Molecular Evolution of Glutamate Receptors: A Primitive Signaling Mechanism that Existed Before Plants and Animals Diverged

Joanna Chiu, Rob DeSalle,† Hon-Ming Lam,‡ Lee Meisel,* and Gloria Coruzzi**

*Department of Biology, New York University; †Department of Entomology, American Museum of Natural History, New York, New York; and ‡Department of Biology, The Chinese University of Hong Kong, Shatin, Hong Kong

We performed a genealogical analysis of the ionotropic glutamate receptor (iGluR) gene family, which includes the animal iGluRs and the newly isolated glutamate receptor-like genes (*GLR*) of plants discovered in *Arabidopsis.* Distance measures firmly placed the plant *GLR* genes within the iGluR clade as opposed to other ion channel clades and indicated that iGluRs may be a primitive signaling mechanism that predated the divergence of animals and plants. Moreover, phylogenetic analyses using both parsimony and neighbor joining indicated that the divergence of animal iGluRs and plant *GLR* genes predated the divergence of iGluR subtypes (NMDA vs. AMPA/KA) in animals. By estimating the congruence of the various glutamate receptor gene regions, we showed that the different functional domains, including the two ligand-binding domains and the transmembrane regions, have coevolved, suggesting that they assembled together before plants and animals diverged. Based on residue conservation and divergence as well as positions of residues with respect to functional domains of iGluR proteins, we attempted to examine structure–function relationships. This analysis defined M3 as the most highly conserved transmembrane domain and identified potential functionally important conserved residues whose function can be examined in future studies.

Introduction

Ionotropic glutamate receptors (iGluRs) were first discovered in vertebrates, in which they have been shown to be involved in mediating fast neuronal responses in excitatory synapses via the amino acid neurotransmitter L-glutamate (Sprengel and Seeburg 1995). Two classes of animal glutamate receptors exist: iGluRs, which are ligand-gated ion channels, and metabotropic glutamate receptors (mGluRs), which are G-protein-linked receptors. Vertebrate iGluRs are pharmacologically classified into three major groups based on their ligand selectivity: (1) AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazole propionate), (2) KA (kainate), and (3) NMDA (*N*-methyl-D-aspartate). Very often, the AMPA and KA receptors are further grouped together as non-NMDA types due to their sequence similarity and cross-reactivity. That is, AMPA receptors can respond to kainate as well as AMPA and vice versa. Besides these three major classes of iGluRs, there are two other classes of iGluRs that are related to the AMPA/KA class with respect to sequence similarity: the delta class (Yamazaki et al. 1992; Lomeli et al. 1993) and the kainate-binding proteins (Gregor et al. 1989; Wada et al. 1989). The delta class includes δ1 and δ2 subunits that were isolated in rat and mouse brain cDNA libraries. Although researchers have yet to demonstrate that the wild-type delta genes encode proteins with ion channel activity, a mutation in δ2 shown to cause a neurodegenerative disorder in Lurcher mice results in a constitutively open ion channel as tested in *Xenopus laevis* oocytes (Zuo et al. 1997). Using electrophysiological techniques, Rosenmund, Stern-Bach, and Stevens (1998) presented data that indicate iGluR channels exist as tetrameric structures, much like the voltage-gated potassium channels (Doyle et al. 1998). In contrast, other groups previously presented data to support a pentameric structure for iGluRs (Premkumar and Auerbach 1997) similar to the acetylcholine receptor, another ligand-gated ion channel. Ionotropic glutamate receptors can either be homomeric or heteromeric. In both cases, each subunit is encoded by a single gene, and depending on the subunit composition of the channel, the functional properties of the channel differ. All identified animal iGluRs contain six conserved domains that are believed to be functionally important. These include the two ligand-binding domains (GlnH1 and GlnH2, also known as S1 and S2) that have similarity to a glutamine-binding protein (GlnH) in *Escherichia coli* (Nakanishi, Shneider, and Axel 1990; Stern-Bach et al. 1994), and the four transmembrane domains (M1–M4) (fig. 1*A*). M2 is believed to be a major part of the pore and is important in the selective filtering of ions (Wo and Oswald 1995). Unlike the other three transmembrane domains, M2 was proposed not to span the membrane, thus exposing the two ligand-binding domains to the extracellular side of the membrane (fig. 1*B*) (Hollmann, Maron, and Heinemann 1994; Bennett and Dingledine 1995). It was suggested that the two ligand-binding domains act in concert on the extracellular side of the membrane to provide a ligand-binding site (Stern-Bach et al. 1994).

While iGluRs were originally discovered in vertebrates, more recently they have been identified in invertebrates, including *Caenorhabditis elegans* (Maricq et al. 1995), and *Drosophila melanogaster* (Schuster et al. 1991; Ultsch et al. 1992, 1993). A deletion mutation in *C. elegans* glr-1 suggests a role for these receptors in mechanosensation and signal transduction (Maricq et al. 1995). Surprisingly, putative iGluRs have also recently been identified in an organism lacking a nervous system, the model plant *Arabidopsis thaliana* (Lam et al. 1998). The similarity between the plant *GLR* genes and the an-

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Address for correspondence and reprints: Gloria Coruzzi, Department of Biology, New York University, 100 Washington Square East, New York, New York 10003.

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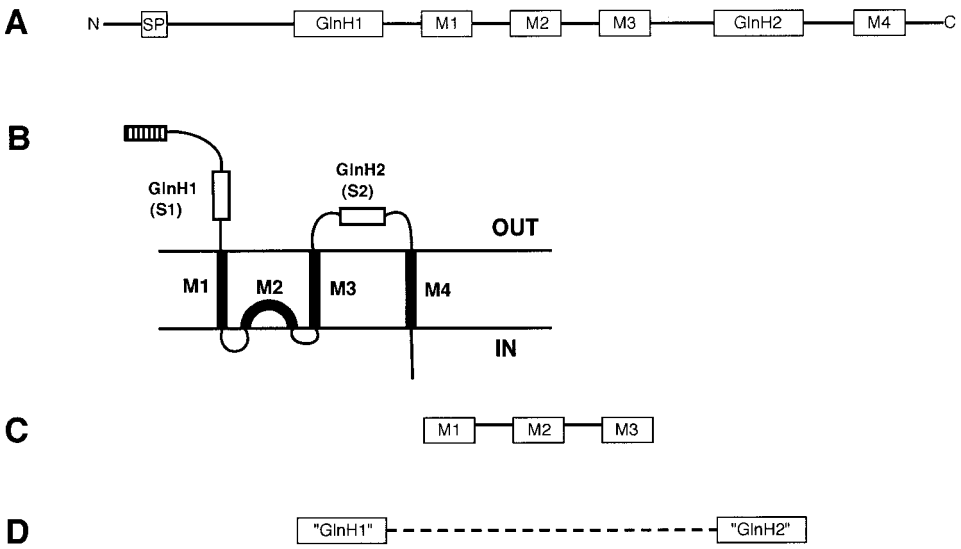

FIG. 1.—*A,* Diagrammatic representation of the iGluR protein showing the important functional domains, the two ligand-binding domains (GlnH1 and GlnH2), and the four transmembrane domains (M1–M4). SP at the N-terminal end represents the signal peptide. N and C represent the amino- and carboxy-terminal ends, respectively. *B,* Diagram showing the current model of the membrane topology of animal iGluRs. *C,* Diagram of the three transmembrane domains used in the ION analysis. *D,* Diagram showing the bacterial periplasmic amino-acid-binding protein and its putative region of homology with the animal iGluR proteins.

imal iGluRs spans all the important domains that are conserved among the animal iGluRs, including the two ligand-binding domains and the four transmembrane segments (M1–M4). The highest similarity between plant *GLR* genes and animal iGluRs is observed in M3, in which the percentage of identity is above 60% (data not shown). Currently, there are at least four plant *GLR*s. Based on sequence similarity, the plant genes seem to be more closely related to the non-NMDA class of animal iGluRs, but the exact classification has yet to be determined functionally in a heterologous expression system. In planta studies using iGluR antagonists suggest plant *GLR*s may be involved in light signal transduction (Lam et al. 1998). The discovery of iGluRs in plants suggests that signaling by excitatory amino acids in human brains has evolved from a primitive signaling mechanism that existed prior to the divergence of plants and animals.

In this study, we performed a phylogenetic analysis of the iGluR gene family of plants and animals. By including the plant *GLR* genes in the analysis, we examined how the plant genes fit into the evolutionary history of these receptors. By examining the genealogy of the iGluR gene family, we also attempted to make predictions concerning the functional properties of the plant receptors based on residue conservation. Moreover, by looking at amino acid character state changes, we identified residues that are invariant or are diagnostic of the various classes of iGluRs. Absolutely conserved residues are likely to be functionally important, while class-specific variations may control subtype specificity for ligand. By correlating this information to the location of the residue within the gene as well as the functional properties of the various receptor classes, we identified possible structure–function relationships.

Materials and Methods

Sequences

The animal glutamate receptor channel genes used in this study were obtained from GenBank. Table 1 lists all of these genes and their GenBank accession numbers. The four putative plant glutamate receptors were isolated by a combination of cDNA screening and genomic and EST sequence analysis as described in Lam et al. (1998) and are included in this study. We used a two-step approach in examining the plant *GLR* genes. First, we aligned and compared them with amino acid sequences of various kinds of ion channels (animal glutamate receptors, potassium channels, acetylcholine receptors, and $GABA_A$ receptors) in the database to determine if reasonable similarity to any ion channel genes could be established at least in the transmembrane domains (ION analysis). These genes are listed in tables 1 and 2 with their GenBank accession numbers. The second step was to limit the study to glutamate receptors only (GLU analysis). Bacterial periplasmic amino-acid-binding protein gene sequences from *Escherichia*, *Salmonella*, and an archaebacteria (also listed in table 1) were obtained and used as outgroups.

Alignment

CLUSTAL was used to align all amino acid sequences. All alignments using the default parameters in CLUSTAL for both the GLU and ION analyses showed large regions of difficult-to-align amino acids. We therefore implemented a ''culling'' procedure (Gatesy, DeSalle, and Wheeler 1993) to remove alignment-ambiguous regions.

In the ION analysis, in which we used several kinds of ion channel genes, only three transmembrane domains were used in our alignments in the following way.

Table 1
GenBank Accession Numbers and Abbreviations for All Glutamate Receptors and Bacterial Periplasmic Amino-Acid-Binding Proteins Examined in this Study

Gene	Species	Accession Number	Abbreviation Used (if different from gene name)
Animal glutamate receptors			
hNMDAR1	*Homo sapiens*	D13515	
rNMDAR1	*Rattus norvegicus*	X63255	
σ1	*Mus musculus*	D10028	
rNR2A	*R. norvegicus*	M91561	
rNR2B	*R. norvegicus*	M91562	
rNR2C	*R. norvegicus*	M91563	
rNR2D	*R. norvegicus*	L31612	
dNMDAR1	*Drosophila melanogaster*	X71790	
NMDAR	*Xenopus laevis*	X94156	frogglur
rδ1	*R. norvegicus*	Z17238	
rδ2	*R. norvegicus*	Z17239	
GluH1	*H. sapiens*	M64752	
α1	*M. musculus*	X57497	
rGluRK2	*R. norvegicus*	X54655	ratk2
rGluRC	*R. norvegicus*	M36420	
rGluR6	*R. norvegicus*	Z11715	
β2	*M. musculus*	D10054	
rKA1	*Rattus rattus*	X59996	
h-EAA2	*H. sapiens*	S40369	humeaa2
γ2	*M. musculus*	D10011	
dGluR1	*D. melanogaster*	M97192	
dGluR2	*D. melanogaster*	M73271	
GLR1	*Caenorhabditis elegans*	U34661	celegansglr1
Putative GluR	*C. elegans*	Z75545	celgansglur
GluR3/C	*Gallus gallus*	X89509	gallusglur3
GluR-II	*Columba livia*	S47031	pigeonglur
GluR4	*Carassius auratus*	U12018	goldfishglur
fGluR2Ac	*Oreochromis nilonica*	L46366	cichlidglur
fGluR2A	*Tilapia nilotica*	L34036	fishglur
GluR	*Lymnaea stagnalis*	ACGAE	snailglur
GluR-K1	*L. stagnalis*	X87404	lymglur
Plant *GLR*s			
GLR1	*Arabidopsis thaliana*	AF079998	
GLR2	*A. thaliana*	AFO79999	
GLR3	*A. thaliana*	AF007271	
GLR4	*A. thaliana*	AC000098	
Bacterial periplasmic amino-acid-binding proteins			
glnH	*Escherichia coli*	X14180	ecoliglnh
glnP	*Archaeoglobus fulgidus*	AE001090	afggln
glnP	*Salmonella typhimurium*	U73111	salglnp

The entire amino acid sequences were trimmed to include only M1–M3 in glutamate receptors and the corresponding transmembrane regions in other ion channel genes (fig. 1*C*). Since no experimental data are available for assigning transmembrane identity to sequences of the plant *GLR*s at the present moment, the transmembrane regions of plant *GLR*s used in this study are predicted based on hydropathy plots and their alignment with animal glutamate receptors (data not shown). These sequences of only approximately 100 amino acids were then aligned using CLUSTAL and a gap-to-change cost of 10. These alignments were of inferior quality in the regions of proposed transmembrane identity as determined by CLUSTAL and showed extremely limited levels of similarity. In the GLU analysis, the entire amino acid sequences of all animal glutamate receptor genes and plant *GLR*s were aligned using three separate sets of alignment parameters (gap to change = 10, 20, 30). These alignments were examined for regions of alignment stability (columns of amino acids that did not change from alignment cost to alignment cost) and ambiguity (as defined in Gatesy, DeSalle, and Wheeler 1993). The alignment-ambiguous amino acid columns were ''culled'' from the data matrix. All of these preliminary alignments are available on request from the authors.

Data Coding

Two data-coding issues involved in the analysis of these sequences were the coding of outgroups and the coding of long gaps. A major problem we encountered with incorporating outgroup sequences into our data matrices was the lack of amino acid similarity in several regions of the genes examined. For the GLU analysis, we chose two bacterial and one archaebacterial periplasmic amino-acid-binding protein genes as outgroups.

Table 2
GenBank Accession Numbers and Abbreviations for Non-Glutamate-Receptor Ion Channel Genes Examined in this Study

Gene	Species	Accession Number	Abbreviation Used (if different from gene name)
Potassium channels			
MBK1 (Kv1.1)	*Mus musculus*	Y00305	
AKT1	*Arabidopsis thaliana*	X62907	
ECOKCH	*Escherichia coli*	L12044	
dShaker	*Drosophila melanogaster*	M17211	
KAT1	*A. thaliana*	M86990	
HuK4 (Kv1.2)	*Homo sapiens*	L02752	
dShab	*D. melanogaster*	M32659	
Raw3 (rKv3.4)	*Rattus rattus*	X62841	
mShal (Kv4.1)	*M. musculus*	M64226	
MIRK1	*M. musculus*	X73052	
GIRK1	*Rattus norvegicus*	L25264	
nIRK1	*Caenorhabditis elegans*	U40947	
PaK1	*Paramecium tetraurelia*	U19907	
Acetylcholine receptors			
α1	*Heliothis virescens*	AJ000399	wormα1
unc-38	*C. elegans*	X98600	
ACR-3	*C. elegans*	Y08637	
α3	*D. melanogaster*	Y15593	dα3
α4-2	*R. norvegicus*	AF007212	rα4-2
α2	*R. norvegicus*	L10077	rα2
γ	*M. musculus*	M30514	mγachr
α6	*Gallus gallus*	X83889	gα6
α8	*G. gallus*	X52296	gα8
δ	*G. gallus*	K02903	gδ
α6	*H. sapiens*	Y16282	hα6
α7	*H. sapiens*	Y08420	hα7
$GABA_A$ receptors			
GABA-ϵ	*H. sapiens*	Y09765	
GABA-ρ1	*M. musculus*	AF024620	
GABA-ρ3	*R. norvegicus*	D50671	
GABA-γ3	*R. norvegicus*	X63324	
GABA-β	*D. melanogaster*	L17436	
GABA-rd1	*Aedes aegypti*	U28803	

Paas (1998) demonstrated that there are two major regions of supportable similarity between these bacterial genes and eukaryotic ionotropic glutamate receptor genes, and we chose to use these two regions as outgroup sequences for rooting the eukaryotic ionotropic glutamate receptor analysis (fig. 1*D*). All other regions of the glutamate receptor genes that were deemed alignment-unambiguous (see above) that are not in these two regions were coded as missing in the outgroups (indicated by question marks in the matrix; fig. 2).

In many cases, long stretches of positions in our alignment showed gaps. We chose to gap code these positions so as not to heavily weigh these regions in phylogenetic analysis. Our gap coding was implemented as unordered, and indel events were coded as distinct on the basis of their lengths (DeSalle and Brower 1997). Figure 2 shows some examples of how we implemented the gap coding.

Phylogenetic Analysis

Phylogenetic analysis using parsimony (PAUP*; Swofford 1998) was used to infer phylogeny. All characters were equally weighted in all analyses. This weighting scheme allowed us to most efficiently and severely test hypotheses of relationships (Kluge 1997) among the genes in this multiple gene family. For the ION analysis, uncorrected absolute distances between amino acid sequences of plant *GLR*s and various types of ion channel genes were calculated in PAUP* based on the data matrix which includes transmembrane regions only (fig. 3). Two different distance measures were generated in this analysis, one for M1–M3, and another one for M3 only. Characters 46–71 in this alignment were excluded in the distance calculation from M1 to M3. Dglur1 has an insertion in this region which created gaps in all other sequences. By excluding this region, we avoid the introduction of similarity due to the gaps present in all of the sequences. The range and mean values for each comparison were then computed using the distances between different plant *GLR*s and the members of each ion channel class.

For the GLU parsimony analysis, we implemented heuristic searches with 10 random-addition searches with TBR branch swapping to explore the tree space and narrowed the possibility that we obtained suboptimal trees. It was not necessary to place a limit on the number of trees saved in each search. When multiple equally parsimonious trees were obtained, we constructed strict-consensus trees to represent our phylogenetic hypothe-

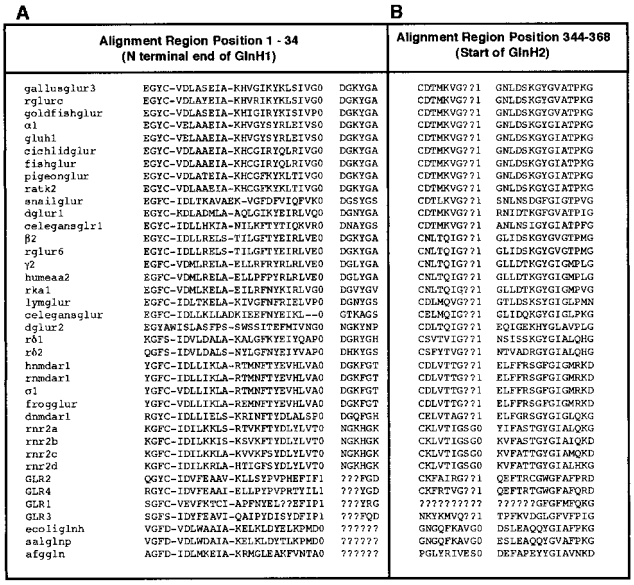

FIG. 2.—Examples of gap coding. Two regions from the glutamate receptor alignments are shown. In both examples, alignment using CLUSTAL resulted in the insertion of gaps longer then a single peptide. Such areas were recoded so as not to outweigh these regions in phylogenetic analysis. *A,* Alignment from position 1 to position 34 (our alignment positions) using CLUSTAL. Question marks denote gaps introduced by CLUSTAL. The three gaps in the plant *GLR* genes right after the space are the gaps in question. The column with 0 and 1 shows the recoded character states for these gaps. 0 indicates that no gaps are present, and 1 indicates the presence of two gaps. All question marks are treated as missing data in the phylogenetic analysis. *B,* Alignment from position 344 to position 368 (our alignment positions). Question marks denote gaps introduced by CLUSTAL. The two gaps in most sequences three positions to the left of the space are the gaps in question, and the column to the right of these two gaps represents the scoring system. (Accession numbers for these genes may be found in table 1.)

sis. The amino acid sequence character matrix used in the maximum-parsimony analysis was also analyzed using neighbor joining (Saitou and Nei 1987). Uncorrected absolute distances were calculated and analyzed by neighbor joining in PAUP*.

Node Robustness

We generated three measures of node robustness for all nodes in our parsimony analysis: bootstrap values (Felsenstein 1985), Bremer (1994) indices, and jackknife estimates (Farris et al. 1996). Bootstrap and jackknife analyses were performed using PAUP*, and Bremer indices were calculated using AUTODECAY (Eriksson 1996). Ten random-addition searches with TBR branch swapping were used for each replicate in bootstrap analysis and for the searches at each node for AUTODECAY analysis. For our neighbor-joining analysis, robustness of nodes was inferred using 1,000 bootstrap replicates performed by PAUP*.

Inhomogeneity Estimates

Estimates of the congruence of different gene regions were obtained through the ILD test developed by Farris et al. (1994, 1995) as implemented in PAUP*. We partitioned our data matrix between transmembrane domains (M), GlnH domains (GlnH), and domains outside of these two areas (INTER) and tested for incongruence of these gene regions to see if there were significantly different phylogenetic signals emanating from the three different regions.

Character Mapping

The final data matrices were transported to MacClade (Maddison and Maddison 1992) and manipulated in that program. By using the ''chart and trace'' option in the program, we were able to examine the number of possible character states at each residue. We targeted regions from the transmembrane domains and the GlnH domains that had previously been determined as functional regions for analysis. In our analysis, we simply looked for regions of conservation as well as significant correlation of character changes with respect to changes in amino acids and functionality in the proteins.

Results and Discussion

Placement of Putative Plant Glutamate Receptors in the Context of Ion Channel Classes

Previous distance measures demonstrated a high degree of identity between plant *GLR*s and animal i-GluRs, but did not accomplish a phylogenetic analysis (Lam et al. 1998). To determine where these plant *GLR* genes fit within the context of ion channel evolution, we constructed a matrix with amino acid sequences for the transmembrane domains from all GluRs in table 1 and all ion channel genes, including both ligand and voltage-gated ion channels, in table 2 (ION analysis). In addition to animal iGluRs which are shown to have extensive identity with the plant *GLR*s (Lam et al. 1998), we chose to examine the relationship of plant *GLR*s and other ion channel classes such as acetylcholine receptors, $GABA_A$ receptors, and potassium channels due to structural similarities suggested in other reports (Hollmann, Maron, and Heinemann 1994; Bennett and Dingledine 1995; Wo and Oswald 1995). Glutamate receptors, nicotinic acetylcholine receptors, glycine receptors, serotonin receptors, γ-aminobutyric acid (GABA) receptors, etc. are all members of the ligand-gated ion channel superfamily (Barnard 1992). Although in the same superfamily, i-GluRs are distinct in terms of primary structure (fig. 3) as well as membrane topology (fig. 4). The initial hypothesis based on generating hydropathy plots indicated that the animal glutamate receptor protein, like the nicotinic acetylcholine receptors, has four transmembrane segments (Hollmann et al. 1989). However, more in-depth investigation has now led to the current model, in which the second transmembrane segment of iGluRs does not actually span the membrane (Hollmann, Maron, and Heinemann 1994; Bennett and Dingledine 1995). This major structural difference distinguishes the iGluRs from other ligand-gated ion channels, but, on the other hand, it suggests a closer structural and functional relationship between iGluRs and other ion channels. For example, membrane-spanning M1 and M3 together with non-membrane-spanning M2 in glutamate receptors fit the description of a structure known as a pore loop,

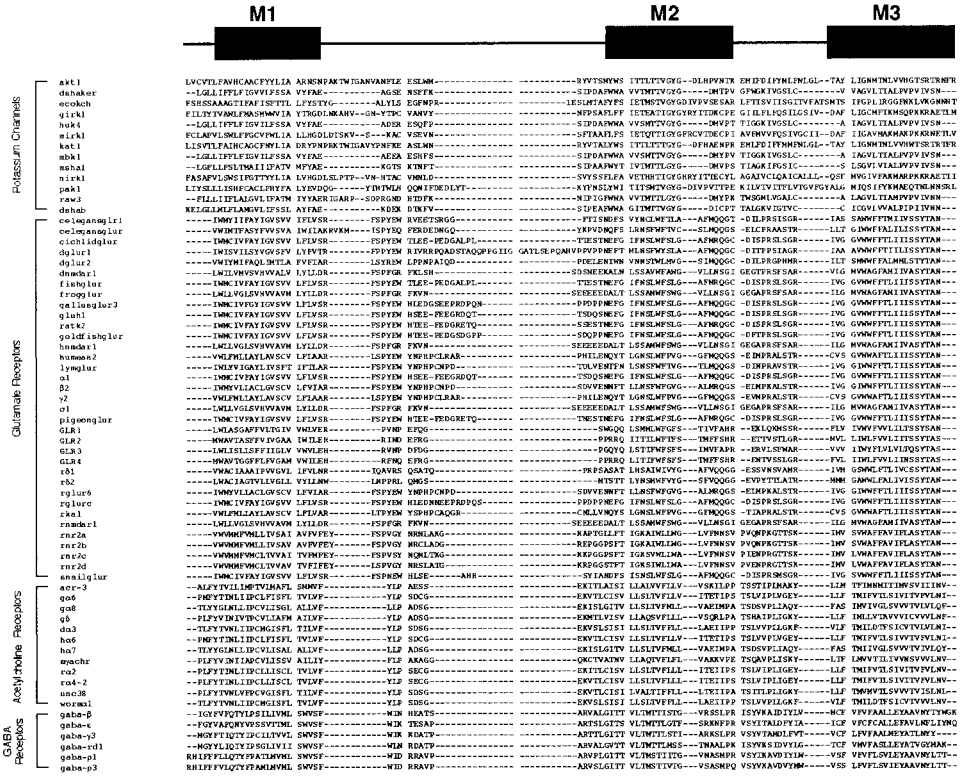

FIG. 3.—Alignment of animal and putative plant glutamate receptor transmembrane regions with several ion channel transmembrane regions that have putative similarity. Transmembrane regions 1–3 of glutamate, acetylcholine, and GABA$_A$ receptors are used in this analysis. For potassium channels, regions S5, P, and S6 are used due to their suggested homology with M1–M3 of animal iGluRs (Wo and Oswald 1995). The three transmembrane regions were aligned by determining the limits of the three regions in all genes in the figure. The three transmembrane regions are labeled using the transmembrane region numbering for animal glutamate receptors. (Accession numbers for these genes may be found in tables 1 and 2.)

which is common in other ion channels, such as voltage-gated potassium, sodium, and calcium channels, as well as inward rectifier potassium channels and nucleotide-gated cation channels (MacKinnon 1995). Wo and Oswald (1995) reported conservation of amino acid residues between glutamate receptors and potassium channels within this functionally important region.

The main purpose of this first analysis was to attempt to discover elements of the plant *GLR* transmembrane sequences that might diagnose the plant *GLR* genes as glutamate receptors compared with other ion channels. We limited our analysis to relevant parts of the proteins which are possibly homologous across all the ion channels, i.e., the characters which make up the pore loop region in the potassium channels and the iGluRs and the corresponding transmembrane segments (M1–M3) in acetylcholine and GABA$_A$ receptors (fig. 4). The resulting alignment (fig. 3) is unstable, as evidenced by drastic changes in alignment columns with slight changes in gap-to-change cost parameters (data not shown). The phylogenetic tree resulting from parsimony analysis is not strongly supported except for the node which groups the plant *GLR*s with the animal iGluRs and the nodes which group the members of the distinct ion channel classes (data not shown). This may indicate different evolutionary origins for the various ion channel classes, or it may simply indicate that it is not possible to homologize amino acid sequences in transmembrane domains of iGluRs and other ion channels. In this respect, it is important to note that although Wo and Oswald (1995) suggested possible homology between transmembrane regions of potassium channels and animal iGluRs, our alignment leads us to suggest that this homology is doubtful. Instead, the similarity in the pore loop structure present in both classes of ion channels may be due to convergent evolution.

Distance calculations of the data matrix aligned in figure 3 are shown in figure 4. The smaller the distance value, the more closely related are the sequences compared. Although the ranges for absolute distances of the different categories overlap slightly when considering M1–M3 in glutamate receptors and the corresponding regions in other ion channels, the mean values indicate that the plant *GLR*s are most closely related (smaller distance values) to animal glutamate receptors. This result is strongly supported by previous analyses, such as hydropathy plots and BLAST searches (data not shown). This suggests that the plant *GLR*s have amino acid residues in their transmembrane domains that are diagnostic of animal glutamate receptor genes rather than other ion channel classes. In addition, when considering only M3 in glutamate receptors and the corresponding transmembrane domain in other ion channels, the absolute distances between plant *GLR*s and animal iGluRs decrease, while those between plant *GLR*s and non-glutamate-receptors increase dramatically.

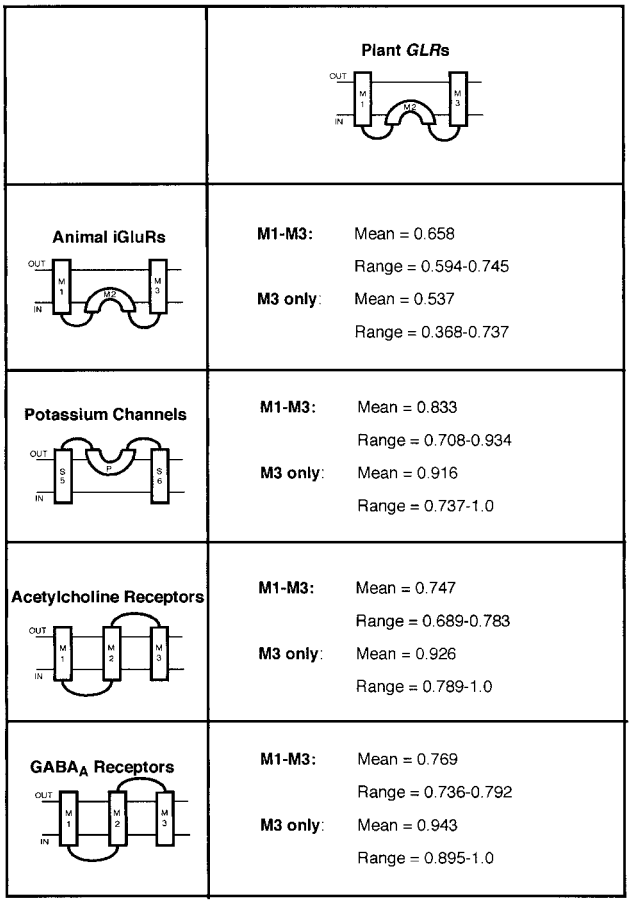

FIG. 4.—Uncorrected absolute distances between plant *GLR*s and four classes of ion channels (animal iGluRs, potassium channels, acetylcholine receptors, and $GABA_A$ receptors) based on the transmembrane region alignment shown in figure 3. The membrane topology for each of the different ion channels in the three transmembrane segments considered in this analysis is also shown here. For distance calculations using M1–M3 of glutamate receptors and the corresponding transmembrane regions in the other ion channels, characters 46–71 from the alignment shown in figure 3 are excluded. In this region, gaps are introduced in the alignment in all sequences due to an insertion in dglur1 (see fig. 3). For distance calculations using only M3 of glutamate receptors and the corresponding regions of other ion channels, characters 1–117 were excluded. In each of the comparisons, uncorrected absolute distances for all different combinations of plant *GLR*s and members of each ion channel class were calculated using PAUP*. The mean value and the range were then computed and are presented. Note: The smaller the distance values, the more related the sequences.

The alignment of the transmembrane regions of the various ion channels brings renewed significance to the similarity observed in the transmembrane regions of the animal iGluRs and plant *GLR*s. Unlike the comparison between the transmembrane segments of glutamate receptor genes and other ion channel genes, which shows very limited similarity, the transmembrane regions of animal iGluRs and plant *GLR*s are similar not only in terms of hydrophobicity, but also in terms of primary sequence. This evidence supports common ancestry of animal iGluRs and plant *GLR* genes rather than convergence.

Based on the information from this analysis, we predict that the membrane topology of the *Arabidopsis GLR*s will be similar to that of animal iGluRs where the second hydrophobic segment of the protein does not span the membrane, thus leaving the two ligand-binding domains on the extracellular side of the cell membrane (fig. 1*B*). This coincides with the prediction of TMPred analysis done on two of the plant *GLR* genes (Lam et al. 1998). Functional assays and membrane topology experiments will be required to confirm this prediction experimentally.

Plant Glutamate Receptor Genes in the Context of Glutamate Receptor Gene Evolution

The ION analysis described above suggests that the plant *GLR* genes belong to the iGluR family. The next step of our analysis was to examine where they fit into the evolutionary history within the iGluR receptor gene family. We therefore constructed a matrix composed of amino acid sequences from the glutamate receptor genes of plants and animals listed in table 1. One of the challenges of this analysis was to choose an appropriate outgroup. This is not straightforward, since no putative iGluRs have been uncovered in the genomes of eubacteria, archaebacteria, or yeast whose entire genomes have been completely sequenced. However, a group of genes known as the bacterial periplasmic amino-acid-binding proteins has been known to share identity with one of the functionally important modules of the animal iGluRs, the ligand-binding domain GlnHs (Nakanishi, Shneider, and Axel 1990). It was suggested that perhaps with the rise of multicellularity, there was increased selection pressure toward the development of membrane proteins necessary for cell–cell signaling (Wo and Oswald 1995). We therefore used three noneukaryotic periplasmic amino-acid-binding protein sequences (two eubacteria and one archaebacteria) that share similarity with the putative ligand-binding domains of iGluRs as outgroups (fig. 1*D*). Visual inspection of the alignments we obtained using the bacterial sequences as outgroups reveals a much greater degree of similarity when compared with the alignment in the ION analysis (fig. 2). However, the regions prior to the conserved GlnH1 and after GlnH2 showed no similarity and were easily ''culled'' (Gatesy, DeSalle, and Wheeler 1993) from the matrix.

Parsimony analysis of this glutamate receptor data matrix using the bacterial sequences as outgroups gave two equally parsimonious trees of 2,686 steps with a consistency index of 0.705 and a retention index of 0.782. Figure 5 shows the consensus parsimony tree with Bremer support and jackknife and bootstrap values. The plant *GLR* genes are consistently placed as the most basal group of glutamate receptors in the gene family, and the support for this arrangement is relatively robust (Bremer support at the node = 7; bootstrap at the node = 98%; jackknife at the node = 97%). Our neighbor-joining analysis generated a tree with a very similar topology and led to identical conclusion in terms of the placement of the plant *GLR*s (fig. 6). This placement indicates that the putative plant receptors diverged from the other animal iGluRs well before the various iGluRs that reside in animals (e.g., AMPA vs. NMDA) began

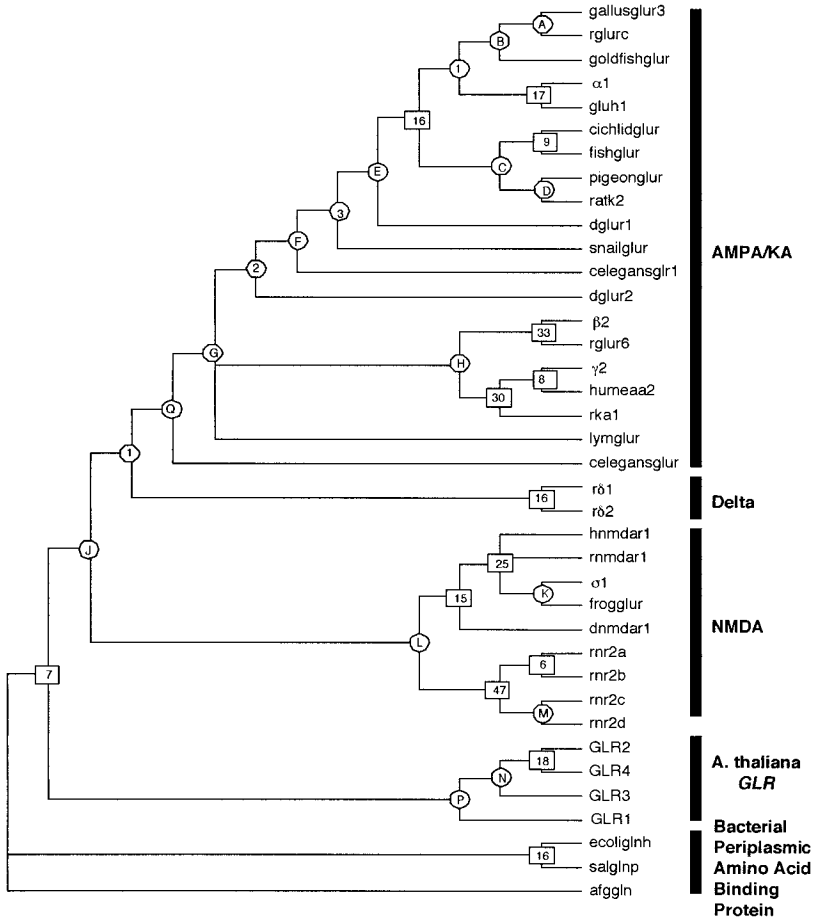

FIG. 5.—Phylogenetic tree generated from parsimony analysis of 35 glutamate receptor genes and 3 bacterial periplasmic amino-acid-binding protein sequences. This is the consensus of two most-parsimonious trees obtained from our search. Nodes are labeled in the following way: Nodes with squares on them are strongly supported. The number in the square is the Bremer support value, and all nodes with squares had bootstrap and jackknife values of 95%–100%. The nodes with circles on them are less strongly supported. Nodes with letters on them had Bremer support values, bootstrap values, and jack-knife values, respectively, as follows: A = 3/96/92, B = 2/72/77, C = 3/82/79, D = 3/85/83, E = 3/67/58, F = 10/84/88, G = 1/59/58, H = 8/80/83, J = 7/86/85, K = 1/60/52, L = 9/92/96, M = 1/74/66, N = 0/50/61, P = 6/97/92, and Q = 20/93/93. All other nodes with circles show the Bremer support value in the circle and at least one of the measures for robustness (bootstrap and jackknife) is less than 50%.

to diverge. In addition to the placement of the plant *GLR*s, the branch lengths in the neighbor-joining tree (fig. 6) also indicate that whereas most of the animal iGluRs, with the exception of snail, *C. elegans*, and *Drosophila* iGluRs, are very closely related, the plant *GLR*s show more significant differences. Moreover, our analysis establishes the relationship among members of the plant *GLR* gene family, in which *GLR2* and *GLR4* are related more closely to each other than to either *GLR1* or *GLR3*. Members of each of the three subdivisions within the plant *GLR* gene family may represent proteins that have different functions, e.g., ligand selectivity, as in the case of animal iGluR subtypes. This hypothesis needs to be tested experimentally in future functional assays. According to this analysis, the members of the various classes of animal iGluRs, AMPA/KA, NMDA, and delta, each reside within their appropriate groups with strong node support. Previously, the cloned glutamate receptor gene (glr-1) from *C. elegans* was classified into the AMPA/KA family due to sequence similarity (Maricq et al. 1995). This analysis supports the hypothesis, although no functional data are available at present.

Due to the early divergence of the plant genes, it is difficult to predict the function of putative glutamate receptors in plants. However, based on our phylogenetic trees (figs. 5 and 6), it is likely that the plant *GLR*s will have ligand-binding capability, since most of the animal GluRs retained the ligand-binding property of the bacterial periplasmic binding proteins. It is interesting to note that no ligand-binding or channel activity has been recorded with a class of animal iGluR genes known as the delta class. Wild-type delta genes showed no channel activity in heterologous systems such as *X. laevis* oocytes, even when glutamate, the natural ligand for glutamate receptors, was added to the bath solutions (Araki et al. 1993; Lomeli et al. 1993; Zuo et al. 1997). However, when a mutated form of the rat δ2 gene that causes the phenotype of the neurodegenerative Lurcher mice was used in similar experiments, the channels seemed to be constitutively open in the presence or absence of ligand (Zuo et al. 1997). This raises two major ques-

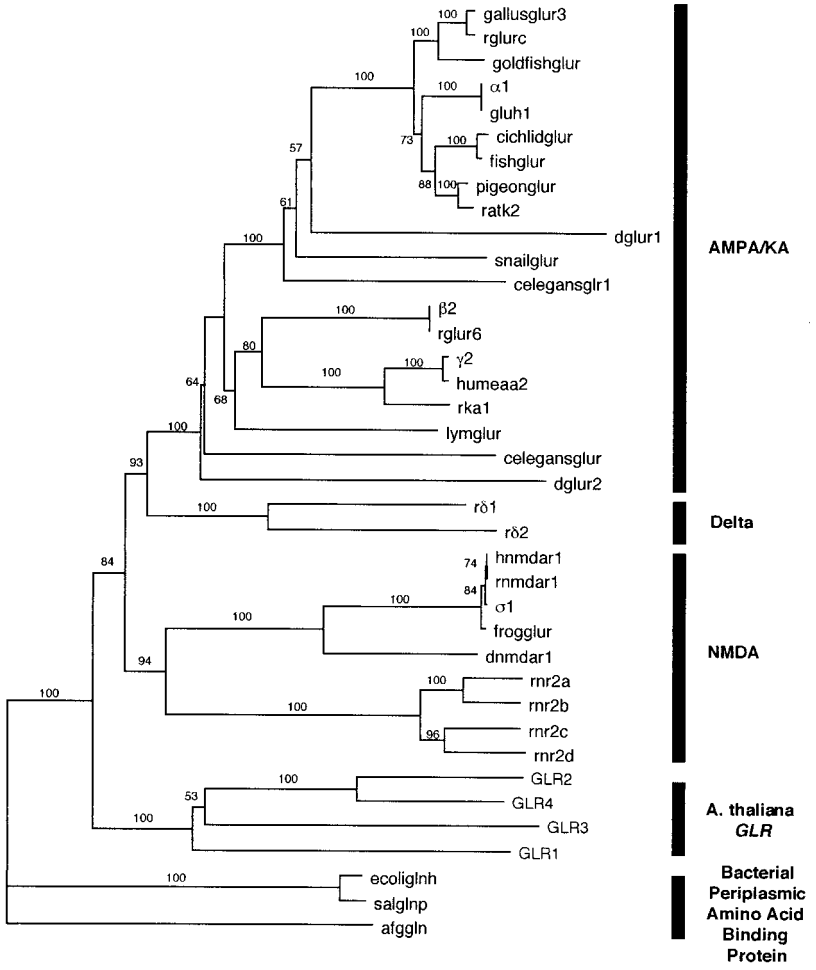

FIG. 6.—Phylogenetic tree generated from neighbor-joining analysis of 35 glutamate receptor genes and 3 bacterial periplasmic amino-acid-binding protein sequences showing branch lengths and bootstrap values. Uncorrected absolute distances were calculated from the same amino acid data matrix used in the maximum-parsimony analysis and analyzed by neighbor-joining in PAUP*. Bootstrap values are also generated in PAUP* and are shown for each of the nodes except one. The node which holds all the AMPA/KA genes except for celegansglur and dglur2 collapses in the tree from the bootstrap analysis and is not shown here.

tions: (1) Do the plant *GLR*s retain ligand-binding ability? (2) Will they respond to the same ligand as the animal receptors, or can they be constitutively open? In the rat δ2 mutants, a single nucleotide alteration causes an alanine in M3 of the wild-type protein to change to a threonine in the mutant (Zuo et al. 1997). While most functional studies of animal iGluRs have concentrated on M2 due to the presence of RNA editing in this region, it is significant that the greatest sequence similarity between animal iGluRs actually resides in M3. The same thing is true when the plant *GLR* genes are aligned with the animal iGluRs (fig. 4). Due to the high sequence conservation of M3 among animal iGluRs and between plant *GLR*s and animal iGluRs, this region is suggested to have functional importance. The location of the mutation in the Lurcher mice (Zuo et al. 1997) in the M3 domain supports this hypothesis. A close examination of the residues in the plant *GLR* genes corresponding to the Lurcher mutation residue raises an interesting speculation. Instead of having a sequence of ''LAA,'' as in wild-type delta proteins, the putative plant receptors have a sequence of ''LTS'' or ''LAS'' at the corresponding location. Like in the case of the rat δ2 mutation, which results in ''LAT'' at that location, the crucial second alanine is altered. However, instead of changing alanine to a threonine, as in the rat δ2 mutant, it is substituted with serine in the plant proteins. The amino acid serine is very closely related to threonine in terms of charge and size and may confer similar functional properties. Although it is not likely that the putative plant channels are constitutively open, as in the case with the rat δ2 mutant, it is possible that they are constitutively open in certain cellular conditions, such as at certain extracellular ion concentrations. This and other possibilities are yet to be tested experimentally. On the other hand, perhaps these plant proteins do not function primarily via channel activity, but via protein–protein interactions. In this case, that would mean that channel activity actually evolved after the divergence of plants and animals.

Homogeneity of the Transmembrane Domains and the GlnH Domains

We tested the homogeneity of the phylogenetic signal in the transmembrane domains versus that in the

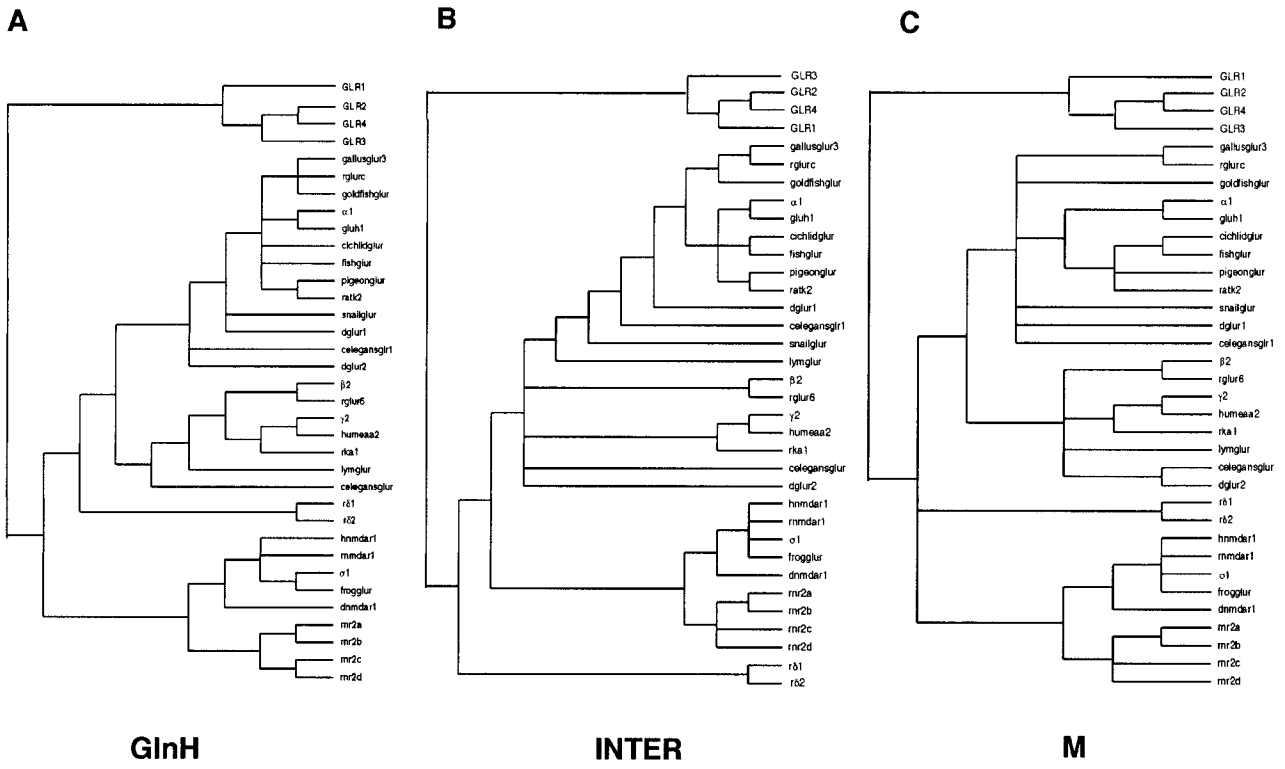

FIG. 7.—*A,* Consensus tree generated using parsimony for the GlnH regions of 35 glutamate receptor genes. There were 16 most-parsimonious trees of length 811; consistency index (CI) = 0.667 and retention index (RI) = 0.755. *B,* Consensus tree generated using parsimony for the regions of 35 glutamate receptor genes that lie between the GlnH and transmembrane domains. There were eight most-parsimonious trees of length 1,036; CI = 0.749 and RI = 0.810. *C,* Consensus tree generated using parsimony for the transmembrane regions of 35 glutamate receptor genes. There were 26 most-parsimonious trees of length 699; CI = 0.735 and RI = 818.

GlnH domains using the ILD test devised by Farris et al. (1994, 1995). This test was implemented to determine if the phylogenetic signals emanating from two (or more) data partitions are homogeneous. We reasoned that an inference of homogeneity of the two kinds of regions would be indicative of a common evolutionary history or linkage. In this case, the hypothesis that these regions were united in an exon-shuffling scenario late in evolution would be rejected. In contrast, inhomogeneity would be indicative of incongruent evolutionary history, and the hypothesis that these two regions of the gene coevolved before plants and animals diverged would be rejected. Figure 7 shows the individual phylogenetic analyses of the three partitions (transmembrane domains [M], ligand-binding domains [GlnH], and the regions [INTER] between the transmembrane domains and GlnH domains) we erected. Visual inspection of these trees indicates some evident topological differences. However, statistical analysis of the incongruence length difference reveals no statistically significant incongruence among the three partitions (M vs. GlnH: $P = 0.87$; INTER vs. M: $P = 0.44$; INTER vs. GlnH: $P = 0.84$). These results indicate that the transmembrane domains and GlnH domains have most likely coevolved since their assembly before the divergence of plants and animals.

Predicting Function from Sequence

By including the plant *GLR*s in our analysis, we have the opportunity to identify residues of the iGluR proteins that were conserved before the divergence of plant *GLR*s and animal iGluRs. Since these residues have not mutated throughout the evolution of iGluRs, they are most likely functionally important residues that are crucial to the basic operations of these receptors. The invariant residues in plant *GLR*s and animal iGluRs are presented in figure 8*A*. Conservation is apparent in a stretch of characters at the end of M3. Several invariant residues are also present in the ligand-binding domains, GlnH1 and GlnH2. In addition, two tryptophan residues (W) are universally conserved in M1 and M2, respectively. Whereas at least one of these residues has been studied in mutagenesis experiments—mutations of R499 result in nonfunctional channels (Uchino et al. 1992)—published data from mutagenesis studies are not available on other invariant residues identified in this study. By conducting mutagenesis studies on these universally invariant residues, one should be able to gain insight into possible structure–function relationships.

On the other hand, by recognizing the difference in the locations of conserved regions in the different classes of iGluRs, we hope to identify regions of the proteins that may cause them to function differently, e.g., ligand and ion selectivity. In addition, by comparing the plant genes with the different classes of animal iGluRs separately (NMDA, AMPA/KA, and delta), we hope to gain insight into the functional properties of the putative plant receptors. Residues that are conserved only between plant *GLR*s and each animal iGluR subclass are

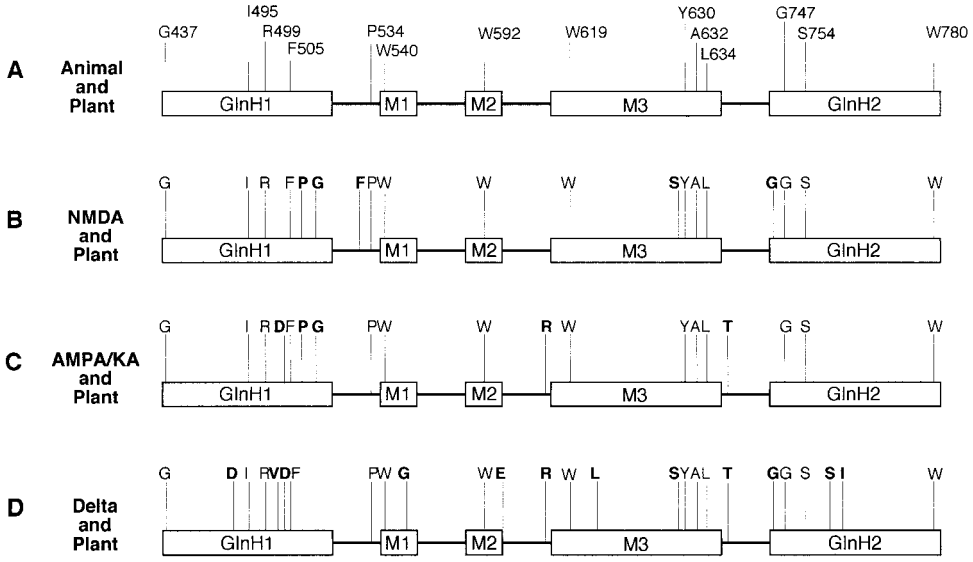

FIG. 8.—Diagrams showing the invariant residues between plant *GLR*s and different animal iGluR classes: all animal iGluRs (*A*), NMDA (*B*), AMPA/KA (*C*), and delta (*D*) iGluRs. In panel *A*, the positions of the invariant residues between plant *GLR*s and all animal iGluRs are given, and the numbers correspond to amino acid positions in the human GluH1 gene (GenBank accession number M64752). The approximate positions of the conserved residues are shown in relation to the functional domains, GlnH1, M1–M3, and GlnH2. In panels *B–D*, residues that are invariant between all plant and animal genes are shown as in panel *A*. In addition, invariant residues between plant *GLR*s and each iGluR subclass (NMDA, AMPA/KA, and delta) are shown in bold. The residues shown in bold can be uniquely invariant between plant *GLR*s and one iGluR subclass or invariant between plant *GLR*s and two of the three iGluR subclasses. By definition, these residues have to be conserved among members of each iGluR subclass as well. In fact, the number of residues that are conserved in each iGluR gene class is underestimated by adding the plant genes to each of the three analyses. The boxes representing the domains do not reflect actual sequence length.

shown in bold in figure 8*B–D*. The phylogenetic analyses shown in figures 5 and 6 failed to classify the plant *GLR*s into one of the gene classes of iGluRs—NMDA, AMPA/KA, or delta—based on a comparison using all of the important functional domains. However, it is still possible that the plant *GLR*s may be more closely related to a certain class of iGluR when looking at individual functional domains. According to figure 8, this is not the case. There appears to be more conservation between the plant *GLR*s and the delta iGluRs in all functional domains. As mentioned earlier, the wild-type delta iGluRs have no channel activity (Araki et al. 1993; Lomeli et al. 1993; Zuo et al. 1997); therefore, it is possible that this is the case for the plant genes. However, it is interesting to note that two residues, proline (P) and glycine (G), present at the end of GlnH1 in NMDA and AMPA/KA are conserved in the plant *GLR*s but altered in the delta iGluRs. GlnH1 is believed to function as part of the ligand-binding site in iGluRs. Since NMDA and AMPA/KA iGluRs are both functional in terms of ligand-binding, the plant *GLR*s may have the capacity for ligand binding as well, but the presence of channel activity that is totally identical to NMDA and AMPA/KA iGluRs is another matter. It would be informative to conduct mutagenesis studies in which proline and glycine are altered in NMDA or AMPA/KA iGluRs to resemble the delta residues, or vice versa. Such studies will give us answers as to whether the delta iGluRs have lost proper ligand-binding ability due to the loss of these two conserved residues, or whether residues other than the two mentioned are also crucial.

Besides looking at residues in the iGluR proteins that are invariant between plants and animals, it is also interesting to look at the differences between the two. To examine more detailed structure–function relationships in this respect, we isolated M3 and examined it more closely. M3 was chosen because it is the most conserved region of the protein, as observed in figures 4, 8 and 9, as well as in previous sequence alignment analyses (data not shown). Whereas it is believed that M2 serves as a major part of the channel pore (Sommer et al. 1991; Köhler et al. 1993), the function of M3 is not exactly clear yet. While most researchers have concentrated their studies on M2 due to its structural importance and RNA-editing property, the analysis presented here could identify residues that may be functionally important in M3. We identified residues that are conserved in all of the animal iGluRs (fig. 9*A*), as well as those that are conserved in both animal iGluRs and plant *GLR*s (fig. 9*B*) in M3. As observed in fig. 9, the longest stretch of conserved residues in animal iGluRs is at the end of M3 (YTANLA), although the sixth residue in M3, tryptophan (W), is strikingly conserved in both plant *GLR*s and animal iGluRs. When the plant *GLR*s are added to the analysis, the conservation generally decreases, but the level of conservation and the properties of the amino acids in the M3 C-terminal region barely change. In other cases, possible amino acid character states added to the animal character states (shown in bold) by the addition of the plant genes are sometimes quite different in terms of amino acid charge and size. This is apparent in characters 1, 9, 14, 15, and 20. For character 1 in M3, two of the four plant *GLR*s (*GLR1* and *GLR4*) have an aromatic phenylalanine (F) instead of a nonaromatic amino acid such as isoleucine (I) or valine (V). Although this character is not extreme-

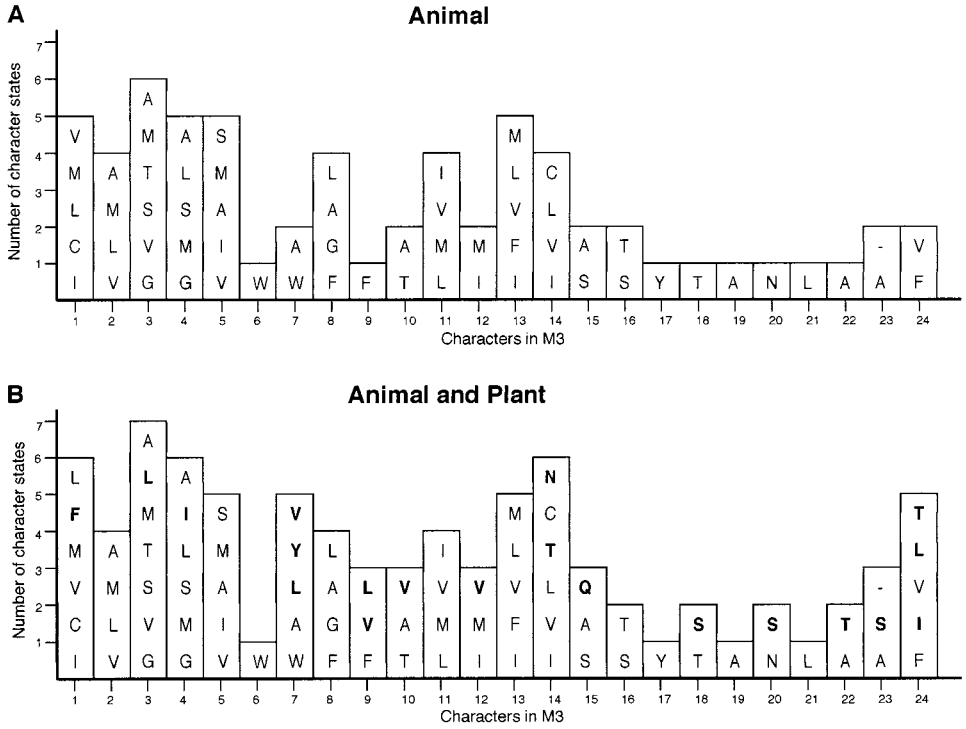

FIG. 9.—Graphs showing the number of possible character states at each amino acid position in M3 in the GLU analysis. The number of possible amino acids is shown on the Y-axis, and the amino acid positions in M3 from the N-terminal to the C-terminal end are shown from left to right on the X-axis. Character 1 in M3 corresponds to amino acid position 614 in the human GluH1 gene (GenBank accession number M64752). The possible amino acids at each character are shown inside the bar. The most common amino acid at each character is located at the bottom of each bar, while the least common is located at the top. *A,* The animal graph was generated by excluding the plant and bacterial genes. By using the trace character function in MacClade (Maddison and Maddison 1992), each character of M3 was examined, and the number of possible amino acids at each position was recorded. *B,* The animal-and-plant graph was generated by excluding only the bacterial genes. In the animal-and-plant graph, the amino acids in bold are added onto the possible character state list with the incorporation of the plant genes in the second graph.

ly conserved even in animal iGluRs with five possible character states, this drastic change in amino acid size and properties may have functional consequences. The case of character 9 in M3 seems to be the opposite of that of character 1 (fig. 9). Instead of having an aromatic phenylalanine (F) that is conserved in all of the animal iGluRs, the plant *GLR*s have nonaromatic leucine (L) or valine (V). Although these amino acids are similar in terms of hydrophobicity, their sizes are different. Therefore, if M3 is a structural part of the channel pore or vestibule, this character change may affect the selectivity and permeability of ions passing through the channels. In general, this part of the analysis points to possible sites for mutagenesis at which residues in animal iGluRs are altered to resemble the corresponding plant residues. Results of these studies can help to uncover the function of residues in M3 as well as the functional properties of the plant *GLR*s.

In conclusion, using the plant *GLR* genes as a tool to identify invariant residues may provide ''fossil'' evidence with which to explore how cell–cell signaling by excitatory amino acids in animal brains evolved from a primitive signaling mechanism that existed before plants and animals diverged. The fact that iGluRs appear to exist in plants and animals and not in the genomes of unicellular organisms whose genomes are completely sequenced suggests that cell–cell signaling in multicellular organisms may predate the divergence of animals and plants.

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