Plant cell biology research in the Chrispeels Laboratory: 1967-2005.

1967-1973. The biosynthesis and secretion of hydroxyproline-rich cell wall glycoproteins (HRGPs or extensins). In the early1960s plant cell walls were discovered to contain hydroxyproline-rich proteins and suspension cultured cells were found to be especially rich in these proteins. The Hyp residues are glycosylated with short Ara sidechains. We found that the biosynthesis of peptidylhydroxyproline involves a post-translational modification carried out by peptidyl prolyl hydroxylase, an enzyme that requires iron and ascorbate for full in vitro activity. The Golgi Apparatus plays an important role in the glycosylation of extensins, and UDP-arabinose arabinosyl transferase is located in the Golgi apparatus. The synthesis of extensins, in spite of the name given is not associated with cell growth but occurs after cell growth stops. HRGP synthesis is also activated when cells are wounded and this accounts for the high level of HRGP in the walls of suspension-culture cells. When these cells are made to differentiate, HRGP synthesis is turned down again.

1974-1980. The mobilization of storage proteins in the cotyledons of young seedlings. When dicot seedlings grow they mobilize the storage proteins contained in their protein bodies or protein storage vacuoles (PSV). We found that mobilization of storage proteins in mungbean cotledons is dependent on the de novo synthesis of an endopeptidase that is transported into the PSVs. During or after its synthesis the enzyme first accumulates in cytoplasmic "foci" (vesicles of either ER or Golgi) before arriving in the PSVs. PSVs function as autophagic organelles internalizing cytoplasmic components for subsequent digestion. To carry out this autophagic function the PSVs need to acquire new hydrolytic enzymes. Germination is also accompanied by a massive reorganization (breakdown and re-synthesis) of the ER. At the start of germinatin the ER is largely tubular, but within a few days a new cisternal ER is formed. Cotyledons contain protease inhibitors that disappear during germination, but these do not inhibit the proteases that are synthesized. Antibodies to the endopeptidase allowed us to confirm that mungbean belongs in the genus *Vigna* and not in the genus *Phaseolus*.

1980-1990. The biosynthesis and deposition of storage proteins in developing seeds. Role of the ER in glycosylation. Germination is accompanied by the synthesis and deposition of some new proteins in the PSVs, but the bulk of protein transport into PSVs occurs during seed formation (embryogenesis). To get a better handle on protein transport into vacuoles and the biosynthetic and post-translational events involved in the synthesis of vacuolar proteins, we therefore switched to this stage of plant development. The major proteins (vicilin, legumin, lectins) in PSVs are synthesized on the rough ER and transported via the Golgi to the PSVs. This was shown by combining pulse-chase experiments with isopycninc sucrose gradients to separate the various organelles. Attachement of high mannose glycans occurs in the ER and these glycans are modified in the Golgi. Only specific Asn residues are glycosylated and in the mature proteins these Asn residues carry either high-mannose or complex glycans. We identified for both vicilin and phytohemagglutinin (from *Phaseolus vulgaris*) the structure of the glycans attached to specific Asn residues. The enzymes that modify high mannose glycans act sequentially. A mutant Arabidopsis thaliana plant that cannot make complex glycans

because it lacks GlcNAc transferase has no visible phenotype indicating that these glycans are not essential. Glycans have no vacuolar targeting information (unlike mammalian lysosomal proteins). Proteolytic processing of vicilin and legumin occurs in the vacuoles and yields the final polypeptide pattern for these proteins found in the mature seeds. Transport to the vacuole occurs in vesicles that lack a clathrin coat. Research on the ER was continued much later when we investigated the nature of the unfolded protein response in plants, using an expression profiling approach.

1986-2004. Analysis of the phytohemagglutinin-amylase inhibitor-arcelin gene family of the common bean *Phaseolus vulgaris*. Phytohemagglutinin (PHA), which makes up about 5 % of total bean seed protein was found to be a convenient protein for the study of glycoprotein synthesis and transport, because it can be purified in one step with a porcine thyroglobulin affinity column. A derived amino acid sequence of PHA was reported in the literature, but we decided that it had to be wrong because it contained several Met residues whereas PHA has none. We cloned both PHA-E and PHA-L. Our work on α-amylase inhibitor (AI) led to the cloning of its gene and the finding that it corresponds to the misidentified PHA gene. Cloning of the arcelin genes (arcelins are storage/defense proteins found in wild accessions of *P. vulgaris*) revealed strong sequence identity between the PHAs, AI and arcelins. Remarkably, these proteins have different biochemical/biological functions. The crystal structure of PHA shows it to be composed of two legume lectin canonical dimers that pack together differently from concanavalin A. This work also led to the cloning of several insect amylases and a study of their interaction with different AIs.

1985-1995. Vacuolar protein targeting. The availability of the PHA genes and the ease of isolating newly synthesized (metabolically labeled) PHA with an affinity resin made it possible to study synthesis and vacuolar targeting of this glycoprotein in heterologous systems. First we showed that when expressed in tobacco, PHA still goes to the vacuole. Targeting to the vacuole requires specific information, whereas secretion appears to be the default pathway. However, when expressed in animal (COS) cells, PHA is secreted. In yeast on the other hand full length PHA is targeted to the yeast vacuole. We were unable to dissect the polypeptide chain and identify the targeting domain of PHA by deleting certain domains or fusing them with other proteins either in yeast or in plants. KDEL works as an ER retention domain, but retention is incomplete. A protein can also be delivered to the vacuole if it has a membrane anchor.

1990-2000. Genetically engineered insect resistant seeds. The availability of the AI cDNA led us to a side-trip into biotechnology. AI-1 inhibits the digestive amylases of mammals and certain seed-eating insects. We discovered in wild accessions of *P. vulgaris* a second AI-2 with slightly different properties (inhibits different insects). Transformation of pea plants with the bean AI cDNA and a seed specific promoter showed that the pea seeds were now completely resistant against the pea weevil (*Bruchus pisorum*). Transformation of azuki beans and chickpeas yielded similar results. Feeding the transformed peas to rats at 30 % of the diet has minimal nutritional effects on the rats. (This work was carried forward by my collaborator TJV Higgins at the CSIRO in Canberra).

1990-1995. Cloning of invertase and use of invertase to modify plant growth. Sucrose hydrolyzing enzymes (invertase or β-fructosidase) are found in the vacuole, the cell wall and the cytoplasm. Cultured carrot cells contain high levels of cell wall invertase a considerable portion of which can be solubilized with high salt. This invertase was purified and antibodies against it combined with expression cloning allowed us to obtain the first plant invertase clone. Overexpression of invertase in the cell wall inhibits plant development probably because it interferes with sucrose unloading. The system was used to demonstrate that glycans are essential for glycoprotein stability. Inhibition of glycan synthesis does not inhibit invertase polypeptide synthesis, rather the polypeptide was synthesized in the presence of tunicamycin and then degraded. This project yielded a valuable reagent. An antiserum to invertase was found to contain antibodies only to the complex glycan and not to the polypeptide. This became a valuable reagent to identify proteins that have complex glycans.

1999-2003. Analysis of the mannose-binding lectin FRIL. A collaboration with Jeff Moore, then working at Imclone, led to the cloning and characterization of a mannose-binding from the common bean and the hyacinth bean. This lectin, which we called FRIL (FLT2 receptor interacting lectin), is a standard legume lectin and also has homology to the PHA family. This discovery was patented by UCSD with Imclone. FRIL acts as a chemoprotectant when injected into mice before chemotherapy. This project was carried forward by Jeff Moore who founded Phylogix LLC to develop FRIL as a pharmaceutical.

1992-2006. Aquaporins and their role in the plant. How does water go through the membranes of plant cells? Diffusion through the lipid bilayer was always thought to be the main path. In 1990 we obtained the cDNA for a major abundant tonoplast protein of 27 kD (we called it TIP). Not knowing what its function might be we presumed it to be a solute transporter. After expressing the protein in Xenopus oocytes and having been advised by Peter Agre to do a “swelling experiment”, a TIP obtained from Artabidopsis turned out to be a water channel or aquaporin. Aquaporins permit the rapid diffusion of water through biological membranes. They are present in the tonoplast (vacuolar membrane) and the plasma membrane. Phosphorylation may regulate the activity of some aquaporins. They constitute a gene family of some 35 members divided into 4 clades with different location or functions. Some aquaporins transport glycerol. In the plant they are responsible for cell-to-cell water transport and their expression responds to a number of signals, including hormonal and environmental. Aquaporins play a role in the recovery from dehydration and the repair of winter embolisms.

2000-2004. G-Protein coupled receptors. Collaboration with G. Colucci at Arena Pharmaceuticals results in a project to examine the function of the only GPCR in plants (GCR1). The expression of GCR1 is cell-cycle regulated and its action intersect with the action of abscisic acid. GCR1 regulates DNA synthesis through the activation of phosphatidyl inositol specific phospolipase C. Research on G-Protein coupled receptors is being continued at Arterra Bioscience srl in Naples, Italy under the leadership of G. Colucci.