This is to certify that the

dissertation entitled

Characterization of Gap Junction-Like Polypeptides in Plant Cells

presented by

Sally Ann Meiners

has been accepted towards fulfillment
of the requirements for

Ph.D. degree in Biochemistry

Melvin Schindler
Major professor

Date April 17, 1991

CHARACTERIZATION OF GAP JUNCTION-LIKE POLYPEPTIDES IN PLANT CELLS

By

Sally Ann Meiners

A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Biochemistry

1991

ABSTRACT

CHARACTERIZATION OF GAP JUNCTION-LIKE POLYPEPTIDES IN PLANT CELLS

by

Sally Ann Meiners

Coordination and synchrony of a variety of cellular activities in tissues of plants and animals occur as a consequence of the transfer of low molecular weight biosynthetic and signaling molecules through specialized structures (plasmodesmata in plant cells and gap junctions in animal cells) that form aqueous channels between contacting cells. Investigations with rat liver demonstrated that cell-cell communication is mediated by a hexameric pore structure in the plasma membrane. Following association with the same structure in a contiguous cell, a trans-double membrane channel is created that has been termed a gap junction. In plant tissue, long tube-like structures called plasmodesmata are suggested to serve a similar cell-cell linking function between cytoplasmic compartments. Although morphologically distinct, dynamic observations suggest similarities in transport properties between gap junctions and plasmodesmata. This dissertation now provides evidence that these functional similarities may reflect a more profound identity between rat liver connexin 32, the polypeptide that comprises rat liver gap junctions, and an immunologically homologous protein found in soybean, *Arabidopsis thaliana*, and perhaps all plants that has been localized in soybean root cells to the plasma membrane/cell wall interface. This localization is consistent with a hypothetical role for the plant protein as a

structural component of plasmodesmata. Gap junction antibodies initially used to identify this plant protein were also used to isolate a cDNA clone from an *Arabidopsis thaliana* expression library. The deduced amino acid sequence obtained from the cDNA demonstrated sequence homology with rat liver connexin 32. The same antibodies, when utilized in an assay to measure cell-cell communication between soybean root cells, demonstrated a retardation effect on intercellular transport, presumably by binding to the plant protein and thereby altering channel accessibility or structure.

Dedicado a mis amigos queridos

ACKNOWLEDGEMENTS

I would like to acknowledge all the past and present members of the Schindler and Wang laboratories for their advice, assistance, and collaborations throughout the years. In particular, I would like to thank my advisor, Dr. Mel Schindler, for his never failing support, for pushing me when I needed to be pushed, and for encouraging the growth and independence of my scientific thought. Special thanks also go to Dr. Siu Cheong Ho, who originally trained me in laboratory work and taught me the value of a good sense of humor, and to An-de Xu and Wenge Zhang for their superb technical assistance.

I would also like to acknowledge Todd Black, Doug Cress, and Aris Economides for sharing their knowledge of molecular biology with me. Their advice and assistance every step of the way was very much appreciated. Thanks, guys.

Finally, thanks to John Kemner for helpful discussions and moral support during the difficult times; to Neera Agrwal for guiding me through my comprehensive examination; to Stacey Kraemer for her assistance with this manuscript; and to my family for putting up with all the highs and lows.

TABLE OF CONTENTS

CHAPTER I

CHAPTER II

CHAPTER III

CHAPTER IV

LIST OF FIGURES

Figure Page

CHAPTER I

CHAPTER II

CHAPTER III

CHAPTER IV

LIST OF ABBREVIATIONS

kDa	kilodalton
TPA	12-0-tetradecanoyl-phorbol-13-acetate
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
DAG	diacylglycerol
ER	endoplasmic reticuluum
IP_3	D-myo-inositol-1,4,5-triphosphate
PMSF	phenylmethylsulfonyl fluoride
NEPHGE	nonequilibrium pH gradient electrophoresis
IgG	immunoglobulin G
CNBr	cyanogen bromide
TFA	trifluoroacetic acid
TBS	tris-buffered saline
PBS	phosphate-buffered saline
BSA	bovine serum albumin
RSA	rabbit serum albumin
kb	kilobase
bp	base pair
MW	molecular weight
TE	tris EDTA buffer
EDTA	ethylenedinitrillo-tetraacetic acid disodium salt
FDA	fluorescein diacetate

FRAP	fluorescence redistribution after photobleaching
FITC	fluorescein-isothio-cyanate
FITC-20, 40, & 70 kDa dextrans	fluorescein-derivatized 20, 40, & 70 kDa dextrans
ACAS	anchored cell analysis and sorting

Chapter I

LITERATURE REVIEW

REVIEW OF LITERATURE

Introduction to Gap Junctions and Plasmodesmata

Cell-cell communication between contiguous tissue-forming cells is vital for control and coordination of cell proliferation, tissue metabolism, and synchrony (1,2). The structural element in the physical linkage connecting the cytoplasmic compartment of animal cells is the low-resistance pathway formed by transmembrane protein channels termed gap junctions (1,2). These structures permit the diffusion mediated transfer of low molecular weight cytoplasmic components ($\leq$ 1.7 kDa as measured with synthetic peptides (2]) that can transmit biochemical signals or metabolically share biosynthetic/energetic precursors (1). The basic unit of the gap junction is the connexon (1,3), which is composed of six polypeptide chains (termed connexins) that form a hexagonally symmetric transbilayer structure containing a single aqueous channel with a diameter of 2 nm (3). A gap junction is formed by the head-to-head association of two connexons between contacting cells (1,3). Intercellular communication between plant cells has been ascribed to trans-wall tubular structures, termed plasmodesmata, which provide intercellular channels for the movement of low molecular weight ($\leq$ 1,000 mol wt) metabolic, biosynthetic, and signaling molecules (4,5,6,7) between contiguous cells. Both gap junctions in animal cells and plasmodesmata in plant cells appear to be the essential structural elements enabling the integration of cellular activity within the synchronous behavior and development of tissues.

The large body of data detailing gap junction-mediated communication in animal cells is contrasted by the limited knowledge of intercellular communication mechanisms in plant tissues. It has been pointed out that the evolutionary development of a cell wall in plants precluded the development of a means for direct communication with vascular systems, e.g. blood and lymph in animals, and therefore, that plant cells must rely heavily on symplastic communication. (Symplast refers to the interconnected protoplasts, all bound by a continuous plasma membrane (4,8].) Plasmodesmata have been implicated as trans-cell wall channels providing a low resistance pathway between plant cytoplasmic/symplastic compartments (5,6). An important aspect of the plant studies has been the observation that the transport properties of plasmodesmata are strikingly similar to those of animal gap junctions (9,10,11,12,13,14). In addition to the similarity in channel size discrimination, intercellular transport via both plasmodesmata and gap junctions can be inhibited by incubation of tissues in divalent calcium or 12-O-tetradecanoyl-phorbol-13-acetate (TPA), a tumor promoter derived from croton oil (9,10,11,12). Furthermore, intercellular transport in animals (13,14) and also, apparently, in plants (O. Baron-Epel, unpublished results) can be reduced by the exposure of tissues to octanol.

Recent work in our laboratory, utilizing an affinity purified antibody that recognizes rat liver connexin 32[1], has demonstrated a homologous protein of 29 kilodaltons (kDa) in the cell membrane/wall fraction of soybean (*Glycine max* [L.] Merr. cv. Mandarin) root cells (SB-1 cell line) grown in callus and suspension culture (15). Immunofluorescence studies utilizing the same monospecific antibody have demonstrated fluorescent spots on the plasma membrane and cell wall of soybean in a pattern reminiscent of plasmodesmatal pitfields (16). These findings suggest that the functional similarities noted between animal gap junctions and plant plasmodesmata may be related to similar protein building blocks that comprise each structure.

ANIMAL GAP JUNCTIONS: BIOCHEMISTRY AND DYNAMIC CHARACTERIZATION

Structural Characterization

The connexons comprising gap junctions have been found in most metazoan phyla and have been demonstrated in most vertebrate tissues. (Exceptions are adult skeletal muscle, lymphoid cells, many nerve cells (17,18,19].) Recently, invertebrate tissues have also been shown to possess morphologically distinct, gap junction-like structures following isolation and electron microscopic analysis (20). Gap junction material isolated by a number of

---

[1] Rat liver connexin 32 derives its name from its molecular mass of 32.007 kDa, which was calculated from the amino acid sequence deduced from cloned cDNA. However, the polypeptide exhibits aberrant migratation on reducing sodium dodecyl sulfate polyacrylamide gels (SDS-PAGE) and is observed with M_r 27,000.

procedures from a variety of sources (e.g. liver, pancreas, stomach, brain) contains, upon analysis by reducing SDS-PAGE, a predominant polypeptide with reported molecular masses of 26 to 29 kDa (18,19,20,21,22). Other reports by various groups show the presence of a polypeptide with M_r 16,000 in invertebrates (20) and a polypeptide with M_r 43,000 in heart (17). Of all the gap junction polypeptides (connexins) reported, the M_r 27,000 rat liver polypeptide is perhaps the most extensively studied and documented. It is now becoming clear by comparative biochemical and immmunological studies that homologies exist between the paradigm rat liver connexin and junctional proteins present in other tissues and organisms (17,18,19).

cDNA clones coding for several gap junction proteins have recently been isolated and characterized (17,23,24,25,26,27). A comparison of the deduced amino acid sequences reveals that these proteins all appear to be related to a greater or lesser extent and that the COOH terminal of the proteins is more variable than the NH_2 terminal. The general trend also seems to indicate that connexins from comparable tissues (e.g. rat, human, and *Xenopus laevis* liver) exhibit considerable sequence homology, while connexins from the same organism but different tissues may exhibit a lesser degree of homology. (For example, the rat liver (23,24) and *Xenopus* liver (27) sequences are 71% identical, and the rat liver and human liver (23) sequences are 98% identical. In contrast, the rat liver and rat heart (17) sequences are only 54% identical at the amino acid level.)

The gap junction protein sequences deduced thus far are characterized by a preponderance of hydrophobic amino acids in the NH_2 terminal. Kyte and Doolittle analyses (28) indicate, in most cases, the presence of four hydrophobic domains which are of sufficient length to traverse a membrane bilayer. (*Xenopus* embryonic connexin 38 (25), with a fifth hydrophobic domain predicted close to the COOH terminal, is an exception.) The COOH terminal, which is suggested to contain the cytoplasmic residing portion of the polypeptide chain, possesses many charged and hydrophilic amino acids.

The gap junction primary sequences demonstrate a diverse and abundant array of consensus sequence sites available for post-translational modifications, including potential sites for asparagine-linked glycosylation and protein kinase C, cAMP-dependent, and tyrosine phosphorylation. The number and position of such sites appears to be quite flexible; the asn-linked glycosylation sites, for example, vary from none in *Xenopus* liver connexin 30 (27) to three in rat heart connexin 43 (19). Whether any of these sites are truly glycosylated has not yet been conclusively established for any gap junction polypeptide. The observed phosphorylation sites are significant given observations that channel state, open or closed, may be controlled by phosphorylation events (29, 30, 31). For example, protein kinase C-mediated phosphorylation has been inferred to modify transport as a result of measurements showing that agents that can activate protein kinase C, e.g. TPA, DAG (diacylglycerol), also inhibit intercellular communication (31,32,33). Phosphorylation by cAMP-dependent protein kinase has also been implicated in the regulation of gap junctional communication (29); but the fact that some gap junction proteins lack the appropriate cAMP-dependent phosphorylation sites (e.g. rat heart connexin 43 (17]) suggests that there may be tissue-specific regulation through these sites, or perhaps they are not directly involved in controlling transport. The relevance of the tyrosine phosphorylation sites is still, at this time, unclear.

Functional Characterization

Past suggestions that gap junctions were indeed the structural elements of an intercellular communication system have rested on predominantly correlative observations relating to transport and the presence of gap junction material (1,2,34,35). More recently, both immunological (36,37) and channel reconstitution experiments (38) have provided the direct link between the gap junction proteins and the site of a trans-bilayer communication channel. Warner *et al.* (36) demonstrated that affinity purified rat liver connexin 32 antibodies could block dye transfer and electrical coupling between the progeny cells when microinjected into an identified cell of the 8-cell stage embryo of *Xenopus*. In a similar manner, rat liver connexin 32 antibodies irreversibly blocked intercellular junctional conductance and dye permeability following injection into pairs of rat ventricular myocytes, hepatocytes, and cultured sympathetic neurons (37). Hertzberg *et al.* (38) demonstrated similar ionic permeabilities and control properties with purified rat liver gap junctions inserted into bilayers as compared to isolated pairs of rat hepatocytes. In light of these direct demonstrations of gap junction-mediated transport, work has now focused on examining how alcohols, tumor promoting substances, and calcium can specifically modify gap junction proteins to alter the channel permeability.

Developmental Significance

Gap junctions have been described as routes by which information is transmitted for the control of embryonic development and differentiation. Gap junctions may permit cellular gradients to develop and so influence cell behavior in such a manner that a spacial patterning of types develops. These gradients could control tissue growth and polarity (36,39,40,41,42,43). This argument has gained support by the demonstration of severe developmental abnormalities which resulted from antibody-mediated inhibition of junctional communication (36,44,45). Microinjection of affinity purified rat liver connexin 32 antibodies into *Xenopus laevis* embryos lead to pronounced defects in the development of dorsioanterior structures in the tadpole, including derivatives of the neural tube (36). The morphogenetic gradient controlling bud position in the body column of *Hydra attenuata* was interfered with after these antibodies were introduced into permeabilized epithelial cells; an increased frequency of budding around the head was noted (44). In another study, blastomeres in the mouse morula did not compact properly after they were microinjected with rat liver connexin 32 antiserum (45). In each of these cases, the antibodies used were subsequently shown to bind specifically to gap junctional domains which reside in the cytoplasm (46,47). The antibodies were also shown to be capable of blocking the conductance of reconstituted gap junction channels *in vitro* (48).

PLANT INTERCELLULAR COMMUNICATION:
PLASMODESMATA AS TRANS-WALL PERMEABLE CHANNELS

Organization and Evidence for a Transport Channel in Plasmodesmata

Plasmodesmata are tubular structures that penetrate the cell walls of higher plants, algae, and some fungi, apparently serving to connect the cytoplasmic compartments of contiguous cells (4,5,49,50,51). In higher plants, they have a diameter of approximately 60 nm and in thin sections appear to be composed of a central cylindrical membrane surrounded by 6 to 9 doughnut-like subunits (5,52). Plasmodesmata to date have not been isolated, and all structural information describing them consists of transmission electron microscopy of fixed sections (5,52,53,54,55,56). This has led to a variety of models (for example, see 53,57,58,59) and precludes a more exact characterization of their structure. The earlier studies, as exemplified by Robards' in 1968 (57), envisaged a desmotubule, i.e. the central membrane in plasmodesmata, running from cell to cell and connecting at each end with endoplasmic reticuluum (ER). The surrounding subunits were thought to be comprised of protein. Later studies (52,58,59) have replaced the proteinaceous model with one in which plasmadesmata are considered to be an extension of the ER membrane. This type of model predicts that the transport structures consist almost entirely of lipid. However, neither the lipid model nore the proteinaceous model before it are based on direct experimental evidence. Investigations by at least one group now suggest that plasmadesmata may be composed largely of protein, as predicted by the original model, wrapped around an ER core. Cooke *et al.* (60) determined that protease treatment of fern (*Onoclea sensibles*) gametophytes could disrupt the overall integrity of plasmodesmata, but that treatment with detergents did not have the similar effect.

Over the whole wall surface, the frequency of plasmodesmata may vary from 0.1 to 10/μm^2 depending on plant and plant cell type (4,61,62,63,64). This density is considerably greater in pitfields, which are specialized areas of high plasmodesmatal content. On a total cell basis, even the smallest meristematic cells have between 1,000 and 10,000 connections with their neighbors (4). Evidence for the role of plasmodesmata as trans-wall, low resistance channels includes: (a) they appear to have an aqueous channel that penetrates through them (4,5,6); (b) transport of plant cellular substances between cells is proportional to the plasmodesmatal frequency (4,63,65,66); (c) ion transport, as represented by AgCl deposits, is found to colocalize with plasmodesmata (67); (d) intercellular transport appears not to exist between cells not connected by plasmodesmata (such as stomatal guard cells) (68,69); (e) plasmolysis, which affects plasmodesmatal organization, alters rates of cell-cell communication (8,70); and (f) there is a strong correlation between the extent of electrical coupling and the frequency of plasmodesmata (5).

Functional Analysis of Intercellular Communication in Plants

Plasmodesmata have been suggested to serve as the structural element for continuity between the membrane-bound cytoplasmic network that is continuous throughout the plant, termed the symplast (4,5,6). The best evidence for symplastic transport is that compounds can move between plant cells without being exchanged with molecules in the external solution (4,5,6); ionic and metabolic coupling occur between plant cells (4,69,72,73); and chemicals that do not pass across membranes may be injected into plant cells and subsequently spread to surrounding cells (6,8,49,74). Employing the latter method, a number of groups have used a variety of fluorescent dyes to establish that hydrophilic molecules $\leq$ 1,000 mol wt may be transported between cells (6,8,11,74,75,76,77).

Baron-Epel *et al.* (11) found that cell-to-cell transport could be reversibly inhibited in soybean cells by divalent calcium and TPA. Phorbol esters such as TPA are diacylglycerol (DAG) analogues and therefore activate protein kinase C directly (78). Therefore, TPA inhibition of dye coupling in soybean cells is consistent with the hypothesis that the DAG-IP_3 (D-myo-inositol-1,4,5-triphosphate) secondary messenger system (78,79) is involved in regulating plasmodesmatal permeability. Interestingly, Tucker (80) found that elevated cytoplasmic levels of IP_3 also caused a reversible blockage of dye transport in the *Setcreasa purpurea* staminal hair system, most probably by mobilizing Ca^{2+} to activate a Ca^{2+}-dependent protein kinase (e.g. protein kinase C). Taken together, the results presented here suggest that one pathway for the down regulation of permeability in plant cells may involve phosphorylation of a plasmodesmatal protein by protein kinase C, much as the down regulation of permeability in animal cells apparently involves protein kinase C-mediated phosphorylation of gap junction connexins (31,32).

Developmental Significance

Different plant cell types have varying numbers of plasmodesmata inserted into their walls. Variations in the number of plasmodesmata between given plant cells may be a potentially significant factor in the regulation of cell growth and development. For example, plasmodesmatal frequency may be a measure of the ability of a cell to maintain cell division. Gunning (81) has shown that the apical cell of *Azolla* (which produces all the cells of the mature root) fails to maintain the same number of plasmodesmata in each successive cell plate. The apical cell therefore becomes more and more isolated symplastically, a feature which could ultimately determine the final length of the root.

Variations in plasmodesmatal size exclusion limits may be a contributing factor in plant cell differentiation. For example, the onset of synchronous cell divisions that precede flowering in the apices of *Silene coeli-rosa* was correlated with a down regulation of the symplastic pathway within these apices (82,83). Experiments with molecular dyes suggested that the size of the molecules that could permeate the plasmodesmata was reduced, but that the actual number of plasmodesmata between the apical cells remained unchanged.

PLASMODESMATA AND GAP JUNCTIONS:
DIFFERENT ARCHITECTURE, SAME BRICK?

Functionally, plasmodesmata appear to be very similar in activity to animal gap junctions. The similarity in molecular size discrimination, ionic transport properties, and control by TPA and/or Ca^{2+} is noteworthy (9,10,11,12,13,14). Structurally, however, there appear at first glance to be considerable architectural differences between plasmodesmata and gap junctions. The gap junction need only span a 2 nm "gap" between cells (Figure 1A,B)(1,3), whereas plasmodesmata must traverse cell walls 70-90 nm in thickness (Figure 1E,F)(4,5).

As previously indicated, a gap junction is formed by the head-to-head association of two connexons between adjacent cells (Figure 1A,B). A connexon is in turn comprised of six identical polypeptide chains (with M_r 27,000 in rat liver) termed connexins (3,84,85). The diameter of the connexon is 8.5-9.0 nm, and the diameter of its central transport channel is 1.5-2.0 nm (Figure 1C,D). Plasmodesmata are long tubular structures (Figure 1E,F) which, in thin sections, demonstrate a "central rod" (suggested to be comprised of ER) surrounded by 6 to 9 irregularly shaped subunits. Each of these subunits has a diameter of 9-10 nm. In addition, each contains a densely staining central area, reminiscent of a pore, which has a diameter of 1-2 nm (Figure 1G,H) (5,52). Therefore, despite their differences in gross morphology, gap junctions and plasmodesmata do seem to share similar structural features. Each plasmodesmatal subunit has approximately the same dimensions as a

Figure 1. Structural comparison of animal gap junctions (A-D) and plant plasmodesmata (E-H). (A) Thin-section electron micrograph of gap junctions between two mouse liver cells. The gap between the membrane is electron-dense because it has been filled with an electron-dense dye (procian brown). Bar = 100 nm (85). (B) Cartoon of gap junctions based on electron microscopy and X-ray diffraction studies. The drawing shows the interacting plasma membranes of two adjacent cells. The apposed bilayers are traversed by protein assemblies termed connexons, each of which is comprised of six polypeptide chains. Two connexons join across the intercellular gap to form a channel (termed a gap junction) connecting the two cells (85). (C) Electron micrograph of isolated rat liver gap junctions negatively stained with phosphotungstate. The connexons are organized in a hexagonal array. Bar = 25 nm (85). (D) Schematic drawing of gap junction connexons. (E) Thick (200 nm) sections of plasmodesmata through the inner tangential endodermal wall of barely (*Hordeum vulgare*) fixed in glutaraldehyde and osmium tetroxide. Bar = 250 nm (52). (F) Schematic drawing of a plasmodesma in the walls of absorbing hairs of *Tillandsea usneoides* (52). (G) Tranverse section of a plasmodesma fixed in a solution containing tannic acid. The "central rod" generally stains more lightly than the surrounding subunits; therefore, it is unclear whether these structures are related. Bar = 25 nm (52). (H) Artist's interpretation of (G), a transverse section of a plasmodesma. The subunits surrounding the central rod are compared to the gap junction connexons in (D). p = plasma membrane; w = wall.

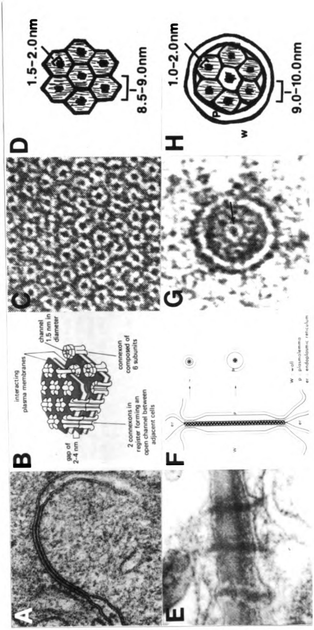

A
B
gap of 2-4 nm
interacting plasma membranes
channel 1.5 nm in diameter
connexon composed of 6 subunits
2 connexons in register forming an open channel between adjacent cells
C
D
1.5–2.0nm
8.5–9.0nm
E
F
w
er
p
W : wall
p : plasmolemma
er : endoplasmic reticulum
G
H
w
p
1.0–2.0nm
9.0–10.0nm

Figure 1

gap junction connexon.

The only structural information which is currently available detailing the organization of plasmodesmata is derived from transmission electron microscopy (5,52,53,54,55,56). Plasmodesmata have not been isolated, nor have they been biochemically characterized. Considering the similarities in function, control of function, and subunit structure between plasmodesmata and gap junctions; and considering other biological structures that can form vastly different organizations even though the same basic element is employed, e.g. actin or tubulin, it may not be inappropriate to speculate that plasmodesmata may be comprised of connexon-like elements stacked in a concentric fashion to form a trans-wall aqueous channel.

That such connexon-like elements may exist in plants is supported by our observation that when soybean root cell (SB-1 cell line) homogenates were electrophoresed on reducing SDS-PAGE gels and transferred to nitrocellulose paper, the resulting immunoblot with rat liver connexin 32 antibody yielded two polypeptide bands migrating with M_r 29,000 and 48,000. This is to be compared with two polypeptide bands migrating with M_r 27,000 and 48,000 for rat liver connexin 32 when immunoblotted in a similar fashion (15). (The M_r 48,000 polypeptide is a putative dimer of the M_r 27,000/29,000 polypeptide (15,86].)

Gap junction homologous polypeptides have also been noted in a number of other plants in addition to soybean, among them daisy (*Chrysanthemum leucanthemum* L.), petunia (*Petunia hybrida*), cucumber (*Cucumis sativus* L.) (16), *Arabidopsis thaliana* (87), and maize (*Zea mays* L.) (88,89). Most significantly, Yahalom *et al.* have localized antigens that are cross-reactive with antibodies directed against both rat liver connexin 32 and rat heart connexin 43 to the plasmodesmata of *Zea mays* L.(89). The investigators conducted their experiments utilizing colloidal gold-derivatized antibodies (90) visualized on thin section tissue preparations by electron microscopy techniques.

Further evidence for connexon-like elements in plants comes from our isolation of a cDNA clone from *Arabidopsis thaliana* using rat liver connexin 32 antibodies as probes (87). The *Arabidopsis* clone codes for a polypeptide which demonstrates approximately 27% identity and 40% similarity with the sequence of the rat liver connexin 32 (16,17). (This may be compared with the reported 32% identity observed between *Xenopus* embryonic connexin 38 and rat liver connexin 32 (25].) The *Arabidopsis* and rat liver sequences also yield similar Kyte-Doolittle hydropathy profiles (28) and Chou-Fasman secondary structure predictions (91).

In his book *Plant and Planet*, Huxley (92) points out that Charles Darwin once wrote in his journal, "prove animals like plants". The discovery of a gap junction-like polypeptide in plant cells may be a step on the road to that vision by suggesting the exciting possibility that gap junctions may be an evolutionary modification of plasmodesmata.

REFERENCES

1. Hertzberg, E.L., Lawrence, T.S., and Gilula, N.B. 1981. Gap junctional communication. Annu. Rev. Physiol. 43: 479-491.
2. Loewenstein, W.R. 1979. Junctional intercellular communication and the control of growth. Biochim. Biophys. Acta 560: 1-65.
3. Unwin, P.N.T., and Zampighi, G. 1980. Structure of the junction between communicating cells. Nature 283: 545-549.
4. Gunning, B.E.S. 1976. Introduction to plasmodesmata. In: Intercellular Communication in Plants: Studies on Plasmodesmata. B.E.S. Gunning and A.W. Robards, eds., pp. 1-13. Springer-Verlag, Berlin.
5. Gunning, B.E.S., and Overall, R.L. 1983. Plasmodesmata and cell to cell transport in plants. Bioscience 33: 260-265.
6. Terry, B.B., and Robards, A.W. 1987. Hydrodynamic radius alone governs the transport of molecules through plasmodesmata. Planta 171: 145-157.
7. Goodwin, P.B. 1983. Molecular size limit for movement in the symplast of the *Elodea* leaf. Planta 157: 124-130.
8. Erwee, M.G., and Goodwin, P.B. 1984. Characterization of *Egeria densa* leaf symplast: response to plasmolysis, deplasmolysis, and to aromatic amino acids. Protoplasma 122: 162-168.

9. Rink, T.J., Tsien, L.W., and Warner, A.E. 1980. Free calcium in *Xenopus* embryos measured with ion-selective electrodes. Nature 283: 658-660.

10. Wade, M.H., Trosko, J.E., and Schindler, M. 1986. A fluoresence photobleaching assay of gap junction-mediated communication between human cells. Science 232: 525-528.

11. Baron-Epel, O., Hernandez, D., Jiang, L.-W., Meiners, S., and Schindler, M. 1988. Dynamic continuity of cytoplasmic and membrane compartments between plant cells. J. Cell Biol. 106: 715-721.

12. Spray, D.C., Stern, J.H., Harris, A.L., and Bennett, M.V.L. 1982. Comparison of sensitivities of gap junctional conductance to H and Ca ions. Proc. Natl. Acad. Sci. USA 79: 441-445.

13. Spray, D.C., Ginzberg, R.D., Morales, E.D., Gatmaitan, Z., and Arias, I.M. 1986. Electrophysiological properties of gap junctions between dissociated pairs of rat hepatocytes. J. Cell Biol. 103: 135-144.

14. Spray, D.C. 1990. Electrophysiological properties of gap junction channels. In: Parallels in Cell to Cell Junctions in Plants and Animals. A.W. Robards, H. Jongsma, W.J. Lucas, J. Pitts, and D.C. Spray, eds., pp. 63-86. Springer-Verlag, Heidelberg.

15. Meiners, S., and Schindler, M. 1987. Immunological evidence for gap junction polypeptide in plant cells. J. Biol. Chem. 262: 951-953.

16. Meiners, S., and Schindler, M. 1989. Characterization of a connexin homologue in cultured soybean cells and diverse plant organs. Planta 179: 148-155.

17. Beyer, E.C., Paul, D.L., and Goodenough, D.A. 1987. Connexin 43: a protein from rat heart homologous to a gap junction protein from liver. J. Cell Biol. 105: 2621-2629.

18. Dermietzel, R., Leibstein, A., Prixen, U., Janssen-Timmen, U., Traub, O., and Willecke, K. 1984. Gap junctions in several tissues share antigenic determinants with liver gap junctions. EMBO (Eur. Molec. Biol. Org.) J. 3: 2261-2270.

19. Hertzberg, E.L., and Skibbens, R.V. 1984. A protein homologous to the 27,000 dalton liver gap junction protein is present in a wide variety of species and tissues. Cell 39: 61-69.

20. Finbow, M.E., Eldridge, T., Buujhens, J., Lane, N.J., Shuttleworth, J., and Pitts, J.B. 1984. Isolation and characterization of arthopod gap junctions. EMBO (Eur. Molec. Biol. Org.) J. 3: 2271-2278.

21. Hertzberg, E.L. 1984. A detergent-independent procedure for the isolation of gap junctions from rat liver. J. Biol. Chem. 259: 9936-9943.

22. Ehrhart, J.C., and Chauveau, J. 1977. The protein component of mouse hepatocyte gap junctions. FEBS (Fed. Eur. Biochem. Soc.) Lett. 78: 295-299.

23. Kumar, N.M., and Gilula, N.B. 1986. Cloning and characterization of human and rat liver cDNAs coding for a gap junction protein. J. Cell Biol. 103: 767-776.

24. Paul, D.L. 1986. Molecular cloning of cDNA for rat liver gap junction protein. J. Cell Biol. 103: 123-134.

25. Ebihara, L., Beyer, E.C., Swenson, K.I., Paul, D.L., and Goodenough, D.A. 1989. Cloning and expression of a *Xenopus* embryonic gap junction protein. Science 243: 1194-1195.

26. Gimlich, R.L., Kumar, N.M., and Gilula, N.B. 1990. Differential regulation of the levels of three gap junction mRNAs in *Xenopus* embryos. J. Cell. Biol. 110: 597-605.

27. Gimlich, R.L., Kumar, N.M., and Gilula, N.B. 1988. Sequence and developmental expression of mRNA coding for a gap junction protein in *Xenopus*. J. Cell Biol. 107: 1065-1073.

28. Kyte, J., and Doolittle, R.F. 1982. A simple method of displaying the hydropathic nature of a protein. J. Mol. Biol. 157: 105-132.

29. Saez, J.C., Spray, D.C., Nairn, A.C., Hertzberg, E.L., Greengard, P., and Bennett, M.V.L. 1986. cAMP increases junctional conductance and stimulates phosphorylation of the 27 kDa principle gap junction polypeptide. Proc. Natl. Acad. Sci. USA 83: 2473-2477.

30. Wiener, E.C., and Loewenstein. W.R. 1983. Correction of cell-cell communication defect by introduction of a protein kinase into mutant cells. Nature 305: 433-435.

31. Spray, D.C., and Burt, J.M. 1990. Structure-activity relations of the cardiac gap junction channel. Am. J. Physiol. 258: C195-C205.

32. Gainer, H.S.C., and Murray, A.W. 1985. Diacylglycerol inhibits gap junctional communication in cultured epidermal cells:

evidence for a role of protein kinase C. Biophys. Res. Commun. 126: 1109-1113.

33. Yada, T., Rose, B., and Loewenstein, W.R. 1985. Diacylglycerol downregulates functional membrane permeability. TMB-8 blocks this effect. J. Membr. Biol. 88: 217-232.

34. Caveney, S. 1985. The role of gap junctions in development. Annu. Rev. Physiol. 47: 319-335.

35. De Laat, S.W., Tertoolen, L.G.H., Dorresteign, A.W.C., and Van den Biggelar, S.A.M. 1980. Intercellular communication patterns are involved in cell determination in early molluscan development. Nature 287: 546-548.

36. Warner, A.E., Guthrie, S.C., and Gilula, N.B. 1984. Antibodies to gap junctional protein selectively disrupt junctional communication in the early amphibian embryo. Nature 311: 127-131.

37. Hertzberg, E.L., Spray, D.C., and Bennett, M.V.L. 1985. Reduction of gap junctional conductance by microinjection of antibodies against the 27 kDa liver gap junction polypeptide. Proc. Natl. Acad. Sci. USA 82: 2412-2416.

38. Spray, D.C., Saez, J.C., Brosius, D., Bennett, M.V.L., and Hertzberg, E.L. 1986. Isolated liver gap junctions: gating of transjunctional currents is similar to that in intact pairs of rat hepatocytes. Proc. Natl. Acad. Sci. USA 83: 5494-5497.

39. Sheridan, J.D., and Atkinson, M.M. 1985. Physiological roles of permeable junctions: some possibilities. Annu. Rev. Physiol. 47: 337- 353.

40. Blackshaw, S.E., and Warner, A.E. 1976. Low resistance junctions between mesoderm cells during development of trunk muscles. J. Physiol. 255: 209-230.

41. Ito, S., and Ikematsu, Y. 1980. Inter- and intra-tissue communication during amphibian development. Dev. Growth Differ. 22: 247-256.

42. Guthrie, S.C. 1984. Pattern of junctional communication in the early amphibian embryo. Nature 311: 149-151.

43. Guthrie, S.C., Turin, L., and Warner, A.E. 1988. Pattern of junctional communication during development of the early amphibian embryo. Development 103: 769-783.

44. Fraser, F.E., Green, C.R., Bode, H.R., and Gilula, N.B. 1987. Selective disruption of gap junctional communication interferes with a patterning process in Hydra. Science 237: 49-55.

45. Lee, S., Gilula, N.B., and Warner, A.E. 1987. Gap junctional communication and compaction during preimplantation stages of mouse development. Cell 51: 851-860.

46. Zimmer, P.B., Green, C.R., Evans, H.W., and Gilula, N.B 1987. Topological analysis of the major protein in isolated intact rat liver gap junctions and gap junction-derived single membrane structures. J. Biol. Chem. 262: 7751-7763.

47. Milks, L.C., Kumar, N.M., Houghten, R., Unwin, N., and Gilula, N.B 1988. Topology of the 32 kDa liver gap junction protein determined by site directed antibody localizations. EMBO (Eur. Molec. Biol. Org.) J. 7: 2967-2975.

48. Young, J.D.-E., Cohn, Z.A., and Gilula, N.B. 1987. Functional assembly of gap junction conductance in lipid bilayers: demonstration that the major 27 kDa protein forms the junctional channel. Cell 48:733-743.

49. Tucker, E.B., and Spanswick, R.M. 1985. Translocation in the staminal hairs of *Setcreasea purpurea*. II. Kinetics of intercellular transport. Protoplasma 128: 167-172.

50. Robards, A.W. 1975. Plasmodesmata. Annu. Rev. Plant Physiol. 26: 13-29.

51. Robards, A.W. 1980. General and molecular cytology. Prog. Bot. 42: 1-15.

52. Robards, A.W. 1976. Plasmodesmata in higher plants. In: Intercellular Communication in Plants: Studies on Plasmodesmata. B.E.S. Gunning and A.W. Robards, eds., pp. 15-37. Springer-Verlag, Berlin.

53. Overall, R.L., Wolfe, J., and Gunning, B.E.S. 1982. Intercellular communication in *Azolla* roots: I. Ultrastructure of the plasmodesmata. Protoplasma 111: 134-150.

54. Evert, R.F., Escrich, W., and Heyser, W. 1977. Distribution and structure of plasmodesmata in mesophyll and bundle sheath cells of *Zea mays*. Planta 136: 77-89.

55. Gunning, B.E.S., and Hughes, J. 1976. Quantitative assessment of symplastic transport of pre-nectar into the trichomes of *Abutilon* nectaries. Aust. J. Plant Physiol. 3: 619-637.

56. Oleson, P. 1979. The neck constriction in plasmodesmata: evidence for a peripheral sphincter-like structure revealed by fixation with tannic acid. Planta 144: 349-358.

57. Robards, A.W. 1968. A new interpretation of plasmodesmatal ultra-structure. Planta 82: 200-210.

58. Oleson, P. 1980. A model of a possible sphincter associated with plasmodesmatal neck regions. Eur. J. Cell Biol. 22: 250.

59. Robards, A.W., and Lucas, W.J. 1990. Plasmodesmata. Annu. Rev. Plant Physiol. Plant Molec. Biol. 41: 369-419.

60. Cooke, T.J., Tilney, M.S., and Tilney, L.G. 1990. The distribution, activity, and structure of plasmodesmata in developing fern gametophytes. (Abstr.) J. Cell Biol. 111: 1451.

61. Livingston, L.J. 1935. The nature and distribution of plasmodesmata in the tobacco plant. Am. J. Bot. 22: 75-87.

62. Kuo, J., O'Brien, T.P., and Canny, M.J. 1974. Pit field distribution, plasmodesmatal frequency, and assimilate flux in the mesotome sheath cells of wheat leaves. Planta 121: 97-118.

63. Sauter, J.J., and Kloth, S. 1986. Plasmodesmatal frequency and radial translocation rates in the ray cells of poplar (*Populus x Canadensis* Moench 'robreta'). Planta 168: 377-380.

64. Robards, A.W. 1982. Cell interactions in plants: a comparative study. In: The Functional Integration of Cells in Animal Tissues. J.D. Pitts and M.E. Finbow, eds., pp. 57-79. Cambridge Univ. Press, London.

65. Robards, A.W., and Clarkson, D.T. 1976. The role of plasmodesmata in the transport of water and nutrients across roots. In: Intercellular Communication in Plants: Studies on Plasmodesmata. B.E.S. Gunning and A.W. Robards, eds., pp. 181-207. Springer-Verlag, Berlin.

66. Clarkson, D.T., Robards, A.W., Stephens, J.E., and Stark, M. 1987. Suberin lamallae in the hypodermis of *Zea mays;* root development and factors affecting the permeability of hypodermal layers. Plant Cell Environ. 10: 83-94.

67. Van Steveninck, R.F.M. 1976. Cytochemical evidence for ion transport through plasmodesmata. In: Intercellular Communication in Plants: Studies on Plasmodesmata. B.E.S. Gunning and A.W. Robards, eds., pp. 132-147. Springer-Verlag, Berlin.

68. Juniper, B.E. 1977. Some speculations on the possible roles of the plasmodesmata in the control of differentiation. J. Theor. Biol. 66: 583-592.

69. Palevitz, B.A., and Hepler, P.K. 1985. Changes in dye coupling of stomatal cells of *Allium* and *Commelina* demonstrated by microinjection of Lucifer yellow. Planta 164: 377-380.

70. Barclay, G.F., Peterson, C.A., and Tyree, M.T. 1982. Transport of fluorescein in trichomes of *Lycopersicon esculentium*. Can. J. Bot. 60: 397-402.

71. Drake, G. 1979. Electrical coupling, potentials, and resistance in oat coleoptiles: effect of azide and cyanide. J. Exp. Bot. 30: 719-725.

72. Iijima, T., and Sibaoka, T. 1982. Propagation of action potential over the traplobes of *Aldrovanda vesiculosa*. Plant Cell Physiol. 28: 679-688.

73. Vorob'ev, L.N., Tarkhanov, K.A., and Vakhmistrov, D.B. 1982. Use of the electrical coupling factor for quantitative

estimation of symplastic communications. Soviet Plant Physiol. 28: 495-502.

74. Goodwin, P.B. 1983. Molecular size limit for movement in the symplast of the *Elodea* leaf. Planta 57: 124-130.

75. Erwee, M.G., Goodwin, P.B., and Van Bel, A.J.E. 1985. Cell-cell communication in the leaves of *Commelina cyanea* and other plants. Plant Cell and Environ. 8: 173-178.

76. Tucker, E.B. 1982. Translocation in the staminal hairs of *Setcreasea purpurea*. I. A study of cell ultrastructure and cell-to-cell passage of molecular probes. Protoplasma 113: 193-201.

77. Stewart, W.W. 1981. Lucifer dyes and highly fluorescent dyes for biological tracing. Nature 292: 17-21.

78. Nishizuka, Y. 1986. Studies and perspectives of protein kinase C. Science 233: 305-312.

79. Pooviah, B.W., Reddy, A.S.N., and McFadden, J.J. 1987. Calcium messenger system: role of protein phosphorylation and inositol biphospholipids. Physiol. Plant 69: 569-573.

80. Tucker, E.B. 1988. Inositol biphosphate and inositol triphosphate inhibit cell-to-cell passage of carboxyfluorescein in staminal hairs of *Setcreasea purpurea*. Protoplasma 137: 140-144.

81. Gunning, B.E.S. 1978. Age-related and origin-related control of the numbers of plasmodesmata in cell walls of developing *Azolla* roots. Planta 143: 181-190.

82. Goodwin, P.B., and Lyndon, R.F. 1983. Synchronization of cell division during transition to flowering in *Silene* apices is

not due to increased symplast permeability. Protoplasma 116: 219-222.

83. Santiago, J., and Goodwin, P.B. 1988. Restricted cell-cell communication in the shoot of *Silene coeli-rosa* during the transition to flowering is associated with a high mitotic index rather than with evocation. Protoplasma 146: 52-60.

84. Hertzberg, E.L., and Gilula, N.B. 1979. Isolation and characterization of gap junctions from rat liver. J. Biol. Chem. 254: 2138-2147.

85. Gilula, N.B. 1983. Gap junctions are comprised of channels that directly connect the interiors of interacting cells. In: Molecular Biology of the Cell. B. Alberts, D. Bray, J. Lewis, M. Raff, K. Roberts, and J.D. Watson, eds., p. 690. New York, N.Y.

86. Nicholson, B.J., Hunkapillar, M.W., Grim, L.B., Hood, L.E., and Revel, J.A. 1981. Rat liver gap junction protein: properties and partial sequence. Proc. Natl. Acad. Sci. USA 78: 7594-7598.

87. Meiners, S., Xu, A., and Schindler, M. 1991. Gap junction protein homologue from *Arabidopsis thaliana*: evidence for connexins in the plant family. Proc. Natl. Acad. Sci. USA, in press.

88. Yahalom, A., Epel, B., Laird, M., Traub, O., and Schindler, M. 1990. Connexin homologous polypeptides are present in maize mesocotyl cell walls. (Abstr.) Plant Physiol. 93: 138.

89. Yahalom, A., Warmbrodt, R., Laird, M., Revel, J.P., Traub, O., and Epel, B. 1991. Maize mesocotyl plasmodesmatal proteins

cross-react with connexon gap junction protein antibodies. The Plant Cell, in press.

90. Binder, M., Tourmente, S., Roth, J., Renaud, M., and Gehring, W.J. 1986. *In situ* hybridization at the electron microscope level: localization of transcripts on ultrathin sections of lowicryl K4M-embedded tissue using biotinylated probes and protein-A gold complexes. J.Cell Biol. 102: 1646-1653.

91. Chou, P.Y., and Fasman, G.D. 1978. Prediction of the secondary structure of proteins from their amino acid sequence. Adv. Enz. 47: 45-147.

92. Huxley, A. 1987. *Plant and Planet*, p. 16. Penguin Books, Harmondsworth, Middlesex, England.

Chapter II

CHARACTERIZATION OF A GAP JUNCTION
PROTEIN HOMOLOGUE
IN
CULTURED SOYBEAN ROOT CELLS
AND
THE ORGANS OF DIVERSE PLANTS

ABSTRACT

Soybean (*Glycine max* [L.] Merr. cv. Mandarin) protoplasts and rat livers were processed in parallel by a detergent-independent procedure originally designed to isolate rat liver connexin 32. This procedure resulted in protein fractions significantly enriched in a 29 kDa polypeptide for soybean and a 27 kDa polypeptide for rat liver (molecular mass determined by migration in SDS-PAGE gels.) Antibodies were prepared against the 27 kDa rat liver polypeptide (rat liver connexin 32) and its 29 kDa counterpart in soybean (operationally termed soybean connexin). (These antibodies will be hereafter referred to as rat liver connexin antibody and soybean connexin antibody.) As judged by specific reactivity on immunoblots, both antibodies were able to demonstrate the immunological relatedness of the 27 kDa (rat liver) and 29 kDa (soybean) polypeptides. The antibodies were utilized in immunofluorescence studies to localize the putative soybean connexin in cultured soybean cells; these experiments yielded a peripherally localized punctate pattern of labeling at areas of contact between cells. Use of rat liver connexin antibody as a probe on immunoblots also demonstrated that connexin-like proteins are present in a large number of different plants and plant organs.

INTRODUCTION

Cellular integration of biological activities in tissues is the role of gap junctions in animal cells (1,2,3) and plasmodesmata in plant cells (4,5). Although quite different in gross macromolecular organization when viewed at the electron-microscopic level (6,7), both structures have been demonstrated to provide hydrophilic conduits for low-molecular weight molecules through the plasma membranes of adjacent cells (2,5,8,9). The transport competency of these channels also appears to be controlled by similar molecules, e.g. 12-O-tetradecanoyl-phorbol-13-acetate (TPA) and Ca^{2+} (10,11). Evidence is presented herein which suggests that such functional similarities may be indicative of a more fundamental identity between the 27 kilodalton (kDa) polypeptide of rat liver gap junction (rat liver connexin 32) and an immunologically related 29 kDa polypeptide isolated from soybean cells (operationally termed soybean connexin). A variety of immunological reagents were used in this study to pursue characterization and localization of soybean connexin. The results demonstrated: (1) immunological and biochemical relatedness between rat liver and soybean connexins; (2) a peripherally localized organization of soybean connexin at contacting regions between cultured soybean cells; and (3) the presence of soybean-type connexin in extracts from the petals, fruits, and leaves of a variety of plants.

MATERIALS AND METHODS

Protein Isolation and Antibody Preparation against Rat Liver and Soybean Connexins

Cells (SB-1 cell line) originally derived from soybean (*Glycine max* [L.] Merr) roots (12) were grown in 1B5C medium, pH 5.5 (basic medium plus 1 mg/ml 2,4-dichlorophenoxyacetic acid and 2 g/l casein hydrolyzate) in the dark (13). Protoplasts were prepared from these cells according to the method of Ohyama (14). Soybean protoplasts and rat livers were processed in parallel by the detergent-independent procedure used by Hertzberg (15) to isolate rat liver gap junction connexins. The resulting protein fractions were then subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions (16), but without boiling. Boiling, variations in extraction procedure, and differences in polyacrylamide gel cross-linking have dramatic affects on the mobility of rat liver connexin 32. Aggregation to 48 kDa and higher forms has frequently been observed (15,17). Reducing SDS-PAGE gels stained with Coomassie brilliant blue demonstrated the presence of protein fractions greatly enriched in 27 kDa (rat liver) and 29 kDa (soybean) polypeptides, in addition to lesser amounts of 48 kDa and 68 kDa polypeptides (Figure 1B,D). (Compare to whole cell homogenates from soybean and rat liver, Figure 1C,D.) The 27 and 29 kDa regions of the gel (representing

Figure 1. SDS-PAGE of fractions obtained during the detergent-independent connexin isolation procedure described by Hertzberg (15). Samples here and elsewhere were diluted 1:1 with β-mercaptoethanol sample buffer (16) and were allowed to stand for 30 min at room temperature. They were subsequently analyzed on SDS-PAGE gels (acrylamide concentration 10%) stained with Coomassie brilliant blue. The contents of the lanes were: (A) soybean cell homogenate derived from suspension culture; (B) soybean connexin fraction; (C) rat liver homogenate; and (D) rat liver connexin fraction. The molecular weight standards were: phosphorylase b, 92,000; bovine serum albumin, 66,000; ovalbumin, 45,000; and carbonic anhydrase, 29,000. Soybean cells in (A) were homogenized with 2 volumes of 1B5C medium, 10 mM PMSF (phenylmethylsulfonyl fluoride) in a Waring blender at 4°C for 10 min. Rat liver in (C) was homogenized with 2 volumes of 1 mM $NaHCO_3$, 10 mM PMSF in a Waring blender at 4°C for 3 min. Approximately 200 µg of protein were loaded in (A) and (C), and approximately 20 µg were loaded in (B) and (D).

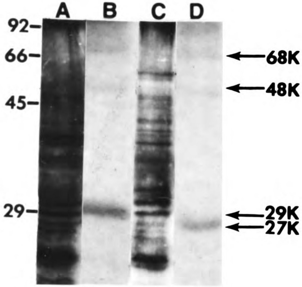


Figure 1

approximately 50 µg of protein) were each excised and homogenized in two volumes of double-distilled water using a Bellco tissue homogenizer (Bellco Glass, Vineland, N.J., USA). They were then suspended in Freund's adjuvant (Sigma Chemical Co., St. Louis, MO, USA) (complete for the initial injection; incomplete for booster injections) and injected into a white New Zealand female rabbit. The antisera obtained against the 27 kDa and 29 kDa polypeptides were affinity purified in the following manner. Approximately 50 µg of either rat liver or soybean connexin fraction were electrophoresed on a preparative SDS-PAGE gel. The polypeptides were transferred from the gel to nitrocellulose paper (18). Following incubation in the corresponding antiserum, the 27 kDa (rat liver) and 29 kDa (soybean) regions were visualized on a portion of the immunoblot by using alkaline phosphatase-conjugated goat anti-rabbit IgG (Sigma). These regions were then excised from the remaining portion of the blot, and the bound antibodies (rat liver connexin antibody and soybean connexin antibody, respectively) were eluted by the method of Smith and Fisher (19).

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis and Two-Dimensional Non-Equilibrium pH Gradient Electrophoresis (NEPHGE) Chromatography

To explore the extent of relatedness between the rat liver 27 kDa polypeptide and the soybean 29 kDa polypeptide, NEPHGE was performed in the first dimension and SDS-PAGE was performed in the second dimension. The procedures described in O'Farrell *et al.* (20) were followed with these modifications: The NEPHGE gels were prepared using pH 2-10 ampholites, and the electrophoresis was performed at 400 mV for 5 h. The basic electrode solution contained 10 mM NaOH and 5 mM $Ca(OH)_2$. Sample buffer contained a final urea concentration of 9.5 M. The second-dimension chromatography was performed with 120 mm separating gels with acrylamide composition 12.5%. Approximately 25 µg of rat liver connexin fraction or soybean connexin fraction were used for each two-dimensional gel. The polypeptides were transferred from the gel to nitrocellulose paper and probed with rat liver connexin antibody (30 µg/ml).

Comparative Peptide Map Analysis

To further explore the extent of homology between the rat liver and soybean connexins, comparative peptide map analyses utilizing either *Staphylococcus aureus* V-8 protease (Miles Laboratories, Naperville, IL, USA) or cyanogen bromide (CNBr) were performed. Rat liver and soybean connexin fractions were separated by SDS-PAGE and transferred to nitrocellulose. The positions of the 27 (rat liver) and 29 kDa (soybean) polypeptides were determined by incubating a portion of the immunoblot with rat liver connexin antibody (30 µg/ml) and then visualizing the bound antibody by using alkaline phosphatase conjugated goat anti-rabbit IgG. The 27 kDa rat liver and the 29 kDa soybean polypeptides were excised from the remaining portion of the blot.

The polypeptides were compared after partial V-8 proteolysis according to the method of Yuen *et al.* (21). The blotted protein bands (representing approximately 20 µg of 27 kDa or 29 kDa polypeptide) were placed in Eppendorf tubes containing 1 ml of 0.5%

(w/v) PVP-40 in 100 mM acetic acid. They were incubated in this solution at 37°C for 30 min. The nitrocellulose paper was then rinsed extensively in double-distilled water (10X, 1 ml), cut into small pieces (2 x 4 mm), and transferred into clean Eppendorf tubes containg 200 µl of 0.1 M Tris-HCl buffer (pH 6.8). A 0.5 mg/ml solution of V-8 protease was prepared in 0.1 M Tris-HCl (pH 6.8), and enough was added to the Eppendorf tubes to give a 1:20 (enzyme:substrate, w/w) ratio. The polypeptides were digested on the nitrocellulose paper for 24 h at 37°C, and the digestion was stopped by acidifying the solution with 3 µl of 10% trifluoroacetic acid (TFA). The supernatants were transferred into clean Eppendorf tubes. The nitrocellulose paper was rinsed with 100 µl of 0.1% TFA, and the combined extracts were dried in a Speed Vac. The samples were redissolved in 25 µl of double-distilled water, separated on SDS-PAGE gels, and transferred to nitrocellulose. The immunoblots were probed with rat liver connexin antibody (30 µg/ml).

In addition to digestion with V-8 protease, blotted rat liver and soybean connexins were cleaved with CNBr. Nitrocellulose containing the blotted protein was cut into 2 x 4 mm pieces and suspended in Eppendorf tubes containing 100-200 µl of 70% formic acid. A CNBr solution (70 mg/ml CNBr in 70% formic acid) was prepared and an appropriate amount (10µl per 10 µg of protein) was added to the tubes. The suspensions were incubated in the dark at room temperature for 24 h. The cleavage solutions were removed, diluted with 10 volumes of double-distilled water, and dried in a Speed Vac. They were redissolved in 25 µl of double-distilled water, separated on SDS-PAGE gels, transferred to nitrocellulose paper, and immunoblotted with rat liver connexin antibody (30 μg/ml).

Tissue Preparation and Immunofluorescence Analysis

Soybean cells (2 d after transfer to fresh 1B5C medium) were pelleted by centrifugation in a clinical centrifuge for 5 min at 460 x g. The cells were resuspended in 1B5C medium containing 0.1 mg/ml pectinase (CalBiochem, La Jolla, CA, USA) and were digested for 30 min at 25°C. This treatment appears to modify the permeability of the cell wall without destroying its structure, allowing greater antibody accessibility to polypeptides not exposed on the cell wall surface (22). To ensure that this treatment did not release large amounts of soybean connexin from the cell wall, cell wall digests (supernatant fractions) were collected following incubation of soybean cells with 0.1 mg/ml pectinase for 30 min at 25°C. Cell wall digests were also collected following incubation of separate samples of cells with pectinase at concentrations of 0.5, 1.0, 5.0, and 10.0 mg/ml for 30 min at 25°C. The cell wall digests were analyzed on SDS-PAGE gels transferred to nitrocellulose paper and probed with rat liver connexin antibody (30 μg/ml).

Subsequent to treatment with 0.1 mg/ml pectinase, soybean cells were washed by pelleting and resuspension in fresh 1B5C medium. They were then fixed in 3.7% formaldehyde in 1B5C medium for 30 min. After fixation, the cells were washed in Tris-buffered saline (TBS: 20 mM Tris-HCl pH 7.5, 0.5 M NaCl) and incubated for 1 h with 60 μg/ml of either rat liver connexin antibody or soybean connexin antibody in TBS containing 3% (w/v) bovine serum albumin (BSA).

They were washed in TBS-3% BSA and incubated for 1 h with 30 µg/ml fluorescein-conjugated goat anti-rabbit immunoglobulin (IgG) (Boehringer Mannheim Biochemicals, Mannheim, FRG) in TBS-3% BSA. The cells were again washed in TBS and mounted on slides in phosphate-buffered saline (PBS: 0.14 M NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, 4.3 mM $NaHPO_4$) containing 5% of the anti-bleaching agent *n*-propyl gallate (Sigma) and 70% glycerol (v/v). The slides were viewed with a Leitz epifluorescence microscope (Leitz, Wetzlar, FRG) using a 25X objective lens. Controls were performed utilizing preimmune sera (60 µg/ml) purified over a protein A affinity column (Sigma).

Immunofluorescence analysis was also performed on freshly excised mouse livers. The livers were cut into 4 pieces and immersed into petroleum ether at -70°C for 2 min. Liver pieces were mounted onto the chuck of a Miles cryostat using "Tissue Tek", an embedding medium for frozen tissues (Miles Laboratories). The liver was sectioned in the -20°C cryostat to a thickness of 8 µm. Liver sections were then placed onto coverslips and immersed in -20°C acetone for 10 min. The coverslips were washed with TBS and incubated in primary and secondary antibodies as described for soybean cells. The coverslips were mounted on slides in the anti-bleaching agent described above and were viewed with a Leitz epifluorescence microscope using a 40X objective lens.

Preparation of Plant Organ Samples

Various plant organs, namely, petals, fruits, and leaves, were obtained from a number of sources and rapidly frozen at -70°C. The frozen material was homogenized with four volumes of 1 mM $NaHCO_3$ in a Bellco tissue homgenizer. The homogenate was suspended 1:1 (v/v) in SDS-PAGE B-mercaptoethanol sample buffer (16). The mixture was allowed to sit for 30 min at 25°C and was then centrifuged for 1 min in an Eppendorf microfuge at 15,000 x g. The pellet was discarded. The supernatant was subjected to SDS-PAGE, and the polypeptides were transferred to nitrocellulose and immunoblotted with rat liver connexin antibody (30 µg/ml). Samples were derived from the following plants: daisy (*Chrysanthemum leucanthemem* L.), rose (*Rosa* sp.), petunia (*Petunia hybrida*), lettuce (*Lactuca sativa* L.), tomato (*Lycopersicon esculentum* [L.] Mill), and cucumber (*Cucumis sativus* L.). Petals were obtained from daisy, rose, and petunia; fruits were obtained from tomato and cucumber; and leaves were obtained from lettuce.

RESULTS

Reactivity of Antibodies to the 27 kDa and 29 kDa Polypeptides of Rat Liver and Soybean Connexins

To examine the immunological relatedness of rat liver and soybean connexins, monospecific antibodies were separately prepared against both proteins, as described in *Materials and Methods*. Each monospecific antibody was used to probe immunoblots of rat liver and soybean connexin fractions. The rat liver connexin antibody recognized 29 and 27 kDa polypeptides in the soybean and rat liver fractions, respectively, and 48 kDa polypeptides in both (Figure 2A,B). Analogous results were obtained with the soybean connexin antibody (Figure 2C,D). Preimmune IgG controls for both immunological reagents failed to recognize any polypeptides (Figure 3). As reported for the 27 kDa rat liver polypeptide (15), the 29 kDa soybean polypeptide forms a putative dimer with M_r 48,000.

Comparison of Rat Liver and Soybean Connexins Using Two-Dimensional Chromatography

Two-dimensional chromatography using NEPHGE in the first dimension and SDS-PAGE in the second dimension was performed to compare rat liver and soybean connexins. Western transfer blots, prepared from the two-dimensional gels, were probed with rat liver connexin antibody. Rat liver connexin 32 was found to have a lower

Figure 2. Immunological characterization of antisera raised against the rat liver 27 kDa polypeptide (rat liver connexin) and the soybean 29 kDa polypeptide (soybean connexin). The antisera were made monospecific against the corresponding antigens as described in *Materials and Methods*. Samples were resolved on SDS-PAGE gels (acrylamide composition 10%) and transferred to nitrocellulose paper (400mA, 90 min). Antibody binding was detected here and for all subsequent immunoblots using alkaline phosphatase-conjugated goat anti-rabbit IgG (Sigma) according to the manufacturer's instructions. The contents of the lanes were: soybean connexin fraction (A) and rat liver connexin fraction (B) immunoblotted with rat liver connexin antibody; and soybean fraction (C) and rat liver fraction (D) immunoblotted with soybean connexin antibody. The molecular weight standards were as described in Figure 1. Approximately 20 μg of protein were loaded in each lane of the gel.

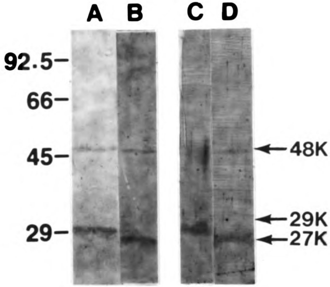


Figure 2

Figure 3. Immunological analysis of preimmune IgG binding to samples resolved on SDS-PAGE gels (acrylamide composition 10%) and transferred to nitrocellulose. The contents of the lanes were: soybean connexin fraction (A) and rat liver connexin fraction (B) immunoblotted with rat liver connexin preimmune IgG; and soybean fraction (C) and rat liver fraction (D) immunoblotted with soybean connexin preimmune IgG. The molecular weight standards were as described in Figure 1. Approximately 20 µg of protein were loaded in each lane of the gel.

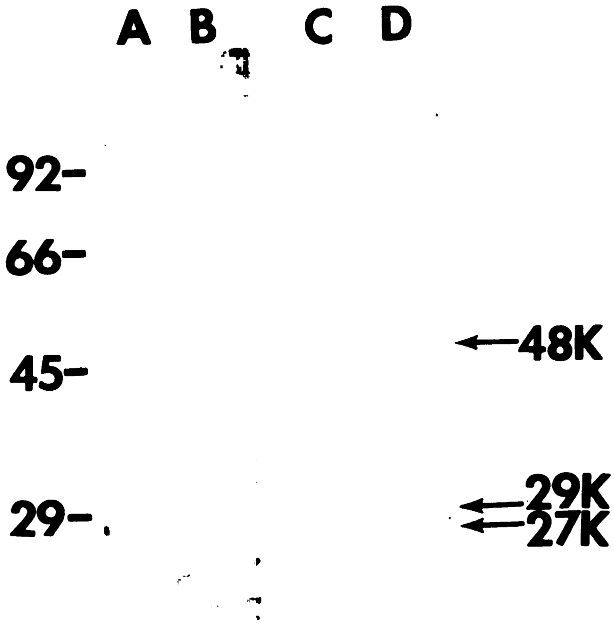


Figure 3

apparent isoelectric point (pH 9.4) (Figure 4A) than soybean connexin (pH 9.8) (Figure 4B). Both, however, exhibited similar patterns of aggregation (48 kDa and 68 kDa spots) (Figure 4A,B).

Comparison of Rat Liver and Soybean Connexins Using Comparative Peptide Map Analysis

In a further effort to explore protein homology, one-dimensional peptide map analyses of the 27 kDa (rat liver) and the 29 kDa (soybean) polypeptides were performed. Rat liver and soybean connexin fractions were electrophoresed on SDS-PAGE gels. The polypeptides were transferred to nitrocellulose, and the 27 kDa and 29 kDa regions were identified and excised from the blot. Pieces of nitrocellulose paper containing the 27 kDa and 29 kDa polypeptides were then individually treated with either *Stapylococcus aureus* V-8 protease or CNBr, and the resulting peptide mixtures were loaded onto another SDS-PAGE gel. The resolved peptides were transferred to nitrocellulose and probed with rat liver connexin antibody. Related patterns of cleavage and aggregation were observed following V-8 (Figure 5) and CNBr (Figure 6) treatment in a comparison of rat liver (Figures 5A,6A) and soybean (Figures 5B,6B) connexins. On the basis of these results, the rat liver and soybean connexins would appear to be related, but not identical.

Figure 4A. Two-dimensional SDS-PAGE and NEPHGE chromatography. (A) Rat liver connexin fraction (25 μg) was subjected to electrophoresis in the first dimension on a non-equilibrium pH gradient gel (acrylamide composition 4%) for 5 h at 400 mV. Electrophoresis was performed in the second dimension on an SDS-PAGE gel (acrylamide composition 12.5%) which was then transferred to nitrocellulose paper.

Figure 4B. Soybean connexin fraction (25 μg) was subjected to electrophoresis as described in (A), and the polypeptides were then transferred to nitrocellulose paper. Both immunoblots were probed with rat liver connexin antibody.

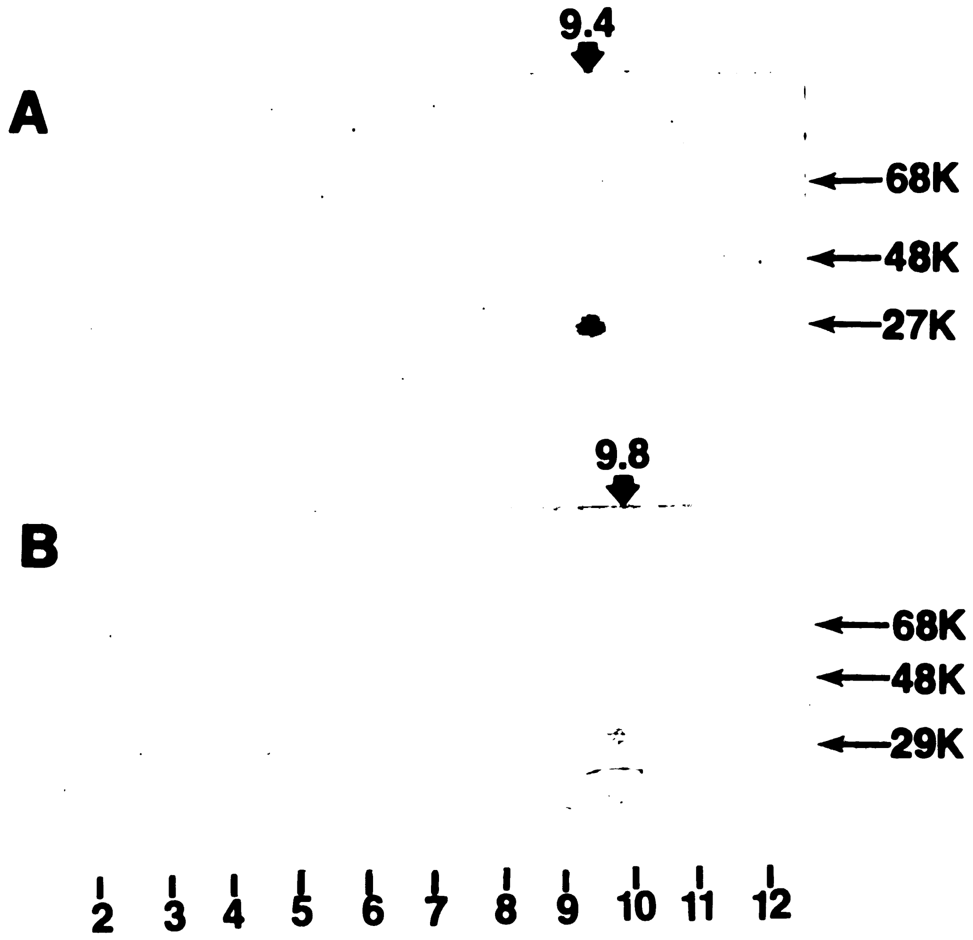


Figure 4

Figure 5. Comparative peptide map analysis of rat liver and soybean connexins after limited V-8 proteolysis. Samples were resolved on SDS-PAGE gels (acrylamide composition 15%), transferred to nitrocellulose, and immunoblotted with rat liver connexin antibody. The contents of the lanes were: (A) rat liver connexin digest; and (B) soybean connexin digest. Approximately 50 µg of protein were loaded in each lane of the gel.

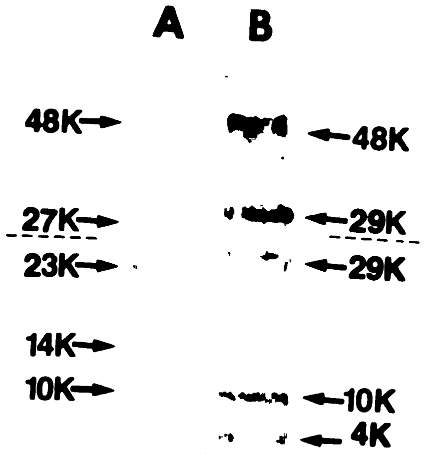


Figure 5

Figure 6. Comparative peptide map analysis of rat liver and soybean connexins after limited CNBr cleavage. Samples were resolved on SDS-PAGE gels (acrylamide composition 15%), transferred to nitrocellulose, and immunoblotted with rat liver connexin antibody. The contents of the lanes were: (A) rat liver connexin digest; and (B) soybean connexin digest. Approximately 50 μg of protein were loaded in each lane of the gel.

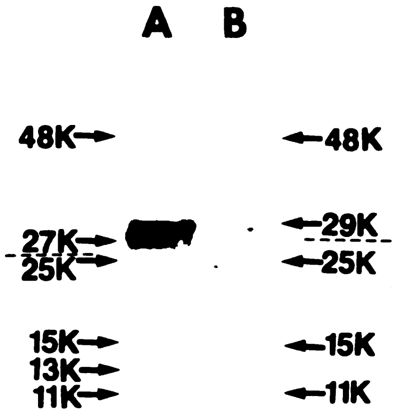


Figure 6

Immunofluorescence Localization of Soybean Connexin in Soybean Cells Grown in Tissue Culture

Soybean cells were grown in suspension culture and labeled as described in *Materials and Methods.* An important element of the labeling process was that the cells had to be mildly treated with pectinase to make the cell wall region accessible to the antibody. Failure to treat cells resulted in no labeling (data not shown). Rat liver and soybean connexin antibodies were utilized on both soybean cells and mouse liver thin sections. (The mouse liver connexin is immunologically homologous to the rat liver connexin (23].) Rat liver connexin antibody (Figure 7A-D) and soybean connexin antibody (Figure 7E-H) demonstrated the same fluorescence pattern for each material employed. Punctate fluorescent spots and patches were observed at the cellular periphery between contacting soybean cells (Figure 7B,D) and contacting mouse liver cells (Figure 7F,G). The punctate labeling of mouse liver reproduces previously reported results (23). Variations in fluorescence patch size were noted for soybean and may be related to differences in the amount of antigen at each site. Such variability at the cell periphery has also been noted for the size of pitfields and the number of plasmodesmata per pit field (24). No labeling of either pectinase treated soybean cells (Figure 8A-D) or mouse liver thin sections (Figure8E-H) was found with preimmune IgG, and the punctate fluorescence patterns yielded by the connexin antibodies also were not observed in reports of soybean cells probed with soybean seed agglutinin antibody (25) or calcofluor, a stain of β(1-4) glucans in the cell wall (13).

Figure 7. Immunofluorescence localization of plant-type connexin in soybean cells. Antibody binding was detected using fluorescein-conjugated goat anit-rabbit IgG (Boehringer Mannheim Biochemicals) according to the manufacturer's instructions. Soybean cells were subjected to pectinase treatment and were then probed with either rat liver connexin antibody (A & B; A, phase, B, fluoresence) or soybean connexin antibody (E & F; E, phase, F, fluorescence). Mouse liver thin sections were also probed with rat liver connexin antibody (C & D; C, phase, D, fluoresence) or soybean connexin antibody (G & H; G, phase, H, fluorescence). Soybean cells were viewed with a Leitz epifluorescence microscope using a 25X objective lens. Mouse liver thin sections were viewed using a 40x objective lens. Bar = 20 µm.

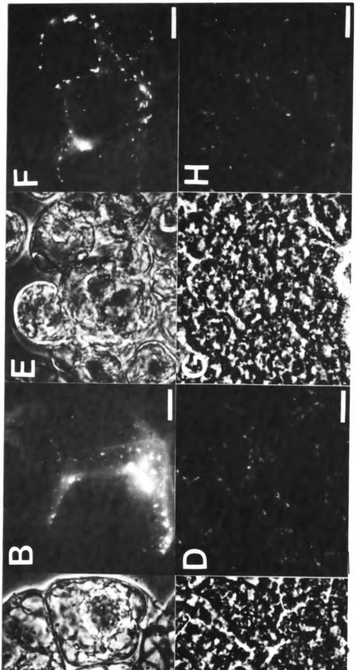


Figure 7

Figure 8. Immunofluorescence preimmune IgG controls. Pectinase treated soybean cells were probed with rat liver connexin preimmune IgG (A & B; A, phase, B, fluoresence) or soybean connexin preimmune IgG (E & F; E, phase, F, fluorescence). Mouse liver thin sections were also probed with rat liver connexin preimmune IgG (C & D; C, phase, D, fluorescence) or soybean connexin preimmune IgG (G & H; G, phase, H, fluorescence). Soybean cells were viewed with a Leitz epifluorescence microscope using a 25X objective lens, and mouse liver thin sections were viewed using a 40X objective lens. Bar = 20 µm.

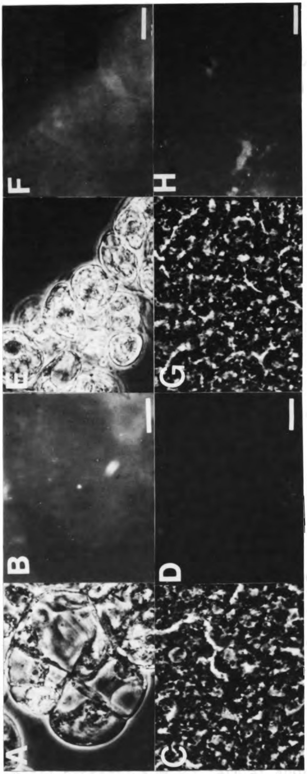


Figure 8

Pectinase-Mediated Release of Soybean Connexin

Labeling of soybean cells requires a pre-incubation step with 0.1 mg/ml pectinase for 15-30 min in order to modify the cell wall, rendering it accessible to antibodies. This treatment was found not to release soybean connexin as judged by immunoblot analysis of cell wall digests using rat liver connexin antibody as a probe (Figure 9C). In contrast, treatment of cells with 10 mg/ml pectinase (Figure 9F), an enzyme concentration known to disrupt plant cell walls (14), released significant amounts of soybean connexin.

Immunological Analysis of Soybean-Type Connexin in Extracts from Plant Organs

Hertzberg and Skibbens (23) and Dermietzel *et al.* (26) demonstrated the existence of a rat liver-type connexin in a variety of animal species and tissues. Recently, work by a number of investigators has indicated other, tissue-specific connexins (27,28,29). To examine the distribution and existence of soybean-type connexin in plants, samples were obtained from the petals, leaves, and fruits of several different species and extracted as described in *Materials and Methods*. The extracts were subsequently analyzed on SDS-PAGE gels which were transferred to nitrocellulose paper. When probed with rat liver connexin antibody, the immunoblots exhibited a similar pattern of labeling for each plant variety and tissue type examined. Two polypeptide bands were observed in each petal extract: a major band at approximately 29 kDa and variable amounts of a less enriched band at approximately 48 kDa (Figure 10B-D). Similar results were obtained for the leaf and

Figure 9. Immunological analysis of rat liver connexin antibody binding to pectinase digests of soybean cell walls. Samples were resolved on SDS-PAGE gels (acrylamide composition 12.5%) and transferred to nitrocellulose. The contents of the lanes were: (A) rat liver connexin fraction; (B) pectinase enzyme control; lanes C-G, cell wall digests prepared with pectinase at a concentration of: (C) 0.1 mg/ml; (D) 0.5 mg/ml; (E) 1.0 mg/ml; (F) 5.0 mg/ml; and (G) 10.0 mg/ml. The molecular weight standards were as described in Figure 1 with the addition of soybean trypsin inhibitor, 22,000. Approximately 20 µg of protein were loaded in (A) and (B), and approximately 100 µg were loaded in (C)-(G).

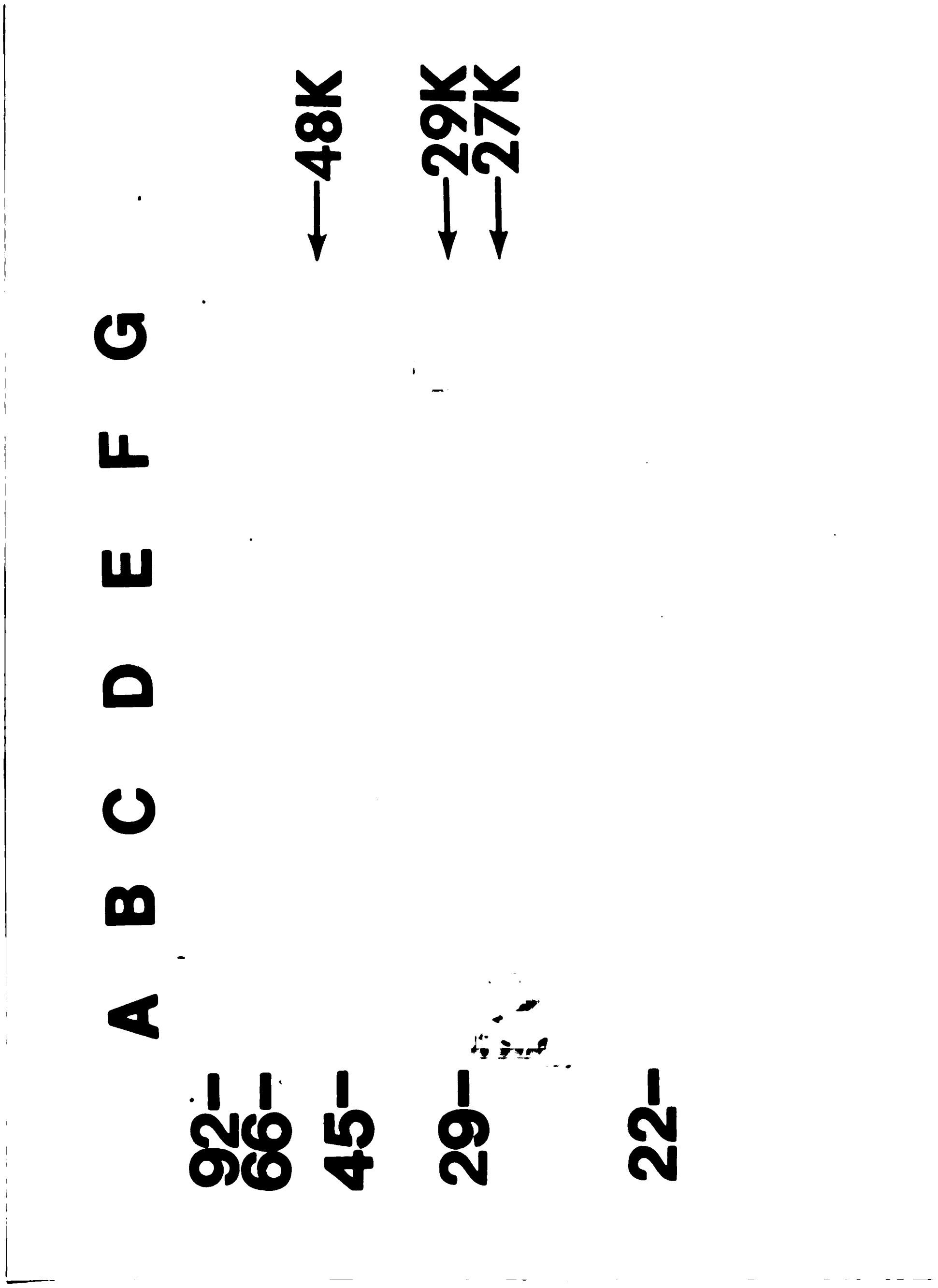


Figure 9

Figure 10. Immunological analysis of soybean-type connexin in petal extracts. Samples were resolved on SDS-PAGE gels (acrylamide composition 12.5%) and transferred to nitrocellulose. The contents of the lanes were: (A) soybean connexin fraction; (B) daisy petal extract; (C) rose petal extract; and (D) petunia petal extract. The molecular weight standards were as described in Figure 9. Approximately 20 µg of protein were loaded in (A), and approximately 200 µg were loaded in (B)-(D). The immunobot was probed with rat liver connexin antibody.

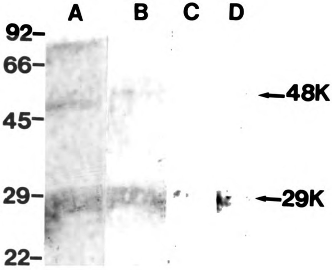


Figure 10

fruit extracts (Figure 11B-D). The slight variations in migration on SDS-PAGE gels for the 29 kDa polypeptide, ranging between 26 and 29 kDa, appear to be related to tissue handling and extraction conditons. The rat liver 27 kDa antibody reacted with 27, 48, and 68 kDa polypeptides in control rat liver connexin fractions (Figure 11A) and with 29, 48, and 68 kDa polypeptides in control soybean connexin fractions (Figure 10A).

Figure 11. Immunological analysis of soybean-type connexin in leaf and fruit extracts. Samples were resolved on SDS-PAGE gels (acrylamide composition 12.5%) and transferred to nitrocellulose. The contents of the lanes were: (A) rat liver connexin fraction; (B) lettuce leaf extract; (C) tomato fruit extract; (D) cucumber fruit extract. The molecular weight standards were as described in Figure 9. Approximately 20 µg of protein were loaded in (A), and approximately 200 µg were loaded in (B)-(D).

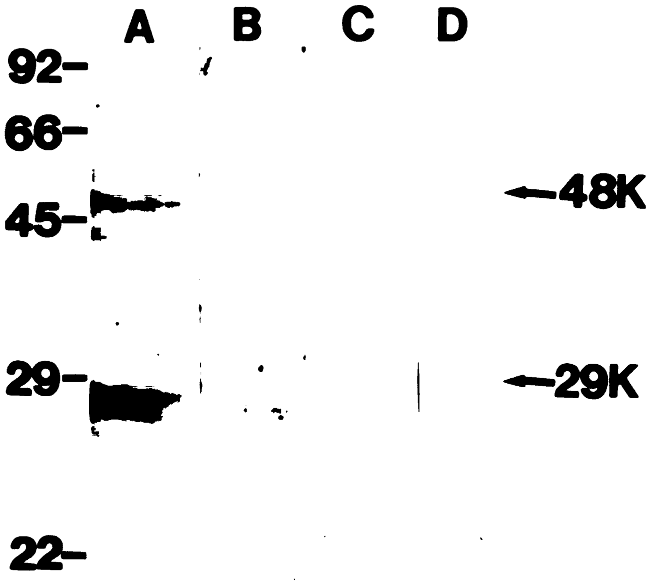


Figure 11

DISCUSSION

This study provides evidence for a 29 kDa polypeptide in soybean root cells (SB-1 cell line) which is immunologically and biochemically similar to the 27 kDa polypeptide of rat liver gap junction (rat liver connexin 32). Antibodies raised against one type of polypeptide cross-react with the other; and the polypeptides share similar isoelectric points, V-8 protease and CNBr-derived peptide maps, and the ability to form high molecular weight species on reducing SDS-PAGE gels.

This study also provides evidence that the soybean-type polypeptide is found in a variety of plants and plant tissue types in addition to soybean root (namely, daisy, rose, and petunia petals, lettuce leaves, and tomato and cucumber fruits). The 29 kDa polypeptide, which has been operationally termed soybean connexin, may be representative of a protein found in all plants that can, by analogy with gap junctional organization and assembly in animal cells, form the structural subunit of plasmodesmata.

Use of antibodies against the rat liver and soybean connexins demonstrated that the soybean protein is appropriately localized topologically to serve as a transport element. This was indicated by: (1) the punctate localization of the soybean connexin between contacting and connected soybean cells in a pattern similar to the observed distribution and size heterogeneity of plasmodesmatal pit fields (assembled in newly-forming cell walls during cell division, or synthesized de novo between contacting cells after cell wall formation is complete (4,30]); and (2) the release of soybean connexin from the cell wall with pectinase under conditions that do not affect cell viability or membrane permeability (22).

Recent work of other investigators supports the view that connexin homologous proteins in plants may construct the plasmdesmatal transport channels between contiguous cells. Yahalom *et al.* (31) demonstrated plasmodesmatal labeling on thin sections of *Zea mays* L. that were probed with either rat heart connexin 43 antibody (Figure 12) or rat liver connexin 32 antibody. Some cytoplasmic labeling was also seen with the rat heart connexin 43 antibody, but the labeling with the rat liver connexin 32 antibody was specific for the plasmodesmata. Preimmune sera controls for both antibodies failed to show any labeling.

The extent of homology between the animal and plant connexins will be further explored in the next chapter.

<u>Figure 12</u>. Thin section electron micrograph of plasmodesmata between two *Zea mays L.* cells. The *Zea mays* L. mesocotyl tissue was fixed in a solution of 4% formaldehyde and 0.5% glutaraldehyde in 100 mM NaH_2PO_4, pH 7.2. Thin sections were labeled with rat heart connexin 43 antibody (31) followed by goat anti-rabbit IgG conjugated to 12-15 nm colloidal gold. p = plasmodesmata; w = wall. This photograph was reproduced with the permission of Dr. B.L. Epel.

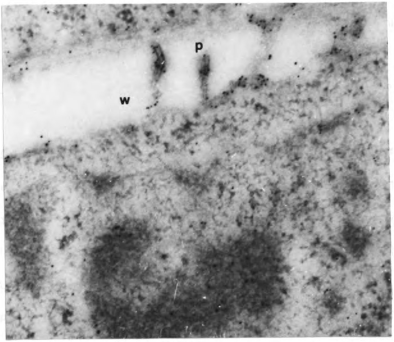


Figure 12

REFERENCES

1. Hertzberg, E.L., Lawrence, T.S., and Gilula, N.B. 1981. Gap junctional communication. Annu. Rev. Physiol. 43: 479-491.
2. Loewenstein, W.R. 1979. Junctional intercellular communication and the control of growth. Biochim. Biophys. Acta 560: 1-65.
3. Loewenstein, W.R. 1987. The cell-to-cell channel of gap junctions. Cell 48: 725-726.
4. Gunning, B.E.S., and Overall, R.L. 1983. Plasmodesmata and cell to cell transport in plants. Bioscience 33: 260-265.
5. Van Steveninck, R.F.M. 1976. Cytological evidence for ion transport through plasmodesmata. In: Intercellular Communication in Plants: Studies on Plasmodesmata. B.E.S. Gunning and A.W. Robards, eds., pp. 132-147. Springer-Verlag, Berlin.
6. Hertzberg, E.L., and Gilula, N.B. 1979. Isolation and characterization of gap junctions from rat liver. J. Biol. Chem. 254: 2134-2147.
7. Robards, A.W. 1976. Plasmodesmata in higher plants. In: Intercellular Communication in Plants: Studies on Plasmodesmata. B.E.S. Gunning and A.W. Robards, eds., pp. 15-57. Springer-Verlag, Berlin.
8. Terry, B.B., and Robards, A.W. 1987. Hydrodynamic radius alone governs the mobility of molecules through plasmodesmata.

Planta 171: 145-157.

9. Erwee, M.G., and Goodwin, P.B. 1983. Characterization of the *Egeria densa* Planch. leaf symplast. Inhibition of the cellular movement of fluorescent probes by group II ions. Planta 158: 320-328.

10. Baron-Epel, O., Hernandez, D., Jiang, L.-W., Meiners, S., and Schindler, M. 1988. Dynamic continuity of the cytoplasmic and membrane compartments between plant cells. J. Cell. Biol. 106: 715-721.

11. Wade, M.H., Trosko, J.E., and Schindler, M. 1986. A fluorescence photobleaching assay of gap junction-mediated communication between human cells. Science 232: 525-528.

12. Gamborg, O.L., Miller, R.A., and Ojima, K. 1968. Nutrient requirements of suspension cultures of soybean root cells. Exp. Cell Res. 50: 151-158.

13. Metcalf, T.N. III, Wang, J.L., Schubert, K.R., and Schindler, M. 1983. Lectin receptors on the plasma membrane of soybean cells. Binding and lateral diffusion of lectins. Biochemistry 22: 3969-3975.

14. Ohyama, K. 1975. Isolation of 5-bromodeoxyuridine-resistant cells from soybean protoplasts. In: Plant Tissue Culture Methods. L.R. Wetter and O.L. Gamborg, eds., pp. 36-39. Prairie Regional Laboratory, Saskatoon, Sask., Canada.

15. Hertzberg, E.L. 1984. A detergent-independent procedure for the isolation of gap junctions from rat liver. J. Biol. Chem. 259: 9936-9943.

16. Laemmli, U.K. 1970. Cleavage of structural proteins during the

assembly of the head of bacteriophage T4. Nature 227: 680-685.

17. Nicholson, B.J., Hunkapillar, M.W., Grim, L.B., Hood, L.E., and Revel, J.P. 1981. Rat liver gap junction protein: properties and partial sequence. Proc. Natl. Acad. Sci. USA 78: 7594-7598.

18. Towbin, H., Staehelin, T., and Gordon, J. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. USA 76: 4350-4354.

19. Smith, D.E., and Fisher, P.A. 1984. Identification, developmental regulation and response to heat shock of two antigenically related forms of a major nuclear envelope protein in *Drosophila* embryos: application of an improved method for affinity purification of antibodies using polypeptides immobilized on nitrocellulose blots. J. Cell Biol. 99: 20-28.

20. O'Farrell, P.Z., Goodman, H.M., and O'Farrell, P.H. 1977. High resolution of two dimensional electrophoresis of basic as well as acidic proteins. Cell 12: 1133-1142.

21. Yuen, S.W., Chin, A.H., Wilson, K.J., and Yuan, P.M. 1989. Microanalysis of SDS-PAGE electroblotted proteins. BioTechniques 7: 74-83.

22. Baron-Epel, O., Gharyal, P.K., and Schindler, M. 1988. Pectins as mediators of wall porosity in soybean cells. Planta 175: 389-395.

23. Hertzberg, E.L., and Skibbens, R.V. 1984. A protein homologous to the 27,000 dalton liver gap junction protein is present in

a wide variety of species and tissues. Cell 39: 61-69.

24. Evert, R.F., Escrich, W., and Heyser, W. 1977. Distribution and structure of the plasmodesmata in mesophyll and bundle sheath cells of *Zea mays* L. Planta 136: 77-89.

25. Ho., S.-C., Malek-Hedayat, S., Wang, J.L., and Schindler, M. 1986. Endogenous lectin from cultured soybean cells: Isolation of a protein immunologically cross-reactive with seed soybean agglutinin and analysis of its role in binding of *Rhizobium japonicum*. J. Cell. Biol. 103: 1043-1053.

26. Dermietzel, R., Leibstein, A., Brixen, U., Janssen-Timmen, U., Traub, O., and Willecke, K. 1984. Gap junctions in several tissues share antigenic determinants with liver gap junctions. EMBO (Eur. Molec. Biol. Org.) J. 3: 2261-2270.

27. Paul, D.L. 1986. Molecular cloning of cDNA for rat liver gap junction protein. J. Cell Biol. 103: 123-134.

28. Beyer, E.C., Paul, D.L., and Goodenough, D.A. 1987. Connexin 43: A protein from rat heart homologous to a gap junction protein from liver. J. Cell Biol. 105: 2621-2629.

29. Ebihara, L., Beyer, E.C., Swensen, K.I., Paul, D.L., and Goodenough, D.A. 1989. Cloning and expression of a *Xenopus* embryonic gap junction protein. Science 243: 1194-1195.

30. Jones, M.G.K. 1976. The origin and development of plasmodesmata. In: Intercellular Communication in Plants: Studies on Plasmodesmata. B.E.S. Gunning and A.W. Robards, eds., pp. 81-105. Springer-Verlag, Berlin.

31. Yahalom, A., Warmbrodt, R., Laird, M., Revel, J.P., Traub, O., and Epel, B. 1991. Maize mesocotyl plasmodesmatal proteins

cross-react with connexon gap junction protein antibodies. The Plant Cell, in press.

Chapter III

GAP JUNCTION PROTEIN HOMOLOGUE
FROM
ARABIDOPSIS THALIANA:
ISOLATION AND CHARACTERIZATION OF A cDNA CLONE

Abstract

An *Arabidopsis thaliana* library constructed in the Bluescript expression vector Lambda Zap II was screened with three affinity purified antibodies raised against (1) rat liver connexin 32; (2) a 29 kDa protein found in soybean cells which is immunologically related to rat liver connexin 32; and (3) a synthetic peptide corresponding to amino acid sequence 98-124 of rat liver connexin 32. A single clone was obtained whose gene product demonstrated immunological cross-reactivity with all three reagents. The cDNA from this clone contained 1,171 base pairs which coded for a protein of 280 amino acids with a calculated molecular mass of 32,339 daltons. Sequence homology observed between the *Arabidopsis* polypeptide and rat liver connexin 32, in conjunction with observed similarities in predicted number and distribution of hydrophobic domains, predicted secondary structure, sites for post-translational modification, and basic pI, provide strong cumulative evidence that the biological range for connexin-type proteins may now be considered to include the plant kingdom.

INTRODUCTION

The cells of most tissues appear to be coupled by low resistance pathways that are permeable to ions and small molecules. Gap junctions and plasmodesmata are thought to be responsible for this coupling in animal and plant cells, respectively (1,2,3,4). In contrast to gap junctions, which have been studied extensively, little is known about the biochemical components and organization of plasmodesmata. Functionally, however, much data suggests that the transport properties of these structures are remarkably similar (3,4,5). In the belief that analogous mechanisms may employ homologous components, albeit in different organizational patterns, a series of experiments was initiated to search for a connexin-like protein(s) in soybean root cells (SB-1 cell line) and various other plants and plant tissue types (6,7). These efforts demonstrated that diverse plant organs contain a polypeptide that is immunologically related to connexin 32 from rat liver (7). The current study is directed toward examining the extent of similarity between the plant protein(s) and the rat liver connexin at the amino acid level. The primary structure of a plant connexin-like protein is presented, which was deduced from the nucleotide sequence of cloned cDNA from an *Arabidopsis thaliana* expression library.

MATERIALS AND METHODS

Preparation and Characterization of Immunological Reagents

Cells (SB-1 cell line) originally derived from soybean (*Glycine max* [L.] Merr.) roots (8) were grown in 1B5C medium, pH 5.5, in the dark (9). Soybean protoplasts were prepared from these cells (10) and processed in parallel with rat liver according to the detergent-independent procedure developed by Hertzberg to isolate rat liver connexins (11). The resulting connexin-enriched fractions were employed for the preparation of antibodies, as discussed in Chapter II. Antisera and affinity purified antibodies were prepared against isolated 27 kDa and 29 kDa bands cut from rat liver and soybean connexin fractions, respectively, which were electrophoresed on denaturing SDS-PAGE gels (6,7,12). Antiserum and affinity purified antibody were also prepared against a hydrophilic loop peptide of rat liver connexin 32 (amino acid sequence 98-124). The peptide was synthesized on an Applied Biosystems, Inc. peptide synthesizer (Foster City, CA, USA). The resultant peptide mixture was chromatographed on a Sephadex G-25 column using 1 M Glycine, pH 3.0 as an eluant. The first peak obtained (as measured by absorption at 280 nm) was utilized for preparing antigen and an immunoaffinity column. Rabbits were immunized with peptide coupled to rabbit serum albumin (RSA) (13) according to the procedures of Milks *et al.* (13). The peptide/RSA complex (3 mg) was emulsified with complete Freund's adjuvant and injected into a white New Zealand female rabbit; booster injections utilized 0.5 mg of complex emulsified with incomplete adjuvant. Immune sera were purified by absorption to the peptide affinity column. The resulting affinity purified antibody will hereafter be referred to as rat liver connexin peptide antibody.

Immunological Analysis of Rat Liver Connexin Peptide Antibody Binding to *Arabiposis thaliana* Leaf Homogenates and Connexin-Enriched Fractions from Rat Liver and Soybean

Leaves from *Arabidopsis thaliana* were homogenized with four volumes of 1 mM $NaHCO_3$ in a Bellco tissue homogenizer (Bellco Glass, Vineland, N.J., USA). The homogenate was extracted for 1 h with 1% SDS in 1 mM $NaHCO_3$ and centrifuged for 1 min in an Eppendorf microfuge. The pellet was discarded and the supernatant was dialyzed against double distilled water for 12 h with two changes of water. All steps were performed at 4°C. The *Arabidopsis* supernatant and connexin-enriched fractions isolated from rat liver and soybean were electrophoresed on SDS-PAGE gels (12) and transferred to nitrocellulose paper (14). They were then immunoblotted with rat liver connexin peptide antibody (25 µg/ml).

Screening of *Arabidopsis thaliana* cDNA Expression Library and Examination of *Arabidopsis* Fusion Protein

An *Arabidopsis thaliana* cDNA library (provided by Dr. M. Thomashow, Department of Crop and Soil Sciences, Michigan State University, E.Lansing, MI, USA) was constructed in the expression vector Lambda Zap II (Stratagene, La Jolla, CA, USA). *Escherichia coli* strain Y1090r$^-$ was infected with the Lambda Zap II phage and grown on agar plates (2.5 x 10^4 plaques/plate) (15). A positive plaque was identified following a sequential screening of the library with rat liver connexin peptide antibody (5 µg/ml) and monospecific reagents prepared against the 27 kDa polypeptide of rat liver connexin and the 29 kDa polypeptide of soybean connexin (10 µg/ml each). The clone was isolated, amplified, and rescreened three times. The pBluescript SK$^-$ plasmid containing the *Arabidopsis* insert was excised from the clone with helper phage R408 (Stratagene) and used to transform *E. coli* strain XL1-Blue (Stratagene) as indicated by the manufacturer.

Fusion protein from the *E. coli* clone (designated clone 73) was visualized in the following manner. Five ml of LB medium (16) were inoculated with a single bacterial colony and grown overnight. The cells (1.5 ml) were centrifuged for 20 sec in a microfuge. The pellet was resuspended in 100 µl TE (Tris-EDTA) buffer (TE: 10 mM Tris-HCl, pH 7.4, 1 mM EDTA), and 200 µl of 0.2 M NaOH/2% SDS were added. The mixture was placed on ice for 10 min and neutralized with HCl. The resulting bacterial lysate was separated by SDS-PAGE, transferred to nitrocellulose, and immunoblotted with either rat liver connexin peptide antibody (25 µg/ml) or mouse *E. coli* β-galactosidase antibody (20 µg/ml) (Accurate Chemical Scientific Corp., Westbury, N.Y., USA).

DNA Sequencing Strategy

Plasmid (pSM73) was isolated from clone 73 and purified on a CsCl gradient. EcoRI (Boehringer Mannheim Biochemicals, Mannheim, FRG) digestion of the plasmid DNA was performed to determine the size of the *Arabidopsis* insert (17). Double-stranded sequencing was performed using a Stratagene sequencing kit and [^{35}S]dATP as described by the manufacturer. The nucleotide sequence was determined by the dideoxy chain termination method of Sanger *et al.* (18). Initial sequencing of the cDNA was performed using T3 and T7 sequencing primers, while subsequent sequencing was performed using synthetic oligonucleotide primers prepared against previously sequenced regions.

Northern Blot Analysis

The cDNA of clone 73 was prepared for detection by nick translation with biotin-7-dATP using the Blu-GENE system (Bethesda Research Laboratories, Bethesda, MD, USA). The hybridized probe was detected colorimetrically by the addition of streptavidin-alkaline phosphatase conjugate and incubation in the substrates nitroblue tetrazolium and 5-bromo-4-chloro-3-indoylphosphate (19). Poly (A)$^+$ RNA was isolated from soybean root cells and *Arabidopsis* leaves and electrophoresed in a 1.2% agarose gel. The RNA was transferred to nitrocellulose by capillary action and probed with the prepared cDNA (20 ng/ml) (20,21).

RESULTS

Reactivity of Antibodies Utilized to Isolate a cDNA Clone (clone 73) from an *Arabidopsis thaliana* Library

Utilizing an immune reagent provided by Dr. E.C. Beyer (Division of Hematology, Washington University School of Medicine, St. Louis, MO, USA) prepared against a peptide loop region of rat liver connexin 32 (amino acid sequence 98-124), we found that, as previously reported, rat liver connexin could be labeled on immunoblots (Figure 1A). More importantly, extracts from both soybean root cells (Figure 1B) and *Arabidopsis* leaves (Figure 1C) demonstrated a polypeptide band in the 29 kDa region. Variable amounts of a 48 kDa polypeptide were also observed which presumably reflect an aggregated species of the 29 kDa protein (6,7) (Figure 1A-C). Antibodies prepared against an amino acid sequence that is uniquely observed in rat heart connexin 43 (amino acid sequence 252-271) (also provided by Dr. E.C. Beyer) demonstrated no labeling (data not shown). As shown in Figure 1D-F, antibodies prepared in our laboratory against this same stretch of rat liver connexin 32 labeled a 27 kDa polypeptide in the lane containing rat liver derived material (Figure 1D) and a 29 kDa polypeptide in lanes containing soybean (Figure 1E) and *Arabidopsis* (Figure 1F) derived material. In all instances, variable amounts of a 48 kDa polypeptide were observed on the immunoblots (Figure 1D-F). These

Figure 1. Immunological analysis of rat liver connexin peptide antibody binding to rat liver connexin fraction, soybean connexin fraction, and *Arabidopsis thaliana* leaf extract. Samples were resolved on SDS-PAGE gels (acrylamide composition 12.5%) and transferred to nitrocellulose. The contents of the lanes were: (A) rat liver connexin fraction, (B) soybean connexin fraction, and (C) *Arabidopsis* leaf extract immunoblotted with rat liver connexin peptide antibody provided by Dr. E. C. Beyer; and (D) rat liver fraction, (E) soybean fraction, and (F) *Arabidopsis* extract immunoblotted with rat liver connexin peptide antibody prepared in our laboratory. The molecular weight standards were: phosphorylase b, 92,000; bovine serum albumin, 66,000; ovalbumin, 45,000; carbonic anhydrase, 29,000; and soybean trypsin inhibitor, 22,000. Approximately 20 µg of protein were loaded in (A), (B), (D), and (E), and approximately 200 µg were loaded in (C) and (F).

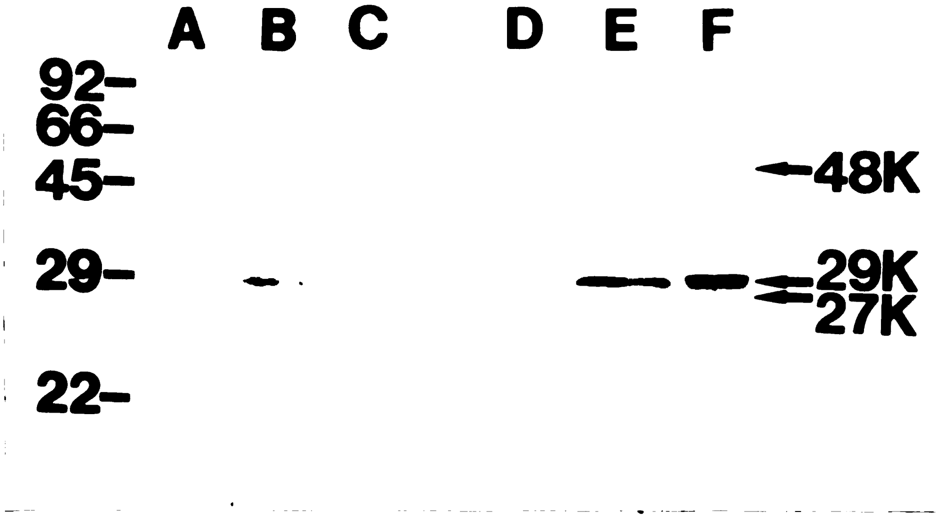


Figure 1

results were identical to those seen previously with antibodies raised against the 27 kDa polypeptide of rat liver connexin 32 and the 29 kDa polypeptide of soybean connexin (data not shown) (7).

Identification of a cDNA Clone

An *Arabidopsis thaliana* cDNA library constructed in the expression vector Lambda Zap II was screened as described in *Materials and Methods*. A lambda clone was identified which demonstrated reactivity to all three immunological reagents utilized (rat liver connexin antibody, soybean connexin antibody, and rat liver connexin peptide antibody). The positive plaque was isolated and replated to purity. The pBluescript plasmid was excised and used to transform *E. coli* strain XL1-Blue. Lysates prepared from the resulting bacterial clone 73 were subjected to electrophoresis on an SDS-PAGE gel; the polypeptides were transferred to nitrocellulose and then probed with either rat liver connexin peptide antibody (25 μg/ml) (Figure 2A) or *E. coli* β-galactosidase antibody (20 μg/ml) (Figure 2B). Immunoreactive bands were observed at approximately 34 kDa and 58 kDa for both antibodies. The fusion protein thus expressed has a somewhat higher molecular weight than that previously demonstrated in an extract from *Arabidopsis* leaves (29 kDa monomer, 48 kDa dimer) (Figure 1C,F). This was presumed to be related to the addition of 36 amino acids from the β-galactosidase gene that are a result of the vector construct.

EcoRI digestion of plasmid DNA isolated from the clone resulted

Figure 2. Immunological analysis of antibody binding to fusion protein derived from a clone (clone 73) expressing a putative *Arabidopsis* connexin homologue. Samples were resolved on SDS-PAGE gels (acrylamide composition 12.5%) and transferred to nitrocellulose. The contents of the lanes were: (A) bacterial lysate derived from clone 73 immunoblotted with rat liver connexin peptide antibody; and (B) bacterial lysate immunoblotted with *E. coli* β-galactosidase antibody. The molecular weight standards were as described in Figure 1. Approximately 100 µg of protein were loaded in each lane of the gel.

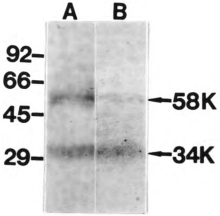


Figure 2

in the release of a single 1.2 kb (kilobase) fragment (Figure 3), which is of sufficient size to encode the entire *Arabidopsis* protein of 29 kDa (Figure 1C,F).

Nucleotide and Deduced Amino Acid Sequence

Double-stranded sequencing was performed as described (*Materials and Methods*). The nucleotide and deduced amino acid sequences are presented in Figure 4. The cDNA contained 1,171 base pairs (bp), and the only open reading frame coded for a polypeptide with a molecular mass of 32,339. (The molecular mass was calculated from the sequence.) The untranslated 5′ sequence contained 49 bp, and a putative ATG initiation codon (boxed) occurred at base 50. A TAA stop codon preceding the presumed initiation codon was observed in the 5′ leader sequence. (This TAA stop codon was probably "read through" by the ribosome when the previously discussed fusion protein was synthesized. Otherwise, a product cross-reactive with both rat liver connexin peptide antibody and *E. coli* β-galactosidase antibody would not have been formed.) Support for the presumed initiation codon may be inferred from the presence of a sequence reading AGGAAGT, which is related to the Shine-Delgarno sequence, AGGAGGT. This sequence occurred immediately adjacent to the TAA codon. There is no evidence for a cleaved leader or signal sequence. The open reading frame of 840 bp was terminated by one stop codon followed by another. The sequence ATAAA was found 19 bp from a tail of 17 adenosines. This sequence may be equivalent to the consensus polyadenylation signal AATAAA, which is typically observed approximately 20 bp from the 3′ tail.

Figure 3. Detection of the cDNA insert of *Arabidopsis* clone 73. Plasmid DNA was digested with EcoRI (Boehringer Mannheim Biochemicals) (10 units/µg DNA, 37°C, 1h) to release the cDNA insert. The digest was separated on a 1.2% agarose gel and visualized by ethidium bromide staining. The contents of the lanes were: (A) undigested pSM73; and (B) DNA digest from pSM73. Approximately 1 µg of DNA was loaded in each lane of the gel. The positions of migration of the DNA size standards are shown on the left.

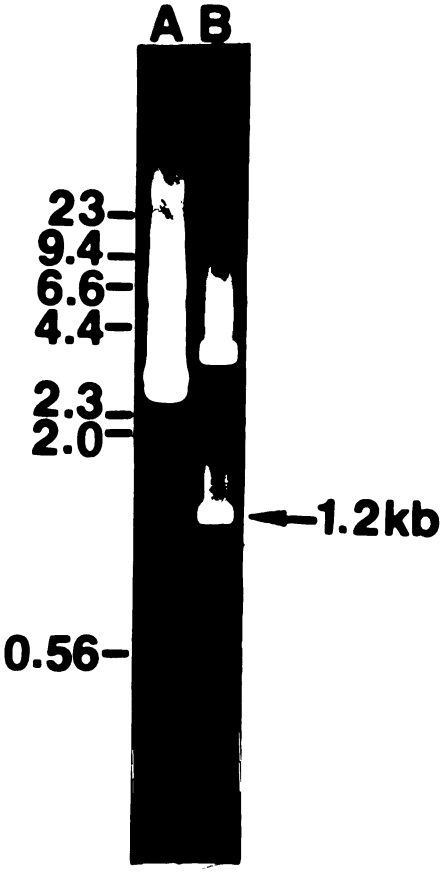


Figure 3

Figure 4. Sequence of cDNA from *Arabidopsis* clone 73. The complete nucleotide sequence of the cDNA from clone 73 is shown with residues numbered on the left side. The deduced amino acid sequence is shown with residues numbered on the right. The predicted molecular mass is 32,339 daltons.

```
                                                                                          met  leu  trp  his  arg
(1)      5' TGAGAGT GTT  CAA  GGA  TTT  GCA  GAG  TGG  CGT  TCA  GGA  AGT  TAA  CTG  GGA  ATG  CTT  TGG  CAC  CGA     6

            asn     leu  val  lys  leu  leu  gly  tyr  cys  arg  glu  asp  lys  ala  leu  leu  leu  val  tyr  glu
(66)        AAT     CTG  GTG  AAG  TTA  TTG  GGA  TAC  TGT  CGT  GAA  GAC  AAA  GCG  CTT  CTC  CTT  GTC  TAC  GAG    26

            phe     ile  pro  lys  glu  val  leu  arg  val  met  phe  leu  arg  arg  asn  asp  pro  phe  pro  trp
(126)       TTC     ATC  CCC  AAA  GAA  GTC  TTG  AGA  GTC  ATG  TTT  CTT  AGA  CGA  AAC  GAT  CCT  TTT  CCT  TGG    46

            asp     leu  arg  ile  lys  ile  val  ile  cys  ala  ala  arg  gly  pro  cys  val  ser  thr  gln  leu
(186)       GAT     TTA  AGG  ATC  AAG  ATT  GTG  ATT  TGT  GCA  GCT  CGT  GGT  CCT  TGC  GTT  TCT  ACA  CAG  CTT    66

            thr     lys  arg  glu  cys  ile  tyr  arg  asp  leu  gln  val  phe  his  ile  leu  leu  asp  leu  ser
(246)       ACA     AAG  AGA  GAA  TGC  ATC  TAT  AGA  GAT  CTT  CAA  GTC  TTC  CAT  ATT  CTT  CTT  GAT  TTG  AGC    86

            tyr     gly  ala  val  leu  ser  arg  val  ser  gly  pro  trp  leu  val  ala  met  glu  gln  gln  asn
(306)       TAT     GGC  GCA  GTG  CTT  TCT  AGA  GTT  TCT  GGT  CCG  TGG  CTG  GTG  GCT  ATG  GAA  CAG  CAG  AAT    106

            arg     glu  val  his  arg  gly  thr  ala  lys  val  his  arg  arg  his  ile  lys  val  met  leu  leu
(366)       AGA     GAA  GTT  CAC  AGA  GGG  ACG  GCT  AAG  GTG  CAT  AGG  CGT  CAT  ATA  AAG  GTT  ATG  CTG  CTC    126

            leu     glu  tyr  ile  ala  gly  his  leu  tyr  val  lys  ser  val  ala  phe  ala  phe  gly  val  val
(426)       CTA     GAA  TAT  ATA  GCA  GGC  CAT  TTA  TAC  GTT  AAG  AGT  GTC  GCA  TTC  GCC  TTT  GGT  GTG  GTT    145

            leu     leu  glu  ile  met  thr  gly  leu  thr  ala  his  asn  thr  lys  arg  pro  arg  gly  gln  ala
(486)       TTA     CTA  GAA  ATA  ATG  ACC  GGA  CTG  ACG  GCG  CAC  AAC  ACA  AAA  CGG  CCC  AGA  GGA  CAA  GCA    166

            glu     asn  his  leu  met  arg  thr  tyr  val  met  asp  asp  lys  his  thr  gln  thr  ala  thr  pro
(546)       GAG     AAT  CAC  TTA  ATG  CGA  ACA  TAC  GTC  ATG  GAT  GAT  AAG  CAC  ACA  CAG  ACA  GCA  ACT  CCA    185

            tyr     tyr  thr  his  lys  arg  thr  glu  ile  glu  glu  gln  asn  asn  glu  ile  lys  gly  ile  asn
(606)       TAC     TAC  ACA  CAC  AAA  CGA  ACA  GAG  ATA  GAA  GAA  CAA  AAT  AAT  GAG  ATC  AAA  GGA  ATC  AAC    206

            lys     val  asn  his  asn  gln  arg  val  ala  gly  thr  arg  leu  gln  phe  ala  leu  arg  his  tyr
(666)       AAG     GTC  AAT  CAC  AAC  CAA  AGA  GTC  GCC  GGA  ACC  AGA  TTA  CAA  TTC  GCG  CTA  CGA  CAT  TAC    225

            thr     leu  leu  leu  val  ile  glu  pro  asp  pro  lys  asn  gln  thr  thr  his  glu  gly  ser  lys
(726)       ACT     CTC  CTT  CTT  GTA  ATC  GAG  CCA  GAC  CCA  AAA  AAC  CAG  ACC  ACC  CAT  GAA  GGA  AGT  AGG    246

            ser     lys  ser  ser  asn  thr  ser  lys  gly  leu  met  leu  ser  his  thr  val  leu  pro  leu  asn
(786)       TCG     AAG  TCC  TCG  AAC  ACA  TCC  AAG  GGC  TTA  ATG  TTG  TCC  CAT  ACC  GTT  CTT  CCA  CTA  AAC    266

            arg     thr  val  ala  asn  ser  ser  arg  ser  ser  pro  his  his  tyr  arg  stop
(846)       AGG     ACC  GTT  GCT  AAC  TCT  TCA  CGT  TCT  TCG  CCA  CAT  CAC  TAT  CGC  TAA  TAA  ACG  TGA  AAC    280

(906)       ACT     TGG  GTC  GTG  AAC  GCA  AGA  GAG  CAA  CGC  TGG  ACG  GTT  TGG  ATC  CAT  GTA  TGA  ATA  TAT

(966)       ATA     AAT  AGT  TGT  TTG  GAC  AAC  CTT  TTT  TTT  GGG  TCT  GGT  ATG  AAG  TGA  TTT  TAG  AGT  TCT

(1026)      TTA     TTG  TTT  CAT  TTC  TCT  TTG  AAT  TAA  TTC  TGT  AAT  TCT  AGA  TAA  ATA  TTT  TCA  TTC  ATA

(1086)      TAT     AAA  ATA  TTT  TTT  TAA  AGT  CTA  TGT  TTA  CAG  AGT  TGA  TAT  TGG  ATA  AAG  TTT  GAG  AAA

(1146)      GTT     TCA  TAC  AAA  AAA  AAA  AAA  AAA  AA                                                      3'
```

Figure 4

Figure 5 depicts the amino acid sequence of the *Arabidopsis* polypeptide manually aligned to demonstrate maximum identity with the sequence of rat liver connexin 32 (22,23). The abundance of basic residues in the *Arabidopsis* protein resulted in a calculated pI of 10.4 (24), which is consistent with the apparent basic nature of connexins; rat heart connexin 43 (25) has a predicted pI of 9.3, while rat liver connexin 32 has a predicted pI of 9.8 when calculated in a similar manner. (This value is in good agreement with our experimentally determined pI of 9.4 for rat liver connexin 32 (Chapter II, Figure 4A].)

The *Arabidopsis* sequence was further analyzed via the procedure of Kyte and Doolittle (26), which predicted four hydrophobic regions (noted as I, III, V, and VII, using the designation in Beyer *et al.* (25]) (Figure 6A). The more hydrophilic interspersing regions were noted as II, IV, VI, and VIII. The hydrophobic domains are of sufficient length to traverse a bilayer (amino acids 18-38, 79-101, 136-156, 217-236). This pattern is similar to that observed for rat liver connexin 32 (Figure 6B), although the rat liver polypeptide has a longer hydrophobic domain VII with a slight position shift in sequence when compared to the *Arabidopsis* polypeptide. Chou-Fasman secondary structure predictions (27) for the two proteins were also similar (data not shown). The hydrophobic domains were predicted to have β-pleated sheet character, whereas the more hydrophilic regions were predicted to have random coil character.

The deduced amino acid sequence of the *Arabidopsis* protein had four potential glycosylation sites (28) showing the Asn-X-Thr/Ser

Figure 5. Comparison of the deduced amino acid sequences of the *Arabidopsis* polypeptide and rat liver connexin 32. Alignment of sequences was manually performed to maximize identity; dashes were introduced to optimize alignment. Shaded regions indicate identity. The *Arabidopsis* sequence is shown on the top.

met	leu	trp	his	arg	asn	leu	val	lys	leu	leu	···	gly	tyr	cys	arg	glu	asp	lys	ala	leu	20
met	asn	trp	thr	gly	···	leu	tyr	thr	leu	leu	ser	gly	val	asn	arg	his	ser	thr	ala	ile	20
leu	leu	val	tyr	glu	···	phe	ile	pro	lys	glu	val	leu	arg	val	met	phe	leu	arg	arg	asn	40
gly	arg	val	trp	leu	ser	val	ile	phe	···	···	ile	phe	arg	ile	met	val	leu	val	val	ala	39
asp	pro	phe	pro	trp	···	asp	leu	arg	ile	lys	ile	val	···	ile	cys	ala	ala	arg	gly	pro	59
ala	glu	ser	val	trp	gly	asp	glu	···	···	lys	ser	ser	phe	ile	cys	asn	thr	leu	gln	pro	58
···	cys	val	ser	thr	gln	leu	thr	lys	arg	glu	cys	ile	tyr	arg	asp	leu	gln	val	phe	his	79
gly	cys	asn	ser	val	cys	tyr	asp	his	phe	phe	pro	ile	ser	···	···	···	···	···	···	his	73
ile	leu	leu	asp	···	leu	ser	tyr	gly	ala	val	leu	ser	arg	val	ser	gly	pro	trp	leu	···	98
val	arg	leu	trp	ser	leu	···	···	···	···	gln	leu	ile	leu	val	ser	thr	pro	ala	leu	leu	90
val	ala	met	glu	gln	gln	asn	arg	glu	val	his	arg	gly	thr	ala	lys	val	his	arg	arg	his	119
val	ala	met	···	···	···	···	···	···	···	his	val	···	···	ala	···	···	his	gln	gln	his	100
ile	···	lys	val	met	leu	leu	leu	glu	tyr	ile	ala	gly	his	···	leu	tyr	val	lys	ser	val	138
ile	glu	lys	lys	met	leu	arg	leu	glu	···	···	···	gly	his	gly	asp	pro	leu	his	leu	glu	118
ala	phe	ala	phe	gly	val	val	leu	leu	glu	ile	met	thr	gly	···	leu	···	···	thr	ala	his	156
glu	val	lys	arg	his	lys	val	his	···	···	ile	···	ser	gly	thr	leu	trp	trp	thr	tyr	val	136
asn	thr	lys	arg	pro	arg	gly	gln	ala	glu	asn	his	leu	met	arg	thr	tyr	val	met	asp	asp	177
ile	ser	val	val	phe	arg	leu	leu	phe	glu	ala	val	phe	met	···	···	tyr	val	phe	tyr	leu	155
lys	his	thr	gln	thr	ala	thr	pro	tyr	tyr	thr	his	lys	arg	thr	glu	ile	glu	glu	gln	asn	198
leu	tyr	pro	gly	tyr	ala	met	val	arg	leu	val	···	lys	cys	···	glu	ala	phe	pro	cys	pro	174
asn	glu	ile	lys	gly	ile	asn	lys	val	asn	his	asn	gln	arg	val	ala	gly	thr	arg	leu	gln	219
asn	thr	val	asp	cys	phe	val	ser	arg	pro	thr	glu	lys	thr	val	···	phe	thr	val	phe	met	194
phe	ala	leu	arg	his	tyr	thr	leu	leu	leu	···	val	ile	glu	pro	asp	pro	lys	asn	gln	thr	239
leu	ala	ala	ser	gly	ile	cys	ile	ile	leu	asn	val	ala	glu	val	val	tyr	leu	ile	ile	arg	215
thr	his	glu	gly	ser	lys	ser	lys	ser	ser	asn	···	thr	ser	···	lys	gly	leu	met	leu	ser	258
ala	cys	ala	arg	arg	ala	gln	arg	arg	ser	asn	pro	pro	ser	arg	lys	gly	ser	gly	phe	gly	236
his	thr	val	leu	···	pro	leu	asn	arg	thr	val	···	ala	asn	ser	ser	arg	ser	ser	pro	his	277
his	arg	···	leu	ser	pro	glu	tyr	lys	gln	asn	glu	ile	asn	lys	leu	leu	ser	glu	gln	asp	256
his	tyr	arg																			280
gly	ser	leu	lys	asp	ile	leu	arg	arg	ser	pro	gly	thr	gly	ala	gly	leu	ala	glu	lys	ser	277
asp	arg	cys	ser	ala	cys																283

Figure 5

Figure 6. Comparison of hydropathicity between the sequences of the *Arabidopsis* polypeptide and rat liver connexin 32. Hydropathicity values were determined with a window of 20 residues. Hydrophobic domains I, II, V, and VII and more hydrophilic interspersing domains II, IV, VI, and VIII are indicated for the *Arabidopsis* (A) and rat liver (B) polypeptides. It is worth noting that the longest hydrophobic segment in the rat liver polypeptide (domain V) is suggested to form the lining of the transmembrane channel (13). In the *Arabidopsis* polypeptide, the longest hydrophobic segment is domain III, which is suggested to have a channel-lining motif (see *Discussion*).

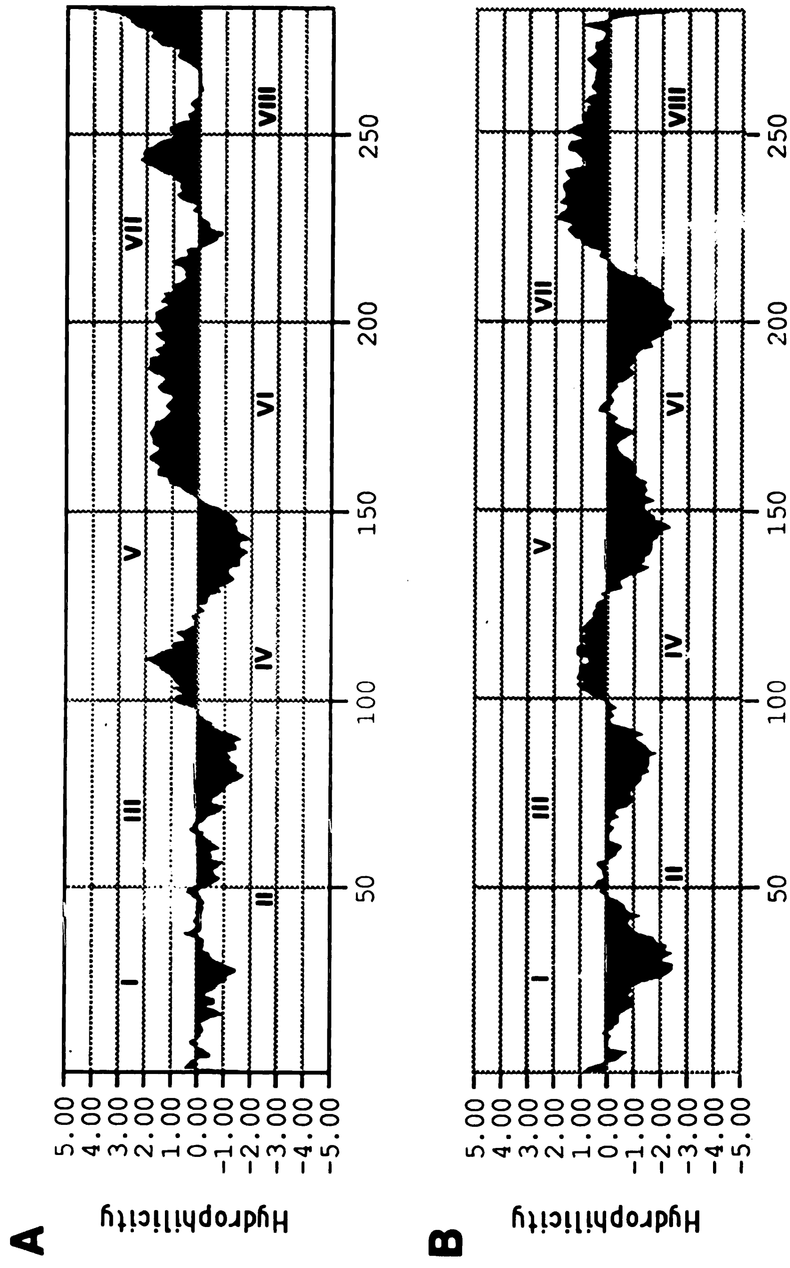

A
B
Hydrophilicity
Hydrophilicity
5.00
4.00
3.00
2.00
1.00
0.00
-1.00
-2.00
-3.00
-4.00
-5.00
I
II
III
IV
V
VI
VII
VIII
50
100
150
200
250

Figure 6

consensus sequence:

237-238-239, 250-251-252, 265-266-267, 270-271-272

Asn-Gln-Thr, Asn-Thr-Ser, Asn-Arg-Thr, Asn-Ser-Ser

This may be compared with three for rat heart connexin 43 (25), two for *Xenopus* embryonic connexin 38 (29), one for rat liver connexin 32 (23,24), and none for *Xenopus* liver connexin 30 (30). Preliminary results in our laboratory suggest that these sites are not glycosylated for the rat liver or *Arabidopsis* polypeptides (data not shown). As demonstrated for rat liver connexin 32, consensus cAMP-dependent phosphorylation sites (amino acid positions 112 and 192) (31), a tyrosine phosphorylation site (position 86) (32), and protein kinase C sites (positions 112, 251, and 271) (33,34) were also observed in the *Arabidopsis* sequence. (The cAMP-dependent protein kinases have a strong preference for serines and threonines that are located two residues to the COOH-terminal side of two basic amino acids (31). Tyrosine phosphorylation may require the presence of a basic amino acid that is seven residues to the NH_2-terminal side of the target tyrosine, together with one or more acidic residues between one and six residues away (32). Protein kinase C phosphorylation appears to favor serines and threonines that are two residues to the NH_2-terminal side of a basic amino acid (33,34].)

Modeling of the predicted transmembrane domain located in region V of rat liver connexin 32 has suggested that this segment provides the appropriate sequence to form the surface of the hydrophilic channel (13). Examination of this domain in connexins from rat heart (25) and *Xenopus* embryonic tissue (29) shows a characteristic sequence of amino acids in which a charged amino acid or an amino acid containing a small or hydroxylated side chain is situated at every fourth position, adjacent to large hydrophobic residues. Such an organization is also observed for transmembrane segment M3 of synaptophysin and is reminiscent of the consensus sequence of the M2 segment of ligand-gated channel proteins (35). Assuming that such a motif is representative for channel-lining transmembrane segments, then the channel-lining domain of the *Arabidopsis* protein would be in hydrophobic region III (amino acids 79-101) (Figure 6A) rather than in region V as suggested for rat liver connexin 32 (Figure 6B).

A comparison of the deduced amino acid sequences of the rat liver and *Arabidopsis* proteins demonstrated an overall identity of ~27% and a similarity of ~40% with conservative substitutions (e.g., Arg/Lys, Thr/Ser, Ile/Leu) and third base changes. (This may be compared to the reported 32% identity observed between *Xenopus* embryonic connexin 38 and rat liver connexin 32 (29].) Two areas of particularly high homology were found, corresponding to amino acids 90-101 *Arabidopsis*/amino acids 81-93 rat liver and amino acids 116-132 *Arabidopsis*/amino acids 97-111 rat liver. These two segments each demonstrated about 60% identity. Significantly, the sequence of one of these regions (amino acids 116-132 *Arabidopsis*) is closely related to the sequence of the rat liver connexin peptide (amino acids 98-124) utilized to prepare a synthetic antigen for immunization. The resulting rat liver connexin peptide antibody was employed as a probe to screen the *Arabidopsis* cDNA library.

Analysis of the *Arabidopsis* Polypeptide mRNA

Poly A(+) mRNA was isolated from soybean root cells and *Arabidopsis* leaves and blot hybridized employing the *Arabidopsis* clone as a probe (Figure 7). mRNA from both sources demonstrated a single species: 1.3 kb for *Arabidopsis* (Figure 7A) and 1.4 kb for soybean (Figure 7B). The size of the mRNA for *Arabidopsis* is somewhat larger than the 1.2 kb cDNA (Figure 3B) and potentially indicates that the cDNA clone has some missing bases in the 5′ end and/or poly As in the 3′ end. The observation of a single hybridized band for soybean (Figure 7B) is consistent with our previous reports demonstrating a connexin homologue in soybean root cells (6, 7, 36). This polypeptide was observed by utilizing the same immunological probes employed to isolate the *Arabidopsis* cDNA clone.

Figure 7. Northern blot analysis of the poly A(+) mRNA fraction derived from *Arabidopsis* leaves and soybean root cells. Samples were electrophoresed in 1.2% agarose gels and transferred to nitrocellulose by capillary action (20). The blot was probed with the cDNA of clone 73. The contents of the lanes were: (A) poly A(+) mRNA isolated from *Arabidopsis* leaves; and (B) poly A(+) mRNA isolated from soybean root cells. Approximately 3 µg of mRNA were loaded in each lane of the gel. The numbers on the left indicate the positions of migration of the DNA size standards.

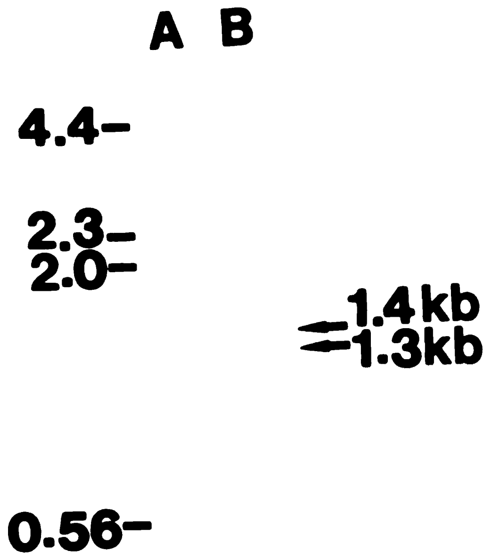


Figure 7

DISCUSSION

Evidence has been presented thus far for a gap junction-homologous polypeptide in plant cells. The existence of such a polypeptide in plants that may be a component of a trans-wall channel between contacting cells has previously received functional support from a variety of transport measurements in diverse plant material (5,37). Experiments have shown, both in terms of size discrimination and response to secondary messengers, i.e. Ca^{2+} and DAG, that the transit channels in plants behave remarkably like gap junctions in animal cells (5,37). However, morphological analysis of the contacting regions between plant cells does not demonstrate the classical gap junction structure for which the animal connexon is the basic unit. Indeed, the membrane bilayers of connected and contacting plant cells are separated by as much as 250 nm of cell wall material, hardly the 2 nm gap that is characteristic for contacting connexons.

Should connexons exist as structural elements of transit channels between plant cells, it would appear that their assembly might be considerably different than the head-to-head association traditionally observed for gap junctions. Such assemblages might not be readily recognizable or capable of being isolated as intact structures. In consideration of these problems of morphological identification, our laboratory has established at least three criteria to demonstrate the existence of a plant-type gap junction polypeptide, or connexin. This plant-type connexin must: (a) demonstrate immunological relatedness to previously identified animal connexins; (b) demonstrate sequence homology to animal connexins; and (c) display chemical similarities and structural motifs that appear to be common to animal connexins.

Immunological Relatedness of Plant-Type and Animal Connexins

As an initial approach to examining immunological relatedness, an antibody was prepared against the 27 kDa polypeptide of rat liver connexin 32. This antibody was shown to cross-react with a 29 kDa polypeptide in a variety of plants and plant tissue types, including soybean root cells and *Arabidopsis* leaves. Immunofluorescence studies utilizing the rat liver connexin antibody as a probe demonstrated that the 29 kDa polypeptide was localized to the cell periphery in soybean cells, specifically in a punctate pattern at regions of cell contact. It was also demonstrated that the 29 kDa polypeptide could be released from soybean cells following mild pectinase treatment under conditions that do not affect cell viability or membrane permeability (6,7,36). These experiments are consistent with a localization for the 29 kDa plant protein at the cell wall/plasma membrane interface, appropriately positioned to be involved in potential cell-cell transport channels.

Comparison of Sequences

An *Arabidopsis thaliana* cDNA library constructed in an expression vector was screened with antibodies raised against (1) the 27 kDa polypeptide of rat liver connexin 32; (2) the 29 kDa polypeptide of soybean connexin; and (3) a synthetic peptide corresponding to amino acid sequence 98-124 of rat liver connexin 32. A cDNA clone was isolated whose gene product demonstrated cross-reactivity with all three immunological probes. A comparison of the deduced amino acid sequences of this *Arabidopsis* protein and rat liver connexin 32 (Figure 5) demonstrated an overall identity of 27% and a similarity of 40%. Regions of higher and lower homology were present throughout the *Arabidopsis* protein, with greater sequence identity observed in the NH_2-terminal portion of the molecule. (A pattern of greater homology in the NH_2-terminal region appears to be a general theme for gap junction connexins.)

The *Arabidopsis* protein did not exhibit a leader or signal sequence. In addition, the sequence for the *Arabidopsis* protein demonstrated the entire array of sites available for post-translational modification that have been reported for other connexins. There were four potential asn-linked glycosylation sites (28) in the COOH-terminal of the polypeptide (amino acid positions 239,252,267, and 272). Consensus sequences were also found for cAMP-dependent protein kinase phosphorylation (31) (positions 112 and 192). The suggested importance of these sites for a controlling role of cAMP-dependent protein kinase phosphorylation in gap junctional regulation has been discussed elsewhere (1,38), but the absence of such phosphorylation sites in rat heart connexin 43 (25) may indicate that tissue-specific regulation occurs through them or that they are not directly involved in controlling transport. It is also worth noting that cAMP-mediated phosphorylation of proteins does not appear to occur in plants (39). Utilizing the criteria of Patschinsky *et al.* (32) for the sequence of amino acids surrounding the sites of tyrosine phosphorylation, a single site could be observed in the *Arabidopsis* sequence (position 86). This site was located within a hydrophobic domain in region III (Figure 5A). This domain is suggested to form the lining of a channel. This is of interest in that such a site is also observed in the hydrophobic domain found in region V of rat liver connexin 32 (position 151)(Figure 5B), which has been suggested to form the channel-lining surface for the transmembrane pore (13). A role for the control of transport by protein kinase C-mediated phosphorylation has been inferred from measurements with both animal (40) and plant cells (5). These measurements demonstrated that agents that can activate protein kinase C (such as Ca^{2+} and DAG) also modify intercellular communication. It was, therefore, intriguing to note a number of consensus protein kinase C sites (33) in the *Arabidopsis* protein (positions 112,251,and 271), particularly in regions of the protein containing a high density of positively charged amino acids. Positive charges surrounding protein kinase C consensus sites have been demonstrated to significantly enhance the phosphorylation of these sites (34).

A general analysis of the *Arabidopsis* sequence in relation to topological consequences, in conjunction with the predictive procedures of Kyte and Doolittle (26), suggest the following features that have also been observed for other connexin proteins: (a) the absence of a cleavable signal sequence that may be interpreted as evidence for a cytoplasmic disposition of the NH_2-terminal; (b) the existence of four putative hydrophobic domains with positions in the *Arabidopsis* sequence that closely match their distribution in rat liver connexin 32; and (c) a predicted hydrophobic domain in region III (Figure 5A) which contains an arrangement of charged, hydroxylated, and small side chain-containing amino acids that has been suggested to form the inner walls of transmembrane channels (35).

Chemical Properties of Connexins

Three particularly unusual chemical features of connexins studied to date are that heating in SDS will cause them to aggregate (11), they have unusually high pI values, and they may be aberrant in their migration on reducing SDS-PAGE gels (23,24). (For example, the molecular weight (MW) of rat liver connexin 32 as judged by migration on SDS-PAGE gels is 27 kDa, but the MW calculated from the cDNA sequence is 32.002 kDa (28,29].) In all these respects, the *Arabidopsis* polypeptide (pI = 10.4, MW determined by migration on SDS-PAGE gels = 29kDa, MW calculated from sequence = 32.339 kDa) is identical in chemical properties to other connexins.

The cumulative experimental data which demonstrates immunological relatedness, sequence homology, similar predicted hydrophobic domains, and related chemical properties between the *Arabidopsis* polypeptide and the animal connexin proteins, in particular rat liver connexin 32, suggests that this polypeptide may be the first representative of a connexin-like protein in plants. However, the results presented thus far do not provide direct experimental evidence as to whether the *Arabidopsis* polypeptide (or any other connexin-like polypeptide in plant cells) is involved in cell-to-cell communication, as has been demonstrated for animal connexins. That question will be addressed in the final chapter of this dissertation.

REFERENCES

1. Hertzberg, E.L., and Johnson, R.G., eds., p. 548. 1988. Gap Junctions. Alan R. Liss, Inc., New York, N.Y.
2. Lowenstein, W.R. 1979. Junctional intercellular communication and the control of growth. Biochim. Biophys. Acta 560: 1-65.
3. Gunning, B.E.S., and Overall, R.L. 1983. Plasmodesmata and cell-to-cell transport in plants. BioScience 33: 260-265.
4. Robards, A.W., and Lucas, W.J. 1990. Plasmodesmata. Ann. Rev. Plant Physiol. Plant Mol. Biol. 41: 369-419.
5. Baron-Epel, O., Hernandez, D., Jiang, L.-W., Meiners, S., and Schindler, M. 1988. Dynamic continuity of cytoplasmic and membrane compartments between plant cells. J. Cell Biol. 106: 715-721.
6. Meiners, S., and Schindler, M. 1987. Immunological evidence for gap junction polypeptide in plant cells. J. Biol. Chem. 262: 951-953.
7. Meiners, S., and Schindler, M. 1989. Characterization of a connexin homologue in cultured soybean cells and diverse plant organs. Planta 179: 148-155.
8. Gamborg, O.L., Miller, R.A., and Ojima, K. 1968. Nutrient requirements of suspension cultures of soybean root cells. Exp. Cell Res. 50: 151-158.

9. Metcalf, T.N., III, Wang, J.L., Schubert, K.R., and Schindler, M. 1983. Lectin receptors on the plasma membrane of soybean cells. Binding and lateral diffusion of lectins. Biochemistry 22: 3969-3975.

10. Ohyama, K. 1975. Isolation of 5-bromodeoxyuridine-resistant cells from soybean protoplasts. In: Plant Tissue Culture Methods. L.R. Wetter and O.L. Gamborg, eds., pp. 36-39. Prairie Regional Laboratory, Saskatoon, Sask., Canada.

11. Hertzberg, E.L. 1984. A detergent-independent procedure for the isolation of gap junctions from rat liver. J. Biol. Chem. 259: 9936-9943.

12. Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227: 680-685.

13. Milks, L.C., Kumar, N.M., Houghten, R., Unwin, N., and Gilula, N.B. 1988. Topology of the 32-kD liver gap junction protein determined by site-directed antibody localizations. EMBO (Eur. Molec. Biol. Org.) 7: 2967-2975.

14. Towbin, H., Staehelin, T., and Gordon, J. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. USA 76: 4350-4354.

15. Young, R.A., and Davis, R.N. 1983. Efficient isolation of genes by using antibody probes. Proc. Natl. Acad. Sci. USA 80: 1194-1198.

16. Miller, H. 1987. Practical aspects of preparing phage and plasmid DNA: growth, maintenance, and storage of bacteria and bacteriophage. In: Guide to Molecular Cloning Techniques. S.L.

Berger and A.R. Jimmel, eds., pp. 145-170. Academic Press, Inc., San Diego, CA.

17. Sambrook, J., Fritsch, E.F., and Maniatis, T. 1987. Extraction and purification of plasmid DNA. In: Molecular Cloning: A Laboratory Manual, Ed. 2. J. Sambrook, E.F. Fritsch, and T. Maniatis, eds., pp. 1.21-1.52. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, Mass.

18. Sanger, F., Nicklen, S., and Coulson, A.R. 1977. DNA sequencing with chain-termination inhibitors. Proc. Natl. Acad. Sci. USA 74: 5463-5467.

19. Amorese, D. 1986. Nonradioactive detection of RNA using the BluGENE system. Focus 8: 6-8.

20. Sambrook, J., Fritsch, E.F., and Maniatis, T. 1989. Extraction, purification, and analysis of messenger RNA from eukaryotic cells. In: Molecular Cloning: A Laboratory Manual, Ed. 2. J. Sambrook, E.F. Fritsch, and T. Maniatis, eds., pp. 7.1-7.57. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, Mass.

21. Stuart, P., Ito, M., Stewart, E., and Conrad, S.E. 1985. Induction of cellular thymidine kinase occurs at the mRNA level. Mol. Cell Biol. 5: 1490-1497.

22. Sillero, A., and Ribeiro, J.M. 1989. Isoelectric points of proteins: theoretical determination. Analytical Biochem. 179: 319-325.

23. Paul, D.L. 1986. Molecular cloning of cDNA for rat liver gap junction protein. J. Cell Biol. 103: 123-134.

24. Kumar, N.M., and Gilula, N.B. 1986. Cloning and characterization of rat liver cDNAs coding for a gap junction protein. J. Cell Biol. 103: 767-776.

25. Beyer, E.C., Paul, D.L., and Goodenough, D. 1987. Connexin 43: A protein from rat heart homologous to a gap junction protein from liver. J. Cell Biol. 105: 2621-2629.

26. Kyte, J., and Doolittle, R.F. 1982. A simple method of displaying the hydropathic nature of a protein. J. Mol. Biol. 157: 105-132.

27. Chou, P.Y., and Fasman, G.D. 1978. Prediction of the secondary structure of proteins from their amino acid sequence. Adv. Enz. 47: 45-1472.

28. Hubbard, S.C., and Ivatt, R.J. 1981. Synthesis and processing of asparagine-linked oligosaccharides. Ann. Rev. Biochem. 50: 555-583.

29. Ebihara, L., Beyer, E.C. Swensen, K.I., Paul, D.L., and Goodenough, D.A. 1989. Cloning and expression of a *Xenopus* embryonic gap junction protein. Science 243: 1194-1195.

30. Gimlich, R.L., Kumar, N.M., and Gilula, N.B. 1990. Differential regulation of the levels of three gap junction mRNAs in *Xenopus* embryos. J. Cell Biol. 110; 597-605.

31. Edelman, A.M., Blumenthal, D.K., and Krebs, E.G. 1987. Protein serine/threonine kinases. Ann. Rev. Biochem. 56: 567-513.

32. Patschinsky, T., Hunter, T., Esch, F.S., Cooper, J.A., and Sefton, B.M. 1982. Analysis of the sequence of amino acids surrounding sites of tyrosine phosphorylation. Proc. Natl. Acad. Sci. USA 79: 973-977.

33. Woodgett, J.R., Gould, K.L., and Hunter, T. 1986. Substrate specificity of protein kinase C. Eur. J. Biochem. 161: 177-184.

34. House, C., Wettenhall, R.E.H., and Kemp, B.E. 1987. The influence of basic residues on the substrate specificity of protein kinase C. J. Cell Biol. 262: 772-777.

35. Betz, H. 1990. Homology and analogy in transmembrane channel design: lessons from synaptic membrane proteins. Biochemistry 29: 3591-3599.

36. Xu, A., Meiners, S., and Schindler, M. 1990. Investigations of immunological relatedness between plant and animal connexin. In: Parallels in Cell-to-Cell Junctions in Plants and Animals. A.W. Robards, H. Jongsma, W.J. Lucas, J. Pitts, and D. Spray, eds., pp. 171-183. Springer-Verlag, Heidelberg.

37. Tucker, J.E., Mauzerall, D., and Tucker, E.B. 1989. Symplastic transport of carboxyfluorescein in staminal hairs of *Setcreasea purpurea* is diffusive and includes loss to the vacuole. Plant Physiol. 90: 1143-1147.

38. Burt, J.M., and Spray, D.C. 1988. Intropic agents modulate gap junctional conductance between myocytes. Am. J. Physiol. 254: H1206-H1210.

39. Salimath, B.P., and Marme, D. 1983. Protein phosphorylation and its regulation by calcium in membrane fractions from zucchini hypocotyls. Planta 158: 560-568.

40. Spray, D.C., and Burt, J.M. 1990. Structure-activity relations of the cardiac gap junction channel. Am. J. Physiol. 258: C195-C205.

Chapter IV

RETARDATION OF SYMPLASTIC TRANSPORT BETWEEN CONTIGUOUS SOYBEAN ROOT CELLS BY ANTIBODIES DIRECTED AGAINST RAT LIVER CONNEXIN 32

ABSTRACT

A technique has been developed that results in the reversible permeabilization of the cell wall and plasma membrane of soybean (*Glycine max* [L.] Merr.) root cells grown in suspension and callus culture. Cells in culture were treated with saponin (0.1 mg/ml) for 15 min at room temperature. They were then incubated with either fluorescently derivatized dextrans (20-70 kDa) or with fluorescein-conjugated goat anti-rabbit IgG to ascertain the exclusion size of macromolecules capable of diffusing across the cell wall and plasma membrane into the cytoplasm. Following an incubation period of 30 min, it was observed by conventional and confocal fluorescence microscopy that all derivatized macromolecules tested (20-150 kDa) could be incorporated into the cytoplasm, but not into the vacuole. This procedure did not appear to affect cell viability adversely, as a normal doubling time for the cells was observed following the permeabilization procedure.

In a separate set of experiments, soybean root cells grown in suspension culture were permeabilized with saponin and then incubated for 45 min with rat liver connexin peptide antibody, an affinity purified antibody raised against a synthetic peptide corresponding to amino acid sequence 98-124 of rat liver connexin 32. During the last 10 min of the incubation period, the cells were labeled with fluorescein diacetate, a fluorescent dye. Some cells

were selectively photobleached, and the rate of diffusion-dependent return of the dye was determined. Fluorescence recovery rates were interpreted as dye transport across plasmodesmata. Results of experiments demonstrated that the recovery rates of soybean cells incubated with rat liver connexin peptide antibody were about 1/2 slower than the recovery rates of control cells. Control cells included (a) cells incubated with no antibody and (b) cells incubated with rat liver connexin peptide antibody plus an excess molar amount of synthetic antigen.

INTRODUCTION

A variety of techniques presently exist to introduce impermeant molecules into the cytoplasm of living cells (1). These methods have, for the most part, been developed for use with either animal cells in tissue culture or with plant protoplasts. The procedures, therefore, are principally designed to, in a temporary and reversible manner, permeabilize the plasma membrane. Plant cells, on the other hand, have provided a more difficult biological material for cytoplasmic incorporation of macromolecules. The impermeant lipid bilayer of the plasma membrane, in conjunction with the molecular sieving properties of the cell wall, have resisted most permeabilization procedures and have required more exotic approaches, such as coating micrometer-sized projectiles with the molecule to be included and then shooting these coated projectiles under high pressure into the cells. This technique has been most successfully employed for the incorporation of DNA (2,3). In a previous effort to examine the wall porosity in soybean cells (4), investigators in our laboratory found that mild treatment of the cells with pectinase could substantially enhance the trans-wall diffusion of macromolecules. Following such treatment, molecules as large as soybean agglutinin (120 kDa) were found to enter the space between the cell wall and the plasma membrane. More importantly, cells treated in this manner retained their viability, as judged by their ability to undergo repeated plasmolysis (4). These observations provided evidence that the molecular sieving properties of the wall could be substantially modified without seriously affecting cell viability. In our initial efforts to incorporate macromolecules into plant cells, this pectinase treatment was utilized simultaneously with saponin, a plant glycoside that perturbs membranes. Such a binary treatment resulted in the incorporation of high molecular weight dextrans and immunoglobulins into the cell cytoplasm. Subsequent work has demonstrated that the pectinase treatment is unnecessary and that saponin (0.1 mg/ml) by itself is capable of transiently and reversibly permeabilizing whole soybean root cells in suspension culture while maintaining cell viability.

The saponin permeabilization technique was utilized to introduce an antibody that recognizes rat liver connexin 32 into the cytoplasm of cultured soybean cells. Evidence has been presented elsewhere in this dissertation for a polypeptide found in soybean root cells that is immunologically and biochemically related to rat liver connexin 32. Based on immunofluorescence results obtained in our laboratory (5), we have hypothesized that the soybean polypeptide, referred to here as soybean connexin, is a component of plasmodesmata. Plasmodesmata are believed to be plant cell-cell communication structures and are therefore the functional analogues of gap junctions in animal cells (6,7,8). This study now demonstrates that, following the incorporation of connexin antibodies into the cytoplasm of soybean root cells, the rate of cell-to-cell dye transfer of fluorescein diacetate (FDA) is reduced to approximately 1/2 its value in control cells. The retardation of dye transfer, as assayed by fluorescence redistribution after photobleaching (FRAP)(9), presumably resulted from the obstruction of plasmodesmatal channels caused by the binding of the antibody to the soybean connexin. This provides further experimental evidence for the plasmodesmatal localization of the soybean connexin and suggests that the protein may play a direct role in forming the plasmodesmatal transport channel.

MATERIALS AND METHODS

Cell Culture, Permeabilization, and Analysis

Cells (SB-1) derived from soybean (*Glycine max* [L.] Merr. cv. Mandarin) roots were grown in 1B5C medium in darkness (10). Five ml of 4 d-old cells were pelleted by centrifugation in a tabletop centrifuge for 2 min at 460 x g. The supernatant was discarded, and the cells were resuspended in 5 ml of fresh 1B5C medium containing 0.1 mg/ml saponin (Sigma Chemical Co., St. Louis, MO, USA). The cells were incubated in the saponin solution for 15 min at room temperature, washed three times by pelleting and resuspension in 1B5C medium, and incubated in a variety of fluorescein (FITC)-derivatized macromolecules or fluorescein diacetate (FDA). The procedure for labeling calli grown on agar was identical except for the elimination of the centrifugation steps. The incubation periods and fluorescent probes employed were as follows: 10 min for FDA (Molecular Probes, Junction City, Oregon, USA); 30 min for FITC-conjugated dextrans (20 kDa, 40 kDa, and 70 kDa) (Sigma); and 30-60 min for FITC-conjugated goat anti-rabbit immunoglobulin G (IgG) Bio-Rad, Richmond, CA, USA). FITC-conjugated goat anti-rabbit IgG was chromatographed on a Sephadex G-25 column prior to use to remove possible contamination with free fluorescein. All fluorescent molecules were used at a concentration of 1 mg/ml, while FDA was used at 10 µg/ml. Following incubation, the cells from suspension culture were again washed in 1B5C medium and mounted on slides in 70% (v/v) glycerol in 1B5C medium containing 5% (w/v) of the anti-bleaching agent *n*-propyl gallate (Sigma). The slides were viewed with a Leitz epifluorescent microscope (Leitz, Wetzlar, FRG) using a 40X objective or 25X objective lens. Labeled calli were placed on coverslips and optical sectioning was performed with a 100X objective lens (oil immersion) utilizing the ACAS (Anchored Cell Analysis and Sorting) 570 Confocal Interactive Laser Cytometer (Meridian Instruments, Okemos, MI, USA).

Cell Proliferation Assay

Twenty ml of a 4 d-old culture of soybean root cells were pelleted by centrifugation at 460 x g for 3 min. The cell pellet was resuspended in 20 ml of fresh 1B5C medium containing 1 mg/ml saponin and was incubated in this solution for 15 min at room temperature. The cells were then washed 5 times by pelleting and resuspension in 1B5C medium and grown for 80 h in darkness. At 0, 5, 10, 20, 30, 35, 50, 60, and 80 h time points, an aliquot of cells in a constant volume (500 µl) was added to a tarred Eppendorf tube. The wet weight was determined, and the dry weight was obtained after dehydration of the cells in a vacuum oven. All time point measurements were made in triplicate.

Incorporation of Antibody and Fluorescence Redistribution After Photobleaching Assay

Five ml of 4 d-old soybean cells were pelleted by centrifugation for 3 min at 460 x g. The cell pellet was resuspended in 5 ml of fresh 1B5C medium containing 0.1 mg/ml saponin. The cells were incubated for 15 min in the saponin solution and washed 3 times by pelleting and resuspension in 1B5C medium. They were then incubated for 45 min with a 1 mg/ml solution of rat liver connexin peptide antibody, an affinity purified antibody prepared against a hydrophilic loop peptide of rat liver connexin 32 (amino acid sequence 98-124) (11,12). Control cells were incubated with 1 mg/ml rat liver connexin peptide antibody plus 1 mg/ml synthetic peptide. (This represents a 40 fold molar excess of peptide versus antibody.) During the last 10 min of the incubation period, the cells were labeled with FDA (10 µg/ml). They were then washed three times in 1B5C medium and mounted on slides.

The slides were placed on the stage of an ACAS 570 workstation (Meridian Instruments, Okemos, MI, USA). The cells were moved on the automated stage in 2.0 µm steps in a two-dimensional grid past a microscope objective (40X). The objective focused the excitation beam (488 nm) of an argon ion laser to a 1 µm diameter beam on the sample. A photomultiplier tube captured the emission intensities at each addressed excitation point. The emitted intensities were color-coded and displayed on a computer video screen as false color images of cellular fluorescence. The intensity of the laser beam was then significantly increased, and a series of bleaches was performed within a single cell to photochemically destroy the cytoplasmically localized dye. After photobleaching, the bleached dye molecules from one cell and the unbleached molecules from an adjacent cell can redistribute through plasmodesmata. The redistribution of these molecules was monitored as a function of time to yield single exponential recovery curves from which rate constants for dye transport were determined. Fluorescence recovery rates were also determined for cells only labeled with FDA.

RESULTS

Incorporation of FITC-Conjugated Dextrans into the Cell Cytoplasm and Preservation of Cell Viability

Soybean root cells (SB-1 cell line) were grown in suspension culture and then incubated with saponin (0.1 mg/ml) and fluorescently derivatized macromolecules as described in *Materials and Methods*. FITC-conjugated dextrans of approximately 20 kDa and 40 kDa were found to incorporate into the cell cytoplasm but not into the vacuole, as observed in Figure 1. Control cells that were saponin-treated without the addition of fluorescent macromolecules were observed to be morphologically intact by phase microscopy (Figure 1A) and exhibited no intracellular fluorescence (Figure 1B). Cells not treated with saponin but incubated with FITC-40 kDa dextran and then washed as described in *Materials and Methods* also appeared normal by phase microscopy (Figure 1C). In addition, these cells did not exhibit intracellular accumulation of fluorescence (Figure 1D). Saponin treatment of cells in the presence of FITC-20 kDa dextran (Figure 1E,F) and FITC-40 kDa dextran (Figure 1G,H) for 30 min now demonstrated the inclusion of both dextran sizes. Note the labeling of the cytoplasmic region (Figure 1F,H) and the exclusion of fluoresence from the vacuolar space. Experiments utilizing FITC-70 kDa dextran (Stokes radius of approximately 5.5 nm (4]) also demonstrated intracellular accumulation (Figure 1I,J).

Figure 1. Fluorescence labeling of soybean cells grown in suspension culture following saponin treatment. Control cells were either treated with saponin without subsequent incubation with FITC-conjugated dextran (A & B; A, phase, B, fluorescence) or incubated with FITC-40 kDa dextran for 30 min without prior treatment with saponin (C & D; C, phase, D, fluorescence). Other cells were treated with saponin and then incubated for 30 min with FITC-20 kDa dextran (E & F; E, phase, F, fluorescence), FITC-40 kDa dextran (G & H; G, phase, H, fluorescence), or FITC-70 kDa dextran (I & J; I, phase, J, fluorescence). Cells in K & L (K, phase, L, fluorescence) were incubated for 10 min with FDA. Soybean cells were viewed with a Leitz epiphase fluorescence microscope using a 40X objective lens.

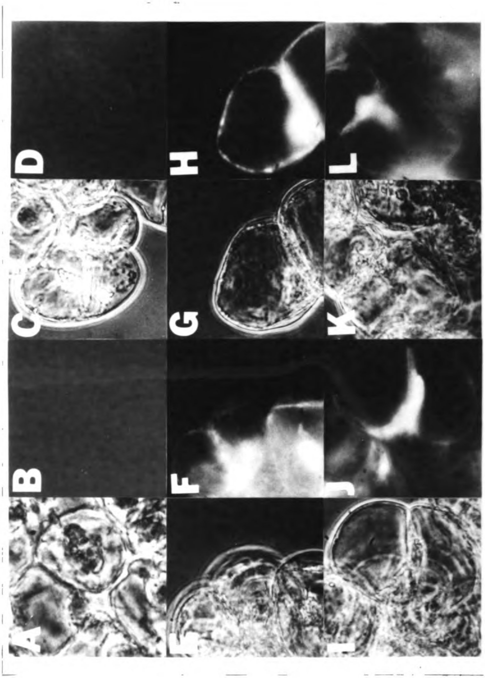


Figure 1

In an effort to conclusively show that the labeling occurred predominantly in the cytoplasmic compartment, two approaches were chosen, as illustrated in Figure 1K,L and Figure 2. Fluorescein diacetate (FDA) has previously been shown to accumulate in the cytoplasm of plant cells (13,14). As can be seen in Figure 1L, the intracellular localization of FDA matched that observed for the fluorescently derivatized dextrans. In Figure 2, cells were plasmolyzed with 0.2 M sorbitol (4) following permeabilization with saponin to provide additional evidence that the incorporated macromolecules were localized to the cytoplasm. As shown in Figure 2C,D for the FITC-40 kDa dextran and in Figure E,F for the FITC-70 kDa dextran, fluorescence was observed in the resulting protoplasts but not in the intracellular space between the cell wall and plasma membrane. A particularly important feature of this permeabilization technique is that it apparently had no serious effect on the viability of treated cells and their ability to proliferate as shown by dry weight (Figure 3) and wet weight determinations (data not shown) as an indication of cell division (15).

Incorporation of FITC-Conjugated Antibodies into the Cytoplasm of Living Cells

Although the dextran-incorporation data provides evidence that macromolecules as large as 5.5 nm in Stokes radius can gain access into the cell cytoplasm, it was important to examine the possibility that the technique could also be used to incorporate antibodies into living cells. An important advantage for the study of structure-function relationships of proteins in animal cells has

Figure 2. Cytoplasmic labeling of plasmolyzed soybean cells. Cells in A & B (A, phase, B, fluorescence) were incubated with FITC-40 kDa dextran for 30 min without prior treatment with saponin and were then plasmolyzed as described in Baron-Epel *et al.* (4). Cells in C & D (C, phase, D, fluorescence) were treated with saponin and incubated with FITC-40 kDa dextran for 30 min, followed by plasmolysis. Cells in E & F (E, phase, F, fluorescence) were treated with saponin and incubated with FITC-70 kDa dextran for 30 min, followed by plasmolysis. Cells were viewed with a 25X objective lens.

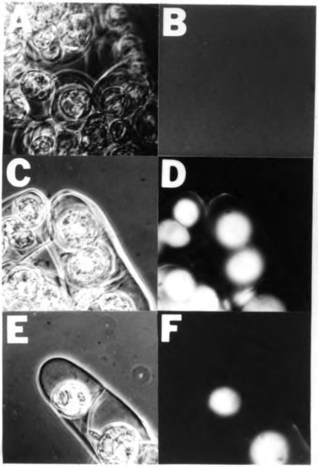


Figure 2

<u>Figure 3</u>. Proliferation of soybean cells grown in suspension culture. Cells were cultured in 1B5C medium with (o) or without (o) prior saponin treatment. Dry weight was used to evaluate growth. Each point consists of the average of three samples. Variability between samples did not exceed 15%.

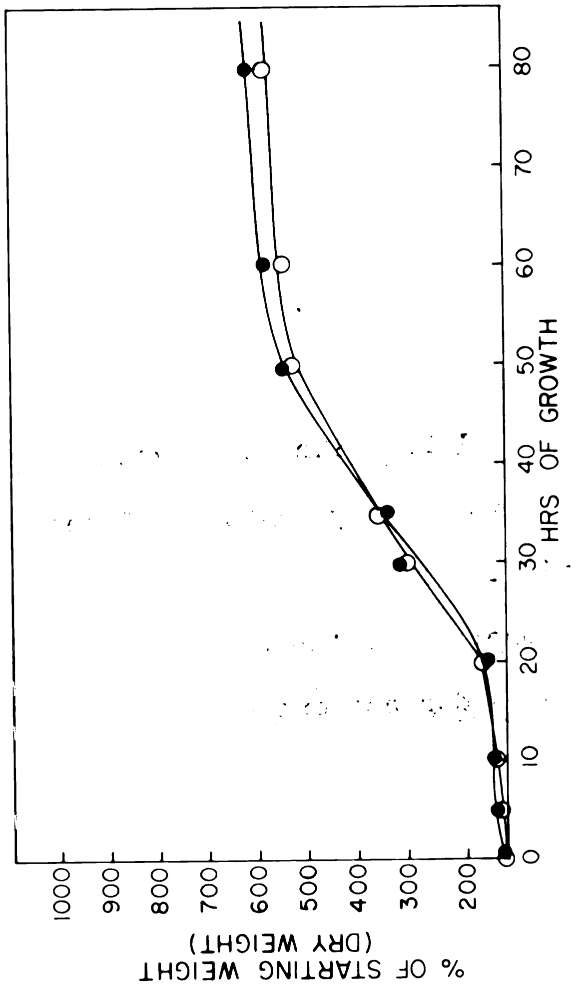

% OF STARTING WEIGHT
(DRY WEIGHT)
1000
900
800
700
600
500
400
300
200
0
10
20
30
40
50
60
70
80
HRS OF GROWTH

Figure 3

been the ability to microinject antibodies that could block the biological activity of a protein in question in viable cells, potentially leading to altered cellular activity (16,17). Such a capability would also be most useful in plant cell research, and evidence is presented in Figure 4 demonstrating the feasibility of such an approach. Control cells are depicted in Figure 4A & B which were incubated for 60 min in FITC-conjugated goat anti-rabbit IgG without prior treatment with saponin. No included fluorescence was observed (Figure 4B). In contrast, incorporation of antibody was observed following saponin treatment and incubation with FITC-conjugated antibody for 30 min (Figure 4C,D) and 60 min (Figure 4E,F).

To provide additional evidence that antibody incorporation had occurred, and that the observed fluorescence was not a result of free fluorescein, confocal fluorescence microscopy was performed on soybean calli that were permeabilized utilizing the saponin procedure. Laser scanning confocal microscopy can significantly enhance images of fluorescence localization in cells and tissues by eliminating the out-of-focus fluorescence from above or below the plane of focus. This has important advantages for providing non-destructive high resolution images within living tissues and also for greatly minimizing the effect of tissue autofluorescence. Optical slices of soybean calli of about 1 µm are presented in Figure 5. As shown in Figure 5A, incorporated fluorescein diacetate (FDA) could partition into the cytoplasm (labeled periphery) and the nucleus (arrow), but not into the vacuole (Figure 5). Incorporated FITC-conjugated goat anti-rabbit IgG, on the other hand, only

Figure 4. Fluorescence labeling of the cytoplasmic compartment of soybean cells with FITC-conjugated goat anti-rabbit IgG. Control cells were incubated with FITC-conjugated goat anti-rabbit IgG for 60 min without prior treatment with saponin (A & B; A, phase, B, fluorescence). Other cells were treated with saponin and incubated with FITC-conjugated goat anti-rabbit IgG for either 30 min (C & D; C, phase, D, fluorescence) or 60 min (E & F; E, phase, F, fluorescence). Soybean cells were viewed using a 40X objective lens.

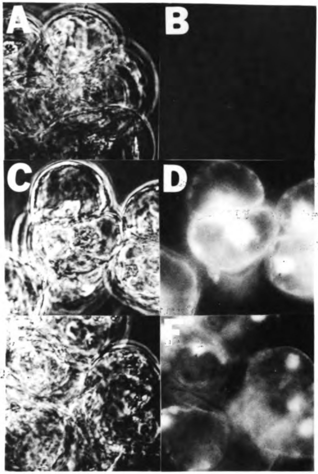


Figure 4

Figure 5. Confocal fluorescence section of cells grown in callus culture. In separate experiments, soybean calli were treated with saponin and then incubated with (A) FDA for 10 min or (B) FITC-conjugated goat anti-rabbit IgG for 30 min. Callus cultures in (C) were incubated with FITC-conjugated goat anti-rabbit IgG for 30 min without prior treatment with saponin. Optical sections were approximately 1 micron thick. Arrow indicates the cell nucleus. Cells were viewed utilizing an ACAS 570 work station with a 100X objective lens. The color code presented relates color to an arbitrary numerical scale of fluorescence intensity.

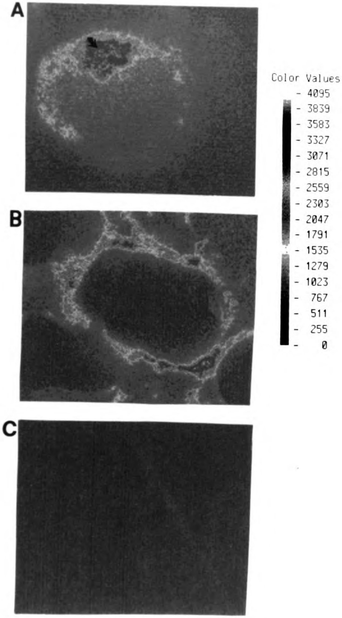

A
B
C
Color Values
4095
3839
3583
3327
3071
2815
2559
2303
2047
1791
1535
1279
1023
767
511
255
0

Figure 5

labeled the cytoplasm (Figure 5B). This is consistent with the known exclusion limit for diffusion-mediated transport of macromolecules into cell nuclei (18). (In contrast, such distinctions between cytoplasmic and nuclear labeling could not be readily discerned for the images presented in Figure 1 and Figure 2 for FITC-derivatized dextrans and in Figure 4 for FITC-conjugated goat anti-rabbit IgG.) A comparison of the two images presented in Figure 5A & B also provides evidence that free fluorescein is not a contaminating facter in the analysis of IgG localization. Figure 5C shows a portion of callus that was incubated with FITC-conjugated goat anti-rabbit IgG without prior saponin treatment. No labeling was observed under these conditions.

Incorporation of Rat Liver Connexin Peptide Antibody and Its Effect on Dye Coupling

Soybean cells grown in suspension culture were permeabilized with 0.1 mg/ml saponin. They were then incubated for 45 min with either rat liver connexin peptide antibody or rat liver connexin peptide antibody plus synthetic antigen. The cells were labeled with fluorescein diacetate (FDA) during the last 10 min of the incubation. FDA is a low molecular weight fluorescent compound which is maintained within the cells following hydrolysis to fluorescein by intracellular esterases (19). Cell-cell communication between the cytoplasmic compartments of the soybean cells was measured by using fluorescence redistribution after photobleaching (FRAP). This technique, which was first employed for the measurement of intercellular communication between animal cells

(9,20), is performed by photobleaching the fluorescent compounds in a cell of interest contacting other similarly labeled cells. Recovery of dye in the bleached cell may occur via a diffusion-dependent rearrangement of the dye molecules between the bleached and unbleached cells. Monitoring this process as a function of time yields a rate constant for dye movement. Single noncontacting cells are photobleached to provide a control demonstrating that recovery is a function of transport between contacting cells, and not the physical consequence of dye reactivation after photobleaching or uptake of dye from the medium.

Computer generated false color images of fluorescence redistribution are shown in Figure 6A and Figure 7A for soybean cells incubated with rat liver connexin peptide antibody or rat liver connexin peptide antibody plus peptide, respectively. Photobleaching resulted in a drop in the fluorescence intensity in the bleached cells (Cell 2 in Figure 6 and cell 4 in Figure 7) and some bleaching in contacting cells (Figure 6A & B, Figure 7A & B). Although FDA recovery occurred for both types of cells (Figure 6C and Figure 7C), the extent of recovery for cells incubated with rat liver connexin peptide antibody alone was lower than that for cells incubated with rat liver connexin peptide antibody plus synthetic antigen.

The redistribution of fluorescence data was analyzed according to the equation:

$$F(-) - F(t)/F(-) - F(0) = e^{-K_1 t_T} \times e^{-K_2 t}$$

where F(-), F(0), and F(t) are fluorescence signals before photobleaching, after, and at time t (21). Typical recovery curves

<u>Figure 6</u>. Fluorescence redistribution after photobeaching in soybean cells permeabilized with saponin, incubated with rat liver connexin peptide antibody, and labeled with FDA. The ACAS 570 workstation was used to photobleach the cells and to record fluorescence distribution as a false color image visualized on a cathode ray tube. Before photobleaching, a strong fluorescence intensity could be seen (A). This intensity was reduced after photobleaching cell 2 (B). (C) depicts fluorescence intensity in the cells 3 min after photobleaching, and (D) depicts a recovery curve for dye transport between cells. Box 2 denotes photobleached cell; box 1 denotes a neighboring contacting cell; and box 3 denotes a noncontacting (control) cell whose fluorescence intensity should remain constant during the course of the experiment. Fluoresence intensities were computer corrected to box 3 to control for long term photobleaching during the monitoring process. The color code presented relates color to an arbitrary numerical scale of fluorescence intensity. The information presented here is representative of 17 such experiments.

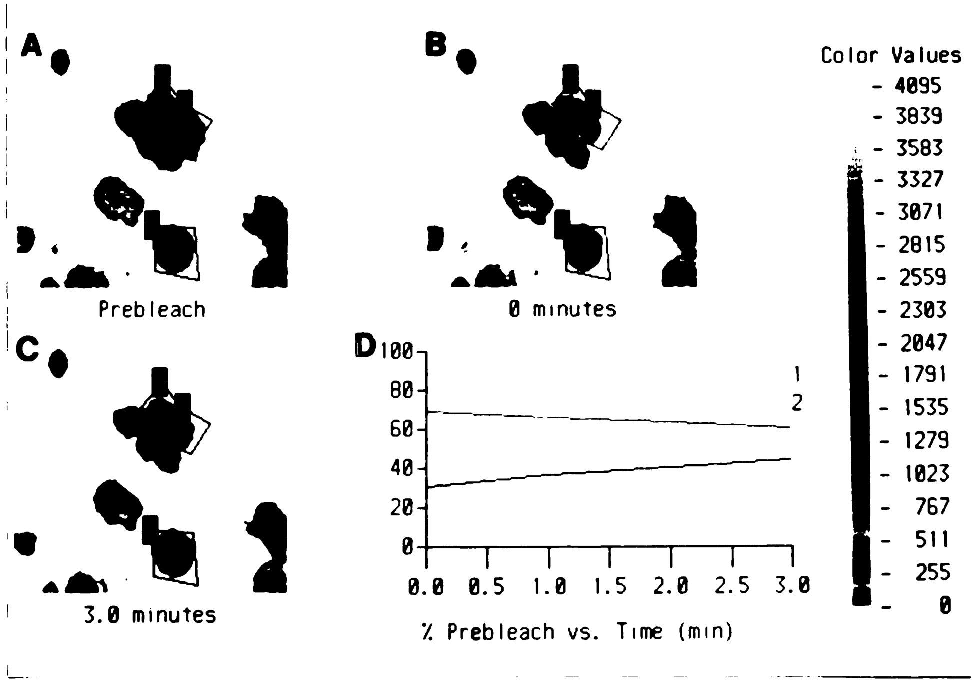
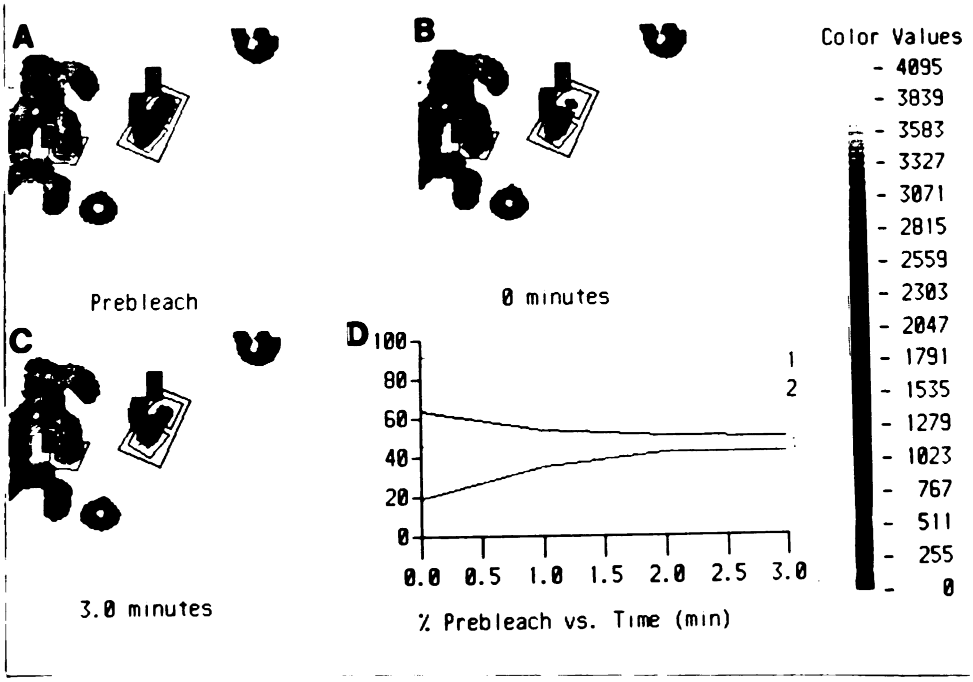

A
Prebleach
B
0 minutes
Color Values
- 4095
- 3839
- 3583
- 3327
- 3071
- 2815
- 2559
- 2303
- 2047
- 1791
- 1535
- 1279
- 1023
- 767
- 511
- 255
- 0
C
3.0 minutes
D
100
80
60
40
20
0
1
2
0.0
0.5
1.0
1.5
2.0
2.5
3.0
% Prebleach vs. Time (min)

Figure 6

Figure 7. Fluorescence redistribution after photobleaching in soybean cells permeabilized with saponin, coincubated with rat liver connexin peptide antibody and synthetic antigen, and labeled with FDA. The experiment was performed as in Figure 6. (A) Prebleach distribution. (B) & (C) The bleached cell immediately and 3 min after photobleaching. (D) Recovery curve for dye transport. Box 4 denotes photobleached cell; box 2 denotes contacting cell; and box 3 denotes noncontacting (control) cell. Box 1, which surrounds boxes 2 & 4, serves to monitor total fluorescence in these cells during the experiment. The information presented here is representative of 13 such experiments.



Prebleach

0 minutes

3.0 minutes

Figure 7

for dye transport are shown in Figure 6D and Figure 7D. The calculated flux rates derived from the recovery curves for cells incubated with rat liver connexin peptide antibody (17 experiments) were 8.7 ± 2.7 arbitrary fluorescence units per minute. Calculated flux rates for cells incubated with rat liver connexin peptide antibody plus synthetic peptide (13 experiments) were 15.5 ± 3.6 arbitrary fluorescence units per minute. The calculated flux rates for cells labeled with FDA but not incubated with any antibody were practically identical to those for cells incubated with antibody plus peptide (data not shown). Therefore, incorporation of rat liver connexin peptide antibody into the cytoplasm of soybean cells specifically resulted in a reduction in the rate of cell-to-cell dye transport to about 1/2 the value in control cells.

DISCUSSION

Symplastic communication between plant cells has been suggested to be the dominant form of plant intercellular communication (22). Morphological evidence has suggested that trans-wall tubular structures, termed plasmodesmata, provide the channel(s) for the intercellular movement of hydrophilic low molecular weight ($\leq$ 1,000 mol wt) metabolic, biosynthetic, and signaling molecules (22,23,24). Evidence has been presented elsewhere in this dissertation for a gap junction-homologous protein which may be a component of plasmodesmata. A series of experiments was initiated to investigate the affect of a gap junction connexin antibody on cell-to-cell transport between soybean cells. The affinity purified antibody (rat liver connexin peptide antibody) used in this investigation was raised against a hydrophilic loop peptide corresponding to amino acids 98-124 of rat liver connexin 32. This peptide has been demonstrated to reside in the cytoplasm of rat hepatocytes (25). In previous attempts to measure the affects of connexin antibodies on cell-cell communication in animal cells, the antibodies, followed by membrane impermeant reporter dyes (e.g. Lucifer yellow), were microinjected into cells, and the movement of dye from the site of microinjection to the surrounding cells was optically monitored (16,26). A potential difficulty with extrapolating these experimental techniques to plant studies may be inferred from the work of Davies and Schuster (27), in which it was found that the properties of wounded plant tissues are markedly different from those of intact tissues. A needle breaching the cell wall may thus lead to altered cellular responses. In an attempt to circumvent this potential problem, a saponin permeabilization procedure was developed to incorporate antibodies into soybean root cells, and fluorescence redistribution after photobleaching (9,13,20) was employed to measure the transport of dye between them. Incubation of the cells with 0.1 mg/ml saponin resulted in the reversible permeabilization of the plant cell wall and plasma membrane under conditions that maintained cell viability and proliferative ability. Macromolecules ranging in molecular mass from 20 kDa to 150 kDa could then be specifically incorporated into the cytoplasm of living soybean cells. FDA was used to label cells permeabilized with saponin and incubated with either 1 mg/ml rat liver connexin peptide antibody or 1 mg/ml antibody plus 1 mg/ml synthetic antigen. (This antibody concentration was chosen because it corresponds to the concentrations used in the animal cell-cell communication studies described above (0.4-1.5 mg/ml) (16,17,26].) FDA is a fluorescent dye which enters cells passively and is trapped inside them following deacetylation by intracellular esterases (19). Therefore, use of saponin to incorporate antibodies and use of FDA as a labeling reagent may be gentler on soybean cells than microinjecting them with antibodies and membrane impermeant dyes.

Incubation of soybean root cells with rat liver connexin peptide antibody resulted in a fluorescence recovery rate for FDA of 8.7 ± 2.7 arbitrary fluorescence units per minute. This is to be compared with a fluorescence recovery rate of 15.5 ± 3.6 arbitrary fluorescence units per minute for cells incubated with rat liver connexin peptide antibody plus synthetic peptide. Since recovery rates are interpreted as dye transport across plasmodesmata, the observed reduction in flux rate for antibody-treated cells is suggested to be due to the binding of the antibody to a plasmodesmatal protein. It has been shown previously that the rat liver connexin peptide antibody recognizes a 29 kDa polypeptide in soybean root cells, which is hypothesized to be a component of plasmodesmata. This polypeptide is representative of a larger group of proteins which collectively have been termed plant connexins (5,11).

Rat liver connexin peptide antibody was used in conjunction with monospecific antibodies prepared against the 27 kDa polypeptide of rat liver connexin 32 and the 29 kDa polypeptide of soybean connexin (molecular weight determined by migration in reducing SDS-PAGE gels) to isolate a cDNA clone from *Arabidopsis thaliana* (5,11). The cDNA coded for a polypeptide that demonstrated 27% overall identity at the amino acid level with rat liver connexin 32. One of the regions of the *Arabidopsis* polypeptide, corresponding to amino acid sequence 116-132, demonstrated approximately 60% identity with rat liver connexin 32 sequence 97-111. This is significant in that rat liver connexin 32 sequence 98-124 was utilized to prepare a synthetic antigen for immunization which resulted in the rat liver connexin peptide antibody used to select the cDNA clone. Scrutiny of amino acid sequence 116-132 in the *Arabidopsis* polypeptide reveals that this region resides partially in a hydrophobic domain (region V, Figure 6A, Chapter III). The corresponding sequence in rat liver connexin 32, on the other hand, resides entirely in a hydrophilic domain (region IV, Figure 6B, Chapter III) which in turn has been suggested to reside in the cytoplasm (25). These observations may provide a clue as to why the rate of dye transport between soybean cells incubated with rat liver connexin peptide antibody was only reduced by about 1/2 as compared to control cells (8.7 ± 2.7 versus 15.5 ± 3.6 arbitrary fluorescence units per minute), whereas transport between animal cells was almost completely inhibited in previous studies of connexin antibody affects on intercellular communication (16,17,26). If the soybean protein is similar to the *Arabidopsis* protein, the portion of the polypeptide recognized by the rat liver connexin peptide antibody may be partially buried in the interior of the protein or in some other way may not be entirely accessible to the antibody. Therefore, binding of the rat liver connexin peptide antibody may partially obstruct but not completely block the plasmodesmatal channel.

REFERENCES

1. McNeil, P.L. 1989. Incorporation of macromolecules into living cells. Methods in Cell Biol. 29: 153-173.
2. Klein, T.M., Harper, E.C., Svals, Z., Sanford, J.D., Fromm, M.E., and Maliga, P. 1988. Stable genetic transformation of *Nicotiana* cells by the particle bombardment process. Proc. Natl. Acad. Sci. USA 85: 8502-8505.
3. McCabe, D.E., Swain, W.F., Martinelli, B.J., and Clinston, P. 1988. Stable transformation of soybean (*Glycine max*) by particle acceleration. BioTechnology 6: 923-926.
4. Baron-Epel, O., Gharyal, P.K., and Schindler, M. 1988. Pectins as modifiers of wall porosity in soybean cells. Planta 175: 389-395.
5. Meiners, S., and Schindler, M. 1989. Characterization of a connexin homologue in cultured soybean cells and diverse plant organs. Planta 179: 148-155.
6. Gunning, B.E.S., and Overall, R.L. 1983. Plasmodesmata and cell-to-cell transport in plants. BioScience 33: 260-265.
7. Van Steveninck, R.F.M. 1976. Cytochemical evidence for ion transport through plasmodesmata. In: Intercellular Communication in Plants: Studies on Plasmodesmata. B.E.S. Gunning and A.W. Robards, eds., pp. 132-147. Springer-Verlag, Berlin.

8. Hertzberg, E.L., Lawrence, T.S., and Gilula, N.B. 1981. Gap junctional communication. Annu. Rev. Physiol. 43: 479-491.

9. Wade, M.H., Trosko, J.E., and Schindler, M. 1986. A fluorescence photobleaching assay of gap junction-mediated communication between human cells. Science 232: 525-528.

10. Ho, S.-C., Malek-Hedayat, S., Wang, J.L., and Schindler, M. 1986. Endogenous lectins from cultured soybean cells: isolation of a protein immunologically cross-reactive with seed soybean agglutinin and analysis of its role in binding of *Rhizobium japonicum*. J. Cell Biol. 103: 1043-1054.

11. Meiners, S., Xu, A., and Schindler, M. 1991. Gap junction protein homologue from *Arabidopsis thaliana*: evidence for connexins in the plant family. Proc. Natl. Acad. Sci. USA, in press.

12. Xu, A., Meiners, S., and Schindler, M. 1990. Investigations of immunological relatedness between plant and animal connexin. In: Parallels in Cell-to-Cell Junctions in Plants and Animals. A.W. Robards, H. Jongsma, W.J. Lucas, J. Pitts, and D. Spray, eds., pp. 171-183. Springer-Verlag, Heidelberg.

13. Baron-Epel, O., Hernandez, D., Jiang, L.-W., Meiners, S., and Schindler, M. 1988. Dynamic continuity of cytoplasmic and membrane compartments between plant cells. J. Cell Biol. 106: 715-721.

14. McCredy, D.W., Harmon, A.C., Pruett, P.L., Fecheimer, M., and Wampler, J.E. 1986. Measurement of subcellular pH in single plant cells (Abstr.). Plant Physiol. 80: 484.

15. Schindler, M., Meiners, S., and Cheresh, D.A. 1989. RGD-

dependent linkage between plant cell wall and plasma membrane: consequences for growth. J. Cell Biol. 108: 1955-1966.

16. Warner, A.E., Guthrie, S.C., and Gilula, N.B. 1984. Antibodies to gap junctional protein selectively disrupt junctional communication in the early amphibian embryo. Nature 311: 127-131.

17. Fraser, S.E., Green, C.R., Bode, H.R., and Gilula, N.B. 1987. Selective disruption of gap junctional communication interferes with a patterning process in *Hydra*. Nature 237: 49-55.

18. Jiang, L.-W., and Schindler, M. 1988. Nuclear transport in 3T3 fibroblasts: effects of growth factors, transformation, and shape. J. Cell Biol. 106: 13-19.

19. Admon, A., Jacoby, B., and Goldschmidt, G. 1980. Assessment of cytoplasmic contaminations in isolated vacuole preparations. Plant Physiol. 65: 85-87.

20. Schindler, M., Trosko, J.E., and Wade, M.H. 1987. Cellular regulators: calcium and lipids. Methods Enzymol. 144: 439-447.

21. Peters, R. 1983. Nuclear envelope permeability measured by fluorescence microphotolysis of single liver cell nuclei. J. Biol. Chem. 258: 11427-11429.

22. Gunning, B.E.S. 1976. Introduction to plasmodesmata. In: Intercellular Communication in Plants: Studies on Plasmodesmata. B.E.S. Gunning and A.W. Robards, eds., pp. 1-13. Springer-Verlag, Berlin.

23. Robards, A.W. 1976. Plasmodesmata in higher plants. In: Intercellular Communication in Plants: Studies on

Plasmodesmata. B.E.S. Gunning and A.W. Robards, eds., pp. 15-57. Springer-Verlag, Berlin.

24. Terry, B.B., and Robards, A.W. 1987. Hydrodynamic radius alone governs the mobility of molecules through plasmodesmata. Planta 171: 145-157.

25. Milks, L.C., Kumar, N.B., Houghten, R., Unwin, N., and Gilula, N.B. 1988. Topology of the 32 kD liver gap junction protein determined by site-directed antibody localization. EMBO (eur. Molec. Biol. Org.) J. 7: 2967-2975.

26. Lee, S., Gilula, N.B., and Warner, A.E. 1987. Gap junctional communication and compaction during preimplantation stages of mouse development. Cell 51: 851-860.

27. Davies, E., and Schuster, A. 1981. Intercellular communication in plants: evidence for a rapidly generated, bidirectionally transmitted wound signal. Proc. Natl. Acad. Sci. USA 78: 2422-2426.

CLOSING STATEMENT

The work described in this dissertation attempted to characterize a 29 kDa protein found in soybean root cells and the organs of diverse plants which is immunologically and biochemically similar to connexin 32 of rat liver gap junction. In addition, attempts were made to study the putative role of the plant protein (operationally termed plant connexin) as a structural element of plasmodesmata, the plant cell-cell communication structures which are the functional analogues of gap junctions in animal cells. The results of these studies may be summarized as follows:

(a) Antibodies raised against the M_r 27,000 polypeptide of rat liver connexin 32 cross-reacted with a M_r 29,000 polypeptide in soybean root cells; daisy, rose, and petunia petals; cucumber and tomato fruits; and lettuce and *Arabidopsis thaliana* leaves. Antibodies raised against the M_r 29,000 polypeptide of soybean connexin cross-reacted with the M_r 27,000 polypeptide of rat liver connexin 32.

(b) The rat liver and soybean connexins demonstrated similar isoelectric points (9.4 versus 9.8) and the ability to form high molecular weight species (suggested to be dimers and trimers) on reducing SDS-PAGE gels.

(c) The rat liver and soybean connexins yielded related patterns of cleavage and aggregation when treated with V-8 protease or CNBr, suggesting that the proteins are similar but not identical.

(d) Immunofluorescence studies utilizing antibodies raised against the 27 kDa polypeptide of rat liver connexin 32 and the 29 kDa polypeptide of soybean connexin demonstrated fluorescent spots on the plasma membrane and cell wall of soybean in a pattern reminiscent of plasmodesmatal pitfields.

(e) A cDNA clone isolated from an *Arabidopsis thaliana* expression library utilizing three different connexin antibodies as probes coded for a protein which demonstrated 27% identity and 40% similarity with the amino acid sequence of rat liver connexin 32. The *Arabidopsis* and rat liver proteins shared similar hydropathy profiles and predicted secondary structure. The antibodies used to isolate the *Arabidopsis* clone were raised against: (1) the 27 kDa polypeptide of rat liver connexin 32; (2) the 29 kDa polypeptide of soybean connexin; and (3) a synthetic peptide corresponding to amino acid sequence 98-124 of rat liver connexin 32.

(f) Soybean cells incubated with antibody raised against the synthetic peptide corresponding to amino acid sequence 98-124 of rat liver connexin 32 showed a reduction in the rate of cell-to-cell dye transfer of FDA as compared to controls. This presumably resulted from the binding of the antibody to a plasmodesmatal protein, thereby altering channel accessibility or structure. The antibody used in this study was demonstrated to cross-react with the 29 kDa polypeptide of soybean connexin; therfore, soybean connexin is suggested to have a plasmodesmatal orientation and to be involved in constructing the plasmodesmatal channel(s).