A PHENOTYPIC SURVEY OF THE *ARABIDOPSIS THALIANA* CYTOKINESIS DEFECTIVE MUTANT *CYT1*

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LIST OF ABBREVIATIONS

AAtDB	An *Arabidopsis thaliana* database (first generation)
ACeDB	A *Caenorhabditis elegans* database
AGP	arabinogalactan proteins (specialized cell wall protein)
ASP	Active Server Pages™, a protocol to render dynamic web pages
AcOH	acetic acid
AtDB	*Arabidopsis thaliana* database (current)
BAP	benzyladenine phosphate, a cytokinin
BSA	bovine serum albumen
cDNA	complementary DNA
cM	centiMorgan, a genetic unit based on recombination frequency
DAPI	2,4-diamino-2-phenylindole (stain for nuclei)
DCB	2,4 dichlorobenzoic acid, an inhibitor of cellulose synthase
DNA	deoxyribonucleic acid
DS	division site
ECM	extracellular matrix
EM	electron microscopy
EMS	ethyl methanesulfonate, an alkylating mutagen
EST	expressed sequence tags (random sequence of expressed RNA)

EtOH	ethanol
F_1	first progeny generation
F_2	second progeny generation
FAA	formalin, acetic acid, alcohol; a fixative
ftp	file transfer protocol, a protocol to transfer files
GB	gigabytes
GC-MS	gas chromatography / mass spectrometry
h	hours
HRGP	hydroxyproline rich glycoproteins
HTML	hypertext markup language, a computer coding protocol
IAA	indole-3-acetic acid, an auxin phytohormone
IIS	Internet Information Server™, a Microsoft computer program
kb	kilobase
LEA	late embryogenesis abundant (classification of proteins)
M_1	first mutagenized generation
M_2	second generation after mutagenesis
MB	megabytes
mRNA	messenger RNA
MSU	Michigan State University
NaOAc	sodium acetate
NASA	National Aeronautics and Space Administration
NP	NANOPURE™ (water filtration system)
PAS	periodic acid-Schiff, a carbohydrate stain

PAW	phenol, acetic acid, water
PbCit	lead citrate, a stain used in electron microscopy
PCR	polymerase chain reaction
pg	picogram
PME	pectin methylesterase
PPB	preprophase band
RNA	ribonucleic acid
RO	reverse osmosis (water purification system)
RT	room temperature
RT-PCR	reverse transcription, followed by PCR
s	seconds
T-DNA	transfer DNA (from *Agrobacterium*)
TC	terminal complex
TTC	1,3,6 triphenyl tetrazolium chloride (stain for metabolic activity)
UA	uranyl acetate, a stain used in electron microscopy
UDP	uridine diphosphate
UV	ultraviolet
VBScript	a Microsoft programming language
WWW	World Wide Web
°C	degrees Celcius

CHAPTER 1: USE OF *ARABIDOPSIS* FOR THE STUDY OF DEVELOPMENT

INTRODUCTION

The study of development has intrigued mankind for centuries. The Greeks evaluated the causes and effects of natural processes using *ex-post-facto* studies and logic, sometimes making impressive observations and discoveries in the absence of sophisticated experimentation. In modern times, we have at our disposal an extensive assortment of biological tools and techniques. We are still hampered, though, by the problem of how best to apply our resources and use the ever-increasing body of knowledge we have accumulated to understand biological processes.

One paradigm of investigation that has been immensely helpful in forwarding biological science is the application of model systems. Instead of dispersing our resources and efforts among a large number of organisms for study, the use of a few systems amenable to intense scrutiny has allowed efficient progress in understanding biological concepts. Investment in the study of these model systems has become a common scientific practice, and application of knowledge gained from these systems towards species of economic interest has been profitable. Fundamental similarities between organisms as a result of

evolutionary heritage has been, and will be for some time to come, something we can exploit in our pursuit of scientific answers about nature.

A model system can be defined as one that fulfills several criteria: it must be applicable to other systems of interest, it must be amenable to experimentation, and it must respond consistently to a set of stimuli or biological or environmental variables. A model system is ideal if it has a life-cycle within a short but manageable time so that data can be collected quickly while at the same time giving the researcher a chance to organize and plan experiments. Also, the expense in terms of purchasing the organism, cost of maintenance, food, and living space are usually a consideration.

Model systems have proven their worth. *Drosophila melanogaster*, the fruit fly, is often the standard to which other model systems are compared. With a generation time of only a few days, living space that consists of a test tube for dozens of individuals, and established manipulation procedures, the fly is cheap and easy to maintain. The nematode *Caenorhabditis elegans* has also become a genetic success story because reliable fate maps have been designed for each cell in the developing organism and its cells are accessible to observation and manipulation because of its transparent body (Hodgkin *et al.*, 1995). Vertebrate studies have been dramatically advanced by studying *Danio rerio*, or zebrafish (Postlethwait and Talbot, 1997; Vascotto *et al.*, 1997). Bacterial model systems have been helpful; the first complete sequence of the genome of a free-living organism, *Haemophilus influenzae,* was published in 1995 (Fleischmann *et al.*, 1995). Recently published was the yeast genome (Goffeau *et al.*, 1996) and a strain of *E. coli* (Blattner *et al.*, 1997). This

information lets us investigate the milieu of interactions for all the genes involved in cellular processes.

The plant kingdom contains a large number of organisms. The incredible diversity in growth aspect, life cycle, and genomic complexity makes the application of model systems even more attractive. Though the genomes of plant species can contain over 100 pg of DNA per cell in the extreme case of *Fritillaria*, the number of expressed genes is probably between 20 000 to 100 000 regardless of the mass of genomic DNA (Lyndon, 1990). *Arabidopsis thaliana, Zea mays,* and *Antirrhinum majus* have become the most utilized systems for genetic studies and of these, *Arabidopsis* has the smallest genome with about 0.1 pg of DNA per cell. This feature has helped *Arabidopsis* become one of the most utilized tools for genetic research in plant development (Scheres and Wolkenfelt, 1998).

ARABIDOPSIS AS A MODEL SYSTEM

Arabidopsis thaliana (L.) Heynh. (Figure 1) is a modest member of the mustard or crucifer family (Brassicaceae or Cruciferae). This family is widely distributed, having approximately 340 genera and 3350 species which are mostly found in temperate climates of the northern hemisphere (Price *et al.*, 1994). Members of this family are concentrated in southwestern and central Asia and the Mediterranean, but secondarily in the arctic, western North America, and mountainous regions of South America (Price *et al.*, 1994). Unlike other Brassicaceae, *Arabidopsis* has no direct economic value. The family has members that are valuable as vegetable crops, oil seeds, spices, and a few as ornamentals. They are usually considered annual herbs and are characterized by their specialized capsular fruits, called siliques (Fig. 1).

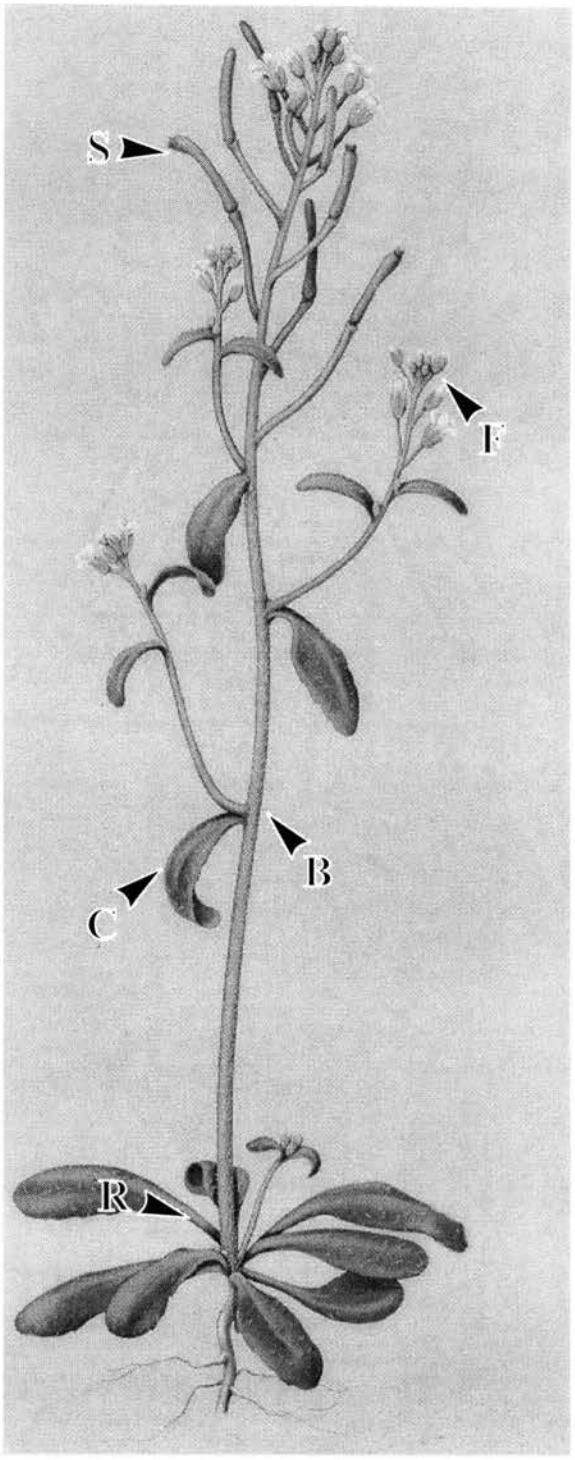


Figure 1: Mature *Arabidopsis thaliana* plant.
Rosette leaves (R) are found at the base of the plant. Bolts (B) extend aerially and bear siliques (S), flowers (F), and cauline leaves (C). (Adapted from Science 282: 663 (October 23, 1998) Genome Maps 9).

Features of *Arabidopsis*

The success of *Arabidopsis* as a model system is because it has a host of desirable features. *Arabidopsis* is a small plant with a short life cycle–a seed grows to a sexually mature stage within 3 weeks–so that a room of moderate size can hold a large number of mutant lines. The stem of *Arabidopsis* supports siliques that range in age from older (basal) to younger (apical). This linear arrangement is of benefit to the researcher studying stages of embryogeny. Early cell division patterns in embryos are predictable as are cell fates, making it possible to identify early mutational departures from normal patterns. Flowers of *Arabidopsis* are complete; they possess both anthers and fused gynoecia. Flowers typically self-pollinate, making stock maintenance easier and eliminating the need to perform every mating required for a genetic experiment. Directed crosses can be performed in a 5 minute procedure where a young flower bud is opened prior to anthesis and emasculated, then rubbed with a detached dehiscent anther from the male parent.

The genome of *Arabidopsis* is also well-suited for molecular biology. There is less repetitive DNA in *Arabidopsis* compared to other plants (Meyerowitz and Pruitt, 1985). This makes it easier to clone a gene through chromosome walking. There are only 5 pairs of chromosomes, which makes assigning each gene to the appropriate chromosome less labor-intensive. Mapping a gene is an important exercise early in investigation of a new mutant to identify ordered contigs from other labs for the region to aid cloning efforts, recognize inadvertent phenotypes caused by double mutations, or identify alleles already the subject of study in other laboratories. The *Arabidopsis* genome is 120 Mb and therefore small enough to be sequenced fairly quickly (Meinke *et al.*, in press).

Mutagenesis of *Arabidopsis* has generated mutants for virtually every aspect of plant growth, environmental response, and development. Different types of mutagenesis treatment can give rise to a variety of phenotypes. For example, X-irradiation of seeds or vegetative meristems causes null mutations (Müller, 1963) and weak alleles (partial loss of function) are often caused by chemical mutagens like ethyl methanesulfonate which can cause point mutations (Feldmann *et al.*, 1994). T-DNA insertional mutagenesis using *Agrobacterium tumefaciens* has allowed rapid identification and cloning of mutant genes as well as a method of mutagenesis (Feldmann and Marks, 1987; Lindsey *et al.*, 1996). Transposon tagging has been established in *Arabidopsis*, and has become a powerful resource for creating tagged mutations for a particular gene (Feldmann *et al.*, 1994). Mutant analysis is regarded as the most powerful way in which gene function can be determined (NSF 90-80, 1990).

The role *Arabidopsis* has played in the understanding of plant development is important. The number of publications each year featuring *Arabidopsis* increased from 19 in 1985 to over 670 in 1994 (NSF 95-43, 1995). This demonstrates the increasing popularity of *Arabidopsis* as a system for plant biological studies. Through advances made with this plant, the molecular, physiological, and developmental biology of other plant systems will be understood more easily, making *Arabidopsis* a valuable model system.

Diversity of research on *Arabidopsis*

Because *Arabidopsis* carries out growth, development and reproduction in a fashion similar to other plants, all of these biological processes can be--and are being--investigated using this plant. Indeed, the growing collection of publications related to all aspects of the life cycle of this economically unimportant plant provides a resource that investigators can

exploit to solve application problems in other systems. It is hard to find any aspect of plant biology that is devoid of impact from the progress being made in *Arabidopsis*.

Basic cellular functions, such as protection from oxidants for example, have recently been analyzed by isolating an *Arabidopsis* homologue of a peroxiredoxin antioxidant, *AtPer1* (Haslekås *et al.*, 1998). The *Per1* homologue was previously characterized only in monocots. Similarities were seen between an EST sequence in an *Arabidopsis* database and a monocot gene already cloned. The investigators used RT-PCR to clone the *Arabidopsis* equivalent and study its expression *in situ*. This study showed that the gene is highly expressed during late embryogenesis and could have an effect on seed dormancy, and that *Arabidopsis* uses gene homologs similar to those found in other plants.

Timing of developmental events is an important aspect of plant growth. Several heterochronic mutations of *Arabidopsis* have been found that transform vegetative leaves into cotyledon-like organs (Conway and Poethig, 1997). In these mutants, vegetative leaves show characteristics of the embryonic leaf, the cotyledon. This study shows that control of the timing of organ formation in plants has a genetic basis which supports models previously constructed for other morphogenetic mutants by identifying a basic developmental pathway (Meinke *et al.*, 1994; Meinke, 1994; 1995). Transcription factors are being sought by investigators because of they can cause dramatic shifts in gene expression in response to developmental or environmental signals. Though the MYB family of transcription factors is rare in animal genomes, plants have over 100 genes that contain MYB-like domains (Martin and Paz-Ares, 1997). PCR using degenerate primers for MYB-like domains revealed a new gene with a putative control function, *AtMYB13* (Kirik *et al.*, 1998).

Expression of this gene is highest during periods of plant stress or high metabolic activity of the plant. It is suspected that *AtMYB13* has a role in causing shoot morphogenesis to be appropriate for the environmental conditions.

A recent paper communicated the effects of decreased oxygen on embryo morphogenesis in a classical physiology approach (Kuang *et al.*, 1998). Plants grown under hypobaric conditions demonstrated a decline in seed germinability. Progression of embryogenesis was proportional to the amount of oxygen supplied to the plants. Though high CO_2 and low O_2 levels are associated with increased carbon fixation, it is now apparent that the metabolically-active process of embryogenesis absolutely requires oxygen. Work of this nature is of interest to NASA in their search for bioreactors suitable to assist astronauts during their extended stays in space.

Identification and elucidation of biochemical pathways is another active field of study. The precursors and enzymes involved in the manufacture of products are economically important and intellectually interesting. Biotin, a compound added to chicken feed to improve the quality of egg production, is synthesized in bacteria and plants. Commercial farming has created a market for this compound which ordinarily is needed only in trace amounts. Over the past decade, the steps needed to produce biotin in plants have been determined (Patton *et al.*, 1998). Two mutants that lack enzymes critical to transform substrates into necessary intermediates along the pathway, *bio1* and *bio2*, have provided information about the biotin metabolic pathway in plants (Schneider *et al.*, 1989; Shellhammer and Meinke, 1990; Patton *et al.*, 1998). In addition, a pathway for vitamin C synthesis has been identified in plants (Wheeler *et al.*, 1998), which is of interest because

vitamin C is essential for the health of animals (including *Homo sapiens*!). Originally characterized as a gene responsible for stress response, the *soz1* gene is now thought to influence the production of vitamin C in plants (Conklin *et al.*, 1996; 1997) and therefore has been renamed *vtc1*. Through these types of studies, alternative and more cost-effective methods to produce vitamin C and biotin for agriculture might be designed.

The studies outlined here are by no means exhaustive; a comprehensive summary of ongoing work on *Arabidopsis* would fill thousands of pages. The overview provided is intended to illustrate some of the breadth of recent work on the plant. Throughout this dissertation, reviews of work completed by other laboratories will be outlined as well. The point is that *Arabidopsis* is suitable for a very large spectrum of studies, and these are being pursued by a scientific community with a great diversity of interests.

Original organization of the *Arabidopsis* community

By 1990, the use of *Arabidopsis* became widespread enough to call for the formation of a steering committee to maximize the effectiveness of the many labs working on this plant. The virtues of *Arabidopsis* as a biological tool were recognized and the ambitious plan to coordinated a project with the mission to completely characterize the structure, function, and regulation of every gene in a plant system was discussed (NSF 90-80, 1990). Advances in gene sequencing technologies and new ways to organize and disseminate genetic information were identified, along with the speed with which new technologies were being introduced. With proper administration of many laboratories, the goal to completely sequence and annotate the *Arabidopsis* genome by the beginning of the next century was deemed realistic.

Labs using *Arabidopsis* for study are located in many different countries. Because research on this plant is intensive, inadvertent redundancy of effort caused by overlapping research is sometimes a problem. In addition, resources for laboratories such as genomic libraries, cDNA libraries, and maps based on molecular or classical genetics are labor-intensive or expensive to create, and would best be used communally. At that time, a set of guidelines for collaboration was drafted and a decision made to form resources centers which provide DNA and seed stocks to researchers.

The Multinational *Arabidopsis* Genome Project

Coordination of the *Arabidopsis* community began when the Multinational *Arabidopsis* Genome Project was established. A steering committee was appointed to oversee the exchange of information between laboratories, identify ways to improve communication and the exchange of resources, support workshops, symposia, and ways to share information about the research focus of member labs. Almost seven years into the collaboration, new resources are still being brought on-line to assist researchers in the community (NSF 97-131).

A computer resource that supported about 100 researchers called "Electronic *Arabidopsis*" was instituted over 10 years ago at Michigan State University (MSU) to provide communication between laboratories (Somerville, 1989). Because paper media is slow to print and distribute, making information available through online resources allows up-to-date exchange of data and ideas. The Unix ACeDB database was modified for *Arabidopsis* researchers to create AAtDB and was made available through computer networking. It contained information about ongoing projects, mutant lines, researcher

contact information, and cloned parts of the genome. Unfortunately, this program demanded a Unix platform which is unpopular with most researchers and suffered from troublesome transmission delays that could extend query sessions for hours.

Since that time, the Internet has grown as a utility and many laboratories have elected to use specialized sites to advertise their research progress. With the advent of the World Wide Web, it is now possible to put the wealth of information from AAtDB onto Web sites that are accessible from many commonly-used platforms. AtDB, the new incarnation of AAtDB, serves the Multinational *Arabidopsis* Research Project and is a widely-used resource for the community, supplying an average of 2 300 different computer users over 20 000 pages of information weekly. Even laboratories of modest size can take advantage of the low-cost, easily maintained web servers that interface with existing university computer networks. The Yanofsky laboratory, for example, has a tutorial that introduces visitors to their research about MADS boxes and how they control gene expression (http://www-biology.ucsd.edu/other/yanofsky/home.html). The Meinke laboratory maintains a site that includes information such as rules for naming new mutant genes, a list of claimed mutant symbols, locus data for genes mapped using classical techniques, and a list of embryo-defective mutants characterized in this laboratory (http://mutant.lse.okstate.edu). The Internet has provided a fast, efficient way to organize laboratories around the world, and has the added bonus of not being restricted by time zone changes!

A newsgroup dedicated to the needs of *Arabidopsis* researchers sends from 3 to 5 messages to the community each day. These messages include pleas for assistance with a particular research problem, requests for DNA or other materials, job announcements, and

indications of the current progress of the Project. Through the newsgroup, the goals of organizing informatics, efficient use of *Arabidopsis* community resources, and exchange of information within the community are addressed.

Each year, the steering committee drafts a summary that indicates the current level of research and re-evaluates the goals for the community, making the collaboration a dynamic, productive enterprise. This summary is distributed throughout the scientific community and provides guidelines to how resources are being used and the progress of the *Arabidopsis* genome sequencing project. The outline indicates to what level resources are being distributed between involved laboratories and the countries that sponsor them. It is anticipated that the entire *Arabidopsis* genome will be sequenced and annotated by the end of 2001. Each annual meeting of *Arabidopsis* researchers is used to update active members of the status of other labs. Additionally, the sequencing project is a great example of how a multinational collaboration can produce an impressive body of scientific work when properly managed.

THE GENETIC BASIS OF DEVELOPMENT IN *ARABIDOPSIS*

Merely knowing the nucleotide sequence of the entire genome of *Arabidopsis* is not sufficient to understand the plant's biology. The products the genes code for and how they are regulated must be understood to assess gene function. It is relatively easy to get the sequence of a small string of a few kilobases of DNA, but *Arabidopsis* has a genome of approximately 100 megabases (Meyerowitz, 1994). Similarity to genes already cloned from other systems aids identification of potential gene function, and the use of mutants defective in identifiable characteristics can give direct clues to the function of that protein.

Genes that direct plant development from the zygote through the stages of embryogenesis and through vegetative and reproductive growth are of special interest to biologists. The genetic controls of differentiation might seem to be too complex to be comprehended. After all, thousands of specialized cells are all spawned from just a single cell, and their fates must be directed at a multitude of control points. However, the influence of several genes together to "program" the fate of a cell and its descendants, as well as the pleiotropic effects of some genes reduces the total number of developmental genes needed to code for an organism (Meyerowitz *et al.,* 1991; Mayer *et al.*, 1991).

Cloning genes from wild-type plants

Because of the universal nature of the genetic code, similar DNA sequences normally code for proteins of similar amino acid composition. Similarity of protein primary sequence suggests similarity of tertiary and quarternary structure and therefore possibly function correlations. Therefore, if segments of DNA between two unrelated organisms are found to be highly similar, there is a good chance that they code for proteins of related function.

Thus, it is possible to apply the lessons learned from other systems where a particular gene is well-characterized. If an investigator is interested in cloning a gene from *Arabidopsis* similar to one that is well-studied in another system, they can use the known sequence to identify related DNA in *Arabidopsis*. Southern blotting can be used to see if the gene is present in the genome. If an appropriate cDNA library is probed with the sequence, it can be determined if the protein product is represented in the tissues used to generate that library. Yeast systems were studied as a eukaryotic model system before *Arabidopsis* and there is a larger knowledge base regarding yeast proteins which can be queried to rapidly identify plant

genes of similar sequence. PCR techniques can also be used to amplify stretches of DNA between primers designed based on characteristics of the desired template. Thus, if similar nitrogenous base sequences are found in *Arabidopsis* and other system genomes, they can be cloned in this fashion.

The Institute for Genomic Research (TIGR) and large-scale projects at MSU and in France, are pursuing the goal of creating an immense database of Expressed Sequence Tags (ESTs). Expressed genes–through their mRNA–are cloned after reverse transcription and partial sequence is obtained from them. Sequence data is entered into a database and researchers can query this to find DNA sequences of interest. When sequence data from a gene in a different model system becomes available, it is a relatively simple matter to see if an ortholog is expressed in *Arabidopsis* and would therefore be a target for a cloning strategy.

Biologists are often curious about the differences in gene expression between different treatments, tissues, or cell types. Differential display has become a useful way to address this question (Brandstatter and Kieber, 1998). With the use of an array of specially constructed random primers as well as primers that hybridize with the polyadenylated tail with a single novel base to anchor the primer to the end of the transcript, comparisons of the relative levels of transcripts between treatments can be made. Differential display can therefore show genes that are upregulated or downregulated under different conditions. The emerging technology of gene chip analysis is becoming accessible to a larger portion of the scientific community, and will provide an even more rapid way to identify genes involved with specific environmental or developmental responses (Kling, 1998).

However, the limitation of these types of investigations is that the true role of the gene is not definitively proven. Hypotheses can be made for gene function, but experiments that prove the function of the gene can sometimes be difficult to design. Compounds that interfere with the gene product activity can be added to help identify the role of the gene. For example, drugs that interfere with a substrate or an antibody from another organism that binds to a particular enzyme's active site can be used to test hypotheses about the encoded protein. Removal of the gene product by creating an antisense construct of the gene and transforming it into wild-type plants is another strategy, though the results of this technique can sometimes be inconclusive because gene product reduction is not necessarily achieved. Site-directed mutagenesis and homologous recombination are still difficult to accomplish in *Arabidopsis*, but these procedures can cause a loss-of-function phenotype to the plant, making construction of hypotheses regarding gene function easier.

Mutagenesis studies

By observing the alteration of an organism's phenotype based a single gene mutation, functions of the wild-type gene can be deduced. This is a powerful tool being used by biologists in a number of systems, and mutant analysis is the most active area in *Arabidopsis* research. Mutants disrupted in biochemical pathways, organ formation, growth, pigmentation, hormone response and indeed almost anything that produces a recognizable phenotype have been collected. In fact, a very subtle phenotype was found in mutant lines with abnormal sterol composition that looked normal, and were identified only through mass screening of pooled plants by GC-MS (Gachotte *et al.*, 1995).

The strategy of mutant analysis is analogous to a hypothetical method of finding out how a machine operates. Suppose you have no manuals regarding the operation or construction of a particular machine, though you have a vast number of these machines. If you were to systematically remove one part from each machine and then analyze how the machine operates, you could eventually construct models for what each part does. Failure for the machine to start allows you to suggest a particular part has a role in starting or maintaining the machine's operation. If the machine runs, but the product is defective in a consistent and recognizable way, you could assign a role for the removed part that explains how it might normally prevent that defect from occurring. When the function of many parts are known, a logical model can be constructed to account for how the parts operate together and thus make the machine function. Mutants represent the machines missing a single part. Through mutant analysis, the roles of each "missing part", or gene, can be identified and the ways in which those parts interact can be used to construct models of how the parts work together normally.

Mutations are created by altering the DNA sequence of wild-type plants. Base substitution, frame shift, deletion or insertion of DNA cause mutations. Some of the first mutants of *Arabidopsis* were produced by X-irradiation of seeds (Müller, 1963) which caused DNA loss ranging from a portion of a gene to a stretch of several adjacent ones. Chemical mutagenesis is the most efficient way to generate point mutations, and thus create alleles for a gene that range from weak (partial loss of function) to null (absence of function) (Meinke, 1985). Transformation of plants with modified T-DNA from *Agrobacterium tumefaciens* can disrupt normal gene function due to the insertion of several kb of foreign

DNA (Feldmann, 1991). This can create mutations that are "tagged". These tags can provide antibiotic or herbicide resistance, making identification of transformants easy, and the presence of a tag flanked by portions of the original gene facilitates cloning of that gene. Transposable element insertion and excision in *Arabidopsis* is being widely used as well and will be a powerful mutagenic procedure with similar advantages as T-DNA tagging but with the added benefit of restoring wild-type morphology if cleanly excised, making confirmation of the mutant locus easier.

Mutant analysis typically begins with large-scale mutagenesis of seeds followed by careful screening of the M_2 generation for defective plants. Several mutant categories are well defined, such as unusual pigmentation, developmental stage at which the defect is observed, or substitution of one structure with another. A large-scale T-DNA based mutagenesis and mutant screen was organized by Ken Feldmann at DuPont, with laboratories interested in different types of mutations invited to search for mutants applicable to their group. Floral mutants were collected and categorized from plants that grew to maturity, shoot and root mutants were likewise analyzed by laboratories involved in that type of research. The Meinke laboratory was already established as a leader in the study of embryo-defective mutants and screened the siliques of the M_1 plants for mutant M_2 seeds. Several classes of embryo-defective mutants were identified in this project.

Complementation of these approaches

Concurrent progress in the genome sequencing project, EST cataloging, and mutant analysis has allowed rapid progress towards the mission of the Multinational *Arabidopsis* Genome Project. Each mutant gene can be mapped to a locus on a chromosome. If an

interesting EST is mapped near that region, it is possible that the EST represents the wild-type gene for that locus, and the mutant corresponds exactly to the EST locus. Sequence analysis of the EST might yield information about identity of the polypeptide and therefore its function. In this manner, both the identity of the gene's sequence and elucidation of gene function can be determined.

EMBRYOGENESIS IN *ARABIDOPSIS*

The study of development is an intriguing and complex field of research. Embryogenesis is especially interesting because of the clear morphological progression from the zygote to the mature, dormant embryo in the dry seed. At the molecular level, however, this progression is brought about only through complex genetic interactions which specify the spacial and temporal development of the embryo. One difficulty of studying embryogenesis is the inaccessibility of the embryo within maternal tissues (Yang *et al.*, 1997). The intriguing ability of plants to undergo somatic embryogenesis not only makes manipulation of embryos easier, it also provides an opportunity to study genetic programs controlling embryo development represented in plants *in vitro*. *Arabidopsis* has been studied extensively to identify genes controlling embryo development and the hormonal and environmental factors which guide their expression.

Stages of embryogenesis

The study of embryogenesis in *Arabidopsis* is aided by the progression of the embryo through consistent and identifiable developmental stages. The pattern generated in the embryo is the basis for the organization of the resulting vegetative plant which develops from it. For example, the polarity and phyllotaxy, if disturbed in the embryo, are altered in the

seedling which grows after germination (Jürgens *et al.*, 1994). Embryogenesis in *Arabidopsis* takes about 12 days and resembles the stereotypic sequence of embryogenetic stages of dicotyledonous plants as originally detailed for *Capsella bursa-pastoris* (Steeves and Sussex, 1989). Whole-mount preparations of cleared seeds can be used to analyze stages of development and identify abnormalities in embryogenesis.

Embryogenic stages are named after the shapes the embryo goes through during development. The zygote is a small cell which divides asymmetrically to establish the apical-basal axis of the embryo. The division is influenced by the unequal distribution of organelles in the cell. The larger basal cell forms the suspensor and hypophysis (which later forms part of the root), while the smaller, apical cell forms most of the embryo proper. The apical cell cleaves twice vertically (parallel to the long axis) and then once horizontally to give rise to the octant stage of embryo development. The first tissue to form is the protoderm which is defined by division planes parallel with the embryo's surface. This 16 cell embryo is termed the globular stage embryo. Elongation of the procambial cells (vascular primordium) just above the hypophysis causes the embryo to stretch along its apical-basal axis. Concurrent cell divisions in the apical region causes lateral spreading. Formation of the embryonic leaves (the cotyledons) gives rise to a heart stage embryo. The hypocotyl grows through cell division and elongation and the hypophysis differentiates into the root apex, forming the torpedo stage of embryogenesis. The cotyledons continue growing and fold down to lie parallel to the hypocotyl as the embryo reaches maturity. Dormancy and desiccation follow.

Mutants defective in embryogenesis have been systematically examined to determine the genetic basis of embryo development (Castle *et al.*, 1993; Franzmann *et al.*, 1995; Meinke, 1995). By classifying embryo-defective mutants based on the stage at which abnormalities are first noticeable, the function of the gene can be inferred based on the assumption that the abnormalities occur at the time the functional gene product is needed (Meinke, 1985). In some cases, phenotypes that were assumed to be lethal were later found to be merely defective because cultured embryos could be rescued (Baus *et al.*, 1986). This significant finding indicates that arrested embryos can be cultured to test the embryo's response to different conditions. By rescuing mutant embryos from *lec1*, *lec2*, and *fus3* lines prior to desiccation, it was determined that the wild-type allele of these genes was involved with initiation of seed maturation programs (Meinke, 1992; Meinke *et al.*, 1994). Failure to initiate late embryogenesis programs caused the embryos to die during desiccation. A biotin-deficient mutant, *bio1*, was rescued by supplementing the medium with a range of biotin precursors (Shellhammer and Meinke, 1990). This allowed the characterization of the biochemical pathway for biotin synthesis in plants.

Somatic embryogenesis and development of plants in culture

Somatic embryos follow similar developmental stages seen in zygotic embryos (Zimmerman, 1993). *Daucus carota* is a model system for somatic embryogenesis studies and genes controlling the process have been characterized (Yang *et al.*, 1997). Unfortunately, hormonal and environmental conditions *in vitro* are sufficiently different from those *in ovulo* that epigenetic changes complicate analysis (Nickle and Yeung, 1993; 1994). An important feature of somatic embryogenesis is that it is a phenomenon which indeed

utilizes genes involved in zygotic embryogenesis (Yang *et al.*, 1997) which can be used to identify genetic components of development and test hypothesized roles of identified genes. Such an *in vitro* culturing technique might also be useful to rescue mutant plants homozygous for a developmentally important gene (Moussian *et al.*, 1998).

Arabidopsis has been recalcitrant to somatic embryogenesis but a recent protocol was designed that allows plant regeneration *in vitro* (Luo and Koop, 1997). The amenability of several cultivars used in *Arabidopsis* research to generate somatic embryos in a variety of media was evaluated. Most had the potential to generate somatic embryos except one line, Ws-2, which did not generate either proembryogenic masses or embryoids. Somatic embryos in other lines recapitulated the stereotypic sequence of zygotic embryogenesis. Discrepancies with other published somatic embryo studies on *Arabidopsis* (Sangwan *et al.*, 1992) relating to cytokinin response and embryo germination were noted. These discrepancies, however, could be explained by epigenetic factors which are well documented in somatic embryo cultures (Nickle and Yeung, 1993). This unpredictability of response generally makes *Arabidopsis* somatic embryogenesis an unsatisfactory system for embryogenic study, though perhaps that it can be used creatively to show expression and induction of embryo-specific genes.

Review of recent literature

The study of embryogenesis in *Arabidopsis* has captured the attention of many laboratories. For recent reviews, see Meyerowitz, 1994, Meinke *et al.*, 1998, or Scheres and Wolkenfelt, 1998. A few of the more interesting studies will instead be presented here.

The *ZWILLE* gene (*ZLL*) has been cloned and shown to play a role in establishing the primary shoot meristem during the transition from embryonic to vegetative growth at the onset of dormancy (Moussian *et al.*, 1998). The molecular identity of *ZLL* is similar to sequences ubiquitous in known eukaryote proteins. Embryos from *zll* mutants do not form the leaf buttresses between cotyledons which are normally present in the mature embryo. Sections through germinated *zll* seedlings show the absence of meristematic tissue in the shoot apical meristem, and mutants which germinate are either shootless, form a strange rod-like organ which lengthens slightly but does not otherwise differentiate, or form a single, terminal leaf. Interestingly, adventitous meristems, which are not inhibited at the axils of the cotyledons, can be used for vegetative growth. The root apical meristem of *zll* mutants was not affected. A different mutant, *shoot meristemless* (*stm*), has a similar phenotype (Long *et al.*, 1996) in which the mutant gene fails to prevent meristem cells from being incorporated into organ primordia. *STM*, a gene that encodes a homeodomain protein responsible for shoot initiation, was shown through *in situ* hybridization to be normally expressed in *zll* mutants so there is no direct interaction between these genes. Models of *ZLL* function suggest that it either prevents differentiation of cells in the shoot meristem or partitions the embryo apex to cause retention of cells fated to remain meristematic during organogenesis.

Another recent study shows that embryos express a novel ribosomal binding protein during the cell proliferation phase of embryogenesis (Hecht *et al.*, 1997). This gene was identified through sequencing of DNA upstream of a gene identified through promoter trapping and named AtRBP37. Comparison of this sequence to those in GenBank showed

that this gene has two consensus sequences similar to that of genes encoding proteins that bind RNA in other organisms. These other proteins did not have more than one of these sequences, making AtRBP37 unique in possessing two consensus sites. Genomic analysis of AtRBP37 shows it is probably a member of an RNA binding protein family represented in the *Arabidopsis* genome. The AtRBP37 protein binds both DNA and RNA *in vitro*. Expression of AtRBP37 showed that it only occurs in young, actively dividing cells and is not associated with tissues that are actively transcribing genes but not dividing. AtRBP37 might have a role in regulating the cell cycle.

A gene similar to one identified as controlling somatic embryogenesis in *Daucus carota* (carrot) somatic embryos was cloned in *Arabidopsis* (Dure *et al.*, 1989). This gene has a high similarity to Late Embryogenesis Abundant (LEA) genes which code for proteins that play a part in controlling water exchange, tolerance to desiccation, or have a protective role on cells as they lose water. The gene is also expressed only during late embryogenesis, supporting the findings of sequence analysis. This report is also interesting because it once again crosses the inter-species gap and shows that information garnered from *Arabidopsis* can be applied to other species. Further, this shows that work done in another model system, somatic embryos formed from *Daucus*, can be applied to a vegetative system.

One aspect of embryogenesis that is particularly interesting in plants is the contribution of maternal gene products to the next generation (Zimmermann, 1993). Unlike animal systems, maternal supply of mRNA or proteins to the next generation is negligible in globular and subsequent stages. The *MEDEA* (*MEA*) gene segregates via an unusual ratio; half of the seeds in a plant heterozygous for the *mea* defect are aborted instead of the

expected 1/4 as predicted by standard inheritance, and inheritance is strictly maternally derived (Grossniklaus *et al.*, 1998). The phenotype of mutants is excessive cell proliferation beginning at the globular stage. A model suggesting a dosage-dependent effect in the triploid endosperm, where two defective *mea* alleles are derived from the mother, was disproven because a cross with a tetraploid male, which would contribute two *MEA* alleles to compensate, did not rescue the mutants. Sequence analysis of *MEA* shows it resembles polycomb transcription factors from *Drosophila*, *C. elegans*, and a plant gene of unknown function. *MEA* thought to function by altering chromatin structure to regulate gene expression. Expression of *MEA* begins during megagametophyte maturation and terminates during seed maturation. It is not known if *MEA* transcripts are extremely stable maternal contributions or if they are also expressed zygotically. This study is significant because it shows a similarity in transcription factor operation between plants and animals and is a good example of parent-of-origin-specific effects whereby the phenotype is transmitted maternally.

Another transcription factor was identified using a technique called virtual subtraction, a sensitive refinement of library subtraction. The cloned gene, *PEI1*, is a zinc finger protein that controls development of the embryo shoot region (Li and Thomas, 1998). Expression is seen only in immature seeds. There is DNA sequence similarity with a transcription factor responsible for the *unkempt* mutation in *Drosophila* (Mohler *et al.*, 1992) and some suspected oncogenes in mice, rats, and humans. To confirm that *PEI1* functions during embryogenesis, an antisense construct was transformed into plants, causing defective seeds containing white, enlarged embryos that did not progress past the globular or early

heart stages. Cultured embryos developed normal-looking roots, but the shoots were aberrant and did not form true leaves. PEI1 protein is seen only in seeds and was reduced in antisense plants. To test whether *PEI1* is a transcription factor, levels of embryo-specific mRNA for storage products were attenuated in antisense plants. Proteins that synthesize chlorophyll and some photosynthesis proteins were not expressed in antisense plants, explaining the white phenotype. Interestingly, arrested white seeds were recovered from plants regenerated from callus formed from cultured antisense plants, suggesting the unique role for *PEI1* in the embryo. It is unclear what role *PEI1* plays in embryogenesis. It is probably responsible for regulation of genes responsible for shoot morphogenesis in the embryo.

The *monopteros* (*mp*) mutant is an embryo mutant found in a screen designed to find patterning defects (Jürgens *et al.*, 1994). Originally thought to be a basal deletion mutant, analysis of the cloned *MP* gene shows that it has features of a transcriptional regulator and homology with *ARF1*, which is thought to regulate genes in response to auxin (Ulmasov *et al.*, 1997). An MP::GUS fusion protein was generated and expressed transiently in onion epidermal cells. The protein was found in the nucleus, supporting its role as a transcription factor, and indicating that at least one of the three predicted nuclear localization signals (NLSs) found in the sequence was active. *In situ* hybridization showed that *MP* is expressed throughout the globular stage embryo but is restricted to the developing provascular system in the heart and subsequent stages. Auxin has been implicated both in the establishment of an apical-basal signaling (Sachs, 1991; Liu *et al.*, 1993) and in establishment of vascular

tissue (Sachs, 1991). The loss of the hypocotyl and root in *mp* plants could be due to disruption of embryo polarity (Hardtke and Berleth, 1998).

These and many other exciting discoveries show that *Arabidopsis* is fulfilling its role as a model system and is a valuable tool for the investigation of embryogenesis. As the entire genomic sequence of *Arabidopsis* becomes known, an explosion of new transcription factors and developmental control genes will be found. Complemented with mutant analysis, the functions of many of these genes will give us a deeper understanding of how plants grow.

Purpose and significance of this dissertation

This dissertation will detail some of my contributions to the Meinke laboratory during my studies here. Work done on the cytokinesis defective mutant *cyt1* is described in the bulk of this dissertaion. Appendices relating to the computer network I designed and the Internet connections I maintained as a graduate student are at the end of this document. Thus, this dissertation might be viewed as a profile of how information is collected at the bench and then disseminated electronically to the community at large.

Investigation of a cytokinesis defective mutant

cyt1 was originally identified as a bloated heart-stage embryo mutant. Sectioning of embryos revealed cellular defects in the form of cell wall gaps and cell wall stubs, excessive polysaccharide in some cell walls, and large and vacuolated cells. Cultured mutants did not grow on callus-inducing or germination media. Fluorescent staining can reveal accumulation of excessive callose in the cell walls (Stone *et al.*, 1984). This staining and electron microscopy of antibody-treated *cyt1* sections showed alterations in the location of de-esterified pectins.

Chapter 2 gives background on cytokinesis and the composition of the plant cell wall. Chapters 3 and 4 contain data which have been included in a manuscript recently published by The Plant Journal (Nickle and Meinke, 1998) which was obtained through a microscopic survey of the mutant phenotype in addition to biochemical analysis of wall components. The function of the *CYT1* protein, which is likely a mannose-1-phosphate guanyltransferase, was identified through the cloning efforts of Drs. Chris Somerville, Wolfgang Lukowitz, and Robert Last, and this will be included in Chapter 5. Chapter 6 is a brief summary.

Interfacing with the Multinational *Arabidopsis* Genome Project

Dr. Meinke's role as curator of mutant gene symbols requires him to maintain an accessible, accurate, and comprehensive list of mutant names and linkage information. Because paper media is not dynamic and usually out of date, we collaborated to design an on-line resource for these data. The Meinke website is now dynamically updated through a linked database and can accept electronic forms for data submission. By maintaining our own server, we are given more control over the content and strategies used for broadcasting and maintaining data.

Customization of data presentation is allowed through the Active Server Pages protocol which is interpreted by Microsoft's Internet Information Server utility. VBScript text is rendered to show the client up-to-date information contained in the database. The system is designed to be low-maintenance, with html code changes required only when new types of data or new presentation templates are desired. Human intervention is required on the part of the curator because information submitted electronically is not automatically

entered into the database. The curator can therefore evaluate the quality of submissions and ensure the information served is accurate.

To assist those who will maintain the Meinke web server in the future, several appendices will show the strategies involved in the construction and modification of the pages. Appendix A outlines how the database is updated and the organization of pages on the server. Appendix B contains annotated source code for ASP elements of the server.

CHAPTER TWO: CYTOKINESIS AND THE PLANT CELL WALL

INTRODUCTION

Multicellular organisms have developed a complex and carefully coordinated program for growth and differentiation. With each mitotic event, a cell partitions into two daughter cells arranged in the proper orientation, each with a full complement of organelles and membrane structures necessary to survive. It has been argued whether the cell is the basic unit of life and some philosophical discussion has been raised debating cell theory vs. organismal theory (Kaplan, 1992, Sitte, 1992). Independent of the conclusions of these types of debate, however, it is agreed that multicellular eukaryotes have a common genetically-driven protocol to follow for mitosis. In animal systems, cells are ordinarily separated only by a plasma membrane while plant cells have a cell wall interposed between their plasma membranes at the conclusion of mitosis. The superficially similar events are, however, undertaken in dramatically different fashions (Kaplan, 1992). Animal cells pinch apart during daughter cell separation; plant cells build a partition that separates daughter cells.

The typical events of mitosis are the duplication of genetic material in the nucleus, separation of duplicated nuclear materials into two units (karyokinesis), and then partitioning

of the subcellular components into two discrete units (cytokinesis). Cytokinesis, however, is a major defect of most of the mutants described here and will be discussed at length. A background for cytokinesis will be presented first. Discussion of the cell wall concludes this chapter.

CYTOKINESIS

The cytosolic components undergo reorganization in preparation for cytokinetic activities after the nucleus has undergone replication during the S-phase of the cell cycle. The cell then completes prophase, metaphase, and enters anaphase. The daughter nuclei become autonomous replicas, each containing a complete diploid genome. A full complement of all other organelles must be established for each new daughter cell. The cellular process that brings about this partitioning is cytokinesis and is performed by all dividing cells. By combining information obtained from a variety of systems, we are only now beginning to understand some of the biochemistry and physiology involved in cytokinesis (Satterwhite *et al.*, 1992).

Cytokinesis of animal and yeast cells

Studies with yeast and animal systems have revealed some clues as to the nature of eukaryotic cytokinesis. In animal cells, karyokinesis is followed by events in which the plasmalemma is constricted at the equator of the cell, causing fission as the cell is partitioned via a gradual narrowing, and finally severing, of the cytoplasmic continuity between the cells (Kaplan, 1992; Doonan, 1996; Foe *et al.*, 1993; Giansanti *et al.*, 1998). Structures such as the contractile ring and the association of actin filaments of the ring with myosin are unique to the animal strategy for daughter cell partitioning (Ostrow *et al.*, 1994).

Cytokinesis in Drosophila larvae

In *Drosophila*, a zygote goes through many mitotic divisions before cellularization (Foe *et al.*, 1993; Miller and Kiehart, 1995). During the 14th round of mitosis, migration of nuclei toward the cell cortex forms a configuration resembling a hollow sphere. Migration of the nuclei proceeds through assembly of microtubules, which serve to simultaneously position each nucleus at the larval periphery and to push yolk particles that impede cleavage deeper towards the medulla. Nuclei are forced against the plasmalemma, causing it to become stippled because of the repositioning of each nucleus. Centrosomes positioned between each nucleus and the plasmalemma organize the microtubule portion of the cytoskeleton. Microfilaments which serve to guide the contractile force generated by myosin assist these movements. When nuclei are in place at the periphery of the larva, the centrosomes take up positions around the nuclei perpendicular to the cell surface (i.e. they move laterally), and as they move, actin forms an annulus that eventually encircles each nucleus and which lies parallel to the plasma membrane. An astral array forms between the centrosomes, setting up a scaffold for a periclinal nuclear division. The plasmalemma external to these cells folds into a surface of complex microvilli and the nuclei undergo their fifteenth mitotic division, yielding over 60 000 presumptive cells. These microvilli are necessary to provide membrane material for cellularization, which occurs in under an hour.

Cellularization begins with invagination of the plasma membrane around each nucleus at the periphery of the cell. The base of these invaginations is demarcated by the annular actin bands. The astral array that allowed karyokinesis in the periclinal division now reorients 90° so it lies perpendicular to the plasma membrane. The dome-shaped elevations

of the plasmalemma above each nucleus are raised up even further; the nuclei apparently become pushed up higher against the cell membrane, and yolk particles are further removed from the cortex, possibly because of microtubules leveraging against the yolk particles (Foe *et al.*, 1993). It is possible that there is some interaction between the actin annular rings and the microtubule cage around each nucleus though the nature of this is unknown. The elevations disappear with the addition of anti-tubulin antibodies to larvae at this stage and it is known that myosin II exerts force on the actin filaments to bring about localized movement (Miller and Kiehart, 1995; Jordan and Karess, 1997).

The activity of myosin and actin as contractile forces has been described in a large number of systems including *Drosophila*, *Saccharomyces cerevisiae*, *Schizosaccaromyces pombe*, *Xenopus*, *Aspergillus nidulans*, and human fibroblasts (Williams *et al.*, 1995; Balasubramanian *et al.*, 1994; Fankhauser and Simanis, 1994; Satterwhite *et al.*, 1992; Mima *et al.*, 1995). Additionally, regulation of myosin II seems to be a common mechanism for timing cytokinesis in all eukaryotic systems examined (Satterwhite *et al.*, 1992). Phosphorylation of myosin II by the $p34^{cdc2}$ protein kinase, also ubiquitous in animal system, is thought to be a mechanism for timing cytokinesis events.

As cellularization of the larval cortex continues, the plasma membrane seems to be drawn down over each nucleus. The nuclei themselves are slightly distorted at the area where the actin annulus constricts them. This annulus moves toward the embryo center, pulling plasmalemma along. As cytokinesis progresses and plasma membrane resources become depleted, microvilli disappear from their location external to each nucleus. This suggests the microvilli provide the additional lipid reserves needed to encapsulate the new

mononucleate cells and their disappearance is probably because they are being stretched tight. Cytokinesis ends when the nuclei and sufficient cytoplasmic reserves are enclosed by the plasma membrane. The new cell remains attached to the yolk syncytium by a narrow stalk which is eventually pinched off to complete separation.

The larvae of *Drosophila* are easily investigated with a dissecting microscope and this makes it an amenable system for the genetic analysis of cell division. Additionally, *Drosophila* is a system rich in genetic work (Miller and Kiehart, 1995; Williams *et al.*, 1995; Satterwhite *et al.*, 1992) and mutations defective in several aspects of cytokinesis have been found, allowing molecular characterization of the cell division program (Miller and Kiehart, 1995). The *sqh* mutant disrupts the regulatory light chain of myosin, causing failure of cytokinesis (Karess *et al.*, 1991) and *pnut* is a septation mutant; the PNUT protein is required in the contractile ring (Cooper and Kiehart, 1996; Neufeld and Rubin, 1994), and many embryo/maternal protein interactions for cytokinesis in the embryo have been documented (Miller and Kiehart, 1995).

It has been found that even at the first stage of cytokinesis which was just described, germ cells undergo division at a different rate than somatic cells, suggesting multiple levels of genetic control (Foe *et al.*, 1993). The essential role of the cytoskeleton and movement proteins are well illustrated in this *Drosophila* example as well.

Cytokinesis in yeast

Yeasts have been used to answer questions about eukaryotic cell functions (Cooper and Kiehart, 1996). Mutants in the model organisms *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* at all stages of their life cycles have been applied to generate

testable models of how they grow and divide. *S. cerevisiae* forms small buds from the mother cell while *S. pombe* grows vegetatively by hyphal tip elongation followed by fission into new cells. Mutants blocked at different stages of mitosis, including cytokinesis events have been isolated in both these organisms (Fankhauser and Simanis, 1994; Amatruda and Cooper, 1992; Balasubramanian *et al.*, 1994).

Cytokinesis in *Saccharomyces cerevisiae*

Bud initiation in *S. cerevisiae* is easily observed under the microscope. A ring of neck filaments aggregates at the point where the daughter cell will bud from the mother. *S. cerevisiae* never buds from the same location twice, and new bud initiation is usually proximal to the most recent bud scar. The neck filaments are a collection of diverse proteins involved in determining the site of bud initiation and also to guide the cellular components through cytokinetic separation of cells. The neck filaments generate the contractile forces needed to separate the mother and daughter cells (Flescher *et al.*, 1993), and consist of several specialized filament-forming proteins that incorporate the actin which interacts with myosin II, causing cell division (Amatruda and Cooper, 1992; Satterwhite *et al.*, 1992).

Mutagenesis studies have allowed the identification of a collection of cell division cycle mutants (*cdc*), of which many show abnormalities in the neck filament region (Cooper and Kiehart, 1996). Many *CDC* genes are 25 - 37% have identical amino acid sequences, suggesting the possibility of overlapping functions (Flescher *et al.*, 1993). Temperature sensitive mutants *cdc3*, *cdc10*, *cdc11* and *cdc12* lack neck filaments at restrictive temperatures, and because of their role in septation, these wild-type proteins of these genes have been assigned to a category called septins. Some *cdc* mutants lose the ability to

separate the mother and daughter cells because of defective cytokinesis, causing filaments of abnormal interconnected chains of cells. Loss of some Cdc functions, however, do not block cytokinesis. For example, in the case of *cdc10* the site of new bud initiation is instead affected (Flescher *et al.*, 1993).

Cytokinesis in *Schizosaccharomyces pombe*

Unlike *S. cerevisiae*, which forms buds during cell division, *S. pombe* forms a mycelium and is therefore termed a fission yeast. Many similar proteins appear to be involved in both systems, and study of each complements the other.

Upon mitotic initiation in *S. pombe*, which is associated with the activation of a $p34^{cdc2}$ protein kinase (Chang and Nurse, 1996), hyphal tip growth stops, and the actin filaments at the growing ends disintegrate. The microtubule element of the cytoskeleton disassembles from the basket conformation that encloses the cell and it repolymerizes at the equator of the long axis of the cell, called the intranuclear spindle. The actin then forms a tight ring in this region, just under the plasmalemma. The ring contracts as the nucleus divides. The septum encroaches into the cell at this region as karyokinesis causes the nuclei to vacate the site of division. Contraction of the ring and plasmalemma and the establishment of primary and secondary septa from the periphery inward (centripetally) yield two daughter cells. Tip growth at the region distal to cell division then is re-established (Fankhauser and Simanis, 1994).

In *S. pombe*, the onset of mitosis primes cytokinesis operations that occur later (Fankhauser and Simanis, 1994). Even if karyokinesis is halted, cytokinesis is able to proceed. Mutants that are unable to initiate mitosis show no signs of attempting septum

formation or other cytoplasm dividing processes. Even yeasts that cannot form a stable spindle, and therefore cannot undergo karyokinesis, form actin rings associated with cell division. One mutant, *cut*, actually cuts the undivided nucleus into two pieces as the cell divides at the equator (Simanis, 1995).

Division mutants in *S. pombe* are also termed *cdc* just as they are in *S. cerevisiae*. For example, the Cdc18 gene in *S. pombe* has high homology with the *S. cerevisiae* Cdc6 gene. Likewise, the *cdc12* mutant in each system is also highly related to that of the other. Interestingly, *cdc12* was named and worked on in independently; the identical number is a coincidence. The *S. pombe* Cdc3 has been characterized as the actin monomer binding protein, profilin (Balasubramanian *et al.*, 1994). Cells with defective *cdc3* are unable to form the actin elements associated with ring contraction. Overexpression of Cdc3 interferes with the actin ring and causes cytokinesis to arrest. The Cdc8 gene encodes a tropomyosin which is a motor protein that interacts with actin, likely driving ring contraction. No tropomyosin has yet been found in *S. cerevisiae*, suggesting the possibility of different proteins involved in the constriction of the division ring, though both likely involve actin binding proteins (Balasubramanian *et al.*, 1992).

Other systems for cytokinesis studies

The role of actin in creating a ring which contracts to liberate two daughter cells is a common theme in the systems described previously. Similar steps in cytokinesis have been described in fibroblast cultures (Roche *et al.*, 1995; Mima *et al.*, 1995) and in *Aspergillus nidulans*, a fungal system which has been scrutinized like the other model systems (Doonan, 1992). Proteins which stabilize an actin network and create contractile force upon this

network are common to all systems so far investigated in detail (Satterwhite *et al.*, 1992; Flescher *et al.*, 1993). After cytokinesis, animal cells may be free to migrate to establish their position to create a functioning organ. This mobility of cells allows organ morphogenesis after cytokinesis, and is not, however, a feature of plant cells (Steeves and Sussex, 1989). Thus, the process of cytokinesis in plants occurs in a substantially different fashion.

Cytokinesis in plants

Cytokinesis in plants includes building a wall between the daughter cells. Animal cells narrow the continuity inwards (centripetally), while plant cells generally build the partition from the center to the edges (centrifugally), though some studies suggest this is not strictly true in all plant species (Sawitzky and Grolig, 1995). Cell wall material is deposited in a centrifugally expanding pattern so that it eventually forms an almost complete boundary between daughter cells. This simple reversal of strategy has profound implications on the underlying mechanisms that lead to cytokinesis. Except for biologically important continuities called plasmodesmata, the partition completely separates the daughter cells (Kragler *et al.*, 1998).

The importance of the orientation and frequency of cell division has been debated extensively. Pattern formation in plants is believed to be established on the basis of how cell division is regulated (Steeves and Sussex, 1989; Giménez-Abián *et al.*, 1998). Interestingly, investigations that attempt to correlate the regularity of cell divisions with the determination of organ size and shape instead show no significant relationship between these elements (Cooke and Lu, 1992; Kaplan, 1992; Traas *et al.*, 1995). Though cytokinesis is itself under

strict genetic controls for initiation and the steps required to effect it, there seems to be an epigenetic component to overall plant structure. The dogma that cell division patterns alone determine the size, structure, and function of plant organs might need to be re-evaluated in the light of this evidence.

The Division Site

During interphase, the microtubule array is organized perpendicular to the long axis of cell expansion. It encloses most of the cell volume (Marc, 1997; Staehelin and Hepler, 1996). As karyokinesis progresses, however, this array constricts towards the region where the cell will separate into daughter cells; the putative Division Site (DS). There is now evidence that a chemical distinction, as yet unknown, is conferred to this region which influences where the new cell plate will be anchored (Verma and Gu, 1996). The preprophase band (PPB), long known to be an indicator of the location of new wall fusion to the old (Traas *et al.*, 1995; Giménez-Abián *et al.*, 1998), seems to be influenced by this site. Changes in actin have been identified in this region (Lloyd, 1991; Staehelin and Hepler, 1996) and there are some data that suggest that this region has components that link the extracellular matrix (ECM) and cellular cortex via a transmembrane association (Verma and Gu, 1996).

The DS seems to be determined at the G2 phase of the cell cycle (Verma and Gu, 1996). Experimental manipulation of the developing cell plate has shown that stable fusion of the new wall can only occur at the region predetermined as the Division Site (Verma and Gu, 1996; Giménez-Abián *et al.*, 1998). Unlike the microtubule network, this site seems to be present throughout mitosis. Microscopy has determined that it is rich in F-actin and is

found at the site of PPB consolidation (Verma and Gu, 1996). Additionally, this region appears to be perturbed when exposed to cytochalasin D. Localized cell wall modifications and thickenings have been reported in this area in some cell types as well as cohesion between the plasmalemma and this region after mild plasmolysis (Verma and Gu, 1996). It is possible that this area provides the cell with some kind of "memory" where the new cell plate should fuse due to the presence of some kind of recognition or cell wall maturation factors.

Consolidation of microtubules at the DS; the preprophase band

A predictable microtubule complex becomes assembled at the DS (Staehelin and Hepler, 1996; Marc, 1997). This is the PPB, which is transiently visible prior to mitosis. The actual role of the PPB is unknown; it is usually used as a landmark for the fusion of the phragmoplast to the mother cell wall (Verma and Gu, 1996; Lloyd, 1991). Experiments have determined that actin filaments, once present throughout the cell cortex, disappear as the PPB is formed. This actin zone of exclusion is a narrow band that persists throughout the development of the new cell wall (Staehelin and Hepler, 1996).

Mutant plants have been obtained that lack a PPB (Traas *et al.*, 1995; McClinton and Sung, 1997). Though cellular organization within these organs is highly disrupted and plants grown from homozygous mutant seeds are inviable, overall organ formation and size are surprisingly normal, supporting the suggestion that cell division itself is not the basis for organ size and shape (Kaplan, 1992; Cooke and Lu, 1992). An experiment that disrupts cytokinesis before the phragmoplast (see next section) becomes established has shown that the PPB marks a region where the phragmoplast will stably join the mother wall, but

variability is allowed. The distance between the phragmoplast and where the PPB was once situated suggests some kind of communication for where the leading edge of the phragmoplast bonds to the mother wall; the phragmoplast will choose the closer of two PPB marked areas (Giménez-Abián *et al.*, 1998).

Formation of the phragmoplast

The phragmoplast is a structure found only during plant cytokinesis and is composed of microtubules, microfilaments, and dictyosome-derived vesicles. The microtubule spindle array which directed karyokinesis migrates towards the middle of the cell, where division will occur (Staehelin and Hepler, 1996). As this array moves equatorially, it also extends laterally in all directions to form an interdigitating complex of microtubules that spans the entire width of the cell (Samuels *et al.*, 1995). As the microtubular structure forms, actin filaments assemble in this region, also with their plus ends proximal to the equator. Plus end motor proteins like kinesin (for microtubules) or myosin II (for microfilaments) would thus be directed toward the site of cell division (Staehelin and Hepler, 1996)

Dictyosome vesicles apparently make use of some form of motor protein (Asada and Collings, 1997) and are seen to aggregate in the center of the microtubule structure. A study that used carefully preserved cryofixed tissue has provided information about the activity of vesicles during cell plate formation (Samuels *et al.*, 1995). Vesicles travel to the site of the forming cell plate and aggregate at the middle. A short-lived tubule (thickness 20 nm) extends from many of these 64 nm thick vesicles and forms a U-shaped hook which intertwine with others to create the tubulo-vesicular network stage of the cell plate. These hooks are postulated to disperse energetic forces which oppose vesicle combination. This

network of intertwined tubules and vesicles develops into the tubular network, defined by when vesicles have combined to form a broad network where individual vesicles are no longer found. The small holes in this network fill in as the fluid structure of the tubular network stabilizes into a more uniform layer. This plate becomes the fenestrated sheet; a broad shroud that extends from the center of the cell equator towards the edges. Strands of endoplasmic reticulum are caught within this sheet and undergo modifications to become primary plasmodesmata. Gradually, the only cytoplasmic continuities through this sheet are where plasmodesmata form (Kragler *et al.*, 1998).

The cell plate grows outward from the center (centrifugal development). The leading edge of this plate is developmentally younger than the central region because new material is added at the edges to extend the plate even while the central portion matures. When the leading edge of this plate contacts the mother wall, usually at the actin-depleted site where the PPB formed, fingerlike projections at this zone of adhesion fuse the mother and daughter cell walls.

A variety of components accumulate in the developing phragmoplast and have unique roles in the development and stability of the wall. Polysaccharides such as cellulose, a polymer of β(1→4)-D-glucose dimers oriented at 180° between each residue (cellobiose), polygalacturonan (pectin), and xyloglucan, a β(1→4)-D-glucan with xylose on many of its residues in a discrete and predictable pattern combine to form the complex that makes up the cell wall. Both callose, a rare β(1→3)glucan, and celluose are synthesized at the new plasma membrane (Cosgrove, 1997). Hemicelluloses and pectins are synthesized in the dictyosome and transported to the phragmoplast for secretion. The membrane borders of these vesicles

contribute to the new plasmalemma, but the large quantity of vesicles means that the amount of membrane lipids are in excess for the phragmoplast; clathrin-coated vesicles have been identified and are thought to fulfil the role of membrane recycling (Samuels *et al.*, 1995).

Genetic analysis has revealed several mutants that are defective in some aspect of cytokinesis and molecular data have been collected that suggest their role in cytokinesis. The *Arabidopsis* mutant *knolle* has been found to be defective in a syntaxin-like gene that probably causes vesicles containing cell wall building material to be targeted to inappropriate areas (Lukowitz *et al.*, 1996). The embryo-defective *emb30* (*gnom*) gene has been cloned and its protein sequence shows high homology to the yeast Sec7 protein which is involved with Golgi vesicle formation (Shevell *et al.*, 1994). Sec7 is a nonclathrin coat protein that directs vesicles from the Golgi to specific intracellular compartments.

Another *Arabidopsis* cytokinesis mutant, *keule*, is apparently defective in cell wall formation, but the defect has not been molecularly characterized. Abnormal planes of division, cell wall gaps and wall stubs, and lethality at the seedling stage are part of the *keule* phenotype. Defects in *keule* resemble defects in caffeine treated plants (Assaad *et al.*, 1996). Caffeine blocks the fusion of vesicles at the cell plate, preventing the stabilization of the structure and disrupting the fusion of the cell plate to the mother cell wall (Hepler and Bonsignore, 1990). Based on these observations, *KEULE* is thought to allow or direct the fusion of vesicles at this stage. *cyd*, a mutant in pea, has also not been characterized at the sequence level, but experiments demonstrate that the phenotype agrees with a defect in vesicle fusion (Liu *et al.*, 1995). The *cyd* defect can also be phenocopied in wild-type plants with the application of caffeine.

Analysis of these mutants will give insight into what processes are involved in cytokinesis and the proteins which are required to carry them out. Interestingly, amino acid sequence commonalities between evolutionarily diverse species have been of great benefit to elucidate gene function. Despite the fact that animal, yeast, and plant cell division occurs in a different fashion, similar genes, such as *EMB30* (Sec7) and *KNOLLE* (syntaxins in neurons and yeast) are found in both systems. The usefulness of sequencing projects in a number of model systems is again underscored.

THE PLANT CELL WALL

The plant cell wall is a dynamic, complex structure that has important physical and chemical properties that impact not only on the growth aspect of the plant itself, but also has ecological implications. The cell wall acts as an exoskeleton for the cells of the plant. It provides support for the plant's growth habit, controls cell size, shape, and attachment to adjacent cells, provides a barrier to pathogens, and contains latent regulatory molecules that can monitor changes in the environment and thereby allow the plant to achieve homeostasis (Fry, 1988). Cell walls can contain substances of limited digestibility, causing them to be a major factor in understory litter in forests, a contributor to soil texture, and a source of industrial waste, particularly during the process of paper manufacture. Fibers of cotton are important economically, and the domestication and transplantation of cotton cultivars, with the intent to process the rich cellulose fibers produced during seed formation, have altered the ecology of Texas and Oklahoma (Supak *et al.*, 1992).

Composition and organization of the plant cell wall

Members of the plant kingdom vary extensively in biochemical composition. Though polysaccharides in cell walls from most plants are somewhat similar, grasses have a makeup that differs substantially from the composition of most plant polymers (Fry, 1988; Gibeaut and Carpita, 1994). Plant cell walls are composed of about 30% (dry weight) cellulose. Though grasses have cells walls composed of only 5% pectin, 4% xyloglucans, 0.5% glycoprotein, and 30% each of arabinoxylan and (1→3), (1→4) mixed glucan, the other monocots and dicots have 35% pectin, 25% xyloglucan, 5% glycoproteins, 5% arabinoxylan, and little if any (1→3), (1,4) glucan (Fry, 1988). These differences in composition suggest that different polymers can serve the same functions in each system. Both grasses and dicots have cell walls that have similar properties and functions. For example, both respond to growth altering stimuli like a decrease in pH (Cosgrove, 1997). Thus, biosynthesis of wall polymers seems to be conserved among the majority of plants.

Cell Wall Composition in Dicots

Dicot cell walls are composed of cellulose, hemicelluloses, pectins, and glycoproteins. The cell wall must be flexible to allow cells to grow, uptake essential substances, and communicate with adjacent cells. Nutrients must be able to reach all living cells within a plant, despite each cell being enclosed within its own wall. Cell walls are utilized as an extracellular route of transportation of substances, known as the apoplasm (Mimura, 1995).

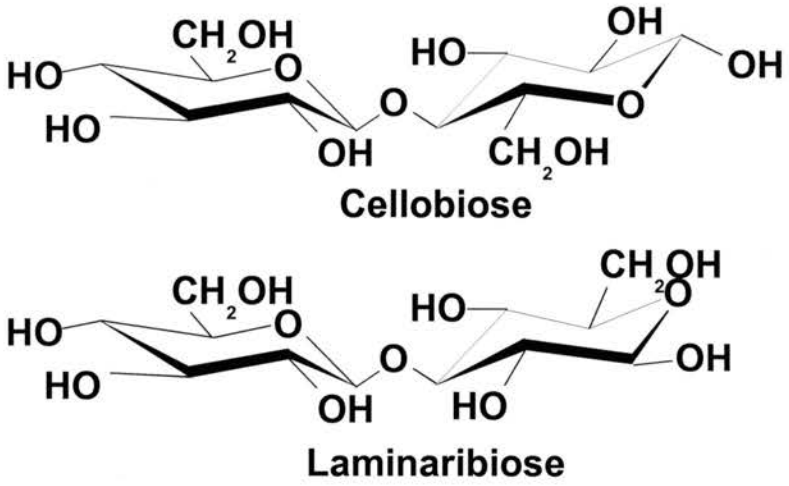
Cellulose is secreted at the plasma membrane by a multienzyme aggregate called the terminal complex (TC). The shape of the TC when viewed with an electron microscope gave

rise to its alternate name: the rosette (Delmer, 1987). The TC's function is to synthesize cellulose and secrete it outside the plasma membrane. To do this, however, it must not only synthesize the glucan, but also pass its product through the plasma membrane and organize parallel strands of glucan so they hydrogen bond into a semicrystalline state (Fig. 2). It is this state that gives cellulose the rigidity to support cell turgor and the growth aspect of the plant. Its ubiquitous presence in plants gives cellulose the distinction of being the most abundant polymer in nature.

The proteins that make up the TC are largely unknown. Cellulose synthase is of course involved, but it is hypothesized that a pore subunit, a crystallization subunit, and a subunit that catalyzes the formation of UDP-glucose which is the substrate for cellulose are parts of the TC (Delmer and Amor, 1995). It is thought that sucrose synthase is the protein that feeds UDP-glucose, which is formed through the hydrolysis of sucrose, to cellulose synthase (Robinson, 1996). Additionally, regulatory subunits and a subunit that interacts with the plasmalemma-associated cytoskeleton are believed to be in the TC (Delmer *et al.*, 1987). These proteins have not yet been identified, and how they interact is still unknown.

One strange phenomenon reported by Delmer (1987) is that isolated plasma membrane fractions of cotton produce callose instead of cellulose *in vitro*. For the last 10 years there was some evidence that cellulose synthase, which was present in the membrane fractions analyzed, would default to callose synthesis when perturbed. The difference between callose and cellulose is that callose is an unbranched $\beta(1\rightarrow3)$glucan and cellulose is a $\beta(1\rightarrow4)$glucan. If cellulose synthase were to adopt an improper conformation, perhaps the active site can catalyze the formation of a callose linkage instead of a cellulose one. Though

a



b

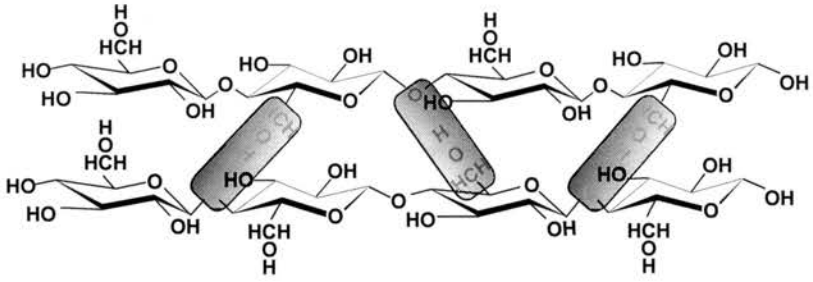


Figure 2: Structure of cell wall glucans.
(a) Structures of the repeating elements of cellulose (cellobiose) and callose (laminaribiose). Note that residues are rotated 180 with respect to each other along the axis of the -O- bond. (b) Hydrogen bonding between adjacent cellulose chains. Elements involved in the bond are indicated by shading.

(a) is adapted from Delmer (1991). (b) is redrawn from Gardner and Blackwell (1974).

callose synthases have been identified in plants, their presence does not preclude the possibility of this form of reaction in cellulose synthase (Škalamera and Heath, 1996).

The plant herbicide dichlobenil (DCB) has been shown to dramatically reduce the amount of cellulose that accumulates in growing plant cell walls (Montezinos and Delmer, 1980). DCB interacts with a component of the TC, and is theorized to interfere with cellulose synthesis (Delmer, 1991). Plants treated with DCB show incomplete cell walls which are enlarged and vacuolated, and callose is also deposited (Vaughn *et al.*, 1996). This also supports the theory that cellulose synthase, when perturbed, causes accumulation of callose.

Acetobacter xylinum, a prokaryote, can extrude cellulose fibers from is plasmalemma when grown under permissive culture conditions. Cellulose synthase in this bacterium has been cloned and analyzed (Pear *et al.*, 1996), but attempts to use this sequence as a probe to detect a similar gene in plants had failed (Delmer and Amor, 1995). Recently, however, orthologs of the bacterial *celA* genes have been found in plants due to the use of hydrophobic cluster analysis (HCA) (Pear *et al.*, 1996). HCA allows for the identification of homologous structure through examination of three dimensional relationships of a folded polypeptide (Haigler and Blanton, 1996). The nucleotide sequence linking these preserved regions differ dramatically from *A. xylinum*, complicating traditional cloning techniques and thus delayed the identification of cellulose synthase in higher plants (Pear *et al.*, 1996).

Further analysis of the putative protein structure of cellulose synthase has shown that the "callose as a default product" model is not likely from the same active site that forms cellulose (Brown *et al.*, 1996; Carpita and Vergara, 1998). The repeating cellobiose residues

are recognizable as linked β(1→4) glucose residues rotated 180° with respect to each other. Catalysis of glucose polymerization therefore would face a rotation barrier if added one unit at a time. Cellulose formation is very rapid and it is likely impossible to re-orient the entire active site in such a way that the catalysis of glucose addition would be possible in such a short time. Thus, the current model is that glucose is appended to the chain in pairs, with each active site that holds a UDP-glucose substrate rotated 180° with respect to the other (Saxena *et al.*, 1995). Thus, the simple tertiary structure conformation change that was hypothesized to cause callose to be formed instead is complicated immensely.

Callose is often not mentioned as a normal cell wall component. Though it is found around the neck of plasmodesmata and is deposited during wound responses, callose is not an abundant component of the healthy cell wall. However, callose is synthesized at the phragmoplast membrane of the fenestrated sheet, long before cellulose accumulates (Samuels *et al.*, 1995; Škalamera and Heath, 1996). Because it does not extensively hydrogen bond with adjacent glucans, callose is thought to supply (or at least allow) the spreading force required to enable the fenestrated sheet to contact and bond to the mother cell wall (Staehelin and Hepler, 1996). Callose is synthesized by a membrane-bound enzyme and appears in the cell plate transiently, during the early vesicle fusion stage.

Another polysaccharide class of the cell wall is that of the hemicelluloses. These include mannans and especially xyloglucans which accounts for a large portion of wall polysaccharides (Lea and Leegood, 1993; Cosgrove, 1997). These can alter the rigidity and fluidity of the wall, creating structural integrity for the cell as well as accommodation of

growth (Cosgrove, 1997). Hemicelluloses are polymerized in the dictyosome and transported to the plasmalemma via vesicles (Samuels *et al.*, 1995; Cosgrove, 1997).

The last abundant class of polysaccharides in the cell wall is pectin. Pectins are found throughout the cell wall in methylesterified form and interweave between other cell wall components (Steele *et al.*, 1997). The middle lamella, where the cell walls of adjacent cells adjoin, contains a de-esterified form of pectin which interacts with calcium. The middle lamella thus "glues" cells together. Pectin polysaccharides consist of a homogalacturonan backbone with polysaccharide side chains. Pectins are characterized as having "hairy" domains, which possess extensive and elongated side chains, and "smooth" regions of about 200 residues that have no side chains (Fry, 1988). The smooth regions can be de-esterified and interact with adjacent de-esterified chains through an interaction with Ca^{++} at the middle lamella. Pectins seem to play a role in cell elongation (Knox *et al.*, 1990).

The polysaccharides are not separated into discrete regions. Though pectins are indicated at the middle lamella, that is true mostly for the de-esterified pectins; esterified polygalacuronans are found throughout the cell wall (Steele *et al.*,1997). The "hairy" regions might serve to anchor adjacent polysaccharide chains. Xyloglucans were once thought to interact with the surface of cellulose microfibrils, causing interfibrillar structural support. However, there is evidence that xyloglucans actually are interwoven within microfibrils, causing small disruptions in their structure and even more firmly intermeshing adjacent microfibrils (Cosgrove, 1998).

Proteins also make up a portion of the cell wall. The hydroxyproline rich glycoproteins (HPRGs), characterized by their repetitive polypeptide sequence abundant in

hydroxyproline, were at first classified as extensins because of a hypothesized role in allowing the cell wall to grow. This function has since been disproved, and the presence of HPRGs probably stabilizes the polysaccharides within the wall. Other proteins have been identified in the cell wall and are puzzling because of their abundance in only one type of tissue (Cosgrove, 1997). Arabinogalactan proteins (AGPs) are heavily glycosylated and soluble (Gibeaut and Carpita, 1991). They are putative shuttle molecules, stabilizing the contents of cell wall vesicles until they combine with the plasmalemma. AGPs are found in multiple forms and in tissue- and cell-specific expression patterns. Additionally, peroxidases, chitinases, and other proteins with a cell defense function have been identified within the cell wall.

Plants with altered cell wall composition

Interestingly, some mutant plants have been found that are altered in their cell wall compositions. Mutants of *Arabidopsis* were screened for a radially swollen phenotype with the idea that a defect in cellulose synthesis would mimic the phenotype of plants treated with the cellulose synthesis inhibitor DCB (Arioli *et al.*,1998). Upon cloning the *rsw1* gene, homology to the catalytic sites in the putative cellulose synthase gene recently characterized in plants (Pear *et al.*, 1997) was noticed. Additionally, careful analysis of wall polysaccharides showed a decrease in cellulose and abnormalities with TC organization. It was further noted that what cellulose that was produced did not become organized into a semicrystalline state, suggesting that *RSW1* also plays a role in organizing the products as they are produced.

Another mutant in *Arabidopsis* that pertains to the composition of the cell wall was noticed in a screen that showed dwarfed growth and weakened stems (Reiter *et al.*,1993; Reiter, 1994). The *mur1* mutant appears to be completely deficient in fucose in plant shoots. This absence is attributed to a defect in fucose synthesis, because mutants grown in the presence of this sugar have wild-type levels of this sugar restored (Reiter *et al.*,1993). Fucose levels are different between the root and shoot of mutant plants, suggesting that there might be isoforms of the enzyme that are activated in different organs. Fucose is a component of xyloglucans, believed to assist in stabilizing adjacent cellulose microfibrils (Reiter *et al.*, 1997). This is supported by the observation that *mur1* plants have a twofold decrease in the force required to break the stems (Reiter *et al.*, 1993).

cyt1 AS A TOOL TO STUDY CELL WALL DEVELOPMENT

It is therefore clear that the cell wall is a complex structure and identification of the genes needed to form a normal cell wall can be accomplished through mutant analysis (Meinke, 1993). A mutant in *Arabidopsis* has been identified that has cell wall gaps, large vacuolated cells, and numerous cell wall defects and might therefore be useful as a means to investigate how the cell wall is formed through genetic analysis. This *cyt1* mutation is lethal because homozygous *cyt1* embryos cannot progress past the heart stage and do not grow in culture. The remainder of this dissertation will outline experimental analysis of the defects in this mutant with the aim of accumulating more information about how the plant cell wall is formed.

CHAPTER 3: INITIAL CHARACTERIZATION OF *CYT1*

INTRODUCTION

Two screens of progeny from an insertional mutagenesis project at DuPont (Feldmann, 1991) yielded 178 embryo defective mutants which fell into six classes (Castle *et al.*, 1993; Castle and Meinke, 1994). These mutants were assigned to linkage groups by crossing plants heterozygous for each isolated embryo defective line with tester lines constructed to facilitate mapping (Patton, 1991). Two mutant lines characterized by fleshy embryos that arrested at the heart stage were first classified as 1054 and 1985 and later renamed *emb101-1* and *emb101-2* respectively because they were found to be allelic. The *emb101* locus maps to position 65 cM on chromosome 2 of the classical genetic map. It is closely linked to *as*, which maps 2 cM south.

The mutant embryos from these 2 alleles differ in size, suggesting different allele strengths, but otherwise resemble puffy, enlarged heart-stage embryos. Initial sections through these embryos indicated cell wall defects consisting of incomplete or thickened cell walls (Yeung, pers. comm.). This suggested that cytokinesis was impaired in this mutant. To reflect this phenotype, the mutants were then re-assigned the names *cyt1-1* and *cyt1-2* to reflect their *cytokinesis defective* phenotypes.

Dry *cyt1* seeds do not germinate when planted on soil or cultured on germination medium. To see if defective seeds could be rescued in culture, young embryos were excised from the testa and cultured on germination media. No growth was observed in any of these preliminary studies.

In this chapter, further characterization of the *cyt1* phenotype is described. Large numbers of embryos were cultured on different media and viability of the mutant embryos was assessed. Light and electron microscopy was used to gather more information on cell wall abnormalities, and rescue of mutant seedlings was attempted by crossing the *cyt1* mutation into another mutant line, *lec1*. Resulting double mutants might therefore precociously begin late embryogenic processes and activate a family member of *CYT1*, if one exists.

METHODS AND MATERIALS

Growth of plant material

Plants grown in pots

Arabidopsis plants were grown at 23 ± 3° C in 3 inch pots, 9 plants each, under 40 W fluorescent lights. The light regime was established with a timer that provided 16 h of light and 8 h dark cycles. Soil was prepared using 12 parts coarse vermiculite (Terra Lite, W.R. Grace Co., Cambridge, MA), 3 parts potting soil (Redi-Earth Peat-Lite, W.R. Grace) and 1 part sterile sand. Plants were watered daily with a fertilizer solution consisting of 1.6 g/l of 7-16-19 All Purpose Hyponex (Hyponex Co., Fort Wayne, IN) and 0.1 g/l 15-16-17 Peat Lite Special (Peters Co., Allentown, PA) in RO water.

Sterile germination of seeds

Sterile cultures were created as described by Baus *et al.* (1986). In short, seeds were sterilized by immersing them first in 70% EtOH for 30 s, 50% Clorox solution (Clorox bleach diluted with RO water) for 5 min, and then rinsed with sterile RO water 5-7 times. 50 sterile seeds each were plated in 100 mm petri dishes containing Murashige and Skoog (MS) inorganic salts (1962), 3% glucose, adjusted to pH 5.7, and solidified with 0.8% Phytagar (Gibco BRL, Grand Island NY).

Culture of embryos

Immature embryos were grown in sterile culture (Patton and Meinke, 1988; Franzmann *et al.*, 1989). Bolts from heterozygous plants were immersed in 70% EtOH for 30s, 30% Clorox solution for 5 min, then rinsed 5-7 times in sterile RO water. Using sterile technique, siliques were opened, the seeds were collected, and their embryos selected either intact (for germination studies) or after mechanical separation of cotyledons from the hypocotyl (for callus induction). Mutant *cyt1* embryos were always cultured intact. Embryos were quickly moved from the opened seed to the medium to ensure minimal desiccation. Media used were as follows: germination medium consisted of MS salts, 3% glucose, 0.8% Phytagar at pH 5.7; callus-inducing medium was made with MS salts, either 0.25% or 3% sucrose, 0.8% Phytagar, 1 mg/L 6-benyzlaminopurine (BAP) and 0.1 mg/L 1-naphthaleneacetic acid (NAA) at pH 5.6. Two concentrations of sugar were used to induce callus on the advice of Farhah Assaad (pers. comm.) because *keule*, another cytokinesis defective mutant, formed callus better on lower sugar media. The vital dye 2, 3, 5-triphenyl tetrazolium chloride (TTC) was added to callus-inducing medium (0.1% w/v, placed in the

dark to prevent photolysis of the reagent) to assess long-term metabolic activity of embryos *in vitro* (Müller *et al.*, 1997).

Microscopy

Dissecting microscope

Seed dissections were performed using a Wild M8 dissecting microscope as described by Meinke (1994). Light was directed onto the stage with desktop spot illuminators and manipulations performed (opening of siliques, seed dissections, etc.) with two pairs of #20 fine forceps. Cultured samples were viewed with the dissecting microscope through the Petri-dish cover.

Viability staining

Fluorescein diacetate (FD) can be used to test viability of plant tissues. Non-fluorescent FD is converted to a fluorescent product (fluorescein) by esterase enzymes present in living cells. UV illumination excites fluorescein which glows a brilliant yellow/orange. The presence of functional esterases suggests an active metabolism and therefore identifies living tissues.

FD solution was prepared immediately before use by mixing 0.1 ml of FD stock (5 mg FD per ml acetone) with 4.9 ml of a 5% sucrose solution. Young immature seeds were placed in drops of FD solution on a microscope slide and a glass coverslip over them was tapped with forceps to cause the embryos to be extruded into the stain. Larger embryos were dissected with fine forceps and placed directly into the stain. Slides were incubated in reduced light for at least 5 min before observation on a Nikon Optiphot epifluorescence microscope equipped with a mercury vapor lamp. Incident light was controlled with a 420-

490 nm blue excitation filter (UV-2A), a 510 nm dichroic mirror, and a 520 nm green barrier filter. Tissues killed by a 10 min immersion in 2% glutaraldehyde, 2% paraformaldehyde solution prior to FD staining were observed as described above, representing a non-metabolizing control.

Embedding of tissue in plastic

Embryos were prepared for fixation by puncturing the seed coat or dissecting the embryo from the testa. These manipulations were performed with the tissue submerged in fixative to prevent desiccation. Samples were fixed for 2 h at RT in 2% glutaraldehyde and 2% paraformaldehyde (0.05 mM PO_4 buffer, pH 7.2) in a light vacuum (-75 kPa), washed 3X in buffer (0.05 mM PO_4 buffer, pH 7.2) and then postfixed 2 h in 1% OsO_4 (0.05 mM PO_4 buffer, pH 7.2) after rinsing three times in PO_4 buffer (pH 7.2), 15 min for each rinse. Samples were rinsed three times in buffer and then dehydrated through an ethanol series: 30%, 50%, 70%, 90%, 95% and 3X 100% EtOH rinses, 15 min per step.

One of two resins was used for embedding samples: Spurr's resin or L.R. White resin. Both resins were purchased from Electron Microscopy Sciences (Fort Washington, PA, USA). Each resin has qualities that better preserve either structural or antigenic characteristics. Spurr's resin has the higher contrast, especially at electron microscope resolution, though it can harden non-uniformly, while L.R. White is easier to handle and preserves the antigenicity of molecules better than Spurr's resin.

One set of samples was infiltrated with a medium hardness Spurr's resin (10 g vinylcyclohexene dioxide, 6 g diglycidylether of polypropyleneglycol, 26 g nonenyl succinic anhydride) after the tissue was submerged in two changes of absolute propylene

oxide as a transition solvent. Infiltration was accomplished for both Spurr's resin and L.R. White with an EtOH:resin series at ratios of 3:1, 2:1, 1:1, 1:2, 1:3, and three pure resin changes, 30 min each as described above for Spurr's resin. Infiltrated tissues were polymerized at 65° C for 16 h in BEEM capsules.

Light microscopy

Sections of embedded tissues were taken using a Leica 2045 Multicut Microtome (Leica, Bellevue, WA). Thickness was set for 3-5 μm and sections were cut with a glass knife freshly prepared using an LKB 7801B KnifeMaker (LKB, Sweden). A boat was affixed to the knife with dental wax so that sections floated as ribbons next to the cutting surface when the boat was filled with water. To prevent sections from floating free during staining, microscope slides were covered with a subbing solution as follows: slides were cleaned overnight in a 70% EtOH solution at pH 2.5 and then dipped (while still wet) in a filtered solution composed of 0.5 g/l chrome alum (chromium potassium sulfate), 5 g/l gelatin, then dried upright overnight in a dust-free area (Yeung, 1984). Ribbons of sections were transferred to these slides and then heated until the water droplets had evaporated and the sections became attached to the slides. These slides were stained as described below.

Electron microscopy

Sample blocks were sectioned at 80 nm thickness (gold-silver) on an MT-6000 Ultramicrotome (Sorvall/DuPont, Wilmington, DE, USA) using a Diatome 45° diamond knife (Diatome US, Fort Washington, PA). Ni and Cu grids (Electron Microscopy Sciences, Fort Washington, PA, USA) were coated with formvar to support the sections as described by Bozolle and Russell (1992). In brief, an EtOH-cleaned and dried glass microscope slide

was submerged into a 3% (w/v) preparation of formvar in ethylene dichloride, withdrawn, and allowed to air dry. The formvar coating was scored and caused to float upon a bath of warm filtered NP water. Grids were placed on the floating film and collected by scooping with a swatch of Parafilm. After drying, the grids were stored in a desiccator before and after being used to collect sections. To collect sections, grids were dipped into the knife boat just enough to touch and adhere to sectioned material which was floating on the water surface.

Staining of sections

Light microscopy

Slides containing sectioned material were rinsed with tap-water and stained with periodic acid-Schiff's reagent (PAS) to reveal total carbohydrate, then counterstained with 1% amido black 10B (Nickle and Yeung, 1992). Slides were preserved using Accumount 280 mounting fluid (Baxter, McGaw Park, IL), and 1½ Fisherbrand coverslips (Fisher Scientific, Pittsburgh, PA). Slides were then observed using Kohler illumination on an Olympus BH-2 microscope.

Electron microscopy

Grids containing sections were immersed in three successive water baths (rendered CO_2-free via 5 min boiling) before being immersed for 1 min in uranyl acetate solution (5% aqueous). Grids were then rinsed three times in CO_2-free water and stained for 2 min in lead citrate (0.3% in CO_2-free, slightly basic NP water). Three rinses in CO_2-free water removed excess stain. The grids were then viewed at 80 keV on a JEOL 100 CX electron microscope.

Whole mount observation of embryos

Whole mounts of wild-type and mutant embryos were examined as described by Castle *et al.* (1993). Immature seeds were fixed with 2% paraformaldehyde in 0.05 M PO_4 (pH 6.5) for 10 min and submerged in Hoyer's solution (7.5 g gum arabic, 100 g chloral hydrate, 5 ml glycerine, 30 ml RO water) then covered with a Fisherbrand 1½ coverslip. After 3-10 hours, slides were viewed with an Olympus BH-2 microscope equipped with Nomarski optics.

Crosses between *lec1* and *cyt1*

Mutant *cyt1* plants were crossed with rescued *lec1/lec1* plants both as the male and female. The *lec1* mutant was originally isolated in the Wassilewskija ecotype and bypasses late embryogenic stages, instead showing seedling characteristics at the embryo stage of development (Meinke, 1992; Meinke *et al.*, 1994). The female parent for each cross was prepared by removing the 3 or 4 youngest post-anthesis flowers and opening and emasculating the 3 or 4 next-youngest closed flower buds. Pollen from the male plant was then deposited on the exposed stigma of flowers prepared in this way. The pollinated flowers were then marked by removing 4 of the flower buds apical to the flowers used in the crosses, and colored thread was tied to the bolts used for the crosses. Plants were then moved to growth chambers until siliques became brown and mature. Resulting F_1 seeds were planted as described above. Plants that showed segregation of both *lec1* and *cyt1* morphologies were identified as double heterozygotes. From these plants, all *cyt1*-appearing embryos were collected at the youngest identifiable stage and plated on callus-inducing medium to determine late embryo viability.

RESULTS

Growth of *cyt1* plants

Plants heterozygous for the *cyt1* mutation appeared normal except that their siliques contained 25% defective seeds following self-pollination. Mutant seeds were easily distinguished when wild-type seeds reached the late torpedo stage of development (Fig 3a), though mutant embryos dissected from the testa could be identified as early as the heart stage. When dry seed was harvested from senescent plants, seeds homozygous for *cyt1* were readily distinguishable because they were shriveled and dark compared to wild-type (Fig 3b). When mutant seeds are planted, they do not germinate, suggesting that *cyt1* mutants are desiccation intolerant (Table 1).

Two allelic *cyt1* mutants were studied in this project. Dissected intact *cyt1-1* and *cyt1-2* embryos are shown in Figure 4 (Fig. 4a, b, respectively). Mutant embryos are from siliques containing mature green wild-type embryos. The *cyt1-1* allele produces embryos that can fill the seed coat by late development, while *cyt1-2* embryos are smaller, suggesting that this might be the stronger allele. Mutant embryos are pale green and appear slightly disorganized at the cellular level, though organ formation occurs since cotyledons and a hypocotyl are distinguishable. Mutant embryos quickly shrink due to desiccation after being removed from the seed coat. Wild-type isolated embryos at the torpedo (Fig. 4c) and cotyledon stage (Fig. 4d) are included for comparison.

In vitro embryo rescue techniques failed to rescue immature *cyt1* embryos. Even when very young heart-stage explants were used, no visible growth of *cyt1* mutants occurred. Media supplemented with phytohormones IAA and BAP, which stimulate rapid

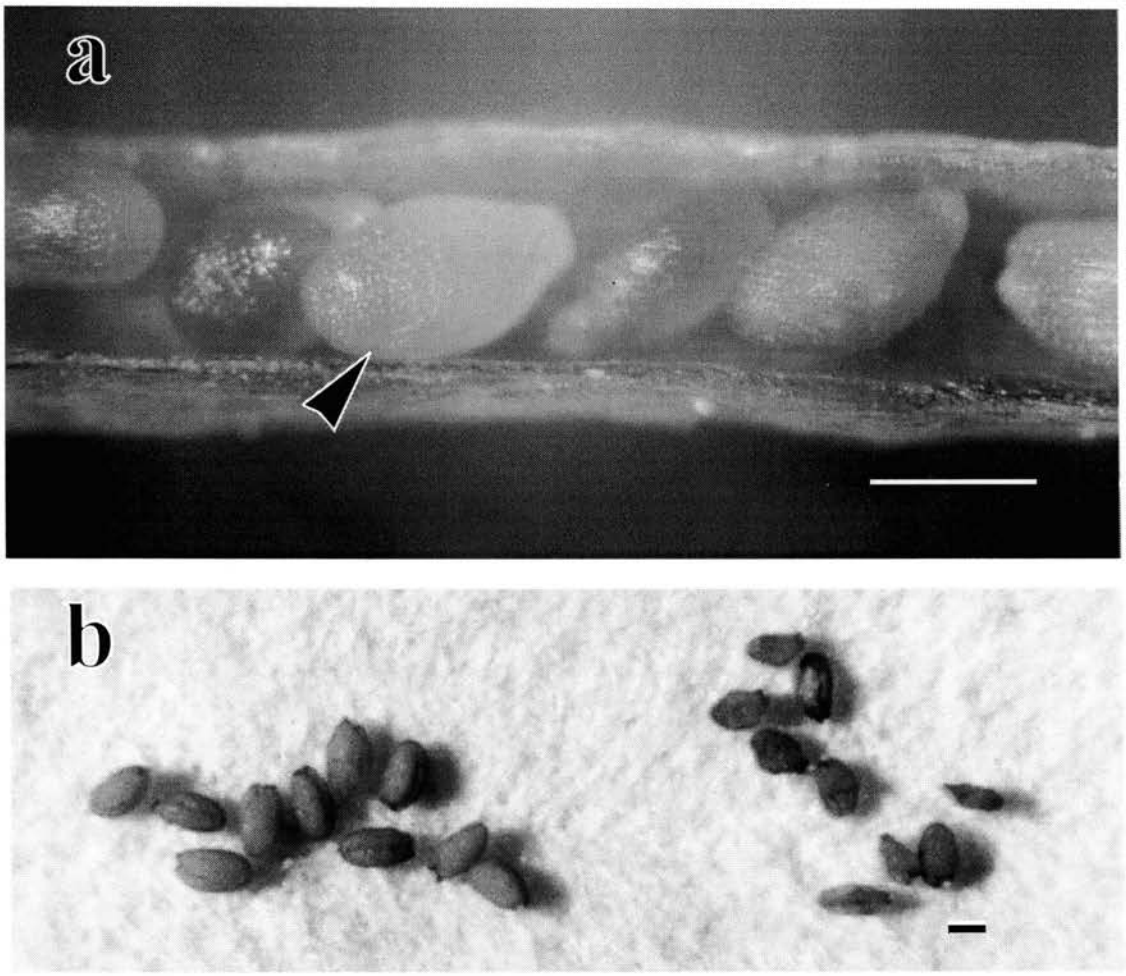


Figure 3: Characteristics of the *cyt1* seed.
An opened silique from a *cyt1-1*/+ heterozygote (a). The mutant seed is pale yellow (arrowhead) compared to surrounding wild-type seeds.
Dry seeds from a *cyt1-1*/+ heterozygote parent (b). Mutant seeds (right) are shrunken and dark brown while wild-type seeds (left) are round and plump.

Scale bars = 0.5 mm.

Table 1. Germination of dry *cyt1* seeds on MS medium

	0.25% Sucrose		3.0% Sucrose	
Genotype	Embryos cultured	Percent response	Embryos cultured	Percent response
wild-type	29	93	27	88
cyt1-1	141	0	135	0
cyt1-2	107	0	112	0

Medium used was MS sucrose medium. Response was considered positive if the radicle emerged from the seed coat and formed root hairs.

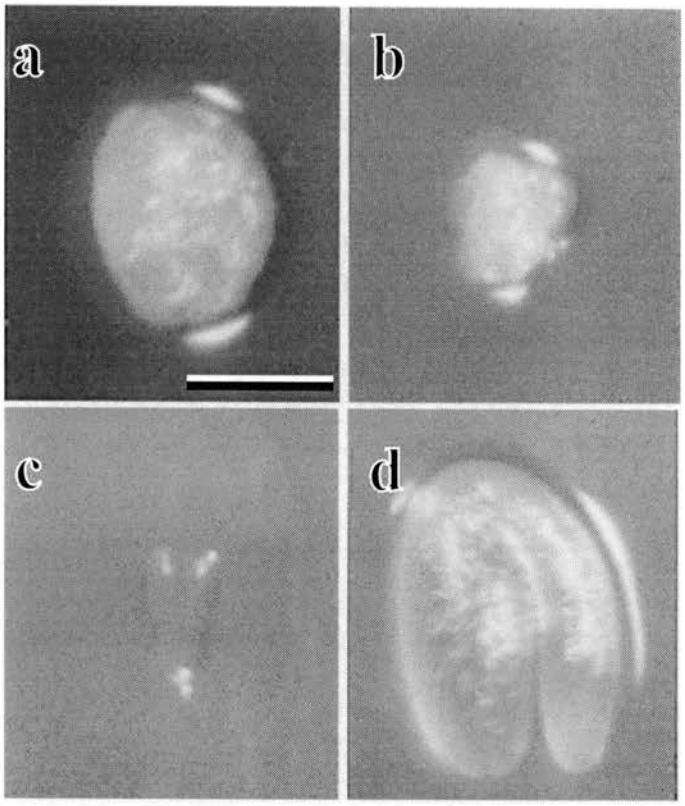


Figure 4: Morphology of excised embryos
Isolated *cyt1-1* (a) and *cyt1-2* (b) embryos seen through the dissecting microscope. These were taken from siliques containing mature green stages of embryos. Wild-type torpedo (c) and mature green (d) embryos at the same stage as (a, b) are shown for comparison.

Scale bar = 200 μm.

establishment of callus from wild-type explants (Patton and Meinke, 1988), also did not elicit any discernible growth from *cyt1-1* or *cyt1-2* despite incubation times spanning months (Table 2). Other cytokinesis defective mutants, *knolle* and *keule*, were able to create healthy-looking callus after two weeks in culture, as were the wild-type controls. Media containing lower sucrose concentrations allowed more rapid callus proliferation with *knolle* explants (data not shown, also agrees with observations by Farhah Assaad, pers. comm.). The absence of *cyt1* response to culture is interesting because other embryo-defective mutant lines which arrested as early as the globular stage have been shown to produce callus (Franzmann *et al.*, 1989). The cell wall defect alone cannot explain the lack of *cyt1* growth in culture because *knolle* and *keule* mutants, which also exhibit defective cell walls, not only grew in culture but also could germinate when dry seed was planted. The lethality of *cyt1* embryos indicates a critical role for *CYT1* in plant cell growth.

Viability of *cyt1* embryos

FD has generally been used for cell viability tests based on the presence of esterases in living tissues. In order to determine if immature *cyt1* mutants are suitable for culture, isolated embryos were submerged in FD to see if these enzymes were functioning. Living tissues showed fluorescence indicating metabolic activity, suggesting they are alive and potentially able to grow under permissive conditions. Figure 5 shows that all cells in young *cyt1* (Fig 5a) and wild-type embryos (Fig 5b) are able to hydrolyze FD and therefore are alive. Older wild-type embryos maintained their viability throughout all tissues (Fig. 5d), but only some cells in older *cyt1* embryos fluoresced (Fig. 5c), suggesting gradual cell death as the embryo matures. Embryos exposed to glutaraldehyde for 10 min prior to vital staining

Table 2. Viability of mutant embryos in culture

	0.25% Sucrose		3.0% Sucrose	
Genotype	Embryos cultured	Percent response	Embryos cultured	Percent response
wild-type	48	90	48	96
cyt1-1	79	0	70	0
cyt1-2	111	0	114	0
knolle	45	80	36	36
keule	41	68	40	60

Embryos were removed from seeds prior to desiccation, placed on callus-inducing media differing in sugar concentration, and classified as responding if they produced callus after 6 weeks in culture.

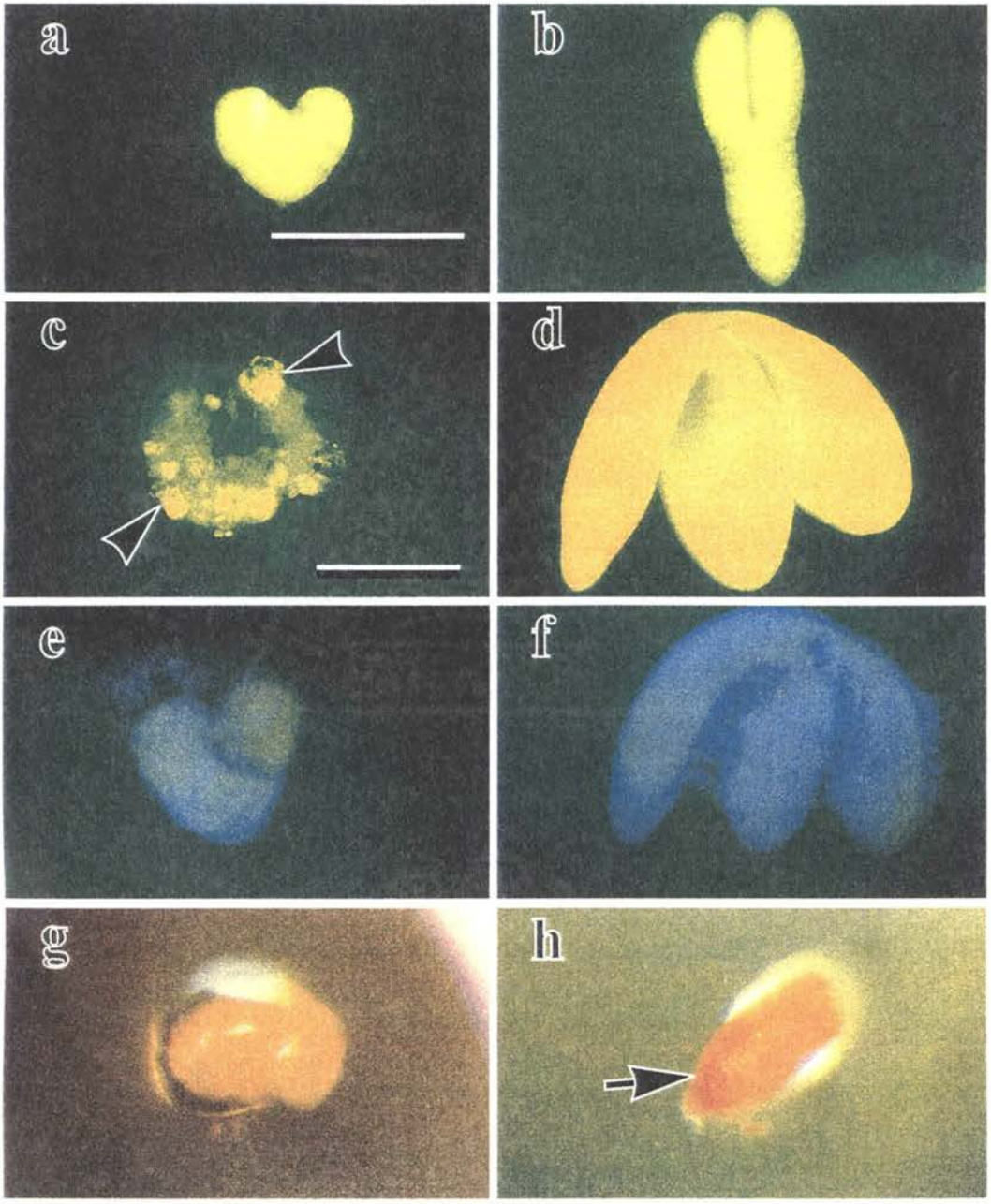


Figure 5: Viability staining of *cyt1* and wild-type embryos.
(a-f) Embryos incubated in fluorescein diacetate. Glowing yellow indicates hydrolysis of the substrate to the fluorescent compound fluorescein. Young isolated embryos from *cyt1-1* and wild-type fluoresce brightly, indicating the presence of metabolic enzymes (a,b). Older wild-type embryos hydrolyze the substrate (d), but *cyt1-1* mutants (c) show progressively less enzyme activity except for localized cell clusters (arrowheads). A 10 min exposure to 2% glutaraldehyde killed both *cyt1-1* (e) and wild-type (f) and eliminated hydrolytic enzyme activity.
(g-h) TTC staining of tissues in culture. Metaboltic enzymes reduce colorless TTC into a red substance. In culture, *cyt1-1* does not become red (g), though wild-type reduces and takes up the dye (h). Root tip is indicated by the arrow. Scale bars =200 μm. (a, b), (c-h) are at the same magnification.

showed a substantial reduction in fluorescence (Fig. 5e, f), confirming that cell death eliminates fluorescence.

Wild-type tissues plated on medium containing TTC turned red within 24 hours of plating (Fig. 5h), indicating that the vital dye was hydrolyzed and taken up by metabolically active tissues. No staining of *cyt1* tissues was seen even after a week on the medium (Fig. 5g). This seems to contrast with the results from FD, but the color change could be more gradual with TTC because it seems to concentrate in tissues more slowly, thus requiring longer for a positive result to be seen. This would make it a better long-term indicator of explant viability in culture. The lack of TTC staining in *cyt1* mutants might be indicative of an intolerance to culture where mutant explants rapidly die due to trauma during embryo excision or an incompatibility with the culture medium.

The cellular phenotype of *cyt1*

Nomarski observation of cleared seeds showed that early development of *cyt1* appeared to be normal. Seeds from siliques containing globular and early heart stage did not consistently show a population of defective embryos that numbered 25% of the whole sample examined, so identification of mutants before the heart stage was unreliable. Variations of development in seeds from heterozygous plants were not significantly different from normal deviations seen in wild-type plants, and the suspensor, protoderm, and hypophysis develop appropriately in all young embryos. Figure 6 illustrates subtle early signs of mutant characteristics (Fig 6a, b) and more readily identifiable late characteristics (Fig 6c, d). Rare early defects can be seen at the heart stage of development; altered cell division patterns can be seen in the protoderm and internal tissues, and some walls appear

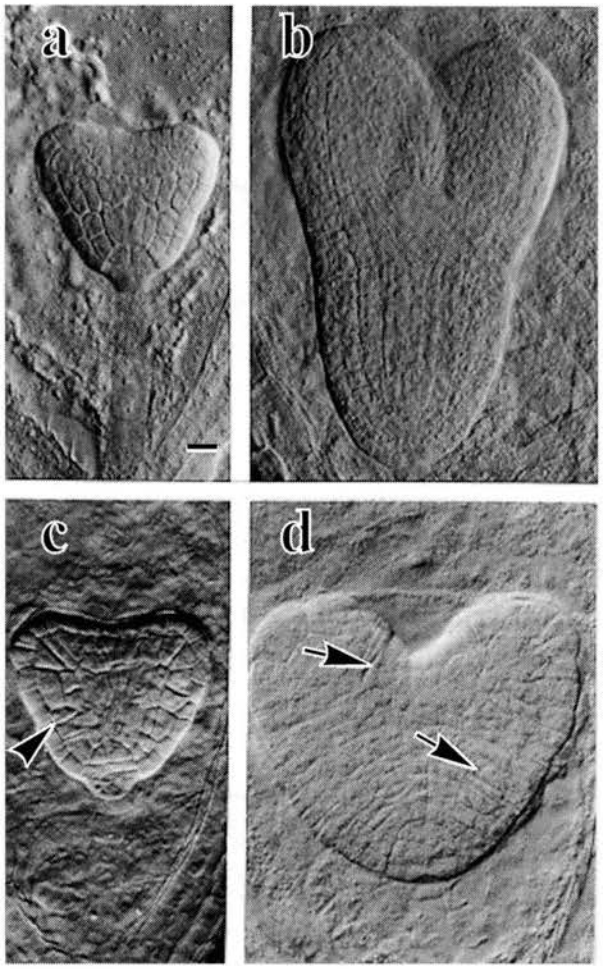


Figure 6: Nomarski photomicrographs of embryos.
At the heart stage, wild-type (a) and *cyt1-1* (c) embryos are similar in shape but mutant embryos exhibit irregular patterns of cell division (arrowhead) and cell walls appear more defined. By the torpedo stage, wild-type embryos (b) show elongation of the hypocotyl and cotyledons whereas *cyt1-1* embryos (d) appear flattened and bloated. Thickened cell walls (arrows) can be seen in mutant embryos at this stage.

Scale bar = 40 μm.

slightly thickened in mutant embryos (Fig 6b). As mutants develop further, the shape becomes distorted and the heart stage embryos become bloated and spread laterally (Fig 6d). The protoderm cells in *cyt1* mutants thicken, becoming columnar. Interestingly, the cytokinesis-defective mutants *knolle* and *keule* show more significant deviation from wild-type much earlier than *cyt1* during development, yet can germinate and form seedlings, while *cyt1* dies without differentiating past the heart stage.

Mutants defective in cytokinesis can be detected by viewing sections through mutant embryos. Cell walls are rendered pink from the periodic acid-Schiff reaction showing cell wall stubs separated by gaps that create cytoplasmic continuities between adjacent cells (Fig. 7a, b). Unlike the *cyd* mutants of pea, where cell wall gaps are restricted to cotyledons (Liu *et al.*, 1995), *cyt1* gaps manifest throughout the embryo without a preference for a particular organ type. Defective cell walls are not found around every cell; many cells appear normal to the extent that they are completely enclosed by a normal-looking cell wall. Cell wall stubs can usually be traced to at least one mother cell wall, indicating that wall fragments do not float unattached within the cytoplasm. Furthermore, cell wall gaps probably exist as one or more perforations in a cell wall. One peculiar cell wall defect in *cyt1* is the extensive thickening of some walls. These thickenings are due to accumulation of polysaccharide material as shown by the intense pink PAS-positive staining. These thickenings are not reported for other cytokinesis-defective mutants (Liu *et al.*, 1995; Assaad *et al.*, 1996, Lukowitz *et al.*, 1996).

Other *cyt1* abnormalities are apparent with light microscope observation. Figure 7 shows that some cells in mutant embryos are quite large and irregular. Many of the cells

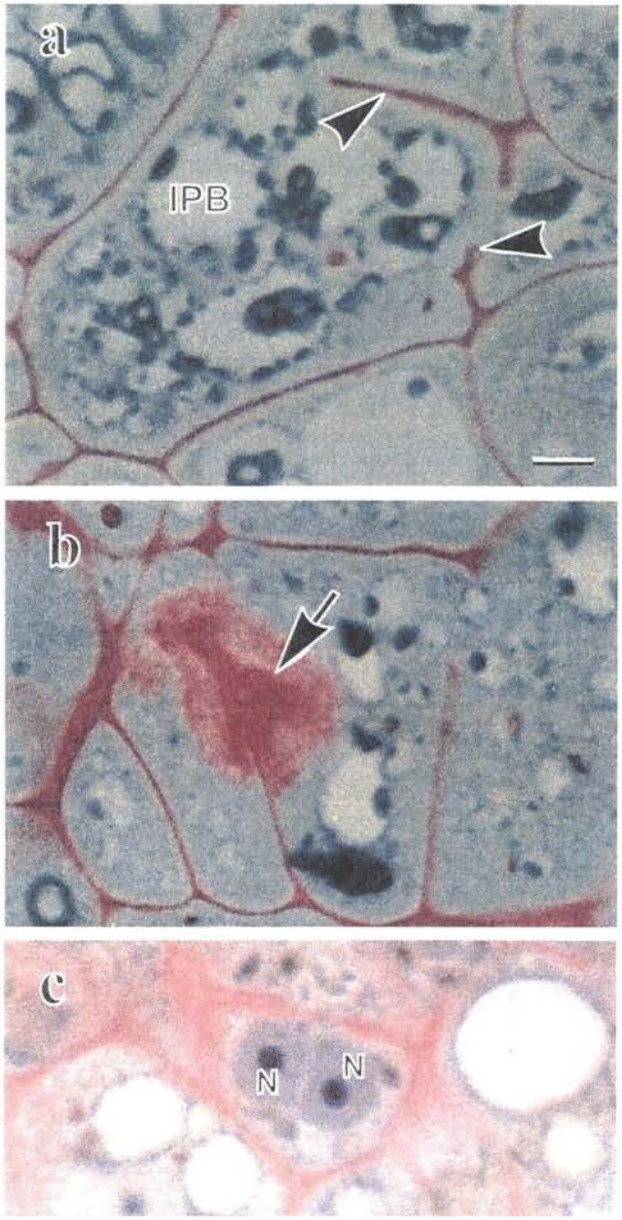


Figure 7: Photomicrographs of cell walls from *cyt1-1*
(a) Cells walls in these light micrographs of sectioned *cyt1-1* embryos stain pink with the PAS reaction. Cell wall stubs (arrowheads) and immature protein bodies (IPB) are evident. (b) Walls of *cyt1-1* mutants can become greatly thickened (arrow). (c) In rare cases, it is possible to identify 2 nuclei (N) within a single cell.

Scale bar = 20 μm.

contain large vacuoles which are uncharacteristic of wild-type embryo cells. In addition, protein bodies that accumulate in embryos do not reach maturity in *cyt1* embryos, suggesting that control of cell differentiation is aberrant in these mutants. These abnormalities indicate that the *cyt1* defect is not limited to the cell wall alone. Rarely, two nuclei can be seen to occupy a single cell when viewing sectioned material (Fig. 7c).

Electron microscopy confirms the presence of cell wall stubs, gaps, and thickenings in the *cyt1* embryo. Wild-type walls are linear and uniform in thickness (Fig. 8a) with a prominent middle lamella that exhibits a dark staining pattern. Cell walls of *cyt1*, in contrast, vary widely in thickness and show a variety of darkly-staining inclusions throughout their width (Fig. 8b, c). Cell wall stubs are rounded in mutants and wall gaps do not appear to contain membrane connections from the stubs. Mutant walls appeared to contain slightly less electron-dense material than wild-type, suggesting that they either contain less protein or are less dense.

Electron microscopy also showed that the majority of organelles appeared normal in *cyt1*. Lipid bodies were of normal size, shape and number, and were situated at the cell periphery. Mitochondria, plastids, and nuclei were present and unremarkable in morphology. These data suggest that fixation protocols were appropriate for sample preservation and that *cyt1* defects did not include these organelles.

***cyt1/lec1* double mutant analysis**

A genetic approach was used to determine if *CYT1* belonged to a family of genes expressed during different stages of development. Because *lec1* mutants exhibit many seedling features *in ovulo* (West *et al.*, 1994), it is possible that a late-functioning homologue

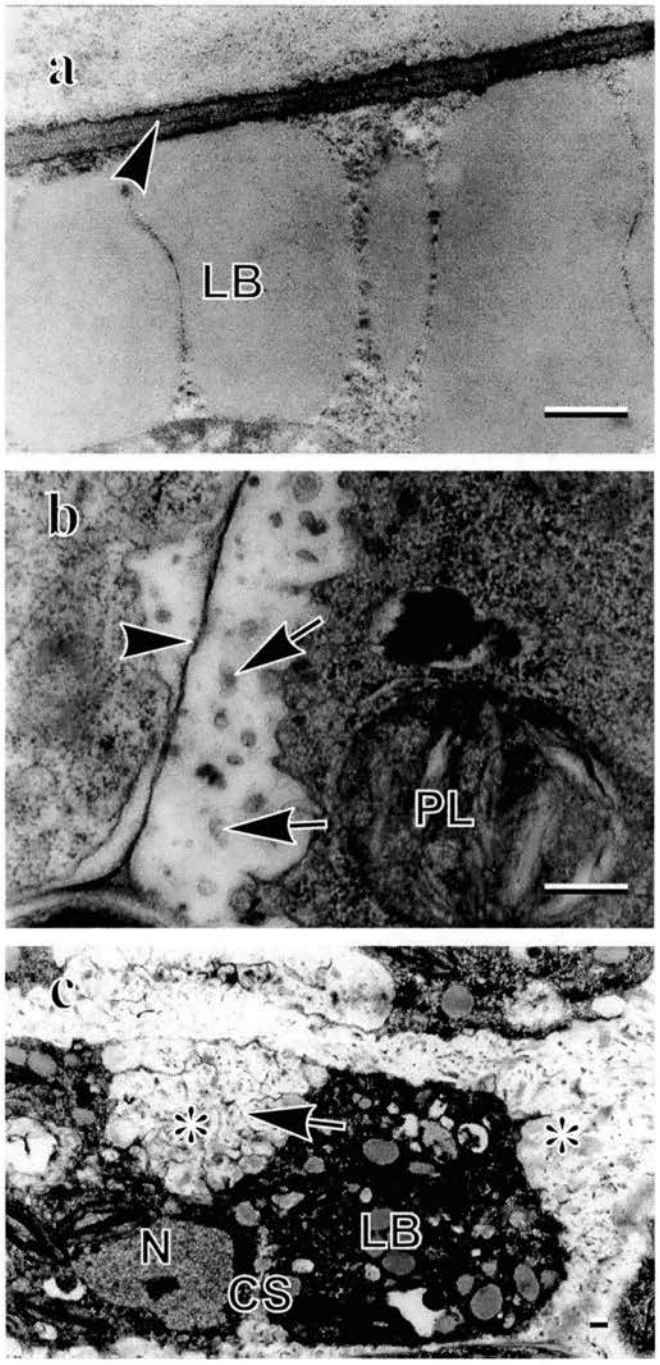


Figure 8: Electron photomicrographs of embryo cell walls.
Wild-type cell wall (a). The wall is straight and regular, and stains evenly. The middle lamella is visible (arrowhead) as are lipid bodies (LB). The cell wall of *cyt1-1*(b). The wall is thickened and uneven. Small inclusions are visible within the cell wall (arrows). The middle lamella is distinct (arrowead). The cell wall of *cyt1-2*(c). Extremely large outgrowths are visible (*) as well as inclusions within the walls (arrows).
N = nucleus, LB = lipid bodies, CS = cell wall stub, PL=plastid. Scale bars = 500 nm.

of *CYT1*, if activated, could rescue these mutants if expressed prior to death caused by the *cyt1* defect. Results are summarized in Table 3. No *CYT1*-like gene seemed to be activated under these conditions, suggesting that *CYT1* might not be a member of a multigene family or that *lec1* is insufficient to activate that member if it exists.

F_2 plants that segregated for both *cyt1* and *lec1* were identified (Fig. 9a). No novel *cyt1/cyt1*, *lec1/lec1* "double" phenotypes were seen. When *cyt1-1/CYT1*, *lec1/LEC1* double heterozygotes were selfed, 238 of 420 seeds appeared wild-type (Fig. 9b, e), 110 were of typical *cyt1* morphology (Fig. 9c, f), and 72 were phenotypically *lec1*(Fig. 9d, g). Double heterozygotes of *cyt1-2/CYT1*, *lec1/LEC1* had 255 normal, 120 *cyt1*-like and 73 *lec1*-like progeny. These results are consistent with a 9:4:3 ratio of wt:*cyt1*:*lec1* ($\chi^2 = 0.8$ for *cyt1-1* and $\chi^2 = 2.0$ for *cyt1-2* P= 0.05) and not significant for 9:3:4 for the same order (χ^2=24.1 for *cyt1-1* and χ^2= 29.1 for *cyt1-2*, P=0.01), indicating that the *cyt1* phenotype is epistatic to *lec1*.

It was decided to test whether the "hidden" *cyt1/lec1* double mutants were capable of developing past the heart stage on callus-inducing media. Of 200 *cyt1-1* and 120 *cyt1-2* mutants (phenotypically) plated on this medium, none produced callus or any other sign of growth. All rescued *lec1* embryos grew on this medium, eliminating the possibility that the *lec1* lesion itself reduces viability. This indicates that *lec1* is unable to rescue the *cyt1* mutants.

Table 3. Segregation data for *lec1* X *cyt1* heterozygotes

	# seeds	# wild-type	# *cyt1*-like	# *lec1*-like	# novel
cyt1-1 X *lec1-1*					
observed:	420	238	110	72	0
expected:		236.25	78.75	78.75	26.25
cyt1-2 X *lec1-1*					
observed:	448	255	120	73	0
expected:		252	84	84	28

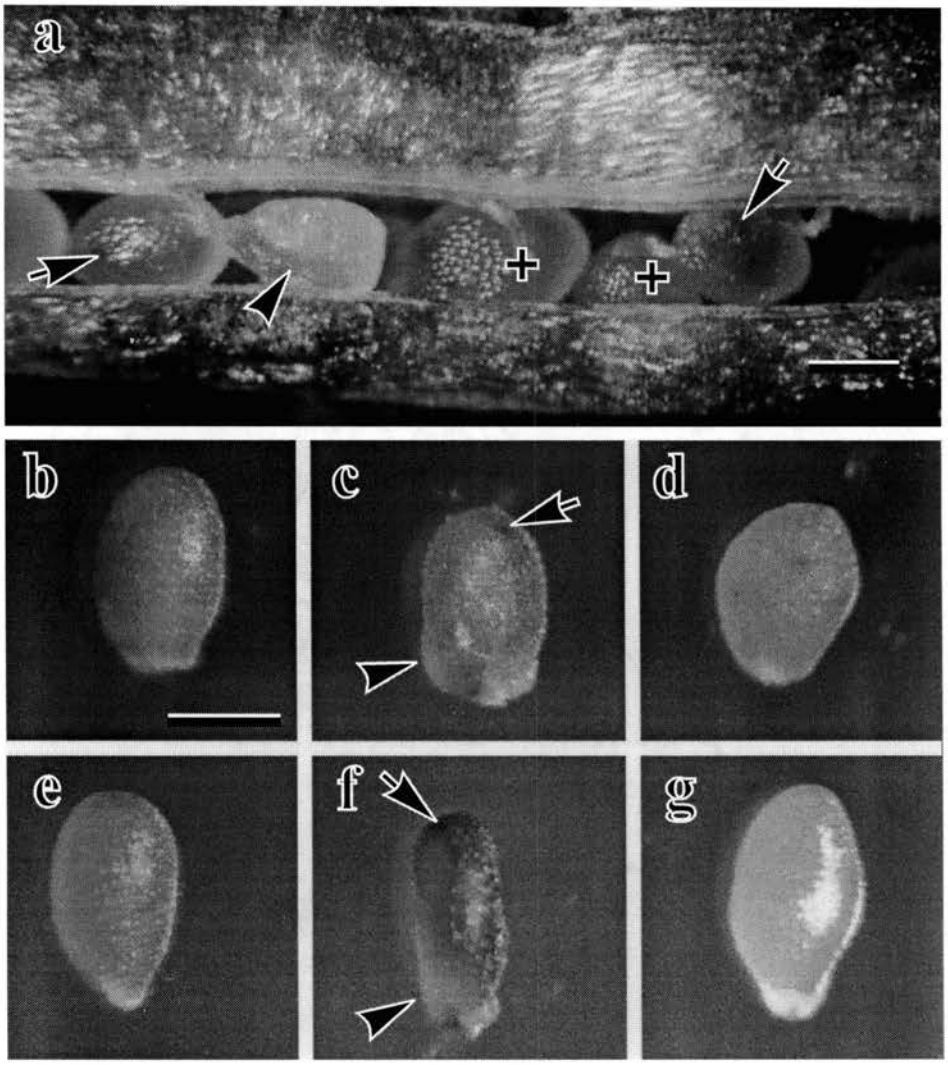


Figure 9: Seeds from a *lec1-1* X *cyt1-1* double heterozygote.
(a) An opened silique from a double heterozygous parent contains seeds segregating for pale *cyt1-1* (arrowhead), *lec1-1* with dark green embryos inside (arrows) and wild-type seeds (+).
(b-g) Examples of mature seeds from a double heterozygous parent prior to desiccation shown from the side (b-d) and rotated 90° around the embryo long axis. Wild-type seeds are isodiametric and plump (b, e) while *lec1-1* embryos are laterally flattened with a prominent bulge where the precociously growing root is situated (arrowheads). Pigment is commonly seen through the seed coats of *lec1-1* mutants (arrows). Pale, deflating seeds are common for *cyt1-1* mutants at this stage (d, g).

Scale bars = 500 μm.

DISCUSSION

cyt1 is defective in cytokinesis

The most striking phenotype of *cyt1* is the abnormal cell walls of the embryo. Large gaps in the cell walls indicate some kind of defect in cytokinesis. The extremely thickened cell walls demonstrate defects in the maintenance of normal wall architecture. Plant cell walls constrain the size and growth of cells (Steeves and Sussex, 1989; Cosgrove, 1997; MacDougall *et al.*, 1997). It is possible that a weak wall could allow enlargement and concurrent vacuolation of cells in *cyt1* mutants. Light microscope observation showed that the cell walls of *cyt1* contain polysaccharides as evidenced by their PAS-positive staining. Electron microscopy confirmed the presence of cell wall stubs and gaps but did not provide histochemical confirmation of wall composition, though the identification of non-uniform inclusions in the cell wall might provide information about the *cyt1* defect.

There are two possibilities for how the cell wall stubs and gaps form in *cyt1*. One is that a primary defect in cytokinesis leads to a termination of the cell plate prior to its fusion to the mother wall. The other possibility is that alteration of the primary cell wall leads to a fragile structure which can be broken at the time of cell expansion. From these primary observations, one cannot distinguish between the two.

Because the cell wall is a complex arrangement of a number of polymers, characterization of these polymers would be a logical undertaking that might allow better elucidation of the role of *CYT1*. Polysaccharides compose the bulk of wall polymers. They are organized in a complex but yet highly controlled conformation to provide stability for

organism structure. More information about *cyt1* cell wall composition would assist the development of models for *CYT1* function.

The *cyt1* phenotype is lethal

One intriguing feature of *cyt1* is its inability to grow in culture. Though cellular and morphological development of *cyt1* are substantially different from wild-type, early defects are not as severe as other embryo-defective mutants which can be rescued in culture (Franzmann *et al.*, 1989) or *knolle* and *keule* which have a limited capacity to germinate and grow as a seedling (Assaad *et al.*, 1996; Lukowitz *et al.*, 1996). Even the application of phytohormones which ordinarily elicit rapid growth in wild-type did not stimulate any identifiable response from cultured mutant embryos.

Young *cyt1* mutant embryos that were freshly isolated appear to be viable as evidenced by the strong fluorescence in FD, but viability is reduced in older embryos. Because the two *cyt1* alleles exhibit morphological differences, partial function of the polypeptide from either allele is likely. The gradual loss of viable cells in the embryo might be due to depletion of either the *cyt1* protein itself of its product if *cyt1* is an enzyme. Alternatively, there might be a maternal pool of *CYT1* product that can sustain the mutant embryos partially through development but gradually becomes exhausted as the embryo reaches the heart stage at which it arrests. This latter hypothesis is also supported by the lack of TTC staining in culture. By removing the embryo from the seed coat and placing it in culture, we might be removing it from the reserves of *CYT1* product that allow it to survive as long as it does *in ovulo*. It is also possible that the rigors of excision and transfer are sufficient to traumatize the tissues enough so they die in culture.

What is clear from these data is that *CYT1* is essential for embryo survival. The fact that cells in young embryos appear to possess the ability to grow and divide, but yet show no response *in vitro* indicates that the genetic defect affects a protein fundamental to normal cell maintenance. Despite other interesting phenotypic aberrations in *cyt1* mutants, any models designed to account for *CYT1* function must account for this lethality. The explanations for these phenotypic features, however, will be useful for us to understand the role of *CYT1*.

lec1 does not rescue _cyt1_

To test whether *CYT1* exists as a member of a family of related genes expressed at different times during development, *lec1* was used to perturb developmental timing and perhaps activate a *CYT1*-like gene at a time that might rescue the *cyt1* mutants. Organ-specific activation of such genes has been documented in the literature (Liu *et al.*, 1995). However, no such family member was apparently activated because double mutants did not grow in culture.

Because crosses did not reveal a phenotype that exhibited both *cyt1* and *lec1* traits, we determined that one trait must be epistatic to the other. Based on our χ^2 tests, *cyt1* is epistatic to *lec1*, indicating that either there is no family of *CYT1* genes or that *lec1* cannot activate a *CYT1*-like gene to rescue cultured embryos. These data suggest that *CYT1* influences the embryo prior to expression of *lec1*.

Putative double mutants were tested for their ability to grow by plating all *cyt1*-appearing mutants on callus-inducing medium. Double mutants included in this sample might be able to demonstrate their ability to grow under these conditions. The 200 plated

cyt1-like mutants should have included approximately 50 *cyt1-1/lec1* double mutants. No growth was seen in any plated embryos (Table 3). From this, I conclude that *lec1* does not have a substantial influence over genetic control of potential *CYT1* homologs. Either *CYT1* is not a member of a family of related genes, or the *lec1* defect is unable to activate such a related gene.

Cell wall defects in *cyt1* are striking

To further examine the *cyt1* defect, more rigorous methods of analyzing the composition of the cell wall were required. To address this need, cytohistochemistry, immunological studies, and more intense biochemical investigations were applied. The next chapter deals with results from analysis of data through these techniques.

CHAPTER 4: FURTHER INVESTIGATION OF THE CELL WALL OF *CYT1* EMBRYOS

INTRODUCTION

Because the gross morphological defects of *cyt1* mutants involve the cell wall, it is logical to focus on the composition of the cell wall to address the fundamental nature of the *cyt1* defect, and ultimately the function of the CYT1 protein itself. The cell wall is composed largely of polysaccharides and is a well organized structure (see Chapter 2). Whether the gaps in the *cyt1* wall result from a defect in cytokinesis or by the substitution of strong wall materials for weaker ones is an important distinction, as the role of *CYT1* would be substantially different for either scenario. If the cell wall composition differs in some large way from wild-type, the chemical nature of that deviation might help with the construction of models for *CYT1* action.

In this chapter, the cell biological analysis of *cyt1* will be reported, particularly with regard to cell wall composition. Immunocytochemical analysis of cell walls was carried out with antibodies developed at the John Innes Centre (Norwich, UK) which allow ultrastructural localization of pectic polysaccharides. In addition, fluorescent dyes specific to certain polysaccharides were used to detect the presence of distinct components of the walls in specimens examined. Herbicides that affect the composition or formation of the cell

wall were applied to wild-type plants to see if disruption of particular cellular processes could phenocopy *cyt1*.

METHODS AND MATERIALS

Microscopy

UV staining solutions

The chemicals used for UV fluorescence microscopy were prepared in advance as stock solutions and then diluted prior to use if necessary. A stock of 0.05% (w/v) aniline blue (Allied Chemicals, Morrisville, NJ) was prepared by dissolving in NP water and was stored at RT in a dark container. A stock of the aniline blue fluorochrome Sirofluor (Biosupplies, Parkville, Australia) was created by dissolving 100 µg of the powder in 1 ml NP water, then stored at 4° C in a dark container. Sirofluor stock was diluted four-fold in an ionic buffer (0.1 M K_3PO_4, pH not adjusted) prior to use. Calcofluor stock was prepared at a concentration of 0.1% (w/v) from Fluorescent Brightener 28 (Sigma, St. Louis, MO) in NP water and was stored at RT in a dark bottle. 2,4-diamidino-2-phenylindole (DAPI) stock (1 mg/ml) was made in NP water and stored at -20° C. The DAPI stock was then diluted 1000 fold in water immediately before use.

Fluorescence microscopy

Whole mounts of wild-type, *cyt1-1*, *cyt1-2*, *knolle*, and *keule* lines embryos were stained with aniline blue or Sirofluor. Embryos were isolated from the testa and placed in fixative (2% formaldehyde, 2% glutaraldehyde in 0.01 M KPO_4 pH 6.5) for 10 minutes. The fixative was drawn off with a pasteur pipette and replaced with aniline blue or Sirofluor. Preparations were observed under UV light as described below.

Embryos embedded in L.R. White were cut at a thickness of 4 μm as described in Chapter 3. In some cases, very young seeds from *cyt1/CYT1* heterozygotes were embedded as a mixture of mutant and wild-type samples and sectioned before the mutant phenotype became apparent. Sections were stained with either PAS/amido black 10B (see Chapter 3) or with aniline blue or Sirofluor (prepared as described in "UV Staining Solutions" above). In some cases, sections stained with PAS/amido black 10B were matched with adjacent sections stained with aniline blue or Sirofluor and photographed so that transmitted light information could be compared with fluorescence data. Fluorescent stains caused bleaching of sections stained with the PAS reaction. Since treating sections with the PAS reaction also causes leaching of previously-applied fluorescent stains, sections could not be observed when stained simultaneously for both UV and transmitted light.

The number of nuclei in each cell was most easily observed in isolated cells. Embryos were treated as described by Liu *et al.* (1995). In brief, embryos were submerged in a modified FAA fixative (50% ethanol, 5% formaldehyde, 6% acetic acid and 5% glycerol by volume) for 2 hours, then transferred to Eppendorf tubes containing 0.5 N HCl for 30 minutes while being vigorously vortexed intermittently to disrupt cell-cell adhesion. Cells were concentrated at the bottom of the tube by centrifuging at 6 000 rpm for 30 s and the supernatant withdrawn. Cells were washed with three changes of 0.1 M KPO_4 buffer (pH4) then stained 5 min with DAPI prepared as described in "UV Staining Solutions" above. Cells were washed once with 0.1 M KPO_4 buffer, spotted onto microscope slides, and then observed under UV light as described below.

Specimens stained with UV-fluorescent dyes were observed using a Nikon Optiphot-2 epifluorescence microscope equipped with a mercury vapor lamp, several excitation filters (UV-2A, 330-380 nm; B-3A, 420-490 nm; GFP 425-475 nm), dichroic mirror (510 nm), and green barrier filter (520 nm). Sirofluor exhibited fluorescence with all excitation filters but was best with the UV-2A combination. DAPI and Calcofluor fluorescence were also detected best with the UV-2A configuration.

Electron microscopy

Specimens for electron microscopy were embedded in L.R. White and sectioned as described in Chapter 3. Silver-gold sections (50 nm) were collected on formvar-coated Ni grids. Sections were blocked in 3% BSA for 1 hr, incubated overnight with diluted primary antibody, washed 3X in NP water, and treated with diluted goat anti-rat conjugated secondary antibodies overnight (15 nm particles; Electron Microscopy Sciences, Fort Washington, PA) as described by Liu *et al.* (1995). Primary monoclonal antibodies used were JIM 5 (antibody generated against de-esterified polygalacturonan), JIM 7 (antibody generated against methylesterified polygalacturonan) and JIM 18 (antibody generated against pea guard cell protoplasts). JIM 18 was not expected to react with antigens in embryo tissue and was included as a negative control. Grids were counterstained in UA and PbCit as described in Chapter 3 following the antibody reactions.

Phenocopy of *cyt1* in wild-type plants by chemical treatment

MS media was modified by adding chemical substances with biological activity that disrupts certain normal processes. The media were mixed and autoclaved as described in Chapter 3 and addenda were introduced through sterile filtration after the media cooled to

about 55°C. The flasks were then rotated to distribute the additives prior to medium hardening. Dichlobenil (DCB; 2,6-dichlorobenzonitrile; Aldrich Chemical, Milwaukee, WI) was prepared as tenfold concentrated stock solutions by dissolving the powder in minimal acetone then bringing up in water, with more acetone being added if the stock became cloudy. Media with final concentrations of 0.1 μM, 1.0 μM, 10.0 μM and 100 μM were prepared in this fashion. Caffeine (Sigma, St. Louis, MO) was diluted in water as 10X stocks (19.4 mg/l or 194 mg/l) and added to media as described for DCB at concentrations of 0.1 μM and 1.0 μM.

Siliques from wild-type plants were sterilized as described in Chapter 3. From these, immature green seeds at the curled-cotyledon stage were selected. Half of these were planted hilum-side down upon control or supplemented media. Embryos were excised from the other half and plated directly upon the media. Plates were cultured for at least two weeks.

At one and two weeks, samples were collected from the plates, fixed, and embedded in L.R. White (see Chapter 3). A portion of these were sectioned for light microscopy and stained with PAS/amido black 10B or Sirofluor as described previously. Another portion was treated with β(1→3) glucanase as described below. The remainder were sectioned for electron microscopy, stained with antibodies as described previously in this chapter, and then observed in the electron microscope.

Enzymatic characterization of cell wall components

β(1→3) glucanase degrades callose. Specimens were treated with β(1→3) glucanase to confirm that callose was the fluorescent compound detected with Sirofluor; when callose is degraded from a specimen, Sirofluor fluorescence of the substrate will be reduced.

Additionally, callose fluorescence from Calcofluor stain interferes with detection of cellulose, so specimens stained with Calcofluor must necessarily be pre-treated with β(1→3) glucanase to remove non-specific staining. Specimens to be treated were first fixed in FAA, rinsed 3X with 0.05 M NaOAc (pH 3.4) over 3 hr, then immersed for 24 h in 200 µl 0.05 M NaOAc (pH 3.4) containing 10 U β(1→3) glucanase (Megazyme, Bray, County Wicklow, Ireland). Tissues were rinsed three times in 0.05 M PO_4 buffer (pH 6.5), post-fixed with 2% OsO_4, dehydrated in an EtOH series, embedded in L.R. White, and sectioned for light microscopy as described in Chapter 3. Sections were stained with either Sirofluor to detect remaining callose content or Calcofluor to detect cellulose in cell walls.

Chromatographic analysis of wall polysaccharides

Andrew Mort has designed a highly sensitive method to evaluate wall components enzymatically liberated from specimens (Mort *et al.*, 1998). Pools of 50 isolated embryos were placed in Eppendorf tubes containing cold 70% EtOH and stored at -20°C. Before analysis, the EtOH was removed and the embryos were washed 3X with fresh 70% EtOH to get rid of free oligosaccharides. The samples were vortexed in PAW buffer (2:1:1 phenol:acetic acid:water) and allowed to sit overnight. The supernatant was discarded after centrifuging for 30s at 10 000 g. The pellets were washed in 1:1 chloroform:methanol and allowed to sit overnight in DMSO at 4°C to get rid of starch. The DMSO was removed and the pellets rinsed 2X in 100% EtOH and then air-dried. The pellets were rehydrated under a light vacuum, centrifuged for 30s at 10 000 g and the supernatant discarded. Five µl 0.05M NH_4OAc (pH 3.5) containing 2 U PME (pectin methylesterase) and PGA (polygalacturonase) (Megazyme, Bray, County Wicklow, Ireland) was added to the pellet and incubated at 40°C

for 3 hr. The supernatant was withdrawn and placed in a new Eppendorf tube and the pellet washed 2X with NP water, which was pooled with the supernatant. The pellets were washed with cold 70% EtOH and later incubated with xylanase, endocellulase, β(1→3) glucanase, and mannanase (all from Megazyme, Bray, County Wicklow, Ireland) as described for the initial PME/PGA digestions, pooling supernatants and rinsates.

Supernatant containing oligosaccharides liberated through enzymatic digestion was concentrated under vacuum, brought up in 3 µl water and transferred to a teflon stub into which several small sample wells were drilled (approx 5 µl capacity per well). Oligosaccharides were derivatized at their reducing end with 4 µl of labeling solution. Labeling solution was made at a ratio of 10:1 ANTS (8-aminonaphthalene-1,3,6-trisufonate; 23 mM in 3% w/w AcOH):sodium cyanoborohydride (1M) and reacted with the sample for 1 h at 90°C. After the reaction, scavenger beads were added to adsorb unreacted ANTS (Mort *et al.*, 1998). Solution was withdrawn and transferred to fresh Eppendorf tubes and the beads rinsed twice with minimal NP water.

Chromatography was performed through a 50 cm long fused silica capillary tube (51.0 ID; Polymicrotechnologies, Phoenix, AZ). The tube was first flushed with 200 mM PO_4 buffer (pH 2.5). The tube was loaded by dipping the end into the Eppendorf tube containing derivatized oligosaccharides for 3 s and then placed in a reservoir that conducts current (20 kV) through the capillary. At least three independently prepared samples were used to gather data for each enzyme regime. When possible, a standard of identified composition was run under the same conditions as the samples. An Edmund 400X International Standard microscope was focused on the capillary at a distance of 26 cm from

the end in which the sample was loaded. ANTS was excited as it passed the objective with an Omnichrome model 100 laser. ANTS fluorescence was quantified by SCION Image software (v1.60a) configured with a PTI IC-1000 camera and the data was rendered graphically with Kaleidagraph (v3.5; Abeldebeck Software). Data collection and interpretation was performed on a Power Macintosh 7200/120.

RESULTS

Cells of *cyt1* can be multinucleate

Because karyokinesis precedes cytokinesis, a defect in cell division logically results in multinucleate cells. I hypothesized that cytokinesis failure would manifest as large, polynucleated cells in *cyt1* mutants. This was not often seen in sections, however (see Fig. 7). Single cells stained with DAPI were examined to determine DNA content. Isolated cells from wild-type embryos each contained only a single nucleus (Fig. 10a). Single cells from both *cyt1-1* and *cyt1-2* demonstrated multiple nuclei (Fig. 10b, c). Most cells in *cyt1* mutant embryos were not multinucleate (Fig. 10d), indicating that many cells carry out cytokinesis at least to the extent of partitioning cellular material into two daughter cells.

Cell walls of *cyt1* mutants accumulate excessive amounts of callose

The composition of the excessively thickened cell walls was of interest. Wall material in dicots consists of cellulose, hemicellulose, and pectic polysaccharides (see Chapter 2). It is possible that the lethality of *cyt1* might be preceded by some kind of stress response. The polysaccharide callose ($\beta(1\rightarrow3)$ glucan) accumulates in stressed plant tissues (Škalamera and Heath, 1996). To see if the wall thickenings represented callose deposition, sections of wild-type, *cyt1-1*, and *cyt1-2* were stained with aniline blue or Sirofluor,

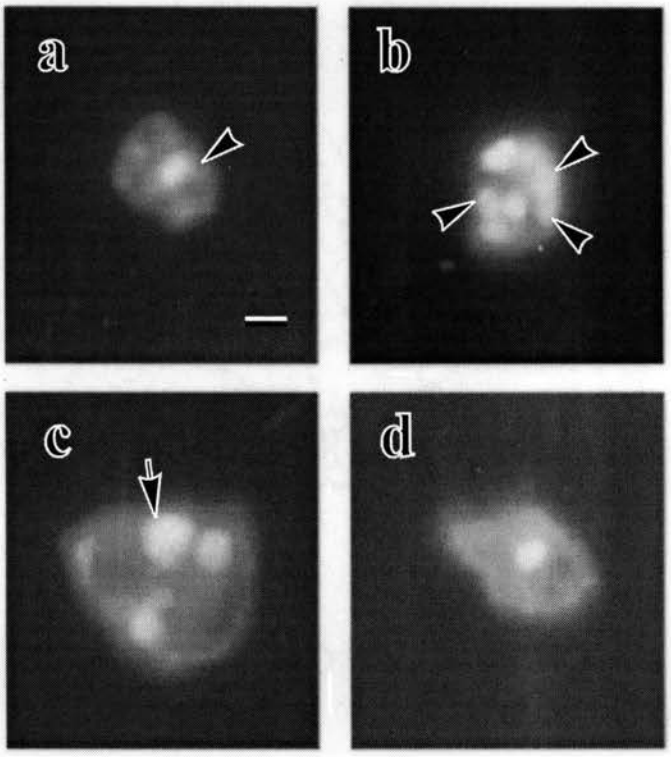


Figure 10: DAPI staining of wild-type and *cyt1* isolated cells. The nuclear material (arrowheads) of wild-type (a), *cyt1-1* (b), and *cyt1-2* cells glows when incubated in DAPI stain and observed with the fluorescence microscope. Note the occasional oversized nucleus (arrow) as seen in (c). Not all *cyt1* cells are multinucleate (d).

Scale bars = 10 μm.

compounds which bind to callose and fluoresce under UV light, and observed with the fluorescence microscope.

Whole mounts were made of tissues submerged in Sirofluor and observed with a fluorescence microscope. Figure 11 shows that though callose could be detected, resolution was not sufficient to show its distribution within the embryo, and chlorophyll showed red autofluorescence which interfered with interpretation. Squashes could be used for quick identification of mutant embryos at early stages but not for detailed analysis of mutants.

A section through a mature cotyledon-stage wild-type embryo was devoid of fluorescence when stained with aniline blue or Sirofluor (Fig. 12a). Wall material of an adjacent section stained pink with the PAS reaction, and cytoplasmic components were blue from the amido black 10B counterstain (Fig. 12b). There was significant fluorescence from cell walls of Sirofluor treated sections of *cyt1-1* (Fig. 12c) and *cyt1-2* (Fig. 12e), especially in the thickened cell wall. Matched sections stained with PAS/amido black 10B showed that fluorescence was restricted only to the locations of cell walls and that wall thickenings were detected by both PAS as well as Sirofluor staining (Figs. 12d, f).

To further determine whether fluorescence was attributed to callose, embryos were treated with $\beta(1\rightarrow3)$ glucanase prior to embedding, sectioning, and staining. Figure 13 shows that fluorescence is considerably reduced in *cyt1-1* embryos pre-treated with glucanase. It is therefore likely that Sirofluor is indeed an accurate and specific dye to test for the presence of callose in sectioned embryos.

Because it was not possible to reliably determine whether an embryo is mutant prior to the heart stage with sectioned or cleared material, globular embryos were sectioned,

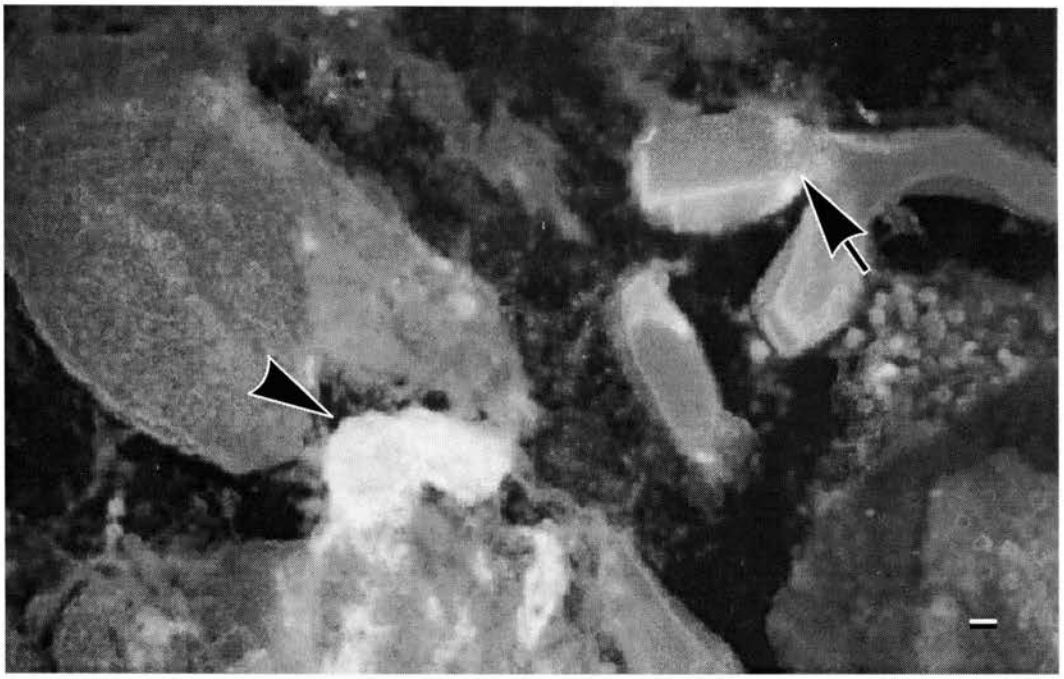


Figure 11: Sirofluor fluorescence of a squashed seed preparation. Fluorescence microscopy reveals fluorescence of a *cyt1-1* embryo (arrowhead) that was extruded from the seed coat by applying pressure on the coverslip with a pair of forceps. Wound-induced callose from a broken cotyledon can be seen in a wild-type embryo (arrow). Red autofluorescence of chlorophyll is seen in the background.

Scale bar = 30 μm.

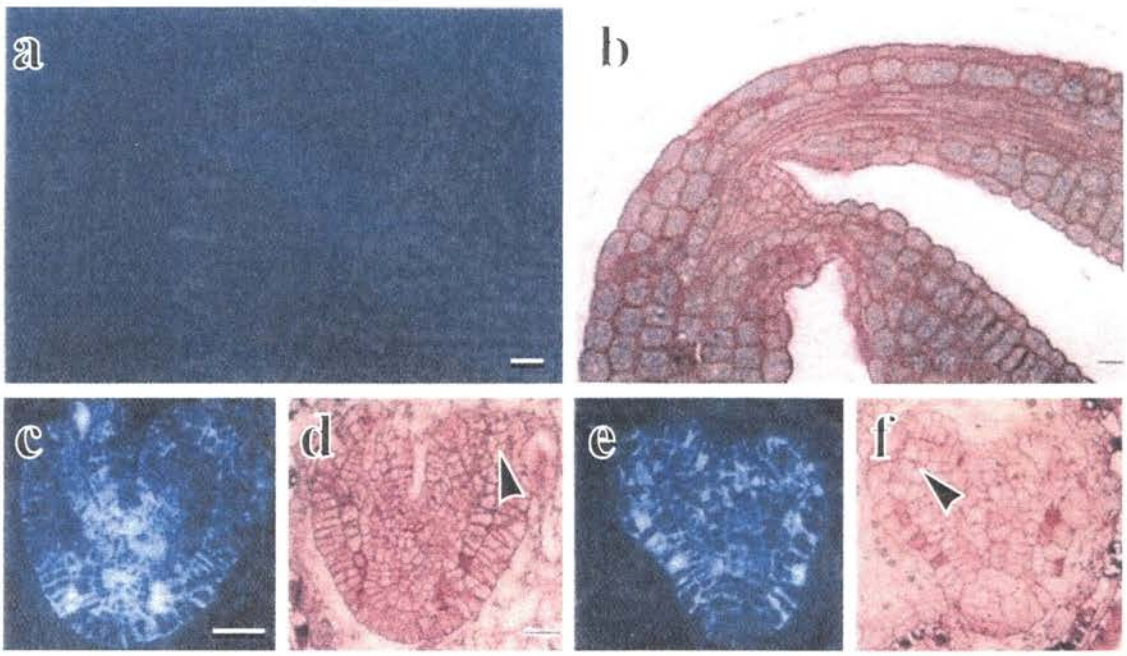


Figure 12: Detection of callose using the fluorochrome Sirofluor.
A wild-type mature embryo (a) shows no fluorescence when stained with Sirofluor and observed under UV light. A matched section shows a well-organized cell pattern (b) as observed with the light microscope. The terminal stage of *cyt1-1* shows distinct fluorescence (c) that lines up with the PAS-positive cell walls seen with light microscopy of an adjacent section (d). A similar pattern is seen with *cyt1-2* (e, f). Note the general disorganization of mutant cell patterns in d, f and incomplete walls (arrowheads).

Scale bars = 25 μm.

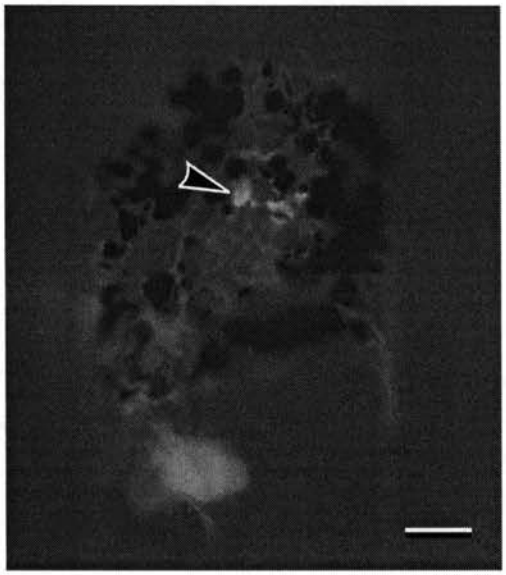


Figure 13: β(1→3) glucanase digests callosic walls of *cyt1*. Sirofluor staining reveals a significant reduction of fluorescence from the cell walls of *cyt1* embryos after 24 hours of incubation with β(1→3) glucanase at 40°. Note that a little residual staining is seen in the most internal region of the embryo (arrowhead), suggesting less accessibility of the enzyme to this region.

Scale bar = 50 μm.

stained with Sirofluor and observed with a fluorescence microscope (Fig. 14). Fluorescent detection is distinct and sensitive, and therefore might assist in the identification of young mutant embryos. Mutant globular embryos were identified in this fashion (Fig. 14a, b). Fluorescence was not apparent throughout all cell walls as was seen in heart stage embryos (see Fig. 12), but instead seemed to originate from several small sources simultaneously. The gradual appearance of callose in the young stage that later became the thick cell walls containing an abundance of callose in older stages suggests that callose-rich cell walls occur through gradual deposition of callose over time, rather than *de novo* construction of callosic walls.

The presence of callose in the cell walls suggests the possibility that glucose is diverted from cellulose synthesis to a callose product, similar to a theory proposed by Delmer (1989; 1991; Delmer and Amor, 1995), who noticed excessive callose from *in vitro* experiments. To see if cellulose was indeed present in *cyt1* embryos, the stain Calcofluor was employed (Galbraith, 1981). Callose fluoresces in the presence of Calcofluor so it was therefore not possible to simply stain sections with Calcofluor and observe them. To remove this confounding staining of callose, embryos were treated with $\beta(1\rightarrow3)$ glucanase and the callose was allowed to digest to completion (about 48 hours). The embryos were then embedded, sectioned, and stained with Calcofluor. Figure 15 shows cellulose fluorescence in wild-type (Fig. 15a), *cyt1-1* (Fig. 15b) and *cyt1-2* (Fig. 15c). Cellulose appears to be located homogeneously throughout cell walls including the thickened walls of *cyt1* mutants. It was not possible to quantify cellulose content on these small samples, so it is unknown whether the cellulose content in *cyt1* is appreciably less than in wild-type.

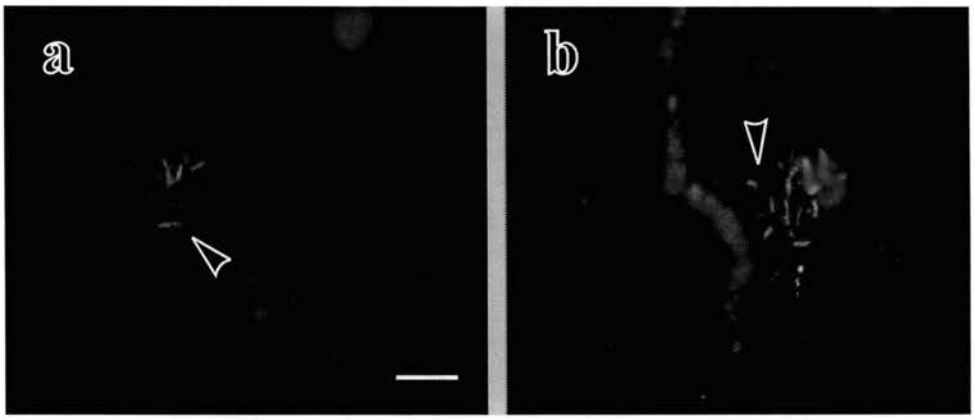


Figure 14: Young *cyt1* mutants stained with Sirofluor.
Despite being morphologically indistinguishable from wild-type embryos at this young stage, preglobular (a) and globular (b) *cyt1-1* embryos fluoresce with Sirofluor when viewed on the fluorescence microscope. Arrowheads indicate callose-rich cell walls.

Scale bar = 40 μm.

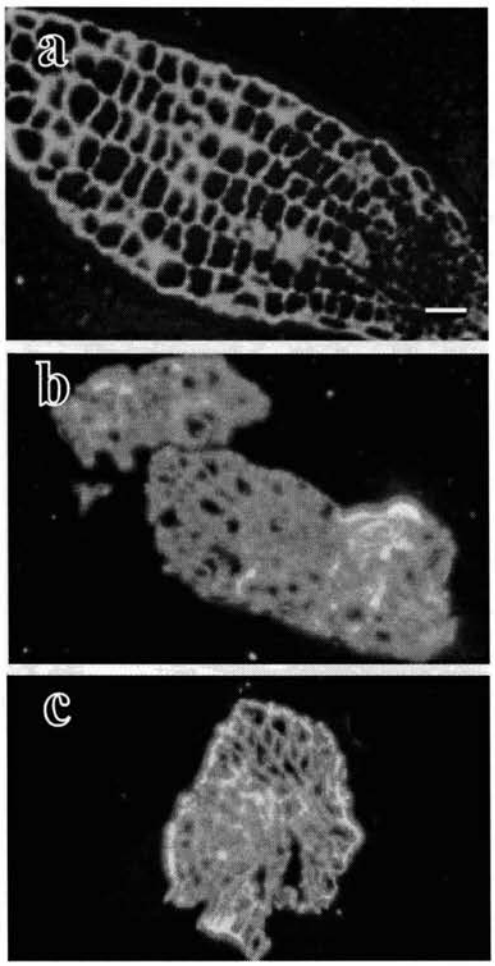


Figure 15: Calcofluor reveals cellulosic walls of wild-type and *cyt1* embryos. Calcofluor causes fluorescence of cellulose in walls of wild-type (a), *cyt1-1* (b), and *cyt1-2* (c). All embryos were previously incubated with β(1→3) glucanase to remove callose, which also fluoresces in the presence of Calcofluor.

Scale bar = 50 μm.

De-esterified pectin location is altered in *cyt1*

Because excessive callose was found in *cyt1*, I decided to evaluate other polysaccharides found in the plant cell wall. VandenBosch *et al.* (1989) used JIM 5 and JIM 7 monoclonal antibodies to look at the distribution of esterified and de-esterified pectins in plant cell walls. De-esterified pectins are are often restricted to the middle lamella of wild-type cell walls (Dolan *et al.*, 1997; Steele *et al.*, 1997) and therefore might provide a way to look at the organization of the cell wall in *cyt1* mutants.

Esterified pectins were visualized by tagging JIM 7 primary antibodies with immunogold-labeled secondary antibodies and were found to be distributed throughout the cell wall in wild-type, *cyt1-1*, and *cyt1-2* embryos (not shown). De-esterified pectins, however, were found to be altered in *cyt1* mutants as compared to wild-type when probed with JIM 5 primary antibodies (Fig. 16 a-c). De-esterified pectins are restricted to the region of the middle lamella in wild-type walls (Fig. 16a). In *cyt1* mutants this class of pectins was distributed throughout the walls (Fig. 16 b, c). It is not clear whether the quantity of de-esterified pectins was altered in mutants. As expected, JIM 18 primary antibodies did not detect antigen in any embryo tissues (not shown).

These data suggest that *cyt1* mutants have a defect in the organization of their cell walls. This is not surprising in light of the wall thicknesses characteristic of *cyt1* mutants; the excessive widths of these walls indicates that some regulatory mechanism of wall morphology is not functioning appropriately. Though a dark-staining middle lamella is apparent in many *cyt1* walls, there appears to be a biochemical defect that is not picked up

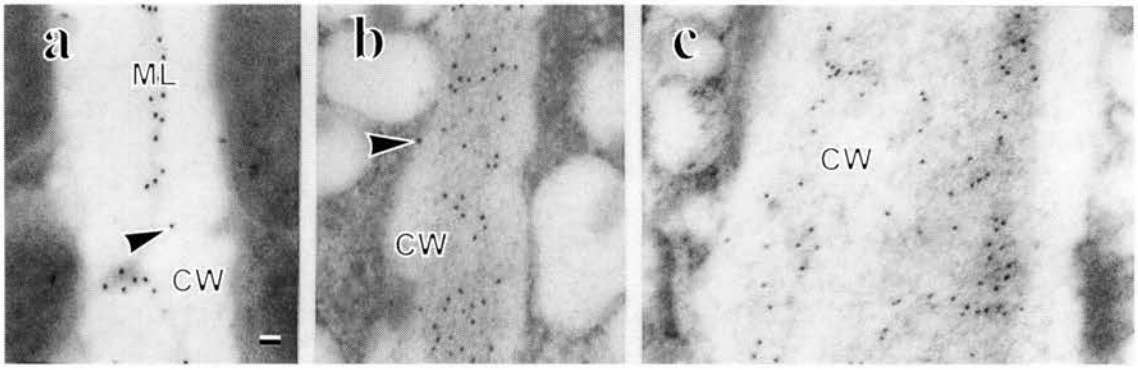


Figure 16: Immunoloabelling of de-esterified pectins in wild-type and *cyt1* walls. The middle lamella of wild-type cell walls (a) is rich in de-esterified polygalacturonic acid as indicated by gold-conjugated antibodies (arrowheads) which are not seen in the peripheral regions of the cell wall. De-esterified pectins are seen throughout *cyt1-1* (b) and *cyt1-2* cell walls (c).

CW, cell wall; ML, middle lamella. Scale bar = 50 nm.

at the morphological level alone. It is unclear whether this defect is sufficient to explain the lethality of the *cyt1* mutation.

Some chemical treatments phenocopy aspects of *cyt1*

A search through the literature showed that the chemical 2,6-dichlorobenzonitrile (DCB) could induce cell wall gaps, an accumulation in callose, and large, vacuolated cells when applied to roots of onion (Vaughn *et al.*, 1996). These features are also characteristics of *cyt1* mutants. Additionally, the mechanism of action of DCB is thought to involve interference with cellulose synthase. Caffeine has been shown to interfere with plant cytokinesis, causing incomplete cell walls (Hepler and Bonsignore, 1990). Caffeine was used to phenocopy abnormalities seen in the cell-wall defective mutant of pea, *cyd* (Liu *et al.*, 1995). DCB and caffeine were tested to see if a *cyt1*-like phenotype could be mimicked in treated wild-type *Arabidopsis* seeds. Because the effects of these substances are known, a close phenotypic match might suggest the nature of the *cyt1* defect.

Three concentrations of DCB, 0.1, 1, and 10 μM, were used to culture immature green seeds of wild-type *Arabidopsis* on semisolid media. A fourth concentration of 100 μM was attempted but came out of solution when media cooled so the effects could not be reliably determined. The severity of morphological aberrations was related to DCB concentration (Fig. 17a). Stunting of roots along with stimulation of adventitious root formation was seen at 0.1 μM, though the plants appeared green and healthy. At 1.0 μM DCB, cotyledons in 1-week old plants were unexpanded and brown, the hypocotyl short and thick, and the root undeveloped. 10.0 μM DCB prevented active germination of cultured seeds, though passive swelling and breakage of the testa did occur. Embryos excised from

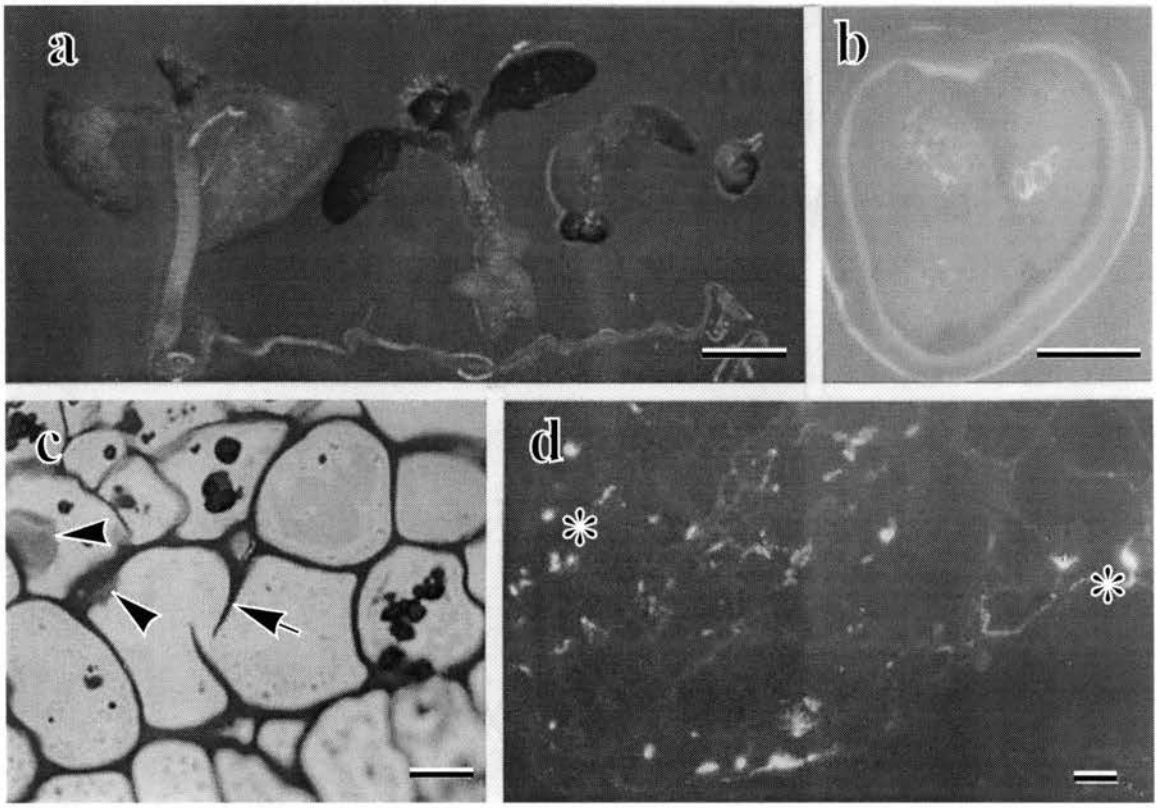


Figure 17: DCB treatment elicits characteristics of *cyt1* in wild-type embryos.
Phenotypes of seedlings grown for 1 week on medium containing 0 (left), 0.1, 1.0 and 10.0 μM DCB (a). An embryo isolated from a young seed cultured for 1 week on 10 μM DCB resembles the *cyt1* terminal phenotype (b, compare with Fig. 4c, d). A section through the hypocotyl of a seedling grown on 1.0 μM DCB has wall stubs (arrow) and thickened cell walls (arrowheads) similar to those found in *cyt1* mutants (c). Sirofluor staining reveals moderate callose accumulation (*) in the root of a wild-type seedling grown on 0.1 μM DCB for 1 week (d).

Scale bars: a = 2 mm; b = 200 μm; c,d = 10 μm.

these ungerminated seeds were large, puffy heart embryos, indicating that DCB did not halt embryogenesis, though it did prevent embryos from passing through torpedo- and later stages, instead allowing the cells to expand and form a giant heart-stage embryo.

When wild-type embryos were excised at the heart stage and placed on 10 μM DCB, they strongly resembled the morphology of *cyt1* mutants (Fig. 17b). Embryo size and the bloated, disorganized appearance of the DCB-treated explants were similar to *cyt1*. Additionally, the DCB treatment caused the wild-type embryo to halt development at the heart stage. These attributes were not as pronounced at lower DCB concentrations.

When sections through an embryo grown on 1.0 μM DCB were examined, wall thickenings and wall stubs were apparent between some cells (Fig. 17c). Cells in these plants were enlarged, vacuolated and did not stain intensely with amido black 10B. Wall gaps were less frequent than those seen in *cyt1* mutants and the stubs were longer than those typically seen in *cyt1*. Callose accumulation was seen in walls of DCB-treated plants, though the amount was far less than that seen in *cyt1* and staining was not uniform nor seen throughout all walls as it was in *cyt1* (Fig. 17d). When DCB-treated embryos were digested with β(1→3) glucanase prior to embedding, sectioning and staining for callose, fluorescence disappeared, confirming that the fluorescence was due to callose (Fig. 18a). Calcofluor staining of this material (Fig. 18b) showed that cellulose was present in DCB-treated samples, though it was not possible to determine if the quantity was decreased. DCB thus demonstrates its ability to partially phenocopy *cyt1* characteristics though there remain significant differences between the phenotypes. There was no concentration of DCB that best caused emulation of all *cyt1*-like defects. Low DCB (0.1 μM) elicited substantial

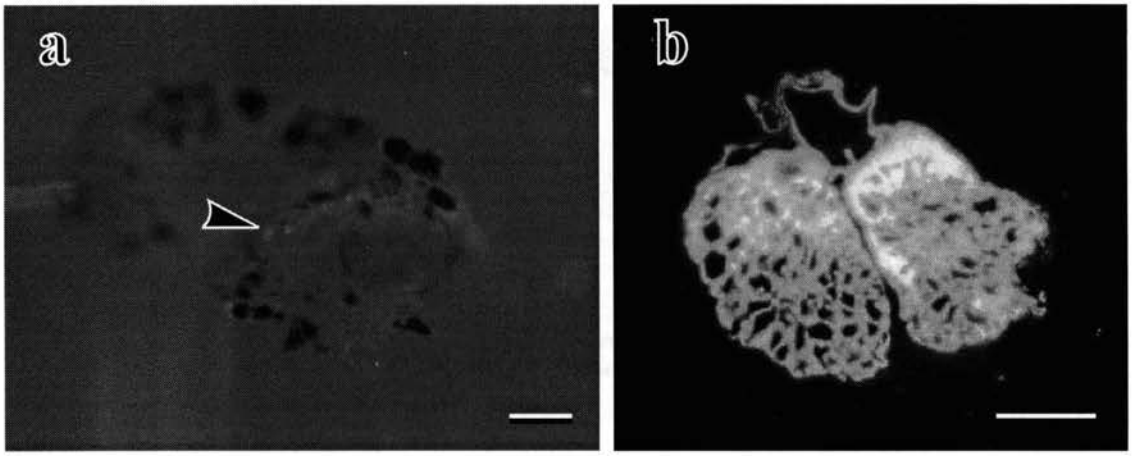


Figure 18: β(1→4) glucanase digests callose from DCB treated embryos.
The hypocotyl from a wild-type seedling (a) cultured for 1 week on 0.1 μM DCB loses Sirofluor fluorescence after being incubated with β(1→4)glucanase. Cells near the center of the embryo are less exposed for digestion and some residual callose remains (arrowhead).
(b) After callose digestion, Calcofluor staining of celluose shows strong fluorescence, indicating that DCB does not prevent cellulose production in cell walls.

Scale bar s= 50 μm.

callose accumulation, while high DCB (10 μM) caused the external morphology to most closely resemble *cyt1*.

To see if DCB is capable of altering pectins in a manner similar to the *cyt1* defect, the sections of treated embryos were decorated with JIM 7 and JIM 5 antibodies. 0.1 and 1.0 μM DCB-treated embryos were examined. Once again, JIM 7 showed an even distribution of esterified pectins throughout the cell wall in a manner similar to wild-type for both concentrations of DCB (data not shown). There is a dose-dependent response for de-esterified pectin distribution. 0.1 μM DCB caused a slight swelling of the cell wall with a modest thickening of the middle lamella, but the de-esterified pectins were not seen in regions of the cell wall peripheral to the middle lamella, suggesting that de-esterified pectin localization is not extremely perturbed in treated embryos (Fig. 19a). At the higher concentration of DCB, de-esterified pectins were distinct throughout the wall (Fig. 19b). It seems that the higher DCB concentration is more effective in mimicking the *cyt1* defect with respect to cell wall organization.

Immature embryos plated on media containing 0.1 μM and 1.0 μM caffeine grew to the seedling stage. Plants appeared healthy, though pale. Sectioned material showed that cell walls were frequently incomplete (Fig. 20a). Wall thickenings were not evident, however. Sirofluor stained sections showed little evidence of callose accumulation (Fig. 20b). Caffeine interferes with dictyosome vesicle fusion, thereby disrupting with early stages of cytokinesis (Hepler and Bonsignore, 1990, Samuels *et al.*, 1995). Though this seems to cause wall gaps, it is apparently not sufficient to elicit a response significant enough to elicit accumulation of callose.

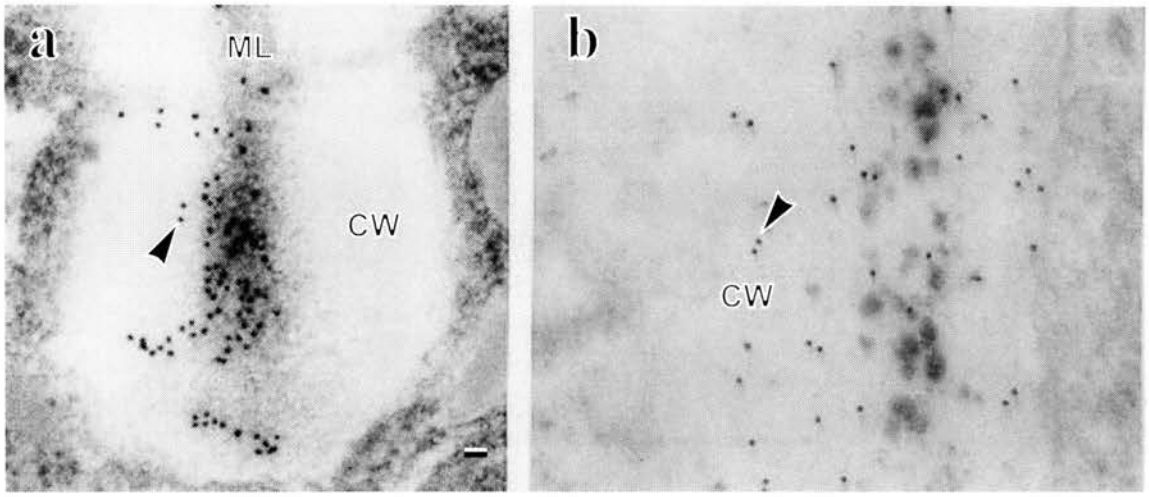


Figure 19: Distribution of de-esterified pectins in DCB-treated walls.
De-esterified pectins are largely restricted to the middle lamella of walls in embryos treated with 0.1 μM DCB (a) labelled with gold-conjugated antibody against the JIM 5 label (arrowheads). Embryos treated with 1.0 μM DCB show that the label is found throughout the cell wall, indicating that the antigen is more dispersed (b).

CW, cell wall; ML, middle lamella. Scale bar = 50 nm.

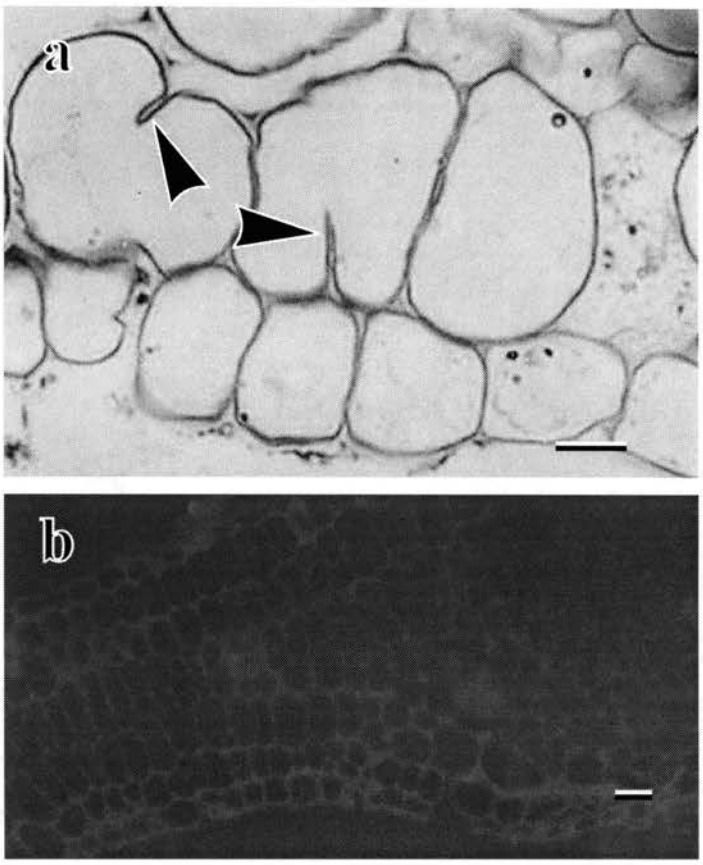


Figure 20: Caffeine causes cell wall defects but not callose deposition.
(a) Light microscopy reveals cell wall stubs (arrowheads) in cell walls of a wild-type seedling grown for 2 weeks on medium containing 1.0 mM caffeine. (b) Sirofluor staining does not reveal callose deposits within hypocotyl cell walls of caffeine-treated tissue.

Scale bars = 50 μm.

Caffeine yields cellular morphology that is less similar to *cyt1* defects than that phenocopied by DCB treatment. It is therefore possible that DCB elicits defects in a manner similar to those caused by a defective *CYT1* product. Incomplete walls caused by caffeine interference of vesicle fusion are less similar and therefore may be of less value for insight into the nature of the wall defects, suggesting that *cyt1* features are not due to abnormalities in vesicle fusion.

Other cell wall defective mutants do not accumulate callose

Except for *cyt1*, several other *Arabidopsis* mutants have been isolated that also demonstrate defective cell walls. The *knolle* and *keule* mutations map to different regions of the genome and list among their deformities wall gaps indicative of a cytokinesis defect. Neither shows cell wall thickenings, though both contain enlarged, vacuolated cells. To further probe the similarities between these mutants and *cyt1*, an evaluation of callose accumulation in *knolle* and *keule* was performed.

Sections of *knolle* and *keule* mutants did not fluoresce with Sirofluor staining (Figs. 21 a, c). They therefore do not accumulate callose, either as primary defect or in response to cell wall gaps. Although *knolle* and *keule* mutants have a more severely distorted embryonic cell pattern at an earlier age than *cyt1* (Figs. 21b, d), *knolle* and *keule* seeds show a low frequency of germination, whereas *cyt1* is completely lethal. The fact that *knolle* and *keule* can germinate while *cyt1* is lethal prior to embryo maturation is another significant difference that further distinguishes these mutants.

The distribution of de-esterified pectins in *keule* is similar to that in the wild-type (Fig. 22a). JIM 5 antibody labeling is found restricted to the region of the middle lamella

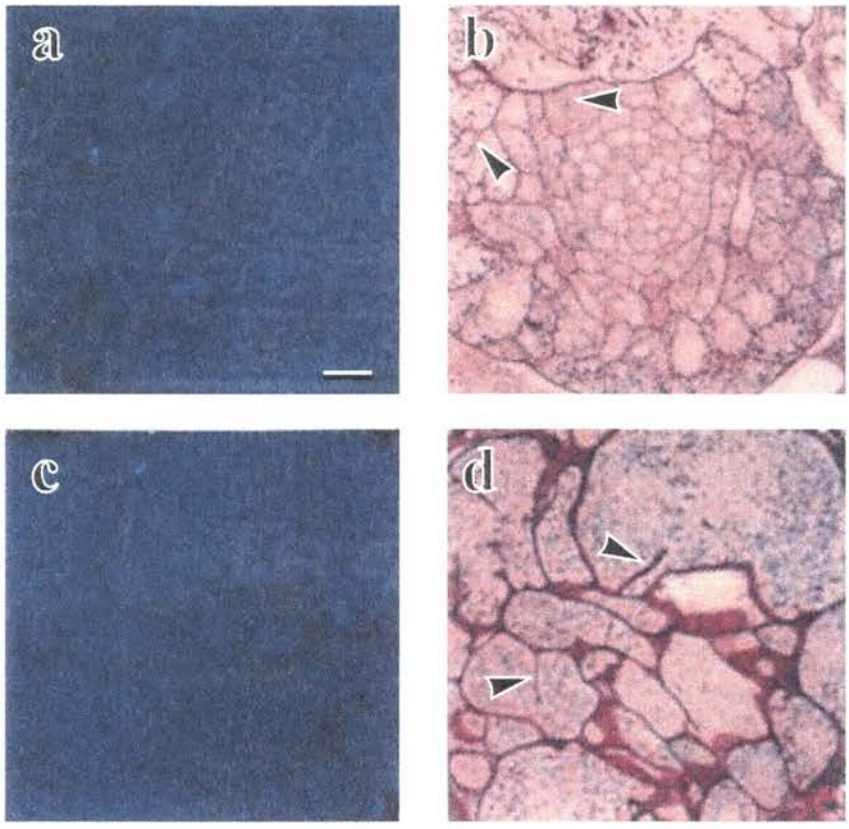


Figure 21: Sirofluor staining of *keule* and *knolle* embryos.
A fluorescence photomicrograph of *keule* (a) shows there is negligible fluorescence, indicating the absence of callose. A matching section viewed with a light microscope (b) shows that the embryo is made of disorganized cells and contains incomplete cell walls (arrowheads). Cell walls of *knolle* are also devoid of callose (c) and light microscopy shows the embryo cell pattern is severely distorted and cell walls are incomplete (d).

Scale bar = 25 μm.

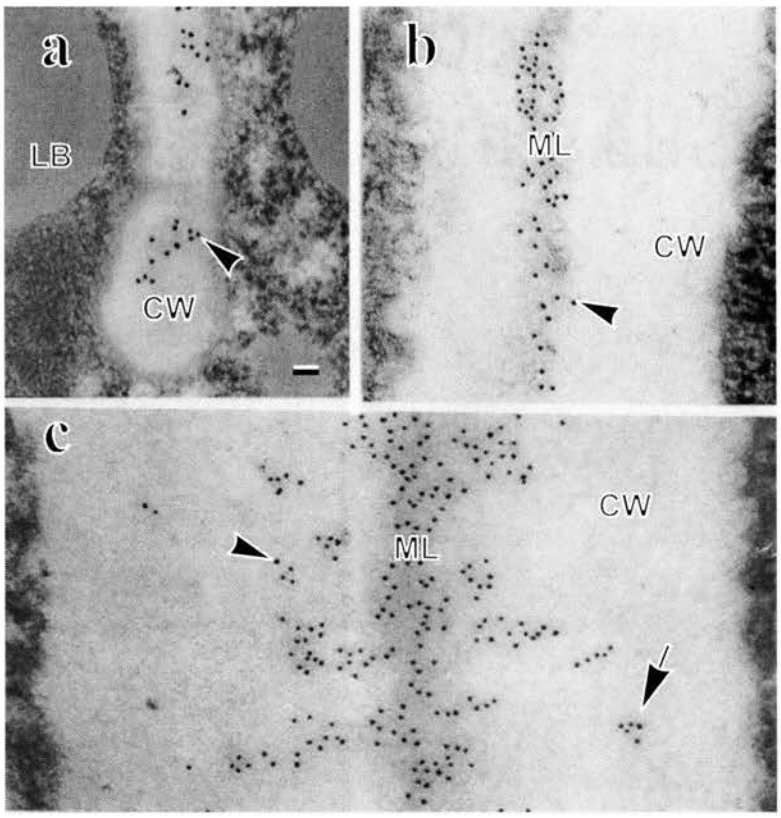


Figure 22: Immunolabelling of de-esterified pectins in *knolle* and *keule* walls.
De-esterified pectins are restricted to the middle lamella of *keule* (a) as shown by the location of gold-conjugated antibody against JIM 5 (arrowheads). Cell walls of *knolle* demonstrated both normal (b) and scattered distribution of de-esterified pectins (c). Note the ectopic clustering of label in the peripheral cell wall (arrow).

LB, lipid body, ML, middle lamella; CW, cell wall. Scale bars = 50 nm.

and not in peripheral areas of the cell wall in this mutant. There is a variable distribution of JIM 5 antigens in *knolle* mutants; they are restricted to the middle lamella in some walls (Fig. 22b) and are found in irregular peripheral positions in others (Fig. 22c). The distribution is not uniform throughout these latter walls but instead appears to aggregate in small clusters. Because *knolle* is defective in a vesicle targeting protein of the syntaxin class, it is possible that vesicles containing pectin modifying enzymes are being inappropriately directed to these peripheral regions of the cell wall, causing de-esterification in unusual locations.

Other wall polysaccharides are altered in *cyt1*

A pilot study of cell walls using chromatography of oligosaccharides liberated through enzymatic digestion of cell wall material seemed sensitive enough to yield information even from the very small sample volumes. A significant difference between wild-type and mutant cell walls was seen in the quantities of labeled oligosaccharides digested with endocellulase (Fig. 23b, c). The chromatograph from a cotton standard (a) were similar to a wild-type sample (b) in terms of ratios detected; both had peaks of similar ratios for 4.65 (peak A), 5.0 (peak B), 5.2 (peak C), and 5.55 min (peak D). The profile for *cyt1* showed a substantial decrease in the height of peak C and peak D was absent. It was not possible to regulate the sample sizes for biochemical manipulation, so only relative ratios should be considered and not the absolute peak height.

Endocellulase digests the glucan backbone of xyloglucans, a major wall component, at residues without a xylan side-chain. Xyloglucans have a repeating structure along the glucan backbone where three consecutive glucose residues have a xylose attached at their 0-6

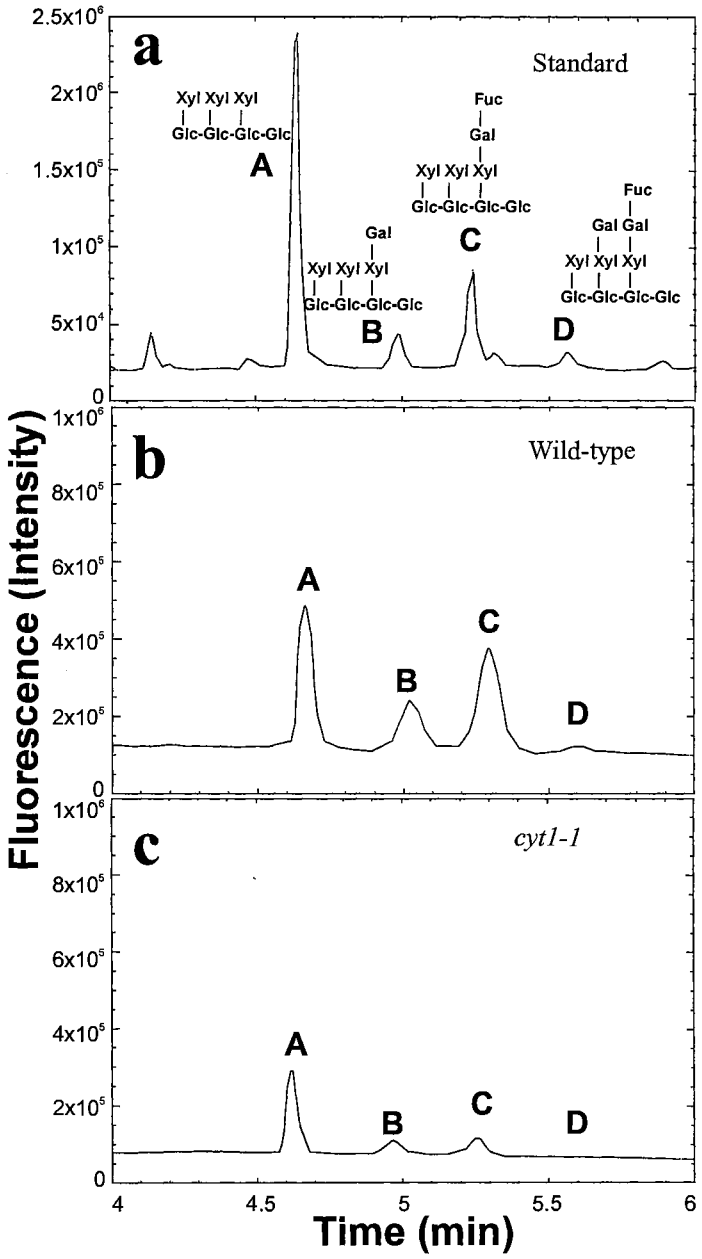


Figure 23: Oligosaccharides released through endocellulase digestion of wild-type and *cyt1* embryos.
A standard from cotton (a) has similar retention times as wild-type (b) and *cyt1-1* material. The peaks represent fragments of the xyloglucan components of the cell walls. Indicated on the standard curve are the identities of the fragments that form each peak. Similar peaks are further identified based on a capital letter (A, B, C, D).

position, followed by an unbranched glucose at the reducing end (Gibeaut and Carpita, 1994). Furthermore, these xylose branches have a characteristic pattern where a galactopyranose residue can have a β(1→2) linkage to the xylose. Some penultimate (from the reducing end) branches also have a terminal fucopyranose linked with a β(1→2) bond. The ratios of these repeated units is reproducible and characteristic of the species being considered (Gibeaut and Carpita, 1994) and consistent between tested samples. Therefore, the change in ratio for *cyt1* mutants is probably related to the mutation.

The common feature for peaks C and D is that both contain a fucose while peaks A and B do not. It is possible that the *cyt1* mutation interferes with the supply of fucose or its addition to xyloglucan branches. Fucose is not related to callose content, so it might be a pleiotropic effect from the *cyt1* mutation or related to the primary defect in a subtle way. Further work on characterization of the *CYT1* locus will probably make more sense of this observation.

DISCUSSION

The phenotype of *cyt1* mutants has several intriguing characteristics including some unique features not detailed in studies of other cytokinesis defective mutants, particularly the excessive accumulation of callose. Some shared aspects include incomplete cell walls as manifested by cell wall gaps and stubs and cells that are large and vacuolated. Table 4 shows comparisons of the *cyt1* phenotype with characteristics of *keule* and *knolle* mutants and those caused by exposure to the herbicide dichlobenil. The distinctive assortment of defects in *cyt1* embryos suggests a unique role for *CYT1* in embryogenesis.

Table 4. Features of mutant and chemical phenocopy treatments

Feature	*cyt1*	*knolle*	*keule*	DCB	caffeine
Phenotype onset	heart stage	proembryo	proembryo	—	—
Enlarged cells	yes	yes	yes	yes	some
Cell wall gaps	yes	yes	yes	yes	yes
Thickened cell walls	yes	no	no	yes	no
Embryos germinate	no	yes	yes	yes[a]	yes
Gene function	unknown	syntaxin	unknown	—	—
Callose deposition	significant	negligible	negligible	moderate	no
JIM 5 localization	abnormal	normal	abnormal	variable	normal

[a] Germination of treated seeds varied with DCB concentration.

Cytokinesis in plants

The *cyt1* phenotype appears to arise from a failure to synthesize a normal cell wall, perhaps interfering with cytokinesis. Models of *CYT1* function should therefore take into account what is known about how cytokinesis occurs and the resulting architecture of the cell wall (Reiter, 1994; Staehelin and Hepler, 1996; McCann and Roberts, 1994). At the initiation of cytokinesis, a phragmoplast composed of microtubules, microfilaments and membrane components becomes established in a plane between the recently duplicated chromosomes. This network is necessary to direct the movement and targeting of vesicles that originate from the dictyosome and contain cell wall materials that form the cell plate (Verma and Gu, 1996). These vesicles rapidly fuse to form a tubulovesicular network that extends peripherally from the cell interior toward the mother cell wall (Samuels *et al.*, 1995). It is this fusion that caffeine treatment blocks, causing cytokinesis defects that resemble the genetic fault in *cyd* mutants of pea (Liu *et al.*, 1995). These defects are similar to those seen in *knolle* (Lukowitz *et al.*, 1996), *keule* (Assaad *et al.*, 1996), and *tso* (Liu *et al.*, 1997) mutants of *Arabidopsis*. Vesicle fusion in *Arabidopsis* requires the function of KNOLLE, a protein in the syntaxin family which facilitates merging of vesicles with target membranes (Lukowitz *et al.*, 1996). *Arabidopsis* has another syntaxin homolog, phragmoplastin, which explains why KNOLLE is not absolutely required by the plant and only results in a defect in cell walls and not lethality (Park *et al.*, 1997). Phragmoplastin associates with vesicles which then become localized at the division plane in mutant cells (Lauber *et al.*, 1997).

After cell plate formation, the tubulovesicular network is consolidated through fusion of lateral elements to form a tubular network. Phragmoplast microtubules disappear and

polysaccharides begin to accumulate in this semifluid structure which is rich in callose (Samuels *et al.*, 1995). The network enters the fenestrated sheet stage and cellulose synthesis is initiated, which stabilizes and makes more rigid this nascent cell wall. Upon contact with the mother wall, attachment occurs at the site of the preprophase band, which transiently appeared earlier in the division cycle (Traas, 1995).

Callose in *cyt1*

Callose is not a substantial component of the final cell wall, and disappears from the cell plate at later stages. As the wall matures, it becomes a matrix of pectins and xyloglucans surrounding an assembly of cellulose microfibrils. The mature wall is an array of cellulose microfibrils, hemicelluloses (xyloglucans), pectins (homogalacturonans and rhamnogalacturonans), modified sugars, glycoproteins (e.g. extensins), and proteoglycans (e.g. arabinogalactan-proteins; AGPs) separated by a middle lamella rich in de-esterified pectins (Cosgrove, 1997).

The accumulation of callose in *cyt1* mutants is dramatic. From the results described here, it seems unlikely that the cell wall defect directly causes callose accumulation in the cell walls; wall gaps caused by caffeine or genetic defects in *knolle* and *keule* do not result in callose accumulation. Samuels *et al.* (1995) suggested that callose provides the "spreading force" for the cell plate to extend toward the mother wall, suggesting that callose is somewhat fluid. Perhaps a hyperabundance of callose in *cyt1* causes regional weaknesses in the new cell wall that can become perforated, leading to wall gaps and stubs which result from cell expansion.

The herbicide DCB elicits callose in treated tissues and is thought to interfere with cellulose synthesis (Vaughn *et al.*, 1996). Cellulose synthase is part of a large membrane-bound complex consisting of several enzymes that coordinate the condensation of glucose monomers to the cellulose polymer (Delmer and Amor, 1995). With a scanning electron microscope, these can be visualized within the plasmalemma as rosettes associated with cellulose microfibrils (Delmer, 1987). Attempts to produce cellulose *in vitro* with isolated membrane fractions of cotton resulted in large yields of callose instead of cellulose, leading to the hypothesis that the rosette, when perturbed, produces callose (Delmer, 1987; Delmer and Amor, 1995). No noticeable reduction in cellulose was apparent in DCB treated specimens through Calcofluor staining, but a means for precise quantification of cellulose in these small specimens is not available at this time. The appearance of callose, however, supports the possibility of the cellulose synthase holoenzyme being compromised, causing an alternate product to form (Delmer, 1987; 1991). If the mechanisms that lead to the similar phenotypes between the DCB phenocopies and the *cyt1* genetic defect are comparable, it could be that *CYT1* is a member of the cellulose synthase holoenzyme and if damaged leads to the production of the callose polymer instead of cellulose.

Models of *CYT1* function

Several models for the molecular basis of the *cyt1* defect can be constructed with this information. It was recently shown that sucrose synthase (SuSy) subunits associate with –or might be a member of– the cellulose synthase complex (Delmer and Amor, 1995). SuSy is an important source of UDP-glucose used for cellulose production *in vivo* (Amor *et al.*, 1995; Carlson and Chourey, 1996). A high concentration of UDP-glucose favors cellulose

production over callose *in vitro*, so it is possible the source of activated glucose for polymerization might bias production of one polymer form over another. Therefore, it is possible to form a model where the *cyt1* defect alters the activity of sucrose synthase in a manner that produces excessive callose. Likewise, the concentration of Ca^{++} can alter the ratio of the cellulose:callose formed *in vitro*, with a high Ca^{++} favoring callose production (Samuels *et al.*, 1995). If *cyt1* affects Ca^{++} levels or the cell's perception of those levels, it could cause callose deposition. Because the cellulose synthase complex is largely still uncharacterized, many scenarios can be formulated that involves disruption of the enzyme. It would not be productive to attempt a comprehensive list because these models cannot be tested.

CYT1 might also coordinate events at the callose-rich stage of cell plate formation. Vesicle targeting and fusion would occur normally, but *cyt1* mutants do not stop producing callose appropriately. This hyperaccumulation of callose might be due to both an overproduction of the polymer and a concurrent lack of removal when it is no longer required. This would explain the unusual wall thickness as well as the gaps, as callose does not provide structural stability of cellulose, which can undergo extensive crosslinking with adjacent strands. What is not addressed in this model is the lethality exhibited by *cyt1* homozygotes.

Another model is that CYT1 is a member of a biochemical pathway or signal transduction network that affects essential functions in addition to cell wall formation. Lethality could result if the function or functions affected were strictly required for embryo survival. General disruption of protein activation or transport could have far-ranging effects

that include vital functions in addition to affecting cell wall formation. A constitutive stress response because of defective environmental sensing, for example, could explain the abundance of callose and could prove fatal to the embryo if cellular reserves were depleted. Again, without more information or more sensitive tests, these models cannot be evaluated.

Because neither *knolle*, *keule*, nor caffeine elicited significant callose formation in addition to the cell wall defects, they might be affected in cell wall formation in a manner fundamentally different from those of DCB or *cyt1*. Caffeine and *knolle* both interfere with proper vesicle function after they leave the dictyosome. It is likely that caffeine therefore bears a good similarity to the defect manifested in *knolle* mutants. Taken together, the lack of callose accumulation in *knolle* and caffeine treatments suggests a mechanism for defects that differs substantially from *cyt1*. The identity of *keule* has not yet been published but is forthcoming (Assaad, pers. comm.). It is likely that *keule* defects occur in a manner different than those of *cyt1*.

While these models can explain some aspects of the *cyt1* phenotype, it is unlikely that they can be tested sufficiently to identify the true nature of the role of *CYT1*. Due to the severe limitation of substrate from mutant embryos, biochemical tests cannot be performed with much accuracy. The lethal phenotype makes sample sizes very small. Without sequence data about the *cyt1* locus and a subsequent comparison of this information with databases containing characterization of similar sequences, it is unlikely that the true role of *CYT1* will be determined only through a classical genetic study.

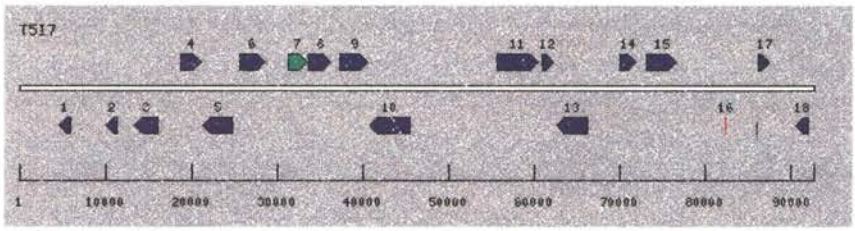
CHAPTER 5: THE IDENTITY OF *CYT1*

Observations of the *cyt1* mutant phenotype and comparisons with manipulated wild-type tissue have allowed the construction of several models for *CYT1* function. A collaboration between the Meinke laboratory and Chris Somerville and Wolfgang Lukowitz at the Carnegie Institute (Stanford, CA) has resulted in a successful chromosome walk that has recently allowed the identification of *CYT1*. The morphological observations shown in this dissertation and recent sequence data on the *CYT1* locus should together provide interesting new information about this gene and allow us to better understand its function.

The *CYT1* locus

As mentioned in Chapter 3, *cyt1* was mapped to the bottom of chromosome 2, within 2 cM of *as*. By using linked markers, the region of interest was first determined to be on the BAC clone T5I7 and identified as transcript #7 as determined by sequencing and annotation performed by TIGR (Rockville, MD) (Fig. 24a). This *CYT1* gene has four exons and four introns, and encodes a putative mannose-1-phosphate guanyltransferase (Fig. 24b) according to sequence similarity. Sequencing of *cyt1-1* and *cyt1-2* by Somerville and Lukowitz revealed defects in two coding regions of this gene, providing further evidence that this locus is the one coding for *CYT1* (Fig. 24c). Interestingly, a mutation affecting vitamin C

a



b

c

cccaaaaaagtcaacattcaacatgtgaataaaaatcaatattggtttctaagtaagtaa
gtaccatattattaaattatttattttggtaaatacgcactcaatttttct*CTCAACGGT*
*GTATATAAACAAAAGGAGTCTCCTTTGGAAAAAACTTGCCT*atcattttgccaacgaacg
ttctttcttcttaatcacagctcagcctgacgcaaccgctcaggctgatctcttccaatt
tacagccatttcccagctcagatctctgatccggtgagatctctctcaaggtaatgcccc
tgcaattttgcttacttctctggttgtgatatgcatgttcttcgaattttcatcgtttgt
gatttgaattctcattttgtatttgctgttgttggtttttaattcgattttccggaacag
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taatcttgaaatgatggacttttatccgatctgggtttaaagctggaattttgattgtgg
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ctcttgttgtctgaattttggaaagaactatttttgttgtatcgttttgatttatttgga
tctgaattcattcacctttttctctgattattgttttgtgtcggttgcatccactttgat
tagatctgaatgaatcatttttttatgtgctcaagttattgtatggattgttctgtttct
agcatgttttggttagacattgttaagatctgacgtttgcattttcaggaaaaggagtta
gagcatcatcaag**ATGAAGGCACTCATTCTTGTTGGAGGCTTCGGCACTCGCTTGAGACC**
ATTGACTCTCAGTTTCCCAAAGCCCCTTGTTGATTTTGCTAATAAACCCATGATCCTTCA
TCAGgtaatctatcttaaatttgccgctttagtctgccagttcttacctatgcctatgtt
tgaaccgaggcatgttttcttgtagATAGAGGCTCTTAAGGCAGTTGGAGTTGATGAAGT
GGTTTTGGCCATCAATTATCAGCCAGAGGTaagatactaatctctcttaacttttttttt
tgcagctattttctgtttacatatgtttgtatttaccatttgctctgtttcgacaggtGA
TGCTGAACTTCTTGAAGGACTTTGAGACCAAGCTGGAAATCAAAATCACTTGCTCACAAG
AGACCGAGCCACTAGGTACCGCTGGTCCTCTGGCTCTAGCGAGAGACAAATTGCTTGATG
GATCTGGAGAGCCCTTCTTTGTTCTTAACAGTGATGTGATTAGTGAGTACCCTCTTAAAG
AAATGCTTGAGTTTCACAAATCTCACGGTGGGGAAGCCTCCATAATGGTAACAAAGGTGa
gattatcgaaacataatactctccagttacgagataagtacgttattcatctaatgtgga
cttgcatgtattggttatataggtgGATGAACCGTCGAAATATGGAGTGGTTGTTATGGA
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GATCAACGCTGGGATTTATCTTCTGAACCCATCTGTTCTTGATAAGATTGAGCTAAGACC
GACTTCAATCGAAAAAGAGACTTTCCCTAAGATTGCAGCAGCGCAAGGGCTCTATGCTAT
GGTGCTACCAGGGTTTTGGATGGACATTGGGCAACCCCGTGACTACATAACGGGTTTGAG
ACTCTACTTAGACTCCCTTAGGAAGAAATCTCCTGCCAAATTAACCAGTGGGCCACACAT
AGTTGGGAATGTTCTTGTTGACGAAACCGCTACAATTGGGGAAGGATGTTTGATTGGACC
AGACGTTGCCATTGGTCCAGGCTGCATTGTTGAGTCAGGAGTCAGACTCTCCCGATGCAC
GGTCATGCGTGGAGTCCGCATCAAGAAGCATGCGTGTATCTCGAGCAGTATCATCGGGTG
GCACTCAACGGTTGGTCAATGGGCCAGGATCGAGAACATGACGATCCTCGGTGAGGATGT
TCATGTGAGCGATGAGATCTATAGCAATGGAGGAGTTGTTTTGCCACACAAGGAGATCAA
ATCAAACATCTTGAAGCCAGAGATAGTGATGTGAAAATGAGATATTATATGTGCAACTTT

Figure 24: Nucleic acid sequence of *CYT1*.
The *CYT1* locus is the seventh ORF in the BAC T5I7 clone (a), shown in green. The *CYT1* gene (b) has 4 introns (thin line), 4 coding regions (green) and three noncoding stretches (blue). Mutations in the transcript (c) are underlined and indicated in color as follows: the position indicated in purple (C) is replaced with T in *vtc1*, the site of T substitution in *cyt1-1* is green (C), and red (C) marks the residue after which a T is inserted in the *cyt1-2* allele. The promoter is indicated in red italic letters and the coding regions of the *CYT1* gene is in blue capital letters. This figure was constructed from data obtained from TIGR and through personal communication with Wolfgang Lukowitz.

biosynthesis was also mapped to this location. This allele, named *vtc1*, was first identified as a mutant causing hypersensitivity to ozone and was named *soz1* (Conklin *et al.*, 1996; 1997). Though morphologically indistinguishable from wild-type plants when grown in optimal conditions, bioassays on these mutants showed that they were deficient in vitamin C, leading to their sensitivity to environmental stress, particularly the oxidizing stress caused by exposure to ozone.

The sites of mutation support our proposal that *cyt1-2* is the stronger allele. A thymidine substitution for cytidine causes an amino acid change from P to L in *cyt1-1*, while the *cyt1-2* mutation leads to a reading frame shift that produces a truncated peptide with an altered amino acid sequence near the COOH terminus. The substitution occurs in a potentially conserved portion of the gene, causing the *cyt1-1* phenotype, while the frameshift, which is often associated with a larger disruption of protein function, leads to the more severe *cyt1-2* phenotype. The mutation caused by the substitution in *cyt1-1* probably disrupts protein folding and therefore reduces the effectiveness of the enzyme's active site in a less severe manner than more radically altered *cyt1-2* polypeptide, causing the difference in phenotypes. A third mutant allele, *vtc1*, was found that involves a C → T substitution near the beginning of the coding sequence, possibly in a less essential region of the polypeptide. This allele will be described later in this chapter.

The putative function of CYT1 as a mannose-1-phosphate guanyltransferase is very similar to that of the MPG1 protein in *S. cerevisiae* which catalyzes the formation of GDP-mannose from GTP and mannose-1-phosphate (Shimma *et al.*, 1997). The amino acid sequence of *CYT1* is 59% identical to *MPG1* (Fig. 25), suggesting this gene was highly

```
CYT1     MKALILVGGFGTRLRPLTLSFPKPLVDFANKPMILHQIEALKAVGVDEVVLAINYQPEVM
MPG1     --G------Y---------TV-----E-G-R----------ANA--TDIA--V--R----
cyt1-1   ------------------------------------------------------------
cyt1-2   ------------------------------------------------------------
vtc1     --------------------S-------------------------------------

CYT1     LNFLKDFETKLEIKITCSQETEPLGTAGPLALARDKLLDGSGEPFFVLNSDVISEYPLKE
MPG1     VET--KY-KEYGVN--F-V----------K--E- V-KKDNS----------C---F--
cyt1-1   ----------------------------L-------------------------------
cyt1-2   ------------------------------------------------------------
vtc1     ------------------------------------------------------------

CYT1     MLEFHKSHGGEASIMVTKVDEPSKYGVVVMEESTGR VEKFVEKPKLYVGNKINAGIYLL
MPG1     LAD---A---KGT-VA----------I-HDIA-PNLIDR------EF---R----L-I-
cyt1-1   ------------------------------------------------------------
cyt1-2   ------------------------------------------------------------
vtc1     ------------------------------------------------------------

CYT1     NPSVLDKIELRPTSIEKETFPKIAAAQGLYAMVLPGFWMDIGQPRDYITGLRLYLDSLRK
MPG1     --E-I-L--MK----------ILVEEKQ--SFD-E-----V---K-FLS-TV---N--A-
cyt1-1   ------------------------------------------------------------
cyt1-2   ------------------------------------------------------------
vtc1     ------------------------------------------------------------

CYT1     KSPAKLTSGPHIVGNVLVDETATIGEGCLIGPDVAIGPGCIVESGVRLSRCTVMRGVRIK
MPG1     RQ-KK-AT-AN----A-I-P--K-SSTAK-----V---NVTIGD---IT-SV-LCNST--
cyt1-1   ------------------------------------------------------------
cyt1-2   ------------------------------------------------------------
vtc1     ------------------------------------------------------------

CYT1     KHACISSSIIGWHSTVGQWARIENMTILGEDVHVSDEIYSNGGVVLPHKEIKSNILKPEI
MPG1     N-SLVK-T-V--N------C-L-GV-V--D--E-K----I---K-----S-SD-VP-EA-
cyt1-1   ------------------------------------------------------------
cyt1-2   -----EQYHRVALNGWSMGQDREHDDPR*
vtc1     ------------------------------------------------------------

CYT1     VM*
MPG1     I-*
cyt1-1   --*
cyt1-2
vtc1     --*
```

Figure 25: Amino acid sequence of *CYT1* , *cyt1-1*, *cyt1-2*, *vtc1*, and the yeast MPG1 protein.

CYT1 shows high homology with *MPG1*, a mannose-1-phosphate guanyltransferase. Locations of identical amino acids are indicated by dashes (-) and the carboxy terminus is shown with an asterisk (*). Data for this figure was generously supplied by Wolfgang Lukowitz and Patricia Conklin.

conserved during evolution. *CYT1* also shows 51% amino acid similarity and 31% identity with a *C. elegans* hypothetical protein.

A query using the genomic sequence of *CYT1* was performed on October 9th, 1998 using the WU-Blast tool set (2.0a19MP-WashU) available from AtDB. Several positive matches were obtained with BLASTN from the *Arabidopsis* GenBank Data Set, but the identification of these indicated they were obtained from EST and genome project submissions that yielded sequence identical to *CYT1* itself and not a different gene of *Arabidopsis*. Based on the evidence available at this time, we conclude that *CYT1* is not a member of a family of related genes. An October 15th, 1998 query using BLASTP with the Non-Redundant *Arabidopsis* Protein Data Set (NRAT) indicated significant matches with three proteins unrelated to the putative mannose-1-phosphate guanyltransferase. A putative translation initiation factor (EIF-2B-ϵ) subunit was between 42 to 48% similar at the amino acid level along the entire sequence. An ADP glucose pyrophosphorylase large and small subunit each had homology between 42 - 59%, possibly due to its similar role in catalyzing a hexose sugar transfer to a nucleotide. Finally, there is approximately 50% amino acid similarity to an acetyltransferase for the 100 amino acids at the N-terminus of *CYT1*.

The genomic sequence of *CYT1* and 3kb 5' upstream sequence showed several possible promoter sequences. The highest probability sequence (97 %) was 701 bases upstream of the initiation codon of *CYT1*. Other possible promoters that were 2493, 2161, and 1102 bases upstream and of lower probability (91, 88, and 85 % respectively) are probably not involved with transcription of the gene. No information was available

regarding whether *CYT1* is constitutive or otherwise preferentially expressed under developmental or environmental conditions based on the promoter sequence.

CYT1 as a mannose-1-phosphate guanyltransferase

Mannose-1-phosphate guanyltransferase mutants have been characterized in *S. cerevisiae* several times and have become known by several different names (*MSN17*, *PSA1*, and *VIG9*) in addition to *MPG1* (Shimma *et al.*, 1997; Hashimoto *et al.*, 1997). Yeasts defective in this gene show glycosylation defects in proteins that affect the cell wall, causing hypersensitivity to antibiotics such as neomycin which do not affect wild-type cells. GDP-mannose is important in both the addition of mannose to core-oligosaccharide synthesis for cell wall proteins which is carried out in the ER and N-glycosylation of proteins carried out in the dictyosome (Roy *et al.*, 1998). Defects in core-oligosaccharide synthesis in yeast cause an increase in cell wall porosity which allows antibiotics to enter the cell and kill it. Defects in N-glycosylation of proteins can cause defects in protein conformation and protein-protein interactions necessary for proper function.

In addition to protein glycosylation, GDP-mannose has recently been identified as a component in the biosynthetic pathway for vitamin C in higher plants (Wheeler *et al.*, 1998). This nicely accounts for the vitamin C phenotype noted in the *cyt1* allele, *vtc1* (Figure 26; Conklin *et al.*, 1996; 1997). The vitamin C content of plant cells can be quite high (Wheeler *et al.*, 1998). It is therefore possible that the weak *vtc1* phenotype (that is, plants are phenotypically normal in the absence of oxidizing stress) arises because GDP-mannose becomes depleted in these plants and therefore reduces ascorbic acid levels to a

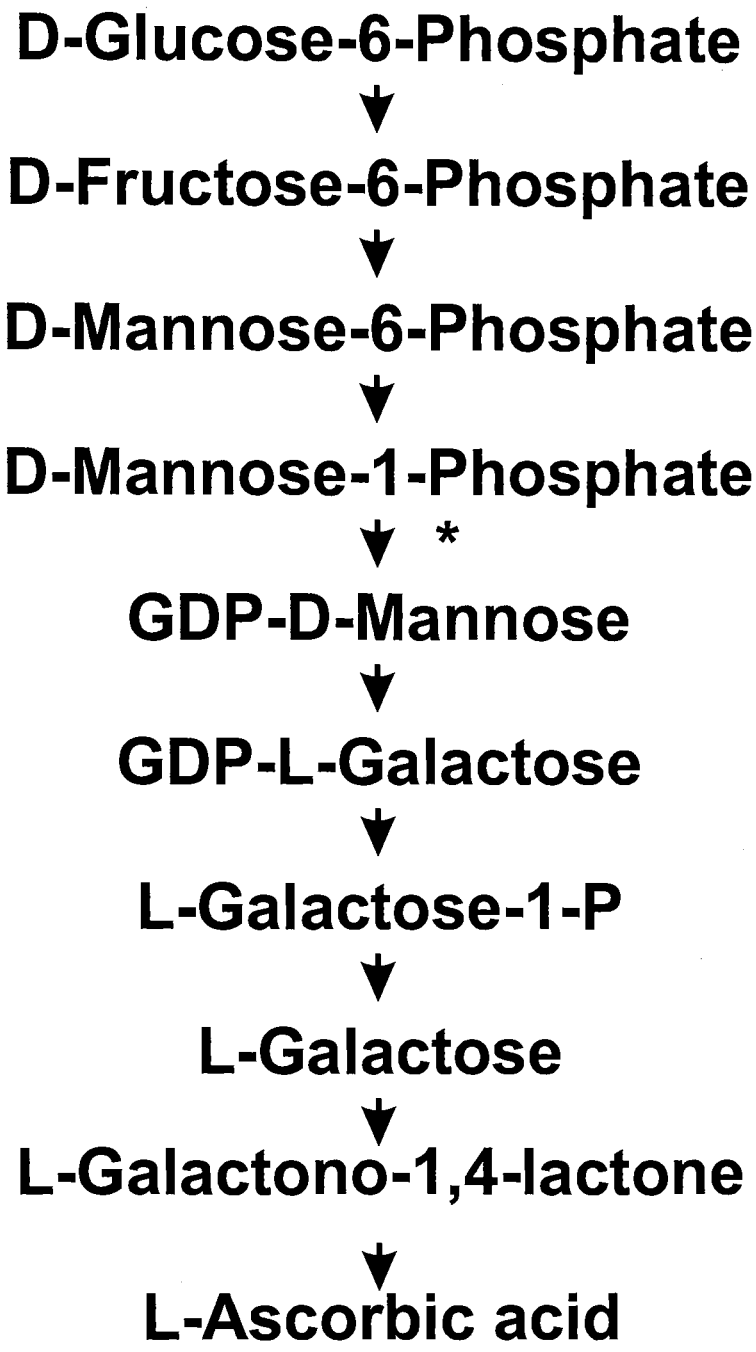

Figure 26: Biosynthetic pathway of vitamin C in plants.
The asterisk (*) shows the catalytic step performed by GDP-D-mannose pyrophosphorylase, the enzyme that is defective in *cyt1* and *vtc1* mutants. This figure is adapted from that proposed by Wheeler *et al.*, 1998).

point where the plant shows hypersensitivity to ozone, though the *vtc1* product is capable of supplying sufficient product to keep the cells healthy under non-stressing conditions.

The relationship between callose deposition in the cell walls and lethality of the embryos is still unclear. However, the *cyt1* character in which xyloglucan composition is altered can be explained. Fucose biosynthesis involves a biochemical pathway which derives from GDP-mannose (Fry, 1987). Therefore, a deficiency of fucose results in an alteration of xyloglucan where side chains are lacking in terminal fucose.

One model that might explain *cyt1* defects is that a defect in protein glycosylation perturbs the proper function of one or more essential proteins. For example, if a defect in the cellulose synthase complex can indeed result in the production of callose as proposed by Delmer (1987; Delmer and Amor, 1995) and the complex is organized in part through interaction of glycosylated amino acids, callose deposition may result due to TC disruption. Further, if callose arises from the TC, it might interfere with crystallization of celluose into microfibrils, causing a lack of wall strength leading to a cytokinesis defect characterized by incomplete cell walls. Because many downstream proteins can be affected by a single lesion that disrupts glycosylations essential for orientation, organization or protein function, a host of other models can also be proposed.

A defect in signal transduction as proposed in Chapter 4 can be invoked to explain the deposition of callose as an excessive stress response. In this model, a constitutive perception of a stressor could result in callose deposition, perhaps also overtaxing resources designed to respond to stresses. The lethality may therefore be a result of starvation due to insufficient cellular resources to accommodate the perceived stress. There are no reports of

"callose toxicity", so this characteristic may be secondary or unrelated to lethality. Also, culture conditions usually involve an excess in nutrients, so starvation before manifestation of growth or cleavage of TTC seems unlikely. Perception of Ca^{++} alters callose/cellulose ratios (Delmer and Amor, 1995). Perturbation of Ca^{++} level perception might also account for *cyt1* defects such as the callose, and additionally might affect essential processes sufficiently to result in embryo lethality.

One idea that correlates cell lethality and callose accumulation is the biomechanical influence of this altered cell wall. Though callose cannot hydrogen bond to adjacent strands as cellulose does, the thickened cell wall may be resistant to factors that allow proper cell expansion. During the course of growth, the cell is unable to respond appropriately and the cytoplasm may be unable to carry out essential functions, leading to the progressive death of older *cyt1* cells which was shown in Figure 5. The cells can still expand because a characteristic of mutant cells is that they are larger than wild-type, but the inability to respond suitably could be a cause of cell death.

Further experiments testing *CYT1* as a mannose-1-phosphate guanyltransferase

To see if ascorbic acid deficiency is the cause of the lack of *cyt1* growth, media containing vitamin C was used to rescue these embryos. Media containing vitamin C exceeding 0.1 μM caused bleaching of wild-type controls, though the reason for this is not known. Mutant *cyt1* embryos did not respond to culture in the presence of several concentrations of ascorbic acid as shown in Table 5. This is not surprising because the CYT1 protein is likely involved in several other functions in addition to vitamin C biosynthesis and low ascorbic acid levels in *vtc1* did not cause a mutant phenotype in the

Table 5. Attempted rescue of *cyt1* mutants with ascorbic acid.

	0.01μM ascorbic acid		0.1μM ascorbic acid	
Genotype	Embryos cultured	Percent response	Embryos cultured	Percent response
wild-type	40	93	48	97
cyt1-1	45	0	51	0
cyt1-2	25	0	25	0

Wild-type embryos bleached when cultured on media containing > 0.1 μM ascorbic acid. Response was considered positive if callus formed from the explant.

absence of environmental stress (Conklin *et al.*, 1996; 1997). Embryos were also cultured in the presence of GDP-mannose (Table 6). A preliminary study showed that embryos grew up to 22% larger in the presence of 0.1 or 1.0 μM GDP-mannose, substantially different from the total lack of growth seen under all other conditions. However, these embryos did not mature morphologically or exhibit substantial growth. A follow-up experiment using a multiwell plate containing identical medium did not produce any growth at all (Table 6). Poor growth response could be caused by a reduced ability of GDP-mannose to be taken up by embryos.

Experiments in progress are further chemical characterization of the cell wall using GC-MS by Wolfgang Lukowitz at Stanford and the production of a *CYT1*::*GFP* reporter line to assess the spatial and temporal expression of *CYT1* in the Meinke laboratory. The callose content of *vtc1* mutants will also be looked at in this laboratory and the phenotype of *cyt1-2/vtc1* trans-heterozygotes, which in an initial study appear morphologically normal during embryogenesis, will be investigated. We now know the cellular phenotype and molecular identity of *CYT1* and can therefore better investigate the relationship between GDP-mannose deficiencies and *cyt1* characteristics.

Table 6. Attempted rescue of *cyt1* embryos with GDP-mannose.

First replicate

	1.0μM GDP-mannose		10.0 μM GDP-mannose	
Genotype	Embryos cultured	Percent response	Embryos cultured	Percent response
wild-type	7	85	6	82
cyt1-1	23	36	16	42
cyt1-2	21	21	12	29

Second replicate

	1.0μM GDP-mannose		10.0 μM GDP-mannose	
Genotype	Embryos cultured	Percent response	Embryos cultured	Percent response
wild-type	21	79	25	89
cyt1-1	44	0	27	0
cyt1-2	18	0	21	0

Response was considered positive if embryo diameter increased > 5%.

CHAPTER 6: SUMMARY AND CONCLUSIONS

The Meinke mutant collection contains many embryo defective mutants demonstrating interesting defects. By choosing mutants with a phenotype suggesting a particular cellular defect, biological questions can be formulated and tested. The *cyt1* mutant was chosen because cell walls in mutants were incomplete and suggested a defect in the control of cell division. The work on *cyt1* was performed to examine the genetic control of cytokinesis in flowering plants.

CYT1 is interesting because of the striking range of phenotypes caused by different mutant alleles. The weakest allele, *vtc1*, does not cause an obvious phenotype in the absence of environmental stress. Seedlings of *vtc1* can grow to maturity and homozygous mutant stock can be maintained. The stronger alleles *cyt1-1* and *cyt1-2* were used in the experiments described in this dissertation and became arrested at the heart stage. Seeds that are homozygous for the *cyt1* defect cannot germinate and the mutation must be maintained in heterozygote plants. Because strong mutant alleles are lethal and cause developmental arrest during embryogenesis, *CYT1* is considered an essential gene during embryogenesis.

In addition to showing incomplete cell walls, sections through mutant seeds demonstrated that cell walls were abnormally thick compared to wild-type cell walls. These

thickenings were irregular and when examined with the electron microscope showed inclusions of variable shape, size and staining intensity. The inclusions of variable size, shape, and staining intensity suggested the architecture of the cell wall was somehow altered from wild-type. Electron microscopy showed that JIM 5, an antibody that immunolabels de-esterified pectins, localized ectopically throughout the cell wall in *cyt1* mutants instead of just the middle lamella. This is evidence that regulation of cell wall organization is altered by the *cyt1* defect.

Mutant embryos were also determined to contain excessive amounts of callose in their cell walls. Callose accumulates in response to some forms of environmental stress and is a product hypothesized to arise from defective cellulose synthesis. Its presence in *cyt1* suggests that the TC, an enzyme complex that localizes to the cell membrane and is responsible for cellulose synthesis, may be perturbed in some way that generates callose instead of cellulose. Much is still not known about the makeup of the TC and because cellulose is an economically important fiber, genetic identification of elements affecting the performance of cellulose synthesis is particularly desirable.

DCB, an inhibitor of cellulose synthesis, was reported in the literature to cause *cyt1*-like defects in treated onion roots. DCB phenocopy of *cyt1*-like defects in wild-type *Arabidopsis* embryos was performed in culture. Exposure of wild-type embryos to DCB caused many *cyt1*-like defects including incomplete cell walls, slight accumulation of callose, and thickened cell walls. When young embryos were cultured on DCB-containing media, they developed into enlarged heart-stage embryos similar to excised *cyt1* embryos. Immunocytochemistry showed that the cell walls did not restrict de-esterified pectins to the

middle lamella but instead had them distributed throughout the cell wall in a manner similar to *cyt1* mutants. DCB is thought to function as an inhibitor of cellulose synthesis and this provides additional support to the model where *cyt1* somehow interferes with normal cellulose production and causes the mutant phenotype.

A genetic experiment attempted to initiate seedling programs early in development. The *lec1* mutation causes seedling-like characteristics to appear during embryogenesis. The rationale for crossing *lec1* with *cyt1* was that if a gene family member of *CYT1* is active during seedling growth, the *lec1*/*cyt1* double mutants might be capable of germination. The cross demonstrated that *cyt1* characters are epistatic to *lec1* features and no phenotypically distinct double mutants were observed. No cultured *cyt1*-appearing mutants, which would include double mutants, were capable of germinating, suggesting that either there is no family member of *cyt1* that is active during the seedling stage or that the *lec1* mutation is incapable of activating it.

Chris Somerville and Wolfgang Lukowitz at the Carnegie Institute (CA) recently cloned the *CYT1* locus through chromosome walking. The amino acid sequence of CYT1 suggests that it is a mannose-1-phosphate guanyltransferase, an enzyme needed to synthesize GDP-mannose. GDP-mannose is needed for ascorbate synthesis and the weak *cyt1* allele, *vtc1*, is deficient in vitamin C, which is consistent for the suggested identity of the *CYT1* gene. GDP-mannose is also critical for N-glycosylation, a process which modifies proteins and affects their structure and interaction with other proteins. It is possible that glycosylation of plasmalemma-associated proteins involved with the synthesis and organization of cell wall materials is essential to their correct function. In this model, the severe *cyt1* phenotypes lack

this glycosylation and therefore are incapable of forming an organized, functional cell wall. Lethality might be due to either the lack of this functional wall or a lack of essential nutrient for which GDP-mannose is a precursor.

The importance of *CYT1* for embryo survival and the possibility that it influences the structure of several downstream proteins make it an exciting mutant for study. What has been done to characterize mutant defects, and the recent acquisition of *CYT1* sequence opens up many more avenues for investigation, such as the regulation of *CYT1*, expression patterns, and the complement of other gene products that are modified by N-glycosylation.

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APPENDIX A: ORGANIZATION OF THE MEINKE LAB NETWORK

The Meinke laboratory network is an intranet that is run by a Digital XL566 computer running Windows NT Advanced Server 4.0 (called **MUTANT**). With this server, peripheral workstations running Windows NT 4.0, Windows 95, Windows 3.1, or any of the MacIntosh Operating Systems can sign on with a password and access files depending on user clearance. Clearance is set through the *Administrator* or *tcnickl* accounts when the Server is being accessed locally (i.e. on MUTANT itself, not through a network connection). User groups that define the types of files that can be accessed are set up through "User Manager for Domains", which also creates and deletes user accounts. MUTANT houses two hard disk drives. The C: drive is 500 MB in size and houses operating system files and programs essential to maintain the computer. A 4 GB hard drive was installed as D: and is used for large storage, ancillary programs, and off-site information storage and retrieval. The 4GB drive has been named **GIG!** and can be mapped as a network drive to trusted intranet workstations. Directory- and file-level access can be set within this directory for security.

When a peripheral workstation is accessed, the user is requested to for their id and password. This information is passed through the network to MUTANT, where it is checked against an encrypted password file, and permissions based on the account's user group status

are then set. In this fashion, sensitive files like the Novartis database cannot be accessed by unauthorized accounts. If a user elects to use a Win 95 or lower computer and bypasses the login, they are restricted to local use of that computer only. NT workstations will not allow unauthorized logon.

In addition to tracking user accounts and authorizing access, MUTANT runs the Internet Information Service® (IIS) on startup. IIS is used to create partitions that can be accessed from off-site (through the Internet). One partition offers a site for anonymous ftp: access, which is directed to the directory GIG!:\anonymous. The root directory for GIG! is not accessible except with special access obtained through a defined user account. Within GIG!:\anonymous, directory- and file- level access can be defined to lock out access except by members of defined groups. Virtual partitions for global ftp: access to the machine have also been set up. A WWW partition was assigned to the directory GIG!:\website. Within GIG!:\website is the file default.htm, which is automatically sent to browsers that request http://mutant.lse.okstate.edu/. Subdirectories within ~\website\ can also house a default.htm document that is automatically sent when that subdirectory is requested. Directory browsing has been disabled for security reasons. The subdirectory ~\website\front_mutant\ contains code for HTML forms and subdirectory ~\website\searchable\ contains databases linked to the web to generate dynamic (ASP) pages.

APPENDIX B: BRIEF LIST OF HTML TAGS

The following page briefly outlines some of the codes used for creating an HTML document. It is presented as a single page for ease of photocopying so it can be used as a reference at the computer. The codes it contains should be sufficient to construct a rudimentary web document but the user should acknowledge that the repertoire of HTML code is ever-expanding.

HTML QUICK LIST

Good sites for basic HTML information:

☺ http://millbury.k12.ma.us/~hs/htmlwrite/html2.html
This is a basic tutorial geared to elementary schools with an emphasis on how to help children design their own pages. Excellent examples and non-technical terminology, with links to other tutorial sites geared to the specific lesson make this one of the best instruction sites I've encountered so far.

☺ http://robot0.ge.uiuc.edu/ela/
Carlos (http://robot0.ge.uiuc.edu/~carlosp/) is fairly well known in web-tutorial circles. He has interactive tutorials which allow you to follow along. They *can* be slow, but sometimes (early in the morning, when the servers aren't too busy) you can move at a good pace. The tutorials are for engineering students, but are still surprisingly non-technical.

CODE LIST

Codes are identified by your browser (Internet Explorer, Netscape) by being surrounded by "pointy brackets" ("<", ">"). If your browser understands them, they are implemented. Codes it doesn't understand are simply ignored. Codes can be in capital letters or lower case. I use caps to make them stand out when I read the code, and will adhere to this convention here.

Codes that "activate" a range (e.g. makes a range of characters bold, or makes a range of characters "hot" as a "link") begin with the code beginning sequence in pointy brackets, followed by the range that is to be manipulated. The code is turned off with a "slash" ("/"), followed by the name of the code to be turned off, again all in pointy brackets (e.g. This is not italic, <I>but this is</I>, and this is not.). Not all codes need to be turned off as they don't define a range. (e.g. <P>,
).

Paragraph Formatting

paragraph end: **<P>**

end of line: **
**

lists:

ordered list (numbered):
```
<OL>
  <LI>...
  <LI>...
</OL>
```

unordered list (bullets):
```
<UL>
  <LI>...
  <LI>...
</UL>
```

indented
```
<BLOCKQUOTE>
...
</BLOCKQUOTE>
```

centered
```
<CENTER>...
</CENTER>
```

Text Formatting

Italic: **<I>italic text</I>**

Bold: **<B>bold text</B>**

Underline: **<U>underlined text</U>**

Color font: **<FONT COLOR="red">text</FONT>**

Size font: **<FONT SIZE="3">font</FONT>**

Note that size and color can be combined into one tag.

Adding Pictures

GIF or JPEG files can be used:

<IMG SRC="filename.gif">

Adding Hyperlinks

Link to file: **<A HREF="filename.ext">text</A>**

Link to site: **<A HREF="http://site.com">text</A>**

Note that the text outside the tags is the linking text.

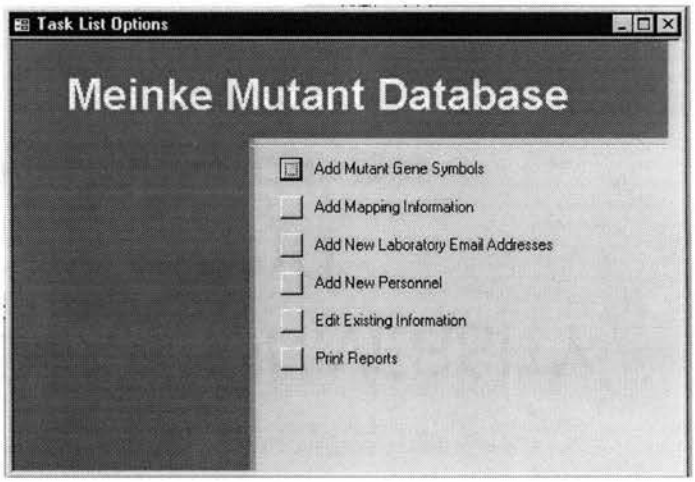
APPENDIX C: GENERAL INFORMATION ABOUT THE MEINKE WEBSITE

To make the task of updating the information in the Meinke website easier and allow more routine maintenance, a database was designed with fill-in forms to direct and error-check input. The database can therefore be kept current with a minimum of effort. The fill-in forms are used to update data tables in the database. These tables can be updated manually or imported as spreadsheets, but the forms direct input with labelled fields and restrict entries to conform to the data types required for the database. The database is queried when a computer client requests current data for either mutant symbols or linkage data. Results to these queries are rendered by Active Server Pages® (ASP) into plain text, tabular, or semicolon-delimited as requested by the client.

This appendix outlines the various interfaces with which a person updating the database will be presented and provides some information about the type of information that is required for each field. The next appendix will detail how Active Server Pages queries the database and presents the information back to the client.

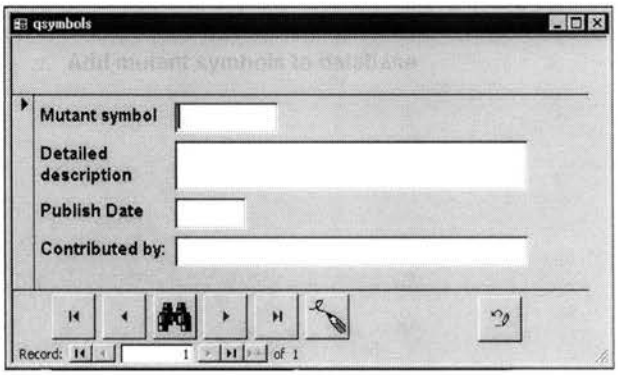
The main switchboard

When Microsoft Access® is started and gig!:\website\searchable\symbolslist.mdb is loaded, the user is presented with the main menu (called a switchboard) which automatically loads with task options. The proper task can be selected by clicking with the mouse on the box to the left of the task to be completed. The main menu lists tasks



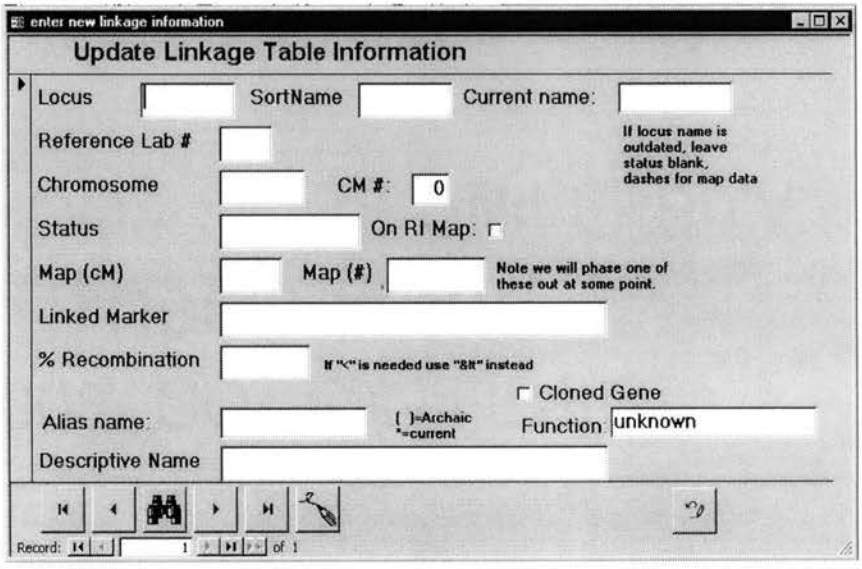
usually associated with adding rather than modifying database information. Gene symbols can be added using the first option, linkage information can be added with the second, and contact information for laboratories contributing information is added with the third. The fourth option allows a page to be generated that lists staff of the Meinke laboratory. The current server renders this information into a web page slowly, so data for staff should be used to create an active server page from which the code is used to create a static page that loads faster. The fifth option allows editing of database information. It brings up a menu that offers selections for the type of data to be edited, but the forms used are otherwise identical to those brought up by the main menu. The reason the "edit" menu is accessed through a second menu is to protect the data from accidental manipulation. It is easier to delete incorrect new data than it is to recover altered data already in the database.

Add mutant symbol



This adds information to the database data table entitled "symbols". The first field requests a mutant symbol, which must be in capital letters only. Lower case letters and numbers are rejected. Symbols should be exactly three letters long, but because some labs have elected to not conform to standardized nomenclature, there is flexibility in the data that can be accepted. The publish date field refers to the year the symbol was submitted. Contributions are indicated by author names in the last field.

Update linkage information



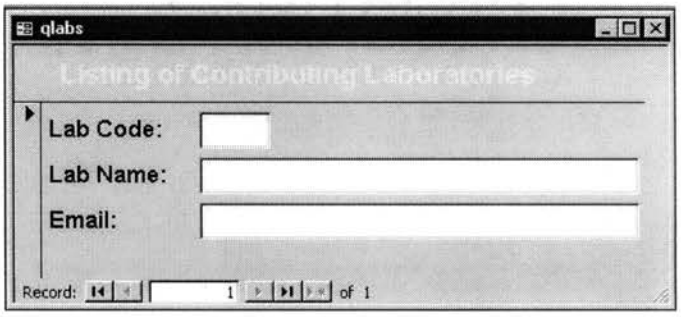
This form allows entry of classical linkage information. The database data table is entitled "linkage_table". The *locus* field is the name of the mutant locus.

Sortname is a modification of *locus* to account for sorting the data when more than one digit follows the locus name. Placeholder zeros move the digit to a correct sorting location: e.g. *emb21* is a member of the *emb* locus name group which has hundreds of members and the sortname would be "emb021" so it would appear in the proper location during a sorted operation. The *current name* field indicates what the current name of the locus and is only used in cases where the locus name has been replaced by another. When this field is filled in (only in the case of an archaic locus) the current status field is left blank and a dash is put in the *Map (cm)* field. *Reference lab* # refers to a code associated with labs in the "linkage_table" data table (to update, see **List of Contributing Laboratories** below). This field accepts only numbers. *Chromosome* and *CM#* are for entering the chromosome for the locus. Two fields are provided; the first also will accept non-numeric entries so that translocations can be indicated and the second must be numeric for sorting purposes. *Status* can be CM, AVM, U, or several other codes but should be left blank if the locus being updated is archaic. *On RI Map* is a checkbox that should be selected if the locus is also found on the RI map. *Map (cM)* and *Map (#)* refer to the map location on the chromosome. Again, two fields are supplied with the first also accepting text (so TOP, BOT, and MID information can be entered) and the second restricted to numbers only. The *linked marker* field holds the name of the locus to which the current record is mapped, and *percent recombination* is the crossover frequency for this locus. Note that if a "<" character is needed, "&lt" should be entered (no quotes around these entries) because "<" and ">" are HTML tags and not printed. The *Cloned gene* and *Function* fields are selected if the locus has been cloned. The *function*

field defaults to "unknown" which can be replaced when the identity of the cloned gene has been determined. The *descriptive name* holds the long form of the locus name.

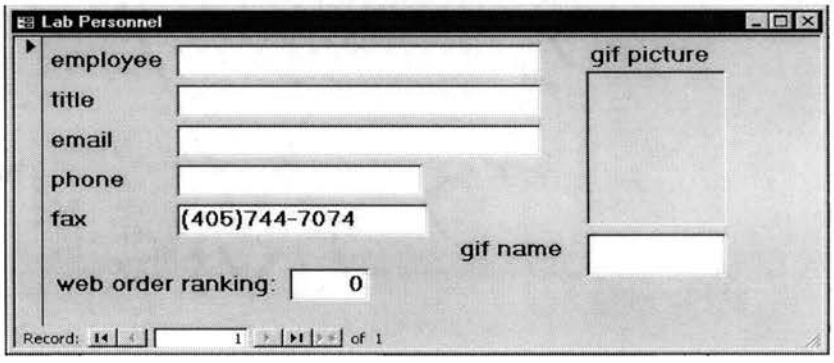
Listing of contributing laboratories

This updates the data table named "LabEmail". Before using this form, make sure the lab is not already represented in the database. *Lab code* holds the number associated with the laboratory. *Lab name* is the last name of the primary investigator for the group submitting the information. The *email* field holds contact information.



Laboratory personnel

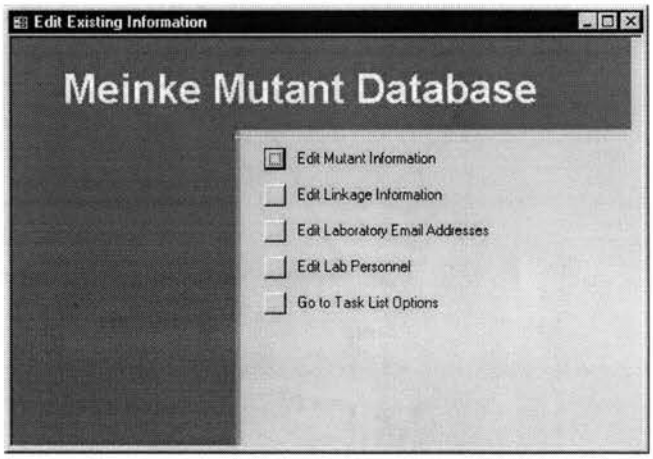
There is a form to update information about current laboratory personnel. Though it is possible to create a "live" version of the personnel page using ASP, this renders slowly due to having gif information



incorporated. However, the fastest way to create the code for a new static page, is done by updating the database, opening the dynamic page "http://mutant.lse.okstate.edu/ourlab/personnel.asp", then copy the commands shown when you "reveal codes" into the file "personnel.html" and saving to gig!:\\website\ourlab\.

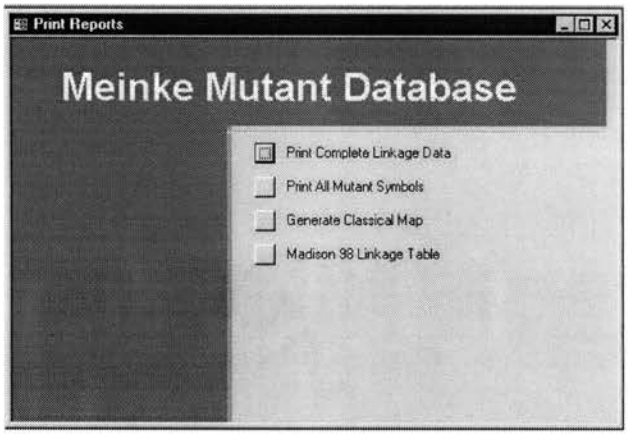
Editing existing information

A second menu can be accessed from the main menu through the second last option. Each selection save the last opens its corresponding data table with a form similar to those described previously in this appendix. However, the navigation controls at the bottom of these forms will indicate that the data in the tables has been loaded. Arrow keys are used by the user to select the record to be edited and updated information can be entered. The final selection on this menu brings back the main menu.



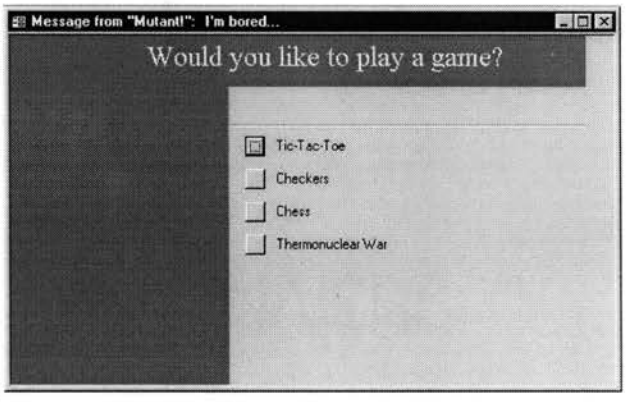
Print records

So that hardcopy can be generated for dissemination or storage, the final selection on the main menu brings up options to print out records. These records are preformatted and provide standardized output that reflects the current state of the database. These options can be altered by a database administrator (for example, the last option was customized for a meeting).



Additional menus

If queried with this final menu, under no circumstances should you select the final option. The computer we call "mutant" may be quirky, but its sense of humor is rather circumspect. Remember, it's left on all night with the lights out, and has permanent connections to the Internet. Treat it with respect and you should get along just fine.



The next appendix shows how ASP renders information selected from the database into html code that suits the client's request.

APPENDIX D: ACTIVE SERVER PAGES FOR THE MEINKE WEBSITE

The Meinke website is a repository for mutant locus name information and classical mapping data. It is designed for a range of browsers and to accommodate the needs of a diverse range of resources in the community. Therefore, the page has been designed without frames and imposes few demands on the client. When on-line forms are provided to the client, alternative methods that do not require forms are also indicated because outdated browsers cannot handle forms. Alternative submission may be in the form of an email address or a page that can be printed out and mailed to the Meinke laboratory so the information can be added to the database underlying the web page.

Data is presented using VBScript that uses SQL commands to establish a connection to the database and query certain fields. Data submission is through forms that are handled by Microsoft Front Page® so that the information saved is formatted and the submitting client receives a confirmation page. I update pages manually through Microsoft Notepad® except for the Front Page form files. A rule of thumb is that any page ending with *.html is a static page or a form, and pages ending with *.asp consist of VBScript code that constructs a web page based on a client request. The following VBScript code is presented here to allow the web page administrator a resource to update and troubleshoot code for the Meinke web page.

First, a few words to those uninitiated in the use of Active Server Pages®. The code combines classic HTML with language that can be executed to produce "HTML-like" output that is rendered into a unique page based on the client request. Essentially, the page is substituting data into the page where appropriate and looping to create long pages with redundant substitutions for various fields. VBScript is identified by being enclosed in "<%" and "%>" tags. This means that anything between those tags is "executed" and returns a value (if appropriate) into the HTML page being rendered. The recurrent theme for the codes shown here are this:

1. Define through HTML the header for the pages (define <TITLE>, <H1>, ... etc.).
2. Make a brief statement to the person using the client about what they are about to see and how to interpret it (and usually a "please wait" because db query can be slow).
3. Define column headings if appropriate.
4. Open the database connection, select appropriate database (use filename as defined in ODBC, which is tuned through "Settings" then "Control Panel" for the operating system), then identify the name of what is to be queried, be it table or a defined query within the database (note; I use queries because they can be sorted. I usually append the letter "q" to the front of my defined queries, such as "qsymbols" which queries the symbols table).
5. Assign data queried from the database to variables. Parse and format these variables for uniformity. Unless an array is defined, when the record is complete, print a line to the new page, then do the same for the next record. Arrays remain in memory until the file has been read and then are printed.

6. When the data have been presented, dump houskeeping variables like counters into formatted explanation strings.
7. All data has now been presented. Show "friendly" features such as navigation buttons, who to contact for problems, etc.

The rest of this appendix will deal with annotated plain examples for their output, with selected code blocks from more sophisticated queries so the administrator reading this will be able to understand underlying logic and be prepared to appropriately modify the code.

VBSCRIPT CODE FOR MUTANT SYMBOLS

Plain list of symbols

The plain query for symbols is the simplest version of the query. It simply opens a connection to the database, identifies the table or query required, assigns the contents of requested fields to variables, then outputs the information into a new page (dynamic page, rendered *de novo* for each client request). Following this example, portions of formatted output which is coded in alternative *.asp pages will be shown.

File: plain_symbol_list.asp (from gig!:\website\genepage)

```
<%@ LANGUAGE = VBScript %>          THIS ESTABLISHES VBSCRIPT AS THE PROGRAMMING LANGUAGE.
<HTML>
<HEAD>
<TITLE>Unformatted Listing of the Symbol Database Information</TITLE>
</HEAD>
(querying database... please wait)
<HR>
<CENTER>
<H1>List of Registered Symbols for Mutant Genes</H1>
</CENTER>
<HR>
<PRE>             PRE MEANS THE TEXT WIDTH IS PRESET (TABS WILL NOW WORK, TEXT WON'T WRAP).
<B><H3>Explanation of Columns:</H3></B><BR>
```

```
<UL>
<LI>Locus:  Gene symbol (CAPS to avoid confusion between letters and numbers).
<LI>Descriptive Name:  Formal name of locus; description of phenotype.
<LI>Date:   Shows date when symbol was reserved or identified.
<UL><LI>YB = Already in previous genome report list (Yellow Book, Year 3).
<LI>M95 = Obtained from abstract book - Arabidopsis meeting, June 1995.
<LI>N96 = Obtained from WWW abstracts - Arabidopsis meeting, June 1996.
<LI>M97 = Obtained from abstracts/pages - Arabidopsis meeting, June 1997.</UL>
<LI>Reference Laboratory:  Source of mutant gene symbol.
</UL>
<P>
<I>Note that this list has no formatting.  Data is "TAB" delimited with spaces used to
create uniform field lengths.  You can save this page as a file and edit it with the
spreadsheet of your choice (follow <A HREF="../toddnickle.html">this link</A> for hints).
This format is offered for browsers that do not handle large tables.</I><P>
                                        THIS LINE OF TEXT ESTABLISHES THE COLUMN HEADINGS.
<B>Symbol</B>              <B>Descriptive Name/Mutant Phenotype</B> <B>Date</B>
<B>Ref_Lab</B><BR>

     THE FOLLOWING CODE ESTABLISHES THE DATABASE LINK AND SETS UP ENVIRONMENTAL VARIABLES.
<FORM ACTION="/advworks/ ........ " METHOD=POST>
<% whitespace = "              " %>  "whitespace" IS A STRING OF SPACES TO PAD FIELD WIDTHS.
<% Dim counter %>          THE COUNTER VARIABLE IS CALLED AND INITIALIZED IN THESE 2 LINES.
<% counter = 0 %>

            THIS IS THE BLOCK THAT CALLS THE SQL CODE ITSELF:  NOTE THAT IT IS TECHNICALLY
               ONE LINE OF CODE BUT LINE BREAKS ARE ESSENTIAL FOR IT TO FUNCTION PROPERLY.
          IT IS CRITICAL THAT THE DATABASE FILE (e.g. "SYMBOLSLIST.MDB") IS DEFINED IN THE
                        SERVER'S "ODBC" UTILITY (IN CONTROL PANEL OF THE OPERATING SYSTEM).
<% Set OBJdbConnection = Server.CreateObject("ADODB.Connection")
OBJdbConnection.Open "symbolslist"                  THIS TARGETS THE FILE "SYMBOLSLIST.MDB".
SQLQuery = "SELECT * FROM qsymbols"    "QSYMBOLS" QUERY IN "SYMBOLSLIST.MDB" IS IDENTIFIED.
Set RSLookup = OBJdbConnection.Execute(SQLQuery)           THE COMMAND RSLOOKUP IS DEFINED.
%>

                                                THIS BLOCK OF CODE QUERIES THE DATABASE.
                                      EACH VARIABLE TO THE LEFT OF THE "=" IS ASSIGNED THE VALUE
                                       QUERIED FROM THE DATABASE (IN QUOTES AND PARENTHESES) AND
                                                  THE WHITESPACE STRING IS APPENDED TO THE END.
                                                   THE VARIABLE IS THEN PARSED TO A SET LENGTH.
          THIS LOOPS TO DO THE SAME TO CONSECUTIVE RECORD UNTIL AN "END OF FILE" IS FOUND.
<% Do While Not RSLookup.EOF %>              DETERMINES LOOP.  NOTE "EOF" = "END OF FILE".
<% symbol = RSLookup("Field1")&whitespace %>ASSIGNS "symbol" THE VALUE IN FIELD1, ADDS PAD.
<% symbol = left(symbol, 6) %>           THIS SNIPS THE LENGTH OF "symbol" TO 6 CHARACTERS.
<% name = RSLookup("Field2")&whitespace %>                                          ...etc...
<% name = left(name, 35) %>
<% dateof = RSLookup("Field3")&whitespace %>
<% dateof = left(dateof, 6) %>
<% lab = RSLookup("Field4")&whitespace %>
<% lab = left(lab, 20) %>
                       THE FOLLOWING LINE PRINTS THE INFORMATION INTO THE DYNAMIC WEB PAGE.
<%= symbol&"              "&name&"    "&dateof&"     "&lab %><BR>
                                                   THE "counter" VARIABLE IS INCREMENTED.
<% counter = counter + 1 %>
             THIS LAST LINE OF THE BLOCK IS SEPARATED PHYSICALLY SO IT FUNCTIONS PROPERLY.
          IT MOVES TO THE NEXT CONSECUTIVE RECORD AND LOOPS TO THE TOP OF THE CODE BLOCK.
<%
RSLookup.MoveNext
Loop
%>

<HR>
               FINALLY, THE VALUE OF "counter" IS PRINTED AFTER TEXT EXPLAINING WHAT IT IS.
<B><I>Total number of symbols in list: <% =counter %></I></B>
```

```
</pre>                                                    END PREFORMATTING.

                                NOW THE USUAL FINAL STUFF TO PUT IN BUTTONS AND DOCUMENT THE PAGE.
<ADDRESS>Contact <A HREF = mailto:meinke@osuunx.ucc.okstate.edu>David Meinke</a> for
corrections or requests for
more information.
</ADDRESS><P>
<A HREF="../default.htm"><IMG SRC="../IMAGES/home_button.gif" ALT="Return to Meinke Welcome
Page" BORDER = 0></A>
<A HREF="genepage.html"><IMG SRC="../IMAGES/mutant_gene_button.gif" ALT="Return to Mutant Gene
Page" BORDER = 0></A>
<BR>
<A HREF="../default.htm">Home</A>, <A HREF="genepage.html">Mutant Genes</A>
</BODY></HTML>
```

Tabular output for mutant symbol queries

Since a formatted table is sometimes more desirable than plain ascii text despite the fact it can take a while to be rendered, the following code shows a modification that allows output to be presented as a table to the client. While the rendered page is considerably larger than the "plain" text shown before, because it is ascii text the transmission time for the information should be negligible. However, slower clients must still interpret the incoming information and tables of 100+ rows can give low-end resources considerable processing difficulties. The computer layman might worry that overtaxing clients might cause hardware problems, but in fact the problem is entirely one of processing time: this code does not damage hardware and only causes long client interpretation times. Tabular output is most popular for preserved hardcopies.

File: query_symbol_list.asp (from gig!:\website\genepage)

```
...                                        CODE PRECEDES THIS, BUT IS SIMILAR TO PREVIOUS EXAMPLE
<TABLE WIDTH=600 BORDER=0>                                  IN THIS CASE, THE HEADER IS A TABLE.
<TR>                 COLOR OF FIELDS AND TEXT PRESENTATION ARE DEFINED, ALONG WITH HEADINGS.
  <TD BGCOLOR="cyan" ALIGN=CENTER>
```

```
  <FONT STYLE="ARIAL" SIZE=3>
  <B>Symbol</B>
</FONT></TD>
  <TD BGCOLOR="cyan" ALIGN=CENTER>
  <FONT STYLE="ARIAL" SIZE=3>
  <B>Descriptive Name / Mutant Phenotype</B>
</FONT></TD>
  <TD BGCOLOR="cyan" ALIGN=CENTER>
  <FONT STYLE="ARIAL" SIZE=3>
  <B>Date</B>
</FONT></TD>
  <TD BGCOLOR="cyan" ALIGN=CENTER>
  <FONT STYLE="ARIAL" SIZE=3>
  <B>Reference Lab</B>
</FONT></TD>
</TR>
```

.ASP MEAT'N'POTATOES: FILES AND QUERIES DEFINED.

```
<FORM ACTION="/advworks/ ........ " METHOD=POST>
<% Dim counter %>
<% counter = 0 %>
<% Set OBJdbConnection = Server.CreateObject("ADODB.Connection")
OBJdbConnection.Open "symbolslist"
SQLQuery = "SELECT * FROM qsymbols"
Set RSLookup = OBJdbConnection.Execute(SQLQuery)
%>
```

DEFINE AND INITIALIZE COUNTER

MY TYPICAL LOOP ACTION: WHILE "END OF FILE" HASN'T APPEARED, DO A LOOP

```
<% Do While Not RSLookup.EOF %>
```

HOWEVER, IN THIS CASE, DATA IS PUT INTO A TABLE DIRECTLY, NOT STORED UNTIL OUTPUT FOR THE RECORD IS COMPLETE. NOTE THAT THE CONTENTS FOUND BY RSLOOKUP ARE IMMEDIATELY RENDERED INTO HTML CODE AND PLACED INTO THE DEVELOPING TABLE.

```
<TR>
  <TD BGCOLOR="f7efde" ALIGN=LEFT>
  <FONT STYLE="ARIAL NARROW" SIZE=2>
  <%=RSLookup("Field1")%>
</FONT></TD>
  <TD BGCOLOR="f7efde" ALIGN=LEFT>
  <FONT STYLE="ARIAL NARROW" SIZE=2>
  <%= RSLookup("Field2")%>
</FONT></TD>
  <TD BGCOLOR="f7efde" ALIGN=CENTER>
  <FONT STYLE="ARIAL NARROW" SIZE=2>
  <%= RSLookup("Field3")%>
</FONT></TD>
  <TD BGCOLOR="f7efde" ALIGN=LEFT>
  <FONT STYLE="ARIAL NARROW" SIZE=2>
  <%= RSLookup("Field4")%>
</FONT></TD>
</TR>
<% counter = counter + 1 %>
```

COUNTER IS INCREMENTED. CHECK NEXT RECORD IN THE DATABASE.

```
<%
RSLookup.MoveNext
Loop
%>
...
```

CODE FOLLOWS, BUT IS SIMILAR TO PREVIOUS EXAMPLE

Specific query for a mutant locus

A form that requests a single symbol that is compared throughout database extracted queries has been devised and is shown here. Previous examples showed code that revealed the complete contents of mutant symbols in the database. Often, investigators are only concerned with a single symbol. Thus the following code shows how the client can specify a string for a symbol that will be compared sequentially through records until a match is made. At each comparison, information associated with the compared field will be cached until overwritten or displayed. Though this is inefficient, it's the best way I know to do it. With the current server, delays caused by this extra demand on the processor are less than two minutes (in rare worst-case situations). Average access times have been clocked (during modem inquiries) to be less than 5s and up to 20 s. Redundant code has again been stripped from these examples so the code responsible for database acquisition, querying, and subsequent output will be highlighted.

The first part of the code is the static modified form code from FrontPage which calls up the *.asp dynamic search VBScript code. They are separate files, but the former errorchecks input from the client using JAVAScript generated by FrontPage and modified by Todd Nickle. The second ASP page is handed the information and generates a return to the client.

File: query_symbol.html (from gig!:\website\front_mutant)

```
<!DOCTYPE HTML PUBLIC "-//IETF//DTD HTML//EN">
<html>
<head>
<meta http-equiv="Content-Type"
content="text/html; charset=iso-8859-1">
<meta name="GENERATOR" content="Todd Nickle (modified from Front Page)">
```

```
<title>Query gene symbols</title>
<meta name="FORMATTER" content="Todd Nickle (modified from Front Page)">
</head>
<body bgcolor="#00FFFF">
<hr>
<h1 align="center">Query Mutant Gene Symbols</h1>
<hr>
                THE FOLLOWING OPENS THE CGI SCRIPT THAT HANDS OVER THE CODE TO THE ASP PAGE
<!--webbot bot="GeneratedScript" preview=" " startspan --><script
language="JavaScript"><!--
function FrontPage_Form1_Validator(theForm)
{
                JAVASCRIPT HERE WON'T LET THE FORM BE SUBMITTED UNLESS FILLED OUT CORRECTLY
  if (theForm.search.value == "")                          ← USER DIDN'T ENTER A SEARCH TERM
  {
    alert("Please enter a value for the \"search\" field.");
    theForm.search.focus();
    return (false);
  }

  if (theForm.search.value.length < 2)                  SEARCH VALUE IS ONLY ONE CHARACTER
  {
    alert("Please enter at least 2 characters in the \"search\" field.");
    theForm.search.focus();
    return (false);
  }

  if (theForm.search.value.length > 3)          SEARCH VALUE IS MORE THAN THREE (SORRY GERD)
  {
    alert("Please enter at most 3 characters in the \"search\" field.");
    theForm.search.focus();
    return (false);
  }
                    EACH VALUE IN THE STRING MUST BE CAPS AND ALPHABETIC TO PASS THIS CHECK
  var checkOK = "ABCDEFGHIJKLMNOPQRSTUVWXYZ";
  var checkStr = theForm.search.value;
  var allValid = true;
  for (i = 0;  i < checkStr.length;  i++)
  {
    ch = checkStr.charAt(i);
    for (j = 0;  j < checkOK.length;  j++)
      if (ch == checkOK.charAt(j))
        break;
    if (j == checkOK.length)
    {
      allValid = false;
      break;
    }
  }
  if (!allValid)       "!" MEANS "NOT", SO IF "ALLVALID" FUNCTION IS NOT TRUE, INDICATE FAIL
  {
    alert("Please enter only letter characters in the \"search\" field.");
    theForm.search.focus();
    return (false);
  }                                                          THIS IS CODED AS AN "ELSE"
  return (true);
}

//--></script><!--webbot bot="GeneratedScript" endspan -->
<form action="one_symbol_result.asp" method="POSTIF CHECKS PASS, INFO→ONE_SYMBOL_RESULT.ASP
onsubmit="return FrontPage_Form1_Validator(this)"
name="FrontPage_Form1">
    <blockquote>
        <blockquote>
```

```
          <p>Symbol to look up: <!--webbot bot="Validation"
          s-data-type="String" b-allow-letters="TRUE"
          b-value-required="TRUE" i-minimum-length="2"          QUERY MUST BE > 1 CHARACTER.
          i-maximum-length="3" --><input type="text" size="4" QUERY MUST BE < 4 CHARACTERS.
          maxlength="3" name="search"> Please use CAPITAL
          LETTERS only.<br>
          </p>
          <pre>
Examples:                                    SEARCH FAILED... ALTERNATE SUBMISSION STYLES AND
Acceptable search terms:   ABA              POSSIBLE ERRORS ARE SUGGESTED IN THIS TEXT BLOCK.
                           CH
Unacceptable search terms: AXR1
                           AXR1-1
                           CH 6
                           aba
                           Aba
</pre>
            <p>Use only one term per search. (ABA ABA1 invalid) </p>
            <p><input type="submit"><input type="reset"> </p>
        </blockquote>
    </blockquote>
</form>

<hr>
                                                  NORMAL BUTTON AND BOTTOM OF PAGE STUFF HERE.
<p><a  href="../default.htm"><img  src="../images/home_button.gif"  border  =  "0"></a><a
href="../genepage/genepage.html">
<img src="../images/mutant_gene_button.gif" border = "0"></a><br>
<a href="../default.htm">Home</a> <a href="../genepage/genepage.html">Mutant Genes Page</a>
</p>
</body>
</html>
```

File: one_symbol_result.asp (from gig!:\website\front_mutant)

```
<%@ LANGUAGE = VBScript %>
<HTML>
<HEAD>
<TITLE>Listing of the Symbol Database Information</TITLE>
</HEAD>
<BODY>
<body bgcolor="#00FFFF">
<HR>
<CENTER>
<H1>Search for Mutant Symbol</H1>
</CENTER>
<HR>
<BLOCKQUOTE><BLOCKQUOTE>
<FORM ACTION="/advworks/ ........ " METHOD=POST>
                                                  CONNECTION TO DATABASE AS BEFORE.
<% Set OBJdbConnection = Server.CreateObject("ADODB.Connection")
OBJdbConnection.Open "symbolslist"                  DATABASE NAMED SYMBOLSLIST.MDB.
SQLQuery = "SELECT * FROM qsymbols"                        QUERY TABLE IS QSYMBOLS.
Set RSLookup = OBJdbConnection.Execute(SQLQuery)
%>

<% searchterm = Request.Form("search") %>  FROM THE FORM IS RECEIVED THE VARIABLE "search".
<% responsestring = "Term "&searchterm&" not found" %>ERROR MESSAGE IN CASE TERM NOT FOUND.
```

```
<% Do While Not RSLookup.EOF %>                          LOOP FOR COMPARISONS NOW STARTED.
<% checkname = RSLookup("Field1") %>      FIELD1 OF QSYMBOLS ASSIGNED TO TEMPORARY VARIABLE.
<% If searchterm = checkname Then %>          COMPARISON WITH TERM AND TEMP VARIABLE MADE.
   <% flag = "set" %>                                        FLAG IS SET IF THEY MATCH.
   <% longdesc = RSLookup("Field2") %>     DATA FOR LONG DESCRIPTION GIVEN TO LONGDESC VBL.
   <% labname = RSLookup("Field4") %>              CONTRIBUTING LAB INFO ASSIGNED TO VBL.
<% End If %>
<%                              DO IT AGAIN. ANOTHER MATCH WILL OVERWRITE POS IDENTIFICATION.
RSLookup.MoveNext
Loop
%>
<H3>Query completed</H3>
<P>
<% If flag = "set" Then %>                              FLAG IS ONLY SET IF A MATCH WAAS MADE.
<B><H2>Term <% =searchterm %> found.</H2></B><BR>            INDICATE THE TERM WAS FOUND.
<B>Description:</B> <% =longdesc %><BR>                      RELEASE DATA AS APPROPRIATE.
<B>Reference Lab:</B> <% =labname %><BR>
</BLOCKQUOTE>
<H3><% =searchterm %> <B>cannot be submitted</B> as a new gene symbol.<P></H3>   MORE INFO.
<% Else %>
<B><H2><% =responsestring %></H2></B><P>                 FLAG NOT SET: NO MATCH WAS SEEN:
Be sure you used only CAPITAL LETTERS without numbers, spaces or special characters.<BR>
<PRE>
Examples:
Acceptable search terms:   ABA
                           CH
Unacceptable search terms: AXR1
                           AXR1-1
                           CH 6
                           aba1
</PRE>
<BR>
If you used an invalid search term, a related symbol may still be represented in the
database.<P>                        END OF WARNING WITH SUGGESTIONS FOR POSSIBLE QUERY GOOFS.
</BLOCKQUOTE>
<H3><% =searchterm %> <B>can be submitted</B> as a new gene symbol if the search term was
acceptable.</H3><P>          BECAUSE THE TERM WAS NOT FOUND, IT *CAN* BE SUBMITTED AS NEW.
<% End If %>
</BLOCKQUOTE>                                                          END OF PAGE STUFF.
<HR>
<A    HREF="../default.htm"><IMG    SRC="../images/home_button.gif"></A><A
HREF="../genepage/genepage.html"><IMG SRC="../images/mutant_gene_button.gif"></A><BR>
<A HREF="../default.htm">Home</A> <A HREF="../genepage/genepage.html">Mutant Genes Page</A>
</BODY>
</HTML>
```

QUERYING RECOMBINATION DATA

Recombination data is another commodity the Multinational *Arabidopsis* Genome Project requires to function efficiently. The database also contains mapping information needed by investigators to choose appropriate actions and experiments to maximize effort and minimize

redundancy. Code that returns values for queried recombination fields is included here... starting with the code for a basic page and culminating with more sophisticated data presentation and searching facilities. It bears noting that information in the database can be for a locus name alone (eg. ABA) or for a locus which is a member of a particular type (eg. ABA1, ABA2, ...) and the person formulating the query may be unaware of the series. So, if a scientist requests information about ABA, not knowing that ABA1 and subsequent members of the group are present, they would have returned to them a "no locus found" page. To minimize this type of confusion, I built into the code options which create alternative search terms which will bring up sufficient information to warn the investigator that the *absolute* term queried was not found, though a potentially useful *alternate* term was seen.

File: plain_linkage_list.html (from gig!:\website\genepage)

```
<%@ LANGUAGE = VBScript %>
<HTML>
<HEAD>
<TITLE>Unformatted List of Mutant Linkage Data</TITLE>
</HEAD>
(querying database... please wait)
<HR>
<CENTER>
<H1>Linkage Table - Mutant Genes</H1>
</CENTER>
<HR>
This is a comprehensive list of mutant genes for which linkage data are available.
Follow <A HREF="mutant_list_exp.html">this link</A> for an explanation of column headings.
Synonyms for mutant genes known by more than one name are included in. E-mail addresses of
laboratories contributing linkage information can be obtained by clicking
on the reference lab heading.
<P>
<I>Note that this list has been formatted to line up.  Data is "TAB" delimited with spaces
used to pad the field sizes so the columns will line up.  Keep this in mind if you save this
page as a file to edit with a spreadsheet (follow <A HREF="../toddnickle.html">this link</A>
for hints).  This format is offered for browsers that do not handle large tables.
</I>
<PRE>                                                    PREFORMATTED TEXT STARTS HERE.
```

```
<B>Locus</B>  <B>#</B> <B>Current</B> <B>Status</B>  <B>Cloned</B>  <B>Map</B>  <B>Linked</B>
        <B>%Rec</B>     <B><A HREF="plain_lab_list.asp">Lab</A></B>     <B>Full Descriptive
Name</B>                    <B>Alias</B><BR>                              SET UP COLUMN HEADINGS.
<FORM ACTION="/advworks/ ........ " METHOD=POST>
<!counters for"unique" loci and number of loci on classical map >          (LIKE IT SAYS...).
<% Dim counter %>
<% counter = 0 %>
<% Dim CMcounter %>
<% CMcounter = 0 %>
                                                                          SET UP DATABASE CONNECTION.
<% Set OBJdbConnection = Server.CreateObject("ADODB.Connection")
OBJdbConnection.Open "symbolslist"                                       SYMBOLSLIST.MDB IS SELECTED.
SQLQuery = "SELECT * FROM qlinkage"                                       QLINKAGE QUERY IS SELECTED.
Set RSLookup = OBJdbConnection.Execute(SQLQuery)
%><% whitespace = "                          " %>  WHITESPACE IS APPENDED AND PARSED TO FORMAT

<% Do While Not RSLookup.EOF %>                INITIATE LOOP TO QUERY EACH RECORD IN THE FILE
<% locus = RSLookup("Locus")&whitespace %><% locus = left(locus, 6) %>
<% chrom = RSLookup("#")&whitespace %><% chrom = left(chrom, 3) %>
<% current = RSLookup("Current")&whitespace%><% current = left(current, 6) %>
<% status = RSLookup("Status")&whitespace %><% status = left(status, 5) %>
<% cloned = RSLookup("Cloned Gene") %>            "Cloned Gene" FIELD QUERIED FOR CONTENT...
<% If cloned = 0 Then %>                                  IF "0", IT MEANS IT'S NOT CLONED...
   <% cloned = " " %>                THEREFORE, PUT SPACE CHARACTER IN THE CLONED POSITION...
<% Else %>
   <% cloned = "+" %>                                    OTHERWISE, PUT A "+" CHARACTER THERE.
<% End If %>
<% map = RSLookup("Map")&whitespace %><% map = left(map, 4) %>
<% marker = RSLookup("Linked Marker")&whitespace %><% marker = left(marker, 15) %>
<% rec = RSLookup("%Rec")&whitespace %><% rec = left(rec, 7) %>
<% If left(rec,1) = "&" Then %><% rec = rec&"  " %><% End If %>
<% lab = RSLookup("Lab")&whitespace %><% lab = left(lab, 4) %>
<% longdesc = RSLookup("Full Descriptive Name of Gene Symbol")&whitespace%><% longdesc =
left(longdesc, 30) %>
<% alias = RSLookup("Alias name") %>
                                                 CONSTRUCT AND OUTPUT STRING FOR QUERIED FIELDS.
<%=locus&"  "&chrom&" "&current&" "&status&"      "&cloned&"     "&map&" "&marker&" "&rec&"
"&lab&"  "&longdesc&" "&alias %><BR>
                                  INCREMENT COUNTER IF THE CHROMOSOME IS NOT A TRANSLOCATION.
<%If chrom <> "=  " Then %><% counter = counter +1 %><% End If %>
                                INCREMENT "CLASSICAL MAP" COUNTER IF THE STATUS READS AS "CM".
<%If left(status, 2) = "CM" Then %><% CMcounter = CMcounter +1 %><% End If %>
<%                                                                        GO TO NEXT RECORD.
RSLookup.MoveNext
Loop
%>
</PRE>                                                                   END PREFORMATTED TEXT
<HR>
<I><B>Total number of loci in list is <% =counter %></B></I><BR>          OUTPUT # TOTAL LOCU
<B>Number of loci on the classical map is <% =CMcounter %></B><P>            OUTPUT # ON MAP
<ADDRESS>Contact  <A  HREF  =  mailto:meinke@osuunx.ucc.okstate.edu>David  Meinke</a>  for
corrections or requests for
more information.
</ADDRESS><P>
<A HREF="../default.htm"><IMG SRC="../IMAGES/home_button.gif" ALT="Return to Meinke Welcome
Page" BORDER = 0></A>
<A HREF="genepage.html"><IMG SRC="../IMAGES/mutant_gene_button.gif" ALT="Return to Mutant Gene
Page" BORDER = 0></A><BR>
<A HREF="../default.htm">Home</A>, <A HREF="genepage.html">Mutant Genes</A>
</BODY></HTML>
```

File: spreadsheet_linkage_list.html
(from gig!:\website\genepage)

```
...                                                    ONLY DATABASE QUERY IS SHOWN HERE
<% Set OBJdbConnection = Server.CreateObject("ADODB.Connection")
OBJdbConnection.Open "symbolslist"
SQLQuery = "SELECT * FROM qlinkage"
Set RSLookup = OBJdbConnection.Execute(SQLQuery)
%>
<% Do While Not RSLookup.EOF %><% locus = RSLookup("Locus") %>
<% chrom = RSLookup("#") %>
<% current = RSLookup("Current") %>
<% status = RSLookup("Status") %>
<% cloned = RSLookup("Cloned Gene") %>
<% If cloned = 0 Then %>                          CLONED STATUS QUERIED AND VALUE ASSIGNED.
   <% cloned = " " %>
<% Else %>
   <% cloned = "+" %>
<% End If %>
<% map = RSLookup("Map") %>                         NOTE THAT WHITESPACE IS NOT USED HERE.
<% marker = RSLookup("Linked Marker") %>
<% rec = RSLookup("%Rec") %>
<% If left(rec,1) = "&" Then %><% rec = rec&"  " %>
<% End If %>
<% lab = RSLookup("Lab") %>
<% longdesc = RSLookup("Full Descriptive Name of Gene Symbol") %>
<% alias = RSLookup("Alias name") %>
                                                   EACH LINE IS OUTPUT HERE DELIMITED BY TABS.
<%=locus&" "&chrom&"      "&current&"       "&status&"     "&cloned&"     "&map&"
"&marker&" "&rec&"        "&lab&"    "&longdesc&"   "&alias %><BR>
<%If chrom <> "=  " Then %><% counter = counter +1 %><% End If %>
<%If left(status, 2) = "CM" Then %><% CMcounter = CMcounter +1 %><% End If %><%
RSLookup.MoveNext
Loop
%>
```

File: alias_linkage_list.html
(from gig!:\website\genepage)

```
...                                    TABLES ARE USED IN THIS EXAMPLE, HEADING NOT SHOWN.
<TABLE WIDTH=700 BORDER=0>                        DEFINE SIZE OF TABLE AND BORDER WIDTH.
<TR>                                               CODE INDICATES THE FIRST ROW OF DATA.
  <TD                                                  <----------------BEGIN FIRST CELL
  BGCOLOR="cyan" ALIGN=CENTER>                  CELL COLOR IS CYAN, TEXT CENTERED WITHIN.
  <FONT STYLE="ARIAL" SIZE=3>                     FONT DEFINED AS ARIAL WITH A SIZE OF 3.
  <B>Locus</B>                                                    TEXT IN CELL, BOLD.
</FONT></TD>                                               <----------------END FIRST CELL
  <TD BGCOLOR="cyan" ALIGN=CENTER>
  <FONT STYLE="ARIAL" SIZE=3>
  <B>#</B>
</FONT></TD>
  <TD BGCOLOR="cyan" ALIGN=CENTER>
  <FONT STYLE="ARIAL" SIZE=3>
  <B>Current</B>
</FONT></TD>
  <TD BGCOLOR="cyan" ALIGN=CENTER>
  <FONT STYLE="ARIAL" SIZE=3>
  <B>Status</B>
</FONT></TD>
  <TD BGCOLOR="cyan" ALIGN=CENTER>
  <FONT STYLE="ARIAL" SIZE=3>
```

```
  <B>Map</B>
</FONT></TD>
  <TD BGCOLOR="cyan" ALIGN=CENTER>
  <FONT STYLE="ARIAL" SIZE=3>
  <B>Linked</B>
</FONT></TD>
  <TD BGCOLOR="cyan" ALIGN=CENTER>
  <FONT STYLE="ARIAL" SIZE=3>
  <B>%Rec</B>
</FONT></TD>
  <TD BGCOLOR="cyan" ALIGN=CENTER>
  <FONT STYLE="ARIAL" SIZE=3>
  <B><A HREF="plain_lab_list.asp">Lab</A></B>        NOTE THAT IN THIS CASE "Lab" IS "HOT".
</FONT></TD>
  <TD BGCOLOR="cyan" ALIGN=CENTER>
  <FONT STYLE="ARIAL" SIZE=3>
  <B>Full Descriptive Name</B>
</FONT></TD>
  <TD BGCOLOR="cyan" ALIGN=CENTER>
  <FONT STYLE="ARIAL" SIZE=3>
  <B>Alias</B>
</FONT></TD>
</TR>                                                                    END FIRST ROW.

<FORM ACTION="/advworks/ ........ " METHOD=POST>
<! counters for number of "unique" loci and loci on classical map >
<% Dim counter %>
<% counter = 0 %>
<% Dim CMcount %>
<% CMcount = 0 %>
                                                    DATABASE CONNECTION ESTABLISHED.
<% Set OBJdbConnection = Server.CreateObject("ADODB.Connection")
OBJdbConnection.Open "symbolslist"
SQLQuery = "SELECT * FROM qlinkage"
Set RSLookup = OBJdbConnection.Execute(SQLQuery)
%>
                                                    LOOP TO MAKE EACH ROW OF DATA.
<% Do While Not RSLookup.EOF %>
<TR>
  <TD BGCOLOR="f7efde" ALIGN=CENTER>
  <FONT STYLE="ARIAL NARROW" SIZE=2>
  <%=RSLookup("Locus")%>
</FONT></TD>
  <TD BGCOLOR="f7efde" ALIGN=CENTER>
  <FONT STYLE="ARIAL NARROW" SIZE=2>
  <%= RSLookup("#")%>
<% chrom = RSLookup("#") %>
</FONT></TD>
  <TD BGCOLOR="f7efde" ALIGN=CENTER>
  <FONT STYLE="ARIAL NARROW" SIZE=2>
  <%= RSLookup("Current")%>
</FONT></TD>
  <TD BGCOLOR="f7efde" ALIGN=CENTER>
  <FONT STYLE="ARIAL NARROW" SIZE=2>
  <%= RSLookup("Status")%>
<% status = RSLookup("Status") %>
</FONT></TD>
  <TD BGCOLOR="f7efde" ALIGN=CENTER>
  <FONT STYLE="ARIAL NARROW" SIZE=2>
  <%= RSLookup("Map")%>
</FONT></TD>
  <TD BGCOLOR="f7efde" ALIGN=CENTER>
  <FONT STYLE="ARIAL NARROW" SIZE=2>
  <%= RSLookup("Linked Marker")%>
```

```
</FONT></TD>
  <TD BGCOLOR="f7efde" ALIGN=CENTER>
  <FONT STYLE="ARIAL NARROW" SIZE=2>
  <%= RSLookup("%Rec")%>
</FONT></TD>
  <TD BGCOLOR="f7efde" ALIGN=CENTER>
  <FONT STYLE="ARIAL NARROW" SIZE=2>
  <%= RSLookup("Lab")%>
</FONT></TD>
  <TD BGCOLOR="f7efde" ALIGN=LEFT>
  <FONT STYLE="ARIAL NARROW" SIZE=2>
  <%= RSLookup("Full Descriptive Name of Gene Symbol")%>
</FONT></TD>
  <TD BGCOLOR="f7efde" ALIGN=CENTER>
  <FONT STYLE="ARIAL NARROW" SIZE=1>
  <%= RSLookup("Alias name")%>
</FONT></TD>
</TR>
<% If chrom <> "=" Then %>
   <% counter = counter + 1 %>
<% End If %>
<% If status = "CM" Then %>
   <% CMcount = CMcount + 1 %>
<% End If %>
<%                                                          GO TO NEXT RECORD.
RSLookup.MoveNext
Loop
%>
</FONT>
<TR>
<TD COLSPAN=3 VALIGN=TOP ALIGN=LEFT>
</TD>
</TR>
</TABLE>                                                    END OF TABLE CODE.
<HR>
<I><B>Number of loci in list is <% =counter %></B></I><BR>
<B>Number of loci on the classical map is <% =CMcount %></B><P>
```

CONSTRUCTION OF THE CLASSICAL MAP

A classical map can be constructed using records in the database with the status of "CM". Though relative distances are not illustrated by maps constructed in this fashion, the data can be sorted by chromosome in order of mapping from top to bottom. Because the number of loci on each chromosome is different and loci are identified by mapping data, not absolute order, data can't be presented simply by constructing a string and dumping to the form. Instead, a two-dimensional array is constructed with loci assigned array positions based on chromosome number and position order. The ASP page is called from genepage.html through

a form, which sends the type of data requested to the dynamic page. In this section, the form that calls the server page is presented, followed by the annotated code that constructs the classical map.

File: genepage.html (from gig!:\website\genepage)

```
...
<p><strong>Classical Genetic Map</strong> This presents the classical map of mutant genes in
a tabular (not graphical) format. </p>
<BLOCKQUOTE>                                                    BLOCKQUOTE INSETS TEXT
Use this form to view the map of:<P>
<form action="classical_map.asp" method="POST">          FORM THAT RECEIVES DATA SELECTED.
    <ul>                                                        UNORDERED LIST INITIATED.
   <li><input type="radio" checked name="Chromosome"       RADIO BUTTONS = SELECT ONE ONLY.
       value="all">  All Chromosomes </li>          "ALL" VALUE IS SET IF ENTIRE MAP WANTED.
        <li><input type="radio" name="Chromosome"
            value=1> Chromosome 1 </li>      VALUE IS SET TO SELECTED CHROMOSOME OTHERWISE.
        <li><input type="radio" name="Chromosome"
            value=2> Chromosome 2 </li>
        <li><input type="radio" name="Chromosome"
            value=3> Chromosome 3 </li>
        <li><input type="radio" name="Chromosome"
            value=4> Chromosome 4</li>
        <li><input type="radio" name="Chromosome"
            value=5> Chromosome 5 </li>
    </ul>
Locus to highlight: <input type="text" size="6" maxlength="6" name="highlightlocus">
(Optionally highlights locus if present on selected chromosome.  Use CAPS only.).<BR>
                                                            FORM BUTTONS ESTABLISHED.
    <p><input type="submit" value="Submit Form"> <input type="reset" value="Clear Form"></p>
                                                            END OF FORM INDICATED.
</form></BLOCKQUOTE>
```

File: classical_map.html (from gig!:\website\genepage)

```
<%@ LANGUAGE = VBScript %>
<HTML>
<HEAD>
<TITLE>Classical Genetic Map</TITLE>
</HEAD>
(querying database... please wait)
<HR>
<CENTER>
<H1>Classical Genetic Map</H1>
</CENTER>
<HR>
Classical map of mutant genes of <I>Arabidopsis</I>.  Last major update: June, 1998.  Data
are organized into columns that show the map location in cM and locus name. Genes marked with
an asterisk (*) were initially placed on the recombinant inbred map based on recombination
```

```
data with molecular markers and then transferred to the classical map as described in
<A HREF="mutant_list_exp.html">this link</A>.  Genes marked with a plus (+) have been
cloned or their protein product identified. Map locations shown here are estimates based
largely on 2-point recombination data. The orders of closely-linked genes may therefore
differ from those shown.

Go to <A HREF="../front_mutant/formsexpl.html">this link</A> to submit new linkage information
or request corrections to the classical genetic map.
<FORM ACTION="/advworks/ ..... " METHOD=POST>
<! set up arrays >                                                    (LIKE IT SAYS...)
<% Dim locus(5,250) %>ESTABLISH "locus" AS A 2-D ARRAY WITH 5 CHROMOSOMES AND MAX 251 LOCI.
<% Dim mapunit(5,250) %>                                          IBID. FOR "mapunit" (cM).
<% Dim rimap(5,250) %>                        "rimap" HOLDS A SYMBOL IF LOCUS IS ON THE RI MAP.
<% Dim cloned(5,250) %>     "clomed" HOLDS A SYMBOL THAT INDICATES WHETHER LOCUS WAS CLONED.
<% Dim maxchrom(5) %>     "maxchrom" HOLDS VALUE FOR MAX NUMBER OF LOCI FOR EACH CHROMOSOME.
<% Dim plocus(5) %>                           "plocus" HOLDS VALUE FOR PRINTING FOR EACH LOCUS.
<% Dim pmap(5) %>                             "pmap" HOLDS VALUE FOR PRINTING FOR MAP LOCATION.
<% Dim primap(5) %>                         "primap" HOLDS VALUE FOR PRINTING FOR RIMAP VALUE.
<% Dim pcloned(5) %>                 "pcloned" HOLDS VALUE FOR PRINTING FOR CLONED CHARACTER.
<% Dim pprint(5) %>       "pprint" HOLDS THE STRING CONSTRUCTED FROM OTHER "p" VALUES ABOVE.
<% Dim count, lastchrom, maxcount %>                                  TRACKING VARIABLES.
<% count = 1 %>                                          INITIALIZE COUNT WITH A VALUE OF "1".
<% lastchrom = 1 %>                                SET VALUE FOR LAST CHROMOSOME QUERIED TO "1".
<% maxcount = 0 %>     "maxcount" TRACKS NUMBER OF LOCI ON "LONGEST" CHROMOSOME (MOST LOCI).
                                                                      CONNECT TO DATABASE.
<% Set OBJdbConnection = Server.CreateObject("ADODB.Connection")
OBJdbConnection.Open "symbolslist"
SQLQuery = "SELECT * FROM qclassic"                                CONNECT TO QUERY qclassic.
Set RSLookup = OBJdbConnection.Execute(SQLQuery)
%>

<% chromosome = Request.Form("chromosome") %>          "chromosome" HOLDS VALUE SENT BY FORM.

<% Do While Not RSLookup.EOF %>                                       USE LOOP TO FILL ARRAY.
   <% chromdata = RSLookup("csome") %>                 CHROMOSOME OF CURRENT RECORD IS LOADED
   <% If lastchrom <> chromdata Then %>               IF RECORD IS FOR THE NEXT CHROMOSOME...
       <% If maxcount < count Then maxcount = count %>         CHECK IF THIS IS "LONGEST"...
       <% maxchrom(lastchrom) = count %>          SET THE MAX NUMBER OF LOCI FOR THIS CHRMSM.
       <% lastchrom = chromdata %>              CHANGE THE VALUE FOR LAST CHROMOSOME QUERIED.
       <% count = 1 %>                                          RESET THE COUNT FOR LOCI.
   <% End If %>
                          ASSIGN DATA TO THE ARRAY.  NOTE "chromdata" IS CURRENT CHROMOSOME
                                                              AND "count" IS LOCUS POSITION
<% locus(chromdata, count) = RSLookup("locus") %>
<% mapunit(chromdata, count) = RSLookup("Numeric Map") %>
                                          SETS A SPACE SO NUMBERS LINEUP IF CHARACTERS < 10.
<% if mapunit(chromdata, count) < 10 Then mapunit(chromdata, count) = mapunit(chromdata,
count)&" " %>

<% If RSLookup("RI") = "True" Then %>                              IF "RI" IS INDICATED...
<% rimap(chromdata, count) = "*" %>                                   PUT IN THE SYMBOL...
<% Else %>
<% rimap(chromdata, count) = " " %>                                    OTHERWISE A SPACE.
<% End If %>

<% If RSLookup("Cloned Gene") = "True" Then %>           IF "Cloned Gene" IS INDICATED...
<% cloned(chromdata, count) = "+" %>                                  PUT IN THE SYMBOL...
<% Else %>
<% cloned(chromdata, count) = " " %>                                   OTHERWISE A SPACE.
<% End If %>

<% count = count + 1 %>                                               INCREMENT LOCUS COUNT.
```

```
<%                                                          LOOP THROUGH TO NEXT RECORD.
RSLookup.MoveNext
Loop
%>

<% maxchrom(lastchrom) = count %>          ASSIGN MAX NUMBER OF LOCI FOR FINAL CHROMOSOME.
                                        CORRECT FOR OVERCOUNTING OF NUMBER OF LOCI. (FUDGE).
<% totalloci = maxchrom(1)-1 + maxchrom(2)-1 + maxchrom(3)-1 + maxchrom(4)-1 + maxchrom(5)-1
%>
<% j = 1 %>                          i AND j ARE INITIALIZED IN PREPARATION TO EXTRACT DATA.
<% i = 1 %>

<% If chromosome = "all" Or chromosome = "" Then %>          IF ENTIRE MAP WAS REQUESTED:
<H2><CENTER><STRONG>Classical Genetic Map of All Chromosomes<BR></STRONG></CENTER></H2><BR>
<FONT SIZE=-1>
<PRE><B><U>Chromosome 1    Chromosome 2       Chromosome 3    Chromosome 4
Chromosome 5</U></B><BR>                                                SET UP COLUMN HEADINGS.
                    "WHILE" THE COUNT IS LESS THAN THAT OF THE CHROMOSOME WITH THE MOST LOCI:
<% Do while j < maxcount %>
<% For i = 1 to 5 %>                       LOOP FOR EACH CHROMOSOME (i) FOR EACH POSITION (j).
<%If j < maxchrom(i) Then%>  IF THE POSITION IS LESS THAN MAX POSITION FOR EACH CHROMOSOME:
<% plocus(i)=locus(i,j) %>                                  SET UP "PRINT" DATA FOR CURRENT ROW.
<% pmap(i)=mapunit(i,j) %>
<% primap(i)=rimap(i,j) %>
<% pcloned(i)=cloned(i,j) %>
                                                          CONSTRUCT THE STRING WITH DATA...
<% pprint(i)=plocus(i)&": "&pmap(i)&" "&primap(i)&" "&pcloned(i)&" " %>
<% Else %>                                                                          OR...
                                                    MAKE A BLANK STRING TO PRESERVE FORMATTING.
<% plocus(i)="      " %><% pmap(i)="      " %><% pprint(i)="           " %>
<% End If %>
<% next %>                                          GO TO NEXT CHROMOSOME AT THE SAME POSITION.

<% For i = 1 to 5 %>                                        OUTPUT STRING TO RENDERED PAGE.
   <%= pprint(i) %>
<% next %><BR>
<%j = j + 1 %>                                                    INCREMENT LOCUS POSITION.
<% Loop %>
</PRE>
<% For i = 1 to 5 %>           START A LOOP TO INDICATE NUMBER OF LOCI FOR EACH CHROMOSOME.
   <B>Number of loci on chromosome <%=i%> is  <%=maxchrom(i)-1%></B><BR>.
<%next%>
<B><I>Total number of loci in entire map is <%= totalloci %></I></B><BR>     INDICATE TOTAL.

                                        REMEMBER, PREVIOUS CODE IS FOR ENTIRE MAP CONSTRUCTION.
<% Else %>                                      "ELSE" INSTEAD USES ONLY SELECTED CHROMOSOME.
<H2><STRONG>Classical Genetic Map of Chromosome <% =chromosome %><BR></STRONG></H2>
<BLOCKQUOTE><BLOCKQUOTE><BLOCKQUOTE>
<PRE><% i = chromosome %>       ASSIGN SELECTED CHROMOSOME TO VERTICAL ARRAY TO BE QUERIED.
<% Do while j < maxchrom(i) %>                        LOOP FOR LENGTH OF DESIRED CHROMOSOME.
<% plocus(i)=locus(i,j) %><% pmap(i)=mapunit(i,j) %><% primap(i)=rimap(i,j) %>
<% pcloned(i)=cloned(i,j) %>                                  ASSIGN DATA FOR PRINT STRING.
                                              CONSTRUCT A COMPOUND STRING FROM ASSIGNED DATA.
<% pprint(i)=plocus(i)&": "&pmap(i)&" "&primap(i)&" "&pcloned(i)&" " %>
<%= pprint(i) %><BR>                                    OUTPUT CONTENTS OF "pprint" STRING.
<%j = j + 1 %>                                                INCREMENT POSITION LOCATION.
<% Loop %>
</PRE>
</BLOCKQUOTE></BLOCKQUOTE></BLOCKQUOTE>
   <B>Number of loci on chromosome <% =i %> is <% =maxchrom(i)-1 %></B><P>
   <B><I>Total number of loci in entire map is <%= totalloci %></I></B><BR>
<% End If %>
</FONT>
```

```
<p><a href="../default.htm"><img src="../IMAGES/home_button.gif" border=0 alt="Meinke Lab
Welcome Page"></a> <a href="genepage.html"><img src="../IMAGES/mutant_gene_button.gif"
border=0 ALT="Return to Mutant Genes Page"></a><br>
<a href="../default.htm">Home</a>, <a href="genepage.html">Mutant Genes</a> </p>
</BODY></HTML>
```

Final Word

From the example code given in these appendices, it should be a small task to modify the ASP ™ VBScript ™ instructions to alter the presentation of the information extracted from the database. A resource that can be used to find the syntax of Visual Basic is the Visual Basic coding books included with some versions of Microsoft Office products. Additionally, copies of Access, Excel, and Word that are installed on a computer often come with help files that contain lookup tables for the language vocabulary and include syntax examples. If these do not suffice, the reader is instructed to find one of the many books dealing with Visual Basic that can be obtained from most bookstores. Simply editing the code with Notepad ™ (available through all Windows ™ operating systems as an accessory) should allow changes to be implemented as soon as the file is saved.

VITA

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Doctor of Philosophy

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