ASPB Pioneer Member

Patricia Zambryski

My research career, accompanied by many amazing mentors, colleagues, students, and postdocs, spans over 50 years of investigating diverse topics in the biological sciences. I began as an undergrad at McGill University in the lab of a renowned fungal geneticist, Etta Käfer, searching for mutants of Aspergillus nidulans. Working in the lab was fun and motivating, prompting my application to graduate schools in Canada and the USA. Patrick O'Farrell and I married in the summer of 1969 and headed off to Boulder, Colorado to pursue doctorates while skiing in the Rocky Mountains. I was fortunate to be Larry Gold's first graduate student, and I learned much conceptually and technically while figuring out novel aspects of bacteriophage T4 gene expression during infection of Escherichia coli. We worked side-by-side while taking "time-points" every 15 seconds to catch when specific proteins first appeared in the short (30 minute) T4 life cycle [Bacterial cells were labeled with 14C-amino acids. As T4 shuts down host protein synthesis, T4 specific proteins were detected by autoradiography following gel electrophoresis.]. I received my Ph. D. in the summer of 1974. During the 1970s, there was much interest in tumor viruses to possibly provide insights and cures for cancer. So, for the next seven years I went off to UCSF for a postdoc in Howard Goodman's lab. Patrick O'Farrell had developed a technique for high resolution of proteins by 2D electrophoresis during his Ph. D.; at UCSF, we collaborated to increase the resolution of this method. My goal was to use this method to detect tumor virus-specific proteins, and to monitor alterations in cellular protein abundance during viral infection or in tumor cell lines. The magnitude of the changes was dumfounding without providing clues about which altered proteins might be most critical. In the late 1970s, hundreds of labs were working on animal cell viruses and tumors; I figured I needed to take a different approach to study tumor biology.

At this juncture, Marc Van Montagu invited me to the University of Gent (Belgium) to study plant tumors called crown galls, which are caused by the soil bacterium Agrobacterium tumefaciens. Again, I compared protein patterns between tumor and non-tumor cells. Again, nature bewildered us; no candidate tumor specific protein(s) were revealed. This was puzzling, as it was known that Agrobacterium transferred a particular segment of DNA from its tumor inducing (Ti) plasmid, the transferred DNA or T-DNA, into plant cell DNA. Obviously, T-DNA was expected to encode proteins, but a few years later it was revealed that T-DNA encodes very low levels of novel enzymes resulting in overproduction of plant growth hormones that cause the "tumor" phenotype.

Nevertheless, I was hooked on this relatively unknown bacterial-plant interaction system. I left Gent carrying genomic DNA extracted from independent culture lines of crown gall tumors. I was going home to UCSF to clone the T-DNA element from tumor DNA, and Howard Goodman's lab was just the place to do this as his lab had superb cloning expertise and had just "cloned" DNA and cDNAs for human insulin and growth hormone. But first a short interlude.

Back at UCSF, Howard asked me to help on another project involving 2D electrophoresis, this time of RNA. He had just cloned a yeast-specific tyrosine tRNA gene that contained an unexpected short sequence of 14 nucleotides in the gene versus the mature tRNA. Howard wanted to know if this extra DNA, now known as an "intron", was transcribed and then processed into the mature tRNA. At the time (1978), introns were just making their debut. Proving the tRNA intron was transcribed involved methods that

would be considered archaic today. [Liquid yeast cultures were labeled with 32P (1 mCurie/ml!), spun down, and incubated with mild denaturing agents that did not lyse the cells. A precipitate of the resulting supernatant (containing small RNAs) was separated by electrophoresis in two dimensions to produced radioactive "spots" of RNA that were digested and separated by high voltage paper electrophoresis to determine their sequences.] Indeed, yeast produced a precursor tRNA with an additional 14 nucleotides in the anticodon loop, and this precursor was processed to the mature tRNA by addition of a yeast cell extract. This was an exciting project, and my last publication under the name Patricia O'Farrell. Henceforth I would use my maiden name, Zambryski.

The native Agrobacterium T-DNA is a 20 kb element within the large 200 kb Ti plasmid. I set out to discover what sequences determined the "ends" of the T-DNA. Fragments of tumor DNA were cloned into lambda vectors and probed with radioactive DNA fragments near the limits of the T-DNA region of the Ti plasmid. Sequence comparisons between clones of Ti plasmid and tumor DNAs revealed the ends of the T-DNA (from independent tumor lines) occurred within identical short direct repeats of 25 bp. We had found the T-DNA borders! As Howard Goodman was moving his lab to Boston, I made the logical decision to continue research on Agrobacterium at the University of Gent, and I stayed in Gent from 1981-1986. I first asked whether the "internal" T-DNA was necessary for DNA transfer to plant cells. I designed a large deletion of the Ti plasmid carrying a few kb overlapping the left and right T-DNA borders, without the internal (20kb) T-DNA sequences. Indeed, this truncated "T-DNA" was transferred and stably integrated into plant DNA! Concomitant research in Gent, and many other labs, tested if one could insert DNA into the T-DNA region on the Ti plasmid for Agrobacterium to transfer to plants. In the early 1980s there were no cloned plant DNAs, so bacterial antibiotic resistance genes under the control of plant-specific promoters were used to test whether "foreign" DNA could be transferred to plant cells. Moreover, since Agrobacterium T-DNA genes are expressed in plant cells, Agrobacterium provided a source of plant promoters for these early studies. Plant genetic engineering had begun! Many researchers developed small T-DNA "vectors". Bacterial plasmids were easily manipulated in E.coli to contain T-DNA borders, selectable marker genes, and "genes of interest" (GOI); these small plasmids were then simply transformed into Agrobacterium enabling transfer of any GOI to plant cells. This was a very exciting period for plant research.

Next, I wanted to know "how" T-DNA transfer occurred. In 1984, Scott Stachel visited the lab in Gent. We had worked together in Howard's lab at UCSF before Howard left for Boston. When I moved to Gent, Scott continued his Agrobacterium research in Seattle in Gene Nester's lab, and he studied another region of the Agrobacterium Ti plasmid. While the T-DNA is a "transferable" element, it does not encode functions for its transfer; instead, the ~ 30 kb virulence (vir) region with 7 polycistronic regions (A,B,C,D,E,F,G) encodes ~ 25 proteins essential for T-DNA transfer. Scott discovered that the vir region was not expressed in vegetatively growing Agrobacterium; instead, vir gene expression was induced in liquid cultures containing plant cells. Soon after Scott's arrival in Gent, we found plant cells were not essential for vir gene expression. Unexpectedly, the medium in which plant cells were grown induced vir gene expression, implying the vir inducing factor was soluble. We grew many liters of plant cell culture and concentrated the conditioned liquid medium. We collaborated with an excellent chemist in the Gent lab, Eric Messens, to identify the vir inducing factor as the small phenolic molecule, acetosyringone (AS) (MW 196). Turns out, AS is very abundant in plant cell wall extracts and is commercially available.

AS allowed efficient/easy induction of vir gene expression, opening the door to investigate T-DNA transfer in detail. Scott Stachel and I showed vir proteins, VirA and VirG, acted as two component regulators to allow transcription of vir genes; in fact, the Agrobacterium specific VirA-VirG system was one of the first two component signaling systems identified in bacteria! [VirA acts as a sensor for the plant phenolic, AS, and VirG acts as the vir gene specific transcriptional activator.] We also used vir gene expression to answer

the question, "what is the transferable T-DNA copy"? Surprisingly, a single stranded (SS) copy of the T-DNA region, dubbed the T-strand, was induced. Genetic analyses revealed vir products, VirD1 and VirD2, created SS breaks in the lower strand of the 25 bp T-DNA border repeats; displacement of the lower strand of the T-DNA produced the T-strand.

These discoveries as a student and postdoctoral scholar led to 30 primary research papers, and I was hired with tenure by the Division of Molecular Plant Biology at UC Berkeley in July 1986. As a "plant" faculty member, I decided I should start some plant research. I chose two topics not widely investigated at the molecular level in plants, cell-to-cell transport via plasmodesmata (PD), and genes essential for flower development in Arabidopsis.

First, I summarize a bit of our early work on Agrobacterium at UC Berkeley. We wondered how a vulnerable SS DNA, the T-strand, might travel from Agrobacterium to the plant cell nucleus without degradation, and how might the T-strand be targeted to the plant cell nucleus? Two vir proteins, VirD2 and VirE2, provided these functions by associating with the T-strand; a) VirD2 is covalently bound to the 5' end of the T-strand after cleavage at the right 25 bp T-DNA border, and b) VirE2 acts as a non-sequence specific SS binding protein implying it likely binds along the length of the T-strand to prevent T-strand degradation. Remarkably, both proteins carry bona fide nuclear localization signal sequences (NLS) to target the T-strand-VirD2-VirE2 complex to the plant nucleus. In the 1990s researchers (outside the plant field) imagined that plant cells must do things differently than animal cells. However, we demonstrated that functional plant NLSs were identical to animal NLSs; unexpectedly, bacterial proteins, Agrobacterium VirD2/VirE2 provided this insight into plant nuclear trafficking.

Getting back to how we began to investigate plant cell-to-cell movement via PD. As plant viruses pirate PD during cell-to-cell movement, we decided to study the model plant virus, tobacco mosaic virus (TMV) and its movement protein (MP). At the time, it was known that plant viruses move their SS genomes cell to cell via PD and that MP was essential for such movement. We speculated that TMV MP might bind to the TMV RNA genome during transit through PD (by analogy to VirE2 binding to the SS T-strand). Indeed, TMV MP acts like a non-sequence specific SS binding protein! It was exciting to provide a (previously unknown) fundamental insight into plant virology, thanks to "cross-feeding" of ideas from our research on Agrobacterium SS DNA movement! Many plant virology labs use viral MPs to probe PD function. Rather than compete with excellent plant virology labs, we studied PD during different stages of plant development (see below).

In the late 1980s, genes essential for the regulation of flower development were discovered using the exceptionally simple genetically tractable plant, Arabidopsis thaliana. The so-called A,B,C floral regulatory genes (and many others) were identified by mutant genes that altered floral development. My lab identified novel floral genes by searching for mutants with phenotypes distinct from those displayed by A,B,C mutants. Remarkably, Agrobacterium again played a critical role in these discoveries. Ken Feldmann (working at Dupont with abundant greenhouse facilities) created an amazingly useful library of thousands of T-DNA insert lines (carrying only an antibiotic resistance gene) displaying a wide variety of mutant phenotypes in essential plant pathways. These T-DNA tagged lines enabled rapid identification/cloning of mutant loci (by homology to T-DNA borders or antibiotic resistance gene probes), and consequently their respective wild type genes. Ken Feldmann generously provided my lab with mutant lines with interesting abnormal floral phenotypes that we named tousled, ettin, and petaless (petaless turned out to be an allele of unusual floral organs (ufo)). TOUSLED encodes a nuclear serine/threonine protein kinase essential for apical gynoecium development, ETTIN encodes an auxin response factor critical for auxin signaling that dramatically affects apical basal patterning in the gynoecium, and UFO encodes an F-box protein critical to regulating antagonistic pathways in petal development. Finally, we characterized a mutant called seuss (seu) that modified the ettin phenotype. SEU encodes a DNA

binding transcriptional cofactor required at different times during plant development. After 15 years of research to identify and characterize genes essential for Arabidopsis floral development, we refocused our attention to the role of PD in development.

We began by studying PD in leaves. In the early 90s, PD were believed to be nearly closed, with small size exclusion limits (SEL) (<1 kDa) allowing transport of plant hormones and micronutrients. SELs were observed following microinjection of different sized fluorescent dextran tracers. Interestingly, larger tracers (up to 20 kDa) could move cell-to-cell if co-injected with viral MPs, presumably because MPs "gate" PD during viral spread. Turns out, microinjection itself is traumatic to cells and causes PD closure. As microinjection of single plant cells was technically challenging, researchers turned to "biolistic bombardment" of plant leaves to introduce fluorescent tracers; however, the results were inconsistent between different labs, presumably due to variable high pressures used to introduce probes. PD research gained a remarkable boost with the availability of GFP, a genetically encoded fluorescent probe. We created GFP probes containing different cellular localization sequences and introduced DNA to express these probes using a "home-made" extremely low-pressure biolistic bombardment system. We demonstrated that native GFP moved extensively cell to cell unless it was fused to a subcellular address such as an NLS or an endoplasmic reticulum targeting sequence. Interestingly, the extent of GFP movement was dependent on leaf age, with younger leaves exhibiting more movement than older leaves. Our results complemented studies in other labs showing plant transcription factors and gene silencing signals moved intercellularly. Thus, macromolecular traffic via PD provided plants with "supracellular" means to regulate their growth and development and a rich area of research.

We also studied PD transport during the transition from vegetative to floral development. Remarkably, meristems stop PD transport from young leaves to the meristem for a short window of time, just when inflorescence meristems begin to appear. PD may restrict movement of signals (from leaves below the meristem) to facilitate the dramatic shift in organogenesis from leaves to flowers. To my knowledge there have been no follow-up studies on these intriguing results, potentially because they are technically challenging to perform.

We performed extensive studies on PD-mediated movement of GFP tracers during Arabidopsis embryogenesis (see also below). Single sized (27 kDa) GFP tracer moves through all cells of embryonic roots and leaves. In later stages, double sized (54 kDa) GPF tracers become restricted to cells of the hypocotyl and embryonic roots, while triple sized (81 kDa) GFP is restricted to meristematic regions. Thus, there are domains of cells with different PD apertures in different regions of the developing embryo.

Our next Agrobacterium project characterized the membrane spanning channel for DNA and protein transport to plant cells, the type IV secretion system (T4SS), that is constructed from eleven proteins (VirB1-B11) encoded by the VirB operon. We created a library of T4SS peptides and tested them for peptide interactions (in yeast) to predict the topology of VirB proteins residing in the inner membrane, periplasm, or outer membrane. T4SS assembly and function is studied by numerous labs around the globe, as T4S is highly conserved in bacteria that transfer proteins, DNA, and toxins from pathogenic bacteria that cause disease in plant and animal cells.

Our major contribution to understanding T4SS function derived from demonstrating that the T4SS localizes to multiple (15-20) evenly spaced distinct foci around the entire circumference of vir induced cells. Numerous T4SS provide Agrobacteria with multiple sites along its length for stable attachment to plant cells. Our results depended on 1) very high-resolution fluorescent microscopes housed in our department, and 2) expression of VirB protein-GFP fusion proteins at very low levels. Previous work suggested that T4SS formed a single focus at the bacterial cell pole; however, these studies used lower resolution microscopy and

fluorescent fusion proteins expressed at high levels. [Notably, bacteria sequester overexpressed proteins at their poles as non-functional aggregates.]

Getting back to PD: Perhaps our most unique and significant contribution to PD research was the design of a genetic screen for mutants with alterations in plant cell-to-cell movement. Since PD are essential, we reasoned that mutants in genes essential for PD function would be dead, and seeds carrying such mutants would not germinate into plants that could be screened for changes in PD transport. Instead, we screened thousands of heterozygous lines of embryo lethal mutants of Arabidopsis; such lines carry wild type and mutant embryos in their seed pods. Our screen was both difficult and time consuming and involved carefully opening seedpods on microscope slides containing fluorescent tracers, followed by fluorescent microscopy to detect mutant embryos with increased or decreased transport of tracer compared to sibling wild type embryos. We identified several lines, dubbed increased exclusion limit (ise) or decreased size exclusion limit (dse). To date we have characterized ise1, ise2, ise3, ise4, and dse1. We originally (perhaps naïvely) hoped our mutants would identify PD localized proteins essential for PD regulation/function. However, instead all the mutations resided in essential plant genes critical to plant cell homeostasis; this makes perfect sense in hindsight. Notably and importantly, we could induce acute loss of function of each gene by viral induced gene silencing in mature plants, and thereby recapitulate increased (or decreased) PD mediated movement of fluorescent tracers! To give a flavor of the essential pathways identified, ise1 and ise2 disrupt chloroplast biogenesis and trigger a retrograde signaling pathway that disrupts PD transport, and ise3 and ise4 disrupt the TARGET OF RAPAMYCIN (TOR) core signaling network that also impacts PD transport. Thus, a challenging genetic screen for altered PD function revealed unexpected insights into how plant cells transport micro- and macromolecules to neighboring cells to carefully regulate overall plant growth in response to the environment.

One final story: Agrobacterium is now a model system for polar growth in bacteria. That the Agrobacterium vir specific T4SS localizes circumferentially to multiple discrete foci around the bacterial perimeter made us wonder how so many T4SSs might be inserted into the bacterial cell wall in the context of the cell cycle. To monitor the cell cycle we made GFP fusions to classic bacterial cell division specific proteins, FtsA and FtsZ, that localize to the mid-cell in model bacteria such as E. coli. Surprisingly, time lapse fluorescence microscopy of GFP fusions to Agrobacterium-specific orthologs of FtsA and FtsZ revealed localization to a single pole during the growth phase of the cell cycle, and briefly to the mid-cell during cell division. The growing end of the cell is called the growth pole (GP), and the non-growing pole is the old pole (OP). Interestingly, and unexpectedly, new GPs form at the site of cell division; this places FtsA and FtsZ at the new GPs in elongating cells. Additional studies identified GP- and OP-specific localized proteins. One novel GP protein, which we named GROWTH POLE RING (GPR) protein, is notable for forming a striking hexameric 200 nm ring structure at the GP that is essential for polar growth and the rod shape of Agrobacteria; deletion of GPR results in round cells and dramatically reduced cell growth. GPR is a large (2115 amino acid) protein with a transmembrane domain to anchor GPR in the bacterial inner membrane. Each monomer of GPR consists of 12 overlapping apo-lipoprotein domains, and the GFP ring structure may act as an organizing center for protein, lipid, and peptidoglycan synthesis during polar growth. We hope other labs will continue the quest to determine how GPR functions. So much remains to be uncovered, as Agrobacterium is truly a powerhouse of fundamental insights into numerous essential bacterial processes.

The ASPB has honored me as a plant scientist. Here, I also highlight my graduate and postdoctoral research as a microbiologist. My lack of formal training in plant science was not an obstacle to creative research in plant biology. Curiosity in new areas of biology was the driving force of my research career. I performed extensive “bench” work as a young scientist, but as a professor I deeply acknowledge

many students and postdocs who were/are creative thinkers and excellent experimentalists. They too had an abundance of curiosity that led to unexpected avenues of research and fundamental insights. I especially thank John Zupan, my lab manager and senior research colleague for over 30 years of stellar scientific investigations, for help making the lab a collegial and creative work environment.

The following persons (listed alphabetically) contributed significantly to the three areas of research performed in my lab in the Department of Plant Biology at UC Berkeley since 1986: (1) Agrobacterium: Julieta Aguilar, James Anderson-Furgeson, Sue Bailey, Christian Baron, Todd Cameron, Vitaly Citovsky, Guido de Vos, Michaeleen Doucleff, Olga Draper, Romain Grangeon, Liz Greene, Zisheng Guo, Cheryl Hackworth, Liz Howard, Gretchen Kuldau, Matxalen Llosa, Gail McLean, Rebecca Middleton, Theodore Muth, Sebastian Robalino-Espinosa, Doyle Ward, Barbara Winsor, John Zupan. (2) Plasmodesmata: Jake Brunkard, Tessa Burch-Smith, Euna Cho, Yuval Cohen, Katrina Crawford, Andreas Gisel, Howard Goodman, Fred Hempel, Insoon Kim, Ken Kobayashi, Michael Mindrinos, Anne Runkel, Solomon Stonebloom, Elisabeth Waigmann, Min Xu. (3) Flower development in Arabidopsis: Tim Durfee, Kyle Serikawa, Jennifer Nemhauser, Jennifer Pfluger, Judith Roe, Allen Sessions.

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