV detection was used as described by Harlow and Lane (1988).

3.6.2.2 Bradford procedure

The dye-binding assay of Bradford (1976) was employed to determine protein concentrations in experimental samples using BSA as a standard.

3.6.3 Protein purification and generation of polyclonal antibodies

3.6.3.1 Protein purification

AIP1 and *AIP3* cDNAs, which were recovered from yeast two-hybrid screening and cloned in-frame into pBI786, a modified His_6-tagged vector derived from pRSETB (Invitrogen, Carlsblad, CA), were obtained from previous work (Kohalmi *et al.*, unpublished) and the recombinant plasmids were used to transform *E. coli* strain NM522. The expressed recombinant proteins were purified using Ni^{2+}-NTA agarose resin (Qiagen, Mississauga, ON) according to the instructions of the manufacturer.

3.6.3.2 Generation of polyclonal antibodies

Recombinant AIP1p and AIP3p were purified to homogeneity as described above and used to generate polyclonal antibodies in New Zealand white rabbits (two rabbits/antigen). Rabbits were injected four times each at two-week intervals with 50 μg of AIP1p or 100 μg of AIP3p. The first injections were done using emulsions of the recombinant proteins in refolding buffer (0.5 M NaCl, 0.4 M arginine-HCl, 10 mM Tris pH 7.5) and Freund's complete adjuvant, whereas subsequent boost injections used incomplete adjuvant. Serum was collected eight weeks following the first injection and the IgG fraction was purified using standard procedures (Harlow and Lane, 1988).

3.6.3.2.1 Cross-reactivity tests

In order to estimate cross-reactivity among the different antibodies, slot blot analysis was performed by spotting varying concentrations of native *E. coli*-derived recombinant ALS, AIP1p and AIP3p proteins to nitrocellulose membrane (Schleicher and Schuell, Inc., Keene, NH). The slot-blotted membranes were then subjected to Western analysis using a chemiluminescence procedure (Amersham Pharmacia Biotech, Piscataway, NJ) using the corresponding polyclonal antibodies as probes. After Western analysis, antibody cross-reactivity was estimated based on the common dilution that yielded a similar detection signal between corresponding and non-corresponding antibodies.

3.6.4 SDS-PAGE electrophoresis and Western blotting

SDS-PAGE electrophoresis, including the preparation of gels and samples, was carried out as described by Sambrook *et al.* (1990). Electrophoresis of samples in parallel with prestained low molecular mass protein markers (BioRad, Hercules, CA) was carried out using a BioRad Mini-Protein gel system at a 180-V constant setting. The gel was stained and dried (when required) using a BioRad GelAir drying system, or used directly for Western blot analysis.

Western blotting was carried out based on protocols described by Sambrook *et al.* (1990) with some modifications. Nitrocellulose membranes were obtained from MicroSeparation Inc. (Westborough, MA) and used as the solid support for electro-transfer. Electro-transfer was carried out using the BioRad Trans-Blot module system at 80 V and 4°C for 2 h. Alternatively, blotting was carried out using a BioRad Trans-Blot

SD semi-dry transfer cell maintained at 15 V for 15 min per mini gel. After transfer was completed, the membrane was blocked in a sufficient volume of TBST buffer (50 mM Tris-HCl pH 7.9, 150 mM NaCl and 0.05% Tween 20) containing 5% (w/v) of non-fat dried milk (NFDM) for 1 h at RT, or overnight at 4°C in blocking solution containing 0.02% sodium azide. The blocked membrane was subsequently washed twice, for 15 min each time, with TBST buffer, followed by a third wash for 10 min. The membrane was then incubated with the indicated primary antibodies diluted in 2% milk TBST for 1 h, after which the membrane was washed three times for 10 min each time. Typically, dilutions of 1:3000 and 1:2500 were used for anti-AIP1p and anti-AIP3p, respectively. Antibody F14, a laboratory stock of purified IgG against *B. napus* ALS but also reactive to *A. thaliana* ALS was used at a concentration of 12.5 μg/mL. After washing, membranes were incubated for 1 h with HRP-conjugated goat-anti-rabbit IgG (secondary antibody) diluted in 2% milk TBST, typically at a 1:20,000 dilution. The membrane was washed three times for 10 min each time, and subsequently detected using ECL™ or ECL+Plus™ enhanced chemiluminescence Western blotting detection reagents according to the instructions of the manufacturer (Amersham Pharmacia Biotech, Piscataway, NJ).

3.6.4.1 Sub-organellar immunolocalization of ALS, AIP1p and AIP3p proteins

Approximately 2 g of 21-25 d-old plants were used for the preparation of chloroplasts, stromal extracts and thylakoid membrane fractions as described by Gualberto *et al.* (1995). Figure 3.1 is a flow chart of the preparation scheme. Aliquots of each fraction containing equal amounts of total protein were subjected to SDS-PAGE

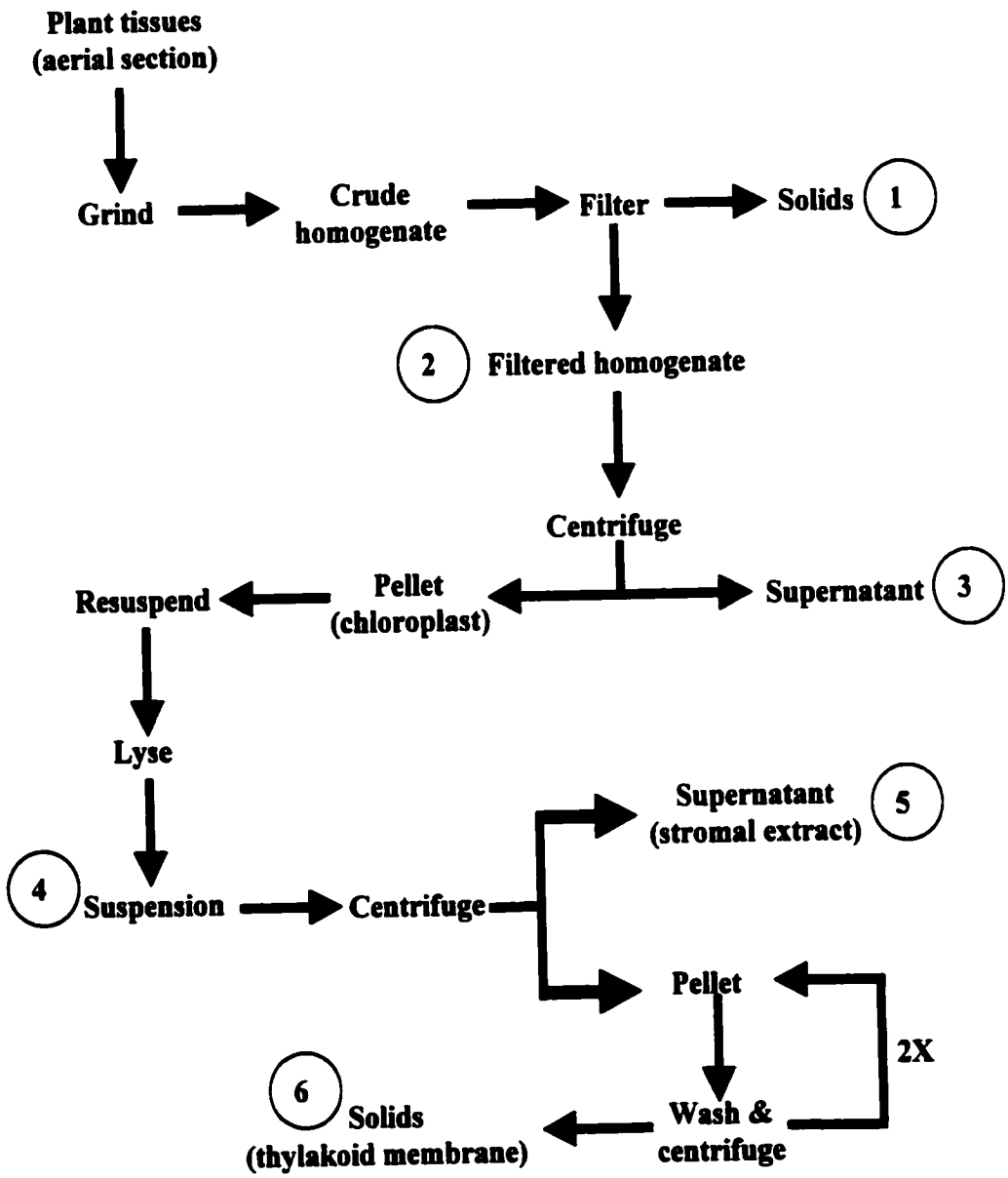


Figure 3.1. A scheme for stromal extract preparation.

and Western blot analysis (as described in Section 3.6.4) to detect the presence of ALS, AIP1p and AIP3p proteins using their corresponding antibodies as probes.

3.6.5 Enzyme activity assays and chlorophyll quantitation

3.6.5.1 ALS activity assays

Fifty-microlitre aliquots of stromal extracts containing 30-50 mg/mL total protein were used for ALS activity assays using the method of Singh *et al.* (1988b). For herbicide and BCAA inhibitory treatments, 50 and 100 nM chlorsulfuron, 1 mM each of isoleucine, leucine and valine, and their combined pool containing 1 mM of each BCAA were incorporated into the assay.

3.6.5.2 Shikimate oxidoreductase (SOR) activity assays

In order to confirm that the stromal extracts used were indeed derived from the stroma compartment of the chloroplast, shikimate oxidoreductase (SOR) activity, a stromal-specific marker enzyme, was determined in stromal extracts (i.e. fraction 5) of known protein concentration and fractions 2, 3, 4 and 6, as shown in Figure 3.1, using the protocol described by Fiedler and Schultz (1985).

3.6.5.3 Chlorophyll quantitation

In order to confirm that fraction 6 (Figure 3.1) was indeed derived from the thylakoid membrane of the chloroplast, chlorophyll contents in fractions 2, 3, 5 and 6 were quantitated. Each fraction (Figure 3.1) was adjusted to 10 mL total volume, after which aliquots of 0.0, 0.1, 0.2, 0.3 and 0.4 mL were combined with 1.0, 0.9, 0.8, 0.7, and

0.6 mL ddH_2O, respectively. Each combined suspension was subsequently mixed with 4 mL acetone, vortexed and centrifuged, after which the absorbance at 652 nm of a 1-mL aliquot of each diluted sample was measured using a Spectronic Genesys 5 spectrophotometer (Milton Roy Co., Rochester, NY).

3.7 Protein-protein interaction experiments

3.7.1 *In vitro* coupled transcription/translation and interaction experiments

Purified recombinant cDNAs of *ALS*, *AIP1* and *AIP3* in the "prey" vector pBI-771 were PCR-amplified using LV009 (GAT AAT ACG ACT CAC TAT AGG GTC GAG GGA CTA CAA GGA C) and LV010 (TCA TTT AGG TGA CAC TAT AGG AGA CTT GAC CAA ACC) primers, where the LV009 contained the T7 RNA polymerase promoter (the underlined sequence), which is required for *in vitro* transcription. The purified PCR fragments were used to express radiolabeled proteins *in vitro* using a coupled transcription/translation system (TNT® Coupled Reticulocyte Lysate Systems, Promega, Madison, WI) containing $^{[35]}$S-methionine. Luciferase cDNA supplied with the kit was also transcribed/translated as the control. After transcription/ translation, the labeled products were electrophoresed and subsequently stained, destained and amplified (to increase signal intensity) using Amplify® solution (Amersham Pharmacia Biotech, Piscataway, NJ). The gel was then dried and exposed to X-ray film. The degree of signal intensity from each of the labeled products was visually compared and the sample volume which would give equivalent signal intensity of all labeled products was determined.

The $^{[35]}$S-met labeled ALS, AIP1p and AIP3p translated products were used in *in vitro* binding assays against several unlabeled *His$_6$*-tagged proteins that were immobilized to Ni^{2+}-NTA beads including, AGAMOUS-LIKE (AGL)2, AGL4 and AGL24, ASK1 (*A. thaliana* Skp1) and *ll*-ASK1 (leaderless ASK1). For the binding assay, a 50-µL aliquot of 50% Ni^{2+}-NTA agarose resin suspension in 30% ethanol was centrifuged to remove the ethanol-containing storage solution and subsequently washed once each with ddH_2O, NETN buffer (20 mM Tris-HCl pH 8.0, 100 mM NaCl, 10 mM NaF, 25 mM β-glycerolphosphate, 20 mM imidazole, and 0.5% NP40) and NETN buffer containing 2 mg/mL BSA. Approximately 5 µg of unlabeled *His$_6$*-tagged protein was immobilized to the resin in a final volume of 400 µL NETN by tumbling for 1 h at 4°C, and subsequently washed once with NETN buffer containing 2 mg/mL BSA. After immobilization of the *His$_6$*-tagged protein, an equivalent (input) volume that would give the same signal intensity of each of the labeled proteins (including luciferase) was added to independent reactions, and the volume brought up to 500 µL with NETN. The binding reaction was incubated by tumbling for 2 h at 4°C, after which the beads were washed twice with NETN buffer containing 2 mg/mL BSA and the resin resuspended in 50 µL of 2x SDS loading buffer in preparation for SDS-PAGE analysis. Labeled input proteins were also analyzed in parallel by SDS-PAGE. After electrophoresis, the gel was treated in the same fashion as was the gel of the translation products described above, and the radiolabeled protein bands were visualized and examined.

3.7.2 *In vivo* protein-protein interaction experiments

3.7.2.1 Co-immunoprecipitation experiments

In preparation for co-immunoprecipitation experiments, IgGs purified from ALS, AIP1p and AIP3p antisera were covalently cross-linked to protein A-sepharose beads using protocols already available (Harlow and Lane, 1988). For co-immunoprecipitation experiments, appropriate volumes of stromal extract normalized to a pre-determined protein concentration were mixed with 50 µL of pre-blocked resin suspension of protein A-sepharose cross-linked to IgGs of anti-ALS (F14), anti-AIP1p or anti-AIP3p, and the volume was brought up to 500 µL with stromal extract buffer. Incubation was then carried out by tumbling for 1 h at 4°C, after which the reactions were briefly centrifuged and the supernatant discarded. The resin was washed twice with stromal extract buffer and subsequently treated with an appropriate volume of 2x SDS loading buffer in preparation for SDS-PAGE and Western blot analysis. Along with corresponding cross-linked IgG-resin, crosslinked-preimmune IgG-resin was analyzed in parallel as a control.

3.7.2.2 Immobilized metal-ion affinity chromatography (IMAC) co-purification experiments

3.7.2.2.1 Immobilized metal-ion affinity chromatography nickel(II) co-purification experiments

In a 500-µL eppendorf tube, 50-µL aliquots of IMAC Ni^{2+} agarose resin (Qiagen, Mississauga, ON) were washed with deionized water and equilibrated with binding buffer (1x stromal extract buffer; 50 mM KPO_4 pH 8.0, 150 mM NaCl, 10 mM FAD plus 0.5x lysis buffer, 10 mM KPO_4 pH 8.0, 2 mM $MgCl_2$). Stromal extracts were prepared from transgenic line DPC712-3 and approximately 200 µg of total protein was

added to the beads in 500 µL of binding buffer. As a control, stromal extracts were prepared from wild type (WT) plants (C24) and treated identically. Binding reactions were performed by tumbling for 1 h at 4°C, followed by centrifugation and two brief (~1 min) washes with binding buffer. For washing, a second approach was used whereby 10-20 mM imidazole was incorporated in the washing buffer. The bound protein complex was eluted with 50 µL of binding buffer containing 50 mM imidazole. The eluted samples were then mixed with 50 µL of 2x SDS loading buffer and boiled for 10 min in preparation for Western blot analysis.

3.7.2.2.2 Immobilized metal-ion affinity chromatography cobalt(II) co-purification experiments

Experiments using IMAC Co^{2+} agarose resin (Clontech, Palo Alto, CA) were performed in the same fashion as experiments using IMAC Ni^{2+} agarose resin, with the exception of each wash being 10 min in duration. Incorporation of imidazole at low a concentration (10 mM) was also done.

3.8 Free amino acid extraction and analysis

Free amino acid extractions from wild type and *CaMV 35S* ectopically-expressing transgenic lines (Section 3.5.2.2) of *A. thaliana* were carried out based on a modified method (A. Shukla, Personal Communication) originally introduced by Bieleski and Turner (1966). Approximately 0.2 g (fresh weight) of plant (22-25 d old) materials was harvested, placed in a mortar, and ground under liquid nitrogen to fine powder. Subsequently, 1 mL of ice cold methanol:chloroform:water (MCW) mixture (12:5:3, v/v/v) was added, mixed for approximately 1 min and transferred to a 1.5-mL

centrifuge tube. The homogenate was thoroughly vortexed for 5 min and subsequently centrifuged for 5 min after which the supernatant was collected and the residue re-extracted with 1 mL of MCW. After centrifugation, the first and second supernatants were combined and designated as "MCW extract." One millilitre of chloroform was added, followed by 1.5 mL of water, to the MCW extract and mixed well. The resulting two-phase mixture was centrifuged to separate the phases, after which the chloroform/lower layer was discarded and the aqueous layer was retained. The twice-extracted residue from the MCW extraction was then re-extracted with 1 mL of 80% (v/v) ethanol using the same time regime and vortexing as above. This EtOH extract and the aqueous layer of the MCW extract were combined and vacuum dried using a Büchi RE 111 Rotavapor (Brinkmann Instruments Inc., Westbury, NY). The dried sample was redissolved in 1 mL of System 6300 high performance amino acid sample dilution buffer (Beckman Instruments Inc., Fullerton, CA) previously spiked with a known amount of an internal standard, norleucine.

Analysis of free amino acids was carried out by the Animal Nutrition and Biotechnology Laboratory of the Department of Animal and Poultry Science, University of Saskatchewan, Saskatoon, SK using a Beckman HPLC System (Beckman Instruments Inc., Fullerton, CA).

3.9 Statistical analysis

All experiments were performed in duplicate at least. Cross-reactivity tests and chlorophyll quantitation were based on single determinations. Average values for,

standard errors (α= 0.05; unless specified otherwise) of, and regression analysis of, data were calculated using Microsoft® Excel 97 (Microsoft Corporation, Redmond, WA).

4. RESULTS

4.1 Characterization of *AIP1* and *AIP3* genes

4.1.1 cDNAs and gene products of *AIP1* and *AIP3*

Previously, *AIP1* and *AIP3* cDNAs from *ALS* two-hybrid experiments were recovered and their sequences partially characterized. In these yeast two-hybrid experiments, ten classes of ALS-interacting proteins (AIPs) were identified; two of these classes (AIP1p and AIP3p) were found to be similar to bacterial ALS regulatory (small) subunits. The *AIP1* and *AIP3* cDNAs have been sequenced, and their deduced protein primary structures determined (Kohalmi and Crosby, unpublished).

In this study, the molecular weights of ALS, AIP1p and AIP3p polypeptides were estimated using immunoblots of *A. thaliana* extracts, and were found to be approximately 66, 53 and 50 kDa, respectively, as shown in Figure 4.1. These AIP1p and AIP3p estimated molecular masses were similar to the predicted sizes (53.8 and 51.8 kDa, respectively) deduced from the full-length protein primary structures conceptually translated from their ORFs, shown in Figures 4.5 and 4.6. *In vitro* translation of AIP1p and AIP3p resulted in two main bands for each protein when they were resolved in SDS-PAGE. Molecular masses of approximately 53 and 49 kDa for AIP1p, and 32 and 30 kDa for AIP3p were observed, as shown in Figure 4.1. The size differences and possible explanations for the presence of two bands arising from *in vitro* translation will be considered in the DISCUSSION section.

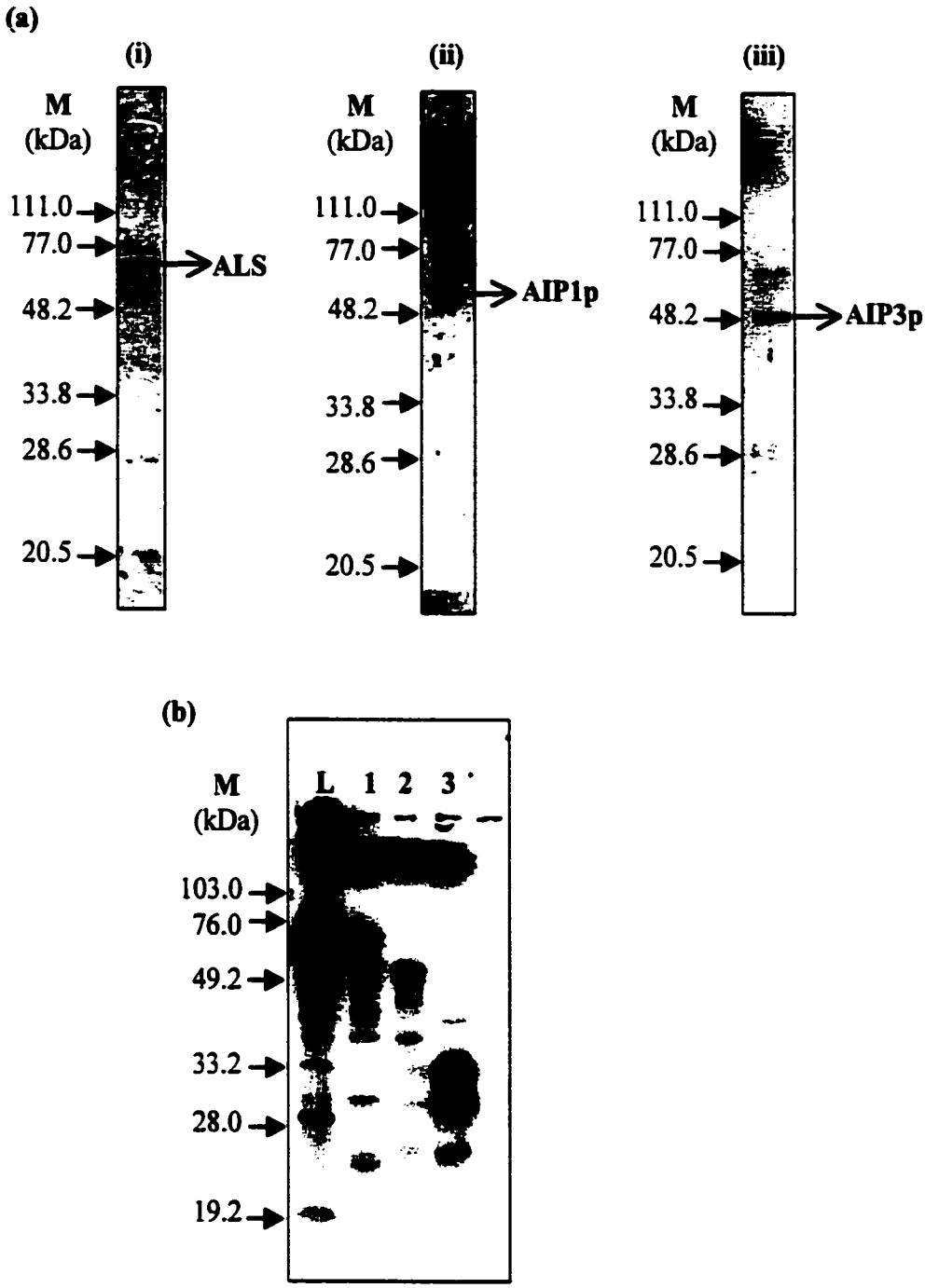


Figure 4.1. Western blots (a) of ALS (i), AIP1p (ii) and AIP3p (iii) detected in wild type stromal extracts using their corresponding antibodies as probes, and *in vitro*-translated proteins (b) of ALS (1), AIP1p (2), AIP3p (3) and luciferase (L) as control. M represents protein markers and their molecular masses.

Subsequent global database searches using *BLAST (Basic Local Alignment Search Tool*, Altschul *et al.*, 1997*)* available at the National Centre for Biotechnology Information, NCBI (Bethesda, MD) (http://www.ncbi.nlm.nih.gov/BLAST/) revealed that AIP1p and AIP3p are variably homologous to several dozen ALS catalytic or regulatory/small subunits from various prokaryotic or eukaryotic species, as summarized in Figures 4.2 and 4.3 and Tables 4.1 and 4.2. Tables 4.1 and 4.2 describe the corresponding protein homologues presented in Figures 4.2 and 4.3, respectively. In either the N- or C-terminal homologous region, twenty-three protein sequences were ≥ 65% homologous to AIP1p, whereas nineteen were ≥65% similar to AIP3p, where homology is defined as similarity at the level of amino acid identity or conservative substitutions between the query AIP1p or AIP3p sequence and elements of the non-redundant (nr) protein database. The S values represented by the coloured and scaled bars in Figures 4.2 and 4.3 are the scores of alignment calculated by summing the scores for each aligned position and the scores for gaps, where scores were determined using a substitution matrix approach (e.g. BLOSUM62) embedded in the *BLAST* program. For example, if two sequences show 65% similarity to a query sequence, they would not necessarily exhibit identical S values if their scores for gaps were different. Although the gaps for this *BLAST* analysis were fixed to a certain value, each sequence was aligned uniquely to the query protein, resulting in different numbers of gaps for the same degree of homology. Therefore, homology percentage was used instead of S value in reporting the data. The "*BLAST* 2 Sequences" analysis revealed that *AIP1* and *AIP3* sequences were approximately 74 and 80% homologous at the nucleotide and deduced amino acid levels, respectively. Specifically, a computer application was developed at

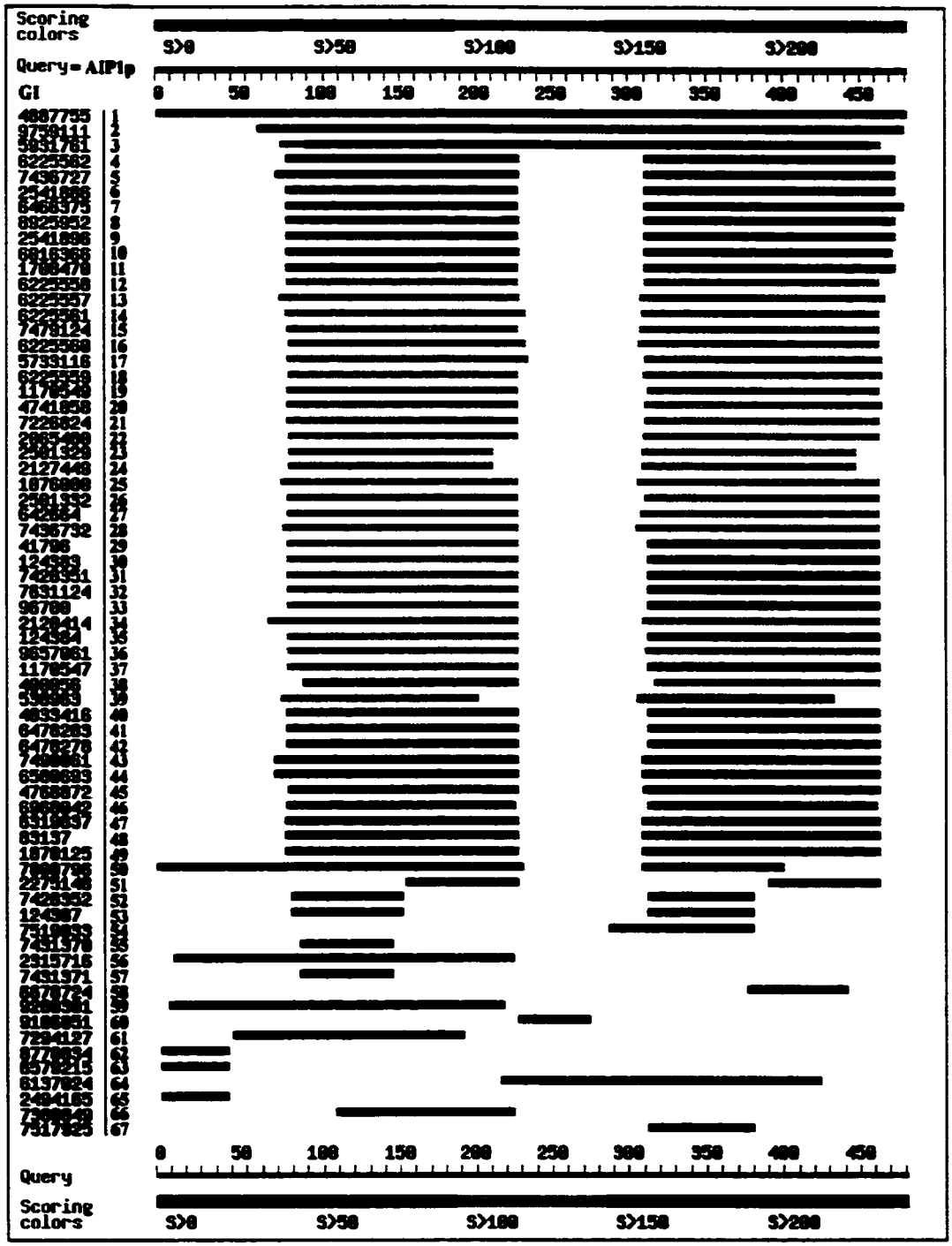


Figure 4.2. Results of database search using *BLAST* for AIP1p. Bars represent various degree of homology between AIP1p and the rest. Analyzed sequences 1-19, 21-23 and 26, which were ≥65% homologous to AIP1p, are described in Table 4.1 of the following page.

Table 4.1. A description of corresponding protein homologues presented in Figure 4.2.

	GenInfo Identifier (GI)	Description	Protein length (a.a)
1	4887755	putative acetolactate synthase of *Arabidopsis thaliana*	484
2	9759111	acetolactate synthase-like protein of *Arabidopsis thaliana*	477
3	5931761	acetolactate synthase small subunit of *Nicotiana plumbaginifolia*	449
4	6225562	acetolactate synthase small subunit *Synechosystis* sp.	172
5	7436727	acetolactate synthase (ALS) of *Synechosystis* sp.	188
6	2541886	acetolactate synthase of *Cyanidioschyzon merolae*	166
7	6466375	unknown; acetohydroxyacid synthase small subunit of *Cyanidium caldarium*	172
8	8925952	acetohydroxy-acid synthase small subunit of *Galdieria sulphuraria*	181
9	2541896	acetolactate synthase *Cyanidium caldarium*	166
10	6016366	acetohydroxyacid synthetase (AHAS) small subunit *Guillardia theta*	169
11	1708470	ILVH_PORPU acetolactate synthase small subunit of *Porphyra purpurea*	174
12	6225558	acetolactate synthase, small subunit (ilvN) of *Archaeoglobus fulgidus*	159
13	6225557	acetolactate synthase of *Aquifex aeolicus*	192
14	6225561	ilvN of *Mycobacterium tuberculosis*	168
15	7479124	acetolactate synthase small subunit of *Streptomyces coelicolor*	174
16	6225560	hypothetical protein of *Mycobacterium leprae*	169
17	5733116	acetohydroxy acid synthase small subunit of *Streptomyces cinnamonensis*	175
18	6225559	acetolactate synthase, small subunit of *Methanobacterium thermoautotrophicum*	168
19	1170549	ILVN_BACSU acetolactate synthase small subunit of *Bacillus subtilis*	174
21	7226824	acetolactate synthase III, small subunit of *Neisseria meningitidis* MC58	163
22	2065480	acetohydroxyacid synthase small subunit of *Methanococcus aeolicus*	169
23	2501329	ILVH_MYCAV acetolactate synthase small subunit of *Mycobacterium avium*	167
26	2501332	ILVH_METJA probable acetolactate synthase small subunit of *Methanococcus jannaschii*	172

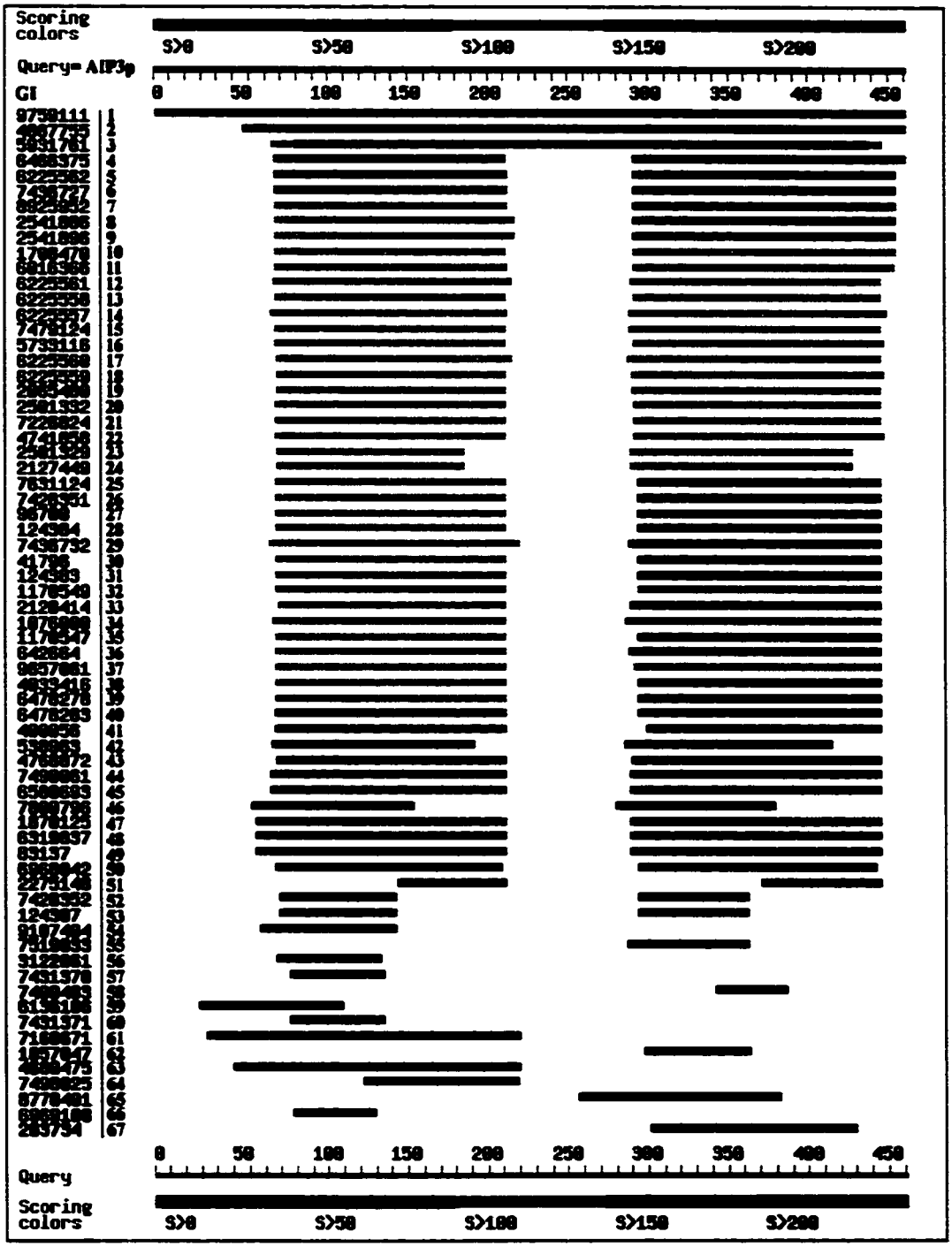


Figure 4.3. Results of database search using *BLAST* for AIP3p. Bars represent various degree of homology between AIP3p and the rest. Analyzed sequences 1-17, 23 and 24, which were ≥65% homologous to AIP3p, are described in Table 4.2 of the following page.

Table 4.2. A description of corresponding protein homologues presented in Figure 4.3.

	GenInfo Identifier (GI)	Description	Protein length (a.a)
1	9759111	acetolactate synthase-like protein of *Arabidopsis thaliana*	477
2	4887755	putative acetolactate synthase of *Arabidopsis thaliana*	484
3	5931761	acetolactate synthase small subunit of *Nicotiana plumbaginifolia*	449
4	6466375	unknown; acetohydroxyacid synthase small subunit of *Cyanidium caldarium*	172
5	6225562	acetolactate synthase small subunit of *Synechosystis* sp.	172
6	7436727	acetolactate synthase (ALS) of *Synechosystis* sp.	188
7	8925952	acetohydroxy-acid synthase small subunit of *Galdieria sulphuraria*	181
8	2541886	acetolactate synthase of *Cyanidioschyzon merolae*	166
9	2541896	acetolactate synthase *Cyanidium caldarium*	166
10	1708470	ILVH_PORPU acetolactate synthase small subunit of *Porphyra purpurea*	174
11	6016366	acetohydroxyacid synthetase (AHAS) small subunit *Guillardia theta*	169
12	6225561	ILVH_MYCTU acetolactate synthase small subunit of *Mycobacterium tuberculosis*	168
13	6225558	acetolactate synthase, small subunit (ilvN) of *Archaeoglobus fulgidus*	159
14	6225557	acetolactate synthase of *Aquifex aeolicus*	192
15	7479124	acetolactate synthase small subunit of *Streptomyces coelicolor*	174
16	5733116	acetohydroxy acid synthase small subunit of *Streptomyces cinnamonensis*	175
17	6225560	hypothetical protein of *Mycobacterium leprae*	169
23	2501329	ILVH_MYCAV acetolactate synthase small subunit of *Mycobacterium avium*	167
24	2127449	acetolactate synthase small chain of *Mycobacterium avium*	167

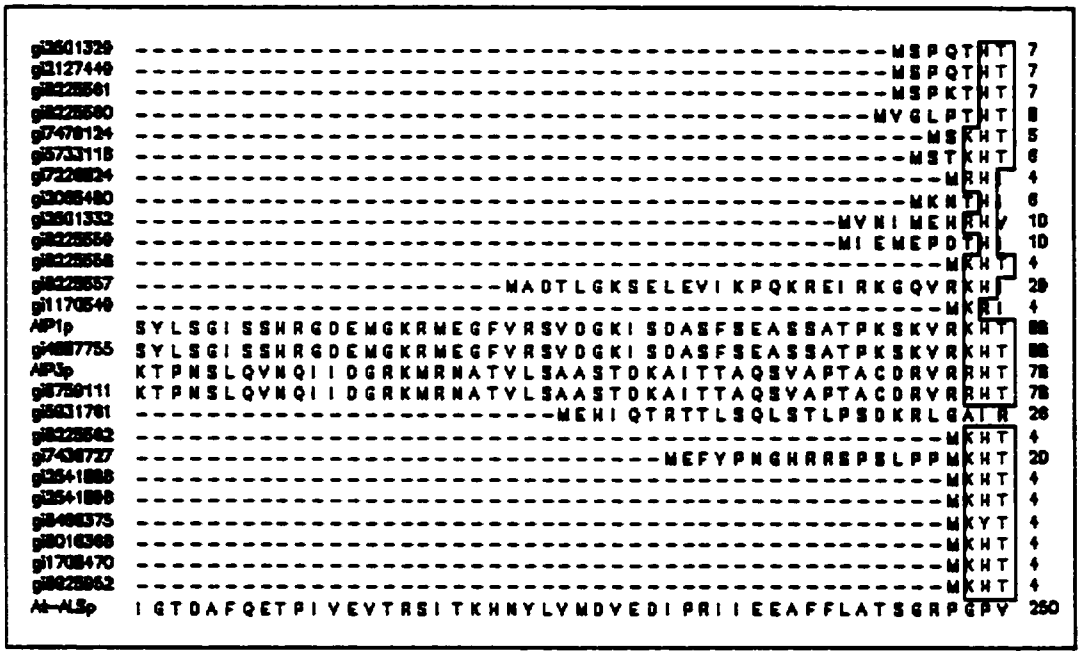
the Bioinformatics Centre of the Plant Biotechnology Institute that combined the existing exon prediction algorithms GRAIL (Genomix, Oak Ridge, TN), Genscan (C. Burge, http://worf.molbiol.ox.ac.uk/documentation/gene_predict/genscan.htm) and MZEF (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY) to predict *AIP3* exons in an 85-kb block of *A. thaliana* genomic sequence previously determined to contain the *AIP3* sequence. In the case of AIP1p, this was not done since the deduced size was consistent with the size of AIP1p seen in both Western blots of plant extracts and *in vitro*-translated AIP1p. The results revealed that the exonic sequence (putative ORF) for *AIP3* was longer than that previously determined based on its cDNA size derived from the two-hybrid system construct i.e. E84 and E229 cDNA clones (Figure 4.6). This result was informative, since the molecular weight deduced from this new putative ORF was more consistent with the size of AIP3p seen in the Western blots of the plant extracts. To support the predicted component of *AIP3*, the putative ORF was subjected to homology assessment using *BLAST* and the results revealed that the additional, predicted exonic sequence was markedly similar to those of ALS regulatory/small subunits from various prokaryotic or eukaryotic species. The homologous domains of AIP1p and AIP3p *vis-à-vis* their prokaryotic counterparts were localized primarily in the N- and C-terminal regions, separated by a largely non-homologous region of 60-70 amino acid residues, whereas the first 70-80 amino acid residues at the N-termini of AIP1p and AIP3p were not homologous at all to their prokaryotic/eukaryotic counterparts, as shown in Figures 4.2 and 4.3.

Genomic and cDNA sequences of AIP1p and AIP3p were also subjected to *BLASTN* and *BLASTX* analyses against the *A. thaliana* genome database to search for gene duplication in the entire *A. thaliana* chromosomes, as described in Section 3.4.12.

No significant of nucleotide or amino acid sequence local aligments were obtained in terms of gene duplicates in any chromosome of *A. thaliana*. The high bit score and low e value were only obtained when *AIP1*/AIP1p aligned to itself or to *AIP3*/AIP3p, and *vice versa*. The other sequence local alignments exhibited a markedly low bit score (20-40) and a high e value (0.006 and higher).

The local alignment (*BLAST*) of AIP1p or AIP3p resulted in the retrieval of dozens of homologous sequences. Based on these results, proteins with sequence similarities of 65% or more to AIP1p and/or AIP3p, together with ALS, AIP1p and AIP3p, were compiled as FASTA files and subjected to the multiple sequence alignment algorithm Clustal W (Thompson *et al.*, 1994), as described in Section 3.4.12. This was to assess common sequence structural positions and/or ancestral residues where the alignment is presented with gaps, enabling matched sequences or patterns to be presented in the same column. Portions of the multiple sequence alignment results are presented in Figure 4.4. With the exception of the ALS catalytic subunit of *A. thaliana* (At-ALSp), all sequences analyzed exhibited a remarkable orthology (i.e. homologous sequences in different species that arose from a common ancestral gene during speciation) that may or may not reflect a similar function. The alignment results showed conserved regions among both prokaryotic and eukaryotic species. The AIP1p and AIP3p proteins are actually identical to GI:4887755 and GI:9759111 proteins, submitted in the protein database with different annotation as putative acetolactate synthase genes in *A. thaliana* (Lin *et al.*, 1999) and acetolactate synthase-like protein of *A. thaliana* (Sato *et al.*, 1997), respectively. Several conserved regions were altered when compared between prokaryotic and eukaryotic genes. For example, the 92nd and 94th amino acid residues of AIP1p and AIP3p were unique to these proteins. Likewise, the 121st and

(a)



(b)

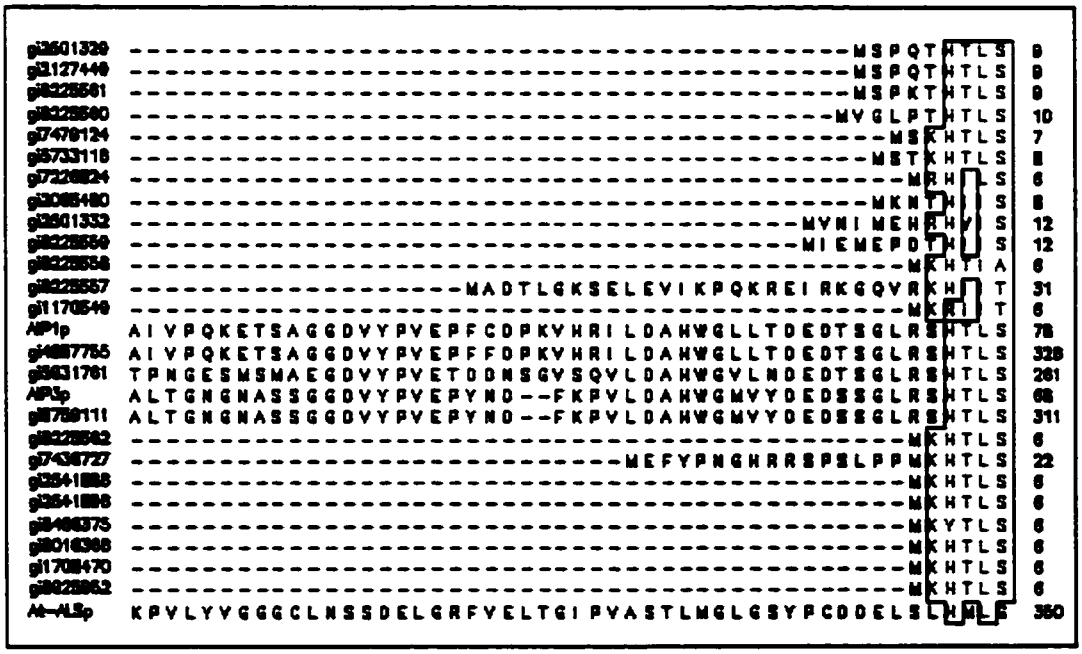


Figure 4.4. Portions of multiple sequence alignments, using Clustal W algorithms (Thompson *et al.*, 1994), among *A. thaliana* ALS catalytic subunit (At-ALSp), N-domains (a) and C-domains (b) of AIP1p and AIP3p, and protein sequences that showed ≥65% similarity to AIP1p and AIP3p proteins. Each GI number corresponds to its description in Table 4.1 and 4.2. The first residues of AIP1p and AIP3p C-domains correspond to residues 246 and 239, respectively. Complete results are presented in Appendices A1 and A2.

(a)

(b)

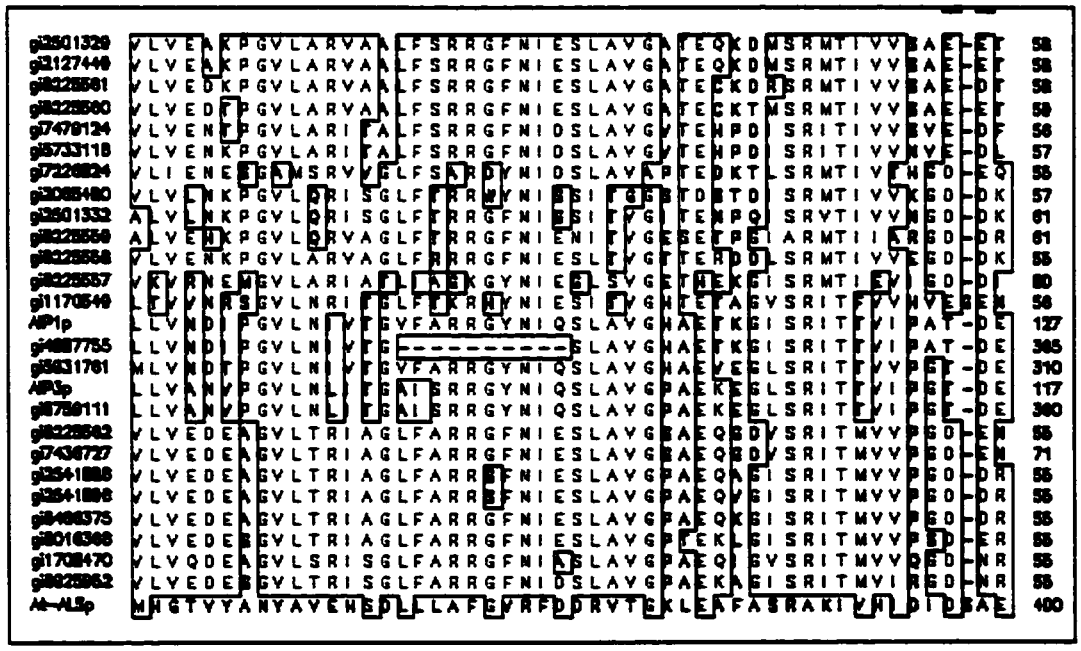


Figure 4.4. Continued...

122nd residues of AIP1p were unique to AIP1p and AIP3p as well as to GI:5931761, which is an acetolactate synthase small subunit from *N. plumbaginifolia* (Hershey *et al.*, 1999).

4.1.2 Genomic *AIP1* and *AIP3* DNAs

The *AIP1* (clone E73) and *AIP3* (clone E84) cDNAs were used as hybridization probes to recover the corresponding chromosomal genes by screening *A. thaliana* genomic lambda libraries (CB4-8, ABRC). *Sal*I fragments of *AIP1* and *AIP3* recovered from the libraries were subcloned into the pSL1180 plasmid (3.42 kbp), and the recombinants transformed to *E. coli* (DH10B) by electroporation. Purified recombinant *AIP1* and *AIP3* DNAs were restriction mapped and sequenced. Sequence data spanned approximately 6.3 and 3.4 kb of the *AIP1* and *AIP3* genes, respectively, plus the 2.1-kb 5' segment for *AIP3* retrieved from the *A. thaliana* database. The sequencing and contig alignment strategies, which utilized a *SeqMan*II computer program, are summarized in Figure 4.5. In the process of final editing of the *AIP1* and *AIP3* sequences, ten of twenty-two contigs of *AIP1* DNA, containing several unspecified nucleotides, were re-sequenced using a set of ten extension primers. The *AIP3* gene was constructed from thirteen contigs in which three contigs exhibited several sequence ambiguities. The sequences of *AIP1* and *AIP3* DNA were compared with the *A. thaliana* genomic database, where a few (four to ten) nucleotide disagreements or unspecified nucleotides were identified and corrected using both sequences as a reference.

The exon/intron arrangements for the genes were determined using a *MegAlign*II computer program to compare and align the genomic sequences with their respective cDNAs. Regions of DNA overlap were defined as an exon region. The genomic *AIP1*

(a)

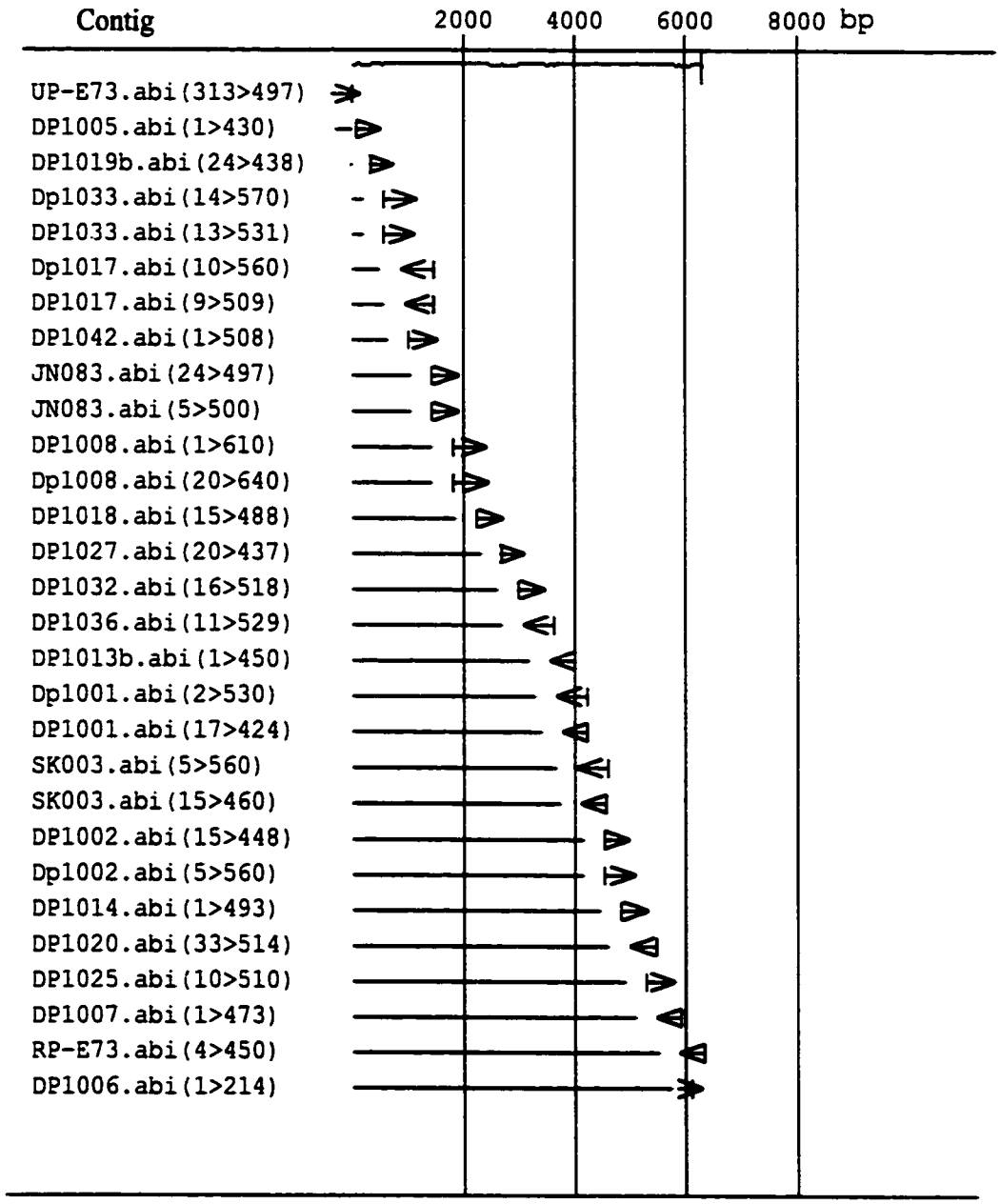


Figure 4.5. Sequencing and contig alignment strategies of *AIP1* (a) and *AIP3* (b) genes. The file name and numbers in brackets represent the primers used and the sequence length analyzed, respectively.

(b)

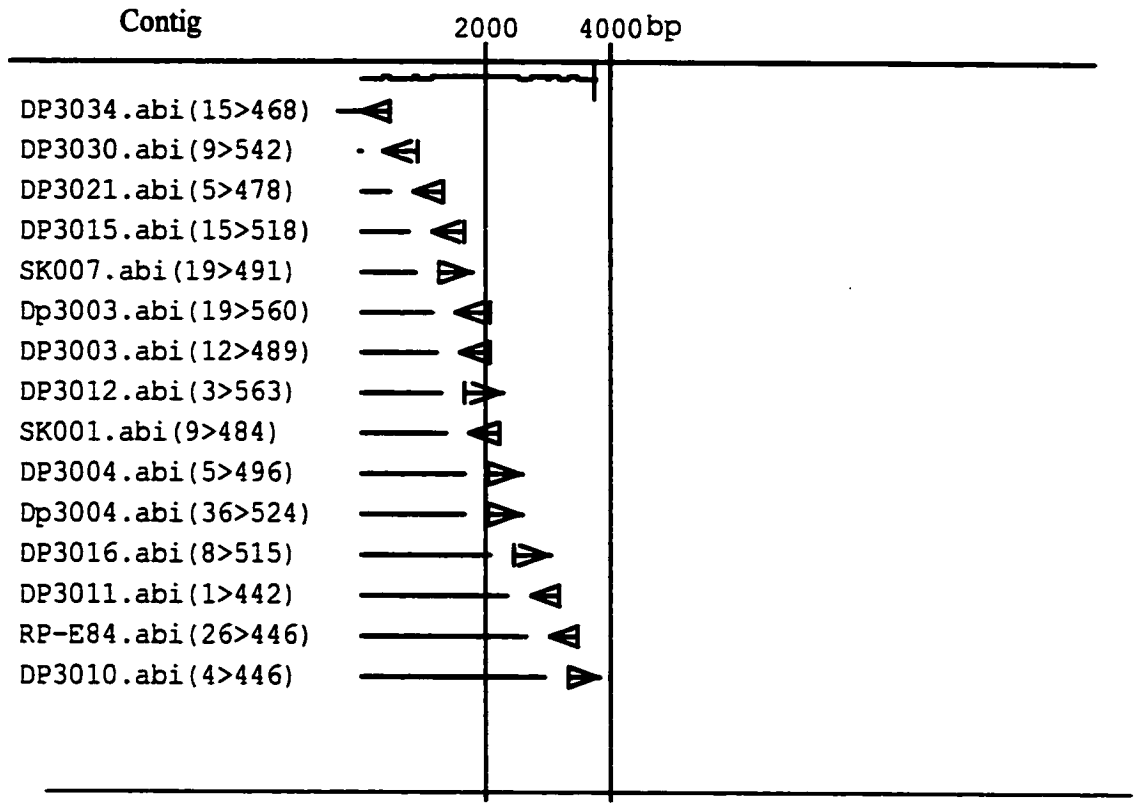
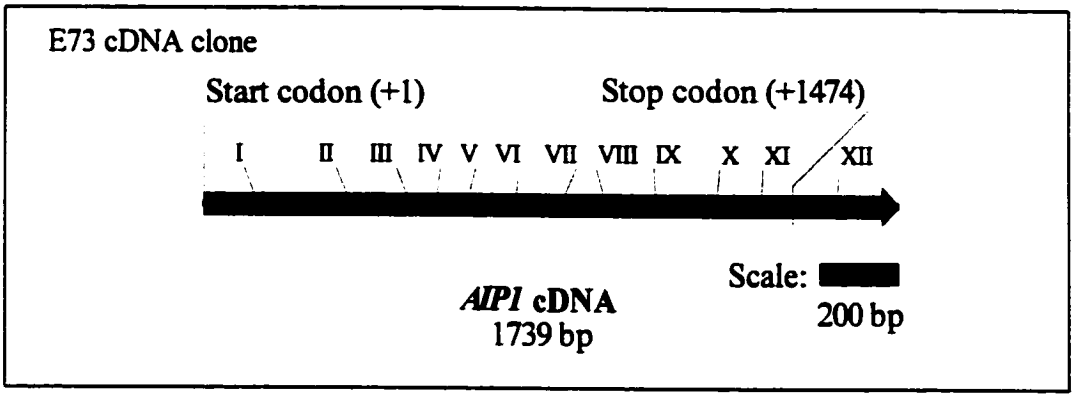


Figure 4.5. Continued...

(i)



(ii)

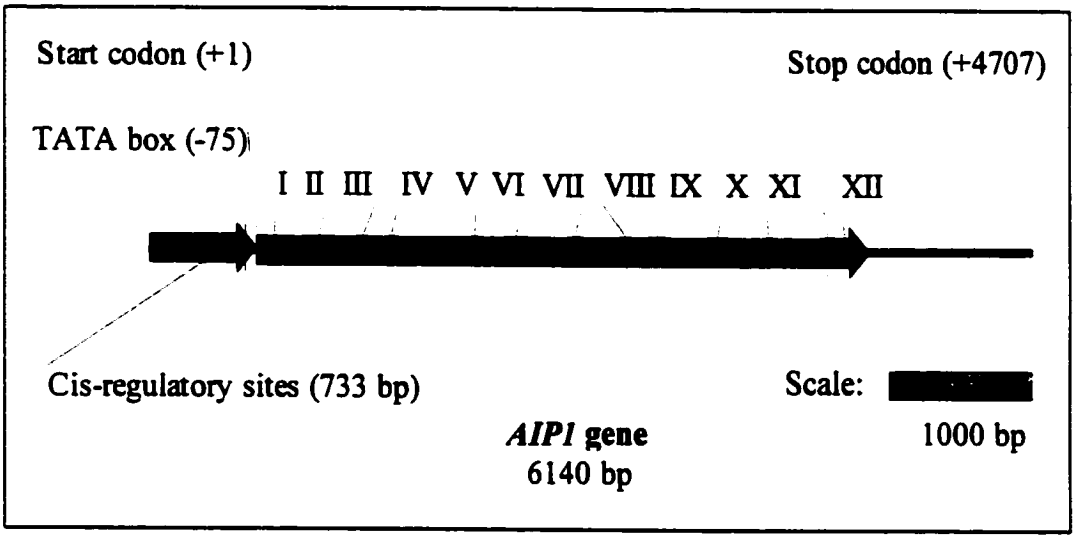


Figure 4.6. *AIP1* cDNA (i) and its chromosomal gene (ii). Bars with Roman numerals and of darker colour represent exons and introns, respectively.

and *AIP3* had twelve exons/eleven introns and thirteen exons/twelve introns, respectively (Figures 4.6 and 4.7). Their TATA boxes and start and stop codons also were predicted by manual/visual surveying of the sequences. As mentioned earlier (Section 4.1.1), the AIP3p exons were predicted to be longer than previously thought. Consequently, the chromosomal exons/introns were further analyzed to approximately 2.1 kb upstream using the available *A. thaliana* database (Figure 4.7). This most recent version of the *AIP3* gene predicted four additional exons and three introns, making a total of thirteen exons and twelve introns, respectively, which were corroborated using the exon/gene prediction tools described in Section 3.4.12. The *cis*-regulatory sites were thus predicted within a 585-bp domain (e.g. TATA box), and start and stop codons were re-inspected and confirmed (Figure 4.7).

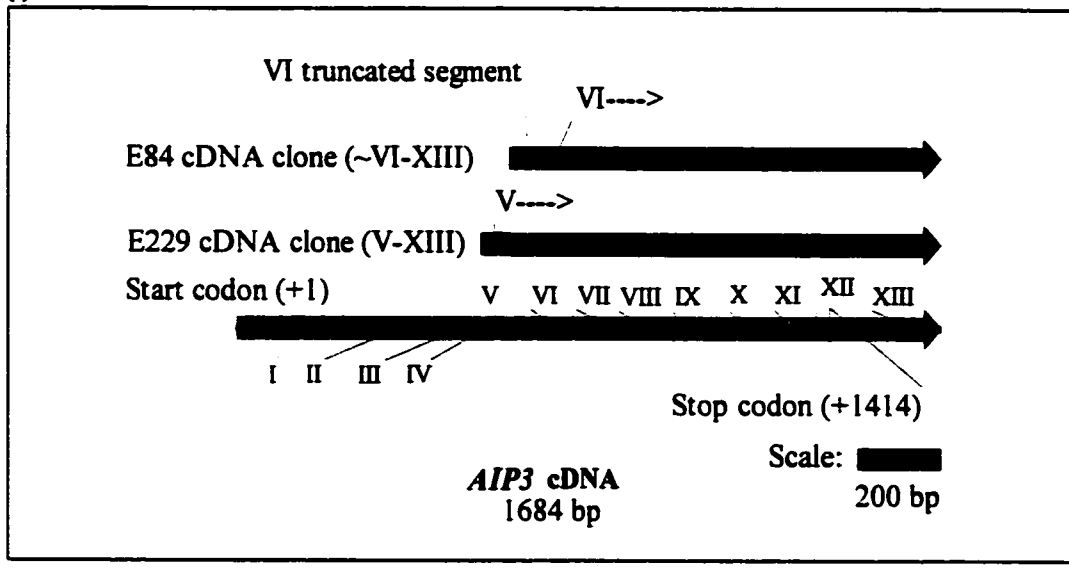
4.1.3 Expression and ectopic expression studies

4.1.3.1 Northern blotting and quantitative RT-PCR

Northern blotting experiments were performed to analyze the transcriptional abundance of mature *ALS*, *AIP1* and *AIP3* transcripts in total RNA isolated from roots, stems, leaves and flowers. The results showed that the abundance of these three transcripts was too low to be detected using standard Northern protocols. As an alternative, a quantitative RT-PCR technique was employed which used single stranded (ss) cDNA templates synthesized from total RNAs of flower, leaf, stem and root and reverse transcriptase (RT) with oligo(dT) as primer, as described in Section 3.4.8.1.

Quantitative RT-PCR was used to determine the presence and relative abundance of *ALS*, *AIP1* and *AIP3* transcripts in *A. thaliana* organs i.e. flower, leaf, stem and root

(i)



(ii)

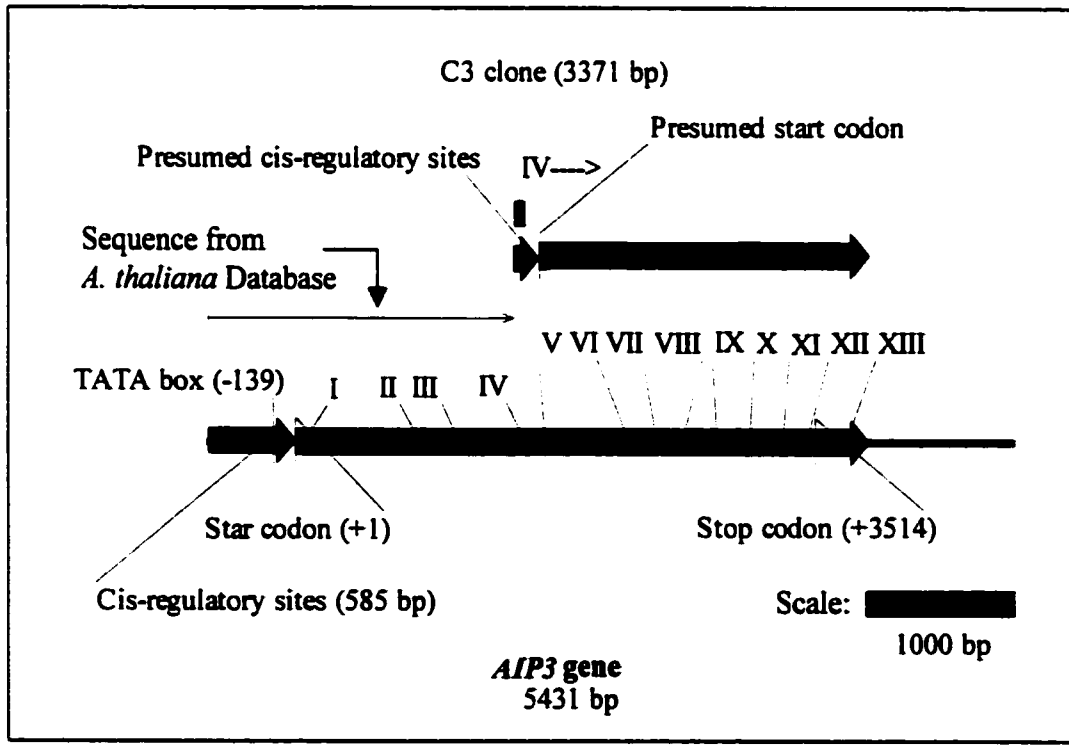
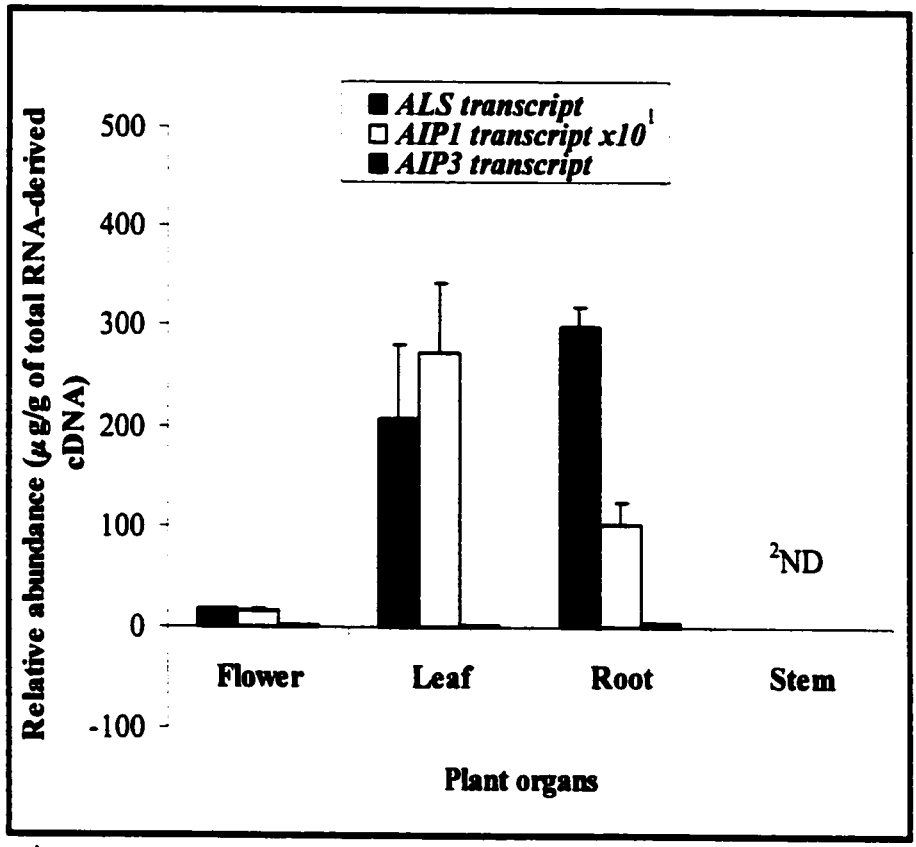


Figure 4.7. *AIP3* cDNA (i) and its chromosomal gene (ii). Bars with Roman numerals and of darker colour represent exons and introns, respectively.

from 40-43 d-old plants (Figure 4.8). All transcripts were present in various degrees of abundance in all *A. thaliana* organs, with the exception of the stem where transcripts were not detected. A relative abundance of 0.2 μg/g, which corresponded to the lowest detectable amount (1 fg) of external standard and was based on 5 ng of total RNA-derived cDNA as template, was considered to be the minimum detectable amount. In all organs, the *AIP1* transcript was markedly higher in abundance, with leaf displaying the highest abundance (2,721 μg/g of total RNA-derived cDNA). The *ALS* catalytic subunit transcript was higher in abundance in all organs relative to the *AIP3* transcript. Among organs with detectable transcripts, *ALS* and *AIP3* transcripts in root displayed the highest abundance at 299 and 4 μg/g of total RNA-derived cDNA, respectively. The *AIP1* transcript was in highest abundance in leaf. Flower displayed the lowest abundance for all transcripts examined; the *AIP3* transcript was present at 0.84 μg/g of total RNA-derived cDNA, the lowest level in flower.

4.1.3.2 Preliminary promoter analysis

Analyses of putative upstream *cis* regulatory regions (promoters) of the genes were performed using promoterless *GUS* fusion expression systems, as described in Section 3.5.2.1. The 5' putative promoter sequences spanning 0.75 and 2.1 kbp of the *AIP1* and *AIP3* genes, respectively, were PCR amplified with *Pfu* DNA polymerase using sense and antisense primers designed to incorporate a terminal 5' *Sal*I site for the *AIP1* gene and a terminal *Xba*I site for the *AIP3* gene. DNA templates for amplifying promoter segments were derived from the recombinant pSL1180-genomic *AIP1* DNA and *AIP3*-containing genomic DNA from *A. thaliana*. Unlike the *AIP1* putative



[1]The *AIP1* transcript relative abundance is tenfold of the actual scale
[2]ND= None detected (i.e. < 0.2 μg/g which corresponded to the minimum detectable amount of external standard)

Figure 4.8. Relative abundance of *ALS*, *AIP1* and *AIP3* transcripts in flower, leaf, stem and root of 40-43 d-old *A. thaliana*. Error bars represent standard errors (α= 0.1).

promoter domain, which arose from this work, the *AIP3* putative promoter domain was obtained through *BLAST* database searches since this work only defined a short segment (approximately 180 bp) of the required sequence. The database search revealed that the P1 clone AB005242 containing 82 kbp of *A. thaliana* genomic DNA and located on chromosome 5 contained the 2.1 kb *AIP3* upstream sequence (Figure 4.7). Accordingly, the design of *AIP3* primers was based on this sequence information. The amplified fragments were subcloned to a high copy number plasmid pSL1180 and subsequently to pBI101.1 using *E. coli* as the host. The recombinant DNAs in *E. coli* were purified and electroporated to *Agrobacterium* for the purpose of subsequent transformation to *A. thaliana* using the *Agrobacterium* flower-infiltration protocol described in Section 3.5.2.1.

The results of GUS activity staining assays of the transformant plants for preliminary promoter analysis of *AIP1* and *AIP3* are presented in Figures 4.9 and 4.10, respectively. Of ten transformants analyzed, nine carrying the *AIP1* promoter construct displayed various GUS activity staining patterns when compared to a transformant (control) expressing GUS activity driven by the *CaMV 35S* promoter. Interestingly, GUS activity in younger organs (e.g. leaf, flower) tended to be less intense than in older organs, although this observation was not apparent in all transformant lines. On the other hand, none of the ten transformants tested for *AIP3* promoter construct displayed any observable GUS activity; instead, they displayed a wild-type appearance in the staining assays (Figure 4.10).

4.1.3.3 Ectopic expression of AIP1p and AIP3p proteins

Over expression of AIP1p and AIP3p proteins in *A. thaliana* using the corresponding cDNAs (E73 and E84 cDNA clones, respectively) driven by the *CaMV 35S* promoter were performed as described in Section 3.5.2.2. Western blots detected the presence (or absence) of the expressed proteins, as shown in Figure 4.11. Ten independent AIP1p and AIP3p transgenic lines were analyzed using Western blotting techniques to determine qualitative levels of expression in each line. For AIP1p transgenic lines, all ten independent transformants survived under kanamycin (100 mg/L) selection, and approximately 250 mg of plant material (pooled from 22-25 d-old plants) was harvested from the selection media (1/2 MS plus kanamycin) and extracts subjected to Western blot analysis. For AIP3p transgenic lines, six of ten transformants survived under the same selection conditions and were subjected to the same Western blot analysis as used for AIP1p lines. The remaining transformed, selected plants were used for analysis of free amino acid content. The results of this analysis are presented in Section 4.4.

As shown in Figure 4.11, panels (a) and (b), ten and six transformant lines of AIP1p and AIP3p, respectively, displayed altered levels of AIP1p and AIP3p protein expression compared to the wild type. For AIP1p protein [panel (a)], lines 2, 5, 6, and 10 exhibited an increase in the level of expression, whereas line 8 exhibited a slight decrease and lines 3, 4, 7, 9 and 11 were devoid of AIP1p signal under the conditions used (approximately 16-20 μg total protein per lane). For AIP3p protein [panel (b)], lines 1, 4, and 6 exhibited a decrease in protein levels, whereas line 5 exhibited a marked reduction, line 7 expression was devoid of AIP3p signal, and line 9 was comparable to

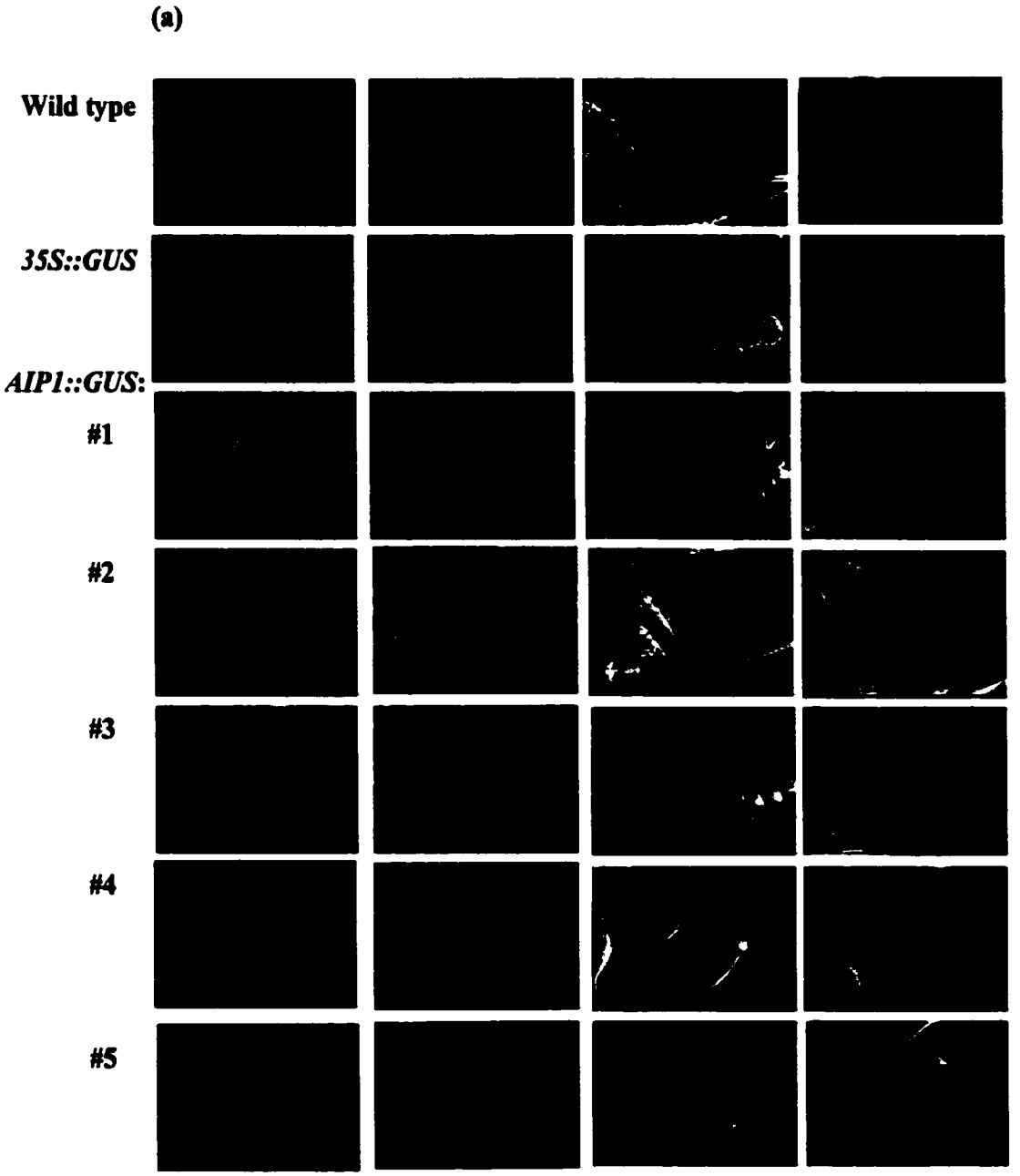


Figure 4.9. GUS expression profiles in *AIP1* promoter driven *GUS* transgenic lines #1-5 (a) and #6-10 (b) as compared to those in wild type and *CaMV 35S* strong promoter driven *GUS* line (*35S::GUS*).

(b)

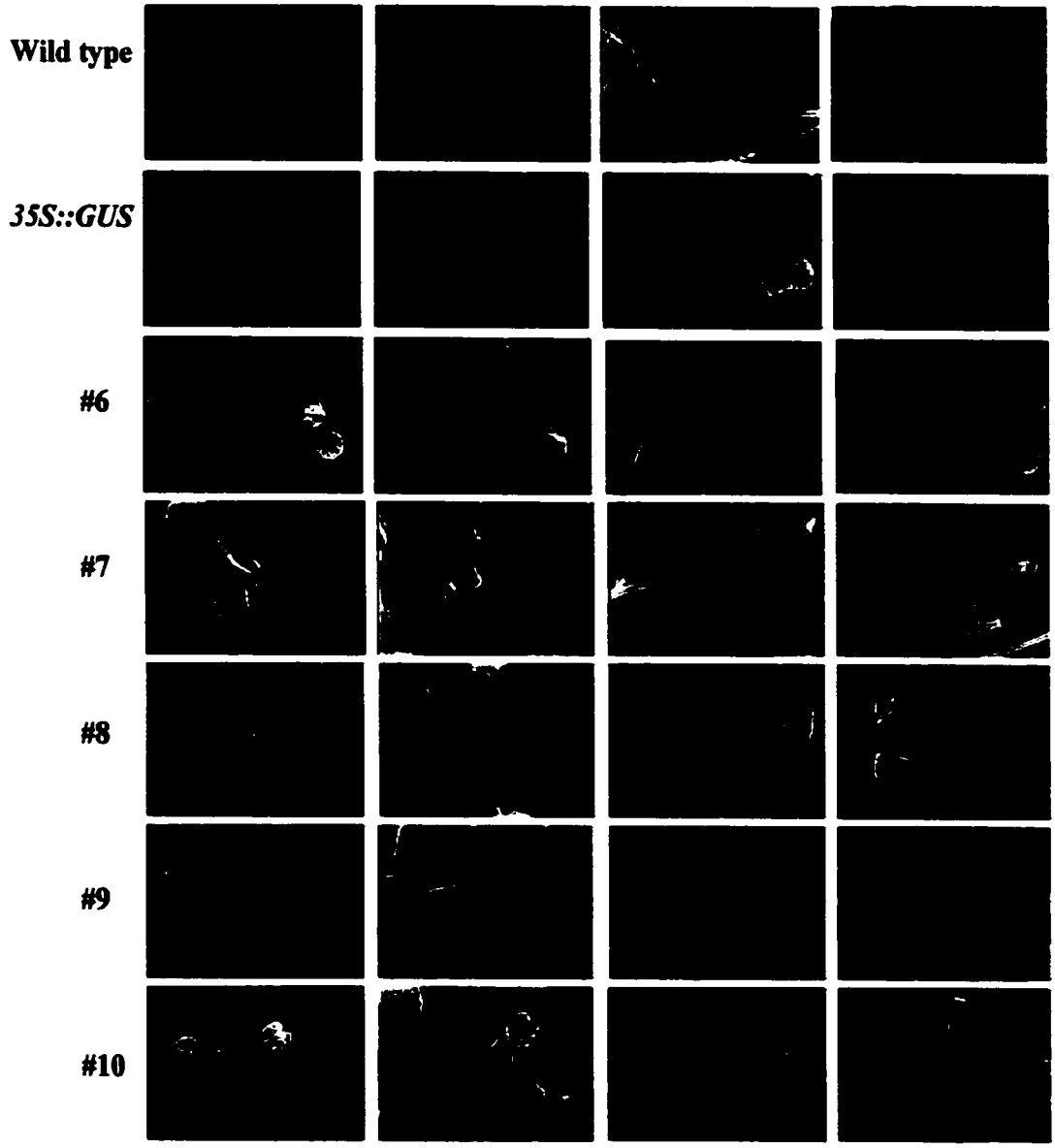


Figure 4.9. Continued...

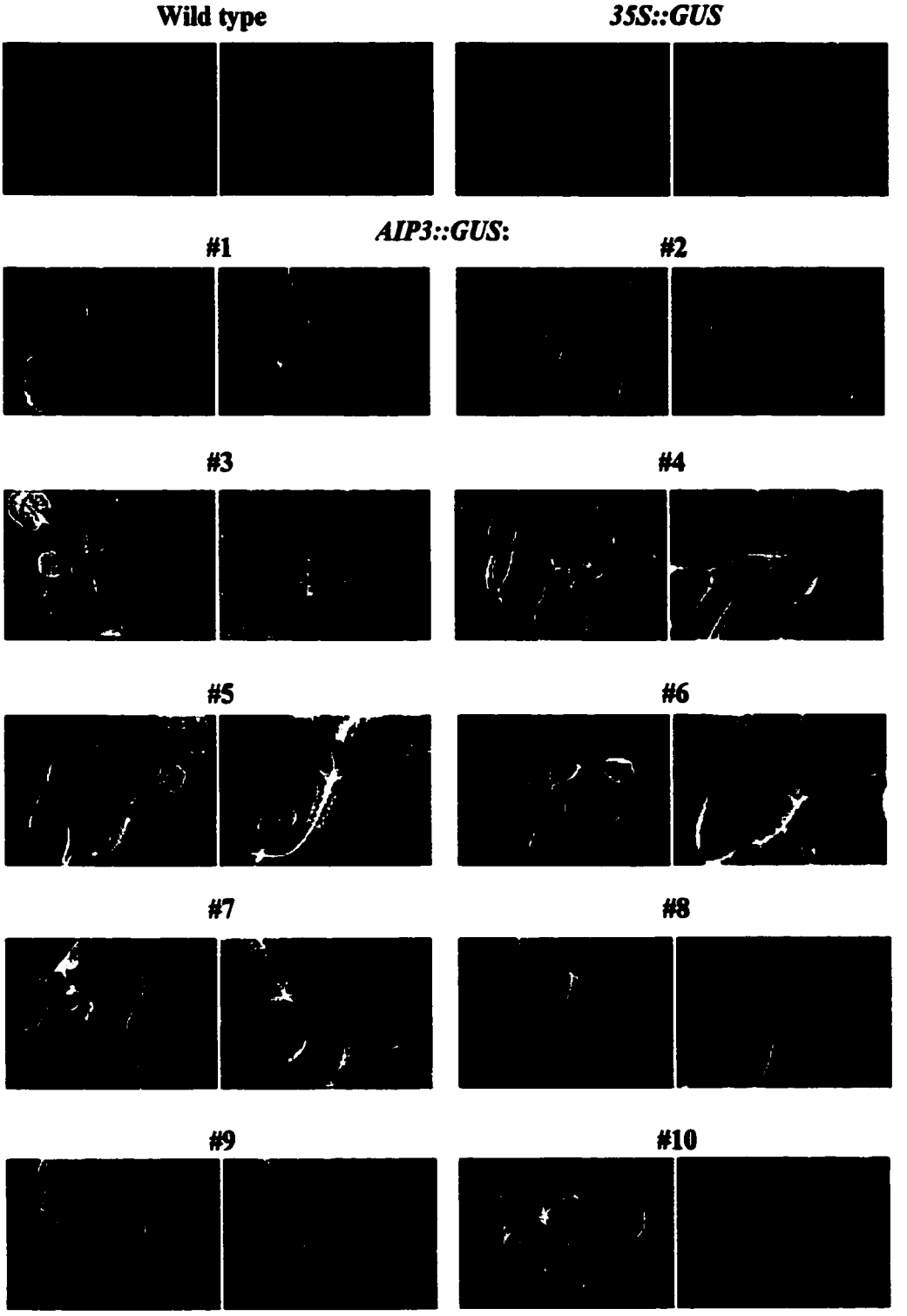


Figure 4.10. GUS expression profiles in *AIP3* promoter driven *GUS* transgenic lines #1-10 as compared to those in wild type and *CaMV 35S* strong promoter driven *GUS* line (*35S::GUS*).

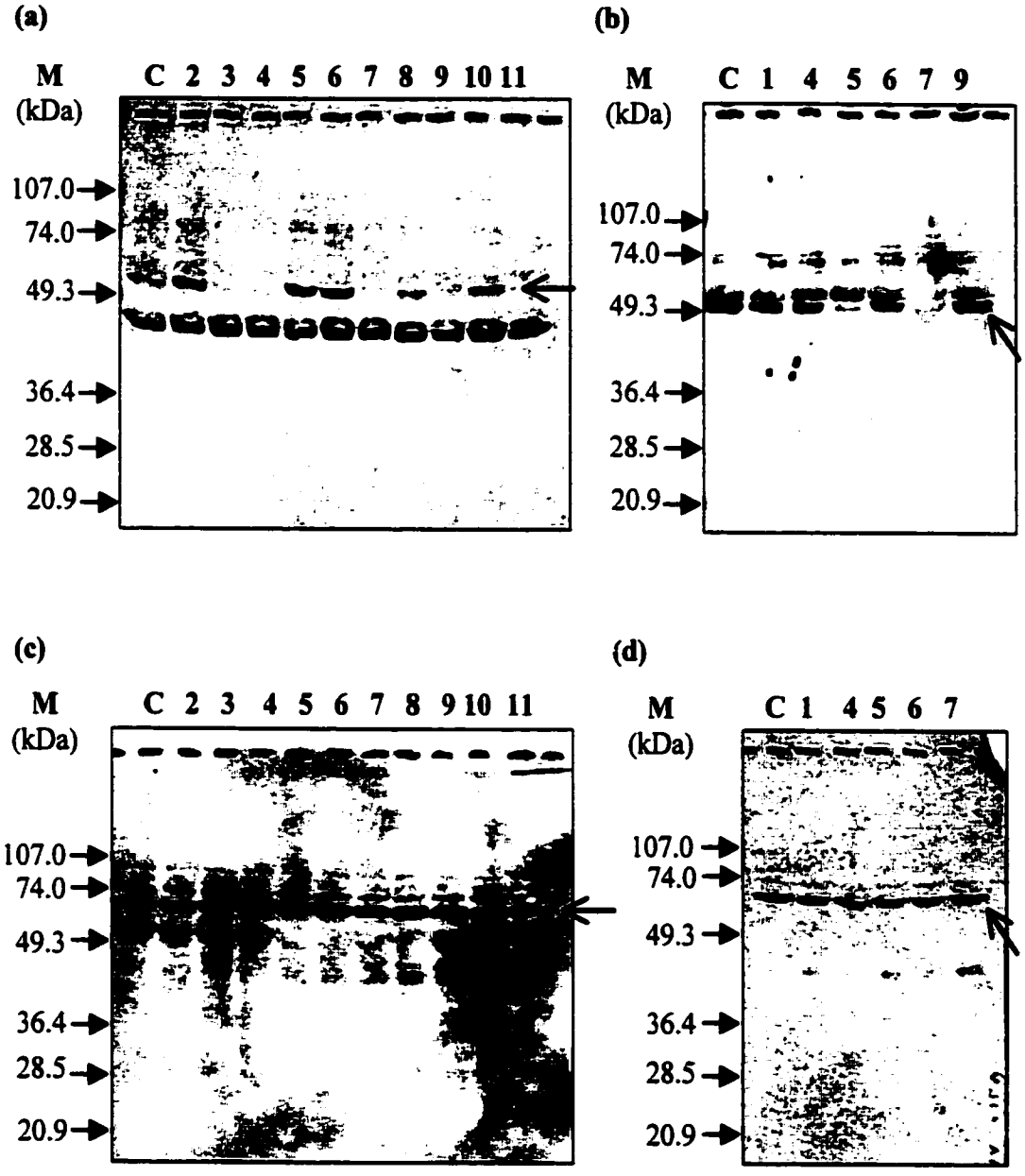


Figure 4.11. Effects of ectopic expression of *AIP1* and *AIP3* transgenes on the level of expression of AIP1p (a) and AIP3p (b), respectively, and ALS (c and d) as indicated each by an open arrow, which were detected in wild type (C) and transgenic lines of AIP1 (c) and AIP3 (d). M represents protein markers and their molecular masses.

the wild type. As shown in panels (c) and (d), ectopic expression of AIP1p and AIP3p proteins, respectively, did not affect the level of ALS expression in the corresponding transformant lines.

An interesting result was observed in that *AIP1* expression affected the level of AIP3p protein expression, and *vice versa*. The results, presented in Figure 4.12, panels (a) and (b), show that ectopic expression of *AIP1* and *AIP3* genes resulted in altered levels of AIP3p and AIP1p protein expression, respectively. In the case of ectopic expression of the *AIP1* gene [panel (a)], transformants 2, 3, 7 and 9 exhibited reductions in the level of AIP3p protein expression, whereas lines 5, 6 and 10 exhibited increases, and lines 4, 8 and 11 slight decreases or were comparable to the wild type. In the case of *AIP3* gene ectopic expression, transformants 1 and 4 exhibited reductions in the level of AIP1p protein expression, line 6 a slight increase, line 5 was devoid of signal, and lines 7 and 9 were comparable to the wild type.

4.2 Cross-reactivity studies of polyclonal antibodies and subcellular localization of ALS catalytic subunit, AIP1p and AIP3p proteins

4.2.1 Cross-reactivity studies of ALS, AIP1p and AIP3p polyclonal antibodies

Prior to their use in Western analysis and co-immunoprecipitation, αALS, αAIP1p and αAIP3p antibodies were subjected to cross-reactivity studies using *E. coli* recombinant His_6-tagged proteins of ALS, AIP1p and AIP3p as antigens. The results of these cross-reactivity studies are presented in Table 4.3. The αALS antibody (F14) reacted with its antigen and cross-reacted with AIP1p antigen following extended exposure times. The αAIP1p antibody was observed to react with its antigen and to cross-react with AIP3p at the same exposure time and amount, and with ALS at the

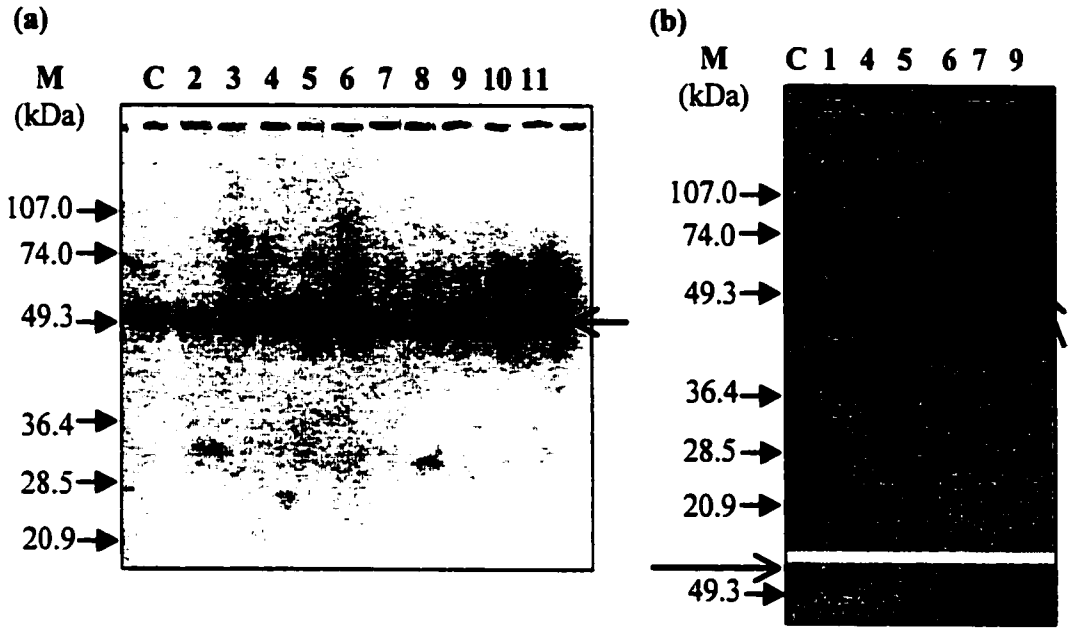


Figure 4.12. Expression profiles of *AIP1* and *AIP3* ectopic expression lines. Expression of AIP3p was detected in *AIP1* ectopic expression lines using αAIP3p as probe (a) and AIP1p expression in *AIP3* ectopic expression lines using αAIP1p as probe (b) as indicated each by a shorter open arrow. C represents wild type and the open arrow indicates AIP1p protein bands which were digitally-enhanced to clearly distinguish the level of expression. M represents protein markers and their molecular masses.

Table 4.3. Cross-reactivity experiment[1] involving polyclonal antibodies used in this research.

Antibody	Exposure time[2] (sec)	*His*$_6$-tagged protein		
		AIP1p	AIP3p	ALS
F14 (αALS)	5	(-)[3]	(-)	+[4] (50 ng)
	30	(-)	(-)	
	60	(-)	(-)	
	300	+ (50 ng)	(-)	
αAIP1p	5	+ (50 ng)	+ (50 ng)	+ (10 ng)
	30			
	60			
	300			
αAIP3p	5	(-)	+ (10 ng)	+ (10 ng)
	30	+ (50 ng)		
	60			
	300			
Secondary antibody only	5	(-)	(-)	(-)
	30	(-)	(-)	(-)
	60	(-)	(-)	(-)
	300	(-)	(-)	(-)

[1]Single determination
[2]X-ray film exposure after secondary antibody incubation
[3](-) = Not cross reactive
[4]+ = Cross reactive or reactive at the specified amount of the antigen

same exposure time, but with a lower amount of ALS. The αAIP3p antibody reacted with its antigen and cross reacted with ALS antigen to a similar extent at the same exposure time, and with AIP1p but to a greater extent over a longer exposure time. None of the antigens was detected when they were probed using only the secondary antibody, which was HRP-conjugated goat-anti-rabbit antibody against the heavy chain domain of rabbit antibody.

4.2.2 Subcellular localization of ALS, AIP1p and AIP3p

Based on a previous report that the ALS catalytic subunit in plants was localized in the chloroplast (Miflin, 1971), the organellar and sub-organellar localizations of ALS, AIP1p and AIP3p were analyzed. Samples from the fractionation scheme presented in Figure 3.1 were subjected to SDS-PAGE followed by Western blotting. The results are presented in Figure 4.13. Upon probing with polyclonal antibodies against ALS, AIP1p or AIP3p antigen, the ALS catalytic subunit was found to co-enrich in the chloroplast stromal fraction (fraction 5 in Figure 3.1) as did both AIP1p and AIP3p (Figure 4.13, lane 5, panels b and c), which were also enriched in the thylakoid membrane fraction (Figure 4.13, lane 6, panels b and c).

Since the stromal extract fraction was used for protein-protein interaction experiments, enzymatic activity assays were performed to confirm that the fraction used indeed corresponded to an enriched stromal extract. The stroma-specific enzyme, shikimate oxidoreductase (SOR), was used as the marker enzyme and the results are presented in Table 4.4. Total chlorophyll content was used as a thylakoid marker, and verified enrichment of the thylakoid fraction used in the localization study. As shown in

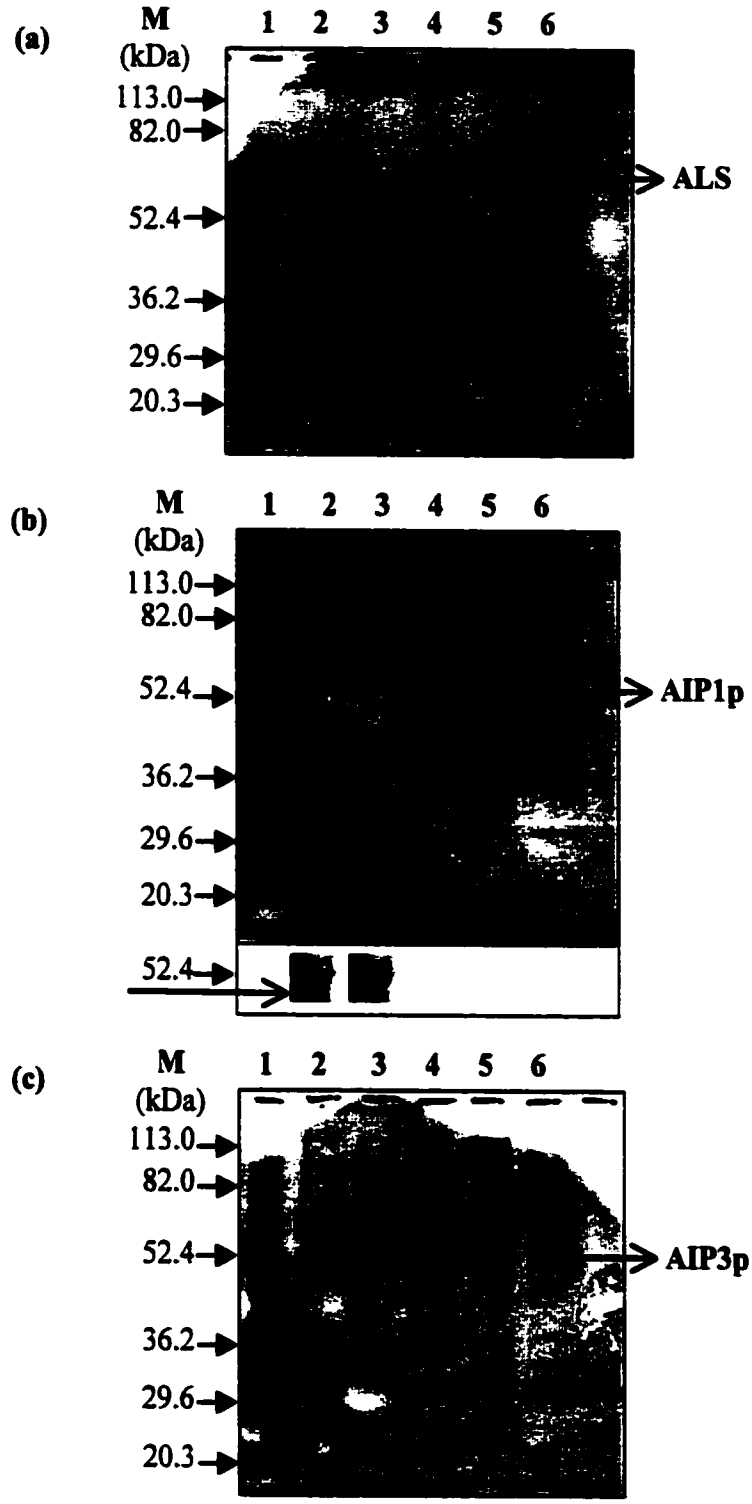


Figure 4.13. Western blots of wild type *A. thaliana* fractionated samples from the stromal extraction scheme (Figure 3.1) probed with αALS-IgG (a), αAIP1p (b) and αAIP3p (c). The longer open arrow indicates AIP1p protein bands of fractions 2 and 3 that were digitally-enhanced, and M represents protein markers and their molecular masses.

Table 4.4. Shikimate oxidoreductase activity and total chlorophyll content of fractions from the stromal preparation scheme.

#	Fractions from stromal preparation scheme	SOR activity assay	Total chorophyll content determination[1]
		Specific activity (SA) (nmol/min/mg protein)	Total Chlorophyll (mg)
1	Solids (cell debris/wall)	NA[2]	NA
2	Filtered homogenate	26.7 ± 3.3[3]	7.81
3	Supernatant	68.9 ± 1.6	2.46
4	Lysed chloroplast (Suspension)	ND[4]	NA
5	Stromal fraction	56.7 ± 2.7	0.12
6	Thylakoid fraction	ND	7.76

[1]Single determination
[2]NA= not analyzed
[3]Standard errors
[4]ND= none detected (the slope i.e. the initial rate) was zero or negative)

Figure 3.1, supernatant fraction 3, obtained after centrifugation of the filtered homogenate, displayed the highest SOR activity, followed by the stromal fraction. As predicted, the thylakoid fraction was enriched in chlorophyll content compared to the stromal fraction.

4.3 Protein-protein interaction experiments involving the ALS catalytic subunit and AIP1p and AIP3p proteins

4.3.1 Co-immunoprecipitation experiments

Co-immunoprecipitation experiments were performed as described in Section 3.6.2.1. These experiments were aimed at assessing the protein-protein interactions involving the ALS catalytic subunit and AIP1p and AIP3p proteins *in vivo*. As described previously in Section 3.7.2.1, each antibody (IgG form of αALS [F14], αAIP1 and αAIP3) was immobilized by cross-linking to protein A-sepharose beads and incubated with stromal extract fractions. From this type of experiment, it was expected that αALS beads would bind to the ALS catalytic subunit in stromal extracts. After washing and centrifugation, the bead contents were subjected to SDS-PAGE and Western analysis using αAIP1p or αAIP3p antibodies, where detection of either or both AIP1p and AIP3p would support the notion that ALS interacts with AIP1p or AIP3p protein, or both, *in vivo*.

No conclusive results with this type of experiment were obtained. The co-immunoprecipitation results showed that strong signals were observed in all cases at approximately 50-55 kDa. This was suspicious since all primary (1^{o}) antibodies bound antigens indiscriminately and were, therefore, concluded to be non-specific signals. To check this result, the same samples were subjected to SDS-PAGE and Western blot

analysis, but instead of incubating with both 1° and secondary (2°) antibodies, only 2° antibody (goat anti-rabbit IgG) was used. The results showed a pattern similar to that obtained previously. This indicated that the antibody heavy chain subunit (55 kDa) was being released from the cross-linked beads and subsequently recognized in Western blots by the goat anti-rabbit 2° antibody, hence masking the primary antibody signals.

4.3.2 *In vitro* coupled transcription/translation and binding assays

The *ALS*, *AIP1* and *AIP3* cDNAs were PCR-amplified from the two-hybrid 'prey' vector pBI-771. The purified PCR fragments were used in *in vitro* $^{[35]}$S-met-coupled transcription/translation using the TNT® Coupled Reticulocyte Lysate System (Promega, Madison, WI), as described in Section 3.7.1. The *ALS*, *AIP1* and *AIP3* cDNAs were successfully translated; for the *AIP3* cDNA, the longer (but not full-length) E229 cDNA clone was used.

The $^{[35]}$S-Met labeled ALS, AIP1p and AIP3p translated products were used in *in vitro* binding assays against several unlabeled *His$_6$*-tagged proteins i.e. AGAMOUS-LIKE (AGL)2p, AGL4p and AGL24p, ASK1p (*A. thaliana* Skp1) and *ll*-ASK1p (leaderless ASK1) immobilized on Ni^{2+}-NTA beads. Over several experiments the $^{[35]}$S-labeled ALS catalytic subunit was observed to non-specifically bind to all proteins (AGL2p, AGL4p, AGL24p, and ASK1p), making it impossible to confirm or refute the existence of a specific protein-protein interaction between ALS and AIP1p or AIP3p.

4.3.3 Generation of transgenic *A. thaliana* containing a His_6 domain at either the N- or C-terminus of the chlorsulfuron-resistant mutant of ALS (*AtALS-csr1-1*)

Based on the results of the *in vivo* co-immunoprecipitation experiments and *in vitro* binding assays, it was impossible to draw conclusions regarding the persistence of ALS - AIP protein interactions *in vivo*. Therefore, a different approach was taken where the chlorsulfuron (CS) resistant mutant allele, At*ALS-csr1-1* (Haughn and Somerville, 1986) was modified to incorporate a six-histidine (His_6) domain and used to generate transgenic plants.

The results of generating independent transgenic lines of *A. thaliana* expressing either N- or C-terminal His_6 fusions of the At*ALS-csr1-1* gene product are presented in Table 4.5. From the C24-ecotype root transformations, a total of 142 independent transformant plants (T1 plants) from a total of 208 calli which exhibited resistance to 100 nM CS grew and survived to maturity, whereupon their seeds (T2) were harvested. T2 seeds from forty-nine lines carrying both N- and C-terminal His_6 fusions were subsequently grown under 100 nM CS, where nineteen of them (i.e. two, twelve and five lines of the N2, C4 and C7 constructs, respectively, Table 4.5) showed a segregation pattern consistent with possible homozygosity for the transgene. T4 seeds from these putative T3 homozygous lines were harvested and again selected in the presence of CS. Eleven (i.e. two, six and three lines of the N2, C4 and C7 constructs, respectively) of nineteen lines (T5 plants) were confirmed to be homozygous as evidenced by 100% survival of progeny seedlings in the presence of 100 nM CS. Visual inspection of the eleven homozygous lines under the microscope revealed no unusual phenotypes for flower, leaf, stem or trichome. Six of the eleven lines (designated as DPN222-2,

Table 4.5. Numbers of putatively-transformed calli/plants, independent plants which survived to maturity and were harvested, and lines subjected to segregation analysis.

		His$_6$-tagged *ALS-csr1-1* construct				Total
	Control (WT[1])	[2]N1	[2]N2	[3]C4	[3]C7	
# of CS[4] resistant calli/plants	0	0	35	105	68	208
# of lines matures and harvested	-	-	26	65	51	142
# of lines subjected to segregation analysis	-	-	9	22	18	49

[1]Wild type
[2]N-terminal construct
[3]C-terminal construct
[4]Chlorsulfuron

DPC411-14, DPC412-5, DPC412-7, DPC711-4 and DPC712-3) were subjected to Western blot analysis using αALS as probe, where all showed different levels of ALS expression. These plants were subsequently used as a source of *His*$_6$-tagged ALS protein to test the interaction potential of AIP1p or AIP3p with ALS using IMAC and Western blot approaches. The transgenic line DPC712-3 consistently showed good ALS expression signals in Western blots and was chosen for use in subsequent interaction experiments. Interestingly, the DPC712-3 line was relatively taller, as shown in Figure 4.14, and earlier to flower than its WT control counterpart (C24-ecotype). However, as presented in Figure 4.15, ALS enzyme activity assays of stromal extracts prepared from wild type, At*ALS-csr1-1* and DPC712-3 lines showed no significant differences, since all lines exhibited approximately 17 nmol/min/mg protein of enzyme specific activity.

4.3.3.1 Effects of inhibitory treatments

The effects of chlorsulfuron and BCAAs on ALS activity in the DPC712-3 line, as compared to the wild type and the *csr1-1* mutant line, are presented in Figure 4.16. Acetolactate synthase enriched from stromal extracts of the DPC712-3 line exhibited reduced inhibition in the presence of 50 and 100 nM CS compared to that from the wild type, but greater sensitivity than ALS from stromal extracts of the *csr1-1* mutant line. In all cases, the 100 nM CS treatment was more inhibitory of ALS activity than the 50 nM CS treatment. The 100 nM CS treatment almost completely inhibited the wild type ALS activity, with approximately 12% of activity remaining under the conditions used; DPC712-3 transgenic and *csr1-1* mutant lines were inhibited by approximately 68 and 30%, respectively.

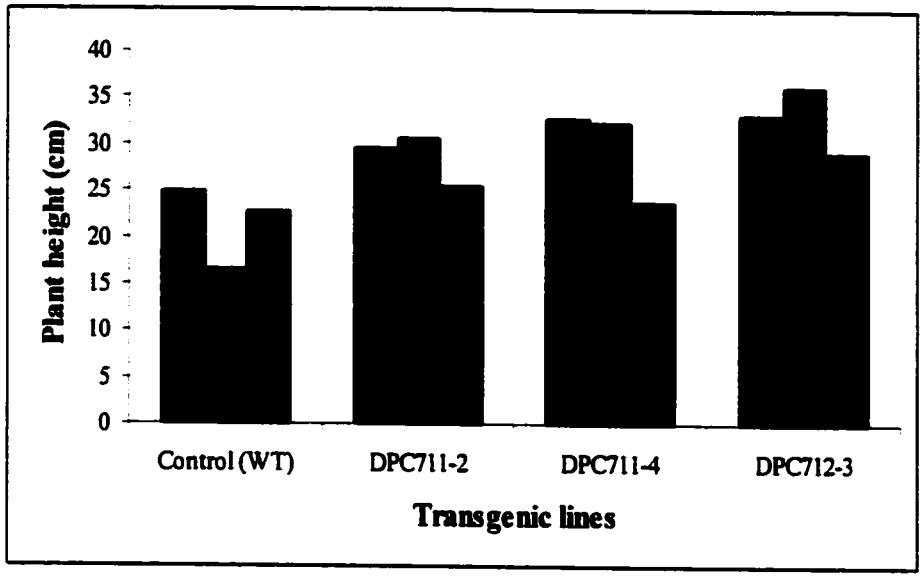


Figure 4.14. Relative plant heights among 34-36 d-old plants of control (wild type) plants and several transgenic lines. Small "plantlets" in ½ MS medium plates were transferred to soil and grown in a growth chamber at 22°C under a 16/8-h light/dark cycle. Each bar represents the height of a single plant.

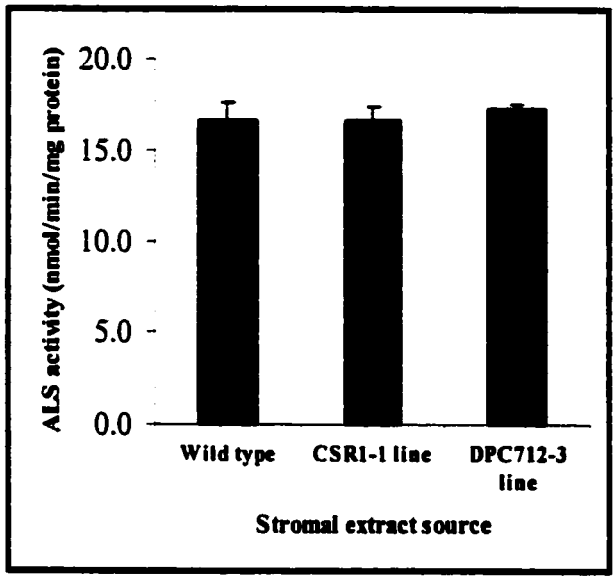


Figure 4.15. ALS enzyme activity of stromal extracts derived from wild type, *csr1-1* and DPC712-3 lines. Error bars represent standard errors.

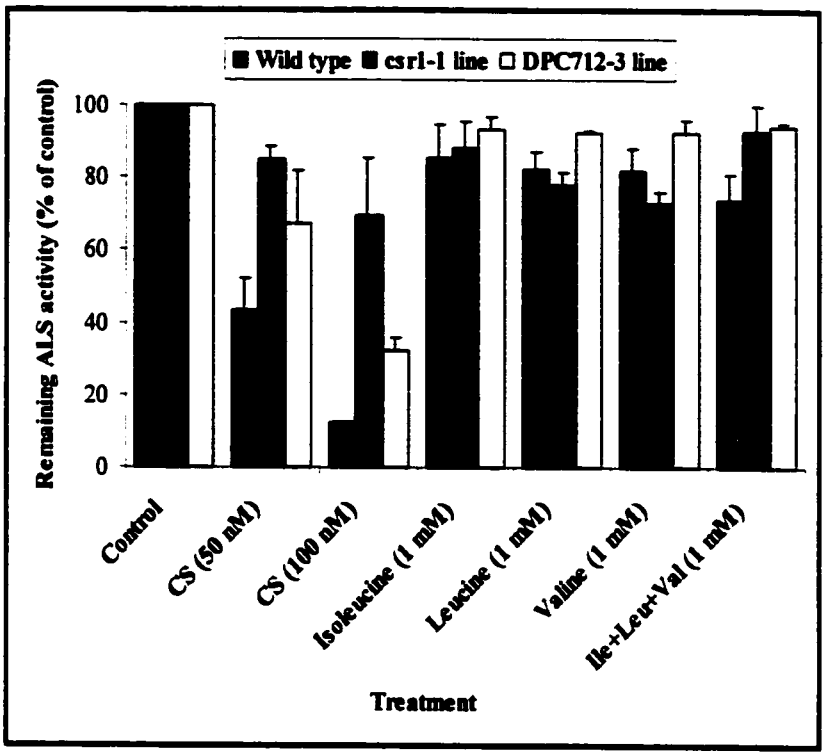


Figure 4.16. Effect of incorporation of chlorsulfuron (CS) or BCAAs in assays of ALS enzyme activity in stromal extracts prepared from wild type, *csr1-1* mutant and DPC712-3 transgenic lines. Error bars represent standard errors.

When 1 mM isoleucine was used as inhibitor, ALS from the wild type, the DPC712-3 transgenic and *csr1-1* mutant lines exhibited similar responses with 85-93% of ALS activity remaining, as shown in Figure 4.16. However, they differed noticeably when 1 mM leucine or valine was used as inhibitor. The ALS from the DPC712-3 line was less sensitive to leucine and valine than that from the wild type, which in turn was less sensitive than that from the *csr1-1* mutant line. When these three BCAAs (1 mM each) were combined and used for inhibition treatments, ALS from the DPC712-3 and *csr1-1* lines exhibited similar responses, but were markedly less sensitive than the wild type. Treatments with valine and BCAA combinations were most effective at inhibiting *csr1-1* and wild type ALS, respectively. On the other hand, each BCCA and their combinations were comparable in inhibiting DPC712-3 ALS activity. The ALS activity from the DPC712-3 line was more resistant to valine and the BCAA combinations than those from the wild type and *csr1-1* lines. The BCAAs were comparable in inhibiting wild type ALS. Isoleucine and BCAA combinations were comparable and least effective in inhibiting CSR1-1 ALS.

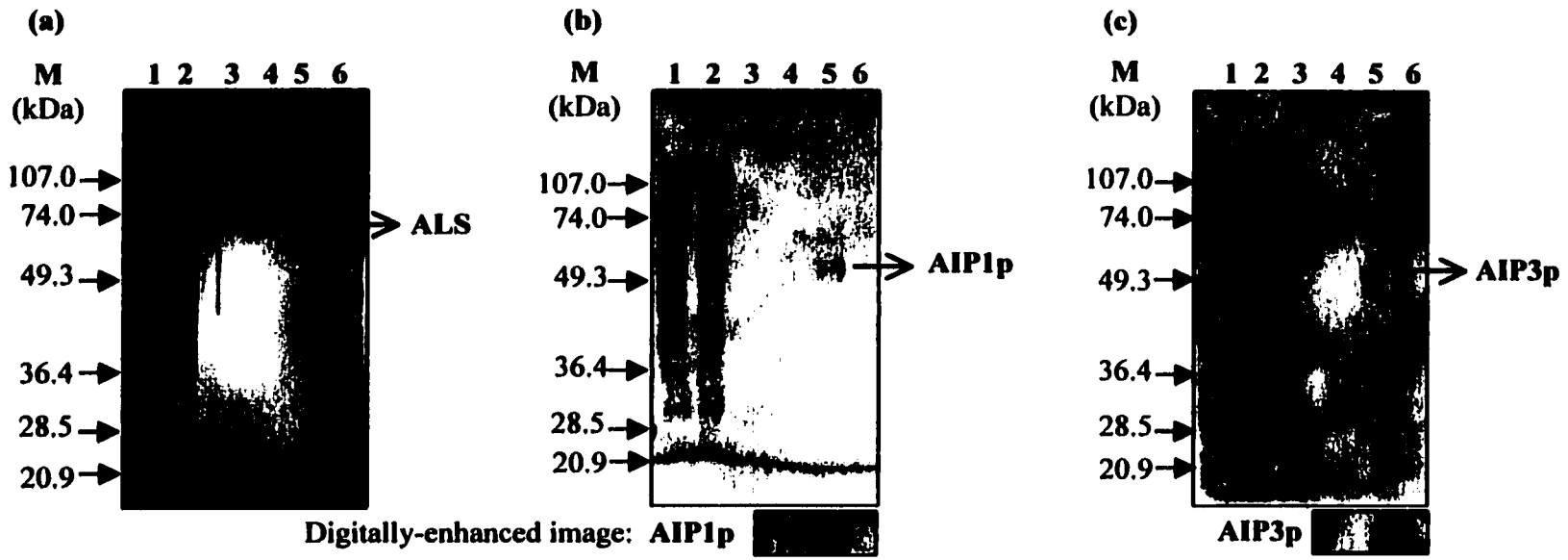
4.3.4 *In vivo* interaction experiments involving the genetically-modified (*gm*) ALS catalytic subunit, AIP1p and AIP3p

Initially, *in vivo* protein-protein interaction experiments were performed using IMAC Ni^{2+} batch chromatography, since the co-immunoprecipitation experiments failed to show the co-precipitation of ALS, AIP1p and AIP3p due to interference by the 1° antibody heavy chain. In these experiments, it was expected that His_6-tagged ALS would be immobilized to the nickel(II) agarose-bead matrix via coordination binding between its six consecutive histidine molecules and the Ni^{2+} ligand. After incubation,

washing, and Western blotting, AIP1p and AIP3p which lacked the *His*$_6$ domain also were assessed for their ability to bind to the beads in a *His*$_6$-tagged ALS-dependent manner.

In summary, these experiments failed to ameliorate the problem of non-specific binding by wild type ALS to Ni^{2+} beads. Stromal extracts from the wild type and DPC712-3 lines were incubated using binding buffers of pH 7.0, 7.5 or 8.0 or incorporation of imidazole at varying concentrations (0, 5, 15, 35 and 55 mM) in the washing buffer, or combinations of both. In other attempts, incorporation of imidazole (15 mM) in the binding buffer or the use of Ni^{2+} agarose- or silica-bead columns and a blocking agent (BSA) was done. Additional attempts were made, including interaction experiments followed by crosslinking using MMPP (magnesium monoperoxyphthalate hexahydrate) as the crosslinking agent, or incorporation of salt or ethylene glycol in the binding and/or washing buffer. In some of these attempts, elutions at low or high pH were also undertaken.

In general, the results suggested that ALS, AIP1p or AIP3p in the wild type stromal extracts bound non-specifically to the agarose or silica bead matrix despite the fact that none of these proteins contained a *His*$_6$ domain. Hence, it was difficult to rule out the possibility that all of these proteins bound non-specifically to the Ni^{2+} ligand. A report by Kasher *et al.* (1993) indicated that nickel IMAC beads exhibited a higher tendency for non-specific binding with non-His tagged proteins than did other divalent metal ions (e.g. Co^{2+}). Accordingly, the use of Co^{2+} instead of Ni^{2+} affinity batch co-purification was investigated. The experiment was conducted in the same fashion as the previous nickel affinity chromatography experiments, except that a binding buffer of pH 8.0, rigorous washings of 2 x 10 min, and elution of samples with 50-mM imidazole



Lanes:

Stromal extract samples: 1= Wild type (C24); 2= Transgenic (DPC712-3)

Eluted samples: 3= C24 - regular washings; 4= C24 - stringent washings (addition of 10 mM imidazole); 5= DPC712-3 - regular washings; 6= DPC712-3 - stringent washings (addition of 10 mM imidazole)

Figure 4.17. Western blots of stromal extract samples or eluted samples from the interaction experiment using Co^{2+} agarose beads. Panels (a), (b) and (c) represent Western blots of the same sample probed with αALS-IgG (F14), αAIP1p and αAIP3p, respectively.

were employed. Results of Western blot analysis of samples eluted from Co^{2+} agarose beads after incubation and washing are presented in Figure 4.17. Acetolactate synthase, AIP1p and AIP3p in the control (wild type) samples were not detected in the immunoblots, as shown in lane 3 of panels a, b and c, respectively. On the other hand, *His*$_6$-tagged ALS, AIP1p and AIP3p were detected exclusively in the transgenic samples when probed with their corresponding antibodies, as shown in lane 5 of panels a, b and c, respectively. Stringent washings resulted in no proteins being detected, as shown in lanes 4 and 6 of all panels. Since wild type ALS, AIP1p or AIP3p was not present in the control samples, this indicated that these proteins did not bind directly to either the Co^{2+} ligand or the agarose-bead matrix. It was concluded that the detection of AIP1p and AIP3p proteins together with *His*$_6$-tagged *gm*ALS in the same eluted sample from the interaction experiment was due to a direct or indirect interaction of AIP1p and AIP3p with the *His*$_6$-tagged ALS. At least two experiments were conducted using different sources of plant material, and the results obtained were consistent with this finding.

4.4 Effects of ectopic *AIP1* and *AIP3* expression

The *AIP1* and *AIP3* cDNAs were fused with *CaMV 35S* strong promoter in a binary vector pRD400 and used to transform *A. thaliana* wild type (Columbia) plants, as described in Section 3.5.2.2. The protein expression profiles of AIP1p and AIP3p were assessed using Western blot analysis; the results are presented in Section 4.1.3.3. In addition, experiments were conducted to study the effect of *AIP1* and *AIP3* ectopic expression on general amino acid metabolism by assessing free (non-protein) amino acid profiles; the results are presented in Figures 4.18, 4.19 and 4.20. Fifteen free amino

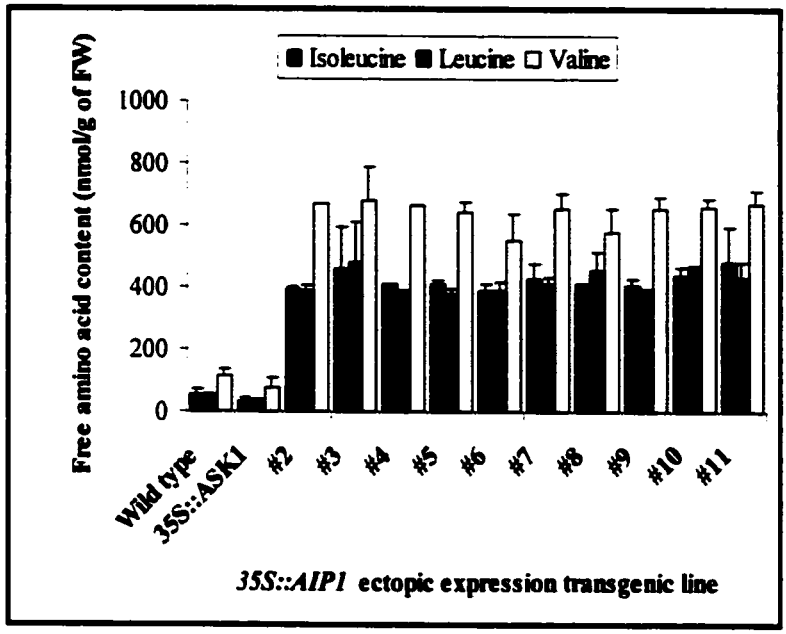
acids were analyzed in wild type and transgenic lines, plus another control line transgenic for a *CaMV 35S::ASK1* construct.

The effect of *AIP1* and *AIP3* ectopic expression on isoleucine, leucine and valine levels (the BCAAs) is presented in Figure 4.18. All *AIP1* and *AIP3* ectopic expression lines exhibited marked changes in their BCAA content, with 5-8 and 7-13 fold increases over the wild type and the ectopically-expressed ASK1p line, respectively. In the wild type, free valine content was the highest relative to other BCAAs, and proportionally increased in all ectopically-expressed lines of *AIP1* and *AIP3*. The levels of both isoleucine and leucine in the ectopically-expressed AIP1p and AIP3p lines examined were also increased, although the increase was not proportional to their levels in wild type i.e. the isoleucine in wild type was higher than leucine in some lines; however, this was not seen in other ectopic expression lines of *AIP1* and *AIP3* where the isoleucine content was the same or lower than leucine.

As shown in Figure 4.19, ectopically-expressed AIP1p lines 2, 4, 5 and 7 and ectopically-expressed AIP3p lines 4 and 6 exhibited levels of methionine comparable to those in the wild type, whereas the remaining lines exhibited higher levels of methionine. All ectopically-expressed AIP1p and AIP3p lines exhibited markedly higher levels of phenylalanine, histidine, lysine, arginine and threonine than did the wild type and the ectopically-expressed ASK1p line. Lysine and arginine in the ectopically-expressed ASK1p line were not detected (the minimum detectable amounts of lysine and arginine were approximately 55 and 185 nmol/g, respectively).

As shown in Figure 4.20, ectopically-expressed AIP1p lines 4 and 8 and lines 1, 7 and 8 exhibited comparable levels of proline and glycine, respectively, to the wild type. Ectopically expressed AIP1p lines 9, 10 and 11 and lines 2, 7 and 8

(a)



(b)

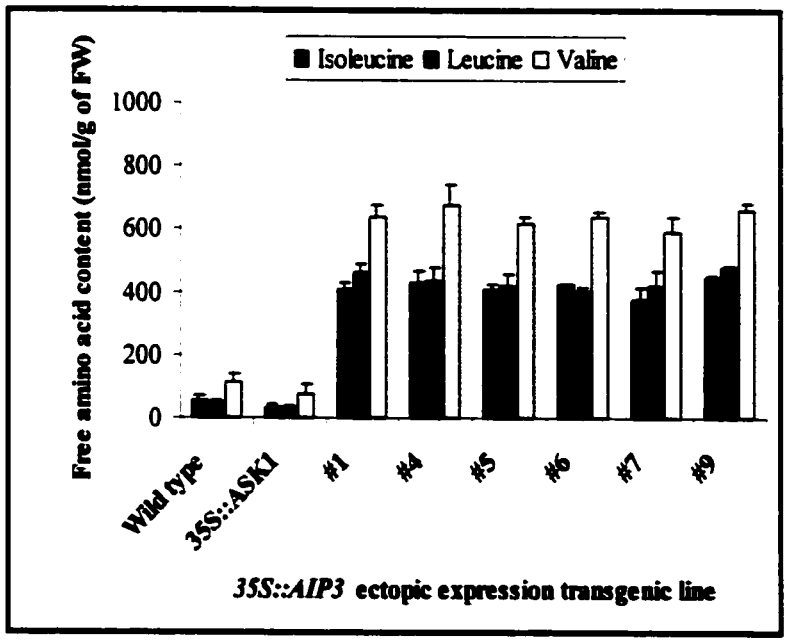


Figure 4.18. The effect of ectopic expression of *AIP1* (a) and *AIP3* (b) transgenes on free amino acid content (BCAAs, isoleucine, leucine and valine). Error bars represent standard errors.

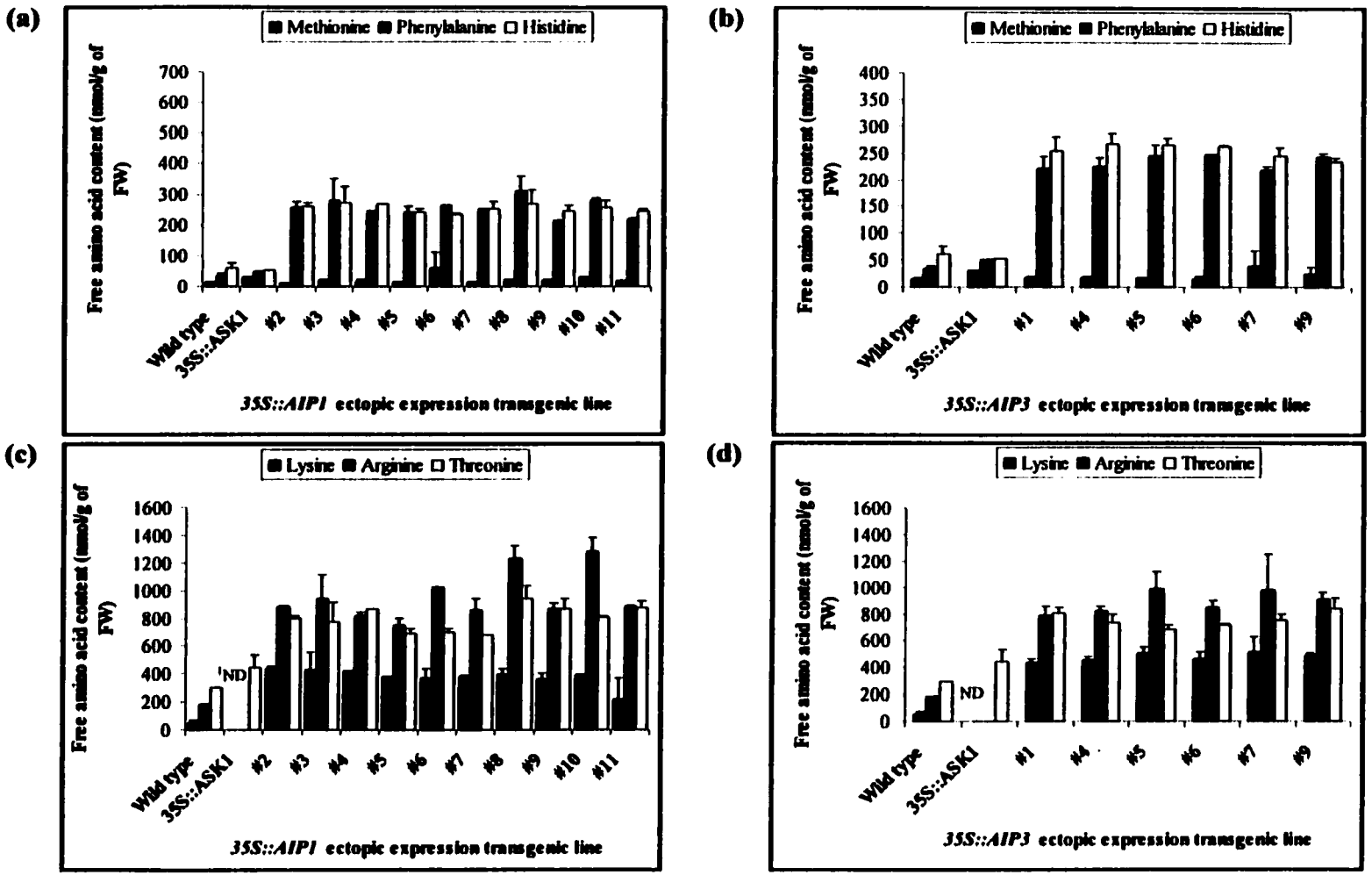


Figure 4.19. The effect of ectopic expression of *AIP1* (a, c) and *AIP3* (b, d) transgenes on the content of methionine, phenylalanine, histidine, lysine, arginine and threonine. [1]ND= None detected (i.e. lysine and arginine in the *35S::ASK1* line). Error bars represent standard errors.

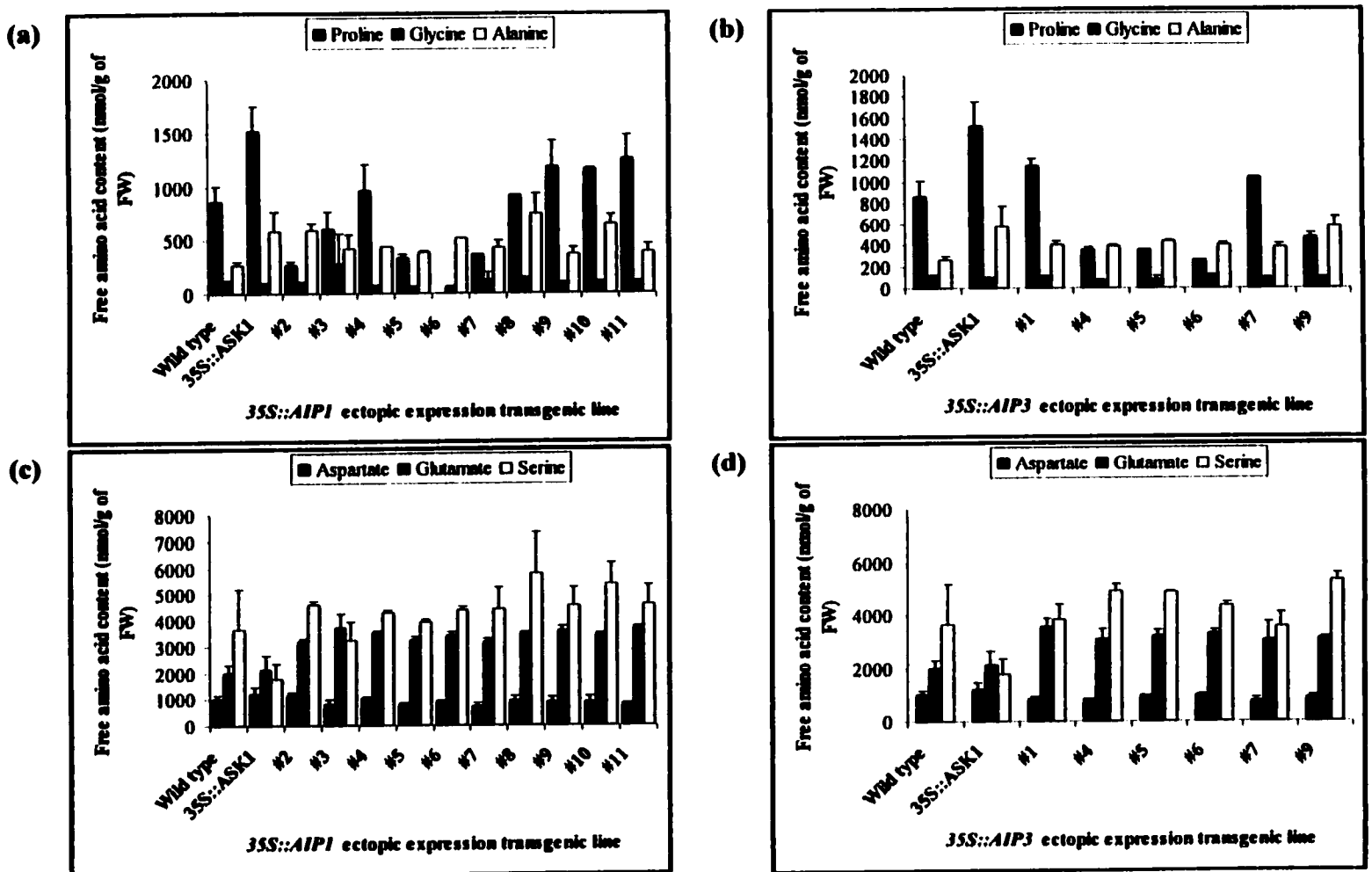
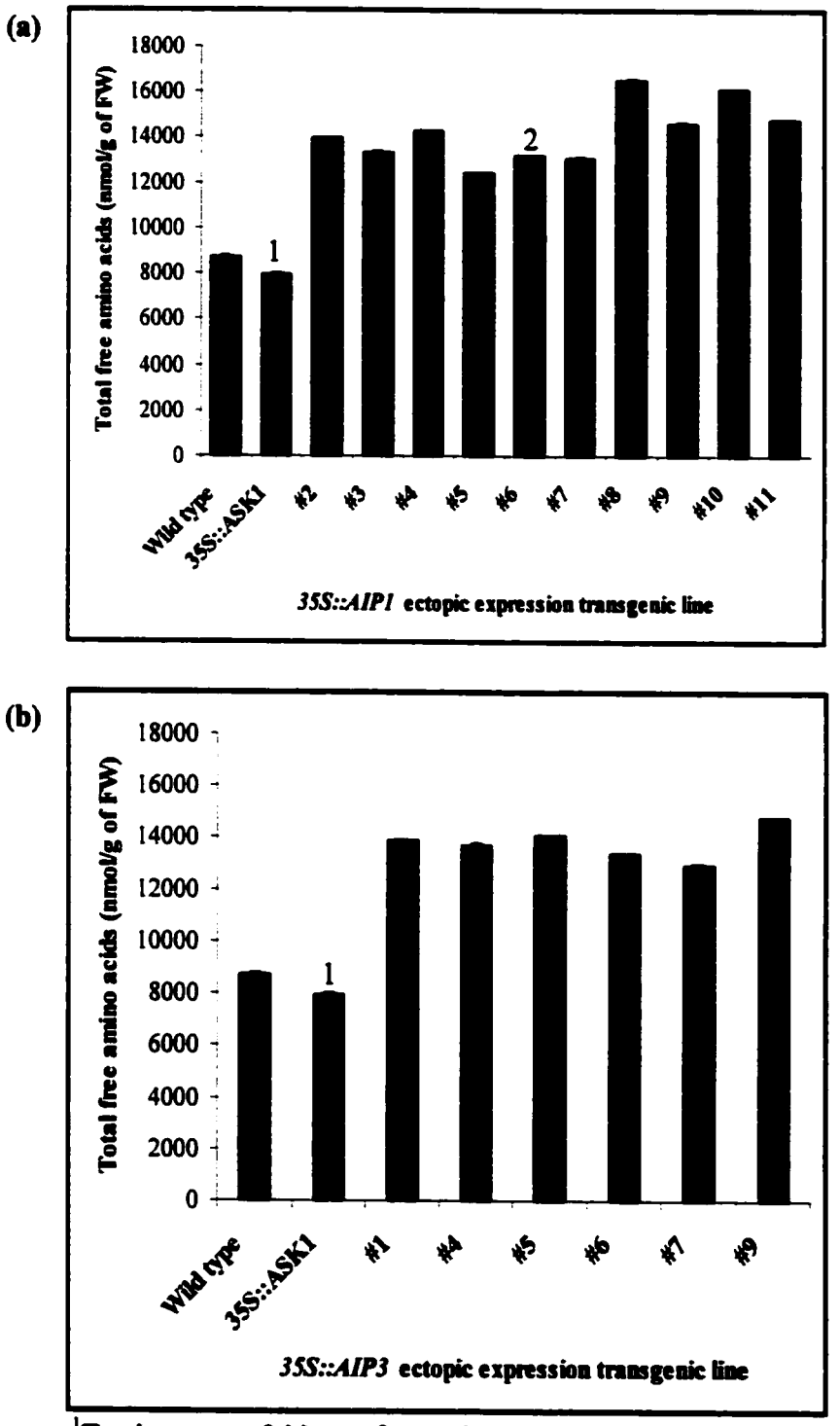


Figure 4.20. The effect of ectopic expression of *AIP1* (a, c) and *AIP3* (b, d) transgenes on the content of proline, glycine, alanine, aspartate, glutamate, serine. Proline was not detected in the *AIP1* #6 line. Error bars represent standard errors.

exhibited higher levels of proline and glycine, respectively, and the remaining lines exhibited lower levels of proline and glycine than did the wild type. Ectopically expressed AIP3p lines 4, 5, 6 and 9 and lines 4, 5, 7 and 9 exhibited lower levels of proline and glycine, respectively, than did the wild type, whereas ectopically-expressed AIP3p lines 1 and 7 and lines 1 and 6 exhibited higher levels of proline and comparable levels of glycine, respectively. Proline was not detected in the ectopically-expressed AIP1p line 6 (the minimum detectable amount of proline was approximately 265 nmol/g). Ectopically expressed AIP1p lines 2, 4, 8, 9 and 10 and lines 2, 3, 4 5, 6, 7, 9 and 11 exhibited comparable levels of aspartate and serine, respectively, whereas ectopically-expressed AIP1p lines 3, 5, 6, 7 and 11 and lines 8 and 10 exhibited lower levels of aspartate and higher levels of serine, respectively, than did the wild type. All ectopically-expressed AIP3p lines and lines 1, 4, 5 and 6 of AIP1p exhibited lower levels of aspartate than did the wild type and comparable levels of serine, respectively, whereas ectopically-expressed AIP3p lines 7 and 9 exhibited lower and higher levels of serine, respectively, than did the wild type. All ectopically-expressed AIP1p and AIP3p lines exhibited higher levels of alanine and glutamate than did the wild type.

In terms of amino acid content in each transgenic line, the ectopically-expressed AIP1p line 8 exhibited higher levels of the amino acids analyzed than did the wild type, with the exception of proline, glycine and aspartate that were comparable to that of the wild type. The ectopically-expressed AIP3p line 1 exhibited higher levels of the amino acids analyzed than did the wild type, with the exception that glycine and serine, and aspartate were, respectively, comparable to and lower than that of the wild type.

As shown in Figure 4.21, all ectopically-expressed AIP1p and AIP3p lines exhibited markedly-higher levels of the fifteen free amino acids analyzed than did the wild type and the ectopically-expressed ASK1p.



[1]Total content of thirteen free amino acids
[2]Total content of fourteen free amino acids

Figure 4.21. The effect of ectopic expression of *AIP1* (a) and *AIP3* (b) transgenes on the total content of fifteen free amino acids. Error bars represent standard errors.

5. DISCUSSION

The results presented in the previous sections provide new perspectives on the molecular biology and cell biology of the ALS catalytic subunit (ALS), ALS-interacting proteins 1 and 3 (AIP1p and AIP3p), and their association *in vivo* leading to the regulation of BCAA metabolism in *A. thaliana*. Both *AIP1* and *AIP3* were characterized at the level of their genes and gene products, where they exhibited both similarities and differences *vis-à-vis* their prokaryotic orthologues with respect to size, chromosomal location, sub-cellular localization and primary sequence homology. Their association with the ALS catalytic subunit was also investigated at the biochemical level. Lastly, the results presented here provide insight into the role of AIP1p and AIP3p in the regulation of BCAA metabolism as indicated by *in vivo* protein-protein interaction experiments, as well as ectopic expression/co-suppression studies involving both genes.

5.1 Acetolactate synthase, *AIP1* and *AIP3* genes and deduced protein structure

Of the ten classes of ALS-interacting proteins (AIPs) previously identified using the yeast two-hybrid system, and partly characterized by Kohalmi *et al.* (unpublished), AIP1p and AIP3p were selected for further study based on the fact that these two proteins exhibited a deduced primary sequence similarity to bacterial ALS regulatory subunits (Figures 4.2 and 4.3). The interaction of these two proteins with the ALS catalytic subunit was confirmed in this study. However, it may be premature to

conclude that AIP1 and AIP3 are functionally redundant as duplicate copies of the same gene, since they exhibit distinct characteristics in terms of their molecular size, chromosomal location (Schnaider *et al.*, unpublished), sub-cellular localization and primary sequence, despite a notable homology of approximately 74 and 80% at the nucleotide and deduced amino acid levels, respectively. In addition, differences in the genomic organization of the two genes, such as the size, sequence content, and number and arrangement of their exon/intron structures (Figures 4.6 and 4.7), further validated the distinctiveness of the two proteins and their corresponding cognate genes.

The *AIP1* cDNA (i.e. E73 cDNA clone shown in Figure 4.6) was identified as an ALS interactor using the yeast two-hybrid system, and recovered from the *A. thaliana* cDNA library. This clone was sequenced and determined to be 1739 bp in length (Kohalmi and Crosby, unpublished) with an ORF of 1474 bp encoding a 491 amino acid deduced gene product of approximately 53 kDa molecular mass. This deduced molecular mass is consistent with that estimated in Western blot experiments using *A. thaliana* extracts, or from *in-vitro*-translated AIP1p produced using the E73 cDNA clone as the template. A minor band beneath the major band was detected in SDS-PAGE analyses of the *in-vitro*-translated AIP1p (Figure 4.1), which could be attributed to a second, partial in-frame translation of this cDNA. Interestingly, this minor band plus bands above the main band was also observed in immunoblots of *A. thaliana* extracts (Figures 4.13 and 4.17). However, this band apparently did not interact with ALS as revealed by protein-protein interaction experiments (Figure 4.17). The origin and identity of this immuno-reactive minor polypeptide has not been determined.

Unlike the *AIP1* cDNA, which has an apparent full-length ORF, the *AIP3* cDNA recovered from the *A. thaliana* cDNA library and represented by two clones - E84 and

E229 - was sequenced (Kohalmi and Crosby, unpublished), determined to be 1030 and 1099 bp in length, respectively, and found out to be incomplete in length (Figure 4.7). A gene database of chromosome 5, to which both of these clones could be aligned, contained the full-length ORF of *AIP3* but was not available until a later stage of the research. Several unsuccessful attempts were made to translate *in vitro* the E84 cDNA clone; after further investigation, it was realized that the failed translation was due to an incomplete coding region sequence. Interestingly, the E229 cDNA clone was successfully translated despite retaining a partial ORF, resulting in two major polypeptide bands of approximately 32 and 30 kDa in molecular mass (Figure 4.1). Sequence data assessment and comparison of the data revealed that the presumed start codon in the E84 cDNA represented a different reading frame from that of the E229 clone, and may explain why the E84 cDNA was not successfully translated. Since the E229 cDNA clone was successfully translated, it was presumed that its start codon was in frame with the downstream ORF of the clone. The assumption turned out to be correct when the full-length ORF of AIP3p was later defined by analysis of the *A. thaliana* genome database using the gene prediction tools described in Section 3.4.12 and its start codon was determined to be in frame with the presumed start codon of the E229 clone. The full-length *AIP3* was determined to be 1684 bp in length with an ORF of 1414 bp encoding a polypeptide (471 a.a.) of approximately 51 kDa in molecular mass. This deduced molecular mass was found to be consistent with that estimated using immunoblots of *A. thaliana* extracts (Figure 4.1).

Although the deduced molecular masses of the AIP1p and AIP3p proteins were consistent with those observed from their corresponding immunoblots, closer examination revealed that the molecular masses estimated from the immunoblots were

approximately 1-2 kDa less than the deduced values. This could be explained by a post-translational modification involving cleavage of an approximately 10-30 a.a. N-terminal signal peptide upon localization to the chloroplast. Since AIP1p and AIP3p were shown to distribute to stromal and thylakoidal compartments of chloroplast, they may be considered as proteins that require signal peptides of approximately 13 to 36 residues at the N terminus, as hypothesized by Blobel *et al.*, which was cited in Voet and Voet (1995).

When AIP1p and AIP3p were subjected to *BLAST* analysis against the non-redundant protein database of all studied organisms and microorganisms, it was revealed that these two proteins were variably homologous to sixty or more ALS catalytic and/or regulatory subunits from various prokaryotic and eukaryotic species, as shown in Figures 4.2 and 4.3. Interestingly, the sequence similarity of both AIP1p and AIP3p to their prokaryotic and eukaryotic counterparts was primarily localized to the N- and C-terminal regions separated by a region of non-homology. Furthermore, the N- and C-homologous domains aligned well with several prokaryotic regulatory subunits, suggesting that AIP1p and AIP3p may consist of two prokaryotic subunit domains that have been duplicated or combined through evolution. The first 70-80 a.a. at the N terminus of both proteins were neither homologous to each other nor to their prokaryotic counterparts, suggesting that these regions might be of more recent evolutionary origin, and may represent a eukaryotic-specific attribute such as a signal peptide for protein targeting, or other structural features and functions that were acquired later in the evolutionary process. Indeed, evidence presented here indicates the AIP1p and AIP3p proteins are nuclear encoded and targeted to plastidic organelles, in turn suggesting a structural requirement for organellar targeting and uptake.

The results of *BLASTN* and *BLASTX* analyses against the *A. thaliana* genome database as part of a search for gene duplication in any *A. thaliana* chromosomes revealed no significant nucleotide or amino acid sequence local aligments in any chromosome of *A. thaliana*. This indicates that *AIP1* and *AIP3* are unique genes i.e. without duplicate copies in the genome of *A. thaliana*.

Local alignment (*BLAST*) was useful in retrieving homologous sequences of AIP1p or AIP3p and in comparing these sequences. However, in order to assess common sequence structural positions and/or ancestral residues of ALS, AIP1p, AIP3p and the retrieved sequences, a multiple sequence alignment (Clustal W algorithm) analysis in which the alignment is presented with gaps enabling matched sequences or patterns to be presented in the same column (Thompson *et al.*, 1994), was employed as described in Section 3.4.12. A complete presentation of the multiple sequence alignment results for both N- and C-terminal domains of AIP1p and AIP3p is shown in Appendices A1 and A2. With the exception of the ALS catalytic subunit of *A. thaliana* (At-ALSp), all sequences analyzed exhibited a remarkable orthology (i.e. homologous sequences in different species that arose from a common ancestral gene during speciation) that may or may not reflect a similar function. The alignment results showed conserved regions among both prokaryotic and eukaryotic species. It was revealed by both *BLAST* and Clustal W analyses that AIP1p and AIP3p exhibited a repeated domain of the prokaryotic polypeptides at the N- and C-domains, although the degree of orthology of these two domains to the various prokaryotic subunits was not exactly identical. The Clustal W analysis also revealed the pervasiveness (twenty-four orthologous proteins were examined, as shown in Appendices A1 and A2) of conserved-region orthology of AIP1p and AIP3p in prokaryotes and eukaryotes, suggesting that

these two proteins may be derived from a common ancestral gene. The AIP1p and AIP3p proteins are actually identical to the GI:4887755 and GI:9759111 proteins, respectively, submitted in the protein database with different annotation as putative acetolactate synthase genes in *A. thaliana* (Lin *et al.*, 1999) and acetolactate synthase-like protein of *A. thaliana* (Sato *et al.*, 1997), respectively. In the GI:4887755 sequence, a conserved region of ten residues starting at the 289th residue of AIP1p is missing, suggesting that the gene prediction tool used by Lin *et al.* (1999) misinterpreted that region as a non-exonic region. Several conserved regions were altered when compared between prokaryotic and eukaryotic genes. For example, the 92nd and 94th amino acid residues of AIP1p and AIP3p were unique to these proteins. Likewise, AIP1p residues 121 and 122 were unique to AIP1p and AIP3p as well as GI:5931761, which is an acetolactate synthase small subunit from *N. plumbaginifolia* (Hershey *et al.*, 1999). Other conserved regions were more recent in evolutionary time since they are unique to eukaryotes.

The chromosomal genes of *AIP1* and *AIP3* were recovered using their corresponding cDNAs as probes and sequence-characterized. The genomic sequences were assessed by manual/visual inspection of their exon/intron arrangement, *cis*-regulatory element (e.g. TATA box) and start and stop codons, as summarized in Figures 4.6 and 4.7. The sequences and their structural arrangements reflected a typical higher eukaryotic organization, including the presence of a complex intron/exon arrangement. Although *AIP1* and *AIP3* exhibited eleven and twelve introns, respectively, their deduced proteins shared remarkable orthology to their prokaryotic counterparts, indicating that domains of prokaryotic origin may serve as the common ancestor of both polypeptides, as mentioned above.

The relative abundance of *ALS*, *AIP1* and *AIP3* transcripts was determined using quantitative RT-PCR (qRT-PCR); the results are presented in Figure 4.8. The data show that *AIP1* transcripts were higher in abundance compared to *ALS* and *AIP3* transcripts in all organs analyzed. Although the primary objective of this experiment was to detect the presence of these transcripts in the main organs of *A. thaliana*, the qRT-PCR experiments enabled quantitation of the relative abundance of these transcripts. The high abundance of the *AIP1* transcript may be explained in several ways. Firstly, although the analysis was done using 40-43 d-old plants, it may be possible that *AIP1* expression reached its peak or that its steady state abundance was highest at this particular stage of plant development. Secondly, consistent with results derived from a *cis*-regulatory element-fusion analysis of *AIP1* expression (detailed further below), transgenic lines displayed high expression, regardless of plant age, of the *GUS* gene under the control of the *AIP1 cis*-regulatory element in most tissues examined. By comparing the stain intensities of *GUS* expression under the control of the *AIP1 cis*-regulatory element and the strong *CaMV 35S* promoter, the *AIP1 cis*-regulatory element was considered to be a moderately strong promoter, since the expression of GUS activity, as evidenced by the stain intensity of GUS-specific substrates, was very similar to that of the *CaMV 35S* promoter in all but one of the transgenic lines examined (Line #2; Figure 4.9). Although the results presented here argue for a relatively high level of AIP1p expression, the high abundance of AIP1 transcripts might not be proportionally reflected in the abundance of the corresponding proteins due to the significant longevity and post-translational persistence of GUS enzyme activity. A similar analysis involving the *AIP3 cis*-regulatory element was conducted but with no meaningful results obtained

due to an incorrect *cis*-regulatory element construct (Figure 4.10), thus preventing a comparative analysis for this gene.

The putative upstream *cis*-regulatory regions (e.g. promoters, enhancers) of *AIP1* and *AIP3* were fused to the *GUS* cassette and transformed to *A. thaliana*; the results are presented in Figures 4.9 and 4.10. Observation of the GUS-staining assay of *A. thaliana* at several developmental stages revealed the expression profile of GUS activity under the control of the *AIP1 cis*-regulatory element. In general, *AIP1::GUS* transgenic lines displayed the expression of GUS activity in most tissues, including root, stem, leaf and flower and its floral organ components. However, upon closer examination, GUS activity was not observed in select tissues among individual lines, with no particular pattern, although older organs tended to be higher in GUS activity than younger organs. For example, GUS activity in some transgenic lines was not observed in petal and some parts of root, leaf and stem. Based on these observations, it seems likely that *AIP1* expression might be subject to one or more supra-regulatory phenomena, such as a chromosomal position effect. It is intriguing to consider that the level of individual BCAAs or the composite pool may play a role in regulating *AIP1* expression. In bacteria, AIP1-like proteins mediate feedback-inhibition regulation of the BCAA biosynthetic pathway in response to the levels of individual BCAAs or pools of them; when the levels of individual BCAAs or their pools increase, ALS catalytic subunit activity is inhibited via an ALS regulatory-subunit-dependent manner, leading to a reduction in BCAA biosynthesis. A similar regulation circuit, but at the level of gene expression, may operate in eukaryotes, where the excess BCAAs or their pools directly or indirectly act to regulate the expression of a postulated negative regulator such as AIP1p, which in turn interacts with the ALS catalytic subunit to down-regulate the

biosynthesis of BCAAs. Accordingly, tissues or organs where GUS activity was observed might be correlated with elevated levels of BCAAs, whereas no observable GUS activity would be correlated with lower BCAA accumulation. It would be interesting to analyze the BCAA biosynthetic profile in select tissues derived from these transgenic lines, and to correlate this data with expression data of *AIP1*, *AIP3* and *ALS* genes and their gene products. Such studies might shed new light on whether BCAA accumulation is directly or indirectly regulated by *AIP1* expression.

Such a scenario could provide a mechanism for the genetic regulation and control of BCAA biosynthesis in eukaryotes, although further studies aimed specifically at regulation of expression of all known genes involved in the first enzymatic step of the BCAA metabolic pathway should be undertaken to support such a hypothesis. It has been reported in the past, as reviewed by Wallsgrove (1990), that the regulation of BCAA biosynthesis in higher plants was not exerted at the gene expression level. However, this report was based on genetic regulation studies of *ALS* catalytic subunit gene expression alone, since no reports at that time described the existence of other genes that might be involved in the regulation of BCAA metabolism.

This study failed to generate transgenic lines carrying an *AIP3*::*GUS* construct due to the late discovery that the *AIP3* gene contained four more 5' exons than was previously realized. As a result, the previously determined 2.1-kb *cis*-regulatory region that had been fused to the *GUS* cassette actually contained three additional 5' introns, resulting in interference with the transcription and translation structures critical for the expression of the *GUS* reporter gene construct.

Ectopic over-expression studies of the AIP1p and AIP3p proteins were carried out where *AIP1* and *AIP3* cDNAs under the control of the *CaMV 35S* promoter were

constructed and transformed to *A. thaliana* plants, as described in Section 3.5.2.2. The results of these studies showed that ectopic expression of *AIP1* and *AIP3* transgenes did affect, to various extents, the expression levels of the corresponding proteins, as shown in Figure 4.11. The results showed that ectopic expression resulted in increased levels of the respective transgenic protein and its endogenous counterpart, whereas suppression constructs were seen to reduce the level of the corresponding endogenous gene products. Interestingly, ectopic expression of *AIP1* was observed to variably regulate the expression level of the AIP3p protein, and *vice versa*, suggesting an interplay of the transcriptional and/or translational regulation of these two genes.

Most of the transgenic lines of *AIP1* and *AIP3* exhibited either a partial decrease in the expression levels of their corresponding proteins, or a complete suppression. These results were likely due to co-suppression between the corresponding transgene and its genomic counterpart, which extended to cross-suppression between the *AIP1* transgene and the *AIP3* endogenous copy (or *vice versa*) due to the high degree of primary sequence similarity between these two genes (Appendices A1 and A2). Indeed, others have shown that transgenes can exert a homology-based suppression phenotype involving both the introduced transgene and the endogene (Napoli *et al.*, 1990; van der Krol *et al.*, 1990). Silencing of gene expression can occur in a *cis* or *trans* manner, where the *trans* event involves an element that triggers silencing of shared homology with either a transgene, an endogenous gene or viruses (Fagard and Vaucheret, 2000). This *trans* phenomenon has been described as homology-dependent gene silencing (HDGS, Meyer, 1996) or co-suppression (Napoli *et al.*, 1990). Homology-dependent gene silencing (HDGS) or co-suppression includes transcriptional gene silencing (TGS), where the homology between the interacting gene is confined to the promoter or

flanking promoter sequences, and post-transcriptional gene silencing (PTGS) where it is confined to the coding region (Muskens *et al.*, 2000). Models for PTGS involve either the production of sense RNA above a threshold level, the production of antisense RNA or the production of aberrant RNA, all leading to the activation of a sequence-specific RNA degradation process.

This HDGS phenomenon accounts for about 5 to 25% of transgenic mutants derived from over-expression of homologous genes (Napoli *et al.*, 1990; van der Krol *et al.*, 1990). Accordingly, the remaining transgenic mutants of *AIP1* and *AIP3* exhibited either the same or an increased level of RNA/protein expression as compared to non-transformed control plants (Figures 4.11 and 4.12). In the case of an increased level of protein expression of AIP1p and AIP3p, this may be attributed to the introduction of the identical corresponding genes (transgenes) into the transgenic lines. Since the degree of co-suppression may vary depending upon the relative position of the transgene and the endogenous gene in the genome (Napoli *et al.*, 1990), it is possible that the same level of expression between the control (wild type) line and transgenic lines may be attributed to mildly exerted co-suppression. This would result in the reduction of expression levels to a point equivalent to the expression level seen in the wild type or, less frequently, the complete blocking of expression of either one of the genes, leaving the gene expressed at the same level as in the wild type.

Cross-suppression of *AIP1* versus *AIP3* transgenes might be predicted on the grounds that these two genes are closely related structurally at the nucleotide level. As reviewed by Meinz, Jr. (2000), approximately 60-70% identity of sequence is necessary and sufficient for post-transcriptional gene silencing (PTGS) to take place. Sequence alignments of the *AIP1* and *AIP3* cDNAs and their corresponding gene products

revealed notable homologies of approximately 74% and 80%, respectively, at the nucleotide and amino acid levels, making it likely that co-suppression was the operative mechanism in moderating the levels of *AIP1* and *AIP3* gene expression. It is important to note that the expression level of ALS was not affected by the ectopic expression of *AIP1* and *AIP3* transgenes, perhaps simply because the *ALS* catalytic subunit gene does not present significant sequence homology to either *AIP1* or *AIP3*, as shown in Figures 4.2 and 4.3.

5.2 Cell biology of ALS, AIP1p and AIP3p

Generation of polyclonal αAIP1p and αAIP3p antibodies was carried out where the antigens were purified from *E. coli* as recombinant His_6-tagged AIP1p and AIP3p proteins. The polyclonal αAIP1p, αAIP3p and αALS (F14) antibodies were each reciprocally tested against all recombinant antigens to determine their degree of cross-reactivity. The results showed that αAIP1p and αAIP3p antibodies exhibited cross-reactivity to various extents against recombinant His_6-tagged AIP1p or AIP3p antigens, as shown in Table 4.3. Anti-ALS antibody cross-reacted against recombinant His_6-tagged AIP1p, but only after prolonged exposure and at a high concentration of the antigen. A possible explanation for the observed cross-reactivity is that the His_6 tag is shared by the recombinant antigens and may be recognized by polyclonal antibodies raised against these proteins.

On the other hand, no significant cross-reactivity was observed when the antibodies were used to detect antigens derived from a plant source, as shown in the Western blot results (Figures 4.1, 4.11 and 4.17). The multiple bands seen in Western

blots of stromal extracts may be due to structurally-related proteins and/or epitopically-similar proteins being detected. However, after co-purification using IMAC Co^{2+} batch chromatography, a single band was observed in the Western blot of AIP1p and AIP3p as shown in Figure 4.17, suggesting each antibody recognized a specific antigen.

As mentioned elsewhere, ALS was found to be localized in the mitochondrion in yeast and fungi (Ryan and Kohlhaw, 1974) and in the chloroplast of algae and higher plants (Miflin, 1974; Reith and Munholland, 1993). In this study, a fractionation study was conducted during the preparation of chloroplast stromal extracts where ALS, AIP1p and AIP3p were detected in all fractions analyzed. Enrichment of the stromal and thylakoidal fractions of chloroplastidic compartments was confirmed by the co-fractionation of chloroplast sub-organelle marker enzyme. Both AIP1p and AIP3p proteins were enriched in the chloroplastidic compartments, i.e. stroma (soluble) and thylakoid (insoluble membrane). The ALS was co-enriched in stromal and thylakoidal fractions as well. Since the AIP1p and AIP3p proteins were enriched in the thylakoid membrane fraction, this suggests that AIP1p and AIP3p may persist, in part, as either integral or surface-bound thylakoidal-membrane proteins. The co-localization of these three proteins in the chloroplast is consistent with the suggestion that a protein-protein interaction involving the ALS catalyitic subunit, AIP1p and AIP3p proteins occurs, with possible functional implications for regulating the metabolic activity of ALS *in planta*.

The modification of the At*ALS-csr1-1* mutant gene product was necessary in order to permit subsequent protein-protein interaction studies. The His_6-tag insertion enabled the capture and purification of ALS using IMAC, which also allowed the identification of co-enriched proteins that may interact with ALS. The use of the chlorsulfuron resistant mutant *ALS* allele was intentional in order to ensure that the

genetically-modified ALS enzyme was functional. Without this distinct property, it would have been difficult to determine whether the endogenous or modified ALS was functional in all respects, including its protein-protein interaction properties. As expected, enzymatic activity of the modified, herbicide-resistant ALS from DPC712-3 line stromal extracts was more tolerant than the wild type ALS to chlorsulfuron inhibitory treatments. Interestingly, the CSR1-1 ALS was more tolerant than the transgenic DPC712-3 ALS. The likely explanation is that the DPC712-3 line expresses a mixture of endogenous herbicide sensitive ALS and the exogenous herbicide tolerant ALS, whereas this would not be the case for the *csr1-1* mutant that expresses exclusively the herbicide-resistant form of the enzyme.

The results of BCAA inhibition studies did not correlate with herbicide inhibition. For example, incorporation of 1 mM isoleucine into *in vitro* assays caused a similar reduction in activity for ALSs derived from all lines examined. A possible explanation is that the herbicide binding site and the BCAA binding site are different, as previously suggested by Schloss (1994) resulting in a different effect on ALS. The effect of isoleucine and valine treatments on wild type *A. thaliana* ALS activity closely resembled that seen for the purified, unfractionated wild type *B. napus* ALS activity (Bekkaoui *et al.*, 1993b), and for the purified wild type enzyme from *Chicorium intybus* L. var. Witloof but not for leucine treatment (Dewaele *et al.*, 1997). Several authors have reported that decreased sensitivity to feedback inhibition by some or all BCAAs was observed in several different herbicide-resistant plants (Eberlein *et al.*, 1997; Rathinasabapathi *et al.*, 1990; Subramanian *et al.*, 1990; Mourad *et al.*, 1995). However, caution should be exercised in examining the data since the ALS activity analyzed was prepared in different ways, or in some cases subjected to purification regimes that may

have impacted its properties. In this study, the association of ALS, AIP1p and/or AIP3p may have functional implications for feedback inhibition of the plant enzyme analogous to ALS feedback inhibition mechanisms in bacteria. It may also be that the effect of BCAA is dependent upon the steady state expression levels of AIP1p and/or AIP3p.

Co-immunoprecipitation and *in vitro* interaction experiments were inconclusive in evaluating protein-protein interactions involving AIP1p, AIP3p and ALS. In co-immunoprecipitation experiments, an approximately 50-55 kDa non-specific band was observed in all cases, suggesting a common binding protein in the fractionated samples. Upon closer examination, it was shown that the common band represented the 55-kDa heavy chain fragment of the IgG that was being released and subsequently detected. Since the size of this protein was approximately the same as AIP1p and AIP3p, it effectively masked the primary antibody signal of these two proteins and prevented their detection by Western blot approaches. *In vitro* interaction experiments revealed non-specific binding of labeled ALS with AIP1p and/or AIP3 as well as IMAC Ni^{2+}-immobilized *His*$_6$-tagged fusion protein variants of AGL2p, AGL4p, AGL24p, ASK1p and *ll*-ASK1p. A possible explanation for this result is that the high concentrations of the immobilized proteins that were required for the experiment may have forced the kinetics of interaction in the direction of non-specific binding.

In vivo interaction experiments using IMAC Ni^{2+} batch chromatography were complicated by a non-specific binding of non *His*$_6$-tagged ALS (wild type ALS) to the Sepharose resin and/or the ligand. This was supported by reports that IMAC Ni^{2+} exhibited a higher tendency of non-specific co-ordination with non-His tagged proteins (Kasher *et al.*, 1993). Accordingly, an alternative approach was taken by using IMAC Co^{2+} batch chromatography, which was reported to exhibit greater specificity for binding

His-tagged proteins than IMAC Ni^{2+} (Kasher *et al.*, 1993). The results showed that IMAC Co^{2+} batch chromatography successfully resolved the problem of non-specific binding and simultaneously showed the existence of protein-protein interactions between ALS and AIP1p and/or AIP3p. This result constitutes a direct biochemical demonstration of the existence of ALS:AIPx protein-protein interactions in plant extracts.

The results confirm a report of the existence of protein-protein interactions using the yeast two-hybrid system (Kohalmi *et al.*, unpublished) in that the ALS catalytic subunit interacts with AIP1p and/or AIP3p. Given the several direct and indirect lines of evidence, including the orthology of AIP1p and AIP3p with other ALS regulatory subunits, it is proposed that AIP1p and AIP3p are derived from a prokaryotic ancestral gene that has been duplicated or combined through evolution and may serve as ALS regulatory subunits in plants. Mutants in the corresponding gene from yeast confirm this suggestion, although AIPx knockouts in *A. thaliana* would be useful in assessing the predicted ALS-regulation phenotype. In bacteria, the ALS holoenzyme has been proposed to assume a tetrameric conformation consisting of two identical catalytic subunits and two identical regulatory subunits, forming an $\alpha_2\beta_2$ structure. In plants, based on the evidence obtained in this study and elsewhere, the ALS holoenzyme likely exhibits a different conformation. Since the plant AIPx 'regulatory' subunits are almost twice the size of their bacterial counterparts and since the catalytic subunit persists as a dimer (Bekkaoui *et al.*, 1993b), it can be proposed that the quaternary content of the ALS holoenzyme may exist as different isoforms, consisting of a single catalytic subunit plus two heterodimer regulatory subunits, thus assuming an $\alpha\beta\beta'$ structure, or a catalytic

homodimer and a regulatory heterodimer, thus assuming an $\alpha_2\beta\beta'$ structure. Other possible quaternary structures that cannot be excluded are $\alpha\beta$, $\alpha\beta'$, $\alpha_2\beta$ and $\alpha_2\beta'$.

In *B. napus* and in wheat, a Stokes radius corresponding to a globular protein of 124-kDa molecular mass (Bekkaoui *et al.*, 1993b) and a native molecular mass of ca. 128 kDa (Southan and Copeland, 1996), respectively, were reported that could be considered closely similar to quaternary structures of $\alpha\beta$ and $\alpha\beta'$ having molecular masses of 118 and 116 kDa, respectively. Quaternary structures of $\alpha_2\beta$ and $\alpha_2\beta'$, which give molecular masses of 183 and 181 kDa, respectively, would resemble native molecular masses of 193 and 200 kDa of ALS from maize (Singh *et al.*, 1988a) and barley (Durner and Böger, 1990), respectively. At higher levels of quaternary structure, 440 kDa molecular masses reported by Durner and Böger (1988) and Muhitch (1988) for barley and maize, respectively, would resemble the 468 kDa oligodimer of $\alpha_2\beta\beta'$. In addition, a 464-kDa homo-octamer of ALS reported by Durner and Böger (1988) would resemble very closely the proposed 468-kDa oligodimer of the $\alpha_2\beta\beta'$ holoenzyme structure. Thus, only the $\alpha\beta\beta'$ heteromer would not be accounted for, since no reports have described a ca. 169-kDa native ALS. Regardless of these accounts, the central issue shown in this research is that ALS exists in association with putative regulatory subunits, although its precise quaternary structure remains to be determined.

The postulate that AIP1p and AIP3p are regulatory subunits was further validated by the study of ectopic expression transgenic lines of AIP1p and AIP3p. The up-regulated or co-suppressed transgenic lines of AIP1p and AIP3p showed, in most cases, a two- to eight- fold increase in free amino acid content as compared to the free amino acid levels observed in the wild type. The ectopic expression of AIP1p or AIP3p

showed a pleiotropic change in free amino acid concentration, although the change was mostly in the direction of an increase in free amino acid content. The introduction of transgenes caused disruption of regulation of the BCAA biosynthetic pathway with a total net increase of free amino acids, regardless of whether the endo- and trans-genes were down- or up-regulated via ectopic expression or co-supression approaches. As postulated in this study, AIP1p and AIP3p are regulatory subunits of ALS and, hence, mediate negative allosteric regulation of BCAA biosynthesis. In the case of down-regulation and co-suppression, these negative regulators are present in small quantity or not at all. Hence, the control of BCAA biosynthesis was reduced, resulting in increased formation of these BCAAs. In the case of up-regulation, it is more difficult to rationalize, although it might be possible that there is a threshold requirement for AIP1p and AIP3p to act as negative regulators. It may be that when both AIP1p and AIP3p concentrations exceed the concentration threshold, then AIP1p/AIP3p become less active due to the dilution of some other unknown essential factors, thus promoting the de-regulation of ALS activity.

It was suggested in the past that because of certain properties of the bacterial ALS enzyme, it might associate with proteins other than the *ilv* regulatory subunits. These suggestions stem from intriguing bacterial mutant *ilv* auxotrophic phenotypes described by McEwen and Silverman (1980a and 1980b) and McEwen *et al.* (1983). In these studies, *E. coli* mutants deficient in conjugal plasmid exchange at the *cpxA* locus were found to exhibit an *ilv* auxotrophic phenotype. The *cpxA* locus did not map to known *ilv* loci, and mutations resulted in a pleiotropic alteration of membrane protein content and stoichiometry. The product of the *cpxA* gene was localized exclusively to the bacterial membrane, and exhibited structural similarities to the first (sensing)

component of prokaryotic two-component signal transduction pathways. Feeding studies involving intermediates in the *ilv* pathway localized the *cpxA* auxotrophic defect to ALS, yet mutant extracts contained normal levels of cytoplasmic ALS enzyme activity. These studies argued, on genetic grounds, that functional ALS in bacteria may exist in association with one or more membrane components.

6. SUMMARY AND CONCLUSIONS

The results presented in this research outline selected aspects of the molecular characterization, genetic and biochemical analyses, and protein-protein interaction assessment of ALS-interacting proteins. This research sheds new light on, and lays a fresh foundation for, the study of BCAA metabolism, particularly on the first common enzymatic step involving eukaryotic acetolactate synthase (ALS).

Chromosomal genes and cDNAs of ALS-interacting proteins 1 and 3 (*AIP1* and *AIP3*), and their gene products, were characterized in terms of their sequence, size (directly derived or deduced) and chromosomal structural features. Differences and similarities between these two entities were also observed, leading to a comparison between these proteins and their prokaryotic counterparts. Although *AIP1* and *AIP3* share a high degree of homology at the cDNA nucleotide and deduced amino acid levels, each exhibits unique characteristics as indicated by their molecular size, chromosomal location and structural organization (e.g. the size, sequence content and arrangement of their exon/intron structure).

Transcripts of *ALS*, *AIP1* and *AIP3* exhibited different levels of abundance, which suggests the expression of these transcripts may be governed by different genetic regulatory mechanisms, which further suggests a functional uniqueness of these two genes/gene products. The *AIP1 cis*-regulatory element fused to the *GUS* reporter gene cassette displayed the properties of a moderately strong promoter, although the post-

translational longevity of the GUS enzyme activity should also be taken into consideration in interpreting this result. However, it could not be compared with the *AIP3* promoter since no results of GUS expression profiles were obtained, although additional characteristics might be revealed in terms of the uniqueness of *AIP3*.

Since the AIP1p and AIP3p deduced proteins share strong homology to their prokaryotic counterparts, it may be concluded that their sequences are derived from a domain of prokaryotic origin that serves as the common evolutionary ancestor of both polypeptides.

Ectopic over-expression studies of the AIP1p and AIP3p protein revealed that *AIP1* and *AIP3* transgenes affect the level of expression of proteins, resulting in a partial decrease/increase or complete suppression. The studies also revealed cross-effects where ectopic expression of the *AIP1* transgene resulted in regulation of the *AIP3* endogene expression level, and *vice versa*. The down regulation and co-suppression of protein levels may be due to post-transcriptional gene silencing (PTGS) effects that involve either the production of sense RNA above a certain threshold, the production of antisense RNA or the production of aberrant RNA that leads to the activation of a sequence-specific RNA degradation process. Whereas the cross-suppression of *AIP1* vs. *AIP3* transgenes may be due to the marked homology between *AIP1* and *AIP3*, this cross-suppression phenomenon was not observed in the case of the *ALS* catalytic subunit gene vs. the *AIP1* and/or *AIP3* genes, presumably because it exhibits negligible sequence homology to either *AIP1* or *AIP3*.

Polyclonal αAIP1p and αAIP3p antibodies partially cross-reacted with both of the *E. coli* recombinant AIP3p and AIP1p antigens, which may be due to the presence of

common *His*$_6$ tags in both recombinant proteins. However, this cross-reactivity was not seen when nascent antigens derived from plant extracts were analyzed following metal-ion affinity co-purification of the antigens. Acetolactate synthase, AIP1p and AIP3p were found to co-localize in both the stromal and thylakoidal compartments of the chloroplast despite their different protein levels. These results suggest that these three proteins may share an important role in regulating the metabolic activity of the ALS holoenzyme *in planta*.

The site-directed *His*$_6$ variant of the At*ALS-csr1-1* transgene enabled the capture and purification of ALS and the identification of co-enriched proteins that interact with ALS. The use of a herbicide resistant *ALS* allele enabled a prior functional assessment of the genetically-modified ALS enzyme. A stromal extract of the transgenic DPC712-3 line was assessed for ALS activity, with and without herbicide and branched-chain amino acid (BCAA) treatments. The ALS enzyme in transgenic stromal extracts exhibited higher herbicide tolerance than that in the wild type, but lower than that in the *csr1-1* mutant.

Inhibition of ALS by one or more BCAAs exhibited differential effects on the wild type, DPC712-3 and *csr1-1* lines. In some cases, the DPC712-3 line exhibited decreased sensitivity to feedback inhibition by individual BCAAs or the combined pool. This decreased sensitivity to feedback inhibition by some or all BCAAs has been also reported for several different herbicide-resistant plants in other studies, although caution should be exercised in examining the data since the ALS activity assessed was prepared in different ways.

The use of co-immuniprecipitation, *in vitro* interaction experiments and *in vivo* interaction experiments using IMAC Ni^{2+} batch chromatography failed to resolve the

protein-protein interaction potential of ALS, AIP1p and/or AIP3p. Factors in the failure include interference by the antibody heavy chain subunit of the IgG-crosslinked sepharose beads in the detection of AIP1p and AIP3p antigens, non-specific binding of the ALS catalytic subunit to several *in-vitro*-translated proteins, and non-specific interactions of wild type ALS with the bead matrix.

The use of IMAC Co^{2+} batch chromatography with plant extracts from transgenic lines containing the herbicide-resistant *His_6-ALS* mutant allele successfully resolved the problem of non-specific interaction and revealed protein-protein interactions between ALS and AIP1p and/or AIP3p. These results constitute direct biochemical evidence in support of the existence of ALS:AIPx protein-protein interactions in plant extracts.

In bacteria, the ALS holoenzyme has been proposed to assume a tetrameric conformation consisting of two identical catalytic subunits and two identical regulatory subunits, forming an $\alpha_2\beta_2$ structure. In plants, based on the evidence obtained in this study and elsewhere, the ALS holoenzyme likely exhibits a different conformation. Thus, ALS holoenzyme quaternary structure may assume several conformations, including $\alpha\beta\beta'$ (i.e. a single catalytic subunit plus two heterodimer regulatory subunits), $\alpha_2\beta\beta'$, $\alpha\beta$, $\alpha\beta'$, $\alpha_2\beta$ and $\alpha_2\beta'$ heteromers. Irrespective of these speculations, the central issue shown in this research is that ALS exists in association with putative regulatory subunits, although its precise quaternary structure remains to be determined.

The postulate that AIP1p and AIP3p are regulatory subunits was further validated by the study of ectopic expression transgenic lines of AIP1p and AIP3p. The introduction of *AIP1* and *AIP3* transgenes caused disruption of regulation of the BCAA biosynthetic pathway with a net increase of total free amino acids, regardless of whether

the endo- and trans-genes were down- or up-regulated via ectopic expression or co-supression approaches. This suggests that the disruption of regulation of BCAA metabolism may be perceived as a direct consequence of AIP1p and AIP3p being regulatory subunits of ALS that mediate negative allosteric regulation of BCAA biosynthesis.

According to Apling (1999), studies of protein-protein interactions may be carried out at three different levels. First, the researcher determines which proteins in the cell physically bind with a particular target protein. Secondly, verification by an independent method is required in order to establish that the novel interaction is indeed biologically important. Lastly, when a protein-protein interaction has been established and verified, studies can be carried out to determine the structure-function basis of the interaction e.g. domains or specific residues that might be responsible for the interaction and the significance of the interaction in the biological process. This study has dealt mostly with aspects at the second level; thus, for the future, conducting research that addresses the third level mentioned above would be of interest.

It may be interesting to determine whether only these three proteins, ALS, AIP1p and AIP3p, are involved in mediating the metabolic activity of the first enzymatic step of the BCAA biosynthethic pathway. One approach would be to use DNA microarray techniques to assess global changes in gene expression, by first generating a mutant that lacks one or more of the regulatory subunits. Furthermore, transgenic lines with altered characteristics (e.g. the *AIP1* and *AIP3* ectopically-expressing transgenic lines) may also be useful as a starting point for similar analyses. The availability of *AIP1* and *AIP3* genes may also open up new avenues for genetic engineering of these two regulatory proteins so as to alter the herbicide resistance profile of the host plant. Similarly, studies

using different plant systems (e.g. agricultural crops) may be useful to assess the potential of altering BCAA content via genetic manipulation of the two regulatory subunits of the crop of interest.

7. BIBLIOGRAPHY

Altschul, S.F., Madden, T.L., Schaffer, A.A., Zhang, J., Zhang, Z., Miller, W. and Lipman, D.J. 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. 25:3389-3402.

Anderson, C.W., Straus, J.W. and Dudoch, B.S. 1983. Preparation of a cell-free protein-synthesizing system from wheat germ. Methods Enzymol. 101:635-644.

Anderson, M. and Roberts, J.A. (eds.). 1998. *Arabidopsis* – Annual Plant Reviews, Vol. 1. Sheffield Academic Press, Inc., Sheffield, England.

Anderson, N.G. 1998. Co-immunoprecipitation: Identification of interacting proteins. In Methods in Molecular Biology, Vol. 88: Protein Targeting Protocols, ed. R.A. Clegg, pp. 35-45. Humana Press, Inc., Totowo, NJ.

Anderson, P.C., Minnetonka, M.N. and Georgeson, M. 1989. Herbicide-tolerant mutants of corn. Genome 31:994-999.

Appling, D.R. 1999. Genetic approaches to the study of protein-protein interaction. Methods: A Companion to Methods Enzymol. 19:338-349.

Azevedo, R.A., Arruda, P., Turner, W.L. and Lea, P.J. 1997. The biosynthesis and metabolism of the aspartate derived amino acids in higher plants. Phytochemistry 46:395-419.

Babic, I. 1991. Elevated Expression of Wild Type and Mutated Forms of a *Brassica napus* Acetolactate Synthase Gene for Herbicide Resistance in *B. napus*. Ph.D. thesis, University of Calgary, Calgary, AB.

Bai, C. and Elledge, S.J. 1996. Gene identification using the yeast two-hybrid system. Methods Enzymol. 273.331-347.

Barak, Z., Calvo, J.M. and Schloss, J.V. 1988. Acetolactate synthase isozyme III from *Escherichia coli*. Methods Enzymol. 166:455-458.

Barak, Z., Chipman, D.M. and Schloss, J.V. (eds.). 1990. Biosynthesis of Branched Chain Amino Acids. VCH Verlagsgesellschaft mBH, Weinheim, Germany.

Bartel, P.L., Chien, C.-T., Sternglanz, R., Fields, S. 1993. Using the two-hybrid system to detect protein-protein interactions. In Cellular Interactions and Development. A Practical Approach, ed. D.A. Hartley, pp. 153-179. IRL Press, NY.

Bekkaoui, F., Babic, I., Condie, J.A., Moloney, M.M. and Crosby, W.L. 1991a. Quantitative PCR assessment of 2 ALS genes from *Brassica napus*. J. Cell Biochem. Suppl. 15A:88-88

Bekkaoui, F., Condie, J.A., Neustaedter, D.A., Moloney, M.M. and Crosby, W.L. 1991b. Isolation, structure and expression of a cDNA for acetolactate synthase from *Brassica napus*. Plant Mol. Biol. 16:741-744.

Bekkaoui, F., Nadin-Davis, S.A. and Crosby, W.L. 1993a. Isolation and structure of an acetolactate synthase gene from *Schizosaccharomyces pombe* and complementation of the *ilv2* mutation in *Saccharomyces cerevisiae*. Curr. Genet. 24:544-547.

Bekkaoui, F., Schorr, P. and Crosby, W.L. 1993b. Acetolactate synthase from *Brassica napus* - immunological characterization and quaternary structure of the native enzyme. Physiol. Plant. 88:475-484.

Bieleski, R.L. and Turner, N.A. 1966. Separation and estimation of amino acids in crude extracts by thin-layer electrophoresis and chromatography. Anal. Biochem. 17:278-293.

Birch, D.E., Kolmodin, L., Laird, W.J., McKinney, N., Wong, J., Young, K.K.Y., Zangenberg, G.A. and Zoccoli, M.A. 1996. Simplified hot start PCR. Nature 381:445-446.

Blatt, J.M., Pledger, W.J. and Umbarger, H.E. 1972. Isoleucine and valine metabolism in *E. coli*. XX. Multiple forms of acetohydroxy acid synthetase. Biochem. Biophys. Res. Commun. 48:444-450.

Blattner, F.R., Plunkett, G., Bloch, C.A., Perna, N.T., Burland, V., Riley, M., Collado-Vides, J., Glasner, J.D., Rode, C.K., Mayhew, G.F., Gregor, J., Davis, N.W., Kirkpatrick, H.A., Goeden, M.A., Rose, D.J., Mau, B. and Shao, Y. 1997. The complete genome sequence of *Escherichia coli* K-12. Science 277:1453-74.

Borstlap, A.C. 1981. Interactions between the branched-chain amino acids in the growth of *Spirodela polyrhiza* aquatic plants. Planta 151:314-319.

Bourgin, J.P., Goujaud, J., Missonier, C. and Pethe, C. 1985. Valine-resistance, a potential marker in plant cell genetics. 1. Distinction between two types of valine-resistant tobacco mutants isolated from protoplast-derived cells. Genetics 109:393-407.

Bowman, J. 1994. Introduction. In *Arabidopsis* - An Atlas of Morphology and Development, ed. J. Bowman, pp. xiii-xv. Springer-Verlag New York Inc., NY.

Boyer, R.F. 1993. Modern Experimental Biochemistry. The Benjamin/Cummings Publishing Company, Inc., Redwood City, CA.

Bradford, M.M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72:248-254.

Bright, S.W.J. 1992. Herbicide-resistant crops. Curr. Topics Plant Physiol. 7:184-194.

Bryan, J.K. 1980. Synthesis of the aspartate family and branched-chain amino acids. In The Biochemistry of Plants, Vol. 5, eds. P.K. Stumpf and E. Conn, pp. 403-452. Academic Press, Inc., NY.

Buday, L. 1999. Membrane-targeting of signaling molecules by SH2/SH3 domain-containing adaptor proteins. Biochim. Biophys. Acta 1422:187-204.

Bult, C.J., White, O., Olsen, G.J., Zhou, L., Fleischmann, R.D., Sutton, G.G., Blake, J.A., Fitzgerald, L.M., Clayton, R.A., Gocayne, J.D., Kerlavage, A.R., Dougherty, B.A., Tomb, J.F., Adams, M.D., Reich, C.I., Overbeek, R., Kirkness, E.F., Weinstock, K.G., Merrick, J.M., Glodek, A., Scott, J.L., Geoghagen, N.S. and Venter, J.C. 1996. Complete genome sequence of the methanogenic archaeon, *Methanococcus jannaschii*. Science 273:1058-1073.

Chaleff, R.S. and Mauvais, C.J. 1984. Acetolactate synthase is the site of action of two sulfonylurea herbicides in higher plants. Science 224:1443-1445.

Chan, D., Lamande, S.R., McQuillan, A. and Bateman, F. 1997. *In vitro* expression analysis of collagen biosynthesis and assembly. J. Biochem. Biophys. Methods 36:11-29.

Chang, A.K. and Duggleby, R.G. 1997. Expression, purification and characterization of *Arabidopsis thaliana* acetohydroxyacid synthase. Biochem. J. 327:161-169.

Chevray, P.M. and Nathans, D. 1992. Protein interaction cloning in yeast - identification of mammalian proteins that react with the leucine zipper of Jun. Proc. Natl. Acad. Sci. USA 89:5789-5793.

Chien, C.-T., Bartel, P.L., Sternglanz, R. and Fields, S. 1991. The two-hybrid system: A method to identify and clone genes or proteins that interact with a protein of interest. Proc. Natl. Acad. Sci. USA 88:8578-9582.

Chipman, D.M., Gollop, N., Damri, B.A. and Barak, Z. 1990. Kinetics and mechanism of acetohydroxyacid synthases. In Biosynthesis of Branched Chain Amino Acids, eds. Z. Barak, D.M. Chipman, and J.V. Schloss, pp. 243-267. VCH Verlagsgesellschaft mBH, Weinheim, Germany.

Chipman, D., Barak, Z.A. and Schloss, J.V. 1998. Biosynthesis of 2-aceto-2-hydroxy acids: acetolactate synthases and acetohydroxyacid synthases. Biochim. Biophys. Acta Protein Struct. Mol. Enzymol. 1385:401-419.

Chothia, C. and Janin, J. 1975. Principles of protein-protein recognition. Nature 256:705-708.

Clough, S.J. and Bent, A.F. 1998. Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. Plant J. 16:735-743.

Cobbett, C.S. 1998. Biochemical genetic analysis of metabolic pathways. In *Arabidopsis* - Annual Plant Reviews, Vol. 1, eds. M. Anderson and J.A. Roberts, pp. 63-98. Academic Press, Inc., Sheffield, England.

Cullin, C., Baudin-Baillieu, A., Guillemet, E. and Ozier-Kalogeropoulos, O. 1996. Functional analysis of *YCL09C*: Evidence for a role as the regulatory subunit of acetolactate synthase. Yeast 12:1511-1518.

De Felice, M., Guardiola, J., Esposito, B. and Iaccarino, M. 1974. Structural genes for a newly recognized acetolactate synthase in *Escherichia coli* K-12. J. Bacteriol. 120:1068-1077.

Devine, M., Duke, S.O. and Fedtke, C. 1993. Physiology of Herbicide Action, pp. 251-294. PTR Prentice-Hall, Inc., Englewood Cliffs, NJ.

Dewaele, E., Forlani, G., Degrande, D., Nielsen, E. and Rambour, S. 1997. Biochemical characterization of chlorsulfuron resistance in *Cichorium intybus* L var Witloof. J. Plant Physiol. 151:109-114.

Duggleby, R.G. 1997. Identification of an acetolactate synthase small subunit gene in two eukaryotes. Gene 190:245-249.

Duquerroy, S., Cherfils, J. and Janin, J. 1991. Protein-protein interaction: an analysis by computer simulation. Ciba Found. Symp.161:237-249.

Durner, J. and Böger, P. 1988. Acetolactate synthase from barley *Hordeum vulgare* L. Purification and partial characterization. Z. Naturforsch Sect. C 43:850-856.

Durner, J. and Böger, P. 1990. Oligomeric forms of plant acetolactate synthase depend on flavin adenine dinucleotide. Plant Physiol. 93:1027-1031.

Durner, J., Gailus, V. and Böger, P. 1991. New aspects on inhibition of plant acetolactate synthase by chlorsulfuron and imazaquin. Plant Physiol. 95:1144-1149.

Eberlein, C.V., Guttieri, M.J., Mallory-Smith, C.A., Thill, D.C. and Baerg., R.J. 1997. Altered acetolactate synthase activity in ALS-inhibitor resistant prickly lettuce (*Lactuca serriola*). Weed Sci. 45:212-217.

Ellenberger, T.E., Brandl, C.J., Struhl, K. and Harrison, S.C. 1992. The GCN4 basic ragion leucine zipper binds DNA as a dimer of uninterrupted α helices: crystal structure of the protein-DNA complex. Cell 71:1226-1237.

Eoyang, L. and Silverman, P.M. 1986. Role of small subunit (*IlvN* polypeptide) of acetohydroxyacid synthase I from *Escherichia coli* K-12 in sensitivity of the enzyme to valine inhibition. J. Bacteriol. 166:901-904.

Fagard, M. and Vaucheret, H. 2000. Systemic silencing signal(s). Plant Mol. Biol. 43:285-293.

Falco, S.C. and Dumas, K.S. 1985. Genetic analysis of mutants of *Saccharomyces cerevisiae* resistant to the herbicide sulfometuron methyl. Genetics 109:21-35.

Falco, S.C., Dumas, K.S. and Livak, K.J. 1985. Nucleotide sequence of the yeast *ILV2* gene which encodes acetolactate synthase. Nucleic Acids Res. 13:4011-4027.

Felder, S., Zhou, M., Hu, P., Urena, J., Ullrich, A., Chaudhuri, M., Shoelson, S.E. and Schlessinger, J. 1993. SH2 domains exhibit high-affinity binding to tyrosine-phosphorylated peptides yet also exhibit rapid dissociation and exchange. Mol. Cell. Biol. 13:1449-1455.

Fersht, A.R. 1984. Basis of biological specificity. Trends Biochem. Sci. 9:145-147.

Fielder, E. and Schultz, G. 1985. Localization, purification, and characterization of shikimate oxidoreductase-dehydroquinate hydrolase from stroma of spinach chloroplasts. Plant Physiol. 79:212-218.

Fields, S. and Song, O. 1989. A novel genetic system to detect protein-protein interactions. Nature 340:245-246.

Fields, S. and Sternglanz, R. 1994. The two-hybrid system: An assay for protein-protein interactions. Trends Genet. 10:286-292.

Fleischmann, R.D., Adams, M.D., White, O., Clayton, R.A., Kirkness, E.F., Kerlavage, A.R., Bult, C.J., Tomb, J., Dougherty, B.A., Merrick, J.M., McKenney, K., Sutton, G. G., Fitzhugh, W., Fields, C.A., Gocayne, J.D., Scott, J.D., Shirley, R., Liu, L.I., Glodek, A., Kelley, J.M., Weidman, J.F., Phillips, C.A., Spriggs, T., Hedblom, E., Cotton, M.D., Utterback, T., Hanna, M.C., Nguyen, D.T., Saudek, D.M., Brandon, R.C., Fine, L.D., Fritchman, J.L., Fuhrmann, J.L., Geoghagen, N.S., Gnehm, C.L., McDonald, L.A., Small, K.V., Fraser, C.M., Smith, H.O. and Venter, J.C. 1995. Whole-genome random sequencing and assembly of *Haemophilus influenzae* Rd. Science 269: 496-512.

Frischauf, A.-M., Lehrach, H., Poustka, A. and Murray, N. 1983. Lambda replacement vectors carrying polylinker sequences. J. Mol. Biol. 170:827-842.

Fritz, C.C. and Green, M.R. 1992. Fishing for partners. Curr. Biol. 2:403-405.

Germino, F.J., Wang, Z.X. and Weissman S.M. 1993. Screening for in vivo protein-protein interactions. Proc. Natl. Acad. Sci. USA 90:933-937.

Gietz, R.D., Triggs-Raine, B., Robbins, A., Graham, K.C. and Woods, R.A. 1997. Identification of proteins that interact with a protein of interest: Applications of the yeast two-hybrid system. Mol. Cell. Biochem. 172:67-79.

Gold, L.M. and Schweiger, M. 1971. Synthesis of bacteriophage-specific enzymes directed by DNA *in vitro*. Methods Enzymol. 20:537-542.

Golemis, E.A and Khazak, V. 1997. Alternative yeast two-hybrid systems. The interaction trap and interaction mating. Methods Mol. Biol. 63:197-218.

Gollop, N., Damri, B., Chipman, D.M. and Barak, Z. 1990. Physiological implications of the substrate specificities of acetohydroxy acid synthases from varied organisms. J. Bacteriol. 172:3444-3449.

Grant, S.G., Jessee, J., Bloom, F.R. and Hanahan, D. 1990. Differential plasmid rescue from transgenic mouse DNAs into *Escherichia coli* methylation-restriction mutants. Proc. Natl. Acad. Sci. USA 874645-4649.

Grula, J.W., Hudspeth, R.L., Hobbs, S.L. and Anderson, D.M. 1995. Organization, inheritance and expression of acetohydroxyacid synthase genes in the cotton allotetraploid *Gossypium hirsutum*. Plant Mol. Biol. 28:837-846.

Gualberto, J.M., Handa, H. and Grienenberger, J.M. 1995. Isolation and fractionation of plant mitochondria and chloroplasts: specific examples. In Methods in Cell Biology Vol. 50, Methods in Plant Cell Biology Part B, eds. D.W. Galbraith, D.P. Bourque and H.J. Bohnert, pp. 161-176. Academic Press, Inc., NY.

Hagelstein, P., Klein, M. and Schulz, G. 1993. Enzymes of leucine synthesis in spinach chloroplasts. Plant Physiol. 102(Suppl):167.

Halpern, Y.S. and Even-Shoshan, A. 1967. Further evidence for two distinct acetolactate synthetases in *Aerobacter aerogenes*. Biochim. Biophy. Acta 139:502-504.

Harlow, E. and Lane, D. 1988. Antibodies, A Laboratory Manual. Cold Spring Harbor Press, Cold Spring Harbor, NY.

Hartnett, M.E., Newcomb, J.R. and Hodson, R.C. 1987. Mutations in *Chlamydomonas reihardtii* conferring resistance to the herbicide sulfometuron methyl. Plant. Physiol. 85:898-901.

Haughn, G.W. and Somerville, C. 1986. Sulfonylurea-resistant mutants of *Arabidopsis thaliana*. Mol. Gen. Genet. 204:430-434.

Haughn, G.W., Smith, J., Mazur, B. and Somerville, C. 1988. Transformation with a mutant *Arabidopsis* acetolactate synthase gene renders tobacco resistant to sulfonylurea herbicides. Mol. Gen. Genet. 211:266-271.

Hawkes, T.J., Howard, L and Pontin, S.E. 1989. Herbicides that inhibit the biosynthesis of branched chain amino acids. In Herbicides and plant metabolism, ed. A. Dodge, pp. 112-126. Cambridge University Press, Cambridge, England.

Hennessey, E.S., Drummond, D.R. and Sparrow, J.C. 1991. Post-translational processing of the amino terminus affects actin function. Eur. J. Biochem. 197:345-352.

Herrlich, P. and Schweiger, M. 1974. DNA- and RNA-directed synthesis in vitro of phage enzymes. Methods Enzymol. 30:654-669.

Hershey, H.P., Schwartz, L.J., Gale, J.P. and Abell, L.M. 1999. Cloning and functional expression of the small subunit of acetolactate synthase from *Nicotiana plumbaginifolia*. Plant Mol. Biol. 40:795-806.

Hervieu, F. and Vaucheret, H. 1996. A single amino acid change in acetolactate synthase confers resistance to valine in tobacco. Mol. Gen. Genet. 251:220-224.

Hill, R.L. and Brew, K. 1975. Lactose synthetase. Adv. Enzymol. 43:411-490.

Hofgen, R., Laber, B., Schuttke, I., Klonus, A.K., Streber, W. and Pohlenz, H.D. 1995. Repression of acetolactate synthase activity through antisense inhibition - Molecular and biochemical analysis of transgenic potato (*Solanum tuberosum* L cv Desiree) plants. Plant Physiol. 107:469-477.

Holtzclaw, W.D. and Chapman, L.F. 1975. Degradative acetolactate synthase of *Bacillus subtilis*: purification and properties. J. Bacteriol. 121:917-922.

Hood, E.E., Gelvin, S.B., Melchers, L.S. and Hoekema, A. 1993. New *agrobacterium* helper plasmids for gene transfer to plants. Transgenic Res. 2:208-218.

Huseby, N.E. and Stormer, F.C. 1971. The pH 6 acetolactate-forming enzyme from *Aerobacter aerogenes*. The effect of 2-oxobutyrate upon the enzyme activity. Eur. J. Biochem. 20:215-217.

Huseby, N.E., Christensen, T.B., Olsen, B.R. and Stormer, F.C. 1971. The pH 6 acetolactate-forming enzyme from *Aerobacter aerogenes*. Subunit structure. Eur. J. Biochem. 20:209-214.

Ito, T., Tashiro, K., Muta, S., Ozawa, R., Chiba, T., Nishizawa, M., Yamamoto, K., Kuhara, S. and Sakaki, Y. 2000. Toward a protein-protein interaction map of the budding yeast: A comprehensive system to examine two-hybrid interactions in all possible combinations between the yeast proteins. Proc. Natl. Acad. Sci. USA 97:1143-1147.

Jefferson, R.A. 1987. Assaying chimeric genes in plants: the GUS fusion system. Plant Mol. Biol. Rep. 5:387-405.

Jefferson, R.A., Bevan, M. and Kavanagh, T.A. 1986. The use of the *Escherichia coli* β-glucuronidase gene as a gene fusion marker for studies of gene expression in higher plants. Biochem. Soc. Trans. 15:17-18.

Johansen, L., Bryn, K. and Stormer, F.C. 1975. Physiological and biochemical role of the butanediol pathway in *Aerobacter (Enterobacter) aerogenes*. J. Bacteriol. 123:1124-1130.

Johnston, M. 1987. A model fungal gene regulatory mechanism: the *GAL* genes of *Saccharomyces cerevisiae*. Microbiol. Rev. 51:458-476.

Jones, S. and Thornton, J.M. 1995. Protein-protein interactions: a review of protein dimer structures. Prog. Biophys. Mol. Biol. 63:31-65.

Jones, S. and Thornton, J.M. 1996. Principles of protein-protein interactions. Proc. Natl. Acad. Sci. USA 93:13-20.

Kaneko, T., Matsubayashi, T., Sugita, M. and Sugiura, M. 1996. Physical and gene maps of the unicellular cyanobacterium *Synechococcus* sp. strain PCC6301 genome. Plant Mol. Biol. 31:193-201.

Kao, H.M., Keller, W.A., Gleddie, S. and Brown, G.G. 1992. Synthesis of *Brassica-oleracea Brassica-napus* somatic hybrid plants with novel organelle DNA compositions. Theor. Appl. Genet. 83:313-320.

Karn, J., Brenner, S., Barnett, L. and Cesareni, G. 1980. Novel bacteriophage lambda cloning vector. Proc. Natl. Acad. Sci. USA 77:5172-5176.

Kasher, M.S., Wakulchik, M., Cook, J.A. and Smith, M.C. 1993. One-step purification of recombinant human papillomavirus type 16 E7 oncoprotein and its binding to the retinoblastoma gene product. Biotechniques 14:630-641.

Keegan, L., Gill, G. and Ptashne, M. 1986. Separation of DNA binding from the transcription-activating function of a eukaryotic regulatory protein. Science 231:699-704.

Keeler, S.J., Sanders, P., Smith, J.K. and Mazur, B.J. 1993. Regulation of tobacco acetolactate synthase gene expression. Plant Physiol. 102:1009-1018.

Kerppola, T.K. and Curran, T. 1991. Fos-Jun heterodimers and Jun homodimers bend DNA in opposite orientations: implication for transcription factor cooperativity. Cell 66:317-326.

Klenk, H.P., Clayton, R.A., Tomb, J.F., White, O., Nelson, K.E., Ketchum, K.A., Dodson, R.J., Gwinn, M., Hickey, E.K., Peterson, J.D., Richardson, D.L., Kerlavage, A.R., Graham, D.E., Kyrpides, N.C., Fleischmann, R.D., Quackenbush, J., Lee, N. H., Sutton, G.G., Gill, S., Kirkness, E.F., Dougherty, B.A., McKenney, K., Adams, M.D., Loftus, B. Peterson, S., Reich, C.I., McNeil, L.K., Badger, J.H., Glodek, A., Zhou, L., Overbeek, R., Gocayne, J.D., Weidman, J.F., McDonald, L., Utterback, T., Cotton, M.D., Spriggs, T., Artiach, P., Kaine, B.P., Sykes, S.M., Sadow, P.W., D'Andrea, K.P., Bowman, C., Fujii, C., Garland, S.A., Mason, T.M., Olsen, G.J., Fraser, C.M., Smith, H.O., Woese, C.R. and Venter, J.C. 1997. The complete genome sequence of the hyperthermophilic, sulphate-reducing archaeon *Archaeoglobus fulgidus*. Nature 390:364-370.

Kleschick, J.V. 1994. Tiazolopyrimidine sulfonanilide herbicides and related compounds. In Herbicides Inhibiting Branched-Chain Amino Acid Biosynthesis - Recent Developments, ed. J. Stetter, pp. 119-144. Springer-Verlag, Berlin.

Klotz, I.M., Darnall, D.W. and Langerman, N.R. 1975. Quarternary structure of proteins. In The proteins, eds. H. Neurath and R.L. Hill, pp. 293-411. Academic Press, Inc., NY.

Koch, C.A., Anderson, D., Moran, M.F., Willis, C. and Pawson, T. 1991. SH2 and SH3 domains: elements that control interactions of cytoplasmic signaling proteins. Science 225:668-674.

Kohalmi, S.E., Nowak, J. and Crosby, W.L. 1995. cDNA library screening with the yeast two-hybrid system. A Laboratory Guide. Gene Expression Group of the Plant Biotechnology Institute, Saskatoon, SK.

Kohalmi, S.E., Nowak, J. and Crosby, W.L. 1997. A practical guide to using the yeast 2-hybrid system. In Differentially Expressed Genes in Plants: A Bench Manual, eds. G. Harper and E. Hansen, pp. 63-82. Taylor & Francis, London.

Kohalmi, S.E., Reader, L.J.V., Samach, A., Nowak, J., Haughn, G.W. and Crosby, W.L. 1998. Identification and characterization of protein interactions using the yeast 2-hybrid system. Plant. Mol. Biol. Man. M1:1-30.

Koncz, C. and Schell, J. 1986. The promoter of TL-DNA gene 5 controls the tissue-specific expression of chimaeric genes carried by a novel type of *Agrobacterium* binary vector. Mol. Gen. Genet. 204:383-396.

Kunst, F., Ogasawara, N., Moszer, I., Albertini, A.M., Alloni, G., Azevedo, V., Bertero, M.G., Bessieres, P., Bolotin, A., Borchert, S., Borriss, R., Boursier, L., Brans, A., Braun, M., Brignell, S.C., Bron, S., Brouillet, S., Bruschi, C.V., Caldwell,B., Capuano, V., Carter, N.M., Choi, S.K., Codani, J.J., Connerton, Cummings, N.J., Daniel, R.A., Denizot, F., Devine, K.M., Duesterhoeft, A., Ehrlich, S.D., Emmerson, P.T., Entian, K.D., Errington, J., Fabret, C., Ferrari, E., Foulger, D., Fritz, C., Fujita, M., Fujita, Y., Fuma, S., Galizzi, A., Galleron, N., Ghim, S.Y., Glaser, P., Goffeau,

A., Golightly, E.J., Grandi, G., Guiseppi, G., Guy, B.J., Haga, K., Haiech, J., Harwood, C.R., Henaut, A., Hilbert, H., Holsappel, S., Hosono, S., Jones, L., Joris, B., Karamata, D., Kasahara, Y., Klaerr-Blanchard, M., Klein, C., Kobayashi, Y., Koetter, P., Koningstein, G., Krogh, S., Kumano, M., Kurita, K., Lapidus, A., Lardinois, S., Lauber, J., Lazarevic, V., Lee, S.M., Levine, A., Liu, H., Masuda, S., Mauel, C., Medigue, C., Medina, N., Mellado, R.P., Mizuno, M., Moestl, D., Nakai, S., Noback, M., Noone, D., O'Reilly, M., Ogawa, K., Ogiwara, A., Oudega, B., Park, S.H., Parro, V., Pohl, T.M., Portetelle, D., Porwollik, S., Prescott, A.M., Presecan, E., Pujic, P., Purnelle, B., Rapoport, G., Rey, M., Reynolds, S., Rieger. M., Rivolta, C., Rocha, E., Roche, B., Rose, M., Sadaie, Y., Sato, T., Scanlan, E., Schleich, S., Schroeter, R., Scoffone, F., Sekiguchi, J., Sekowska, A., Seror, S.J., Serror, P., Shin, B.S., Soldo, B., Sorokin, A., Tacconi, E., Takagi, T., Takahashi, H., Takemaru, K., Takeuchi, M., Tamakoshi, A., Tanaka, T., Terpstra, P., Tognoni, A., Tosato, V., Uchiyama, S., Vandenbol, M., Vannier, F., Vassarotti, A., Viari, A., Wambutt, R., Wedler, E., Wedler., Weitzenegger, T., Winters, P., Wipat, A., Yamamoto, H., Yamane, K., Yasumoto, K., Yata, K., Yoshida, K., Yoshikawa, H.F., Zumstein, E., Yoshikawa, H. and Danchin, A. 1997. The complete genome sequence of the gram-positive bacterium *Bacillus subtilis*. Nature 390:249-256.

Ladner, D.W. 1994. Recent studies of imidazolinone herbicides and related compounds. In Herbicides Inhibiting Branched-Chain Amino Acid Biosynthesis - Recent Developments, ed. J. Stetter, pp. 83-118. Springer-Verlag, Berlin.

Landschulz, W.H., Johnson, P.F. and McKnight, S.L. 1988. The leucine zipper: a hypothetical structure common to a new class of DNA binding proteins. Science 240:1759-1764.

LaRossa, R.A. and Schloss, J.V. 1984. The sulfonylurea herbicide sulfometuron methyl is an extremely potent and selective inhibitor of acetolactate synthase in *Salmonella typhimurium*. J. Biol. Chem. 259:8753-8757.

Lee, K.Y., Townsend, J., Black, M., Chui, C.-F., Mazur, B., Dunsmuir, P. and Bedbrook, J. 1988. The molecular basis of sulfonylurea herbicide resistance in tobacco. EMBO J. 7:1241-1248.

Leutwiler, L.S., Hough-Evans, B.R. and Meyerowitz, E.M. 1984. The DNA of *Arabidopsis thaliana*. Mol. Gen. Genet. 194:15-23.

Lin, X., Kaul, S., Rounsley, S.D., Shea, T.P., Benito, M.I., Town, C.D., Fujii, C.Y., Mason, T.M., Bowman, C.L., Barnstead, M.E., Feldblyum, T.V., Buell, C.R., Ketchum, K.A., Lee, J.J., Ronning, C.M., Koo, H., Moffat, K.S., Cronin, L.A., Shen, M., Van Aken, S.E., Umayam, L., Tallon, L.J., Gill, J.E., Adams, M.D., Carrera, A.J., Creasy, T.H., Goodman, H.M., Somerville, C.R., Copenhaver, G.P., Preuss, D., Nierman, W.C., White, O., Eisen, J.A., Salzberg, S.L., Fraser, C.M. and Venter, J.C. 1999. Sequence and analysis of chromosome 2 of the plant *Arabidopsis thaliana*. Nature 402:761-768.

Liu, H.M., Booth, T.F. and Roy, P. 1992. Interactions between bluetongue virus core and capsid proteins translated *in vitro*. J. Gen. Virol. 73:2577-2584.

Ma, J. and Ptashne, M. 1987. A new class of yeast transcriptional activators. Cell 51:113-119.

Malthe-Sorenssen, D. and Stormer, F.C. 1970. The pH 6 acetolactate-forming enzyme from *Serratia marcescens*. Purification and properties. Eur. J. Biochem. 14:127-132.

Mayer, B.J., Hamaguchi, M. and Hanafusa, H. 1988. A novel viral oncogene with structural similarity to phospholipase C. Nature 332:272-275.

Mazur, B.J., Chui, C.-F. and Smith, J.K. 1987. Isolation and characterization of plant genes coding for acetolactate synthase, the target enzyme for two classes of herbicides. Plant Physiol. 85:1110-1117.

McAlister-Henn, L., Gibson, N. and Panisko, E. 1999. Applications of the yeast two-hybrid system. Methods: A Companion to Methods Enzymol. 19:330-337.

McEwen, J. and Silverman, P. 1980a. Chromosomal mutations of *Escherichia coli* that alter expression of conjugative plasmid functions. Proc. Natl. Acad. Sci. USA 77:513-517.

McEwen, J. and Silverman, P. 1980b. Mutations in Genes *cpxA* and *cpxB* of *Escherichia coli* K-12 cause a defect in isoleucine and valine syntheses. J. Bacteriol. 144:68-73.

McEwen, J., Sambucetti, L. and Silverman, P. 1983. Synthesis of outer membrane proteins in *cpxA cpxB* mutants of *Escherichia coli* K-12. J. Bacteriol. 154:375-382.

McGee, S. and Hay, J.V. 1994. Recent developments in the chemistry of sulfonylurea herbicides. In Herbicides Inhibiting Branched-Chain Amino Acid Biosynthesis - Recent Developments, ed. J. Stetter, pp. 15-46. Springer-Verlag, Berlin.

McNabb, D.S. and Guarente, L. 1996. Genetic and biochemical probes for protein-protein interactions. Curr. Opin. Biotechnol. 7:554-559.

McSheffrey, S.A., McHughen, A. and Devine, M.D. 1992. Characterization of transgenic sulfonylurea-resistant flax (*Linum usitatissimum*). Theor. Appl. Genet. 84:480-486.

Meinke, D. and Koornneef, M. 1997. Community standards for *Arabidopsis* genetics. Plant J. 12:247-253.

Meinz, Jr. F. 2000. RNA degradation and models for post-transcriptional gene silencing. Plant Mol. Biol. 43:261-273.

Mendelsohn, A.R. and Brent, R. 1994. Protein interaction methods-toward an endgame. Science 284:1948-1954.

Meyer, P. 1996. Repeat-induced gene silencing: Common mechanisms in plants and fungi. Biol. Chem. (Hoppe-Seyler) 377:87-95.

Meyerowitz, E.M. and Somerville, C.R. 1994. Introduction. In *Arabidopsis*, eds. E.M. Meyerowitz and C.R. Somerville, pp. 1-6. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Miflin, B.J. 1969. Acetolactate synthase from barley seedlings. Phytochemistry 8:2271-2276.

Miflin, B.J. 1971. Cooperative feedback control of barley acetohydroxyacid synthetase by leucine, isoleucine, and valine. Arch. Biochem. Biophys. 146:542-550.

Miflin, B.J. 1974. The location of nitrite reductase and other enzymes related to amino acid metabolism in the plastids of root and leaves. Plant Physiol. 54:550-555.

Miflin, B.J. and Cave, P. 1972. The control of leucine, isoleucine and valine biosynthesis in a range of higher plants. J. Exp. Bot. 23:511-516.

Miller, J.H. 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Moran, M.F., Koch, C.A., Anderson, D., Ellis, C., England, L., Martin, G.S. and Pawson, T. 1990. Src homology region 2 domains direct protein-protein interactions in signal transduction. Proc. Natl. Acad. Sci. USA 87:8622-8626.

Mourad, G., Williams, D. and King, J. 1995. A double mutant allele, *csr1-4*, of *Arabidopsis thaliana* encodes an acetolactate synthase with altered kinetics. Planta 196:64-68.

Muhitch, M.J. 1988. Acetolactate synthase activity in developing maize (*Zea mays* L.) kernels. Plant Physiol. 86:23-27.

Muhitch, M.J., Shaner, D.L. and Stidham, M.A. 1987. Imidazolinones and acetohydroxyacid synthase from higher plants. Properties of the enzyme from maize suspension culture cells and evidence for the binding of Imazapyr to acetohydroxyacid synthase *in vivo*. Plant Physiol. 83:451-456.

Mullis, K.B., Faloona, F.A., Scharf, S.J., Saiki, R.K., Horn, G.T. and Erlich, H.A. 1986. Specific enzymatic amplification of DNA *in vitro*: the polymerase chain reaction. Cold Spring Harb. Symp. Quant. Biol. 51:263-273.

Murashige, T. and Skoog, F. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. 15:473-479.

Muskens, M.W.M., Vissers, A.P.A., Joseph, N.M. and Kooter, J.M. 2000. Role of inverted DNA repeats in transcriptional and post-transcriptional gene silencing. Plant Mol. Biol. 43:243-260.

Napoli, C., Lemieux, C. and Jorgensen, R. 1990. Introduction of a chimeric chalcone synthase gene into Petunia results in a reversible co-suppression of homologous genes in trans. Plant Cell 2:279-290.

O'Neill, J.P. and Freundlich, M. 1972. Two forms of biosynthetic acetohydroxy acid synthetase in *Salmonella typhimurium*. Biochem. Biophys. Res. Commun. 48:437-443.

O'Shea, E.K., Klemm, J.D., Kim, P.S. and Alber, T. 1991. X-ray structure of the GCN4 leucine zipper, a two stranded, parallel coiled coil. Science 254:539-544.

Ott, K.H., Kwagh, J.G., Stockton, G.W., Sidorov, V. and Kakefuda, G. 1996. Rational molecular design and genetic engineering of herbicide resistant crops by structure modeling and site-directed mutagenesis of acetohydroxyacid synthase. J. Mol. Biol. 263:359-368.

Otto, J.J. and Lee, S.-W. 1993. Immunoprecipitation methods. In Methods in Cell Biology, Vol. 37, ed. D. J. Asai, pp. 119-126. Academic Press, Inc., NY.

Ouellet, T., Rutledge, R.G. and Miki, B.L. 1992. Members of the acetohydroxyacid synthase multigene family of *Brassica napus* have divergent patterns of expression. Plant J. 2:321-330.

Pang, S.S. and Duggleby, R.G. 1999. Expression, purification, characterization, and reconstitution of the large-and small subunits of yeast acetohydroxyacid synthase. Biochem. 38:5222-5231.

Parkhill, J., Wren, B.W., Mungall, K., Ketley, J.M., Churcher, C., Basham, D., Chillingworth, T., Davies, R.M., Feltwell, T., Holroyd, S., Jagels, K., Karlyshev, A., Moule, S., Pallen, M.J., Penn, C.W., Quail, M., Rajandream, M.A., Rutherford, K.M., VanVliet, A., Whitehead, S. and Barrell, B.G. 2000a. The genome sequence of the food-borne pathogen *Campylobacter jejuni* reveals hypervariable sequences. Nature 403:665-668.

Parkhill, J., Achtman, M., James, K.D., Bentley, S.D., Churcher, C., Klee, S.R., Morelli, G., Basham, D., Brown, D., Chillingworth, T., Davies, R.M., Davis, P., Devlin, K., Feltwell, T., Hamlin, N., Holroyd, S., Jagels, K., Leather, S., Moule, S., Mungall, K., Quail, M.A., Rajandream, M.A., Rutherford, K.M., Simmonds, M., Skelton, J., Whitehead, S., Spratt, B.G. and Barrell, B.G. 2000b. Complete DNA sequence of a serogroup A strain of *Neisseria menigitidis* Z2491. Nature 404:502-506.

Pawson, A.J. (ed.). 1998. Protein Modules in Signal Transduction. Springer-Verlag, NY.

Peng, H.L., Wang, P.Y., Wu, C.M., Hwang, D.C. and Chang, H.Y. 1992. Cloning, sequencing and heterologous expression of a *Klebsiella pneumoniae* gene encoding an FAD-independent acetolactate synthase. Gene 117:125-130.

Phizicky, E.M. and Fields, S. 1995. Protein-protein interactions: Methods for detection and analysis. Microbiol. Rev. 59:94-123.

Porath, J. 1985. Immobilized metal ion affinity chromatography - a powerful method for protein purification. In Modern Method in Protein Chemistry, Vol. 2, ed. H. Tschesche, p. 85. Walter de Gruyter, NY.

Porath, J. 1988a. IMAC-immobilized metal ion affinity based chromatography. Trends Anal. Chem. 7: 254-259.

Porath, J. 1988b. High-performance immobilized-metal-ion affinity chromatography of peptides and proteins. J. Chromatogr. 443:3-11.

Porath, J. 1992. Immobilized metal ion affinity chromatography. Protein Expres. Puri. 3:263-281.

Porath, J., Carlsson, J., Olsson, J. and Belfrage, G. 1975. Methal chelate affinity chromatography, a new approach to protein fractionation. Nature 258:598-599.

Porpaczy, Z., Sumegi, B. and Alkonyi, I. 1983. Association between the α-ketoglutarate dehydrogenase complex and succinate thiokinase. Biochim. Biophys. Acta 749:172-179.

Poulsen, C. and Stougaard, P. 1989. Purification and properties of *Saccharomyces cerevisiae* acetolactate synthase from recombinant *Escherichia coli*. Eur. J. Biochem. 185:433-439.

Prelich, G., Tan, C.K., Kotura, M., Mathews, M.B., So, A.G., Downey, K.M. and Stillman, B. 1989. Functional identity of proliferating cell nuclear antigen and a DNA polymerase-δ auxiliary protein. Nature 326:517-520.

Pruitt, R.E. and Meyerowitz, E.M. 1986. Characterization of the genome of *Arabidopsis thaliana*. J. Mol. Biol. 187:169-183.

Pruitt, R.E., Chang, C., Pang, P.P.Y. and Meyerowitz, E.M. 1987. Molecular genetics and development of *Arabidopsis*. In Genetic Regulation of Development, ed. W.F. Loomis, pp. 327-338. Alan R. Liss, NY.

Ranganathan, G. and Kern, P.A. 1999. *In vitro* transcription and translation of lipoprotein lipase. In Methods in Molecular Biology, Vol. 109: Lipase and Phospholipase Protocols, eds. M.H. Doolittle and K. Reue, pp. 337-341. Humana Press, Inc., Totowo, NJ.

Rathinasabapathi, B. and King, J. 1991. Herbicide Resistance in *Datura Innoxia* - kinetic characterization of acetolactate synthase from wild-type and sulfonylurea-resistant cell variants. Plant Physiol. 96:255-261.

Rathinasabapathi, B., Williams, D. and King, J. 1990. Altered feedback sensitivity to valine, leucine and isoleucine of acetolactate synthase from herbicide-resistant variants of *Datura innoxia*. Plant Sci. 67:1-6.

Ray, T.B. 1984. Site of action of chlorsulfuron: inhibition of valine and isoleucine biosynthesis in plants. Plant Physiol. 75:827-831.

Rédei, G.P. 1992. A heuristic glance at the past of *Arabidopsis* genetics. In Methods in *Arabidopsis* Research, eds. C. Koncz, N-H. Chua and J. Schell, pp. 1-15. World Scientific Publishing Co., River Edge, NJ.

Reith, M. and Munholland, J. 1993. Two amino-acid biosynthetic genes are encoded on the plastid genome of the red alga *Porphyra umbilicalis*. Curr. Genet. 23:59-65.

Relton, J.M., Wallsgrove, R.M., Bourgin, J.P. and Bright, S.W.J. 1986. Altered feedback sensitivity of acetohydroxyacid synthase from valine-resistant mutants of tobacco *Nicotiana-tabacum*. Planta 169:46-50.

Rhodes, D., Hogan, A.L., Deal, L., Jamieson, G.C. and Haworth, P. 1987. Amino acid metabolism in *Lemna minor* L. I Responses to chlorsulforon. Plant Physiol. 84:775-780.

Rutledge, R.G., Ouellet, T., Hattori, J. and Miki, B.L. 1991. Molecular characterization and genetic origin of the *Brassica napus* acetohydroxyacid synthase multigene family. Mol. Gen. Genet. 229:31-40.

Ryan, E.D. and Kohlhaw, G.B. 1974. Subcellular localization of isoleucine-valine biosynthetic enzymes in yeast. J. Bacteriol. 120:631-637.

Sadowski, I., Stone, J.C and Pawson, T. 1986. A noncatalytic domain conserved among cytoplasmic protein-tyrosine kinases modifies the kinase function and transforming activity of Fujinami sarcoma virus $P130^{gag\text{-}fps}$. Mol. Cell. Biol. 6:4396-4408.

Sambrook, J., Fritsch, E.F. and Maniatis, T. 1990. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Sato, S., Kotani, H., Nakamura, Y., Kaneko, T., Asamizu, E., Fukami, M., Miyajima, N. and Tabata, S. 1997. Structural analysis of *Arabidopsis thaliana* chromosome 5. I. Sequence features of the 1.6 Mb regions covered by twenty physically assigned P1 clones. DNA Res. 4:215-230.

Schloss, J.V. 1994. Recent advances in understanding the mechanism and inhibition of acetolactate synthase. In Herbicides Inhibiting Branched-Chain Amino Acid Biosynthesis - Recent Developments, ed. J. Stetter, pp. 3-14. Springer-Verlag, Berlin.

Schloss, J.V., Ciskanik, L., Pai, E.F. and Thorpe, C. 1991. Acetolactate synthase: a deviant flavoprotein. In Flavins and Flavoproteins, eds. B. Curti, S. Ronchi and G. Zanetti, pp. 907-914. Walter de Gruyter & Co, NY.

Schloss, J.V., Van Dyk, D.E., Vasta, J.F. and Kutny, R.M. 1985. Purification and properties of *Salmonella typhimurium* acetolactate synthase isozyme II from *Escherichia coli* HB101/pDU9. Biochem. 24:4952-4959.

Schloss, J.V. 1992. Acetolactate synthase. In Chemistry and Biochemistry of Flavo enzymes, Vol. III, ed. F. Müller, pp. 532-542. CRC Press Inc., Boca Raton, FL.

Schloss, J.V. and Aulabaugh, A. 1990. Acetolactate synthase and ketol-acid reductoisomerase. In Biosynthesis of Branched Chain Amino Acids, eds. Z. Barak, D. M. Chipman and J.V. Schloss, pp. 329-356. VCH Verlagsgesellschaft mBH, Weinheim, Germany.

Schmid, E.L., Keller, T.A., Dienes, Z. and Vogel, H. 1997. Reversible oriented surface immobilization of functional proteins on oxide surfaces. Anal. Chem. 69:1979-1985.

Schmidt, R. 1998. The *Arabidopsis thaliana* genome: towards a complete physical map. In *Arabidopsis* - Annual Plant Reviews, Vol. 1, eds. M. Anderson and J.A. Roberts, pp. 1-30. Academic Press, Inc., Sheffield, England.

Shaner, D.L. and Singh, B.K. 1991. Imidazolinone-induced loss of acetohydroxyacid synthase activity in maize is not due to the enzyme degradation. Plant Physiol. 97:1339-1341.

Shaner, D.L. and Singh, B.K. 1993. Phytotoxicity of acetohydroxyacid synthase inhibitors is not due to accumulation of 2-ketobutyrate and or 2- aminobutyrate. Plant Physiol. 103:1221-1226.

Shaner, D.L., Anderson, P.C. and Stidham, M.A. 1984. Imidazolinones; potent inhibitors of acetohydroxyacid synthase. Plant Physiol. 76:545-546.

Shaw, K.J., Berg, C.M. and Sobol, T.J. 1980. *Salmonella typhimurium* mutants defective in acetohydroxy acid synthases I and II. J. Bacteriol. 141:1258-1263.

Singh, B.K., Lumanglas, A. and Wang, B.S. 1991a. Production of monocot-specific monoclonal antibody against acetohydroxyacid synthase and its use in the purification and characterization of the enzyme. Proc. Natl. Acad. Sci. USA 88:4572-4576.

Singh, B.K., Schmitt, G., Lilis, M., Hand, J.M. and Misra, R. 1991b. Overexpression of acetohydroxyacid synthase from *Arabidopsis* as an inducible fusion protein in *Escherichia coli*. Production of polyclonal antibody, and immunological characterization of the enzyme. Plant Physiol. 97:657-662.

Singh, B.K., Stidham, M.A. and Shaner, D.L. 1988a. Separation and characterization of two from of acetohydroxy acid synthase from Black Mexican Sweet corn cells. J. Chromatogr. 444:251-261.

Singh, B.K., Stidham, M.A. and Shaner, D.L. 1988b. Assay of acetohydroxy synthase. Anal. Biochem. 171:173-179.

Singh, B., Szamosi, I., Hand, J.M. and Misra, R. 1992. *Arabidopsis* acetohydroxyacid synthase expressed in *Escherichia coli* is insensitive to the feedback inhibitors. Plant Physiol. 99:812-816.

Smith, D.R., Doucette-Stamm, L.A., Deloughery, C., Lee, H.-M., Dubois, J., Aldredge, T., Bashirzadeh, R., Blakely, D., Cook, R., Gilbert, K., Harrison, D., Hoang, L., Keagle, P., Lumm, W., Pothier, B., Qiu, D., Spadafora, R., Vicare, R., Wang, Y., Wierzbowski, J., Gibson, R., Jiwani, N., Caruso, A., Bush, D., Safer, H., Patwell, D., Prabhakar, S., McDougall, S., Shimer, G., Goyal, A., Pietrovski, S., Church, G.M., Daniels, C.J., Mao, J.-i., Rice, P., Nolling, J. and Reeve, J.N. 1997. Complete genome sequence of *Methanobacterium thermoautotrophicum* deltaH: functional analysis and comparative genomics. J. Bacteriol. 179:7135-7155.

Somerville, C.R. 1993. New opportunities to dissect and manipulate plant processes. Philos. Trans. R. Soc. Lond. (Biol.) 339:199-206.

Southan, M.D. and Copeland, L. 1996. Physical and kinetic properties of acetohydroxyacid synthase from wheat leaves. Physiol. Plant. 98:824-832.

Southern, E.M. 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. 98:503-517.

Srere, P.A. 1987. Complexes of sequential metabolic enzymes. Annu. Rev. Biochem. 56:89-124.

Srere, P.A. 1999. Editorial. Methods: A Companion to Methods Enzymol. 19:193.

Stiege, W. and Erdmann, V.A. 1995. The potentials of the *in vitro* protein biosynthesis system. J. Biotechnol. 41:81-90.

Stormer, F.C. 1967. Isolation of crystalline pH 6 acetolactate-forming enzyme from *Aerobacter aerogenes*. J. Biol. Chem. 242:1756-1759.

Stormer, F.C. 1968a. The pH 6 acetolactate-forming enzyme from *Aerobacter aerogenes*. II. Evidence that it is not a flavoprotein. J. Biol. Chem. 243:3740-3741.

Stormer, F.C. 1968b. The pH 6 acetolactate-forming enzyme from *Aerobacter aerogenes*. I. Kinetic studies. J. Biol. Chem. 243:3735-3739.

Stormer, F.C. and Umbarger, H.E. 1964. The requirement for flavin adenine dinucleotide in the formation of acetolactate by *Salmonella typhimurium* extracts. Biochem. Biophys. Res. Commun. 17:587.

Subramanian, M.V., Hung, H.Y., Dias, J.M., Miner, V.W., Butler, J.H. and Jachetta, J.J. 1990. Properties of mutant acetolactate synthases resistant to triazolopyrimidine sulfonanilide. Plant Physiol. 94:239-244.

Sulkowski, E. 1989. The saga of IMAC and MIT. Bioessays 10(5):170-175.

Swanson, E.B., Herrgesell, M.J., Arnoldo, M., Sippell, D.W. and Wong, R.S. 1989. Microspore mutagenesis and selection. Canola plants with field tolerance to the imidazolinones. Theor. Appl. Genet. 78:525-530.

Tachibana, K. 1987. Herbicide characteristics of bialaphos. In Pesticide Science and Biotechnology, eds. R. Greenhalgh and T.R. Roberts, pp. 145-148. Blackwell Science Publications, Oxford.

Tanaka, H. and Kuwana, H. 1984. A basal unit of valine-sensitive acetolactate synthase of *Neurospora crassa*. Biochem Biophys. Res. Commun. 123:418-423.

Tatusova, T.A. and Madden, T.L. 1999. BLAST 2 Sequences, a new tool for comparing protein and nucleotide sequences. FEMS Microbiol. Lett. 174:247-250.

The Arabidopsis Genome Initiative. Analysis of the genome sequence of the flowering plant *Arabidopsis thaliana*. Nature 408:796-815.

Thompson, J.D., Higgins, D.G. and Gibson, T.J. 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, positions-specific gap penalties and weight matrix choice. Nucleic Acids Res. 22:4673-4680.

Tsui, P. and Freundlich, M. 1990. Antitermination of transcription at the *ilvBN* attentuator requires integration host factor. In Biosynthesis of Branched Chain Amino Acids, eds. Z. Barak, D.M. Chipman, and J.V. Schloss, pp. 53-67. VCH Verlagsgesellschaft mBH, Weinheim, Germany.

Turner, R. and Foster, G.D. 1998. *In vitro* transcription and translation. In Methods in Molecular Biology, Vol. 81: Plant Virology Protocols, eds. G.D. Foster and S.C. Taylor, pp. 293-299. Humana Press, Inc., Totowa, NJ.

Umbarger, H.E. 1978. Amino acid biosynthesis and its regulation. Annu. Rev. Biochem. 47:532-606.

Umbarger, H.E. 1987. *Escherichia coli* and *Salmonella typhimurium*. In Cellular and Molucular Biology, Vol. 1, eds. F.C. Neidhardt, J.L. Ingraham, B.L. Low, B. Magasanik, M. Schaechter, H.E. Umbarger, pp.352-367. American Society for Microbiology Press, Washington DC.

Umbarger, H.E. 1996. Biosynthesis of the branched-chain amino acids. In *Escherichia coli* and *Salmonella*, eds. F.C. Neidhardt, R. Curtiss III, J.L. Ingraham, E.C.C. Lin, K.B. Low, B. Magasanik, W.S. Reznikoff, M. Riley, M. Schaechter, and H.E. Umbarger, pp. 442-457. American Society for Microbiology Press, Washington DC.

Van der Krol, A.R., Mur, L.A., Beld, M., Mol, J.N.M. and Stuitje, A.R. 1990. Flavonoid genes in petunia - addition of a limited number of gene copies may lead to a suppression of gene expression. Plant Cell 2:291-299.

Velot, C., Mixon, M.B., Teige, M. and Srere, P.A. 1997. Model of a quinary structure between Krebs TCA cycle enzymes: a model for the metabolon. Biochemistry 36:14271-14276.

Vergunst, A.C., de Waal, E.C. and Hooykaas, P.J.J. 1998. Root transformation by *Agrobacterium tumefaciens*. In *Arabidopsis* Protocol - Methods in Molecular Biology, Vol. 82, eds. J.M. Martinez-Zapater and J. Salinas, pp. 227-244. Humana Press, Inc., Totowa, NJ.

Voet, D. and Voet, J.G. 1995. Biochemistry. J. Wiley & Sons, NY.

Vyazmensky, M., Sella, C., Barak, Z. and Chipman, D. 1996. Isolation and characterization of subunits of acetohydroxy acid synthase isozyme III and reconstitution of holoenzyme. Biochemistry 35:10339-10346.

Wallsgrove, R.M. 1990. The biochemistry and genetics of branched-chain amino acid biosynthesis in higher plants. In Biosynthesis of Branched Chain Amino Acids, eds. Z. Barak, D.M. Chipman, and J.V. Schloss, pp. 43-51. VCH Verlagsgesellschaft mBH, Weinheim, Germany.

Weber, J., Lee, R.S.F., Wilke-Mounts, S., Grell, E. and Senior, A.E. 1993. Combined application of side directed mutagenesis, 2-azido-ATP labeling, and lin-benzo-ATP binding to study the noncatalystic sites of *Escherichia coli* F_1-ATPase. J. Biol. Chem. 268:6241-6247.

White, R.J. and Jackson, S.P. 1992. The TATA-binding protein - A central role in transcription by RNA Polymerase-I, Polymerase-II and Polymerase-III. Trends. Genet. 8:284-288.

Wiersma, P.A., Schmiemann, M.G., Condie, J.A., Crosby, W.L., and Moloney, M.M. 1989. Isolation, expression and phylogenetic inheritance of an acetolactate synthase gene from *Brassica napus*. Mol. Gen. Genet. 219:413-420.

Wittenbach, V.A., Rayner, D.R. and Schloss, J.V. 1992. Pressure points in the biosynthetic pathway for branched-chain amino acids. In Biosynthesis and Molecular Regulation of Amino Acids in Plants, eds. B.K. Singh, H.E. Flores, and J.C. Shannon, pp. 69-88. American Society of Plant Physiologists, Rockville, MD.

Woodward, W.R., Ivey, J.L. and Herbert, E. 1974. Protein synthesis with rabbit reticulocyte preparations. Methods Enzymol. 30:724-731.

Wu, K., Mourad, G. and King, J. 1994. A valine-resistant mutant of *Arabidopsis thaliana* displays an acetolactate synthase with altered feedback control. Planta 192:249-255.

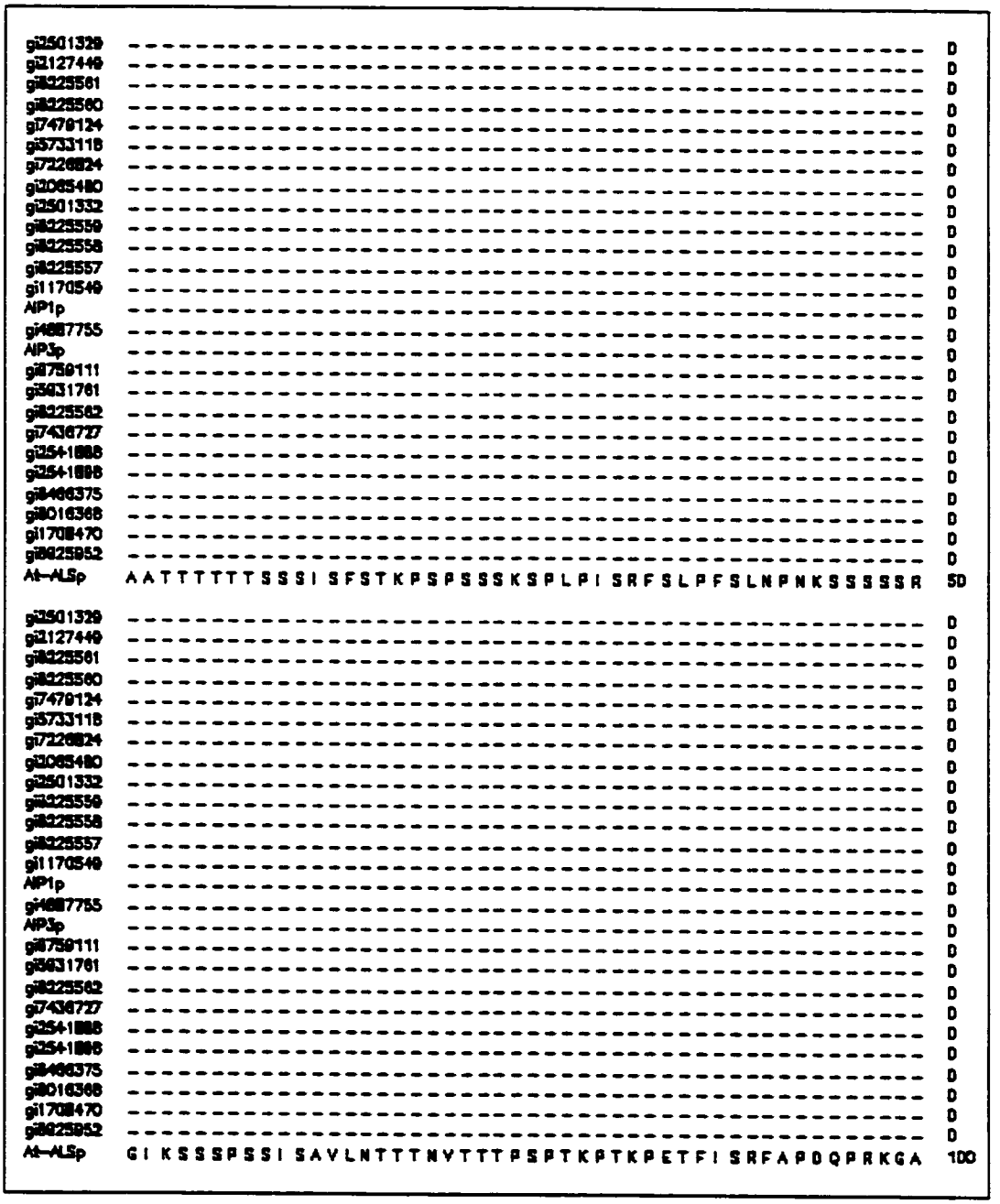
Yip, T.T. and Hutchens, T.W. 1994. Immobilized metal ion affinity chromatography. Mol. Biotechnol. 1:151-164.

Zambrysky, P. 1997. Genetic manipulation of plants. In Exploring Genetic Mechanisms, eds. M. Singer and P. Berg, p. 597. University Science Books, Sausalito, CA.

Zubay, G. 1973. *In-vitro* synthesis of protein in microbial system. Annu. Rev. Genet. 7:267-287.

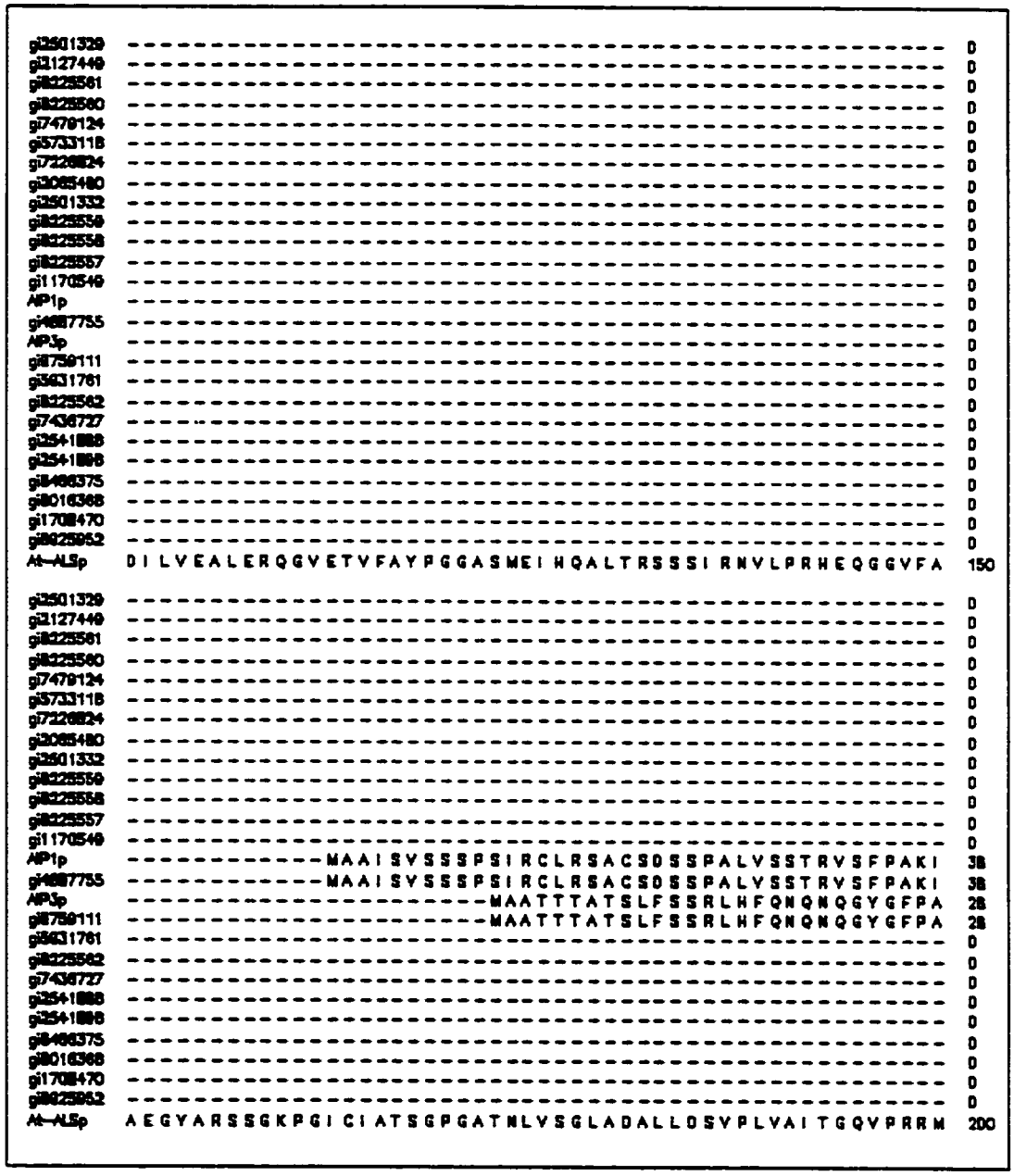
APPENDICES

Appendix A1



Appendix A1. Multiple sequence alignments using Clustal W algorithms (Thompson *et al.*, 1994) of ALS (At-ALSp), N-domains of AIP1p, AIP3 and protein sequences that showed ≥65% similarity to AIP1p and AIP3p. Each GI number corresponds to its description in Table 4.1 and 4.2.

Appendix A1 continued.



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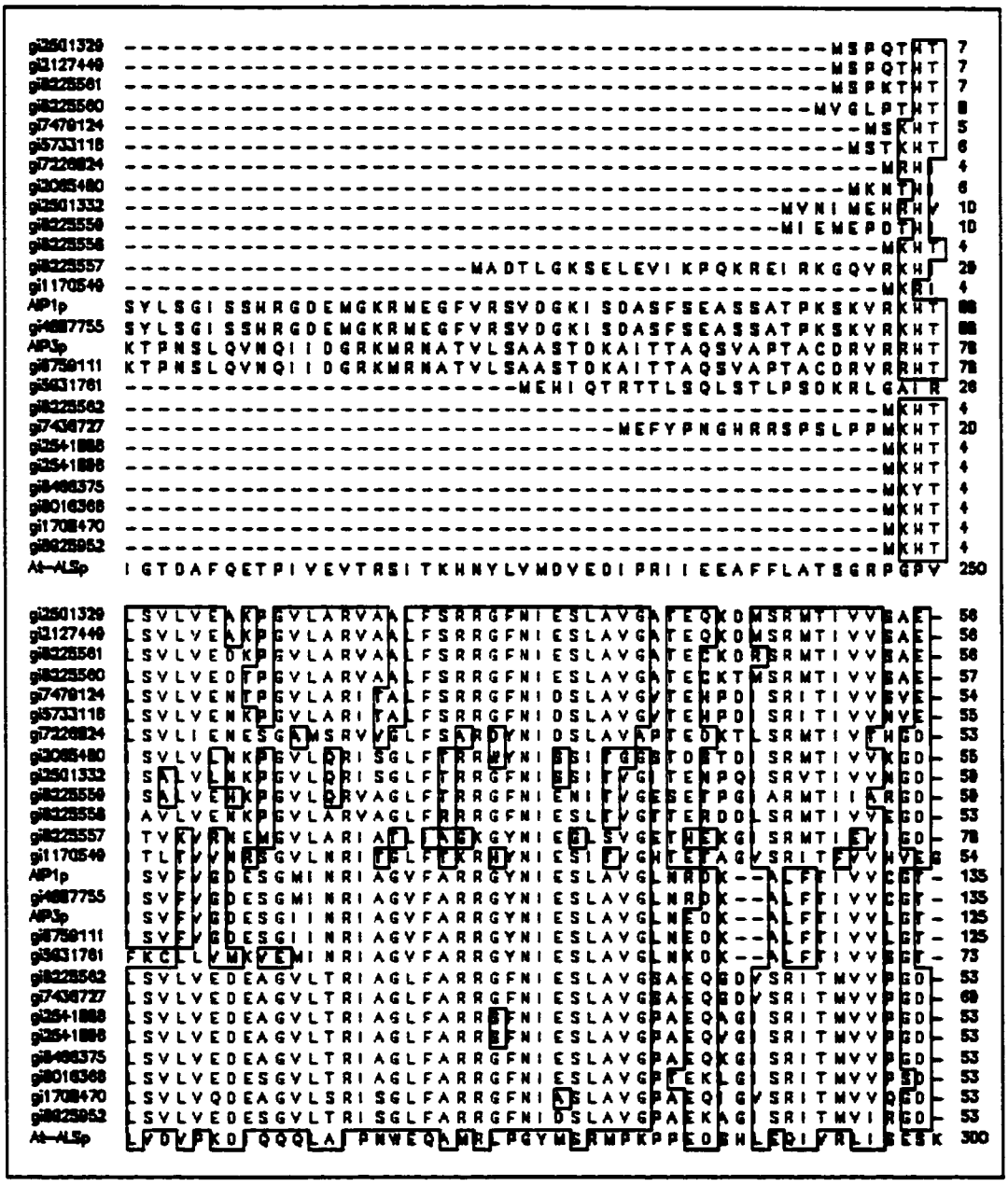

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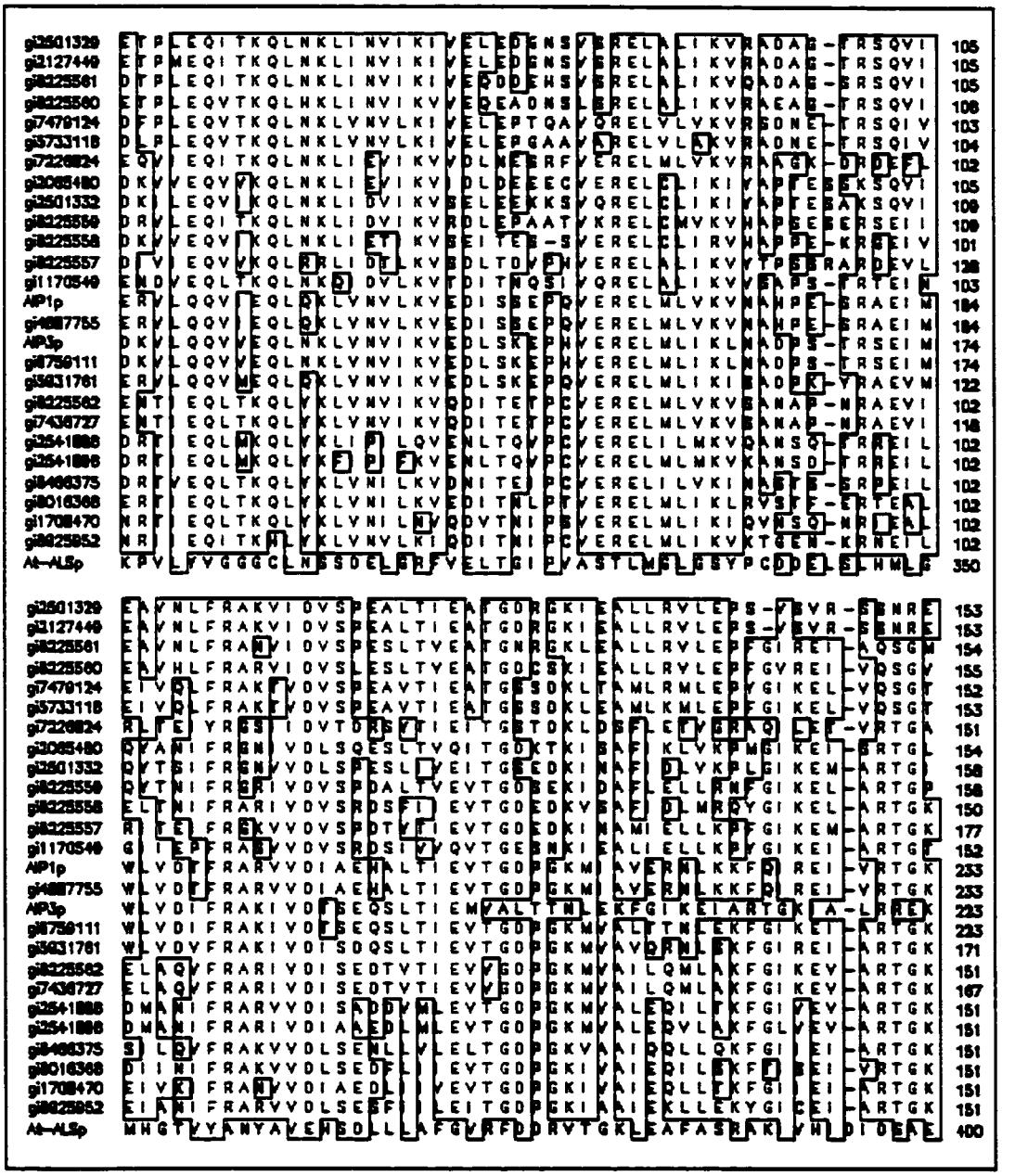

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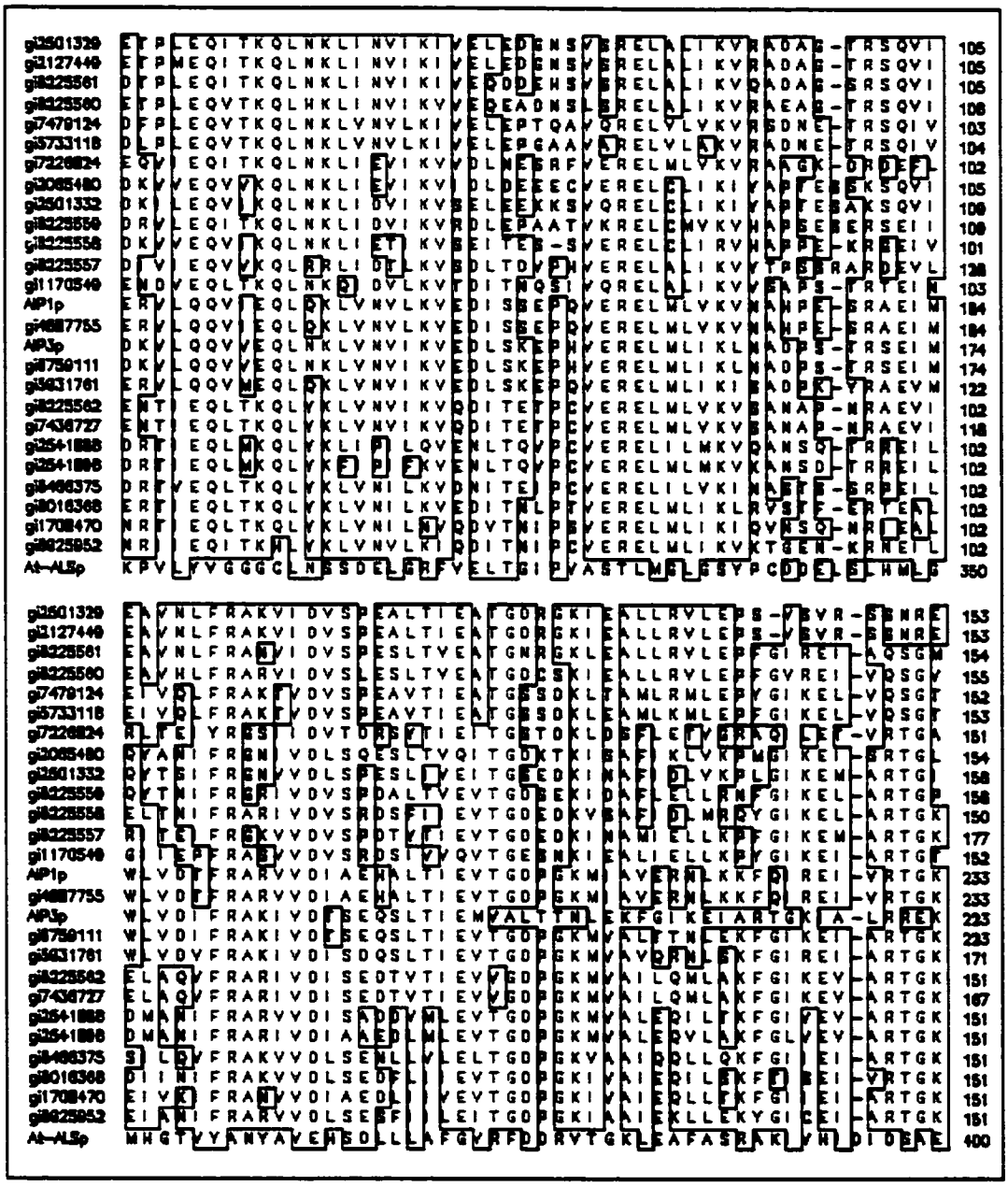

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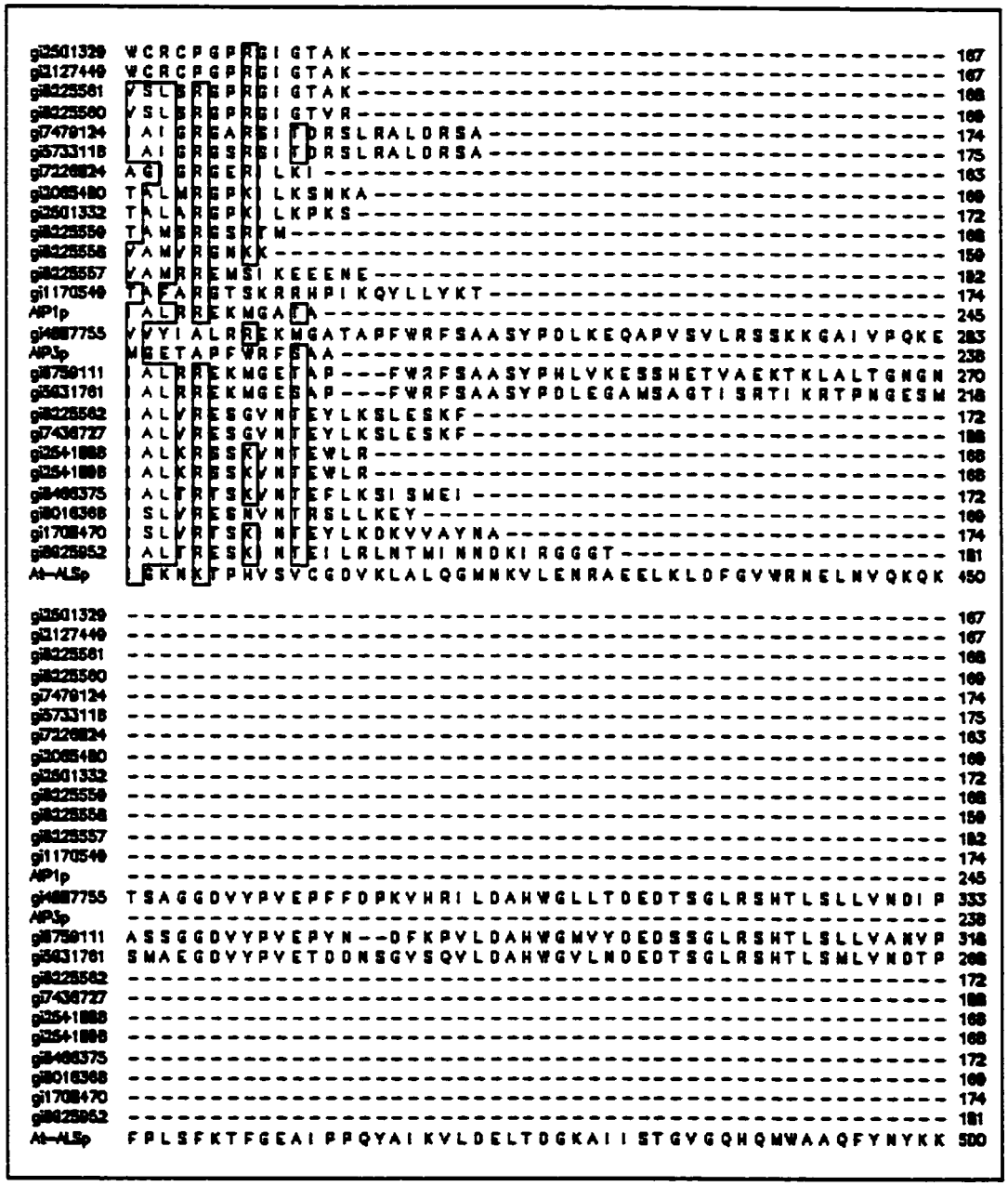

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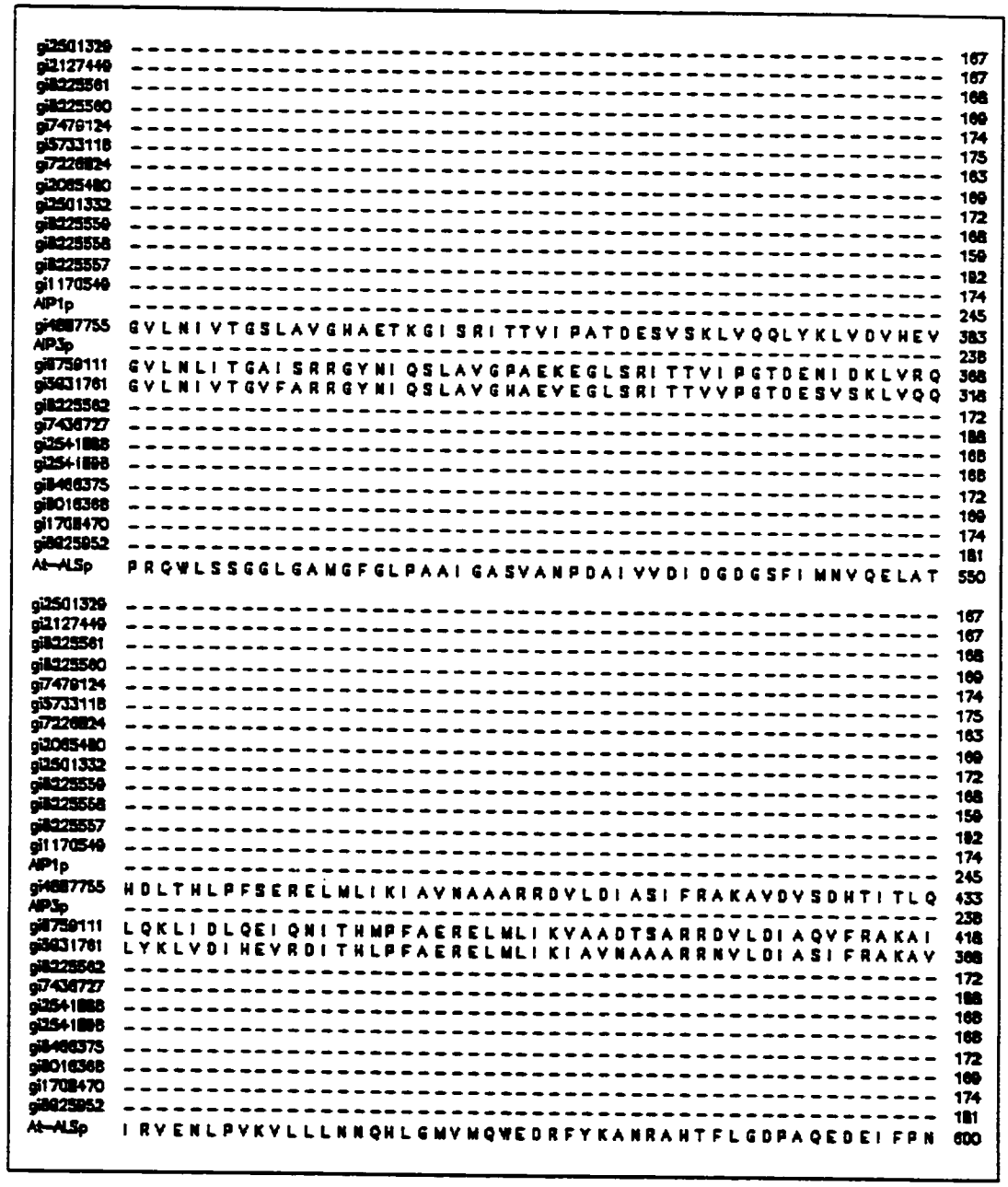

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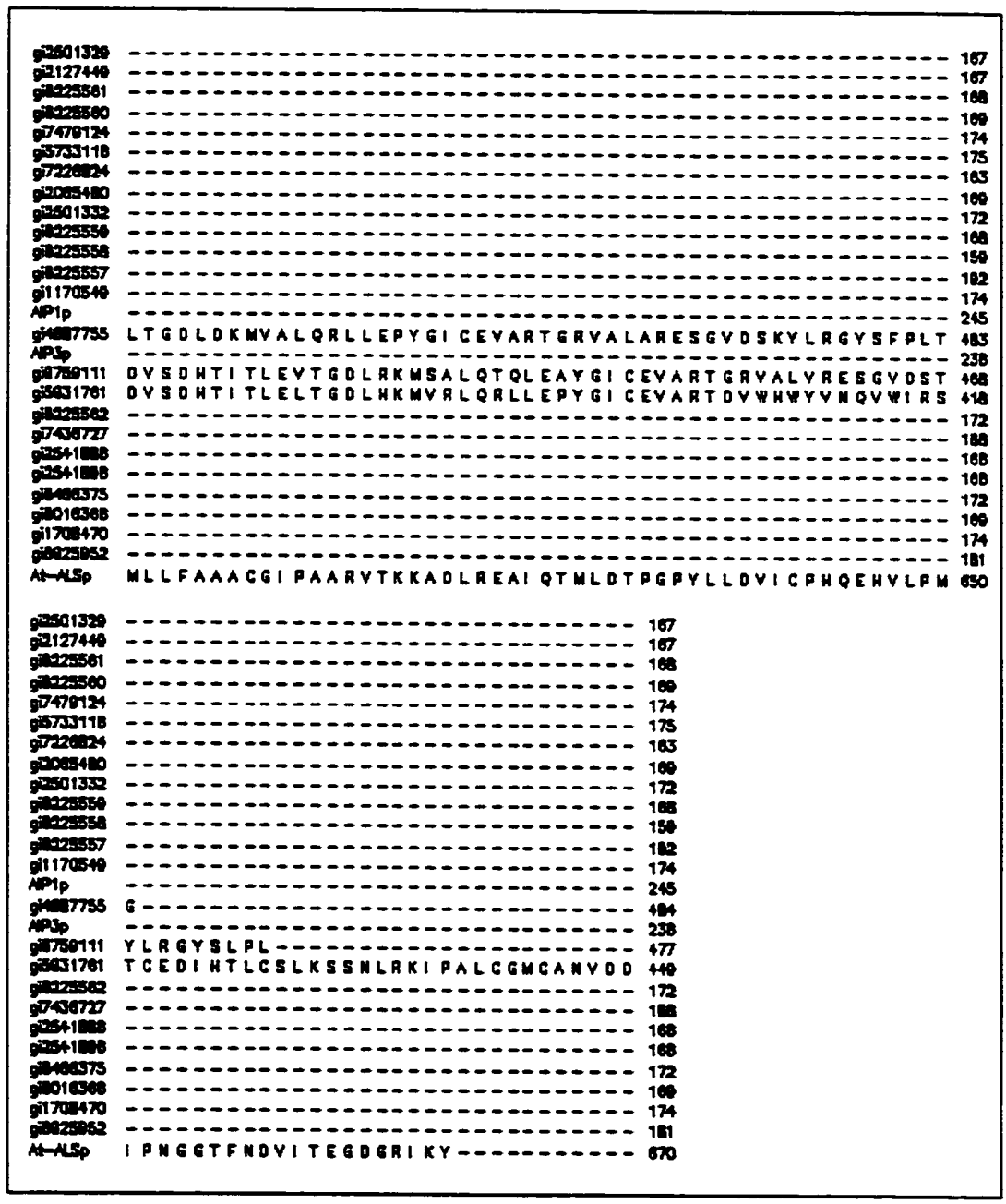
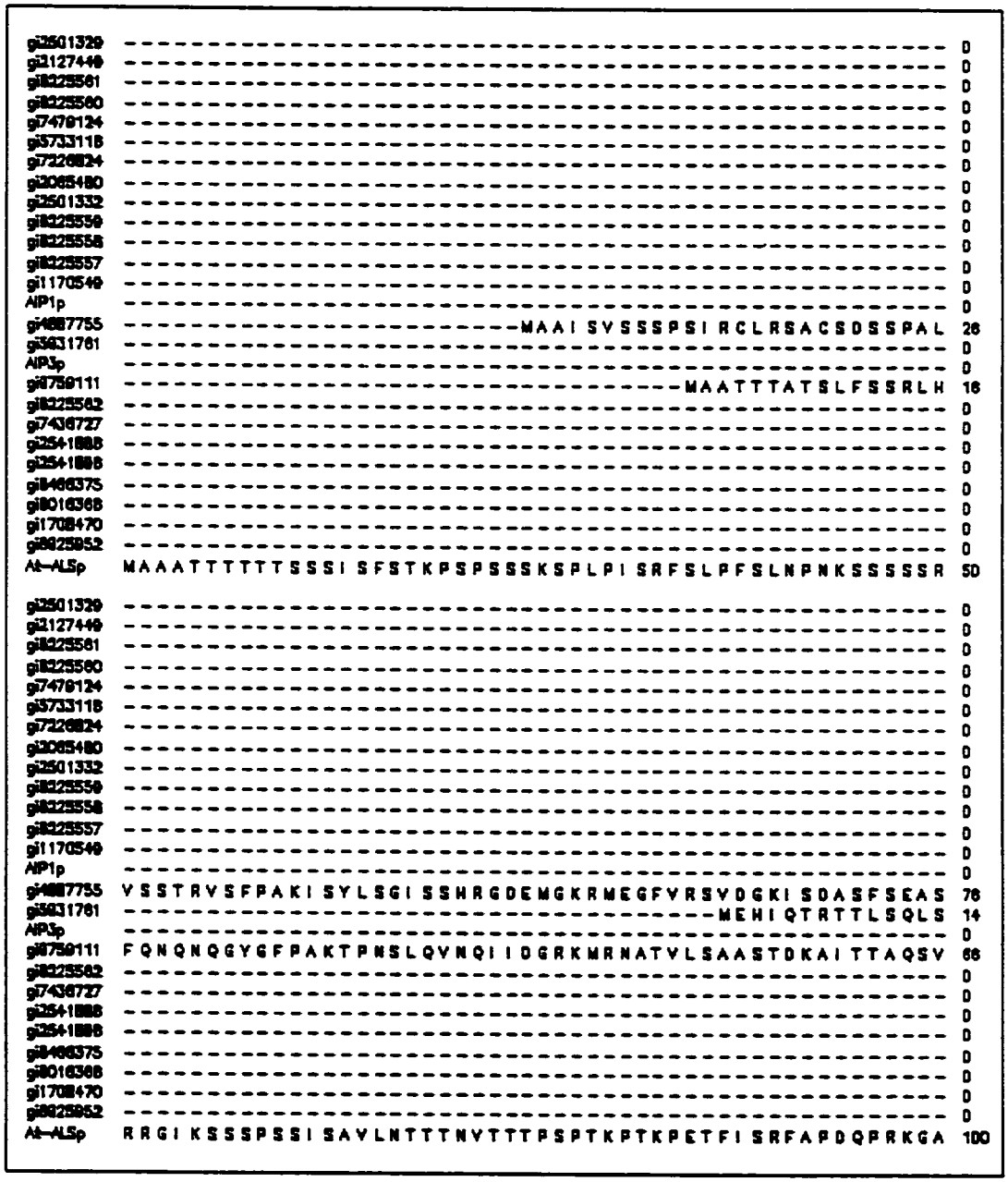
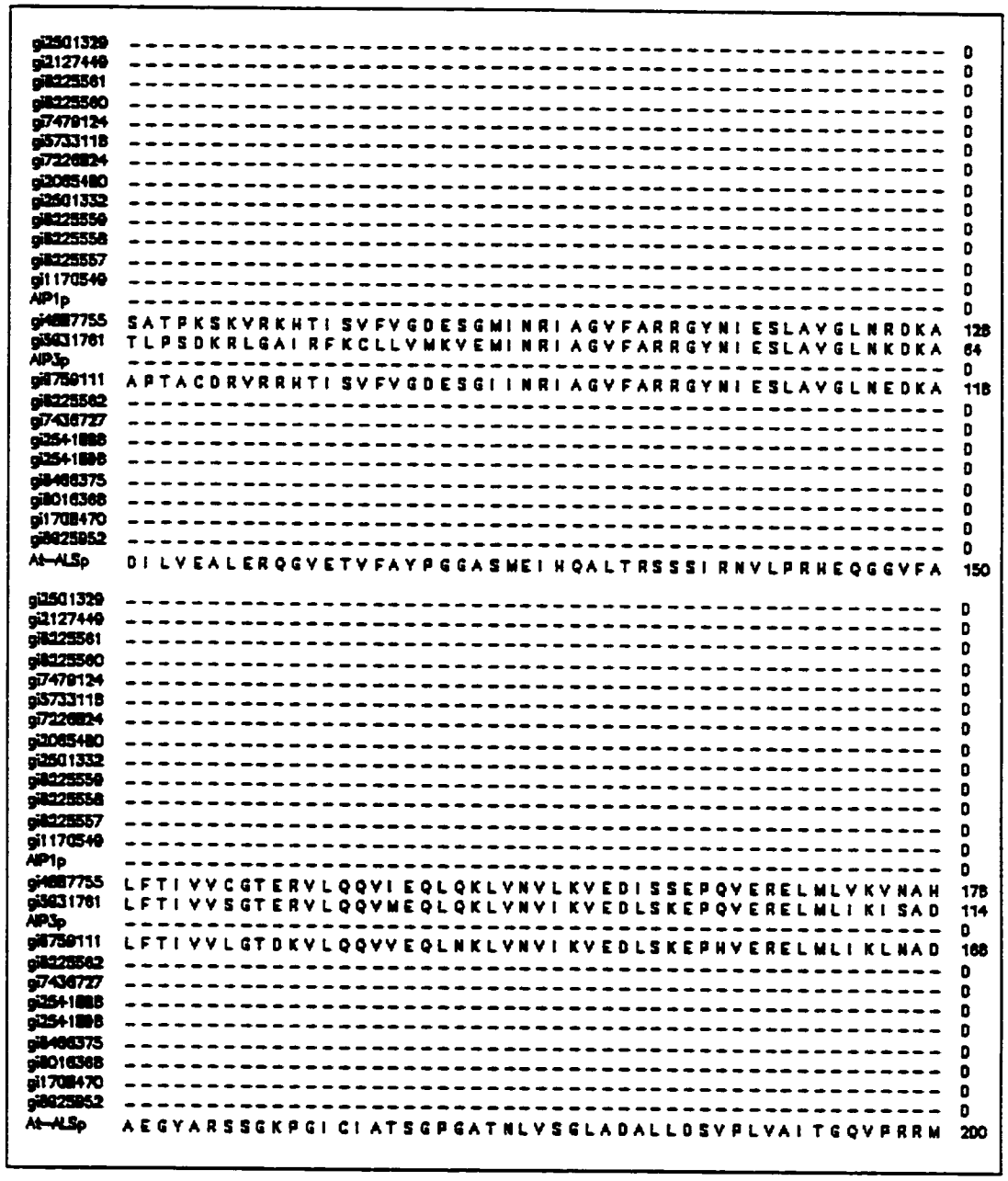


Appendix A2



Appendix A2. Multiple sequence alignments using Clustal W algorithms (Thompson *et al.*, 1994) of ALS (At-ALSp), C-domains of AIP1p, AIP3 and protein sequences that showed ≥65% similarity to AIP1p and AIP3p. Each GI number corresponds to its description in Table 4.1 and 4.2.

Appendix A2 continued



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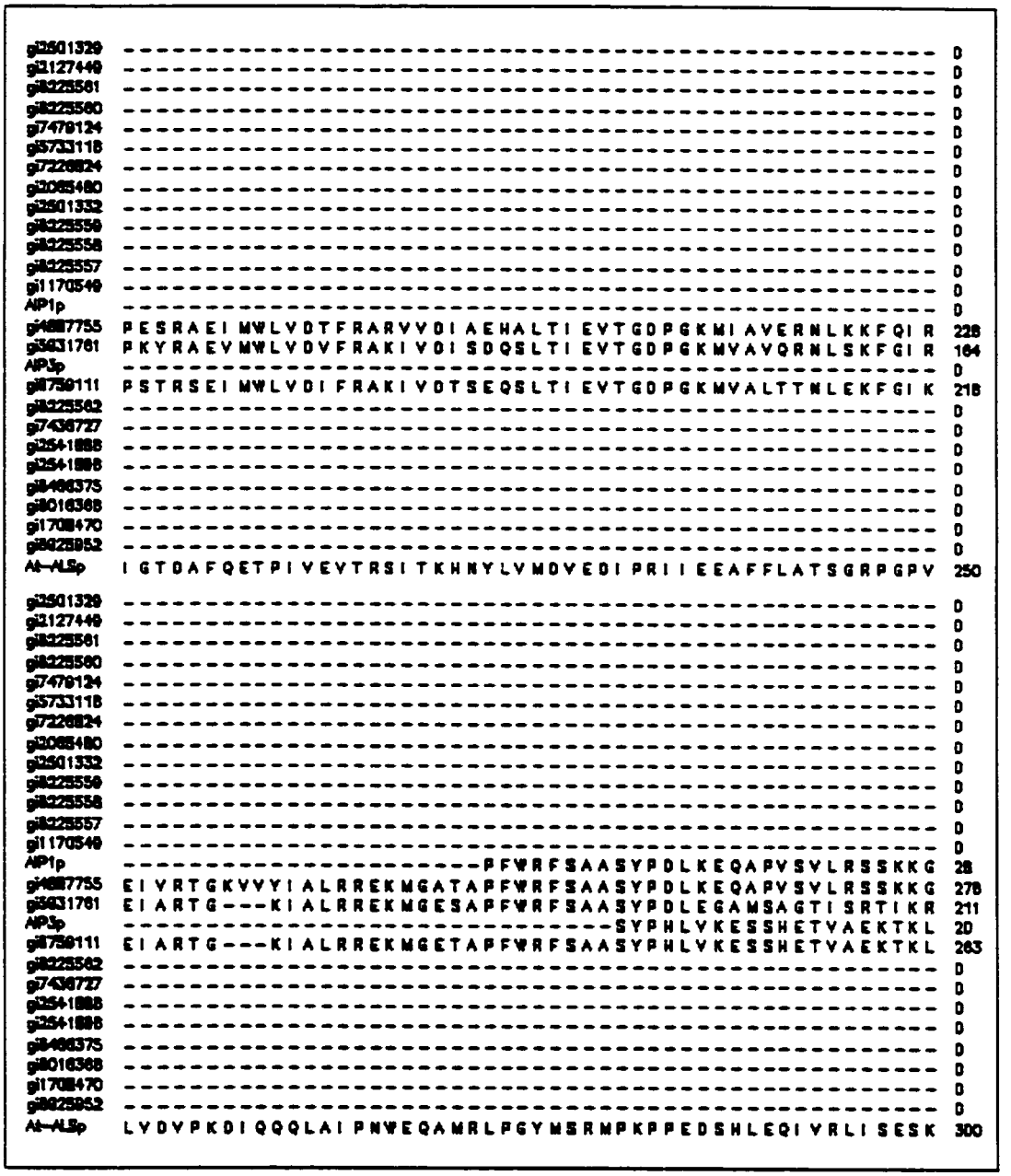

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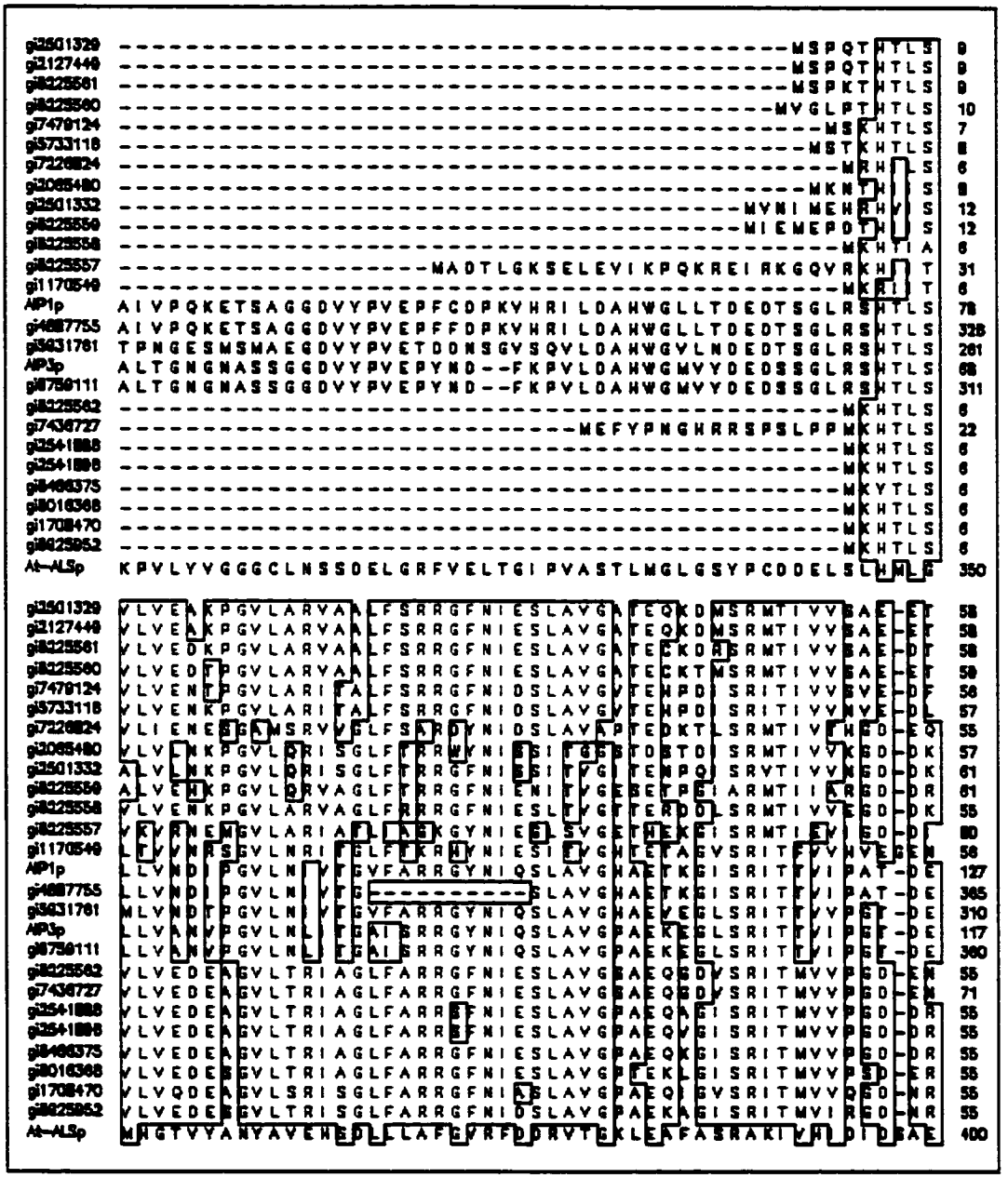

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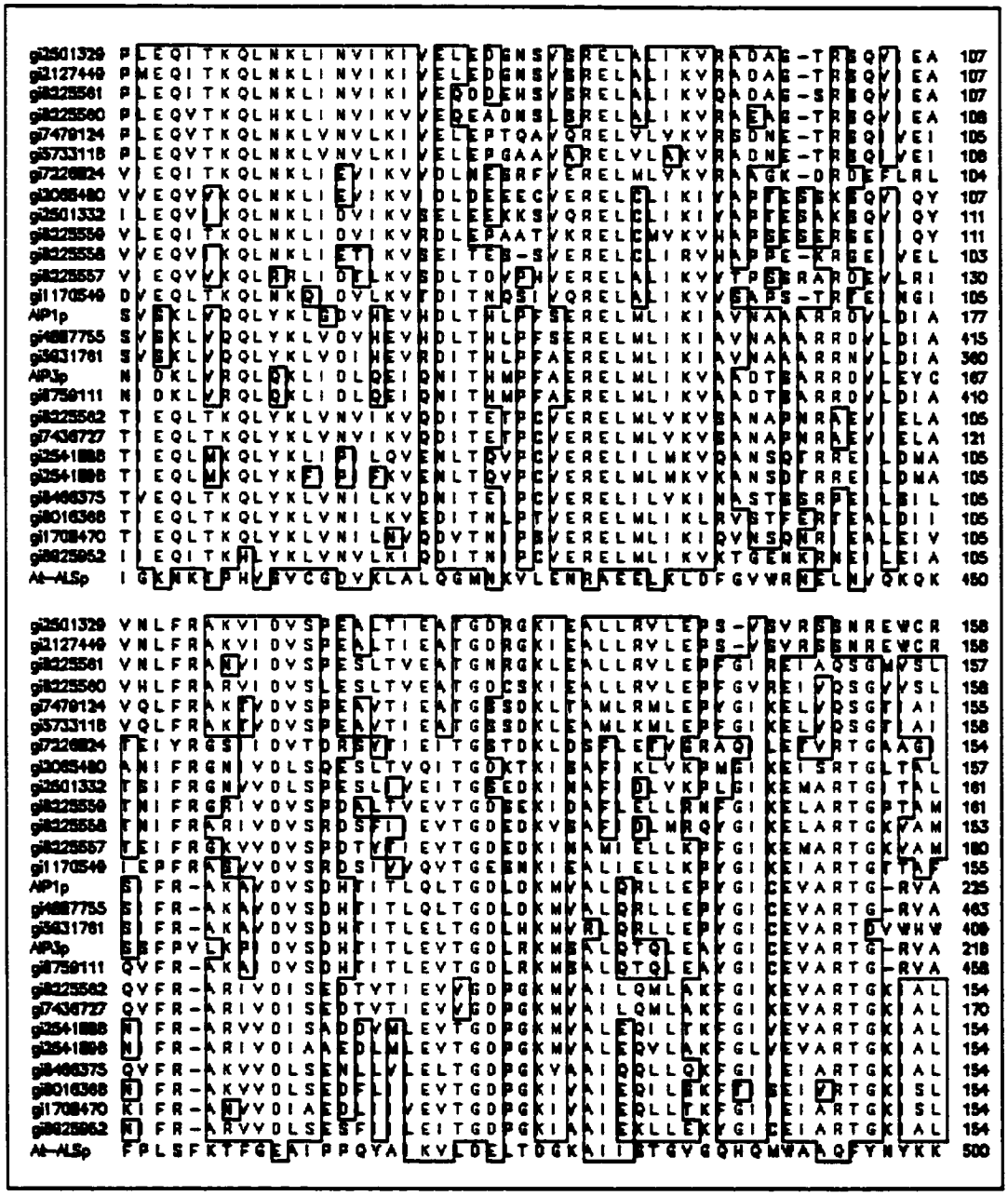

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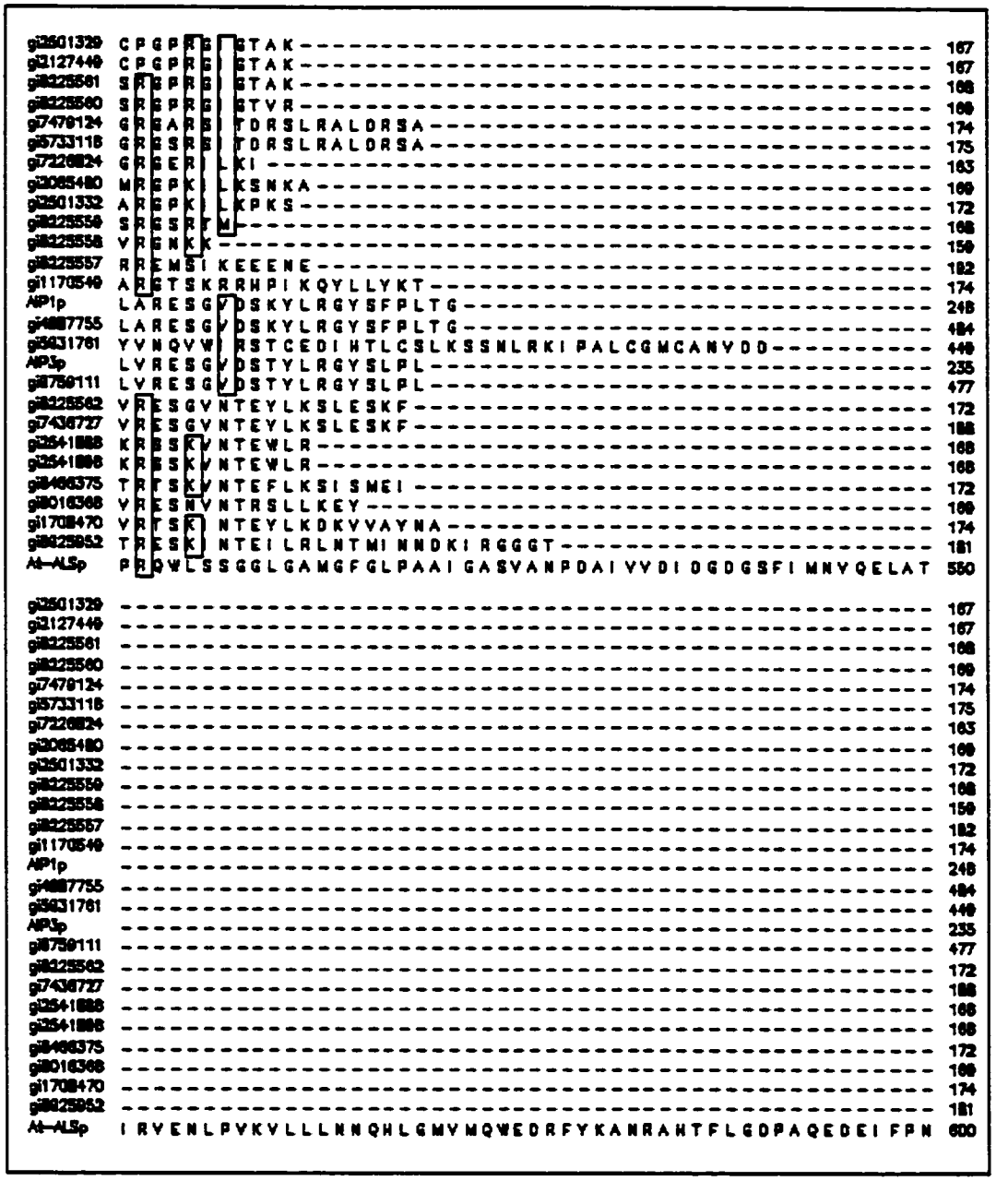

Appendix A2 continued