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Isolation and Characterization of Plant Genes Coding for Acetolactate Synthase, the Target Enzyme for Two Classes of Herbicides

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ABSTRACT

Acetolactate synthase (ALS) is the first common enzyme in the biosynthetic pathways to valine, isoleucine, and leucine. It is the target of two structurally unrelated classes of herbicides, the sulfonylureas and the imidazolinones. Genomic clones encoding ALS have been isolated from the higher plants *Arabidopsis thaliana* and *Nicotiana tabacum*, using a yeast ALS gene as a heterologous hybridization probe. Clones were positively identified by the homology of their deduced amino acid sequences with those of yeast and bacterial ALS isozymes. The tobacco and *Arabidopsis* ALS genes have approximately 70% nucleotide homology, and encode mature proteins which are approximately 85% homologous. Little homology is seen between the amino acid sequences of the presumptive N-terminal chloroplast transit peptides. Both plant genes lack introns. The tobacco ALS gene was isolated from a line of tobacco which is resistant to the sulfonylurea herbicides due to an alteration in ALS. The tobacco gene which was isolated codes for an ALS that is sensitive to the herbicides, as assayed by transformation of the gene into sensitive tobacco cells.

ALS[1] (EC 4.1.3.18) catalyzes the first common step in the biosynthesis of the essential amino acids isoleucine, leucine, and valine. ALS has attracted enhanced interest recently in that it has been demonstrated to be the target of two new and structurally unrelated classes of herbicides, the sulfonylureas (1, 4, 12, 24) and the imidazolinones (29). Both classes of compounds are notable for their high herbicidal potencies, their low mammalian toxicities, and, for some analogs, their selective toxicity to weed species as compared to crop species (16, 29). These compounds inhibit plant growth by inactivating an enzyme in an essential amino acid biosynthetic pathway, rather than by the common alternate herbicidal mode of inactivating a component in a photosynthetic pathway. Because essential amino acids are not produced by mammals, they lack the target enzyme for these compounds, which presumably contributes to the low mammalian toxicities of these herbicides. The selective toxicity to weeds of certain of these compounds is due to their metabolism by crop plants; analogs have been synthesized which are metabolically inactivated by particular crop species, but not by most weed species (32, 28).

Tobacco mutants resistant to the sulfonylurea herbicides chlorsulfuron (Glean; Du Pont) and sulfometuron methyl (Oust; Du Pont) have been isolated by selection for herbicide resistant haploid cell lines in tissue culture. Diploid plants were regenerated from these lines, and the resistance trait was shown to be expressed at the whole plant level. Through genetic crosses it was then established that the resistance was due to single semidominant nuclear mutations (2). The mode of action of the sulfonylurea herbicides was deduced in bacteria (12), when it was found that some bacteria are sensitive to sulfonylurea herbicides when grown on minimal medium, but not when grown on rich medium, and that this sensitivity could be reversed by supplementing minimal growth media with branched chain amino acids. It was then demonstrated that these compounds specifically inhibit acetolactate synthase. Biochemical and genetic studies were used to demonstrate that ALS is also the target of the sulfonylurea herbicides in tobacco and peas (1, 24) and in yeast (4). Imidazolinones have been shown to inhibit maize ALS (29).

ALS genes and proteins have been characterized in bacteria and fungi. Three ALS isozymes have been found in *Escherichia coli* and in *Salmonella typhimurium*. The genes coding for these isozymes were cloned from *E. coli* by complementation of auxotrophic mutants; they are the *ilv*GM, *ilv*IH, and *ilv*BN genes, coding for isozymes II, III, and I, respectively (13, 23, 30). These genes have recently been sequenced (6, 14, 31, 33). Ongoing work has indicated that in these bacteria ALS is a tetramer, composed of two large and two small subunits. The large subunits have mol wt of about 60,000, while the recently recognized small subunits have mol wt of 10 to 20,000 (3, 7, 27). In the yeast *Saccharomyces cerevisiae* an ALS gene clone was isolated from a plasmid library by virtue of its ability to rescue host cells exposed to minimal inhibitory concentrations of herbicide; because the gene was amplified on a multicopy plasmid, cells receiving the cloned gene could produce sufficient ALS to overcome the inhibition of the herbicide (4). Herbicide resistant mutants were subsequently isolated, and both mutant and wild type genes have been sequenced (5, 34). Replacement of the chromosomal copy of the wild type *ILV2* gene with a deletion mutant resulted in Ilv auxotrophy, indicating that only one functional ALS gene exists in yeast (4). A small subunit has not been identified for yeast ALS.

We wished to clone ALS genes from higher plants and, in particular, from a herbicide resistant line of tobacco. Using the yeast ALS gene as a heterologous hybridization probe, we were able to clone ALS genes from the dicotyledonous higher plants *Arabidopsis thaliana* and *Nicotiana tabacum*, or tobacco. The *Arabidopsis* gene was cloned from a herbicide sensitive line, and thus should encode a herbicide sensitive ALS. The tobacco gene was cloned from a herbicide resistant line, although from an allotetraploid tobacco species. Thus, the cloned tobacco gene

[1] Abbreviations: ALS, acetolactate synthase; bp, base pairs.

could encode either a herbicide sensitive or a herbicide resistant ALS enzyme.

MATERIALS AND METHODS

Genomic DNA Libraries. Construction of the tobacco *Sau* 3A genomic DNA library, in the vector λ EMBL 4, has been described (20). The tobacco DNA was prepared from *Nicotiana tabacum* cv Xanthi callus tissue; this line carries a homozygous mutation, S4, which renders the tobacco resistant to sulfometuron methyl (Oust, Du Pont) and chlorsulfuron (Glean, Du Pont) (2). The *Arabidopsis thaliana* cv Columbia DNA library, in the vector λ sep6, was the generous gift of Elliot Meyerowitz (California Institute of Technology, Pasadena, CA; 15). The *Anabaena* 7120 library has been described (25). The phage libraries were routinely plated on the host strain *E. coli* LE392 (18).

ALS Gene Probes. The plasmid pKD1-2 was used as a convenient source of the yeast ALS gene (4). A 2.0 kb *Eco* RI fragment was isolated from pKD1-2 which begins 133 bp before the initial ATG codon of the protein, and ends 170 bp before the TGA termination codon of the protein. For use as a hybridization probe, the fragment was purified by preparative electrophoresis through 0.7% agarose gels, and labeled by the nick translation reaction to a specific activity of 10^8 cpm/μg. The plasmid pDU9 was used as a *Salmonella typhimurium* ALS gene probe (27).

Hybridization Conditions. Lambda phage libraries were grown on the host LE392, and plated to give 5000 plaques on 90 mm Petri dishes, or 50000 plaques on 150 mm Petri dishes. Procedures for plaque lifts and filter hybridizations followed the methods outlined in Maniatis *et al.* (18). Plaque lifts were prehybridized for approximately 4 h in 6X SSPE-0.5% SDS-100 μg/ml denatured calf thymus DNA-1X Denhardt's solution, at 56°C. A fresh aliquot of the same solution was added for the hybridization, together with the ^{32}P-labeled ALS gene probe. Hybridization was allowed to proceed for 24 to 48 h at 56°C. The filters were rinsed for 4 h in 6X SSPE-0.5% SDS at 56°C, and then three times for 20 min each in 2X SSPE at room temperature. The filters were dried and exposed to film, with a Du Pont Lightning Plus intensifying screen at −70°C, for 2 to 3 d. Plaques corresponding to the darkest spots were picked, replated, and rescreened, using freshly prepared probe.

DNA Preparation and Sequencing. DNA was made from plaque purified phage according to the mini-lysate procedure (18). Restriction digests of the DNA were subjected to electrophoresis through 0.7% agarose gels, and the DNA was then blotted onto nitrocellulose. Fragments carrying the putative ALS gene were identified by hybridization, using the conditions described above. These fragments were then isolated from preparative gels, and subcloned into the vectors pBR322, M13mp9, M13mp18, and M13mp19. DNA prepared from M13 phage was sequenced using oligonucleotide primers in the dideoxy chain-termination method.

RESULTS

Hybridization of a Yeast ALS Gene to Plant DNA. We had found that when cloned *S. typhimurium* and yeast ALS genes were hybridized to each other under reduced stringency conditions, homology could be detected (not shown). Subsequent comparisons of the deduced amino acid sequences of the yeast and *E. coli* ALS enzymes also indicated homology, and revealed three conserved domains (5). We therefore reasoned that this homology might extend to other species, and attempted to clone plant ALS genes by the method of heterologous hybridization.

To establish the generality of the cross-hybridization, and to determine the preferred segment of the yeast ALS gene to use as a probe, subclones of the yeast ALS gene were hybridized to genomic DNA blots from the prokaryotic blue green alga, or cyanobacterium, *Anabaena* 7120. Hybridization to the genomic DNA blots was weak but detectable, using the reduced stringency hybridization conditions of 6X SSPE and 56°C. A 2.0 kb *Eco* RI yeast DNA fragment which corresponds closely to the ALS coding sequence was found to be the preferred hybridization probe. This probe was used to isolate a putative ALS gene clone from a phage library of *Anabaena* DNA. Its identity was subsequently confirmed by partial DNA sequence analysis and comparison to the known microbial ALS sequences (not shown).

Having established that unrelated ALS genes could be detected with the yeast ALS gene probe, we next used the yeast gene to try to isolate ALS genes from two dicotyledonous plants, *A. thaliana*, and *N. tabacum* (tobacco). When the yeast ALS gene was used as a probe against tobacco genomic DNA blots, no specific hybridization was detected. However, when the probe was used against a genomic library of tobacco DNA, the DNA amplification in the phage plaques was sufficient for a hybridization signal to be detected. Similarly, when the yeast ALS gene was used as a probe against a genomic library of *Arabidopsis* DNA, hybridizing plaques could be identified.

Mapping and Sequencing of Plant ALS Genes. The tobacco and *Arabidopsis* phage carrying presumptive ALS genes were characterized by restriction enzyme mapping and by hybridizations with the yeast ALS gene probe. These studies indicated that for both the tobacco and the *Arabidopsis* genes, hybridization to the yeast gene extended to two contiguous cloned fragments and spanned a region approximately 2 kb in length (Fig. 1). The finding that the hybridizations extended to contiguous large fragments suggested that the hybridizations were indeed specific, while the finding that the hybridizations centered around 2 kb regions suggested that either the plant genes diverged from the yeast gene at their ends, or that they lacked extensive introns. DNA sequence analysis was used to differentiate between these possibilities.

The tobacco and *Arabidopsis* ALS genes were each sequenced on both strands using a series of oligonucleotide primers to extend the sequence in overlapping segments. The sequences of these two genes, and of their cognate deduced proteins, are shown in Figure 2.

The coding region of the *Arabidopsis* gene has 2013 nucleotides, corresponding to 670 amino acids, while the coding segment of the tobacco gene has 2004 nucleotides, corresponding to 667 amino acids. Both genes appear to lack introns. The *Arabidopsis* ALS gene codes for a protein of mol wt 72593, while the tobacco gene codes for a protein of mol wt 72877. The three additional amino acids found in the *Arabidopsis* protein, relative to the tobacco protein, are found near the N-terminus. Although there is some codon usage bias, all codons are used in both genes. The initial ATG of the tobacco gene is flanked by the sequence ACAATGG, which deviates only at the −1 position from the

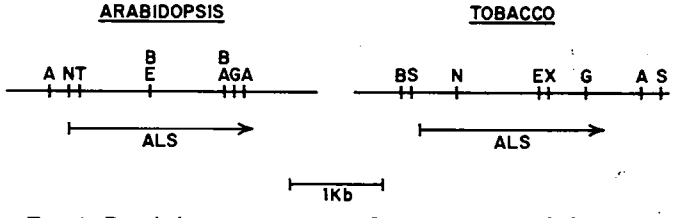
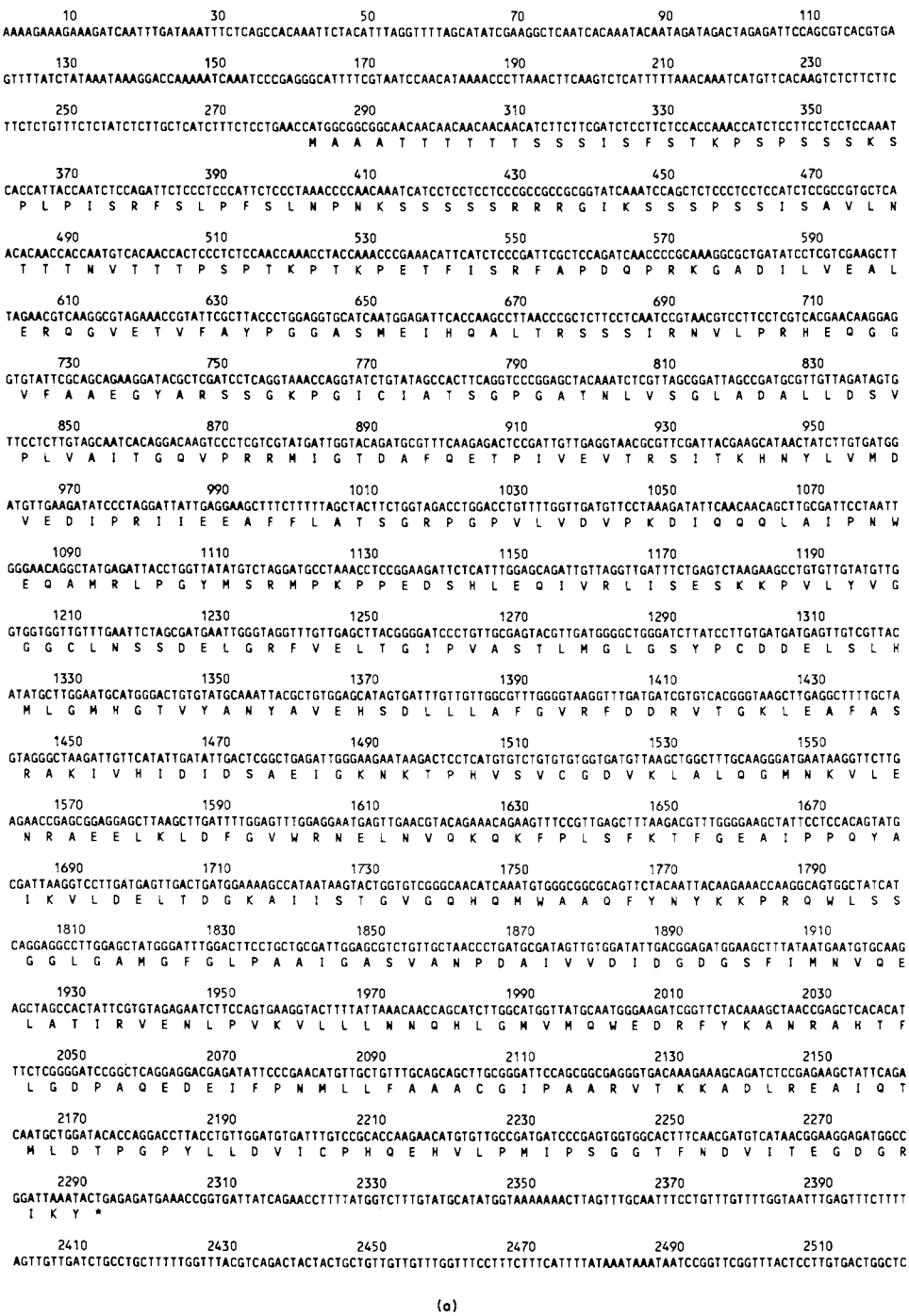


FIG. 1. Restriction enzyme maps of tobacco and *Arabidopsis* ALS genes. Phage λ genomic DNA clones carrying tobacco or *Arabidopsis* ALS genes were digested with restriction enzymes, fractionated by gel electrophoresis, transferred to filters, and probed with a labeled yeast ALS gene. Maps consistent with the observed fragment sizes were inferred from the data. The sites shown were subsequently confirmed by DNA sequence analysis. A, *Ava* I; B, *Bam* HI; E, *Eco* RI; G, *Bgl* II; N, *Nco* I; S, *Ssp* I; T, *Sst* II; X, *Xba* I.



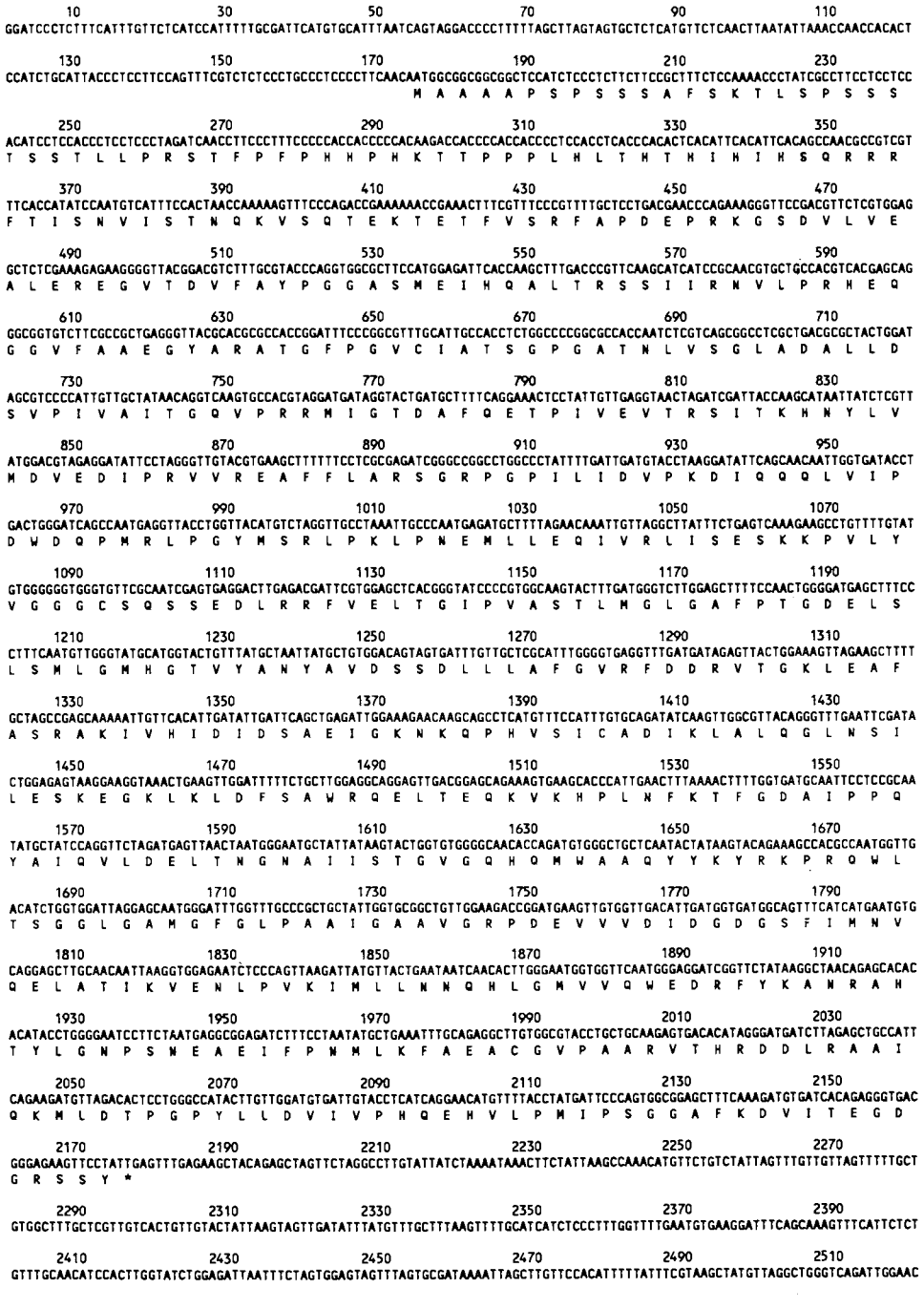
(a)

FIG. 2. Nucleotide and deduced amino acid sequences of the *Arabidopsis* (a) and tobacco (b) ALS genes.

preferred eukaryotic translational start sequence; the *Arabidopsis* gene initiates in the context ACCATGG, which is congruent with the preferred translational initiation sequence (11). This latter sequence conveniently includes an *Nco* I restriction enzyme site.

The deduced amino acid sequences of these proteins can be aligned with those of the yeast and *E. coli* ALS proteins, as shown in Figure 3. Similar alignments of the DNA sequences have allowed us to identify blocks of homology which may be important for the cross-hybridization between the plant and yeast genes. Both plant genes share scattered blocks of homology with the yeast gene. Several of these blocks extend for 20 to 30 nucleotides with few mismatches, and have high GC contents. The most prominent blocks have 36/40 nucleotides conserved between the yeast and tobacco genes, and 27/31 nucleotides conserved between the yeast and *Arabidopsis* genes. Each pair of genes also has several dispersed blocks of 10 to 20 identical nucleotides. We expect that some of these sequences will also be conserved in other ALS genes.

Chloroplast Transit Sequences. Because ALS has been shown to be nuclear encoded but chloroplast localized in plants (2, 9, 22), we assume that these proteins include a chloroplast transit sequence, and that the mol wt of the processed, mature proteins, are reduced. Predicting the proteolytic cleavage site is, however, difficult. Although both proteins begin with the sequence MAAA, they then diverge. Neither putative transit sequence closely resembles those that have been determined for the small subunits of ribulose bis-phosphate carboxylase-oxygenase, the Chl *a/b* proteins, plastocyanin, or ferrodoxin, nor do they correspond well to the consensus transit sequence framework amino acids



(b)

FIG. 2b.

which have been identified for these proteins (10). Because transit sequences generally lack acidic residues, and because homology between the two proteins begins around amino acid 85 (Fig. 3), the transit sequences may extend to amino acid 85. However, if the homology which begins at amino acid 85 is due to conservation of amino acids near the transit peptide processing site, the mature proteins may begin further downstream from residue 85. The first amino acid that is conserved in all ALS genes which have been sequenced occurs at residue 99 (Fig. 3). Because ALS is present in low intracellular concentrations, the N-terminal amino acid sequence of the *in vivo* mature protein has not yet been determined.

Conservation of Plant ALS Sequences. As shown in Figure 3, the amino acid sequences of the tobacco and *Arabidopsis* ALS proteins are highly conserved. If the mature protein is arbitrarily assumed to begin at amino acid 85, then 84% of the amino acids of the mature protein and 73% of the nucleotides are conserved. Modifying the boundaries of the transit peptide does not significantly affect these numbers. Yet while the sequence conservation of the mature proteins from tobacco and *Arabidopsis* is striking, the conservation of the putative transit sequences is not. The transit sequences are in fact barely conserved, with identical amino acids at only 27% of the residues. The nucleotide sequences encoding the transit peptides are more conserved than the amino acid sequences; 50% of the nucleotides are identical between the two transit sequences. Thus, for the mature protein, the amino acid sequences are more conserved than are the nucleotide sequences, as would be expected if constraints on mutation are exercised at the amino acid level. For the transit peptides, however, the nucleotide sequences are more conserved

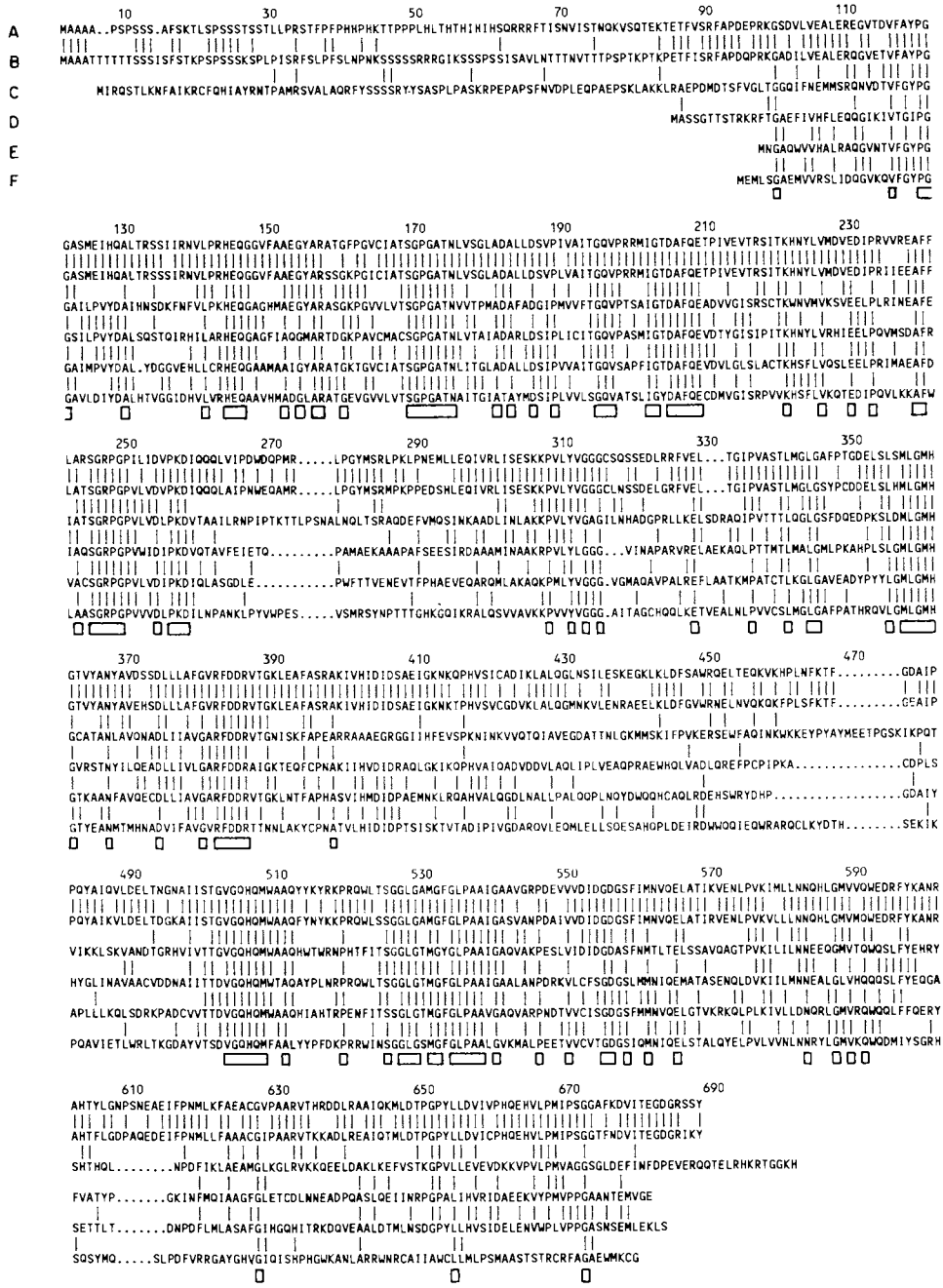


FIG. 3. Comparison of known ALS amino acid sequences. The deduced amino acid sequences of ALS genes are shown for lines A, tobacco; B, *Arabidopsis*; C, yeast (5); D, *E. coli* isozyme I (6, 33); E, *E. coli* isozyme II (14); F, *E. coli* isozyme III (31). Vertical lines indicate amino acid residues which are conserved between two adjacent ALS sequences. Boxes indicate residues which are conserved in all ALS sequences shown.

than are the amino acid sequences, indicating that there are fewer constraints on the amino acid sequence of the transit peptides. Hydropathy analysis of the deduced transit peptide sequences indicates that they share a number of similar hydrophobic and hydrophilic domains, despite their low amino acid sequence homology, thus implying that the polarity of the amino acids in these regions may be more important than their primary structure.

Homologies between Plant, Fungal, and Bacterial Enzymes. In contrast to the high homology seen throughout the plant ALS sequences, alignment of the three *E. coli* ALS sequences has shown that these isozymes have four nonconserved structural domains which are interspersed with three conserved structural domains (6, 14, 31, 33). The homology between the large subunits of any two *E. coli* ALS proteins is only on the order of 40% (33). When the *E. coli* proteins were compared with yeast ALS, the three conserved domains of homology were found to be maintained (5). The two plant proteins have also retained these common regions of homology, as shown in Figure 3. However, even within the nonconserved bacterial and yeast domains, the two plant proteins have maintained strict sequence conservation relative to each other. Because the plant transit sequences do diverge substantially, this conservation of their mature protein sequences may be functionally significant, and not simply due to lesser opportunities for evolution to occur in plants than in bacteria and yeast.

Transcriptional Signals. At the nucleotide level, the locations of the tobacco transcription regulatory sequences are somewhat ambiguous; they have not yet been experimentally identified. Because the ALS gene is not highly expressed, its regulatory sequences might be expected to deviate from the described consensus plant regulatory sequences, which have been largely derived from abundantly expressed genes (21). We expect that the tobacco ALS TATA box is included in the sequence which

begins at base 99 in tobacco, TTAATATTAAAC. The CAAT box appears to be in the sequence CATGTGCATT, starting at nucleotide 40. An apparent poly A addition signal is at base 2221, AAAATAAA.

The putative *Arabidopsis* transcriptional control elements do not share homology with those of tobacco. At the 5′ end of the gene, there is a potential TATA box starting at base 208 with the sequence TTTAAACA, and a CAAT box starting at base 176 with the sequence CAACATAAAAC. Alternative TATA and CAAT boxes are found upstream of these sequences in the *Arabidopsis* gene, at nucleotides 127, TCTATAAATA, and 87, CAATAGAT, respectively, but there is an out-of-frame ATG downstream from them. A consensus poly(A) addition signal is further downstream in the *Arabidopsis* sequence; the sequence AAATAAA is found beginning at base 2480. Both genes appear to have GT-cluster transcription termination signals scattered throughout the 3′ ends of their sequences. More definite identification of these sites must await analyses of the ALS mRNAs.

Genomic Organization of Tobacco and *Arabidopsis* ALS Genes. *Nicotiana tabacum* is an allotetraploid plant, formed from the fusion of two diploid progenitors, *Nicotiana sylvestris* and *Nicotiana tomentosiformis*. This allotetraploidy, coupled with the identification of two genetic loci that could mutate to yield herbicide resistant ALS enzymes (2), lead us to expect that there would be two sets of ALS genes in *N. tabacum*. Filter blot analyses of tobacco genomic DNA confirmed this prediction. Tobacco DNA was digested with either *Eco* RI or *Nco* I restriction enzymes, size fractionated by electrophoresis through agarose gels, and transferred to nylon filters. After hybridization with labeled tobacco ALS gene probes, two 5′ and two 3′ sets of fragments could be detected by autoradiography, as shown in Figure 4a. One set of fragments corresponds to the gene described here; the other is assumed to represent the second genetic locus.

The cloned tobacco ALS gene was isolated from a mutant line that carries one ALS gene which specifies a herbicide resistant enzyme, and one which specifies a wild type enzyme. The cloned gene was reintroduced into tobacco to determine whether it encoded the herbicide resistant or herbicide sensitive form of ALS. This experiment indicated that the cloned gene specifies a herbicide sensitive form of ALS (manuscript in preparation).

In contrast to tobacco, *Arabidopsis* has but a single ALS gene, as shown in Figure 4b. *Arabidopsis* DNA was digested with *Eco* RI or *Xba* I, size fractionated by agarose gel electrophoresis, transferred to a filter, and probed with a labeled, intact *Arabidopsis* ALS gene. A single *Xba* I fragment and two *Eco* RI fragments hybridized to the probe, a pattern consistent with *Arabidopsis* having a single ALS gene.

DISCUSSION

We have isolated plant ALS genes by heterologous hybridizations with a yeast ALS gene. That the yeast gene was a successful probe for the *Anabaena*, *Arabidopsis*, and tobacco genes indicates that ALS genes have been conserved across species boundaries. Such conservation is consistent with the structural homologies that had been seen between the bacterial and yeast ALS enzymes, and that are seen here with the plant enzymes. It is also consistent with the sensitivity to sulfonylurea herbicides of ALS enzymes from a broad range of organisms. While tobacco and *Arabidopsis* are both dicotyledonous plants, they are not classified in the same plant family. The high homology seen between their ALS enzymes leads us to expect that other plant ALS proteins will also be highly conserved. Although heterologous hybridizations have been employed to isolate genes from related species, and to isolate genes coding for highly conserved proteins from evolutionarily diverse sources, this study further extends the utility of the method. Conservation of 'housekeeping genes' across broad species boundaries may in fact be more general than has been assumed, and heterologous hybridizations using yeast and/or bacterial probes may be a generally applicable method for cloning such genes from higher organisms.

The finding that both plant genes apparently lack introns was unexpected, given the general prevalence of introns in plant genes that have been sequenced thus far. We had anticipated that introns might interrupt the plant ALS genes at positions corresponding to the nonconserved domains of the microbial ALS genes. This was not the case, however; the two plant genes are in fact highly conserved across these nonconserved domains. Thus, if the ancestral ALS gene was formed through exon shuffling, introns may have been lost from it prior to the prokaryotic/eukaryotic division (19). We would therefore expect other plant ALS genes also to lack introns.

These ALS genes will be useful for exploring a number of questions of basic interest. Mutant alleles of these genes may be useful as dominant selectable markers for both heterologous and homologous transformations of plants, bacteria, and fungi; each of the species from which we isolated ALS genes is naturally sensitive to a sulfonylurea compound. Sequence analyses of these genes is allowing us to gain insight into the evolution of ALS across a range of species. In yeast, ALS is encoded in the nucleus, but localized in the mitochondria (17, 26), while in plants ALS is nuclear encoded, but localized in the chloroplasts (2, 9, 22). Thus, identification of the domains of specificity for subcellular localization in these sequences will be of interest, as will be expression of these genes in species in which ALS is localized in a heterologous organelle. For example, by expressing plant ALS genes in yeast, it may be possible to determine if the plant ALS chloroplast transit sequences can be recognized by yeast mitochondria. Studying the regulation of the tobacco ALS genes will allow us to begin to elucidate the roles of the different tobacco ALS isozymes. Expression of the plant genes in bacteria should allow enough plant ALS to be accumulated to permit biochemical and structural analyses of substantial quantities of purified enzyme. These studies, coupled with sequence analyses of herbicide sensitive and resistant alleles, should allow us to begin to understand the modes of protein-herbicide interactions of ALS and its inhibitors.

Our success in isolating plant ALS genes will allow us to carry out experiments of agronomic interest. The tobacco gene described here does not confer herbicide resistance in transgenic plants. We assume that this is because it specifies a herbicide sensitive ALS, and that the second tobacco gene encodes the herbicide insensitive ALS. Another possibility is that the plant enzyme, like the bacterial enzymes, has a small subunit, and that the small subunit carries the mutation that confers herbicide resistance. We consider this possibility less likely, in that the bacterial and yeast ALS mutations which have been identified are in the large subunit of the enzyme (34). The cloned tobacco and *Arabidopsis* genes can now be used to isolate the genes specifying resistant forms of ALS from herbicide resistant lines of these plants (2, 8). The cloned genes should also be useful probes for isolating ALS genes from other plant species. ALS genes isolated from sensitive species can then be specifically mutated in order to confer herbicide resistance in transformed crops. Thus, the cloned ALS genes described here should permit the isolation of ALS genes from diverse crop species, and the subsequent transformation of these species to herbicide resistance.

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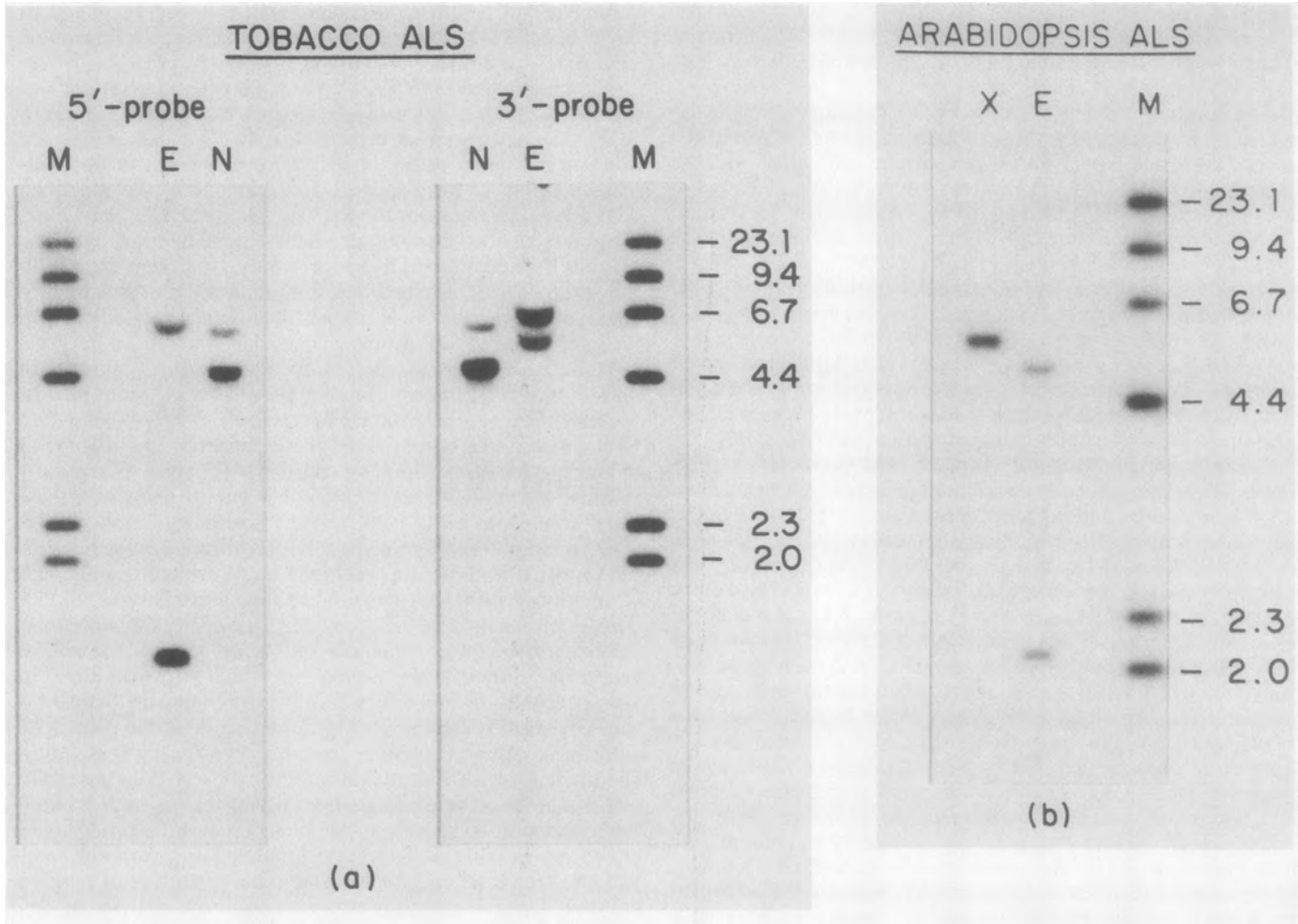

FIG. 4. Hybridization of cloned ALS genes to genomic filter blots of tobacco and *Arabidopsis* DNA. a, Tobacco DNA was digested with *Eco* RI or *Nco* I and fractionated by electrophoresis on an agarose gel. After transfer to a nylon filter, the DNA fragments were hybridized with labeled RNA probes generated from either the 5′ or the 3′ halves of the tobacco ALS gene. b, *Arabidopsis* DNA was digested with *Eco* RI or *Xba* I restriction enzymes and fractionated by electrophoresis on an agarose gel. After transfer to a nylon filter, the DNA fragments were hybridized with a nick-translated internal fragment from the *Arabidopsis* ALS gene.

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