WOODY PLANTS

AND THE

APPLICATION OF MOLECULAR TOOLS

By

Anne Edith Plovanich

A DISSERTATION

Submitted to

Michigan State University

in partial fulfillment of the requirements

for the degree of

DOCTOR OF PHILOSOPHY

Department of Botany and Plant Pathology

2001

ABSTRACT

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Using various molecular biology tools three woody plant genera, *Populus*, *Quercus*, and *Montanoa* were chosen as experimental systems to elucidate heretofor unanswered questions.

To elucidate genetic mechanisms of control of vascular cambial differentiation, hybrid poplar clone 47-174 (*Populus deltoides* x *trichocarpa*) was used as a model to examine gene expression in wood forming tissue and as a result of wind stress or mechanical perturbation (MP). RNA isolated from stressed and non-stressed poplar stems was examined using differential display of PCR products. One hundred thirty differential display gel bands from stem tissue were cloned and sequenced. Northern analysis and BLAST searches confirmed that more than seventy of these clones represent expressed sequence tags (ESTs) for poplar genes expressed in wood forming tissue. An abundance of

representation of stress related genes indicate the ESTs are from abiotic induced mechanical perturbation.

In the second study, DNA fingerprinting techniques were used for the identification of cultivars of *Quercus* x *hispanica*, the Lucombe oak. Using inter-simple sequence repeat (ISSRs) primers, 66 *Q.* x *hispanica* (and related) samples were analyzed and compared for band pattern differences to establish identities. DNA evidence revealed that some named cultivars had identical banding patterns, which then aided in identification of other named or unnamed cultivars with the same DNA banding patterns.

A phylogeny of the genus *Montanoa* based on the Internal Transcribed Spacer (ITS) and the External Transcribed Spacer (ETS) was created. The combined dataset supports the monophyly of the genus and an early evolutionary split that coincides with geographic distribution. One lineage is composed mostly of central and southern Mexican species whereas the other lineage contains those species endemic to Mesoamerica and South America. The relationships of *Montanoa* to other genera in the Heliantheae are briefly discussed.

DEDICATION

Morrie Schwarz

Roland Barthes

Red Dinsmore

A.M.D.G.

ACKNOWLEDGMENTS

At the beginning and at the end are my roots, my beloved parents, Edith Ann Forst Plovanich, who loved all plants and Joseph Paul Plovanich, who loved trees. They left me a legacy and inspiration I cannot define.

John Scott-Craig has been and continues to be my mentor, my guide, my inspiration and my friend. His patient support, encouragement and instruction have been the foundation for this dissertation and the accomplishment of my experiments. Without John, none of this would have happened.

I have also had the privilege of the support and guidance of an extraordinary committee. Frank Telewski and I plotted our course of collaboration based on one strong bond, our love of trees. From the *Aeschylus parviflora* of our first meeting, through the *Pinus ponderosa*, the *Pinus albicaulis*, and the *Populus trichocarpa x deltoides*, it has always been the trees that helped us through the trials, successes and failures of this dissertation project. I have been truly honored and blessed to have his mentorship and friendship.

John Ohlrogge has always been my support, my encouragement, my mentor and my friend. Knowing that John has always believed that this degree was going to happen has given me the strength to persevere. His assistance in the database searching and presentation of this EST collection was invaluable. Alan Prather stepped in to an ongoing dissertation project and provided the advice, knowledge and guidance to publish the Oak chapter and help prepare the Montanoa chapter for this dissertation. He helped fill in all the gaps in my background about systematics and taxonomy, particularly helpful for my reviewers from the Royal Horticultural Society on the Oak work. Michael Thomashow has been an inspiration to me, always. I was honored to have him serve on my committee, and he did not disappoint. His thoughtful evaluation of the poplar chapter has helped prepare for the publication of this project. Mike always challenges me to think more clearly, and to do better than my best.

My committee has always believed in me, encouraged and supported me and has been there every step of the way for guidance when I needed it. I knew that from every one of them, the words, "I will help you out with this" were

always there. Without any member of this committee, my degree would not have happened.

Many people were there to assist in my science with teaching of techniques, discussions of results, experimental design or lab space and equipment. I will only elaborate on one, Ann Greene. My dear beloved and departed friend, "my Ann with no 'e'". Ann taught me how to do my first plasmid mini-preps, make my first solutions and encourage me when molecular experiments don't work! She is missed.

Richard Allison, Kyrstoff Szyzglowski, Dirk Hamburger, Frans DeBruijn and the DeBruijn lab, Jonathon Walton, John Pitkin, Kerry Pedley, John Tonukari and the Walton lab, ShengYang He and the He lab, Tom Newman, Sue Stoltzfus, Barb Christian, Ambo VanHoof, Anna Wiese, Li Ping, Neal Dittmer, Ken Keegstra (whose leadership and generous spirit inspires and guides the department), and in Sweden Anneli Stendberg, Bjorn Sundberg and Magnus Hertzeberg. This has been my science support group.

Data Basing and Graphics for my figures, publications and defense seminar include Paula Lee Hauck, Julie Zwiesler-

Vollick, Abe Koo, Diem T. Hoa, Uwe Rossbach, Kurt Stepnitz and Marlene Cameron.

Those who have provided academic encouragement and inspiration have been Richard Allison, Ken Nadler, Dr. Phu Nguyen, Dr. Jim Flore, Hannah Mathers, Sarah Gilmour and Christoff Benning. Each of them were there at crucial points to foster inspiration.

My financial support has come from Frank Telewski, ShengYang He, Jose Panero, the National Science Foundation, Michigan State University AURIG, the MSU Graduate School and the Horticulture Department.

I cannot fail to mention the incredible moral support of my friends. When one does not have a family, friends achieve a greater importance in one's life. These friends have been and are my family: Julie Zwiesler-Vollick, Anita Davelos, Michele Pruyn, Paula Lee Hauck, Ray Pacovsky, Glen Jones, Karen Bird, John Scott-Craig, Linda Danhoff, The PRL Office Staff, M.O.M., my beloved other mother and my spirit guide, Judy Ferris.

And for those who understand, A.M.D.G.

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INTRODUCTION

WOODY PLANTS AND MOLECULAR TOOLS?

DO THEY HAVE A FUTURE TOGETHER?

Plants that have woody stems (secondary growth or secondary xylem) have traditionally presented problems for scientists wishing to work with them. They take up a large amount of space, most of them grow slowly, are difficult to propagate and maintain in a greenhouse, many are slow to reproduce, flower, or bear fruit; and the woody stems themselves often present a formidable challenge to experimentation. These problems were difficult to overcome, but with the advent of molecular biology, the problems became compounded. In addition to the "normal" associated problems, add to that large genome size, (*Populus* 550 mpb, *Pinus taeda* 25,000 mpb compared to *A.thaliana* 125 mbp), enormous challenges to transformability, and difficulty of extraction of DNA and RNA. There is also a general lack of funding for what may

be considered high risk projects because of the aforementioned difficulties as well as the reluctance of forestry industries to fund projects that may take years to reach marketable products. Genetically modified trees may just not be cost effective.

A further consideration in using molecular tools for woody plant modification is the growing opposition to genetically modified organisms (GMOs) by an increasing vocal and fear motivated public. Genetically modified trees, growing for years in the field versus short-term annual agricultural crops represent an even greater perceived threat to natural forests. Steve Strauss at Oregon State University has had two genetically modified poplar plantations cut down, while Toby Bradshaw at University of Washington, Seattle has had his lab (as well as others in the same building) bombed. This opposition to genetic engineering of trees may further impact funding potential.

Why then, one could ask, would anyone wish to undertake the use of molecular tools to work on woody plants? For the same reason that one would use molecular tools for study of any other organisms: these tools furnish answers to questions that have not previously been able to be

addressed, adding additional layers of knowledge to longstanding questions. And secondly, trees are merely a longer growing crop, planted and harvested like any other plant crop, such as wheat, corn, rice; however maintained in the field for longer periods of time. The world market for wood products, lumber, and paper has as much an increasing demand as the world market for edible resources.

I propose to address some of these questions and furnish some limited answers. In this thesis I apply a number of molecular techniques to DNA and/or RNA extracted from three different woody plant genera, *Quercus, Montanoa,* and *Populus* to furnish answers to problems that have previously not had resolution.

The "Lucombe" oak, first named over two hundred and fifty years ago is grown throughout the UK and Europe. Enormous confusion exists over the true identity of this famous oak. In this dissertation I will look at molecular markers that make it possible to distinguish among cultivated varieties within a taxonomic group of named oak tree cultivars heretofore indistinguishable using traditional morphological characters.

I also use molecular tools to examine the phylogeny of an unusual and spectacular woody genus of Mexico, the *Montanoas*, ("tree daisies"), shedding new light on the relationships of the twenty-five species, and the evolution and radiation of this genus. Previous studies of the genus have relied on morphological and histological studies to establish relationships between the twenty-five species, while failing to identify any related Composite species.

And finally, molecular tools were used to elucidate a number of putative genes present in the cambial region of *Populus* (poplar) that contribute to growth and wood formation. A poplar cultivar (47-174 *P. trichocarpa* x *P. deltoides*) was used to find genes that may be expressed to due wind stress or mechanical perturbation. cDNAs have been cloned and sequenced that are found in stems of this poplar clone.

CHAPTER 1

OVERVIEW OF FOREST BIOTECHNOLOGY

THE IMPORTANCE OF *POPULUS* (Poplar) IN FOREST BIOTECHNOLOGY

WIND STRESS IN POPLARS AND ITS SIGNIFICANCE

IN WOODY PLANT MODIFICATION

CURRENT TRENDS IN FORESTRY BIOTECHNOLOGY

"Have you got a license for that tree (and can you afford to use it?)" is the title of a paper at the next forest biotechnology conference in Washington this July (Bryson, 2001). Research into genetic modification of woody plants has become a timely, controversial and much discussed topic. The current studies relating to genetic modification of trees are on the agenda at the upcoming meeting of the International Union of Forest Research Organization (IUFRO).

Consider trees as crops that remain in the field for many seasons before harvest, rather than merely one. Then consider the many stresses that the crops have to endure to survive over these many seasons. In addition to survival, there are expectations for high yield and good wood quality for designated purposes. All of the biotechnological improvements that science hopes to accomplish with single season crops are just as important, maybe more so, for a plantation of trees. Directed modification of many characteristics of forest trees, regarding wood quality, growth and development, resistance to pathogens and insect vectors, and response to environmental stresses is a direct challenge to scientists seeking to insure and improve yield. A single "crop" failure can cost the land owner years of investment.

To respond to this challenge, it is necessary to have an understanding of the genes that affect these characteristics as well as an understanding of the genetic variation in desirable phenotypes of commercially or biologically important trees. By using genetic modification, improvements in plantation forestry can occur much more rapidly than in long-term field trials, so called "classical" breeding. Genetic tools can also aid in the

conservation of forest species, preservation of biodiversity and better understanding of forest ecology. These genetic tools achieve even greater importance in the light of increasing land pressures and demands for wood products.

The International Union of Forest Research Organizations (IUFRO) is a non-profit, non-governmental network of scientists involved in forestry and forest products research. The section on Molecular Biology (Genetics) of Forest Trees has convened meetings since 1985 (29 attendees) reflecting the early efforts of gene transfer into plants. The last IUFRO section meeting held in 1999 at Oxford, United Kingdom entitled "Forest Biotechnology '99, A Working Party on Molecular Biology of Forest Trees" was attended by participants from thirty different countries (UK 168 attendees). An impressive array of sessions and posters was presented by investigators attempting to explore molecular applications and woody plants. Table 1 presents a summary of the topics and species being studied that were presented as papers at the meeting. Table 2 is a summary of the posters.

Area of focus	Topic	Woody species
Transformation and propagation	Somatic embryogenesis	Norway spruce *Eucalyptus globulus* *Theobroma cacao* *Pinus pinaster* Acacia *Liquidambar sytraciflua*
	Agrobacterium tumefaciens transformation	Teak (*Tectona grandis*) White pine
	Transgene stability/gene expression	Elm Aspen/*Populus* Loblolly pine Scots pine *Casuarina glauca*
Directed wood modification	Cellulose synthesis	Aspen, and various non-woody species
	Lignin biosynthesis	Poplar Spruce Yellow-poplar *Eucalyptus gunnii* Lodgepole pine
Transgenic trees in the field	Risks and controls Outcrossing	 Poplar Wild cherry
	Increased viral susceptibility	Reduced lignin in tobacco
	Flower regulation	Poplar Birch Conifers Eucalyptus *Bambusa edulis*
Genomics	Genome sequencing projects; ESTs, RAPDs, QTLs	Loblolly pine Poplar Eucalyptus Japanese black pine White spruce *Pinus sylvestris* Maritime pine Larch
Conservation	Genomics and biodiversity	Norway spruce *Swietenia humilis* (mahogany)
	Population studies	Pedunculate oak Wild cherry *Populus euphratica*

Table 1. Conference session - Paper abstracts

Area of focus	**Topic**	**Woody species**
Transformation and propagation	Somatic embryogenesis	White spruce Maritime spruce *Quercus suber Quercus robur* *Pinus elliottii x P.caribaea* Eucalyptus Scots pine *Pinus radiata* *Picea abies* *Passiflora* species *Prunus avium* *Sorbus aucuparia*
	Protoplast isolation Biolistic transformation Tissue specific transgene	Elms *Pinus radiata* Apple
Directed wood modification	Lignin plasticity	CAD-deficient pine
	Lignin gene combinations Laccases CAD and Ozone COMT and reduced lignin Peroxidase/reduced lignin	Tobacco *Arabidopsis* Poplar Poplar Hybrid aspen
Transgenic trees in the field	Herbicide resistance	*Pinus radiata* *Picea abies* Black locust
	Field trials Meristem activity Salicylic acid (increased disease resistance)	Norway spruce (5yrs) Eucalyptus Poplar
	Oxidative stress Wounding,drought, cold stress dehydrin *Bacillus thuringensis* ROLC transgene Mycorrizae Long distance pollinators Early flower induction	Poplar White spruce Hybrid larch Aspen/*populus* Transgenic poplar Eucalyptus Birch (MADS box)
Genomics	RAPDs	*Avicennia marina* *Chimonanthus*
	DNA extraction Marker aided selection Genomic organization of repetitive DNA	*Maytenus ilicifolia* *Eucalyptus grandis* Norway spruce

Conservation	Population studies	*Abies alba* *Picea rubens* *Pinus mariana*
	Genetic diversity	*Betula pendula* Norway maple (Finland)
	Endangered species	*Gomortega keule* (Chile)

Table 2. Conference poster abstracts

The upcoming conference in July 2001 that will be held in Stevenson, Washington will address all aspects of molecular techniques applied to the study and manipulation of forest trees. Topics that are germane to this section include: use of molecular markers for ecogenetic studies, DNA marker-based breeding and selection, molecular and genomic studies of tree physiology and development, *in-vitro* culture and asexual gene transfer methods and silvicultural studies of genetically modified trees. The conference in Washington State has several topics that expand the scope of the meeting at Oxford. One area deals with the advent of high-throughput facilities for sequencing and microarrays that has led to an explosion of whole genome and Expressed Sequence Tag (EST) projects. The Swedish group plans to make publicly available 50,000 poplar ESTs at the meeting (Sandberg, 2001). The EST collections from this group as well as others are augmented by expression

data using other techniques such as Serial Analysis of Gene Expression (SAGE) and gene enhancer traps (Lorenz, 2001) (Groover, 2001). Of particular interest is the EST collection from mRNA from tension wood formation in poplar using Amplified Fragment Length Polymorphisms (AFLPS) (Leple, 1999). Jean-Charles LePle, Giles Pilate, and Florian Lafarguette have been working for three years to develop differentially expressed ESTs that are specific to wood tissue deposited in response to gravitropism; wood formed on the upper layer of stems and branches of angiosperms. They are using a poplar hybrid INRI #717-1-B4. This model system will potentially identify genes important for lignin non-deposition in the S3G layer, control of microfibril angle, and cambial activity (LePle personal communication). The genes found as important to tension wood formation may have many similarities to those found in response to mechanical perturbation. Reaction wood (formed in MP) has both tension and compression elements (Telewski, 1995).

However the most striking difference between the two conferences is the attention given to the ecological and social issues that are everywhere the subject of much debate and analysis. The two principal organizers of the

conference have been the victims of eco-terrorism. Toby Bradshaw, University of Washington, has had his laboratory burned in late May, while Steve Strauss, Oregon State, Corvallis, has had two transgenic poplar plantations cut down. Ironically Steve Strauss' work has emphasis on the reduction or elimination of flowering in transgenic poplars to prevent environmental spillover while the bombing in Seattle destroyed the work of a scientist who has dedicated his research to conservation efforts. They are not alone as many countries report the eco-terrorism directed at genetic modification of woody plants. Since woody plants remain in the field for an extended period, their impact on the environment presents different issues than short rotation crops. Two papers that caught immediate attention were "A policy perspective on transgenic trees in Canada" (Bonfils, 2001); and the previously cited: "Have you got a license for that tree (and can you afford to use it)? which will be presented by a law firm from Washington, D.C. Ecological, social, ethical and legal considerations of forest biotechnology have achieved paramount importance. The first two days of the conference are dedicated to an "eco-social" symposium to enlist dialogue on these issues.

Mindful of the risks involved in woody plant modification, scientists world-wide are continuing with their research because of inherent overall benefits. Woody plants share genetic features with herbaceous plants regarding growth and development, metabolic pathways and responses to biotic and abiotic stresses. However genomic approaches to important model tree species offer an understanding of the differences of woody plants from other model plant systems. Secondary growth and wood deposition cannot be understood using *Arabidopsis thaliana* as a model system. "Many aspects of tree development are radically different, and parallel genomics research for several commercial tree species will be required over the next decade." (Robinson, 1999b) Other non-woody model plant systems which explore lignin deposition, such as *Arabidopsis*, Zinnia and *Nicotiana* can have a supporting role in hypothesis development and in bioinformatic searches of sequenced plant genomes. However, trees must ultimately be used as the model systems for testing of such hypotheses. (Chaffey, 1999b) Consequently studies of woody plants at the molecular level, despite difficulties, limitations, and risks are of major importance if plant scientists are to meet the challenges presented.

Trees as laboratory subjects present some difficulties and limitations. They are slow growing, many months before they reach experimental size, sometimes requiring years to achieve flowering and fruiting, and they require a significant amount of space when compared to the number of *Arabidopsis* that can be grown on a Petri dish. The woody stems present a formidable barrier to many laboratory experiments, and in many species, dormancy forces cessation of experimentation. These are the problems faced with traditional scientific research on trees. With molecular techniques the problems are compounded by often large and repetitive genomes: the plant model system *Arabidopsis thaliana* has a relatively small genome size of 125 mbp, , while poplar is 550 mbp, *Pinus taeda* 125,000 mbp. It is suggested that it would take twenty two years to sequence given the techniques used in the Human Genome Project with a genome size of 600 mbp. (Bradshaw and Stettler, 1993; Kinlaw and Neale, 1997; Marie and Brown, 1993; Robinson, 1999a; Wakamiya et al., 1993).

There are the added difficulties of extraction of DNA and RNA, complexity of genetic transformation and tissue culture, longevity and tissue specificity of the transgene in long-lived plants, and the dearth of mutants that can

help elucidate genetic differences. Actually many potentially useful mutants are regularly discarded in traditional breeding programs or relegated as horticultural cultivars. Funding for molecular research on woody plants is scarce as forestry industries are reluctant to invest in technology that may or may not be economically beneficial, certainly not in the short term. Compounding these difficulties is the potential environmental contamination by transgenics (Greene, 1995; Strauss et al., 1999), the threat of eco-terrorism and public acceptance of genetically modified trees.

All of these problems must be addressed by the scientists in the field of forest biotechnology. The history of molecular applications to forest trees has been a short one. The earliest efforts were directed at mapping of desirable traits for the purposes of "classical" tree breeding. Much early work had to do with attempts at gene transfer and of subsequent *in vitro* propagation. Twenty-eight speakers at the symposium entitled, "Genetic Manipulation of Woody Plants" held at Michigan State University in June of 1987 focused on tissue culture and development of gene transfer systems (Hanover, 1987). The first transgenic tree, a poplar, was created in 1987

(Fillatti, 1987) compared to the first transgenic plant, in 1980 (Hernalsteens et al., 1980). Transformation and propagation of woody plants continues to be a considerable challenge that remains elusive for many tree species. The genera that have been used in molecular studies, have been selected primarily because of their economic or conservation value. One genus comes to the forefront in molecular studies not only because of its socio-economic value, but also because of the relative ease with which it can be studied at the molecular level. That is of course, *Populus*, which may well be the first tree species designated for genome sequencing.

POPULUS (POPLAR) AS A MODEL TREE SYSTEM

For scientists who study trees, the diversity of woody plant genera under investigation using molecular tools is of amazing breadth as evidenced in the papers and posters presented at the IUFRO meetings. However one genus of woody plants appears in almost every category of investigation excepting that of propagation and

transformation, the genus *Populus* (Poplar). The latter two categories have become fairly routine for poplar researchers.

In the Northern hemisphere (Eucalyptus being the counterpart in the Southern) poplars are being used as the model system for non-molecular and molecular research pertaining to angiosperm wood formation (Stettler, 1996). There are many reasons for this. Biologically they are easy to propagate from green or woody cuttings, and they are easy to maintain in a greenhouse. In winter under lights, growth will continue without dormancy. They are representative of many other woody plant species, pedigrees of various lines are available and many mutants responsive to a variety of conditions are being maintained.

As a cultivated species poplars have achieved important commercial, conservation, and remediation potential. Poplar can be used for biomass production, the wood used for pulpwood, lumber, veneer, matchwood, and firewood. They can be used as shade trees, windscreens, and to stabilize sites such as steep banks, landfills, spoil banks or borrow pits. Hybrid poplars exhibit different crown and leaf shapes due to varied parentage (Demeritt, ?). They are

rapid growing, amenable to use on marginal soils, and almost circumpolar in adaptation to a variety of climates. To cite a few of the newer uses of poplar worldwide: They provide a fast growing renewable energy source, in Hungary ongoing trials on marginal soils provide biomass energy with only a four year cutting cycle (Marosvolgyi et al., 1999), in India poplar plantations intercropped with chamomile were successful on partially reclaimed marginal soil also for biomass production (Misra and Tewari, 1999). Chinese use of flood tolerant poplar clones has proven successful for reforestation projects (Cao and Conner, 1999). In New Zealand, poplar (which are exotic there) have been introduced successfully for erosion control schemes (Wilkinson, 1999).

An interesting concept has been the use of poplar clones that differ in resistance to air pollutants, SO_2 and O_3, which would make the poplars effective bioindicators (Ballach, 1997). Poplars have previously been shown to react to ozone, varying from sensitive to tolerant (Cao and Conner, 1999) (Wood and Coppolino, 1972). Milt Gordon's lab at the University of Washington in Seattle has studied many aspects of poplars applied to bioremediation. Untransformed Poplar hybrids will metabolize carbon

tetrachloride (CT), perchloroethylene and trichloroethylene (TCE). They have been shown to detoxify atrazine and TNT. potential has been shown to be able to metabolize some isomers of polychlorinated biphenyls (PCBs) (Gordon, 2001). Indeed there is a poplar clone for all purposes and all seasons.

POPLARS AS A MODEL SYSTEM FOR WOODY PLANT MODIFICATION

The ease with which poplars can be clonally propagated from cuttings, which is not the case in most woody plants, was a good indicator that they might by a likely candidate for ease of transformation. That has proven to be the case. Although biolistic projectile bombardment with a gene of interest is possible in many woody plants, transformation using the *Agrobacterium tumefaciens* system is much preferable. The *Agrobacterium* mediated gene transfer is the method of choice because of single-copy and single-locus insertion compared to other plant transformation techniques. Although use of *Agrobacterium* for transformation must be optimized with a number of variables, the choice of strain, the growth media, inducers and the type of plant material, poplars became the obvious

choice for the first woody plant transformation (Hanover, 1987).

THE EARLY DAYS OF TRANSGENIC POPLARS

The development of a transgenic poplar resistant to the herbicide glyphosate was pioneered in the laboratory of Don Riemenschneider of the Forestry Sciences Laboratory of the USDA in Rhinelander, Wisconsin in collaboration with Calgene, Inc., of Davis, California. By using the *Agrobacterium* transformation vector, a bacterial *aro*A gene that conferred resistance was expressed in *Populus* (Fillatti, 1987). This was a highly desirable modification as plantation losses due to weed competition were enormous.

CURRENT TRENDS FOR THE USE OF TRANSGENIC POPLARS

The current research into the use of transformed poplars has focused on two important aspects of poplars, their adaptability to diverse climates, and harsh environments,

(often planted on polluted, barren and exposed sites) and their rapid growth for biomass with short rotation.

Transformed poplars in bioremediation

Genetically modified poplars are being studied in terms of their adaptability to stress situations such as tolerance to heavy metals, ozone, atmospheric H2S. (Arisi et al., 1998; Arisi et al., 2000; Herschbach et al., 2000; Koch et al., 2000; Tyystjarvi et al., 1999) Trials are under way with poplar containing the mammalian P450 insert to further understand the mechanisms of detoxification of many harmful organic pollutants (Ohkawa et al., 1997; Ohkawa et al., 1998).

There are many research groups that have sought modifications of poplars for their eventual downstream uses. To achieve this end, there have been studies of poplar genetic modifications to render the trees herbicide resistant, to improve resistance to insect and fungal pathogens, and to expand their tolerance to environmental abiotic stresses. However the largest group of researchers in this field has concentrated their efforts on lignin modification.

Transformed Poplars and Wood Modification

As a result of their rapid growth and use for pulpwood, there is great interest in modification of the lignin content in poplars to reduce the cost and environmentally deleterious effects of the pulping process. Any discussion of genetic engineering of poplars must include the current trends in lignin modification.

LIGNIN MODIFICATION AND WOODY PLANTS

Lignin - An Overview

Lignin is a complex polymer of phenylpropanoid units mainly deposited in plant secondary tissues that contribute to functions of support and conductivity. It is the second (only to cellulose) most abundant organic polymer on the planet (Zhong et al., 2000). Lignin is primarily deposited in secondary cell walls in conductive and support tissues

of vascular plants. It is present in some herbaceous plants where its negative effect on the digestive process of ruminants is well characterized. The covalent linkages between lignin and polysaccharides render the cell walls of grasses resistant to digestion (Stone, 1997). However the high content of lignin in woody tissues used for paper becomes even more undesirable because of the stringent methods used to remove it in the pulping process. Chemical pulping consists of chemical hydrolysis and solubilization of lignin, by either acid (sulfite) or alkaline (sulfate) pulping while lignin is degraded at very high temperatures and extreme pH. The alkaline pulping or kraft pulping is the most utilized world wide (Baucher et al., 1998). Genetic manipulation of woody plants for even small amounts of lignin reduction is highly advantageous. Lignin content and composition vary from angiosperm to gymnosperm, from one species to another varying among tree types (ring-porous, and diffuse porous, storied and non-storied cambium, etc.) and even varying seasonally and developmentally (Chaffey, 1999b). Characterization of lignin and the process of lignin deposition in woody plants are not well understood.

Role of lignin in plant physiology

Attempts at lignin modification should include an understanding of the physiological function that lignin has in plants and woody plants in particular. Any modification of lignin must include not only the quantitative results of lignin produced and pulping characteristics, but also the effects that this modification may have to the plant and any derived wood products.

Lignification has played an important role in the adaptation of plants to life on land. Lignin has made possible the development of conductive tissues, strengthening them to bear extreme negative pressures while conducting water. It also assists in maintaining the hydrophilic nature of the cell wall. Lignin provides mechanical support strengthening the stem/trunk to uphold the weight of the foliage and canopy and has been suggested to generate an internal strain to reorient the tree to stress as a component of compression wood (Timell, 1986). The lignin polymer provides a barrier of protection against biotic vectors, making the woody stem resistant to decay and can be synthesized *de novo* in response to wounding or pathogen infection (Lewis and Yamamoto, 1990). Recent

studies confirm that enzymes in the lignin pathway are correlated with biotic and abiotic stresses (Cabane, 1999; Enebak et al., 1997). These qualities of impermeability and decay resistance are primary factors for the high pollution associated with the pulping process as harsh chemicals are needed to remove the lignin.

Lignin analysis - quantity and quality

The analytic methods to evaluate lignin composition and content are also problematical (Dean, 1997). It has been described as a recalcitrant subject with no absolutes for any perspective researcher. Each technique has limitations. Quantitation methods include "Klason lignin" for insoluble (in acid) and Acetyl bromide or Thioglycolic acid for soluble lignins. Characterization of the composition of lignin includes Nitrobenzene/Cupric oxide degradation and *in situ* techniques of pyrolysis-GC/MS, nuclear magnetic resonance spectroscopy and others. No current method provides complete or comprehensive information and may be prone to error and false conclusions. However improved methods to determine wood

quality are being developed as part of the effort to modify lignin content (Tuskan et al., 1999).

Lignin biosynthesis

The biosynthesis of lignin is equally not well understood. Generally speaking it begins with the phenylpropanoid pathway, starting with deamination of phenylalanine by phenylalanine ammonia-lyase (PAL) and concluding with the generation of differing amounts of three monomeric subunits, hydroxyphenyl (H), guaiacyl (G), and syringyl (S) units differing from each other by their degree of methylation (Whetten et al., 1998).

(See Figure 1, page 30 for generally accepted lignin pathway).

It is stated that the various levels of the three units vary from plant to plant, even within the same plant, from cell to cell (Chen et al., 1999). Content and composition of lignin vary developmentally and seasonally as necessity

for lignin deposition changes during growth. Storage, transport and synthesis of lignin vary. The biosynthetic pathway itself may vary among different plant families (Baucher et al., 1998).

Attempts at genetic modification of the lignin pathway and the discovery of lignin mutants have resulted in unforeseen diverse and unknown forms of lignin. A loblolly pine (*Pinus taeda*) was found to be mutant in the lignin pathway. The xylem is described as red-brown (similar to brown mid-rib mutants) and still maintains vascular function and mechanical support for the tree (Ralph et al., 1997). However the expression of the gene encoding cinnamyl alcohol dehydrogenase (CAD) is severely reduced and has reduced lignin content (MacKay et al., 1997). In 1999 the lab of Kazuhiko Fukushima provided evidence of a novel biosynthetic pathway in lignin in differentiating xylem of *Magnolia kobus* (Chen et al., 1999). It is not surprising that a component so essential to plant defense and strength would have alternative and redundant systems to insure the production of that component.

CURRENT TRENDS IN LIGNIN MODIFICATION AND POPLAR

Many labs that now study woody plant lignin modification began by looking at herbaceous subjects. The plants of choice have usually been *Arabidopsis*, Zinnia or *Nicotiana* (tobacco)(Chaffey, 1999a; Dharmawardhana et al., 1992; Ye et al., 1994). *Arabidopsis* as a model for lignification studies continues in the labs of Catherine Lapierre of INRA, Versailles, France (Jounin, 1999). Using *Arabidopsis* gene arrays and EST collectiong combined with poplar ESTs have become the combined focus in research in Umea, Sweden (Regan, 1999) and in Genome Canada.

Current research into lignin modification of woody plants, particularly, poplars (and loblolly pine - Sederoff, North Carolina Tree Biotechnology) has concentrated on modification of some of the important and committed steps in the biosynthetic pathway. The review of Wout Boerjan's group from Belgium presents a table (17) showing the modification of lignin genes in various plants and results of those mutations (Baucher et al., 1998). The article also presents the resulting effects in composition and impact on the pulping process.

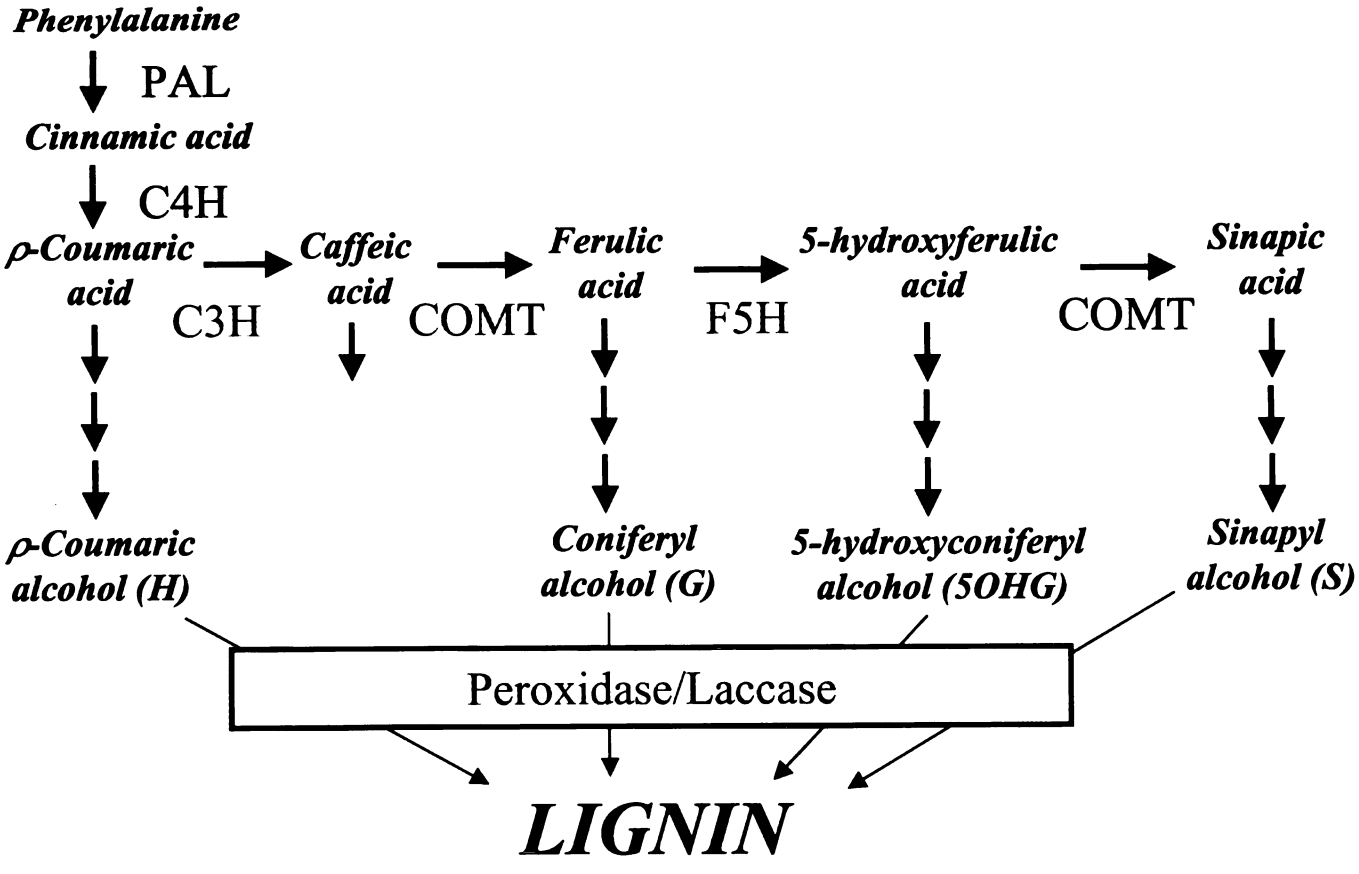
Phenylalanine
PAL
Cinnamic acid
C4H
ρ-Coumaric acid
C3H
Caffeic acid
COMT
Ferulic acid
F5H
5-hydroxyferulic acid
COMT
Sinapic acid
ρ-Coumaric alcohol (H)
Coniferyl alcohol (G)
5-hydroxyconiferyl alcohol (5OHG)
Sinapyl alcohol (S)
Peroxidase/Laccase
LIGNIN

Figure 1.

Figure 1. Legend

Figure 1. Monolignol biosynthetic pathway. The enzymes that have been targeted for modification are Phenylalanine ammonia lyase (**PAL**), Cinnamic Acid 4-Hydroxylase (**C4H**), Caffeic Acid *O*-methyltransferase (**COMT**), Ferulic Acid 5-hydroxylase (**F5H**), 4-Coumarate:CoA Ligase (**4CL**), Cinnamyl alcohol dehydrogenase (**CAD**), and Cinnamoyl-CoA reductase (**CCR**). The peroxidases and laccases which have been proposed to be the enzymes in the final polymerization step are also being modified.

The first modification of the lignin pathway in a woody plant was achieved in 1995 (Vandoorsseleare et al., 1995). *Populus tremula x P.alba* was modified with a reduction in COMT resulting in a substantial (95%) reduction of enzyme activity. No change was observed in the lignin content, a change was observed in the monomeric composition.

The progress in lignin modification till early 1998 was reported in the article from the Boerjan group (Baucher et al., 1998). Since that time numerous groups have studied one or more of the important enzymes in the lignin pathway using transgenic woody plants, in most cases, poplars.

CAD:	Boudet group, Toulousse, France
	Bourjan group, Belgium
	Jouanin group, INRA, Cedex, France
COMT	Chiang group, Houghton, Michigan
	Bourjan group, Belgium
	Lapierre group, Cedex, France
	Douglas/Ellis group, Vancouver, Canada
	Ye group, Athens, Georgia
F5H	Chapple group, West Lafayette, Indiana
	Jouanin group, INRA, Cedex, France
	Chiang group, Houghton, Michigan
Peroxidases/Laccases	
	Nippon paper industries, Japan
	Ellis group, Vancouver, Canada
	Dean group, laccases, Athens, Georgia
	McDougall group laccases, Dundee, Scotland

Table 3. Enzymes in the lignin pathway targeted for genetic modification in woody plants.

Global control of lignin biosynthesis - transcription factor, homeoboxes, and gene silencing

cis-acting regulatory elements that are thought to control aspects of lignin formation have been identified in *Arabidopsis* (Morelli, 1999), poplar (Hertzberg, 1998), loblolly pine (Campbell, 1999), and eucalyptus (Bossinger, 1999).

In tobacco an AC-rich motif, PAL box (Ntlm1), thought to be important in cis-regulation of phenylalanine biosynthesis was isolated (Kawaoka et al., 2000). Lignin expressing the antisense Ntlim1 showed a decrease of 70% as compared with control plants. Post transcriptional gene silencing of PAL expression has resulted in striking differences in lignin content and composition (Korth et al., 2001; Reddy et al., 2000). Characterization of these global regulators to modulate downstream expression of lignin genes for the purpose of lignin modification is ongoing.

The (at times) heated discussion of the best choice of genes to modify in forest trees to modify lignin composition and content is a long way from reaching resolution. But strides have been made to understand a

complex process that is only reaching initial comprehension. Optimism is high that the results so far indicate that the goal is attainable.

MODIFIED POPLARS AND THE ENVIRONMENT

The potential risks of a long-lived transgenic plantation and its potential impact on the environment is now of primary importance in public perception. Steve Strauss at Oregon State University in Corvallis has ongoing research into prevention of reproduction of such transgenics through the control of flowering. Using the *Populus trichocarpa* homologue of LEAFY and FLORICAULA from *Arabidopsis* a gene called PTLF was cloned to examine its expression in a tree species. It would appear that these genes may provide tools for delayed or inhibited flowering in woody plants. (Rottmann et al., 2000)

This same group at Oregon State is using population genetics to measure the gene flow from hybrid poplar plantations into surrounding native poplar stands (DiFazio,

2001). Hybrid poplars, a result of "classical" breeding programs represent a significant number of short rotation commercial plantations, and have done so for many years. The question is a good parallel to see if a "genetically modified" forest (through selective breeding) can impact a natural population.

As ongoing research looks into the effects of transgenic poplars on the environment, it is also beneficial to consider changing poplars to be even more adaptive to adverse environmental factors. Many attempts at poplar modification have directed efforts at resistance to biotic vectors, but little directed at abiotic stresses. Although as a genus they are remarkably versatile in their ability to grow on inhospitable sites, extending their tolerance of poor sites and conditions is equally desirable. The Institute of Plant Sciences and Genetics at The Hebrew University of Jerusalem, Rehovot, Israel has overexpressed a drought/cold/ABA related protein in poplar that shows increased tolerance to salt stress. (Altman, 2001) Open, exposed areas are often available for plantations, poplars are planted, and a situation is prime for failure of the plantation before harvest due to wind stress.

WIND STRESS IN POPLARS

Why study wind stress in poplars? What does this abiotic stress do to trees, and why is this relevant to the genetics of wood development? And how can studying the genes involved in cambial growth in poplar stems resulting from wind stress contribute to a better understanding of wood formation and tree improvement for field plantations?

WIND STRESS AS AN IMPORTANT ABIOTIC ENVIRONMENTAL STRESS IN TREES

The effects of wind stress can be influenced by many factors including canopy architecture, planting methods, root structures, soils, morphology and wind velocity (Couts, 1995). The scope of this study refers to the aspect of flexure of the stem of the tree and/or plant. Wind or MP refers to the temporary displacement or sway of a plant stem from the vertical caused by the wind or by other physical or mechanical means. Repeated flexures, either naturally induced by wind or artificially induced by flexing in laboratory experiments characteristically

results in a shortening and thickening of the stem, and reduced leaf area.

Genetic modification of woody plants and wind stress are topics that are usually not mentioned in the same sentence. In fact wind stress in trees is usually omitted in discussions of abiotic plant stress in general, particularly at the level of gene expression.

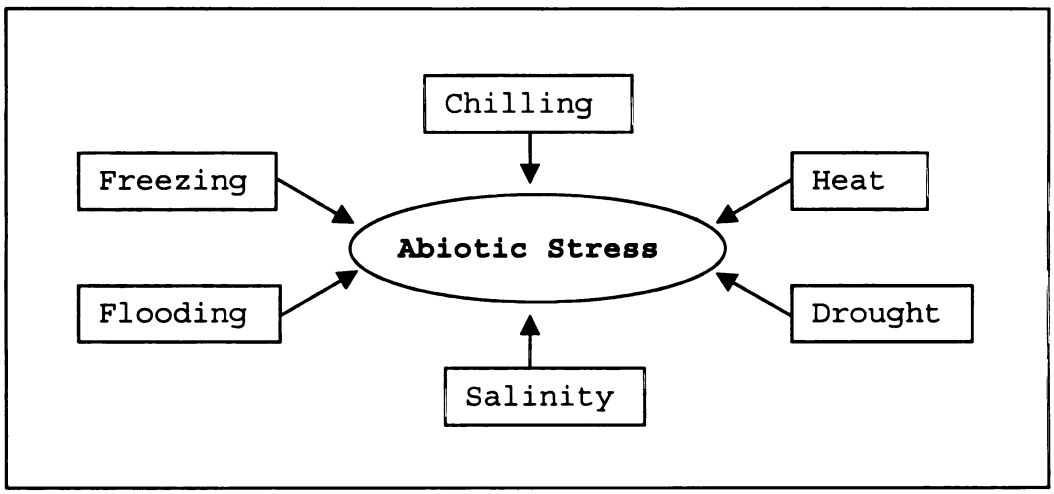

Figure 2. Abiotic environmental plant stresses

The above diagram is a listing of abiotic stresses on plants (Holmberg and Bulow, 1998). Holmerg does not mention wind stress, nor did Neuman in his description of abiotic stresses on poplars (Neuman, 1996). Wind stress or mechanical perturbation (MP) is frequently overlooked.

Plants being stationary objects must provide a defense against wind forces if they are not to blow over or to snap, just as they have developed defenses against other stresses. Although physiological and morphologic effects of the stem flexure (mechanical perturbation - MP) due to wind forces have been well characterized (Telewski, 1995), gene expression has not, with a few exceptions (Janet Braam 1990, 1992,1995, 1997, 1998), (Mizoguchi 1996), (Botella 1995, 1996), (Mauch 1997) and (Depege 1997). As wind stress in trees is responsible for the loss of thousands of acres of tree plantations worldwide, poplars, [Harrington, 1993 #268], *Pinus sylvestris* - in the UK, *Pinus radiata* in New Zealand (Somerville, 1995), rubber trees in the tropics, it is an abiotic stress worthy of more detailed study (Savill, 1983).

Thigmomorphogenesis has been defined (Jaffe, 1973) as a change in growth pattern response or allometry due to touch or flexure. Such flexure will effect transient change in tension and compression of constituent cells within the plane of bending (Biddington, 1986). This is not to be confused with rubbing the plant, vibrating the plant, nor wounding the plant and subsequent breakage of the cell

walls. Although these latter stresses may involve some of the same genetic mechanisms of plant defense, we are particularly concerned with those aspects of the wind which result in trees characterized by an increase in stem taper, decreased height and/or increased radial growth and a decrease in leaf area due to flexure of the stem. Staked trees, even with the wind blowing their leaves do not exhibit these physiological changes (Burton, 1973; Holbrook, 1989; Jacobs, 1954), and mechanical bending during dormancy still increases stem diameter (Valinger et al., 1994).

A wind stressed tree will be shorter in height, with shorter branches, smaller leaves and increased growth at branch bases, stems and at branch nodes (Telewski, 1995).

Table 4 is a proposed model of tree response to wind stress from the time of perception to the time of increased division of the cambium. From initial sensing of the mechanical flexure in the stem a cascade of events is initiated. Within twenty four hours there is increased division of the vascular cambium. In Sweden I observed that the physiological changes in the xylem tissue responding to gravitropic stress in poplar stems were

perceptible with microscopic observation three days after induction.

WIND OR MECHANICAL PERTURBATION (primary stress) FLEXURE OF STEM TISSUE (primary strain)	
Within first second(E)	Cytosolic calcium ion accumlutation (H) Decrease in phloem transport (H) Calmodulin transcription (H)
Within 10-30 min	Calmodulin synthesis (H)
Within 2 hrs (R)	Callose (β-glucan) accumulation (H,C)
Peak with 9 hrs (R)	
	Ethylene synthesis (H, C)
Peak within 15 hrs	Callose reabsorption (H, C)
Within 24 hrs (R)	Increased division of vascular cambium
Unknown time	Increased tracheids/Radial file (C, H) Shorter Tracheid length (C, A) Increased Cellulose Microfibril angle in 2ndary cell wall (C)

Table 4. Suggested model of tree response, suggested at the cambial level to wind or MP based on woody and herbaceous species (C=conifers, H=herbaceous, A=woody angiosperm) Times are estimates (E), or real (R) recorded periods for responses in woody plants. Telewski, unpublished.

Modification of stem taper, additional wood deposition, and changes in lignin content and composition (Pruyn, 1997) (Berlyn, 1979) are direct effects of wind stress. Knowledge of the genetic mechanisms that are involved in changes in stem architecture may be a very important contribution to ongoing research into lignin engineering in woody plants.

From the physiological evidence of tree responses to wind as outlined in the proposed model (Table X), gene expression in response to this stress can also be expected. Physiological and biochemical changes that have been experimentally demonstrated will have correlated gene expression. From the table above those genes should be expressed relating to calcium ion channel sensing, calmodulin, changes in hormone levels, and those involved in cambial development and secondary wall deposition, including lignin. Since plant response to wind is an abiotic stress, defense pathways might also be anticipated. Has any such gene expression been found?

GENE EXPRESSION IN RESPONSE TO WIND STRESS OR MP

Environmental cues are perceived by plant sensory mechanisms, triggering a cascade of internal events to respond to the stress. Wind, which is first perceived as flexure, is known to activate the TOUCH (TCH) genes as characterized by Janet Braam. When she described the touch (TCH) genes that became activated due to touch, wind and water spray, it was also found that TCH gene expression also occurred in response to darkness and temperature shocks (Braam and Davis, 1990). Several studies have discovered genes that might be common to wind stress while studying other stresses such as cold and drought. (Gilmour, 1998; Mizoguchi et al., 1996).

Previous molecular studies directed at touch gene expression have been conducted solely on non-woody plants: *Arabidopsis thaliana*, (Braam and Davis, 1990) (Braam, 1992), mung bean (*Vigna radiata)(Botella et al., 1995)*, wheat (Mauch et al., 1997), and tomato (Depege et al., 1997). In all cases except the mung bean, "touch" was defined as a mechanical flexure (MP) of a selected internode that was moved (or rubbed) back and forth, with hand, glove or glass rod. In the case of wheat, both the flexure and a wind-mimicking fan treatment were used to stress the plants. The mung bean treatment consisted of a

torque applied to the leaves by manually bending them downward for a number of repetitions.

RELATING GENE EXPRESSION DUE TO WIND STRESS WITH OTHER ABIOTIC STRESSES

As cited previously TCH gene expression also occurred in response to darkness and temperature shocks (Braam et al., 1997). Many genes have been shown to be ubiquitous to many abiotic and biotic stress responses as plants arm themselves with defense strategies. Among these are the calcium-channel related genes, implicated as second messengers (Bush, 1995; Haley et al., 1995) and calmodulin genes (Roberts and Harmon, 1992; Sinclair and Trewavas, 1997). Ethylene acts as a signal in many plant processes including transcription of defense genes (Bleecker and Kende, 2000; Ohme-Takagi et al., 2000). Various plant defense genes such as those in the phenylpropanoid pathway, and pathogenesis related (PR) genes (Barin and Zambryski, 1995; Koch et al., 2000; Mauch et al., 1997) also are expressed in response to a variety of stresses.

When wind or MP causes a stem flexure, there are changes in the shape and turgor pressure within the cells that is similar to changes in turgor pressure that is observed in dehydration and cold stress. Mechanosensory pathways such as those activated by wind stress have been linked to dehydration and cold stress, (Cowan et al., 1997). Absisic acid (ABA) may regulate gene expression in cold and desiccation tolerance (Chandler and Robertson, 1994) which also relates to turgor sensing mechanisms such as changes in cell shape, dehydrin and dehydration response genes, the late embryogenesis (LEA) protein family. The cold response (COR) genes and in particular a transcription factor CBF controls transcripts that accumulate in response to mechanical stimulation (Gilmour, 1998) Mizoguchi et al. in 1996 report simultaneous induction of three genes in response to cold, wind, and drought stress, two MAPKs (mitogen-activated protein kinases) and an S6 ribosomal protein kinase (Mizoguchi et al., 1996). Recent work of this same lab of Kazuo Shinozaki has monitored expression of 1300 full-length *Arabidopsis* genes under drought and cold stress using cDNA microarray (Seki et al., 2001). The pattern of gene expression in response to various stresses

is complex, has many pathways that are inter-related and involve the same genes or gene families. It is apparent that analysis of wind stress at the molecular level shows some similarity to gene response to other environmental stresses and bears further investigation. The genes described in response to wind or mechanical stress and those mentioned in studies above have been primarily conducted on herbaceous plants. Where are the studies of gene expression of abiotic stresses of woody plants? Again the use of poplar as a model system for such study is advantageous.

WIND STRESS AND *POPULUS* (POPLAR)

Poplar is well established as a model angiosperm tree system in molecular studies. The advantages of using poplar as previously described are well-documented, and include small genome size, ease of propagation and transformation and easy extraction of DNA and RNA. For the purposes of studying wind stress in trees as well as cambial development, poplar is a very attractive model. Well-documented field studies have identified wind resistant and wind susceptible clones. This is the same as

having *Arabidopsis* mutants that do not respond to a stress with the same phenotype as the wild type. Mutant *Arabidopsis* are conserved and valued, mutant trees that are intolerant of certain conditions or with undesirable growth form are usually destroyed. It is fortuitous that these poplar clones have been saved and propagated. A study of the gene expression in the stems of a wind tolerant poplar clone would contribute to the general knowledge of genes involved in modification of stem architecture and growth, give a greater understanding of how the involved genes have a role in stem function, and may provide tools for the better, stronger and more resilient poplar for the future.

CONCLUSION

If as a society we choose to use the knowledge we gain from the genetic study of cambial development and of wind stress in trees to genetically modify trees for economic or conservation purposes, we must make an investment in understanding and managing our forest resources more wisely.

Whether or not we are totally opposed to modification of woody plants or we are actively engaged in the attempt, we must recognize that our forests are human-dominated ecosystems. (Noble, 1997) Even if they are managed only moderately, or for hunting/gathering, humans are the stewards of global forests. Knowledge of how trees function at the genetic level is vitally important to future forests. The knowledge must be used with interactions among scientists, ecologists, social scientists, community representatives and economists.

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CHAPTER 2

GENE EXPRESSION IN WOOD FORMING TISSUE OF HYBRID *POPULUS* (POPLAR)

ABSTRACT

In trees, wood is produced from the vascular cambium. Until recently little was known about the control of cambial differentiation at the molecular level. In this study, hybrid poplar clone 47-174 (*Populus deltoides* x *trichocarpa*) was used as a model to examine gene expression in wood forming tissue and as a result of wind stress or mechanical perturbation (MP). Poplar clone 47-174 was stressed with MP to simulate the stem flexure of wind stress. RNA isolated from stressed and non-stressed poplar stems was examined using differential display of PCR products. One hundred thirty differential display gel bands from stem tissue were cloned and sequenced. Northern analysis and BLAST searches confirmed that more than seventy of these clones represent expressed sequence tags

(ESTs) for are poplar genes expressed in wood forming tissue. The evidence to support that this collection of poplar ESTs is derived from genes responding to MP stress in stem tissue is discussed.

INTRODUCTION

The importance of wood

Most woody plants are grown for their stems or trunks, for lumber, for paper pulp and other wood products, while others are grown for fruit or nuts and a few grown for essential oils and secondary metabolites, (citrus, eucalyptus, conifers and the like). Demand for these products continues to grow while land suitable for cultivation of trees shrinks. The acreage dedicated to first growth forests is diminishing and pressures to preserve those remaining are imperative. Demand for forest products has never been greater, thus increasing the need for renewable woody crops. Understanding the mechanisms

that contribute to wood formation is necessary as we strive to improve yield and quality of forestry plantations.

Wood development and the function of xylem

In a review in 1952, Bailey described six stages of wood development. Wood is formed in the stems of plants undergoing secondary growth from the vascular cambial zone during xylogenesis. Cells derived from the cambium initials divide, (zone of division), enlarge, (zone of elongation) and differentiate and mature (zone of maturation). Mature xylem then changes from conductive sapwood, from sapwood to heartwood and to the inner core of heartwood (Bailey, 1952; Larson, 1994; Telewski, 1996).

The functions of xylem are diverse. As wood is produced it gives strength and support to the stem that carries a significant weight of foliage, flowers and eventually fruit. The stem contains conductive tissue and provides

strength for the transport of water under extreme negative pressure to the upper most reaches of the plant (Zimmermann et al., 1994). The stem provides protection against a barrage of biotic and abiotic stresses, withstands decay, and reacts and forms wood to reorient the tree with respect to gravity or strengthen it in response to wind flexure. The cell wall of woody plants develops an impervious hydrophobic protective layer, a wall composed of a complex composite of cellulose and lignin. This woody stem that has such an important function for the tree, has also recently become the focus of molecular modifications in attempt to alter its composition.

Methods of woody plant modification

Traditional breeding programs have either crossed or grafted trees in an effort to increase yield and select for desirable traits, or enhance adaptability to diverse environmental conditions. An example of this is the cultivated tree *Gleditsia tricanthos forma inermis,* "Sunburst" honey locust. It is a honey locust that has

been selected from years of effort to reduce the number of thorns and seed pods normally produced in that species. One can now purchase a grafted cultivar named "Sunburst" locust that has been vegetatively propagated from the original selected tree. It will produce few seed pods and few thorns. As a selection "Sunburst" is commercially very successful. However, the process to develop this tree was time consuming.

The advent of molecular technology applied to the modification of woody plants presents an opportunity to shorten the time necessary to introduce or modify desirable traits, increase adaptability and improve wood and fiber quality For example, it is highly beneficial to modify trees to reduce lignin content with the end product being wood fiber for paper. This particular trait has never been a target for traditional breeding methods. However, lower lignin content would facilitate increased efficiency in pulp production, at the same time reducing deleterious pollutants resulting from delignification. Modification of lignin content is one aspect of tree improvement that is desired. Many labs have ongoing research directed toward this end. (Bourjan, Belgium, Jouanin, INRA, Cedex, France,

Chiang, Houghton, Michigan Lapierre, Cedex, France, Douglas/Ellis, Vancouver, Canada, Ye, Athens, Georgia and Chapple, West Lafayette, Indiana).

Growing plantation trees with increased environmental adaptability, or increased yield without sacrificing tree integrity and wood quality would be equally beneficial. Efforts to modify trees will be aided by a better understanding of gene expression during xylogenesis in the vascular cambial zone of woody plants. We must seek to understand gene function while integrating this with an evaluation of the effects of genetic modification on the whole tree.

Gene expression in developing cambium

The development of molecular techniques has enabled us to examine wood formation from another perspective. We are now able to identify some of the genes that are activated during the various phases of xylogenesis. It is possible

to discover and characterize those genes and to integrate them with what we know of the anatomical, biochemical and morphological changes during this process. In studies of herbaceous species, Zinnia, *Arabidopsis thaliana*, and *Nicotiana* (tobacco) [Dharmawardhana, 1992 #38; Ye, 1994 #169; Chaffey, 1999 #223; Jounin, 1999 #314] some knowledge of xylogenesis has been gained. However in herbaceous plants there is no (or minimal) vascular cambium, and no (or minimal) secondary xylem development. Until recently little has been known about the genetic control of the wood forming process. We may now be able to examine the changes that occur at the most basic cellular levels of xylogenesis in the cambial zone and understand more fully the functions that these genes play in woody plants. Studies are being made world-wide in a number of woody plant species to elucidate the molecular mechanisms of wood formation. One of the most widely studied tree genera is *Populus* (poplar).

Populus (Poplar) as a model system for the application of molecular techniques

Poplar has already proven to be a good model system for molecular modification. Poplars are easy to propagate both vegetatively from cuttings, and in tissue culture. They transform easily with *Agrobacterium tumefaciens* plasmid insertion, their genome size is relatively small (Arumuganathan, 1991), and extraction of DNA is comparatively easy (1997b).

In fact, poplar was the first tree to have successfully been genetically modified. A transgenic poplar resistant to the herbicide glyphosate was developed in the laboratory of Don Riemenschneider of the Forestry Sciences Laboratory of the USDA in Rhinelander, Wisconsin in collaboration with Calgene, Inc., of Davis, California. By using the *Agrobacterium* transformation vector, a bacterial *aro*A gene that conferred resistance was expressed in *Populus* (Fillatti, 1987). This was a highly desirable modification as plantation losses due to weed competition were enormous.

Since that first engineered poplar, there have been many genetic modifications proposed. Efforts at altering genes in poplars have been attempted for the purposes of increased herbicide tolerance, resistance to fungal and insect vectors, and augmented adaptability to environmental

sites which are unsuitable for other purposes, reclaimed from mining, polluted, or in extreme climatic situations. It is the desire to cultivate poplars for plantation harvesting in land that might be unsuited for anything else that has led in part to the examination of gene expression due to wind stress.

Wind stress and mechanical flexure

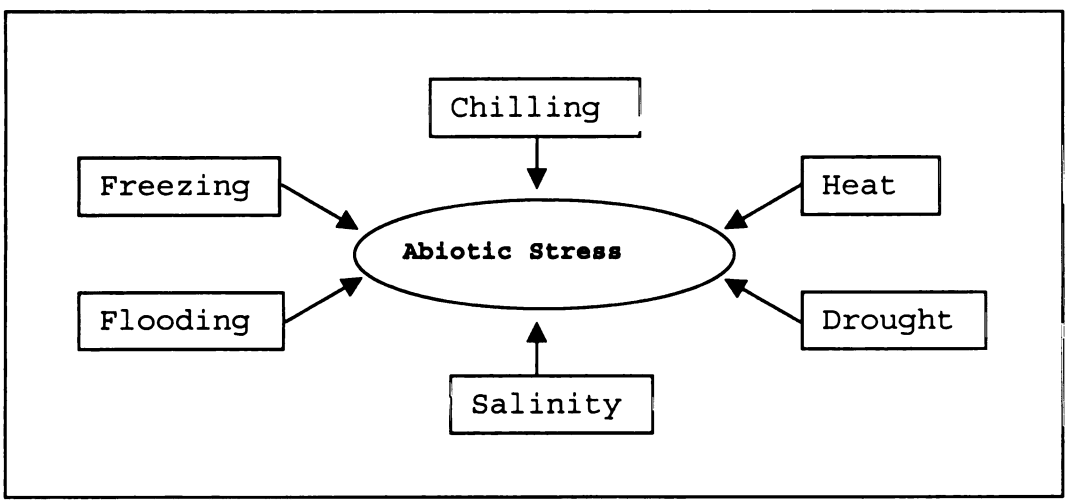

Figure 3. Plant abiotic environmental stresses

The above diagram is a listing of abiotic stresses on plants (Holmberg and Bulow, 1998). Holmerg does not mention wind stress, nor did Neuman in his description of abiotic

stresses on poplars (Neuman, 1996). Wind stress is at least as prevalent as any of those mentioned in Figure 1. and is frequently overlooked (Stanton, 1997). Plants being stationary must provide a defense against wind forces if they are not to blow over or to snap just as they have developed defenses against other stresses. Although physiological and morphologic effects of the stem flexure (MP) due to wind forces have been well characterized (Pruyn et al., 2000; Telewski, 1995), gene expression has not. There are some studies of gene expression due to touch, or flexure, notably those studies of Janet Braam (Braam, 1992; Braam and Davis, 1990; Braam et al., 1997). However there have been no studies at the molecular level of gene expression due to wind or MP in plants with secondary growth.

Wind stress in trees is responsible for the loss of thousands of acres of tree plantations worldwide (poplars, Harrington and DeBell [Harrington, 1993 #268], *Pinus sylvestris* in the UK (Quine, 1995), *Pinus radiata* in New Zealand (Somerville, 1995) rubber trees in the tropics (Clement-Demange and Doumbia, 1995). It is an abiotic stress worthy of more detailed study (Couts, 1995).

Modification of stem taper and additional wood deposition including lignin (Berlyn, 1979; Pruyn, 1997; Pruyn et al., 2000) are direct effects of wind stress. Knowledge of the genetic mechanisms that are involved in changes in tree stems in response to mechanical flexure may be a very important contribution to ongoing research into modification of woody plants.

Gene expression and wood development

Since additional wood deposition is one of the morphological responses to mechanical flexure of a woody stem, genes that are specifically expressed in developing cambium and xylem are expected. One source for identifying these genes would be EST databases being generated from poplar cambium and developing xylem tissues (Sterky et al., 1998). Some of these ESTs are now available on the web at *Populus*DBase. Several groups are sequencing EST collections from tension wood in poplar. Tension wood is that which is formed on the upper side of stems and branches in angiosperms due to gravitropic displacement. Additional tissue specific libraries from the Swedish

consortium will be made available later this summer (Sandberg, 2001).

It should be mentioned that databases are being established for gymnosperm wood formation as well. Collections of *Pinus taeda* (loblolly pine) ESTs are being made at North Carolina under the direction of the Forest Biotechnology Group at the University of North Carolina in the lab of Ron Sederoff. Recently the Canadian government has instituted funding for Genome Canada, the forestry division in collaboration with the University of British Columbia. Genome Canada will be creating EST databases of *Pseudotsuga menziesii* and *Abies grandis*.

Gene expression and mechanosensory perception

Previous molecular studies of touch or movement on non-woody plants are: *Arabidopsis thaliana*, (Braam 1990, 1992) mung bean (*Vigna radiata)*, (Botella 1996) wheat (Mauch 1997), and tomato (Depege et al., 1997). In all cases except the mung bean, "touch" was defined as a mechanical

flexure (MP) of a selected internode that was moved (or rubbed) back and forth, with hand, glove or glass rod. In the case of wheat, both the flexure and a wind-mimicking fan treatment were used to stress the plants. The treatment applied to mung bean consisted of a torque applied to the leaves by manually bending them downward for a number of repetitions.

The genes that have been associated with touch (wind and/or MP) are: the Touch (TCH) genes of Janet Braam, calmodulin or calmodulin-like genes (TCH 1,2,3), and xyloglucan endotransglycosylase (XET, TCH 4). XET has been shown to be related to cell wall deposition, which is to be expected in a stem that is depositing new cells at the point of flexure. Botella isolated, 1-aminocyclopropane-1-carboxylic acid synthase (ACC) synthase involved in ethylene biosynthesis and a calcium dependent protein kinase due to mechanical strain (Botella et al., 1995). Mizoguchi found MAPKKK (mitogen-activated protein kinase kinase kinase) (Mizoguchi et al., 1996) activated by touch, cold and water stress in *Arabidopsis* while Mauch described a lipoxygenase (LOX) induced by touch (MP), wind and wounding in wheat (Mauch et al., 1997). LOX is part of a family of enzymes implicated in mobilization of lipid

reserves in wound responses. The calcium channel related calmodulins, some protein kinases and the MAPKK from other plants have been implicated as early responses to a number of stresses in plant signal transduction pathways (Gilmour, 1998).

The discovery of genes that are expressed due to wind or mechanical flexure has occurred exclusively in herbaceous plants. There have been no molecular studies of MP stress conducted on trees and it is the impact of wind on tree plantations that is increasingly important.

Purpose and experimental design for this study

The worldwide use of poplar for biomass and its planting on marginal soils in windswept sites (Zsuffa, 1996) creates an imperative for understanding the response of various poplar clones to wind stress.

I have investigated gene expression in the stems of hybrid poplar and those expressed in response to mechanical perturbation (MP) during cambial development. Although

wind stress is responsible for the loss of thousands of acres of trees worldwide (Quine, 1995), very little is known of this abiotic stress at the molecular level in woody plants. It was anticipated that I would find genes with similarity to some of the above-described genes, as well as some that would be novel. This is the first systematic exploration of this type of stress in stem tissue of a woody plant.

Design of experiments

The advantages of using poplar as a model system for study of this particular abiotic stress include well-documented field studies which identify wind resistant and wind susceptible clones (Harrington, 1996) and the plantation failures at James River Corporation, Lower Columbia River Fiber Farm (Kaiser, 1997). These clones were available for study in laboratory conditions.

To further elucidate molecular mechanisms of plant response to wind or mechanical perturbation (MP), I have chosen hybrid poplar (*Populus deltoides* x *trichocarpa*) as a model

system. The cross resulting from these two species has produced many clones that have been used extensively in field plantations. *Heterosis* often results in many of the progeny of the cross possessing hybrid vigor (Stettler, 1996). In this instance the word "clone" pertains to vegetatively reproduced and named cultivated trees that result from selection of the F1 seedlings.

In this study I have stress tested a wind resistant clone, poplar hybrid clone 47-174 with mechanical perturbation. My goal was to use the technique of differential display of PCR products to make a comparison of the gene expression of the stressed trees to those of unstressed control trees. To this end I have generated a collection of cDNAs from the mechanically perturbed poplar stems which represent genes expressed in the cambium and developing xylem, including those genes specifically related to the environmental stress.

MATERIALS AND METHODS

PLANT MATERIAL AND GROWTH CONDITIONS

Poplar hybrid clone 47-174 designated as wind resistant (Harrington, 1996) was received from James River Corporation. Cuttings were rooted in woody plant cutting mix at Beaumont Nursery, MSU and kept under mist until roots were established, then transplanted into one gallon pots. Plants were moved to an environmentally controlled room, as free as possible of biotic and abiotic stresses. Plants were grown under Agro Grow High Sodium Lights (18 inches above the final height of the trees) with 16 hour days and fertilized with Peters 20-20-20 at 100 parts per million. Temperature during the 16 hour days was 26C and 21C during dark hours. One central leader was established, trees were staked and no pruning nor movement of stems or foliage was permitted for one week prior to testing.

MECHANICAL PERTURBATION AND HARVESTING

One group of 4 trees of clone 47-174 was set to one side of the bench when the plants were moved into the testing room,

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care was taken not to disturb this control set in subsequent watering. An additional group of 16 to 20 trees, specified for flexure tests was also moved into the testing room. The room was maintained as free as possible from biotic or abiotic stress. The trees were never moved, and great care was taken not to move the leaves or stems while watering. One week prior to harvest, the second group of 47-174s were pre-conditioned with 30 flexures of the stem at a selected internode above a mature leaf (Larson, 1994). The stems were displaced 60 degrees from the vertical in both directions. This was done at the same time every day for seven consecutive days. On the eighth day of testing, several control trees were stripped of leaves and stem segments frozen in liquid nitrogen. The stressed trees were given one final treatment and then a time course of stem tissue of all trees was harvested at T = 0, 1, 2, 4, 8, 12 and 24 hours after the final stress. Two control trees, never having received flexures were harvested at time 0 and 24 hours after initial harvest. Stem segments above, below, and including the node of stress were frozen in liquid nitrogen and stored at -80 degrees C. These experiments were replicated eight times and the stem tissues for the same time points were pooled. Different plant tissues were harvested and frozen in liquid

nitrogen: stems, leaves, meristems, roots, internode of stress, internode above and below stress.

RNA ISOLATION AND DIFFERENTIAL DISPLAY

RNA EXTRACTION METHODS

The method used to extract RNA was modified a number of times throughout the experimentation. The reason for these changes was the need to extract sufficient quality and quantity RNA for use in northern analysis. Extraction of RNA from leaf tissue of poplars has been demonstrated to be comparatively easy, whereas extraction of RNA from woody stems has not. There is little living tissue, there is an abundance of cellulose, polysaccharides and fibers as well as other metabolites rendering extraction difficult.

RNA EXTRACTION METHODS

The following is a list of the different methods that were evaluated to extract RNA, either total or messenger RNA, from the poplar stem sections.

1) Current Protocols - Phenol/SDS extraction, Lithium chloride (1997a)
2) Molecular cloning, A Laboratory Manual (1989)
3) Current Protocols - Guanidine thiocyanate (1997a)
4) Hot phenol (1989) as modified by Rujin Chen 11/93
5) RNAgents Total Isolation System, Promega
6) TRIzol Reagent - GIBCO BRL
7) Rneasy Total RNA Extraction kit - Qiagen
8) mRNA Isolation, Dynabeads, Dynal Inc.
9) Boiling phenol (DeVries, 1988)
10) RNA purification from woody branches and needles of spruce (Wang, 2000)
11) RNA purification from mature conifer needles and phloem tissue (Alosi, 2000)
12) RNA purification from Poplar stem tissue, modification of #10 and #11 - aepjones

RNA extraction from poplar stem - Protocol #12

Five grams of poplar stems were ground in a coffee bean grinder that was RNAse treated, and rinsed with DEPC water. 25 mls of extraction buffer (#10 above) was put into a RNAse treated mortar and pestle. The ground stem powder was added and thoroughly homogenized. This was frozen at -80C in a weigh boat. Removed from the freezer, this weigh boat was floated in a 37°C water bath and when melted transferred to an OakRidge tube to which 5 gm of 8.5M KoAC was added.

The protocol continues as in #10 above with the exception that all spins were done in an RC5B Sorvall centrifuge with a SS34 rotor at 10,000 rpms. Lithium chloride precipitation was done overnight, the ethanol precipitation on the second day was also left overnight at -20C. On the third day the resulting pellet was resuspended in 200 µl of DEPC water, the OD read on a spectrophotometer, and then frozen at -80. The modifications combine the triple detergents (cationic, anionic, and neutral) of protocol #10 with the high molarity potassium acetate precipitation of protocol #11. In addition the high speed spins in OakRidge, Saarstedt threaded closure, and Corex tubes done in a Sorvall RC5B centrifuge were able to guarantee a

pellet in each step, while discarding the unwanted cell components.

The hot phenol method (#4) was the method used for RNA extraction for the differential display experiments. Differential display is a PCR based method and the RNA extracted with the hot phenol method was of sufficiently good quality to successfully execute the display of PCR products. Northern analysis and hybridization using the hot phenol method proved inadequate. The recently published protocols #10 and #11 and their subsequent modifications resulted in better quality RNA and was used in hybridizations after this time period.

After RNA extraction using any of the above mentioned protocols the product was quantified on a spectrophotometer, run on denaturing formaldehyde gels and probed to evaluate quality and quantity.

DIFFERENTIAL DISPLAY OF PCR PRODUCTS

The technique of differential display of PCR products was developed in 1992 by Liang and Pardee to visually provide a

side-by-side display of gene expression of the same organism under two different conditions (Liang et al., 1993; Liang and Pardee, 1992).

RNA from stem tissue of mechanically stressed and unstressed hybrid poplar stems was extracted using the hot phenol method. After quantification by spectrophotometer and verification of the quality of RNA by separation on a denaturing formaldehyde gel, the RNA was DNAse I (BMB) treated to remove any residual DNA. The RNA was then reverse transcribed using MMLV Reverse Transcriptase and the cDNA again quantified. The Reverse Transcriptase, the primers and other components of the differential display reaction were obtained from GeneHunter Corporation. 0.2 µg of the DNAse treated total RNA was reverse transcribed using primers with oligo-dTs with an anchor of G, C, or A to obtain single strand DNA. The products of these three reactions were then used in a second PCR reaction using the same oligo-dT primer in the presence of a second 10mer arbitrary primer. Thirty-two different arbitrary primers (listed in Appendix B) from Gene Hunter Corporation were used to cover an estimated 60 percent of the eukaryotic messages (Liang, 1996). The PCR reaction incorporated ^{33}P dATP into the final double stranded cDNAs. These products

were visualized on a 6% denaturing polyacrylamide sequencing gel. Control samples which used no reverse transcriptase in the first PCR were also amplified in separate reactions as negative controls; while duplicate samples of the Control unstressed, 2 hour, 4 hour, and 12 hour stressed samples were also amplified and visualized.

After gel electrophoresis the polyacrylamide gel was transferred onto Whatman #3 paper, dried for one hour on a gel dryer and the resulting gel and paper were exposed to autoradiograph film overnight. After development of the x-ray film the side-by-side display of control tissue with the stressed samples was possible. Bands that showed a difference in the 2 h, 4h, 8h,and 12h samples from the two control unstressed lanes were marked and then excised through the x-ray film with an Exacto knife cutting the dried acrylamide and filter paper.

To extract the cDNAs, the small dried excised slices were resuspended in 100 µL water, soaked for ten minutes and boiled for 15 minutes. After spinning for two minutes to collect condensation and pellet the gel and paper, the supernatant was transferred to a new microfuge tube. cDNA was then precipitated with 10µL 3M sodium acetate, 5µL

glycogen (10mg/mL) and 450µL of 100% ethanol overnight and spun in a microfuge for thirty minutes in the cold room. This was resuspended in 10µL of water.

CLONING AND SEQUENCING OF DIFFERENTIAL DISPLAY BANDS

One hundred forty-five cDNA bands were excised from the differential display gel. 140 were successfully reamplified and were cloned using TA vector cloning. This method takes advantage of the single base A overhang generated by most DNA polymerases in the PCR reaction. By using a vector that has been generated with a T overhang, the PCR product is ligated into the cloning vector (Hadjeb, 1996) and subsequently transformed using Rubidium chloride prepared DH5α *E. coli* competent cells (Hanahan, 1993). Twenty-four colonies of DH5α *E. coli* were selected on LB/Ampicillin plates using blue/white screening for selection. These were replicated in microtiter plates and stored at -80°C for future screening (over 3500 colonies in duplicate). Two clones of each of 140 bands were grown in

liquid LB/Amp culture and plasmid DNA was isolated from the bacteria. After verification of the correct size insert with PCR evaluation and *BSSHI* enzymatic digestion, one copy of each of the 140 remaining cDNAs were prepared for sequencing. Applied Biosystems (ABI) Prism™ Dye Terminator reactions were performed in an MJResearch thermal cycler, cleaned with BIORAD Corporation spin columns and sequenced using an ABI 373 sequencing machine.

REVERSE NORTHERNS

To elucidate which of the cloned differential display bands was expressed due to MP, the 144 PCR products were dot blotted in three concentrations on duplicate sets of membranes. Restriction digests of the plasmids containing the cloned PCRs were also blotted onto duplicate filters. The RNA from the differential display experiments was reverse transcribed while incorporating a radioactive label. This was used to probe the dot blots and the restriction digest filters. Autoradiography and scanning with a Phosphorimager (Molecular Dynamics) were used to evaluate results.

NORTHERN ANALYSIS

Following RNA extraction (using hot phenol (Method #4) for all gels prior to June 2000, and using the Method #12 protocol subsequently), 20 micrograms of total RNA was run on a denaturing formaldehyde gel. A control sample of RNA from unstressed stem tissue was included with time points as in the differential display, 2, 4, 8 h. The RNA gel was transferred to Millipore Immobilon™-N transfer membrane using with 10 X SSC (1989). Following overnight transfer, the filter was crosslinked using a Strategene Cross Linker. Probes of 70 cDNAs from PCR products from the cloned differential display bands were made using P32. Prehybridization and hybridization solutions contained Dextran sulfate and Heparin to increase incorporation (Singh, 1984). Hybridized filters were exposed to autoradiograph film (Kodak, Rochester, N.Y.) overnight and subsequently developed. Some filters were re-exposed to x-ray film for one week prior to development.

DATABASE SEARCHES

Three internet web servers with search engines were used for data analysis: The *Arabidopsis* Information Resource (TAIR), a publicly available website was the primary search engine used. TAIR BLAST search was done using BLASTx against all protein sequences from the *Arabidopsis* Genome Initiative (AGI)and total genome protein dataset. TAIR Blastn was used to search again the all higher plant (Viridiplantae) sequence database. The Finch (Geospiza Corp) server is available by assigned password from the Molecular Highthroughput Array Facility at Michigan State University. Using the Finch server, 132 sequences were "batch blasted" periodically using the nucleotide BLAST and protein BLAST default algorithms against various NCBI BLAST databases and dBest. PopulusDB, a *Populus tremula x tremuloides* genomic sequence database from Sweden was searched one time for all sequence homologies. Last updated in February 1999, the database contains 5,692 EST's, 4,809 cambium EST's and 883 xylem EST's with average lengths of 440 nucleotides. This database has the capability of performing only nucleotide searches.

PHYLOGENETIC ANALYSIS AND BOX SHADE ALIGNMENT

BioNavigator, a web based bioinformatics program from Entigen, Corporation, California was used for protein sequence analysis. Proteins from database searches were imported into BioNavigator. Protein sets were created and then aligned using ClustalW(Fast) GCG. Pretty Box and Boxshade were used to show protein alignment in specific areas.

BioNavigator Macro #26373 was used to create a phylogenetic tree for β-glucosidases. Protein sets were aligned in ClustalW (Fast), then evaluated in ProtDist as a distance measure of relatedness. The Neighbor Joining algorithm then established the phylogeny. Finally DrawGram graphically depicted the relationship of the proteins in a phylogentic tree.

RESULTS AND DISCUSSION

RESULTS FROM DIFFERENTIAL DISPLAY

141 of 144 excised bands successfully cloned

Total RNA isolated from stems subjected to mechanical flexures was compared to unstressed control 47-174 poplar trees using differential display. 144 bands that appeared to be differentially expressed were excised. Based on the autoradiographs only bands that were visually of greater intensity than the two control lanes, or were not present in the control lanes were selected. In addition, these bands were present in two or more of the four time points (2,4,8,12 hrs). 141 of the 144 were successfully reamplified and subsequently cloned into BlueScriptII. One colony of each of the 141 cloned PCR products was selected (after verification for correct size) and sequenced.

SEQUENCING RESULTS

129 Mechanically perturbed/wind induced (Wimps) sequenced

Of 141 cDNA clones that were sequenced, thirteen produced no signal or unreadable sequence. These thirteen were submitted for repeat sequencing. Of the thirteen that were resequenced only the sequence of Wimp 53 produced readable good quality sequence. Eleven other sequences were of poor quality, however were still used for homology searches. One hundred sequences with less than 2% ambiguity, ranging in size from 200 to 450 bases, will be submitted to GenBank. They are named Wimps, an acronym for **WI**nd or **M**echanically **P**erturbed poplar expressed sequence tags (ESTs).

RESULTS FROM DATABASE SEARCHES

Putative identity of genes identified by differential display of poplar clone 47-174

Table 1 is a presentation of information about 65 ESTs from poplar clone 47-174 which had significant BLASTx or DBest

similarities found from database searches. The number of the Wimp EST is in the column that begins with the two *Populus* Actin clones and is labeled WIMP#, followed by the sequence length in the adjoining column. The BLASTx score from the TAIR search against the *Arabidopsis* genome initiative (AGI) and Total Genome database from their web site is given in order of probability score. For those twenty-nine with BLASTx hits whose expectation values are <1.00E-4, a putative identity is given. Of these twenty-nine, only 5 had homologies to ESTs from the *Populus* database. When homologous *Populus* ESTs and their corresponding WIMPs are compared to the non-redundant protein database using BLASTx, the high hits are a match. This would indicate not only that the *Populus* ESTs and their corresponding WIMPs are indeed matches, but also that the results are consistent. Putative identity of those *Populus* ESTs was the same as those of the putative identity for those Wimps.

Results of northern hybridization of probes made from those Wimps with high BLASTx scores are listed in the last column. "C" indicates that the hybridization signal was constitutive; "N" indicates there was no apparent hybridization and "2 to 4" represents the up regulation of

Wimps #59 β-Glucosidase and #68 Dehydration response protein. If nothing is listed in this column, no northern hybridization was done. .

Other Wimps, (#31-36 in numerical order) had significant (<5.00E-7) homologies to sequences from the *Populus*DBase and the putative identities listed for #s 31-36 are for the *Populus*Dbase sequence. The *Populus*DBase information gives no 3'PolyA terminus for any of their sequences.

On pages thirty-seven and thirty-eight are listed those Wimp #s that had high sequence similiarity to sequences from dbEST, a database of expressed sequence tags. These Wimps had no significant hits with BLASTx or other database searches.

Database searches reveal some Wimps related to plant defense response

Grey-shaded areas indicate database hits that are similar to stress induced or defense genes, or described in MIPS as pertaining to functional categories that relate to plant stress response. For example Wimp68, the putative dehydration response protein showed highest similarity to

Arabidopsis thaliana dehydration induced protein RD22. The second grey-shaded group is the WIMPS with strong sequence similarity to ESTs from stress induced plant libraries.

Sequences with no hits, or no significant hits are not included in Table 5, however are listed and described in Appendix B.

TABLE 5.

ESTS FROM WOOD FORMING TISSUE DATABASE SEARCHES

	WIMP #	LENGTH	TAIR BLAST X AGI/Total GENOME	PUTATIVE IDENTITY	*POPULUS* DB	NORTHERN HYBRIDIZATION
			Score		Score	
1	P.actin2	711	3.00E-38	Actin	e-115	C
2	P.actin1	709	5.00E-67	Actin	505 e-144	C
3	119	317	6.00E-34	SARI/GTP binding	4.00E-23	C
4	36	303	1.00E-30	Aminopeptidase	None	C
5	49	352	1.00E-22	Ribosomal protein	e-113	
6	79	354	1.00E-16	Putative esterase	None	C
7	130	353	1.00E-13	Rubisco activase	None	
8	123	201	3.00E-13	Unknown protein (*A.t.*)	None	
9	126	294	7.00E-13	Putative protein (*A.t.*)	None	
10	30	292	1.00E-11	Putative protein (*A.t.*)	None	N
11	136	195	2.00E-11	Hydroxymethyltransferase	None	C
12	24	284	3.00E-11	Putative protein (*A.t.*)	None	C
13	85	292	4.00E-11	SINA(seven in absentia)	None	C
14	18	235	3.00E-09	Unknown protein (*A.t.*)	None	C
15	59	192	5.00E-09	Beta-glucosidase	None	2 TO 4
16	95	174	4.00E-08	S-receptor kinase	None	C
17	128	259	6.00E-08	Aquaporin	7.00E-38	C
18	62	244	7.00E-08	Subtilisin	None	N
19	92	200	7.00E-08	Ascorbate peroxidase	None	C
20	114	304	7.00E-07	40S Ribosomal protein	None	

TABLE 5. (Continued)

	WIMP #	LENGTH	TAIR BLAST X AGI/Total GENOME	PUTATIVE IDENTITY	*POPULUS* DB	NORTHERN HYBRIDIZATION		
21	96	273	3.00E-06	Selenium binding protein	None	C		
22	108	295	6.00E-06	Putative protein (*A.t.*)	None	C		
23	113	335	1.00E-05	Putative protein (*A.t.*)	None			
24	23	207	5.00E-05	Hypothetical protein(*A.t.*)	None			
25	6	218	1.00E-05	Kinesin	None	C		
26	68	391	8.00E-05	Dehydration ind protein	None	2 TO 4		
27	93	200	0.0001	GTP binding protein	None			
28	66	340	3.00E-04	RNA polymerase II	5.00E-29			
29	77	300	0.001	Thaumatin/Osmotin	3.00E-30	C		
30	117	302	N.S.		None			
31	21	271	N.S.	Sodium/Ca exchanger	2.00E-15	N		
32	132	262	N.S.	Glucose dehydratase	5.00E-07			
33	71	250	N.S.	Phloem specific protein	4.00E-50			
34	89	223	N.S.	Aldehyde dehyrogenase	7.00E-12			
35	109	214	N.S.	60S Ribosomal protein	4.00E-41	C		
36	116	177	N.S.					

TABLE 5. (Continued)

	WIMP #	LENGTH	TAIR BLAST X	NCBI dbEST Score	*POPULUS* DB	NORTHERN HYBRIDIZATION
37	93	200	N.S.	9e-12 tomato mix elicitor	None	N
38	106	256	N.S.	8e-10 P.taeda xylem inclined	None	N
39	22	240	N.S.	7e-78 Roots A.thaliana	None	N
40	34	215	N.S.	7e-10 tomato nutrient def. Roots	None	N
41	9	215	N.S.	7e-10 Six day cotton fiber	None	C
42	55		N.S.	7e-10 Medicago innoculated	None	
43	53 (twice)	221	N.S.	6e-11 P.taeda inclined xylem	None	
44	10	200	N.S.	6e-10 Moss library	None	C
45	33	189	N.S.	6e-10 Ice plant NaCl	None	C
46	75	172	N.S.	6e-10 Glycine max	None	
47	109	238	N.S.	5e-21 Hybrid aspen lib	None	C
48	27	265	N.S.	5e-11 tomato elicit.fruit	None	C
49	11	136	N.S.	4e-10 Hordeum vulgare leaf	None	N
50	89	223	N.S.	3e-18 Poplar xylem 3e-10 drought Medicago t.	None	
51	1	238	N.S.	3e-09 Rice panicle	None	C

TABLE 5. (Continued)

	WIMP #	LENGTH	TAIR	NCBI	*POPULUS* DB	NORTHERN		
			BLAST X	dbEST		HYBRIDIZATION		
52	70	249	N.S.	3e-09 Medicago	None			
53	20	189	N.S.	2e-9 Sorghum Pathogen induced	None	N		
54	13	154	N.S.	2e-9 Sorghum pathogen ind	None	N		
55	71	230	N.S.	2e-56 Hybrid aspen	None			
56	66	340	N.S.	2e-35 Hybrid aspen RNA Pol II	None	N		
57	140	138	N.S.	2e-18 Medicago	None			
58	16	221	N.S.	2e-10 wild tomato	None	C		
59	19	211	N.S.	2e-10 Pinus taeda clone	None	C		
60	59B	138	N.S.	2e-09 P taeda compression wood incl.	None			
61	63	256	N.S.	2e-09 Ice plant NaCl	None			
62	3	292	N.S.	2e-09 Barley leaf library	None	N		
63	87	223	N.S.	2e-0 Glycine max clone	None			
64	50	231	N.S.	1e-49 drought Medicago s.	None			
65	110	192	N.S.	1e-08 Glycine max	None	C		

A detailed presentation of results for each sequence is presented in Appendix A. This appendix includes the sequence, primer combination used to amplify the sequence, homologies, protein alignments and scanned autoradiographs of northern hybridizations.

It must be pointed out that the 3' terminus of eukaryotic genes most often consists of an untranscribed region (UTR) of one to three hundred bp. Many of the Wimps are short sequences, average length of 240 nucleotides, which limits success in homology searches and because of the use of polyA primer for PCR, the sequences are largely derived from the 3' end of the mRNA. The average length for Wimps that resulted in high BLASTx similarities is 240 bases (80 amino acids in translation).

NORTHERN ANALYSIS

65% of Wimp probes hybridize to poplar RNA

The most promising cDNAs, those with BLASTs homologies, 3.00e-04 and lower, or those visualized with reverse northern screening of dot blots of PCR products or of filters with digested plasmids containing the clones, were used to make individual probes for hybridization to blots of stem RNA. PCR products from seventy cloned bands were used to make probes which were used individually to hybridize blots with immobilized RNA from *Populus* of four to eight different time points and control RNA from unstressed poplar stems. Forty-five of the seventy autoradiographs showed hybridization. Only two of the forty-five had apparent two to four-fold differences in gene expression, Wimps 59 and 68. The other forty-three were apparent constitutive messages in the poplar stems.

Hybridization signal varied a great deal from very low to adequate, although incorporation of radioactivity in probe labeling was consistent. Hybridization to a consitituve probe, Actin was also consistent and hybridized strongly to all filters evenly in the control and stressed samples.

A number of different strategies was used to increase Northern blot signals. Different pre- and post-hybridization washes were evaluated, varying quantities of

RNA on the filters, mRNA on the filters, leaving the film exposed for long periods (one week) and evaluation of results with a PhosphorImager (Molecular Dynamics). Experiments to improve the quality of the RNA continued for three years and eventually led to significant improvement in hybridization results.

Putative identity by BLASTx searches of 14 Wimps with hybridization to northerns

Putative identity	Accession # of Similar protein	Wimp#	BLAST X score
SARI/GTP binding protein	At4g02080	119	6.00E-34
Aminopeptidase	At1g63770	36	1.00E-30
Putative esterase	At2g41530	79	1.00E-16
Hydroxymethyltransferase	At4g13930	136	2.00E-11
SINA(seven in absentia)	At5g53360	85	4.00E-11
(developmental protein)			
Beta-glucosidase	At3g18080	59	5.00-9
S-receptor kinase	At5g03700	95	4.00E-08
Aquaporin	At2616850	128	6.00E-08
Ascorbate peroxidase	At1g77490	92	7.00-08
Selenium binding protein	At4g14030	96	3.00E-06
Kinesin	At3g44730	6	1.00E-05
Dehydration induced protein	At5g25610	68	8.00E-05
Thaumatin/Osmotin	At2g28790	77	1.00E-03
60S Ribosomal protein	GI16951	109	4.00E-41

Table 6. Wimps that hybridized to poplar RNA with similarity to proteins in GenBank

DATABASE SEARCHES SUPPORT HYPOTHESIS THAT WIMPS ARE EXPRESSED DUE TO STRESS RESPONSE

Thirty of the one hundred thirty Wimp EST sequences had BLASTx ((probability scores of >3e-04) similarity to known proteins. However of these thirty, only five were found in the poplar EST collection from Sweden. This poplar EST collection was made from sequences randomly amplified using vector primers. As mentioned previously, Wimps are not random, they were cloned from the 3' polyA end of each message and they are directional, and cloned from PCRs from stressed tissue.

In addition to the five Wimps that amino acid similarity to known proteins and in the PopulusDB, a further five Wimps had high homologies in the *Populus*DB, but **not** in other public databases. An additional release of 4,000 poplar sequences from a leaf library were searched using NCBI dbEST. No further Wimps were found in the new release as of July 1, 2001, so only ten Wimps were homologous to existing poplar sequences.

Lack of similarity to the *Populus* database

The two libraries used to construct the poplar database (*Populus*DBase) were made from developing cambium and developing xylem. These poplars were not induced by any stress. The poplar library of 5,692 ESTs represents a total of 3,719 unique transcripts (Sterky et al., 1998). The average size of their cloned messages was estimated at 1.2 kb. The readable sequence generated from vector primer (averaging 400 base pairs) from the library was non-directional and occurred randomly throughout the 1.2 kb region. Therefore there exist 800 possible placements for the 400 base pair sequence (within the 1.2 kb clone). **If** the poplar ESTs overlap the Wimps (average 250 bp) by 50 bases at the 3' end of their 1200 base pair average message **then** that overlap would give a significant BLAST score. By chance only 200 out of 800 positions (1/4) would result in a significant overlap. Twenty-five percent of the 130 Wimps or **33 Wimps** should be found in the *Populus*DBase.

The fact that only ten Wimps were found to have high homologies in the *Populus* database would support a hypothesis that these two collections of ESTS, those from Sweden and the Wimps were from RNA populations derived from different methods and from tissue harvested under different plant conditions. Given that conclusion, it is not surprising that only ten of the 132 Wimps were found in the poplar database. The Wimps were cloned from trees that were mechanically stressed. The two Wimps that were found to have two to four-fold up regulation of expression were not found in the Swedish database..

Wimps with similarity to stress induced proteins

Although function of the 37 Wimps that had high BLASTX scores has not been verified, the profile of the putative identities suggests some of them are stress related proteins (Kasuga et al., 1999; Seki et al., 2001). Wimp 92, ascorbate peroxidase, Wimp 68, dehydration response protein, Wimp 136, hydroxymethyl-transferase, Wimp 62, subtilisin, Wimp 77, thaumatin/osmotin, and Wimp 59 β-

glucosidase represent genes which are stress related. The above-mentioned proteins have been characterized in previous studies (Kasuga, etc. mentioned above) as plant defense, or stress response genes. The dehydration response protein and osmotin are related to changes in cell tension in response to cold acclimation and dehydration. (citations) The changes in cell wall shape during mechanical flexure, alternating between tension and expansion as the stem is flexed, could suggest a similar type of stress response as those of cold, salt and dehydration. All four stresses result in changes in turgor pressure and cell-wall changes.

Over twenty Wimps (in addition to the 37 mentioned above with BLASTx protein similarity) had high hits in dbEST datasets from libraries created from stress induced plants: drought, bacterial pathogen, cold or salt stress (see Table 1). Although this is not conclusive evidence that the Wimps are expressed due to MP, it would support the hypothesis that genes expressed due to mechanical perturbation could be similar to those genes found in response to cold, drought, or salt stress where there is also a change in turgor pressure within the plant cells. **It also supports the hypothesis that mechanical**

perturbation is an abiotic stress of plants that induces a number of stress response genes. Although the data from the northerns would indicate that the Wimp ESTs are largely constitutive, the Wimps were cloned from PCR products from poplars under stress conditions. From the database searches there is an abundance of representation of genes whose function is stress related.

Wimps not found in the developing cambium library

Further evidence that the Wimps represent a collection of stress induced CDNAs comes from results of screening of the Swedish library. A library screen of six different Wimps using the Swedish developing cambium library (from Magnus Herzberg, Department of Forest Genetics and Plant Physiology, Umea, Sweden) failed to hybridize to plaques, while Wimp 128, putative aquaporin, a highly expressed constitutive gene did yield colonies from the Swedish library.

In summary, of 130 Wimps, greater than half are putatively expressed in developing xylem and phloem and putative identity of these cDNAs suggests that a number are implicated in plant stress response pathways.

WIMPS OF PARTICULAR INTEREST

Several Wimps had similarity to proteins that could be of particular interest. Two of these had two- to four-fold upregulation in repeated Northern analysis, Wimp 59, β-glucosidase, and Wimp 68, dehydration response protein, (Figures 4 and 5).

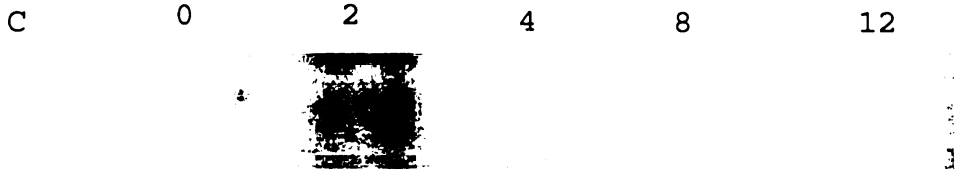

Figure 4. Wimp 59 ß-glucosidase: The photo shows northern hybridization of Wimp 59 to Poplar mRNA. Lanes are control, 0, 2,4,8, and 12 hours after MP.

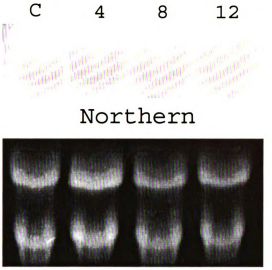

Figure 5. Wimp 68 dehydration response protein: The top photo shows northern hybridization of Wimp 68 to total Poplar total RNA. Lanes are control, 4, 8 and 12 hours after MP. The bottom photo shows the Poplar total RNA gel (used to transfer) stained with ethidium bromide. The graph shows relative intensity of Wimp 68 hybridization after normalization of the ethidium bromide stained gel.

LIGNIN BIOSYNTHESIS AND WIMPS 59 AND 62

Two Wimps that could be involved in lignin precursor storage and polymerization are Wimp 59 - β-glucosidase and Wimp 62, Subtilisin. Figure 6 indicates proposed pathways for the possible transfer, storage and polymerization of the monolignol alcohols (Whetten et al., 1998). These compounds are volatile, they degrade quickly and are toxic to the plant. They must be polymerized rapidly, in other words there must be *de novo* synthesis as required or they must be converted to glucosides and stored. Although this has been proven in the case of conifers (Dharmawardhana et al., 1995) there is little evidence to support this pathway in angiosperms. Noritsugu Terashima has studied the behaviour of monolignol glucosides in Magnolia and Ginkgo lignin, but no β-glucosidase has been cloned specific to this pathway (Matsui et al., 1994).

There is also a lack of knowledge of the polymerization process in angiosperms or gymnosperms. The research of Gordon McDougall at the University of Dundee has focused on the enzymes involved in lignin polymerization, particularly

laccases (Richardson, 2000; Richardson et al., 1997). An oxidase found in his lab contained two polypeptides responsible for the oxidase activity, the larger of the two was homologous to a number of plant subtilisin-like serine proteinases. Wimp 62 shares homology with this group.

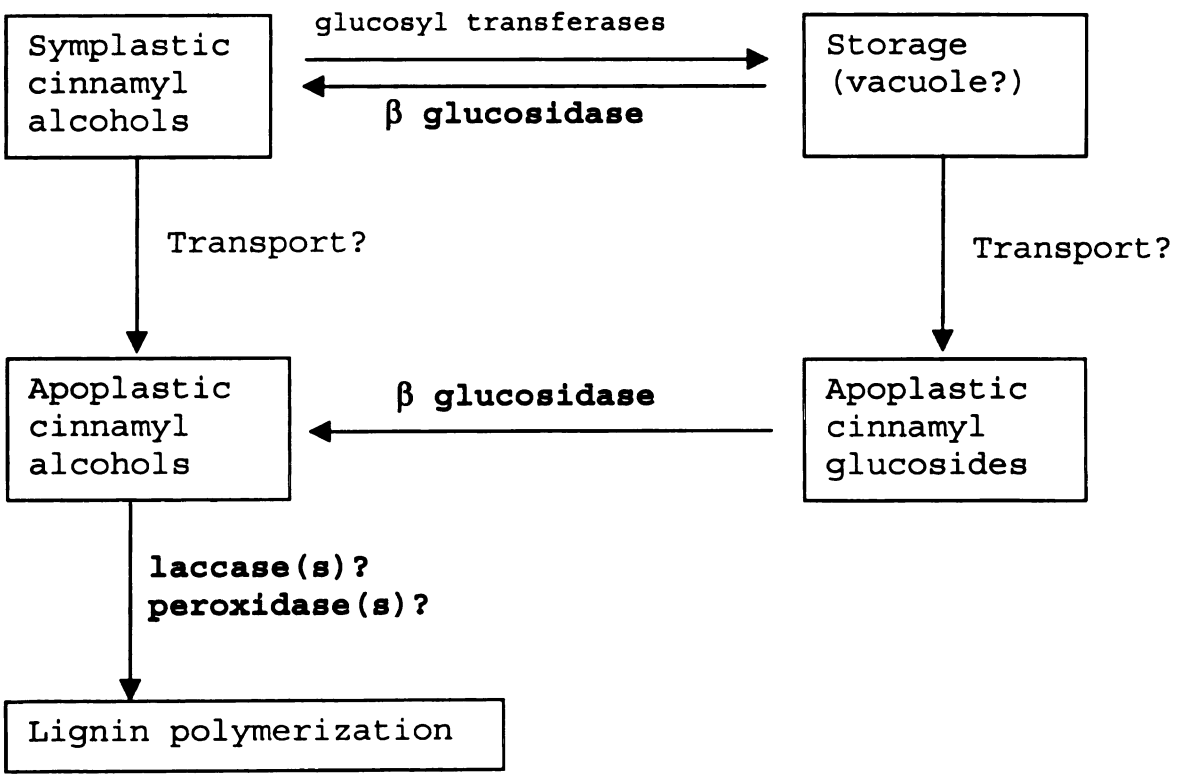

Figure 6. Different storage, transport and deglycosylation pathways for monolignol alcohols (Whetton and Sederoff, 1995)

WIMP 59 - POSSIBLE ROLE IN LIGNIN PATHWAY

After the two- to four-fold up regulation seen on the autoradiograph of Wimp 59, numerous attempts were made to obtain a full length clone. They were not successful. However ClustalW alignment, BoxShade, and phylogenetic analysis of plant β-glucosidases reveal that Wimp 59 is different from other *Populus* β-glucosidases found at present, either in Sweden or in British Columbia in the lab of Brian Ellis (Figures 7 and 8). It is more closely related to those cloned from *Hordeum vulgare* (barley) and *Oryza sativa* (rice). β-glucosidases are a large family of enzymes that catalyze the hydrolysis of glycosidic linkages and are found in plants, fungi, animals, and bacteria (Esen, 1993). Swiss-Prot lists one hundred eighty two β-glucosidases, while a BLINKS (Blast Links) search revealed 32 bacterial, 63 metazoan, 5 fungal and 100 plant β-glucosidases. Plant β-glucosidases have been studied in many metabolic events, particularly in defense against pathogens. However the function of the many plant β-glucosidases has not been well studied.

Wimp 59 is certainly a member of this large family, it is expressed due to mechanical flexure in wood forming tissue, and it does not have homology to any poplar β-glucosidases

found previously. It is a good candidate for further study.

Figure 7. Alignment of ß-glucosidases. ClustalW (fast) (GCG) was used to generate the alignment of the carboxy terminus. Poplar UBC1 represents an unpublished ß-glucosidase sequence from the lab of Brian Ellis (UBC). All other protein sequences were from the non-redundant (nr) protein database at NCBI. The grey-shaded area denotes areas of highly conserved residues.

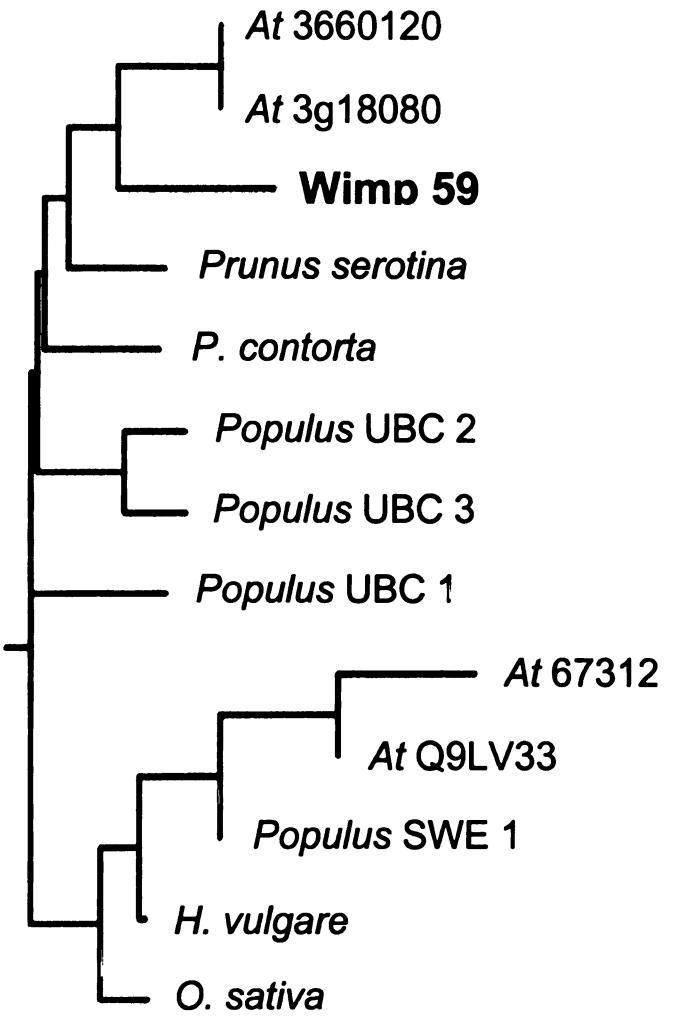

Figure 8. Plant ß-glucosidase protein family. This figure shows a phylogenetic tree created using BioNavigator (see methods). *Populus* UBCs are unpublished ß-glucosidase sequences from the lab of Brian Ellis (UBC). The *Populus* SWE 1 sequence is from *Populus*DBase. All other protein sequences were from the non-redundant (nr) protein database at NCBI.

Wimp 62 - Subtilisin, another possible candidate in the lignin pathway

Wimp 62, with homology to subtilisin is also interesting. Subtilases have been demonstrated to be serine proteases. Gordon McDougall's lab has postulated that such proteinases could have a role in wall modification events in enlarging cambial cells or in maturing xylem elements. The following is the protein alignment of Wimp 62 with an *Arabidopsis thaliana* subtilisin that is the highest hit for the Wimp 62 translation.

```
3'  AYQVNTEYSFGSLTWTDGVHIVRSPLSVRTEFLQPYI    (Wimp62)
       VNT++ FGSL WTDGVH V  P+SVRT+F++ Y+
3'  SHRVNTDFYFGSLCWTDGVHNVTIPVSVRTKFMRNYV    (At Subtilisin)
```

Figure 9. Protein alignment of Wimp 62 Subtilisin

The laccase purified from developing xylem tissue of *Picea sitchensis* (Sitka spruce) is differentially regulated in compression and non-compression wood branches (McDougall, 2000). One domain of this laccase in the N-terminus has protein homology to the subtilisin family and is implicated in lignin polymerization. The research group in Dundee, Scotland has also purified an angiosperm laccase from poplar from lignifying tissue (Ranocha et al., 1999). Wimp

62, which has protein similarity to this family is another candidate for further characterization.

Wimp 68 - Dehydration induced protein

A class of proteins first described in 1998 shares a conserved domain at the C-terminus with Wimp 68. Called the BURP domain for the four members of the group in which they were first identified: BNM2 from *Brassica napus* microspore derived embryo, USPs, abundant non-storage seed proteins, RD 22, a drought induced protein from *Arabidopsis*, and PG1β, the β-subunit of polygalacturonase isozymes (Hattori et al., 1998). The group shares some structural features, but no patterns of tissue specificity nor functional similarity. Wimp 68 has the highest similarity to RD-22 which is expressed due to drought, and as characterized by Shinozaki, is mediated by abscisic acid (ABA) (Yamaguchishinozaki and Shinozaki, 1993).

```
3'    AYQVXNVKPGTVPVCHFXLQDHVVW  (Wimp 68)
      A++V  VKPGTVPVCHF  + HVVW
3'    AFKVLKVKPGTVPVCHFLPETHVVW   (At RD22)
```

Figure 10. Protein alignment of Wimp 68 Dehydration response protein.

The above alignment of Wimp 68 and *Arabidopsis thaliana* RD22 shows grey-shading for 100% amino acid similarity to the conserved amino acids of the BURP domain. It can be concluded that Wimp 68 is expressed in developing cambium, shows two to four-fold up regulation on a northern and is part of a family of plant genes characterized by a BURP domain. It shares protein similarity to a gene from *Arabidopsis* that is induced under stress conditions. Wimp 68 should be further characterized.

DISCUSSION OF NORTHERNS

Radiolabelled probes were prepared from seventy different Wimp cDNAs and used to probe northern blots. There are three categories of results: **twenty-five which had no hybridization** (after being left on film for a week), **forty-five with hybridization** (in some cases, weak), and of these forty-five, **three** indicated a slight difference in gene regulation (two to three fold difference from the control lane). Two of these Wimps, numbers 59 and 68 were examined repeatedly, up to ten times each in an effort to maximize

hybridization. These two showed the greatest difference in hybridization intensity; as well as being putatively linked to stress proteins and/or the lignin pathway. The remaining **forty-two** appeared to be constitutive, showing equal hybridization of control lanes to those in the stressed lanes. It should be mentioned that Northern analysis was ongoing over three years as different protocols were used for RNA extraction in an attempt to test for up regulation.

CLONED BANDS HAVING NO HYBRIDIZATION TO NORTHERN BLOTS

Of the seventy cloned Wimps that were chosen for hybridization to Northern blots, twenty-five yielded no signal after exposure to autoradiograph film for one week (Wimps 2,3,4,8,10,11,12,13,15,20,25,28,58A,62,66,101, 106,115,130). Appendix A presents a detailed history of these bands including sequence data, primers used, database similarity results and northern hybridizations. These bands likely are expressed in poplar stems, however the methods used for verification of gene expression are not

sensitive enough to detect messages with very low expression.

NORTHERNS, GENE EXPRESSION, AND UP-REGULATION OF MP GENES

Although there were apparent differences (two to four-fold) in gene expression on autoradiographs for two Wimps, the majority of the forty-five hybridizing Wimps appeared to be consitutively expressed genes. If these are truly constitutive, then the technique of differential display and the experimental design failed to discriminate among genes that were expressed due to the stress and constitutively expressed genes. Alternatively, a greater number of the 141 cloned cDNAs may represent MP induced genes but the methods used to detect differential expression were not adequate. This could be due to a number of reasons: a) the quantity of message in the harvested stems, b) rapid growth of both stressed and unstressed plant material, and c) quality of the RNA transferred to the nylon membrane. These factors could cast doubt on the results of the northerns.

RNA EXTRACTION

Some discussion of the difficulty that I encountered in the extraction of the poplar RNA may provide guidance for future workers. From the number of articles published dealing with poplars, it would now seem that this should not have been such a problem. However there is a variability in the type of poplar clone, or species being used for extraction. In Sweden, I observed the extraction of RNA from a hybrid cross of Populus tremula x P. tremuloides. The stem sections frozen in liquid nitrogen were then slit vertically through the bark which was then peeled back. A scalpel was used to scrape the bark section (cambial), and in a second scraping of the middle core (xylem). This is the method described in creation of the Poplar cDNA library (Sterky et al., 1998). RNA extraction was then done using Dynal magnetic polyT beads. The stems of the Swedish hybrids were the same age and height of the 47-174 clones that were used in the experiments at Michigan State University, however they were almost an inch or more in diameter making cambial scrapings an option. The 47-174 stems were often only 1/4 inch in diameter and could not be

used for cambial scrapings except in field grown poplars of this clone. Field grown poplars could not be used in these experiments because a variety of biotic and abiotic stresses exist under field conditions. Wind stress comprises elements other than stem flexure such as canopy size and shape, wind direction and speed. These compounded factors could not be segregated in field grown poplars from the exclusive aspect of stem flexure.

Because of the selection of poplar clones, for purposes stated above, it was necessary to develop methods to successfully use these clones for the MP experiments. Over a three year period I continually tested different methods to optimize results of extractions. After the modification I made to protocols #11 and #12, the RNA was of good quality. However, time constraints became a limiting factor. It took six months from rooting of cuttings to harvesting stressed tissue and sufficient plant material was not available.

Since the development of my extraction modifications, my protocol has been requested by a USDA Forest Service laboratory performing RNA extraction on elms and

experiencing problems. (Jennifer Koch, USDA Forest Service, personal communication)

All of the factors mentioned above may have contributed to the lack of conclusive evidence of up-regulation for most Wimp genes. It is my opinion that the combination of the above limitations coupled with the difficulty of extracting high quality RNA that led to the difficult demonstration of upregulation.

An additional factor is the rapid growth of the poplar clones during the experiments. The stems of poplar clone 47-174 (control and stressed) grew almost a foot in height from the time of pre-stressing to one week later at harvest. Cambial development would be similar in both control and stressed stems, the differences in the two samples could be difficult to detect. The stressed stems should be depositing additional xylem at the internode of stress. Although in one set of experiments RNA was extracted from the specific internode of stress (and the internodes above and below the stressed internode), there was no apparent difference in gene expression. The gene expression may be at constitutive or basal levels in all samples, the differences being undetectable with current

technology. It is a matter of allocation of resources, a change in the deposition of material that is almost certainly being continually deposited.

LIMITS OF DIFFERENTIAL DISPLAY

The differential display procedure as applied here will in theory screen sixty percent of the *Populus* genome (product literature of GeneHunter Corporation; Liang and Pardee 1963). Because of cost limitations, each set of primers was used in only one differential display reaction. However as there were two control lanes and four time points represented in the use of each set of primers used, this was considered sufficient to act as a control to limit false positive reactions. If a band was observed in two or more of the time points, and not in the controls, it was considered to be significant and selected for further analysis. Despite measures to decrease false positives, a well-documented pitfall (Bauer, 1993; Callard, 1994) in differential display, is the PCR re-amplification at low stringencies (42°C) that can result in non-specific

amplification. Those bands that did not re-amplify, or produced poor quality unreadable sequence could be the result of nonspecific amplification.

In the population of bands that were sequenced it is unknown the number which truly are not poplar bands, nor specifically expressed to the stress conditions. Only fifty of the seventy cloned bands that were used for radiolabeled probes showed hybridization to poplar RNA. An additional twelve (which were never made into probes) had homology to sequenced ESTs from the *Populus* database, confirming that sixty-two were indeed poplar genes. However a lack of hybridization in the twenty Wimps that did not hybridize to the RNA filters could represent the difference between a PCR based technique (differential display) and northern hybridization. It cannot be ruled out that these differential display bands were the result of false positive selection.

Until the emergence of microarray technology to monitor global gene expression, differential display was widely used by researchers in human medicine and in other fields to analyze differences in gene expression in two different tissue types, or the same tissue type under different

conditions (Liang, 1996). It is still a powerful and valuable tool in organisms that do not have, nor will have in the near future a sequenced genome or collection of expressed sequence tags (ESTs). For certain physiological conditions it remains a useful technique (Liang, 2000). Microarrays made from cDNA libraries that are not normalized to guard against the bias of abundant transcripts may not detect many low abundance messages. Microarrays in general lack sensitivity for low abundance transcripts (Goldberg, 1986). Differential display may sample rarer products more efficiently by reducing the 'C_0t effect' (MathieuDaude et al., 1996).

Two sequences revealed high homology to *Pseudomonas putida* flagellin, Wimp 121 (BlastX 7e-30) and Wimp 124 (9e-30). Interestingly a cDNA probe of Wmp 121 revealed hybridization to all lanes of poplar total RNA. No probe was made of Wmp 124. These high homologies to a bacterial protein sequence could indicate the presence of bacterial contamination from the surface of the poplar tissues used for RNA extraction. This could only be possible if bacterial RNA was extracted along with the plant RNA for both the differential display and in the RNA that was blotted onto a filter for the Northern hybridization.

Although it is unlikely that bacterial RNA could be reverse transcribed in the first strand synthesis since oligo-dT primers were used in these reactions, in rare cases some bacterial RNA does have polyA 3' termini. (Marie Edmunds citation) However an alternative explanation is that bacterial RNA survived the isolation and was PCR amplified from poly A rich regions.

It can therefore be concluded that at least half of the Wimps (sixty-two) that were sequenced from isolated differential display bands are ESTs from poplar stems in developing xylem and cambium. This is supported by Northern hybridization (fifty) and by significant BLAST scores within published Poplar EST sequences (twelve).

An additional thirty-five sequenced Wimps had significant hits to plant ESTs in the NCBI database. This is strong indirect evidence that these Wimps are indeed poplar genes from the developing cambium/xylem region.

Twenty-seven Wimps for which Northern analysis was not done revealed either "no hits", nor significant hits with TAIR, *Populus*DB or Finch searches.

SUMMARY

It can be concluded from database searches and from northern hybridizations that the majority of Wimps are derived from the developing cambium and xylem of poplar. . The Wimps were generated from poplar tissue that was mechanically stressed using PCR techniques. The differential display which uses PCR to amplify and display tissue under different conditions may represent a level of sensitivity in gene expression that cannot be verified with northerns. The majority of the northerns that were done showed constitutive levels of expression, yet sequence similarity to genes of known functional categories reveals a contrasting picture. It can be concluded from database searches that there is an abundance of representation of stress related genes that bear sequence or protein similarity to a majority (65) of the Wimps.

FUTURE DIRECTIONS

Given time and increased funds a more complete coverage of the RNA population could be accomplished with increased efforts to reduce the incidence of false positives. Using a complete set of primer combinations, replication of the differential displays and using a gel apparatus to obtain longer sequences would all improve the significance of the results. Using hybrid poplar clones that were more amenable to cambial scraping and RNA isolation would be desireable, but at the expense of being able to use field evaluated wind tolerant poplars. The technology in the laboratory of Bjorn Sundberg permits sampling of RNAs from a cell specific gradient across the poplar stem. These improvements would optimize results. Having full length clones or obtaining sequences of greater length would also be an improvement. A differential display of cDNAs from proven wind tolerant versus wind intolerant poplar clones would be highly desirable.

These Wimps are a contribution to the public domain databases. It has been shown that a majority of the sequenced Wimps are from plants, and most are from hybrid

poplar stem tissue. Some show evidence that they are representative of genes that would be expressed under wind stress conditions. This collection should be expanded. All of the poplar ESTs, including those from the Swedish database, which is being expanded, the French tension wood database, and hopefully the newly funded Canadian EST collections should be combined on a comprehensive poplar microarray chip. An EST collection of cDNAs from mechanically perturbed poplar stems, akin to the ones that I have cloned, should be included. Then a comparison could be made of poplar hybrid clones shown to be wind tolerant versus those that snap in a strong windstorm. Only in this way will be able to understand the genetic mechanisms underlying tree response to wind stress.

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CHAPTER 3

CULTIVARS OF *QUERCUS CERRIS* x *QUERCUS SUBER*: *Q.* x *HISPANICA*, THE LUCOMBE OAK AND INTER-SIMPLE SEQUENCE REPEATS (ISSRs)

ABSTRACT

Morphological and chromosomal evidence for the identification of cultivars of *Quercus* x *hispanica*, the Lucombe oak has often been inconclusive. The use of DNA fingerprinting techniques has proved helpful for such identifications. Using inter-simple sequence repeat (ISSRs) primers, 66 *Q.* x *hispanica* (and related) samples were analyzed and compared for band pattern differences to establish identities. DNA evidence revealed that some named cultivars had identical banding patterns, which then aided in identification of other named or unnamed cultivars with the same DNA banding patterns. The cultivar named 'Lucombeana' with nine different grafted samples showed no

two had identical banding patterns, while two grafted trees on the grounds of the University of Exeter, and dating in age approximately to the time of William Lucombe, did have the same banding pattern and may indeed be of the original clonally propagated 'Lucombeana'.

INTRODUCTION

From the time that William Lucombe of Exeter discovered a different looking oak seedling in his *Quercus cerris L.* seedbed (probably from a *Q.cerris* pollinated by *Q. suber*) in 1763, (Elwes & Henry 1906) there has been confusion about the name and identity of that oak. Called *Q. lucombeana* by Sweet, in 1826, it has subsequently been called the Lucumbe oak, the Lucombe oak, *Quercus* x *hispanica* Lam.var. *lucombeana*, and the Exeter oak among other names. Within a short time Lucombe clonally propagated thousands of ramets of this oak by grafting onto *Q. cerris* rootstock and distributed them, while the original may have been cut down for Lucombe's coffin boards when the tree was about twenty years old (Elwes & Henry 1906). Lucombe's son named a group

of seedlings from the original tree, which are now regarded as cultivars: 'Crispa', 'Suberosa', 'Incisa', 'Heterophylla', and 'Dentata'. Subsequent cultivars originating from other crosses of *Q.* x *cerris* and *Q.* x *suber (Q.* x *hispanica)* include 'Cana major' of 1849 from the Hammersmith Nursery, 'Diversifolia' from the Smith Nursery) and perhaps the most famous other than that of Lucombe, 'Fulhamensis' Loudon 1838,from the Whitley and Osborne Nursery at Fulham, England. (Bean 1976)

Morphological characteristics, such as corky bark formation, leaf form, leaf retention, height at maturity, acorn size, etc., are quite variable. Chromosome studies of the number and morphology of the chromosomes showed no variation among the forms examined (Caldwell 1953). Because of these difficulties in identification, it is more than inappropriately named.

It was decided to use DNA fingerprinting on a group of *Q.* x *hispanica* cultivars in an attempt to shed light upon the confusion surrounding the Lucombe oak. Are the many cultivars named 'Lucombeana' vegetatively identical, and is it possible to establish their lineage as the same as that original cloned (grafted) material of Lucombe? Although

many fingerprinting techniques have been successfully used on plant material (Weising 1995) and on oaks specifically (Dow 1995 and 1996), it was decided to use ISSRs that had been used successfully in population studies of oaks (Marquardt personal communication). ISSR markers are generated from single-primer PCR reactions where the primer is designed from di- or trinucleotide repeat motifs with anchoring sequences of one to three nucleotides. (Wolfe 1998) These occur in all eukaryotes· Using polymerase chain reaction (PCR) primers created to bind to these repeated regions, the intervening areas are amplified allowing variation to be detected between samples. Plant cultivars that are vegetatively propagated should all have an identical banding pattern. All of the clonally propagated oaks from Lucombe's nursery and all those that were grafted later from this first cross will exhibit an identical, unique banding pattern for each primer used. It should be noted that seed propagated cultivars (e.g. Oilseed Rape), will have a wide range of band patterns, as can individuals within the same species.

MATERIALS AND METHODS

Sixty-four different plant samples of *Q.* x *hispanica* and related materials were collected, see Table 1. Other genera from the *Fagaceae* Dumortier (*Castanea* and *Fagus*) and one outgroup (*Rhododendron*) were used to evaluate various extraction methods and choice of primers (data not shown) Samples from various sources in the United Kingdom (UK) (see Table 7 for specific locations and accession numbers) were mailed to Michigan State University, Michigan, USA in plastic bags through regular postal channels. One sample, *Q.hinckleyi*, was obtained from a herbarium voucher at MSC Herbarium (Plovanich-Jones 002). DNA was extracted using the method of Scott *et al.* 1996 that was developed for use on 'rain forest' plant species but which has often proved quite useful for samples high in polysaccharides and secondary metabolites. The posted samples proved difficult to extract with other methods because of long transit storage conditions.

Name	Accession #	Name	Accession #
Castanea dentata (MSU)		*Q. x hispanica 'Ambrozyana'*	80.0280A
Castanea sativa (Hillier's)		*Q. x hispanica 'Ambrozyana'*	82.0296A
Q.cerris *'Laciniata'*	44.0004	*Q. x hispanica 'Ambrozyana'*	77.4402
Q.cerris L.	14.0079	*Q. x hispanica 'Ambrozyana'*	77.2213
Q.cerris *L.*	75.0105	*Q. x hispanica 'Cana major'*	27.0057
Q.cerris 'Wodan'	89.2595	*Q. x hispanica 'Cana major*	27.0056
Exeter lower		*Q. x hispanica 'Cana major'*	27.0055
Exeter graft union (*Q. robur* L.)		*Q. x hispanica 'Cana major'*	43.0457
Exeter middle		*Q. x hispanica 'Cana major'*	43.0455
Fagus sylvatica L.(MSU)		*Q. x hispanica 'Cana major'*	27.0058
Kensington "A" West Ham Park		*Q. x hispanica 'Crispa'*	43.0463
Kensington "B" West Ham Park		*Q. x hispanica 'Crispa'*	43.0464
Kensington "C" Kensington Gardens		*Q. x hispanica 'Crispa'*	43.0465
Kensington "D" Chiswick House		*Q. x hispanica 'diversifolia'*	87.2194
Q x hispanica *Lam.*	95.0694B	*Q. x hispanica 'diversifolia'*	08.0320
Q x hispanica *Lam.*	95.0694A	*Q. x hispanica 'Fulhamensis'*	86.2228
Q x hispanica *Lam.*	04.0434	*Q. x hispanica 'Fulhamensis'*	82.0142
Q x hispanica *Lam.*	43.0472	*Q. x hispanica 'Fulhamensis'*	78.1942
Q x hispanica Lam.	77.5535	*Q. x hispanica 'Hemelryjk'*	93.0030
Q x hispanica Lam.	44.0403	*Q. x hispanica 'Hemelryjk'*	93.0030B
Q x hispanica Lam.	77.1306	*Q. x hispanica 'heterophylla'*	95.0454
Q x hispanica Lam.	09.0047	*Q. x hispanica 'Lucombeana'*	77.5412
Q x hispanica Lam.	95.60695B	*Q. x hispanica 'Lucombeana'*	14.0133
Q x hispanica Lam.	77.1306	*Q. x hispanica 'Lucombeana'*	56.0039
Q. laceyi Small(Texas)		*Q. x hispanica 'Lucombeana'*	43.0384
Q. hinckleyi C.H. Muell.(Texas)		*Q. x hispanica 'Lucombeana'*	77.7380
Quercus robur L. (MSU)		*Q. x hispanica 'Lucombeana'*	88.0329
Rhododendron species		*Q. x hispanica 'Lucombeana'*	44.0037
Q.suber L.	79.9326	*Q. x hispanica 'Lucombeana'*	17.0048
Q.suber L.	77.4783	*Q. x hispanica 'Lucombeana'*	41.0425
Q.suber L.	43.174	*Q. x hispanica 'suberosa'*	95.1629
Q.suber L.	78.3131	*Q. x hispanica 'Waasland'*	97.0075
Q.suber L.	76.9326	*Q. x hispanica 'Wageningen'*	89.2597
		Q. x hispanica 'Wageningen'	89.2596

Table 7: Plant samples used for DNA microsatellite analysis. Accession numbers for some samples are those of the Arboretum furnishing the plant material.

PCR conditions: 30ul PCR reactions were performed with final concentrations of 1X PCR buffer, 12% sucrose, 0.2nM cresol red, 200uM each dNTP, 0.07U/ul Amplitaq, 30 ng total DNA, 1.8uM UBC primer. An MJ Research, Inc. PTC 100

thermocycler was used with the following conditions: denaturation at 94°C for 2 min. (using a hot start, 3.5 mM $MgCl_2$ was added when temperature exceeded 80°C) 29 cycles at 92°C, 30 sec., 52°C for 45 sec., and 72°C for 2 min. A 2% agarose gel was used for resolution of the PCR products. 25 µl was loaded into each well (the addition of the Cresol red dye to the PCR reaction obviated the need for any further loading or running dye) and run at 50 V for 16 hours using a cooling, re-circulating tank maintained at 12°C.

Bands were visualized by staining gels with Ethidium bromide for thirty minutes and destaining in 0.5X TAE for an additional thirty minutes. DNA Proscan, (Nashville, Tennessee) was used for scoring of bands and evaluation of results. All samples were evaluated with a minimum of two gels for each UBC primer. Four UBC primers (UBC 807, 811, 834 and 841) were used for each sample. Although UBC primer 807 can produce a number of bands that will also appear using primer 834 likewise for 811 and 841, it will also produce many unique bands. The four primers will produce four different banding patterns for each clonally unique cultivar. Samples of 'Lucombeana' from twenty different PCR reactions and the four primers were repeated as the results

were somewhat startling and this was the focal point of the study. Results for all gels were consistent and repeatable.

RESULTS

The ISSR banding patterns of 'Ambrozyana', and 'Fulhamensis' and the two trees from the University of Exeter were identical within each sample group. (Figure 11 Panels a and b) Banding patterns are identical if the same banding phenotype is obtained with all primers used. Figure 1a shows four samples of 'Ambrozyana' using UBC 834, the bands are identical for all four samples. 'Ambrozyana' band patterns for the other UBC primers were consistent and identical within the four. This was also the case with the two samples from the University of Exeter shown in Figure 1b. Again using UBC primer 834 in Figure 11c , three samples of 'Fulhamensis' Lanes A, B, and C, match one of the unnamed trees from Kensington, London, Lane D.

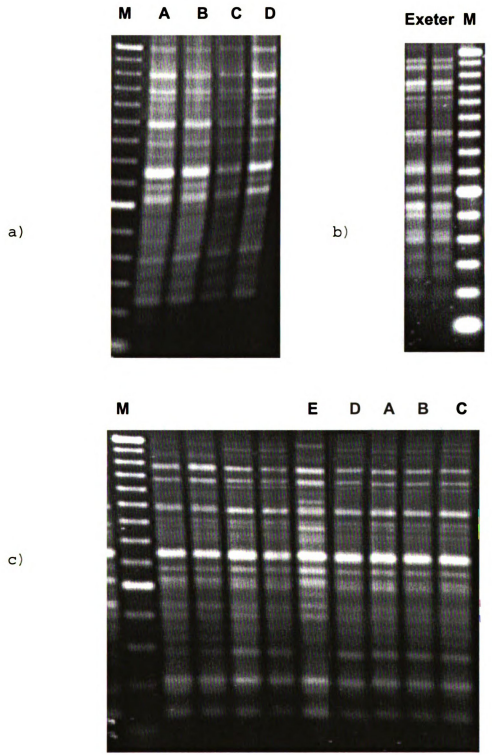
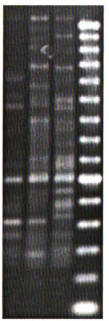


Figure 11: Banding pattern UBC 834: a) '*Ambrozyana*' Lanes A-D, M = Gibco 100 bp molecular weight marker b) E = Exeter oaks, c) '*Fulhamensis*' Lanes A, B, C, and unnamed oak from Chiswick House, London, Lane D, Exeter, Lane E

The cultivars labeled 'Crispa' and 'Cana Major' were different from each other and furthermore no 'Crispa' was the same as the other two Crispas, (Figure 12) and no 'Cana Major' identical to the other five Cana Majors. (Figure 13)

a)



b)

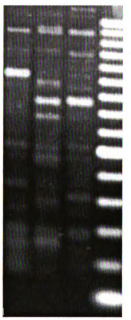


Figure 12. Banding pattern a) UBC 811 and b) UBC 841 of three cultivars of '*Crispa*'

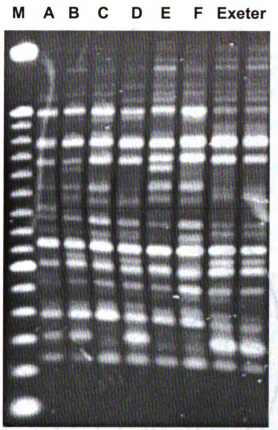


Figure 13: Banding pattern UBC 834 for six samples of var. '*Cana major*'

'Lucombeana' showed that none of the nine different samples all named 'Lucombeana' had identical banding patterns (Figure 14). It should be noted that a different banding pattern always had more than two bands different from another sample being considered with the same name. There

were no instances of samples being different by only one band.

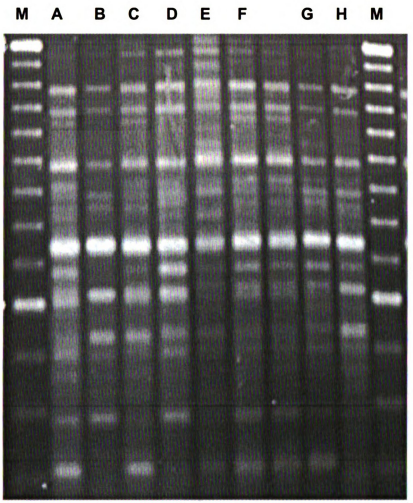


Figure 14. Banding pattern UBC 834 for nine samples of '*Lucombeana*'. One lane is unmarked due to confusion on the label of the posted sample.

The banding pattern from the three samples of 'Fulhamensis' matched those of three of the samples from the London area, Kensington 1, 2, and 4 and that of one sample named 'Lucombeana' #88.0329 (Figure 15).

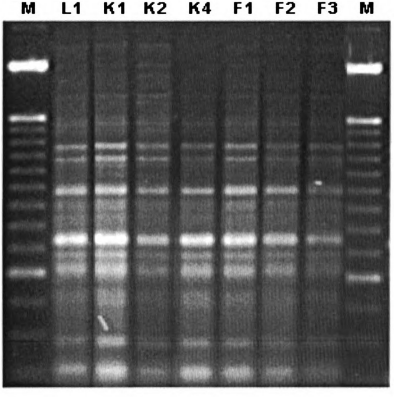


Figure 15: Lanes marked M are molecular weight standard, all other lanes are banding patterns from UBC 841. Lane L1 '*Lucombeana*' 88.0329, Lanes K1, K2, and K4, unnamed oaks from the London area, Lanes F1, F2, and F3 '*Fulhamensis*'

The two accessions of 'Diversifolia' were identical to each other, as were those of 'Hemelrijk' and the 'Wageningen'. These latter two cultivars, often referred to as *Q.* x *hispanica* differed from each other as well as the third Dutch cultivar, 'Waasland'. (These data are not shown). These cultivated varieties have been named more recently and are still being propagated and sold in the nurseries in which they originated. The ten *Q.* x *hispanica* with unspecified cultivar name showed no similarities to each other nor to any other named cultivars. These data are also not shown. It is more than likely that these were propagated from acorns from any of the Lucombe oaks across the United Kingdom.

DISCUSSION

It can be concluded from gel evidence that in the case where banding patterns are identical in multiple different samples of the same named cultivar (four or five minimum) that the given taxonomic designation is correct. Other unnamed samples with the same ISSR profile as the accepted cultivar can subsequently be named. In the case of

Ambrozyana numbers 80.0280A, 82.0296A, 77.4402 and 77.2213, the different samples are clonally identical and this cultivar is almost certainly a valid one. The three 'Fulhamensis' samples, 86.2228, 82.0142 and 78.1942 are identical and the unnamed samples from West Ham Park and Chiswick House (K1, K2, and K4) that match them can be considered the same clone. And one clone from Hillier's Garden and Arboretum, Accession #88.0329, is incorrectly named as 'Lucombeana', it is most definitely 'Fulhamensis'.

Certain cultivars remain problematical, 'Cana Major', 'Crispa' and 'Lucombeana' revealed no banding pattern that was identical within each sample group of the same name. Cultivars that had no more than one or two samples could be said to be not identical to other cultivars if their banding patterns using the same primer (and others) did not match banding patterns of any other cultivar. These include 'Laciniata' 'Suberosa', 'Diversifolia and 'Wodan' which are different from each other and other named cultivars. The ten unnamed *Q.* x *hispanica* samples did not match any named samples and could possibly have grown from acorns (seed propagated), rather than have been vegetatively propagated.

The case of the Lucombe oak is particularly interesting. As no consensus was found from the samples received as 'Lucombeana', we would offer the following suggestion. A much larger sample set of 'Lucombeana'should be collected including those from historical collections (such as Kew, Cambridge, etc.) which date from the time of Lucombe and are vegetatively propagated, a graft union being visibly obvious. The remaining tree from Exeter should be evaluated and included in this collection. Additional primers (7 - 10) should be used to evaluate the larger 'Lucombeana' sample set. A minimum number of samples with identical banding patterns for all primers used should be established as a criterion for the identification of a cultivar. When such a number is determined, then one could establish what is validly a 'Lucombe' oak. Such a proposal is not of course our decision and until the time that determination has been made, we will still be in search of the Lucombe oak.

ACKNOWLEDGMENTS

The following persons aided in the collection of samples, photographs, DNA extractions or PCR ISSR evaluations.

Frank W. Telewski, Beal Botanical Garden, Michigan State University, USA;

Hugh C. Angus, Westonbirt Arboretum, UK;

Steven Scarr - University of Exeter, UK;

Elinor Wiltshire - Carroll House, London, UK;

Glen T. Jones and Jan Rademaker. Michigan State University, USA;

Paula Marquardt - USDA, University of Wisconsin, Rhinelander,USA;

Bryan Epperson - Department of Forestry, Michigan State University, USA;

M.V. Ashley - University of Illinois at Chicago, USA,

MSU US DOE Sequencing Facility - Michigan State University, USA.

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CHAPTER 4

A PHYLOGENY OF THE INTERNAL TRANSCRIBED SPACER (ITS) AND EXTERNAL TRANSCRIBED SPACER (ETS) REGIONS FOR *MONTANOA* (ASTERACEAE: HELIANTHEAE).

ABSTRACT

A phylogeny of the genus *Montanoa* based on the Internal Transcribed Spacer (ITS) and the External Transcribed Spacer (ETS) is presented. The combined dataset supports the monophyly of the genus and an early evolutionary split that coincides with geographic distribution. The two clades revealed by parsimony analysis have each approximately half of the number of species in the genus. One lineage is composed mostly of central and southern Mexican species whereas the other lineage contains those species endemic to Mesoamerica and South America. The molecular phylogeny is compared to previous phylogenetic

hypotheses based on morphological characters. Key features in the structure of the capitulum of *Montanoa*, such as pale morphology, heavily used in the past to construct hypotheses of relationship within the genus, are viewed as of minimal value to circumscribe natural groups. The relationships of *Montanoa* to other genera in the Heliantheae are briefly discussed.

Introduction

The genus represents one of the most conspicuous genera of Asteraceae of mountainous Mesoamerica and northern South America. Some species are ruderal and abundant in recently disturbed areas in tropical deciduous forests whereas others such as *M. revealii* are large buttressed trees up to 20 m in the cloud forests of western Mexico. The shrubby to arborescent habit of most species of *Montanoa* along with its dichasial capitulescences combine to create a spectacular display of white flowers along roads and areas with secondary vegetation. *Montanoa* is a member of the mostly Neotropical tribe Heliantheae which is characterized by heads with receptacular bracts or pales and trinerved

leaves. Most of the 25 species of *Montanoa* are found in Mexico and Central America with five species endemic to northern South America. The genus is characterized by its sterile white ligules, white to yellow, rarely black disc corollas, a chromosome number of $x = 19$, and pales that continue to grow and expand after anthesis (acrescent pales) turning the flowering head into a spiny fruiting ball.

The phylogenetic position of *Montanoa* within Heliantheae has always been enigmatic and controversial. Funk (Funk, 1982) revised the genus and clarified the taxonomy by recognizing 25 species from 125 available names but was unable to identify the sister taxon of *Montanoa* nor clarify the relationships of *Montanoa* within Heliantheae. Robinson(Robinson, 1981) believed the genus to have an isolated position in the Heliantheae and placed it in its own monotypic subtribe Montanoinae in his tribal classification scheme. Both authors relied on morphological characters traditionally used in Asteraceae classification to identify potential sister taxa. According to Robinson(Robinson, 1981) the sterile ray flowers of *Montanoa* tend to group the genus with members of subtribe Helianthinae and Ecliptinae whereas its cypselae

characteristics point to a close relationship to Melampodium and allies. Funk (Funk, 1982) hypothesized that given that *Montanoa* has an unusual chromosome number in the Heliantheae of x = 19, genera sharing this chromosome number could be close relatives of the genus. She compared salient morphological features of *Montanoa* to genera such as Jaumea, Venegasia, Villanova, Synedrella, Actinospermum, Amblyolepis, Gaillaria, Calea, and Podachaenium concluding that these genera did not form a monophyletic group nor any of them was the sister taxon to *Montanoa*. *Rojasianthe* superba was believed to be distantly related to *Montanoa* in spite of shared features such as acrescent pales, an x = 19 chromosome number, white ligules, and opposite leaves. Given that no outgroup could be identified, Funk (1982) polarized characters by using functional groups within *Montanoa* based on pale morphology. This exercise produced three groups or lineages, which were in turn used as reciprocal outgroups to each other.

Molecular studies based on approximately 20,500 bp of the chloroplast genome for 124 genera of Heliantheae have been recently completed (Panero et al. unpublished). Results from these studies indicate that *Montanoa* is the basalmost lineage of tribe Ecliptinae and sister to *Rojasianthe*.

Based on these results we have chosen *Rojasianthe* and *Idiopappus* (subtribe Ecliptinae) as outgroups.

We initiated this molecular study of the genus *Montanoa* with the primary interest of testing the phylogeny based on morphological characters advanced by Funk (1982) in her revision of the genus. Species relationships and other infrageneric groupings previously recognized are based on cladistic analyses of morphological features with a special emphasis on character state series describing various pale characteristics. We wanted to ascertain if pale morphology is an important predictor of relationships in the genus. Secondly we were interested in understanding the origin and evolution of *Montanoa*. To test these hypotheses we built a phylogeny based on the Internal Transcribed Spacer region (ITS) and the External Transcribed Spacer region (ETS). Several studies have shown the ETS to be useful in increasing the phylogenetic signal and support for monophyletic groups in phylogenies based on the ITS region (Baldwin and Markos, 1998; Clevinger and Panero, 2000; Markos and Baldwin, 2001).

MATERIALS AND METHODS

Plant Samples

Thirty-one samples corresponding to 22 species of *Montanoa*, *Rojasianthe superba*, and *Idiopappus quitensis* were collected in the field. Herbarium specimens were used for DNA extraction of *M. atriplicifolia*, *M. pteropoda*, and *M. echinacea*. Table 8 lists the samples used, and GenBank accession numbers.

	GENBANK		
TAXON	ITS 1	ITS 2	ETS
Idiopappus quitensis	AY038116	AY038149	AY038083
Montanoa angulata	AY038117	AY038150	AY038084
Montanoa atriplicifolia	AY038118	AY038150	AY038085
Montanoa atriplicifolia	AY038119	AY038152	AY038086
Montanoa bipinnatifida	AY038120	AY038153	AY038087
Montanoa echinacea	AY038121	AY038154	AY038088
Montanoa fragrans	AY038122	AY038155	AY038089
Montanoa frutescens	AY038123	AY038156	AY038090
Montanoa frutescens	AY038124	AY038157	AY038091
Montanoa grandiflora	AY038125	AY038158	AY038092
Montanoa guatemalensis	AY038126	AY038159	AY038093
Montanoa hexagona	AY038127	AY038160	AY038094
Montanoa hibiscifolia	AY038128	AY038161	AY038095
Montanoa imbricata	AY038129	AY038162	AY038096
Montanoa karwinski	AY038130	AY038163	AY038097
Montanoa karwinski	AY038131	AY038164	AY038098
Montanoa laskowski	AY038132	AY038165	AY038099
Montanoa leucantha subsp arborescens	AY038133	AY038166	AY038100
Montanoa leucantha subsp leucantha	AY038134	AY038167	AY038101
Montanoa liebmannii	AY038135	AY038168	AY038102
Montanoa liebmannii	AY038136	AY038169	AY038103
Montanoa mollissima	AY038137	AY038170	AY038104
Montanoa ovalifolia	AY038138	AY038171	AY038105
Montanoa pteropoda	AY038139	AY038172	AY038106
Montanoa pteropoda	AY038140	AY0381173	AY038107
Montanoa quadrangularis	AY038141	None	AY038108
Montanoa revealii	AY038142	AY038174	AY038109
Montanoa revealii	AY038143	AY038175	AY038110
Montanoa speciosa	AY038144	AY038176	AY038111
Montanoa standleyi	AY038145	AY038177	AY038112
Montanoa tomentosa	AY038146	AY038178	AY038113
Montanoa tomentosa subsp microcephala	AY038147	AY038179	AY038114
Montanoa tomentosa subsp. tomentosa	AY038148	AY038180	AY038115
Rojasianthe superba	AF171947	AF171986	AF172025

TABLE 8. List of taxa used in phylogenetic studies. All specimens collected by J. Panero and deposited at TEX except when noted.

DNA isolation

Total genomic DNA was isolated from fresh leaf tissue collected in the field, stored in liquid nitrogen or silica. The method outlined by (Saghai et al., 1984) and modified by Doyle & Doyle (1987) was used to isolate DNA from fresh and silica dried material. Herbarium samples were extracted using either the method of (Paabo, 1993) and gel purified or the rainforest method (Kirsten and Playford, 1996).

Amplification of the ITS and ETS regions

The ITS and ETS regions were amplified using Polymerase chain reaction (PCR) in either 50 or 100 µl reactions. ITS 5 and ITS 4 primers of White et al. (1990) were used to amplify a region of approximately 700 bp. A primer was developed upstream of the 5′ end of the ITS region to improve amplification efficiency of *M. echinacea* and *M. bipinnatifida* (Primer 7.5: 5′ GAGTCATCAGCTCGCGTTGACTA 3′).

ETS primers 18S-E and ETS-Hel-1 developed by Baldwin & Markos ((Baldwin and Markos, 1998) were used to amplify a region of approximately 400 bp. Amplification of the ITS and ETS region was performed under the following conditions: one cycle of 4 min denaturation at 95°C, primer annealing at 48°C for 45 sec, primer extension at 72°C for 1 minute followed by 32 cycles with similar conditions to initial cycle except for 1 minute denaturation and an additional 2 seconds for every successive extension. This was followed by a final extension of 10 min at 72 °C. PCR products were cleaned and concentrated with Ultrafree-MC filters (Millipore Corporation) prior to sequencing. Dye terminator sequencing was done at the Michigan State University DNA sequencing facility and the University of Texas sequencing facility following manufacturers instructions and protocols. Internal primers 2 and 3 of the ITS (White et al., 1990) and 18S-E for the ETS (Baldwin and Markos, 1998; Markos and Baldwin, 2001) were used in sequencing reactions.

Sequence alignment and phylogenetic analysis

Sequences were assembled into contig files and aligned manually using Sequencher (Gencodes). Parsimony analyses were performed using PAUP 4.0b6b (Swofford, 2001). Heuristic searches were performed with 100 random entries for the combined ITS-ETS data matrix using ACCTRAN, MULPARS and TBR options. Support for monophyletic groups was assessed using 100 bootstrap replicates (Felsenstein, 1985). Tree statistics such as the consistency index (Kluge and Farris, 1969) and the retention index (Farris, 1989) were calculated by PAUP.

RESULTS

Maximum Parsimony analysis of the ITS data matrix produced 136 equally parsimonious trees, tree length 262, CI excluding uninformative characters of 0.85 and a RI of 0.89. Similar analyses of the ETS data matrix produced seven trees of 146 steps, CI excluding uninformative

characters 0.85 and a RI of 0.92. The combined data matrix produced twelve trees of 410 steps, CI excluding uninformative characters of 0.85 and a RI of 0.90. The strict consensus tree from the combined data matrix is shown in Figure 16. Sequence length is comparable to that of other Asteraceae. The combined ITS1 and ITS2 region of *Montanoa* varied in length from 480 to 483 bp. There was little variation in length in the ETS region among the species of *Montanoa* but the outgroup genera have smaller ETS regions. The ETS of *Montanoa* ranged in length from 413 to 415 bp whereas the ETS of *Rojasianthe* is 384 bp and that of *Idiopappus* is 339 bp long.

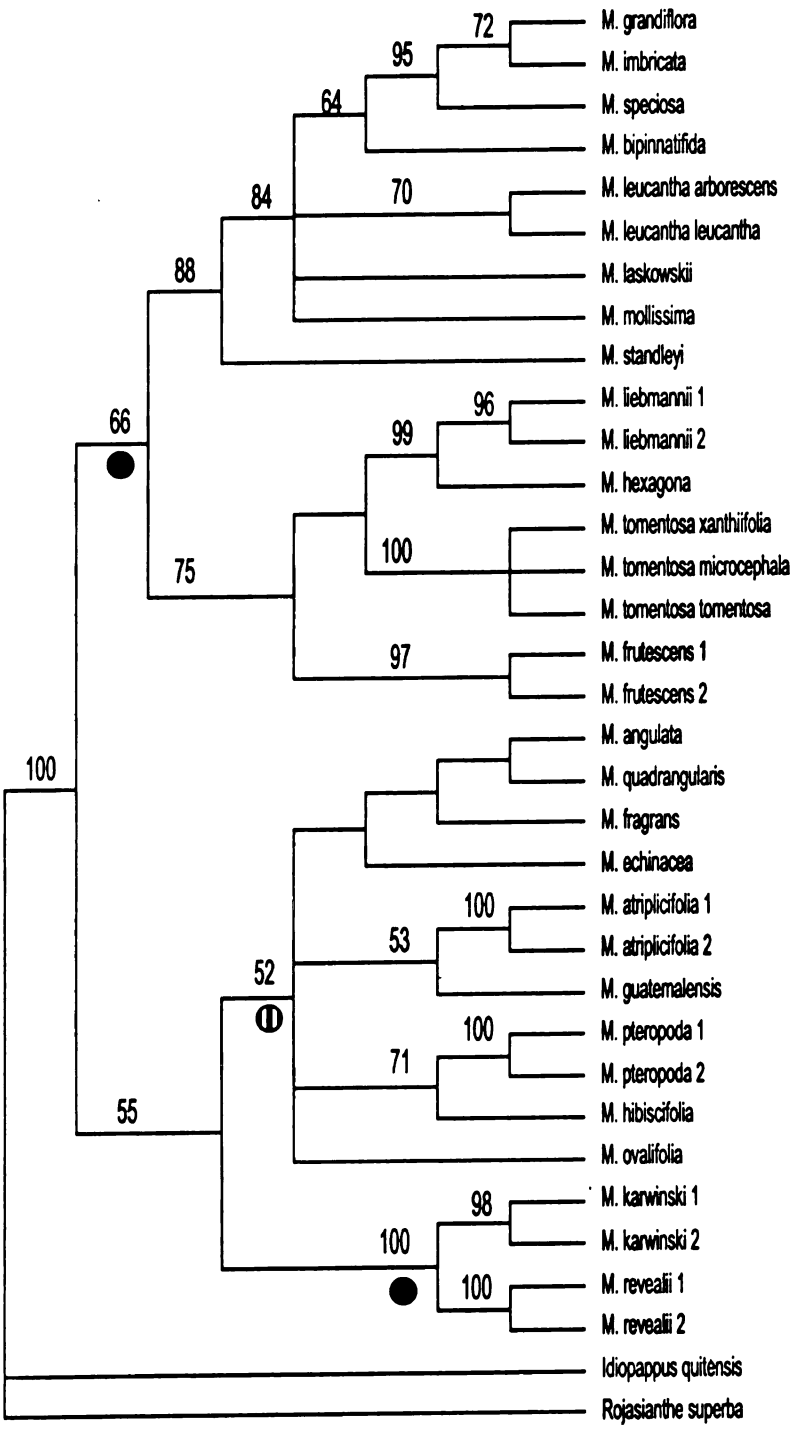

Figure 16. Phylogeny of ITS and ETS of Genus *Montanoa*

● Mexican species

◐ MesoAmerican and Northern South American species

The monophyly of the genus *Montanoa* is well supported with a bootstrap value of 100. The *Montanoa* species are distributed in essentially equal proportions between two main clades. The first clade contains only Mexican species and has moderate support with a bootstrap value of 66. This clade contains two clades that have moderate to strong support. The first clade contains most species from central Mexico that have large heads and showy white ligules and has a bootstrap support of 88. The cloud forest species *M. standleyi* is the basalmost lineage. There is no resolution between four lineages represented by the two samples of *M. leucantha, M. mollissima, M. laskowski,* and the clade composed of the species *M. bipinnatifida, M. speciosa, M. imbricata* and *M. grandiflora*. The other clade contains three lineages in a trichotomy and has a bootstrap support of 75. The two samples of *M. frutescens* are in the same clade with a bootstrap support of 97, the three samples of *M. tomentosa*, cluster together in a trichotomy with bootstrap support of 100, and the third lineage contains *M. hexagona* as sister to the two samples of *M. liebmannii*. The bootstrap support for this clade is 99.

The second main clade includes Mexican, Central and South American species. Relationships among the different species of this clade are not supported in the bootstrap analyses. The Mexican species *M. karwinskii* and *M. revealii* are sister taxa and this relationships is supported by a bootstrap value of 100. This clade is sister to a clade that has weak support with a bootstrap value of 52. This clade contains four lineages. The western Andean species *Montanoa ovalifolia* represents a single lineage. The second lineage is represented by two samples of *M. atriplicifolia* that are sister to *M. guatemalensis*; this clade has weak support with a bootstrap value of 53. The Mesoamerican species *M. pteropoda* and *M. hibiscifolia* are sister with a bootstrap value of 71. The Mesoamerican species *M. echinacea* is sister to the Venezuelan species *M. angulata, M. fragrans* and *M. quadrangularis*, however, the relationships shown in this clade have no bootstrap support.

DISCUSSION

The genus *Montanoa* has always been regarded as one of the most distinctive genera in the Heliantheae and its monophyly has never been questioned. The molecular data presented supports this view. *Montanoa* and *Rojasianthe* are the only genera in the Heliantheae that have pales that grow after anthesis. Pales have been used to circumscribe species and infrageneric groups in *Montanoa* (Funk, 1982). *Montanoa* has two types of pales characterized by the shape of their apical halves. Some species have tapered or pointed pales, whereas others have cuneate or truncate pales. Truncate pales have small pointed or tapered tips and only differ from tapered pales by differential growth and vascularization of the pale body. The distinctive morphology of the pales was used by Funk (Funk, 1982) to place species in two subgenera. Subgenus *Montanoa* has all the species with tapered pales whereas subgenus *Acanthocarpae* has all the species with truncate pales. Results from our study show that pale morphology is not a good indicator of relationships as each pale type has arisen multiple times in the evolution of the genus. We believe this structure is extremely useful in the identification of species and in the construction of phenetic treatments, that is to say those based on morphological features, phenotypic treatments.

Our studies reveal that there was an early split in the evolution of the genus with two lineages containing each approximately half the species in the genus. One clade contains species from central Mexico (Mexican clade) whereas the other clade contains Mesoamerican or southern Mexican species and the South American taxa (Mesoamerican/South American clade). The species relationships revealed by our molecular study do not agree with relationships suggested by cladistic analysis of morphological features in Funk (1982).

The Mexican clade is characterized by two main lineages. All the species in the first clade, with the exception of *M. hexagona*, have tapered pales. The four taxa in this clade are represented by multiple samples of species from different areas of Mexico. The three samples of *M. tomentosa* are sister to *M. hexagona* and *M. liebmanii*. *Montanoa hexagona* was placed by Funk (Funk, 1982) in series *Hibiscifoliae* of subgenus *Acanthocarpae* because it shares with *M. hibiscifolia* a distinctive cypselae wall ornamentation. In our study *M. hexagona* is sister to the diploid species *M. liebmannii*, a weak shrub or perennial herb from central Oaxaca. *Montanoa hexagona* is an

octaploid species and therefore may include multiple genome combinations.

Montanoa tomentosa was placed by Funk (Funk, 1982) in its own series within subgenus *Montanoa* as it is the only species in the genus with densely pubescent pales. In addition, the head of *M. tomentosa* contains only one fertile, central flower whose cypselae is shed as a unit along with all the extended pales of the senescing head. *Montanoa tomentosa* is a variable and abundant species of central Mexico. Funk (1982) placed multiple taxa in the synonymy of *M. tomentosa* that appear to be variations of leaf forms from specific regions of Mexico. She did not provide a hypothesis of relationship for this taxon except for the fact that it represented a distinctive member of subgenus *Montanoa*. We sampled three of the four varieties of the species and they cluster together with high bootstrap support.

Montanoa frutescens from central Mexico is characterized by its greenish disk corollas and reflexed pales. *Montanoa frutescens*, like *M. liebmannii* and *M. tomentosa*, has tapered pales. Funk (Funk, 1982) allies this taxon to *M. guatemalensis* and *M. mollissima*.

The second lineage of the Mexican clade is composed of species that have large heads with ligules ranging from creamy white to bright white and orange disc corollas. *Montanoa standleyi* is the basalmost lineage of this clade. This species, like *M. andersonii* inhabits cloud forests and is endemic to the states of Chiapas and Oaxaca. It can be easily distinguished by its distinctive palmate leaves and recurved pales. *Montanoa standleyi* shares several features with *M. andersonii* including similar pale and head morphologies. *Montanoa mollissima*, *M. laskowski*, and *M. leucantha* each represent single lineages in a polychotmy that also includes the clade of large headed species of central Mexico including *M. bipinnatifida*, *M. grandiflora*, *M. imbricata*, and *M. speciosa*. *Montanoa mollissima*, except for its tapered pales, has a similar habit and overall morphology to *M. leucantha*. This species is a small shrub of xeric areas immediately north and south of the neovolcanic range of central Mexico. *Montanoa mollissima* is further distinguished by its ovate ligules that like those of *M. leucantha*, can vary from creamy to bright white. *Montanoa laskowski* is unusual in *Montanoa* in that it grows in the scrub forest of the Pacific coast of the states of Colima and Jalisco. Like *M. mollissima* it has

bright white oval ligules. *Montanoa leucantha* is one of the most abundant species of *Montanoa*, variety *arborescens* being a common treelet of disturbed, montane areas of central and southern Mexico. The nominal variety differs from variety arborescens by its bright white ligules and smaller shrubby habit. The two varieties of *M. leucantha* were sampled and both taxa are in a sister relationship with a moderate bootstrap support of 70.

The large headed species of *Montanoa* are clustered together in a clade that has a moderate bootstrap support of 64. *Montanoa bipinnatifida* is the basalmost lineage, with *M. speciosa, M. imbricata,* and *M. grandiflora* grouped in a terminal clade with a strong bootstrap support of 95. *Montanoa imbricata* and *M. grandiflora* are sister and characterized by having large white ligules and capitulescences. They grow in ruderal areas, especially along roads and abandoned agricultural fields.

The second clade contains species from western Mexico, Mesoamerica and South America and is characterized by two main lineages with species north and south of the Isthmus of Tehuantepec. The first clade contains two distinctive species from the Pacific coast of central and southern

Mexico north of the Isthmus. *Montanoa revealii* is one of the most spectacular species in the genus. It is a large tree of the cloud forest of the states of Guerrero and Oaxaca. This is a very distinctive taxon that can hardly be confused with any other species in the genus. *Montanoa revealii* is a hexaploid species with tapered pales. Funk (1982) placed this taxon in series *Apertae* Funk as the only member of this group. *Montanoa karwinskii* is another distinctive species in the genus that can be easily recognized by its leaf morphology in which the blade starts at the point of divergence of the three main veins of the leaf. This condition is sometimes observed in *M. revealii*. *Montanoa karwinskii* has truncate pales and therefore, it is a member of subgenus *Acanthocarpae*. It has a large geographic range along the coast and intermountain valleys of western Mexico. The strong relationship shown in our analyses between *M. karwinskii* and *M. revealii* is puzzling given the morphology of key taxonomic features that will support relationships to other species in the genus. It is possible that *M. revealii* represents an allopolyploid of complex origin in which one or two additional species of *Montanoa* may have contributed to its genetic makeup.

The other clade contains species that grow exclusively south of the Isthmus of Tehuantepec. Most of these species are endemic to the montane regions of Central and South America. Four lineages, most of these with weak bootstrap support were revealed by our analysis. The first clade has the Mesoamerican species *M. echinacea* as the basalmost lineage of a clade containing three of the four South American species sampled. None of the relationships shown in the strict consensus tree have bootstrap support. *Montanoa ovalifolia* subsp. *australis* a native of southern Ecuador and northern Peru did not cluster with the other South American species in our analysis. In some of the twelve equally parsimonious trees this species is basal to the clade containing *M. guatemalensis* and *M. atriplicifolia*.

Montanoa guatemalensis is a dodecaploid tree from Central America. This taxon is sister to the diploid species *M. atriplicifolia*, a variable taxon from Mesoamerica. The relationship between these two species is weak as the clade has a low bootstrap support of 53. Funk (Funk, 1982) placed these two species in different subgenera and consequently her discussion of their potential relationships is compromised by pale morphology

considerations. The two species are parapatric in some areas of their respective geographic ranges. Funk (1982) considered *M. atriplicifolia* to be sister to *M. pteropoda*. She based this assumption in the fact that both species share a scandent habit, rather small disk flowers, and a distinctive pale morphology. In our studies *M. pteropoda* is sister to *M. hibiscifolia*. All these species, with the exception of *M. guatemalensis*, were considered by Funk (1982) to be closely related.

The results obtained in this study show that the geographical distribution of species should be regarded as an important consideration in the circumscription of infrageneric taxa above the species level. We believe *Montanoa* to be of Mesoamerican origin and that most of the species we observe today are the result of subsequent radiations into the southern and northern areas of the montane Neotropical region. The Isthmus of Tehuantepec appears to have been an effective barrier to the movement of certain groups of taxa of the Mexican flora and apparently impacted the course of evolution in the genus *Montanoa*. The role of the Isthmus of Tehuantepec as an effective barrier or filter for certain groups of taxa has

been much discussed by several students of the Mexican flora (Miranda, 1952; Rzedowski, 1983).

Finally, the significant incongruences in the phylogenetic relationships depicted by the molecular and morphological studies may be the result of emphasizing pale morphological features in the classification of the genus. Pales are structures that apparently play a role in the protection of developing cypselae (Stuessy and Spooner, 1988) and therefore, may have a limited use in reconstructing phylogenies in *Montanoa* and possibly other composites.

ACKNOWLEDGMENTS

I wish to thank Jose Panero for the opportunity to study such an amazing group of woody plants. Through Jose's love of the Composites of the New World, I gained an appreciation, wonder and respect for this flora through his enthusiastic descriptions. His unbounded excitement about these incredible plants is contagious. It is due to Jose's confidence in me and support that I was able to undertake this project. I also want to thank Sue Stoltzfus for her

help and encouragement with our sequencing effort and Anna Weise for sequences as well. I also wish to thank the support and assistance of Dr. Alan Prather in preparation of this chapter. His continued encouragement and support with this and in revisions of the oak project has been generous and without personal benefit. I owe a great deal of my knowledge of systematics to Alan Prather and his patient teaching. The study and aepj salary was supported by NSF grants DEB 94-96174 and 99-093800 to Jose Luis Panero.

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CONCLUSION

DO WOODY PLANTS AND MOLECULAR TOOLS HAVE A FUTURE TOGETHER?

Problems associated with woody plants and traditional (non molecular) experiments

In the introduction I stated that there were difficulties involved with experimentation on woody plants because of their very nature. Traditional studies are made more difficult due to the slow growth habit, their size and space requirements, dormancy, the difficulty of propagation and length of time for flowering. In addition, the woody stems are a significant barrier to some experiments and the presence of many secondary metabolites are also problematical.

I have worked on three woody plant genera, oaks, *Montanoas*, and poplars and to a great degree I have had difficulties due to some of the above-mentioned problems. The oaks were dormant during five months of the year, and to receive the leaf samples from the UK sometimes took waiting an additional six months for some leaf samples. Although none of the other problems were an issue for the oaks, the high phenolic content was a problem for shipping of the samples.

The *Montanoas* proved a difficult woody subject for another reason; not having to do with their woodiness. I was determined to include all twenty-five species of the genus in the study. One species, *Montanoa joseii* is only found in a remote mountainous area in northern Colombia. I have wanted to go and get a speciment of this plant, however the presence of armed conflict due to the drug wars has made this a challenge. It has only been collected twice, by Jose Cuatrocasas (for whom it is named) and by Vicki Funk. DNA extraction from a herbarium specimen resulted in truncated segments.

The poplars did present a number of traditional difficulties. It took two years to develop a successful method of propagation. At one time we had to wait until

the dormant season in Washington State to obtain specimens. At that time, rooting cutting from green tissue was thought to be undesirable. Tissue culture, mist propagation, different media and different greenhouses were all tried. At the conclusion of these studies, a success rate of 90% of cuttings rooted. Indeed the poplars did occasionally go dormant in the greenhouse at the onset of short days. However under the Agro-Gro lights it was possible to stimulate them to break dormancy. The idea of slow growth is almost a contradiction in terms referring to poplars. While growing, they can elongate up to a foot per week. However from the time of cuttings till the time of stress initiation, it was six months. Space was equally a problem. Only a limited number of trees could be stress tested during one set of experiments. The maximum was twenty trees in one gallon pots.

Length of time for flowering in woody plants was not an issue for these studies, since no flowers needed to be collected, no crosses needed to be done. All material was vegetatively propagated, or collected from stems or leaves.

Problems associated with woody plants and molecular experimentation

Were these molecular studies made more difficult by the fact that I used woody plants? The problems associated with woody plants and molecular tools often refer to the size of their genomes, the difficulty of transformability and problems associated with introduction of transgenes into a long-lived species. Extraction of DNA and RNA for some woody plants is often mentioned as an additional problem.

Since my studies did not involve transformation and introduction of a transgene, many of the issues raised about experimentation on woody plants were not involved. Only the issue of extraction proved problematical.

With the oaks and the *Montanoas,* the molecular studies used DNA extracted from leaf tissue. This was no further problem of extraction than many other plant tissue types. In the years of working with Jose Panero on DNA studies from many exotic and unusual plant specimens, the oak and *Montanoa* DNA extractions were simple by comparison. Occasionally the oak DNA was brownish in color due to the

presence of phenolic residues, but did not present difficulty for further enzymatic manipulation.

The poplar RNA proved to be a real problem. I routinely isolated poplar RNA from leaf samples with ease, but the stem sections proved a formidable barrier. It is with some satisfaction that I have received requests for my modified protocol for RNA extraction from poplar stem tissue.

The methods that are being used around the world in poplar studies on stem sections either are not available for use here, or because of the selection of poplar clones could not be applied.

Despite difficulties, I have been able to reach satisfactory answers to two of my questions, and a partial answer for the third. I have been able to identify cloned cultivars of the "Lucombe" oak, and shown conclusively that the eleven samples received from the UK which are called "Lucombe" oak are not identical cultivars.

I have answered the question of the relatedness of the genus *Montanoa* to other new world composites. And I have been able to show that its evolution has developed

geographically as it has radiated from a MesoAmerican center both north from Central America to Mexico and radiated south from Central America to northern South America.

I have been able to hypothesize that wind stress on poplar trees does induce gene expression; and the genes that appear induced indicates a relation to other stress induced genes. I have been able to identify a set of cDNAs that are expressed in the lignifying tissue of poplar stem tissue.

Answer: Woody plants and molecular tools do have a future together

Despite the difficulties involved working with woody plants, the problems can be surmounted. Each challenge that is unique to using molecular tools on trees can be met with patience, effort, and resources. There must be a commitment to each of these. Working with woody plants is not easy, however, as more questions are answered, it becomes easier.

The need for the preservation, conservation, yet continued harvesting of this very precious resource has never been greater.

So go out, and hug a tree! But only pick up your pippettor and think about using your molecular tool kit if you have real dedication!

APPENDICES

APPENDIX A

COMPLETE WIMP RESULTS:

PRIMERS USED IN DIFFERENTIAL DISPLAY,

SEQENCES, DATABASE SEQUENCE SIMILARITIES,

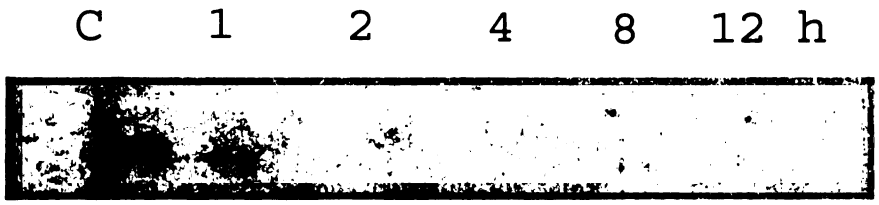
NORTHERN DATA

WIMP 1 - PUTATIVE IDENTITY - UNKNOWN

PRIMERS

5′ -AAGCT$_{11}$G-3′ and 5′-AAGCTTTGGTCAG-3′

>wimp1
TTTTTTGCGTTGGAGAGAAAATGAGTTTCCTTAAATNAAAATTAACTTGGCATTACAAT
ACATATATTAAATAATNGCAATCANNAATAANAAAAGAAAANNGGATGGCCTTATTTTT
GNCTCAGCAAATTGCTTTTGAGGCCTTGTTTTATTCTTCTCTTCTGCTGGAATATACAT
TCCAGCANGGATTCNCCATGGGCATGGCATTNGNAGTNNAGTNTCNTGGCNATCCAGCT
T



Band 1

WIMP 2 - SEQUENCED TWICE - POOR SEQUENCE

PRIMERS

5′ -AAGCT$_{11}$C-3′ and 5′-AAGCTTTGGTCAG-3′

WIMP 3 - PUTATIVE IDENTITY - UNKNOWN

PRIMERS

5′ -AAGCT$_{11}$C-3′ and 5′-AAGCTTTGGTCAG-3′

>Wimp3
CAAAAGACAAAAATATCTCACAGCCATTAAGTTTATATGGTATCGACATA
CAAGCTGAACTGAAATACGTTGGACAGCATGTTTTACATATAACTCGATA
ACATATTTACAAGGATAAGGAAACTACTATAGTACATCCTTTGTATTTGA
TCCTTTCGGCAATCAAGCTTAATCAAGCTT

WIMP 4 - PUTATIVE IDENTITY - UNKNOWN

PRIMERS

5′ -AAGCT$_{11}$C-3′ and 5′ -AAGCTTTGGTCAG- 3′

>Wimp 4
AAGCTTTGGTCAGGAAACCCCATATCAAGTCGTGGCATCTATGAAAGCTC
TTGGGGCATCGTACATGTGCTTTAACATTCTTCATAATAAGTGCGGTCCG
ATTATACTGCCTGGTTCGCTTCACAACAAAATTAGGCGATGTAGNTTTGT
ATATTTTCATAGCTTTGAGTAGTTGTCAAAATTGATCCAATTTGACTCCA
AATCATTTGAGGCTGTCCTGTTACATTTGTTGCTATATCAATCAATTGGT
ACTCAGAGTCGGTACTCGTTCTCGAAAAAAAAAAAG

WIMP 5 - PUTATIVE IDENTITY CYTOCHROME f

PRIMERS

5′ -AAGCT$_{11}$C-3′ and 5′ -AAGCTTTGGTCAG- 3′

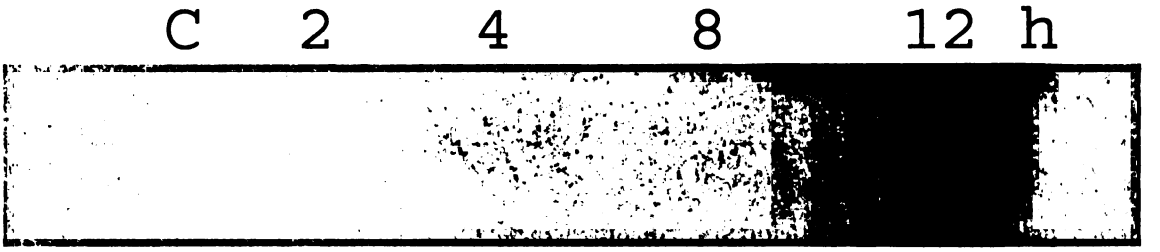
>Wimp 5
AAGCTTTGGTCAGGGAGACGCANAAATAGTACTTCAAGACCCATTACGTG
TCCAAGGCCTTTTGTTCTTCTTGGCATCCGTTATTTNGGCACAAATCTTT
TTGGTTCTTAAAAAGAAACAGTTTGAGAAGGTTCAATTGTCCGAAATGAA
TTTCTAGATTGCGAATTTATCAACATCAAGTTCTTATCTTTTCTTAATTT
CTTAAAAAGAACAAAATTTTTGTTGGAAATTCTGTATGAAAAAAAAAAA

BLASTx homology

>gi|6723761|emb|CAB67170.1| (AJ271079) cytochrome f [Oenothera elata subsp. hookeri]

Score = 94.0 bits (230), Expect = 3e-19

Query: > 5 FGQGDAXIVLQDPLRVQGLLFFLASVIXAQIFLVLKKKQFEKVQLSEMNF 154
FGQGDA +VLQDPLRVQGLLFFLASVI AQIFLVLKKKQFEKVQLSEMNF
Sbjct: 269 FGQGDAEVVLQDPLRVQGLLFFLASVILAQIFLVLKKKQFEKVQLSEMNF 318



Band 5

WIMP 6 - PUTATIVE IDENTITY KINESIN LIKE PROTEIN

PRIMERS

5′ -AAGCT$_{11}$C-3′ and 5′ -AAGCTTTGGTCAG- 3′

>Wimp6
AAGCTTTGGTCAGCAAGGTGGTTAAGGGATTTCTCAGCATTGCTTGTTTC
TCAGGGCACACAGCTTGGACTGCTCCTGAAGAAAATTTTGAAGGGCGATA
TTGGTTCCCTATCAAAGACTGAATTCATAGAAGCCATTTCCCAATATCTT
AGACAAAGAACTANTTTGGCTTCAAGTGATTTCTCCAAGTTTTGTGTCTG
TGGTGGGAAAAAAAAAAA

BLASTx homology

>gi|2392771|gb|AAB70034.1|AAB70034 (AC002534) putative kinesin-like protein [Arabidopsis thaliana]

Score = 61.3 bits (146), Expect = 1e-09

```
Query:  > 64  LGLLLKKILKGDIGSLSKTEFIEAISQYLRQRTXLASSDFSKFCVCGGK 210
              LG  L+KILK D G LS++EF+ A+ +YL+ R  L S +FSKFC CGGK
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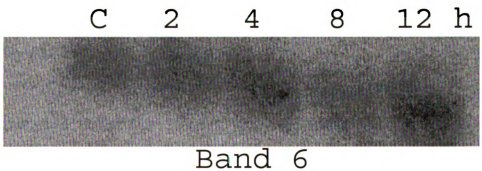


Band 6

WIMP 7 PUTATIVE IDENTITY - UNKNOWN

PRIMERS

5′ -AAGCT$_{11}$C- 3′ and 5′ -AAGCTTCGACTGT- 3′

>Wimp7
TTTTTTTTTTTCAAGAAAGAACAAATTAGACATCCATAAAGCAAGATTCA
GGTTTCCATAAATAAATTACTCTCTTCTTCTACTACTACTACGACTACTA
CAATATTATCTATGGATGACAGTAAGAAAACAAAGCCCTTACATAGGAAA
TATCTTTTGCAGATTCTACCTAATTGAGAATTACAACTGACACATTGAAA
GTCCTGAATGAGGGATGAAAACAAAACGTAGCTGCCACCTCCTGTTGCTT
ATTCTGAACTCATCAAGCACTCTGACCAAAGCTATCAAGCTT

WIMP 8 PUTATIVE IDENTITY - UNKNOWN

PRIMERS

5′ -AAGCT$_{11}$C- 3′ and 5′ -AAGCTTTGGTCAG- 3′

>Wimp8
AAGCTTTGGTCAGCAGTCCATGTGTGCGTGTTTGACATTACAATTGAAAT
CGATGTTGATTGAAAAAGCATCCTCTTGATGCATTTCACATTTCCAATCT
CAGTCCCAAATGGGTTTAGAGCCTGTTTGATTTTTAATAGTATTTTTGAA
AAAAAAAAA

WIMP 9 - PUTATIVE IDENTITY - UNKNOWN

PRIMERS

5′ -AAGCT$_{11}$A- 3′ and 5′ -AAGCTTCGACTGT- 3′

>Wimp9
CTTTTTTTTTTTAANANCATNANACTCTTTGATTTGGTCATTAATTTTAT
TTTTTCTCGTCTTTATNTATTAATCTCTACAACCTTCTCTCATAATTTGT
GCTACNTACCTTTATATCCATGGACAATACACATACCTTCAAGCCACTAN
AGCTATGCTCTCTCCCAATGAAATGTATAACGGTGTATGGAGAAGCCTTA
CTACANTCGAAGCTT

WIMP 10 - PUTATIVE IDENTITY - UNKNOWN

PRIMERS

5′ -AAGCT$_{11}$A- 3′ and 5′ -AAGCTTCGACTGT- 3′

>Wimp10
TTTTTTTTTTTATAAGAACTACGCAATCACATCATTGTAATTATATATTA
ATGCGGGAATTACATTAGTTGTCTATGATAGCATGACATTACACAAACAT
GATCGCTCTATTTTATATGACTAAATTTCTGTCAAGTGTCTGTAATCTCT
GCATTGAACAAGATATGCATAAAATATACAGTCGAAGCTTAATCAAGCTT

WIMP 11 - PUTATIVE IDENTITY - UNKNOWN

PRIMERS

5′ -AAGCT$_{11}$A- 3′ and 5′ -AAGCTTCGACTGT- 3′

>Wimp11
TTTTTTTTTTTATAGCAAAATCATCATTTTCATCCATTCAGCTCACATAC
GATCATCCCATATAATCACTGTTCTCTTTTACGTCTACATGCATAGAATT
ATGTGCATAATTTTAAACATATAACAGTCGAAGCTT

WIMP 12 - SEQUENCED TWICE - POOR SEQUENCE

PRIMERS

5′ -AAGCT$_{11}$G- 3′ and 5′ -AAGCTTCTCAACG- 3′

WIMP 13 - PUTATIVE IDENTITY - UNKNOWN

PRIMERS

5′ -AAGCT$_{11}$G- 3′ and 5′ -AAGCTTCTCAACG

>Wimp13
AAGCTTCTCAACGAGGACAGGTATGTTTTGCTATTTGTGAACCTGTATGC
CCAGACTTTTTGATAAGAACTTCCCTCCCATAACTACTGAGTTTGAATAT
CTAAGCAGCGATGAAGAAGACACAGCTTCTGCATCCCATACCCAAAAAAA
AAAA

WIMP 14 - PUTATIVE IDENTITY - UNKNOWN

PRIMERS

5′ -AAGCT$_{11}$G- 3′ and 5′ -AAGCTTAGTAGGC- 3′

>Wimp14
AAGCTTAGTAGGCAGATTATGAGCACTGCAAGTTTGGCTTATGTAACTCT
AGCGTATTGTTGTCTCGGCATAGGCATTGGTATTGCCGCTTTTATGATTT
CTGCCATTGGATGCTTCATGTTAGGATTTTGATTGTCTAATGTGTAGCCT
TTCATAGAGCTAAATCATATGGAAGACCGCATTACCATTTTTTAATAAAG
ACATTATCGTTTGAATTAATGCAAGTTTTGATGGTTATTTTGGCTTCAAA
AAAAAAAA

WIMP 15 - PUTATIVE IDENTITY - UNKNOWN

PRIMERS

5′ -AAGCT$_{11}$G- 3′ and 5′ -AAGCTTAGTAGGC- 3′

>Wimp15
TTTTTTTTTTTGGCCCAGCAAAGCTACTTATATTTGTTCCACAGATATAT
TCACAGACAAAATAATGACAGGGAACATTTATTAGTTAAAACATTATCAG
AAGTGGTCATAAACCACTCAAATAACCACCAATAGCCTACTAAGCTT

WIMP 16 - PUTATIVE IDENTITY - UNKNOWN

PRIMERS

5′ -AAGCT_{11}C- 3′ and 5′ -AAGCTTCTCAACG- 3′

>Wimp16
TTTTTTTTTTTCAACTGCTTGCAAGAAAAGATCATTGAACTATTAATTAT
TAAAAGAGAAGCCTTTAAAGCACTGTGCATGCATGATACCCTCAAGACTG
CATGTTTACTTTTCCAGGCAAGAACTGAAGGTGAGTTGGGGTTTTGACTA
CAAGCTGAAATATCAAAGTAAAGCGCTAATTCTAGGCTAACATGATTCAT
GGCGAAGAAAATCCAGTGCTT

WIMP 17 - PUTATIVE IDENTITY - UNKNOWN

PRIMERS

5′ -AAGCT_{11}C- 3′ and 5′ -AAGCTTCTCAACG- 3′

>Wimp17
ANAATAGTCTCCATGGCTATAAATCATTTAACACGTTGAGAAGCTTAATC
NAGCTTATCNATACCGTCGACCTCTAGGGGGGGCCCNGTNNCNAATTCTC
CCTANAATGANTCCTANTANCCCCCCTCNCTGGNCTTCCTCTTNCTANAT
CGTGACTGGNAANACNCTGCCTTTACCCANCTTNATCCNACCTCCTAGGG
GNGGCCCTNTCNCCAC

WIMP 18 - PUTATIVE IDENTITY - HYPOTHETICAL PROTEIN

PRIMERS

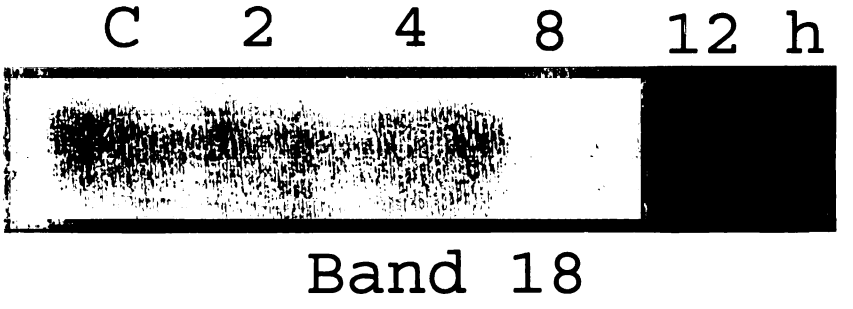
5′ -AAGCT$_{11}$C- 3′ and 5′ -AAGCTTGCACCAT- 3′

>Wimp18
TTTTTTTTTTTCGAGAAAGACAAACCATGAAACAACCATTTTGCTTAAGG
TACAATGTACTGATTTGTTCGTGATTGATTTTGGATTATATGCCATCTGT
GTTTCCAACGATAAGAGCACACTGAACGATCCCAGCAAGGATAAACATTC
ACACTATACTAGGCGGATCCCCCACCCAATTGCAAACTGCACGAGACCAG
CGAGTTCTAATACAGTCCACTAATGGTGCAAGCTT

BLASTx homology

>gi|7523680|gb|AAF63119.1|AC009526_4 (AC009526) Hypothetical protein
[Arabidopsis thaliana]
Score = 56.6 bits (134), Expect = 5e-08

Query: < 229 APLVDCIRTRWSRAVCNWVGDPPSIV 152
APLVDCIRTRWSRA C+W GDPPSIV
Sbjct: 369 APLVDCIRTRWSRAACSWSGDPPSIV 394



Band 18

WIMP 19 - PUTATIVE IDENTITY - UNKNOWN

PRIMERS

5′ -AAGCT_{11}C- 3′ and 5′ -AAGCTTGCACCAT- 3′

>Wimp19
AANCTTGCACCATGATCATAATAAAACTGCCTAAATAGGTGTTCGCCTCT
ATTGATGGGTCTCCACTANGGCGAGTCCCCCATTCAGTCTTGTGTCGTTG
TCTTTTTTTTGTTTTTTTAATGAGTANCATGTGTCGTTATCTATGTATCC
TTTGGCGGTATGAAATATGTAATGCAAATAAAATTCTACTATTTTGTTGA
AAAAAAAAAAG

WIMP 20 - PUTATIVE IDENTITY - UNKNOWN

PRIMERS

5′ -AAGCT_{11}C- 3′ and 5′ -AAGCTTGCACCAT

>Wimp20
AAGCTTGCACCATGTACTGTAGTTGAAGAGTCTAAAGTAGCTTTAGCAGT
GATAATGATGATGGTTATGTATGCCATCGATTGTGATGCCCTTCTCGGAC
CCTCCTTTGGAATCATTACCACTTTTGGTCTTCTGTTTTTTTGACATGCA
ATCGATTGCCACTCCCTTTTCCTTTCCGAAAAAAAAAAA

WIMP 21 - PUTATIVE IDENTITY UNKNOWN

PRIMERS

5′ -AAGCT_{11}A- 3′and 5′ -AAGCTTCTCAACG- 3′

>Wimp21
ATAAGAAGGGTCACCTCAATAATGCTCGACAATCAAGTCCGATCTACAAA
TCAGGGCAAAAAGTCTAACAAAAACATCACTGCAAGTACACTCGTATCCG
GTCAGATTTTCTCTCCATAAGTTGTCGTTGAGAAGCTTAATCAAGCTTAT
CGATACCGTCGACCTCGAGGGGGGGCCCGGTACCCAATTCGCCCTATAGT
GAGTCGTATTACGCGCGCTCACTGGCCGTCGTTTTACAACGTCGTGACTG
GGAAAACCCTGGCGTTACCCA

WIMP 22 - PUTATIVE IDENTITY - Ribosomal protein 18S

PRIMERS

5′ -AAGCT$_{11}$G- 3′ and 5′ -AAGCTTAACGAGG- 3′

>Wimp22
TTTTTTTTTTTNATTAATNAAAACNTCCTTGGCAANTGCTTTCNCANTTN
TNCNTCTTTCATAANTCCAAAAATTTCACCTCTGACTATNAANTACAAAT
GCCCCCNACTGTCCCTGTTAATCATTACNCCNATCCCNAAGGCCAACACA
ATAGGATCNAANTCCTATNATTTTNTCCCATGCTAATNTNTCCAAAGCNT
AGGCTTGCTTTNANCACTCTANTTTCTTCAAATTAACAGC

WIMP 23 - PUTATIVE IDENTITY - HYPOTHETICAL PROTEIN

PRIMERS

5′ -AAGCT$_{11}$G- 3′ and 5′ -AAGCTTAACGAGG- 3′

>Wimp23
TTTTTTTTTTTGGTTACTAAAATNTTTCANTTCGCCAGGTTGTCTCTTGC
CTGCCCATGGATTCGGCAGCANTTTGAAAGGTTAACCTATTCGGGAATCT
CCGGATCTACNCTTATTTTCAACTCCCCNAAGCATTTCGTCGCTTACTAC
GCCCTTCCTCGTCTCTGGGTGCCTAGGTATCCACCGTAAGCCTTTCCTCG
TTAAGCT

BLASTx homology

>gi|6723805|emb|CAB67216.1| (AJ271079) hypothetical protein [Oenothera elata subsp. hookeri]
 Score = 46.9 bits (109), Expect = 3e-05

Query: > 5   FFWLLKXFXSPGCLLPAHGFGSXLKG 82
              F WLL+ F SPGCLLPAHGF S KG
Sbjct: 29   FLWLLRCFSSPGCLLPAHGFSSSSKG 54

WIMP 24 - PUTATIVE IDENTITY - ENOLASE PHOSPHATASE

PRIMERS

5′ -AAGCT$_{11}$G- 3′and 5′ -AAGCTTTTACCGC- 3′

>Wimp24
TTTTTTTTTTTGCAAAGGAAACAGCCGCATTTTCTTTTAAAAGCGACATT
TTTATTTATGTGTGCGTTGTGAAAAGCATTTCATACATTTGCAATAACAC
AAGTACATATCTGCTTTCAGATTTCCAAGCCAAACTTAGCGTGATAAAAA
TTGCATACATATCTGCCATCAGATTTCAGCGAAGGAGGTGATTGTCTTGA
AACCGTGATTCTCTGGAAGAGGTGCATTTCCTGGCCGGATAGAAATCATC
ACATCCAAACCTGCTCCTTTTGCGGTAAAAGCTT

BLASTx homology - Wimp 24

>gi|10177247|dbj|BAB10715.1| (AB007644) contains similarity to enolase-phosphatase-gene_id:K19P17.1 [Arabidopsis thaliana]

Score = 64.0 bits (153), Expect = 4e-10

```
Query:  < 282 AFTAKGAGLDVMISIRPGNAPLPENHGFKTITSFAEI 172
              A  AK AGL+ +ISIRPGNAPLPENHGFKT+TSF++I
Sbjct:    471 AVAAKAAGLEAIISIRPGNAPLPENHGFKTVTSFSQI 507
```

WIMP 25 NO SIGNAL ON SEQUENCE

PRIMERS

5′ -AAGCT$_{11}$G- 3′and 5′ -AAGCTTTTACCGC- 3′

WIMP 26 SEQUENCED TWICE - POOR SEQUENCE

PRIMERS

5′ -AAGCT$_{11}$G- 3′and 5- -AAGCTTCATTCCG- 3′

WIMP 27 - PUTATIVE IDENTITY - UNKNOWN

PRIMERS

5′ -AAGCT_{11}G- 3′and 5- -AAGCTTCATTCCG- 3′

>Wimp27
AAGCTTCATTCCGTACTTACCAAAGAGATGGCACTTCTTCAGGGCCATTT
GTTGAACTGAGCCTTTTTTCACCGAGTCCAGATGCCACTCCGCCAGAGTC
ACCTCCGCCTCTAGGCTGGCCTATGCGCCCGCTTGTGGACCCGAAAACTG
GGCAAGGAACTGATTTCTCGCAGTGAATATCAATTTTGTGAAAAAGAAAA
ACTGATTGTAAAGGGTTAGTCTCCATAAGGATTTTTATTACTGGTTTGTT
TTCCAAAAAAAAAAA

WIMP 28 - PUTATIVE IDENTITY - UNKNOWN

PRIMERS

5′ -AAGCT_{11}G- 3′and 5- -AAGCTTCATTCCG- 3′

>Wimp28
TAAGCTTCATTCCGCTACTGGGTTAAGTACTTACGTTTCTGGGAGCAGTC
TCTGGTGTGGCCTTTCTNAGGCCTNGGTCTTTGTNCATTTTCTCTTTATC
ACCNAAAGAGTTATCATGTGAAACGTTTGATCTTTAGTCAAAATATTCTG
NCATGCTTCTTACAAAAAAAAAAA

WIMP 29 - NEVER REAMPLIFIED NOR CLONED

WIMP 30 PUTATIVE IDENTITY - UNKNOWN PROTEIN

PRIMERS

5′ -AAGCT$_{11}$G- 3′and 5′ -AAGCTTCCACGTA- 3′

>Wimp30
TAAGCTTCCACGTATAATATGAATGGGATCAGACGGAAGTTCTTGGTTCA
GTGGGTTTGGAACCATTTCGTTAGTTACTGTACTGGCCCTAATGCCCTTG
ATCAGTAGTGGTATTTTCCTATTTTGACCTTGTTTAATTTCTGTAATTTG
GGGTTTTATCGCTCTTTTTTTTGTTGGGTTTTGGATTTTGGGGTTATTAA
TGCATTAAGGTAGTTAAGGTTGTTAAATCTGTAATAATCTCTATTATACT
ATTTTTGTACACCACTTTGCTGTCTTCTCCCAAAAAAAAAAA

BLASTx homology

>gi|9758197|dbj|BAB08671.1| (AB018109)
gb|AAD25141.1~gene_id:K17N15.10~similar to unknown protein
[Arabidopsis thaliana] Score = 65.2 bits (156), Expect = 2e-10

Query: > 7 STYNMNGIRRKFLVQWVWNHFVSYCTGPNALD 102
            +TYN+NGIRR++L+QW+W+H VSYCTGPNALD
Sbjct: 306 ATYNVNGIRRRYLIQWLWSHVVSYCTGPNALD 337

WIMP 31 - PUTATIVE IDENTITY - UNKNOWN

PRIMERS

5′ -AAGCT$_{11}$G- 3′and 5′ -AAGCTTCCACGTA- 3′

>Wimp31
TTTTTTGGAAATATCAAGGGTGGTCCTTCATTTTCTTTTATGANTAAGAT
TNTGCTGGAAAAAACTCAAACCAATTGTCTAACCATAGTCATCTTCTTCN
TACTTTCAAATAATTTAAGACTANAATAATTTCAATATCTAATACGTANA
TAATCNTACTGGAAACTANAATCTTATTTACGTTAATTATAGTACTCAAA
GCCCATACACTGGGTAATTCANTCCANTAATCAATCTCAATGACTACCAT
ACACCTCACAATGCCTTNCGTGTGAAGCTTT

WIMP 32 - PUTATIVE IDENTITY - UNKNOWN

PRIMERS

5′ -AAGCT_{11}G- 3′and 5′ -AAGCTTCCACGTA- 3′

>Wimp32
TCCACGTATGTTATTGAAGAATATAAAATCTACTGCATTCTAGTTTGTAT
TGCGGGTAAATGAACTTCACTAGGATATCNACAAAAGTTCCTCTTTACTC
TCATCTNGTTAAAAAGTACGAATATTGTTGATGAACGGGGAAAGCTNGCT
AGTANTTAAAATCTGTTCTGTTCTGGGTTTGTTTNCATTACGTNGTANNN
NCCACTAATTATTGTTGCTTGCATGTTTCAGTTATTTTTTCCAAAAAAAA
A

WIMP 33 - PUTATIVE IDENTITY - UNKNOWN

PRIMERS

5′ -AAGCT_{11}G- 3′and 5′ -AAGCTTCGGGTAA- 3′

>Wimp33
AAGCTTCGGGTAATGATGTAATCTTATGTAGACAATTCAAGCTGCTGACA
TTTTTCAAGCAGCCTCCTACAACTGGAAAGTTTTAGATATAAATAGCCAT
TCTATCAAGAGGAAGCTTGTGGTTAAATCAATTTTCAGTGCTGCTACTGT
CTTGAAATCAAATGATATATCACGTTGAGTCTGCAGATG

WIMP 34 - PUTATIVE IDENTITY - UNKNOWN

PRIMERS

5′ -AAGCT_{11}G- 3′and 5′ -AAGCTTCGGGTAA- 3′

>Wimp34
TNCCTCCCTCAGTTCTAGAACGATCNATNACCTTCGGGTAAATGAACTTC
ACTAGGATATCAAGAAAAGTTCCTCTTTTCTCTCATCTAGTTNAAAAGTT
CGAATATTGTNGATGAAGGGGGAAAGCTTGCTACTAGTTAAAATCTGTTC
TGTTCTGGGTTTGTTTTCATTTTGTTGTAATATCCANTAATTATTGTTGC
TTGCAAAAAAAAAAA

WIMP 35 - PUTATIVE IDENTITY - UNKNOWN

PRIMER COMBINATION

5′ -AAGCT$_{11}$G- 3′and 5′ -AAGCTTCGGGTAA- 3′

>Wimp35
AAGCTTCGGGTAATGCAGTGAAGATTTGAAAGAGGATTAGTTTAATCTTC
TTACCTCTTTGCTTTTGGCTGCTAATGCTTTACACCTGTTTCATGCCTTC
TCTTGTTACTTAAAATCTTGCTCTTGTTTCCTTTATGACTTTGATTTGGT
CATTTACTCAAAAAAAAAAA

WIMP 36 - PUTATIVE IDENTITY AMINO PEPTIDASE

PRIMER COMBINATION

5′ -AAGCT$_{11}$C- 3′ and 5- -AAGCTTCATTCCG- 3′

>Wimp36
CTTTTTTTTTTTCCAGAATGGAGATTATCATTTGGAATCACGCCATTTAA
CAATCCTATCACCACCTTCTGGTAAATTTACGTTGGAAATTGTTACTGAG
ATATATCCCCAGAAGAACACATCATTGGAGGGACTTTACAAGTCATCTGG
GAATTTCTGCACTCAATGTGAAGCAGAGGGTTTCCGCAAAATTACATATT
ATCAGGATCGCCCTGATATAATGGCAAAATACACTGTCCGGAATGAAGCT
TAATCAAGCTTATGGAAAAAAAAAGGCNGGCGGTTTTTTCCCCTTCCNCC
CCC

WIMP 37 - PUTATIVE IDENTITY - UNKNOWN

PRIMER COMBINATION

5′ -AAGCT$_{11}$C- 3′ and 5- -AAGCTTCATTCCG- 3′

>Wimp37
CTTTTTTTTTTTCCATTACAAAAACGTTTTGCCTCGTTGAACATATCTAG
CCACTTGTTACAGGAAACAACATTTTGCCAATATTTCACCAAATTTGGGG
CACCAAAGACCACATCATCTTGAACACAAGAGTACCAGAGTACCAAATAA
ATGACATATAGTGTTTCCTTTTCCCCGGAATGAAGCTTAATCAAGCTT

WIMP 38 - PUTATIVE IDENTITY - UNKNOWN

PRIMER COMBINATION

5′ -AAGCT$_{11}$C- 3′ and 5- -AAGCTTCATTCCG- 3′

>Wimp38
TTTTTTTTTTTCCATCACATTCAAACATCCATTAAAGTTGGGATTTGGCA
AACACAAGTGACAAAGATAATCCCATCTCCTATCAAAATTCTAAACAAAA
TTAAGGGGAAAAAACCCAACACACCACACATTTTACGGAATGAAGCTTAA
TCAAGCTT

WIMP 39 - NEVER EXISTED

PRIMER COMBINATION

NONE

WIMP 40 - NEVER REAMPLIFIED

PRIMER COMBINATION

5′ -AAGCT$_{11}$C- 3′ and 5- -AAGCTTCATTCCG- 3′

WIMP 41 - PUTATIVE IDENTITY - UNKNOWN

PRIMER COMBINATION

5′ -AAGCT$_{11}$C- 3′ and 5′ -AAGCTTCCACGTA- 3′

>Wimp41
TTTTTTTTTTTCGATACCCAAGAGGCAAATAAAAATGCCTTCATAAACTA
TAGAACACTATCACACACATACATATATATATAAATATTTAAGGAAAAAA

GGTCCAAAAGCTACATCACTCAAAATTCTTTATGATACATAAACAGTAAA
ACCAAGGCTCAACAAGAGGTAACCCAGATCGTTTTCAAGAAGCTTGGCCA
AGAAAAAGGACTCCAATGGCCAGTAGCCGCAGCAGCTACTTAATTAGTAC
GTGGAAGCTTAATCAAGCTT

WIMP 42 - PUTATIVE IDENTITY - UNKNOWN

PRIMER COMBINATION

5′ -AAGCT_{11}C- 3′ and 5′ -AAGCTTCGGGTAA- 3′

>Wimp42
CCACACAGTAACAATTAAATATATACCATTCAACTAAAGCATAGAGTATT
TGCTAACTTTAGACAGAACGCAAACCTAGAACTTGGGCTGTGATCATGTC
ACTACTGTCAGAAGAGGTCTAATTTTTCATTGTTTCTCATCATTGTTGGC
GTCCTCATCAAAAGCACCCAGCAGCTCCTGCACATCTCTCTCCTTTATAC
TTGATTTCTTCACATTGCATCTCTCTCCTTCGCATTTATGAACAAGCCAC
AACATCTCTCTTACCCGAAGCTT

WIMP 43 - PUTATIVE IDENTITY - UNKNOWN

PRIMER COMBINATION

5′ -AAGCT_{11}C- 3′ and 5′ -AAGCTTCGGGTAA- 3′

>Wimp43
TTTTTTTTTTTCGACCAAACCAATTCGAATTTTCCAACTTCTGTAGCCCA
AATCCCTATAATTCTGTTCTCGTTTTTACAGTGATCCGCTATGCCAAAAC
TATTTTATAGAAAAAACTCAACAAGAAAACCCAGTCAATGGCAAACCTTC
CAATGTCATCAGCACTGAGACCCCTGTTTACCACCTTTGTCCAGTTTCAG
AGACTTAGGATCAAGTGGGGATAGTACCCACTGTTAGATTACCCGAAGCT
TAATCAAAGCTT

WIMP 44 - PUTATIVE IDENTITY - UNKNOWN

PRIMER COMBINATION

5′ -AAGCT$_{11}$C- 3′ and 5′ -AAGCTTCGGGTAA- 3′

>Wimp44
CTGCAGGATTCGATAGCTTCGGGTAAAATGAAATATGTCGCCTTTGCTTC
ATGCTATGTGACAATTGCTACCATAAAGTTGCTGCATCTTTGTGATAAAC
AGCTGGAATTCTCTTCTGTTTCTCCTTTTCTTCTTGTGTAATTGTGAACA
AAATTTGGTTGAAAAAAAAAAAG

WIMP 45 - PUTATIVE IDENTITY - UNKNOWN

PRIMER COMBINATION

5′ -AAGCT$_{11}$A- 3′ and 5- -AAGCTTCATTCCG- 3′

>Wimp45
AAATTGAGCAGCTGGCATGCATGGCAGGAAGGAGGTTCCTTTTCTAATGA
CACAGATCACCATATATTCATTTTTGTTTTTTGATTCGTGATTTTTTACC
CAATGATGAAAGTTGTAGTTCCTGTGACGTAGATTTGTTTTGCAACAAAC
GAATTGAAGATTCAATTCTAAAGCCATTATTTTATAAAAAAAAAAA

WIMP 46 - PUTATIVE IDENTITY - UNKNOWN

PRIMER COMBINATION

5′ -AAGCT$_{11}$A- 3′ and 5- -AAGCTTCATTCCG- 3′

>Wimp46
AAGCTTCATTCCGGGGGAAGCTATGTTAAAGTGTACATATTATTTGTGCA
GTTCATTTGTAGTTTATTATTATTATTAGGGTGTACCTTGCTGTATTTGA
TAAAAAGAATTCCAATGCTCTGCATTCAATAATTCAGGTTGCATGTGTAA
AAAAAAAAA

WIMP 47 - PUTATIVE IDENTITY - UNKNOWN

PRIMER COMBINATION

5′ -AAGCT$_{11}$A- 3′ and 5′ -AAGCTTCCACGTA- 3′

>Wimp47
TTTTTTTTTTTAGGGATTGATAATGTTGATGTCAAATTTACTATTCGAAT
GTGACATTGTTTGAAGACATTGGGAGATAAAACGACACATGGACATGCCT
ATCAACTTTCATGCATTCGTTGAAATTTAGATCTGCGTGGTATACATGAC
TTCCGTTGGAGCATCGTGACCATGTACGTGGAAGCTTAATCAAGCTT

WIMP 47A - PUTATIVE IDENTITY - UNKNOWN

PRIMER COMBINATION

5′ -AAGCT$_{11}$A- 3′ and 5′ -AAGCTTCGGGTAA- 3′

>Wimp47A
CTTTTTTTTTTTAAGAAAAATTGGATATTCATTACAGGTTACAATTACAT
TATTCTCAAAGCATAAACATGGGATGACAATACTACTGCAACAAAGTAAA
AAATTGTAGAACCTGGTACCACTTTTGGATGTTACCCGAAGCTTAATCAA
GCTT

WIMP 48 - PUTATIVE IDENTITY - UNKNOWN

PRIMER COMBINATION

5′ -AAGCT$_{11}$G- 3′ and 5′ -AAGCTTCGGCATA- 3′

>Wimp48
TTTTTTTTTTTGGAAATTATGAGTGCAGGCCTACAACTTGGAATGCTGTA
ACATCAAAAACAAAACAATGTGAAGAAAAATCAAAACAACATCCTCCCCC
CTATAAAATTCTCTAGGCGAACGTTCTTATGTTGGTGCCATTTGAACACC
CCTCCCCCCTTTCTCTCTCTCAGGGATAAAGGTGAAACTTCTATTGGCCT
ACTTTTTAACGTCCAAGACCTTGCCCAAGTTCCTGTCATCTGAAGGGAGC
AGGGAGCANGATTGGACGCAACAATAGTTAAATCAAACTAGATTACACTT
AGCTTGCAGACACCTTCTTCCGAACAACCCTCTTTCTACGAACCTTTGGT
GCTGGTGATTCCCCATTAATGGAATCAGTATTGCCTTGTTTTATGCCGAA
AGCTT

WIMP 49 - PUTATIVE IDENTITY - RIBOSOMAL PROTEIN

PRIMER COMBINATION

5′ -AAGCT$_{11}$G- 3′ and 5′ -AAGCTTCGGCATA- 3′

>Wimp49
TTTTTTTTTTTGGAAAACGGATACTGAAATTAGAAACAAAGTCCGTCCAT
CATTCTCAGCTCATACGATATTTAAACTTAGCTTATAAAAAGTAAAACGA
CAATCCCTACAAGACATCGAATGGACCCAAGCTGCTACCTGTAAAGAGCC
TGAGGAACTCAAATGTTGCTGGGATACATGAAAACTCTTACACGACATCC
CATGGACTTTGGAGGCAAGTTGGACTTGAACTTAGCTCTGACAACACCAC
TGTTTCCATGAGGCCTTGTGACCTTGCCCCAAATGCAGCGATAGTGGGTC
CCATCCCTCTTCACCTTGGCCTTGTATATGTATGCCGAAGCTTATCAAGC
TT

BLASTx homology

>gi|8954039|gb|AAF82213.1|AC067971_21 (AC067971) Strong similarity to a ribosomal protein from Arabidopsis thaliana gb|AL161667.
 Score = 116 bits (288), Expect = 9e-26

```
Query:  < 335   AYIYKAKVKRDGTHYRCIWGKVTRPHGNSGVVRAKFKSNLPPKSMGCRVRVFMYPSNI 162
               AYIYKAK K++G+HYRCIWGKVTRPHGNSGVVRAKF SNLPPKSMG RVRVFMYPSNI
Sbjct:    55   AYIYKAKTKKNGSHYRCIWGKVTRPHGNSGVVRAKFTSNLPPKSMGSRVRVFMYPSNI 112
```

WIMP 50 - PUTATIVE IDENTITY - UNKNOWN

PRIMER COMBINATION

5′ -AAGCT$_{11}$G- 3′ and 5′ -AAGCTTCGGCATA- 3′

>Wimp50
CTTTTTTTTTTTGGCGAGACAAGCTACCTTTAGCTAGGAGCCTCTTTTTC
CTTCTGCCCGAAAACAACGTTCGACTTGCATGTGTTAAGCATATAGCTAG
CCTTCCTTCTGAGCCAGGATCAAACTCGTCTTTTGAGCATGATCACGCCC
TGCAGTGGTAGAACCTAGTGAACCAGACGTACGACTTCTGAACCCGAAGC
TCTTCTCTTATGCCGAAGCTTATCAAGCTTA

WIMP 51 - PUTATIVE IDENTITY - UNKNOWN

PRIMERS

5′ -AAGCT$_{11}$G- 3′ and 5′ -AAGCTTCGGCATA- 3′

>Wimp51
AAGCTTCGGCATAGAAGAAAGTTGGAATGAACAGAGTAATACAGTGAGTT
AGAATTGACTAGGCTGAGCTACTCTTTTGAAGTGAAATTGTGACCTCAAC
AGTGTGTTGTATATCGCGAGCAAGATCATGTATATATGAAAATCTCTTTC
TTTCGATGCTGCAAAAAAAAAAA

WIMP 52 - PUTATIVE IDENTITY - UNKNOWN

PRIMER COMBINATION

5′ -AAGCT$_{11}$C- 3′ and 5′ -AAGCTTGAGTGCT- 3′

>Wimp52
GCTTGAGTGCTGTGCATTGGTTATGTGGTGAGTTGATTGACTGCTACGTA
GTTTATCGATGCAAATATGGTATGAGCCATTTTTATTGTTATTTGTAGAG
TGTGAGATTATTAGTCGTAGTGGTGTTAATTCCCTAGAGTCGTTTCTGCA
ATCATGTCCAATGAGGTTTATAATAAGGAAATTTCAATTACTTGATGTAG
TTGAGAAGCCATCTCAGACAAGTTGATATATTTTGTGTTGGTCAAGTTTA
GGATTCATGTACAGAAACCGTATTATGTTGCAGGTAATGAATTCATATTT
CCTTGAAAAAAAAAAA

WIMP 53 - PUTATIVE IDENTITY - UNKNOWN

PRIMER COMBINATION

5′ -AAGCT$_{11}$C- 3′ and 5′ -AAGCTTGAGTGCT- 3′

>Wimp53
CTTTTTTTTTTTCGAGGTAATATGTTGATTTAATTTAGCCATTTAAAAAA
GGATAGAAAATANTTAATTGCATCCATTTTACCTGCAAGAAATAATTATT
AGCATGTTTTTCAAATTCCAATGCCAAACTAATCATCTAACTCAATCATC
ATGACCAACTCCATCCTCTTCCTCTTTCTCTTCCCACCCTTTATTTCGGG
TCCAGTTTCTACAAGTGCTGGTTGGAACGGAGCAACCAGTGCTTGAACCC
TCAGCTACTTGAGATACCTCTAATTGAGCACTCAAGCTTAATCAAGCTT

WIMP 54 - PUTATIVE IDENTITY - UNKNOWN

PRIMER COMBINATION

5′ -AAGCT$_{11}$C- 3′ and 5′ -AAGCTTGAGTGCT- 3′

>Wimp54
TTTTTTTTTTTCCAGAGAATAGATTGGTCATGCAACTTCAGTTGGGAAAT
CAAATATTTGTATGTAAATATTTAGAGTTTCTTTTACTAAAATAAAAAAC
CCCCCCTTCTTGGGAATGAAACAATATTTTGAAGTCTATCCTTTCCATTT
ATGTAGTTCCTCAGCACTCAAGCTTAATCAAGCTT

WIMP 55 - PUTATIVE IDENTITY - UNKNOWN

PRIMER COMBINATION

5′ -AAGCT$_{11}$C- 3′ and 5′ -AAGCTTCGGCATA- 3′

>Wimp55
TTTTTTTTTTTCCCAAAACGAAAACCGTGATCATTATCTGAAATATATCA
AATCAGTTGCACCCAACATGCATATTCCCAGCAAGTCGATGCTATATACC
AGAATGACTAGTTTAGCAAACTTGGAAGATTTGTTTCCTCGAGTGGCTAG
TTAGTACAGTTACTTCATCCTAAAACAAGTACTAAATTCTTGCCAGTGTA
TGCCGAAGCTTAATCAAGCTT

WIMP 56/57 - PUTATIVE IDENTITY - UNKNOWN

PRIMER COMBINATION

5′ -AAGCT$_{11}$C- 3′ and 5′ -AAGCTTCGGCATA- 3′

>Wimp56/57
CTTTTTTTTTTTCATACAACTCCACGAAAAGCCAAGTTCAAAACCATATA
TAGATATCCATACTGATAGCTATCACCCCAAGATATGCACAGAAGGTGCA
GGTTACAACGAATTCTAAATGGTTCACTGAAGGGTCTGATGGCCACCAGT
TCTTCCCTTGATTAAGATATGCCGAAGCTTAATCAAGCTTATCCCTCNNG
GCTTTGGGNTTTCGGAATTNCGNACCTTTCC

WIMP 58 - PUTATIVE IDENTITY - UNKNOWN

PRIMER COMBINATION

5′ -AAGCT$_{11}$C- 3′ and 5′ -AAGCTTCGGCATA- 3′

>Wimp58
AAGCTTCGGCATACAGATGATGAACTGTAAGATCCAAAACGGCGTCATTT
GGCATGGAAAATGACAAATTGTAGTTTGGTCCCTGATGTTTTGAAATGTA
TCAGTTGGGTCCCTTAATCAATAAATTTTAATTTTGCCCCTTGAAAAAAA
AAAAG

WIMP 58A - PUTATIVE IDENTITY - UNKNOWN

PRIMER COMBINATION

5′ -AAGCT$_{11}$A- 3′ and 5′ -AAGCTTCGGCATA- 3′

>Wimp58A
AAGCTTCGGCATAGTATATGTAGTTTCTTCTCCAGTGTAGATTTCATGGA
TTTACAATTTGGGGCACTTGTCTATTTTATATCAAAGTAGAGGCATTGGT
CGAAAAAAAAAAA

WIMP 59 - PUTATIVE IDENTITY - β-GLUCOSIDASE

PRIMER COMBINATION

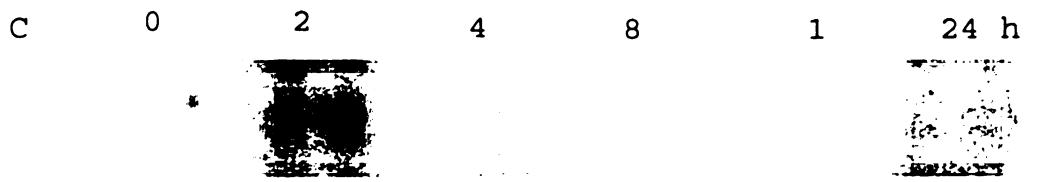
5′ -AAGCT$_{11}$C- 3′ and 5′ -AAGCTTCGGCATA- 3′

>Wimp59
AAGCTTCGGCATAGTATATGTTGATTACACCAACCTAAAGAGGTACCCCA
AAATGTCTGCCTATTGGTTCAAGAAACTACTGGAGCGGAACAAACACTAA
ACATAGCAGCCAGGCTTTCCTTTCGATTTAGTTGTCTGGAAATTAATATT
ATTACAGTAAAGCTATCAAGAACATTGTGTTAAAAAAAAAAA

BLASTx homology

>gi|9294063|dbj|BAB02020.1| (AB020749) beta-glucosidase [Arabidopsis thaliana] Score = 55.4 bits (131), Expect = 8e-08

Query: > 5 FGIVYVDYTNLKRYPKMSAYWFKKLLERN 91
FGIVYVDY LKRYPKMSA WFK+LL+RN
Sbjct: 482 FGIVYVDYKTLKRYPKMSAQWFKQLLKRN 510



Wimp 59 Probe 2/12/00

WIMP 59A - PUTATIVE IDENTITY - UNKNOWN

PRIMER COMBINATION

5′ -AAGCT$_{11}$C- 3′ and 5′ -AAGCTTCGGCATA- 3′

>Wimp59A
AAGCTTCGGCATAATGATCGCAAGTTTAAATAGCTACATTCTTTCAATCC
ATTTTTCCTTGTAAGTTATGTCAATTTTATGCATGTAAATTTCGTTTTTA
TACATGTTTTTCCCCTCCACAATCAATTACCGTTATAATTCCCCTTTGAA
AAAAAAAAA

WIMP 59B - PUTATIVE IDENTITY - UNKNOWN

PRIMER COMBINATION

5′ -AAGCT$_{11}$A- 3′and 5′ -AAGCTTCGGCATA- 3′

>Wimp59B
TTTTTTTTTTTAGCAATTGATGACATGCATTAGAAGTAAATCATTTAGTT
TCCTCAGAATCTAACAGGCGCTACTTGATCAAAACAATACATATTATTCA
TAATTGTACACTATGTATGCCGAAGCTTAATCAAGCTT

WIMP 60 - PUTATIVE IDENTITY - UNKNOWN

PRIMER COMBINATION

5′ -AAGCT$_{11}$A- 3′and 5′ -AAGCTTTAGAGCG- 3′

>Wimp60
AAGCTTTAGAGCGGCAGTACTAGAAATCGAACGTTGGAGATGTAAAACGA
AGCAACATTTTTGGTTTCTGTCCCATTTCGATCATGGATTGGTTGCCATT
CATGGATAACGAACTCTCCCAATAATTCTTTTCATATGTGATACTGTGAG
TTGCCATCAACAAGATGTTTATTTATTTATTTTTACAGAAATGGATGCAT
GGATAGGCTAATAAAAAAAAAAA

WIMP 61 - PUTATIVE IDENTITY - GLYCINE/PROLINE RICH PROTEIN

PRIMER COMBINATION

5′ -AAGCT$_{11}$G- 3′ and 5′ -AAGCTTACCAGGT- 3′

>Wimp61
TTTTTTTTTTGGAAGCAGAAATCAGACATATATATATTATGTTTATGTT
GGACCAGCAATGTAATCATCAAAGTCAGCTAAAAAGGTTAGCTGGAGATG
GTAAAAAGTAAGATTTATTCAAAGAAACCAGAATCTGCTAATGCGTAGCC
AATCAAGACTAAATCCAGCTGACATGCAGGATGATCACTTCCACTTCTTA
AATTTCCCTCCTCCGTGCTTCTTCCCAAACTTCCCACGCTTGAACTTTCC
ACCGCCGTGCTTGCCGCCGTGCTTGAATTTTCCATGACCACCACCATAGA
ATCAAGAAAGAGCTCTCAGTCTGTCAATCCTTACTATGTCTGGACCTGGT
AAGCTTAATCAAGCTT

BLASTx homology

>gi|2129603|pir||S65780 glycine/proline-rich protein GPRP - Arabidopsis thaliana>gi|1465364|emb|CAA59059.1|
Score = 54.3 bits (128), Expect = 6e-07

```
Query:  < 298 YGGGHGKFKHGGKHGGGKFKRGKFGKKHGGGKFKKWK 188
              YG GHGKFKH GKH  GKFK GK G   GGGKFKKWK
Sbjct:    145 YGHGHGKFKH-GKH--GKFKHGKHG-MFGGGKFKKWK 177
```

NORTHERN - NO HYBRIDIZATION

WIMP 62 - PUTATIVE IDENTITY - A. THALIANA SUBTILISIN-LIKE PROTEIN, P. ABIES ANTIFREEZE-LIKE PROTEIN

PRIMER COMBINATION

5′ -AAGCT$_{11}$G- 3′ and 5′ -AAGCTTACCAGGT- 3′

>Wimp62
TTTTTTTTTTTGGAAACATAGAATGAATAGATACATATGAAAGAAATTAC
TATTTAAATTGAATTTGTCTATGTGAAATAATGATCCTCGTGTGGAAGCA
AGTGTTTCTTAGTTTTCATCAAATATAAGGCTGTAAGAATTCTGTCCTGA
CAGACAAGGGACTTCTCACAATATGCACTCCATCAGTCCATGTAAGGCTC
CCAAAAGAGTACTCCGTGTTCACCTGGTAAGCTTAATCAAGCTT

BLASTx homology

>gi|12323571|gb|AAG51764.1|AC066691_4 (AC066691) subtilisin-like protein; 10849-13974 [Arabidopsis thaliana]
Score = 52.3 bits (123), Expect = 1e-06

Query: < 232 AYQVNTEYSFGSLTWTDGVHIVRSPLSVRTEFLQPYI 122
+++VNT++ FGSL WTDGVH V P+SVRT+F++ Y+
Sbjct: 717 SHRVNTDFYFGSLCWTDGVHNVTIPVSVRTKFMRNYV 753

NORTHERN HYBRIDIZATION - NO SIGNAL

WIMP 63 - PUTATIVE IDENTITY - UNKNOWN

PRIMER COMBINATION

5′ -AAGCT$_{11}$G- 3′ and 5′ -AAGCTTACCAGGT- 3′

>Wimp63
AAGCTTACCAGGTGAAAGGTAGAGTGCTAGATACATAGTCAGTGTAAATA
TGTACTGACATGTTTTGTGCTGTGTGAAACTAGCTGTATTTGCAATATAA
TTATTATTGCTTTCTTTTACTTGCAGCCAAAAAAAAAAA

WIMP 64 - PUTATIVE IDENTITY - UNKNOWN

PRIMER COMBINATION

5′ -AAGCT$_{11}$G- 3′ and 5′ -AAGCTTAGAGGCA- 3′

>Wimp64
CTTTTTTTTTTTGGGAGAGTAAGATCACCGTATTATTAGCTTAAAACATA
TGTACAAAAGCATGCAATTGAACTAAAAAAACATCCAGCAAAATAGAAAG
GGCATCACAAACAACATAGTTGTAAGTAATGTTATTAACTACAAAATACA
TTTGTGAATAATAAAGTTCTAGGCATAATTCATTCAGGAATAACTAAACT
TATTGCTACTTCACCAAGTGACATCCGAACAGCTCTAGTTGATCAAATTG
AGATTCAGTTGGTGTTTCGCTTGTTTCGCTTTTCTTGATTCTACATGAAA
TTCACATGTTTGGATAAGCCCCGATGCTAATTTTCTAAGGAATATGACCT
ATCCTTCCTCATCCTCAGATTGAGACGTGTTACTTGTGGTAAGTTATCTG
CCTCTAACTTAATCAAGCTTA

WIMP 65 - PUTATIVE IDENTITY - UNKNOWN

PRIMER COMBINATION

5′ -AAGCT$_{11}$G- 3′ and 5′ -AAGCTTAGAGGCA- 3′

>Wimp65
TTTTTTTTTTTGAAACTCAATGCAGATTAGATTAACCATATACAACGTAG
CGAGAAGACAAACAGCAATAAAATCAACAAAGATGACTCCGATGCCCTCT
CATTTGCCCAAATGAGTGAACCCATGTTCAGATTAAGATTACAATATTAT
AGCCAGCAATGAAGTCGACAAATTTTCTCCATTAGAGTGATGCCAACAGA
GGCTTCACCATAGGAACTTAATTTGTGTGATCTCCAAGAGACGAAAATTC
ATCCACTTCACCACTTGAGGCAATCCATTTATTACATTGCAAATTGTCTA
AAGTTATTATAATGCTCATCGCCCAGCAAGTGCTCTGTAGCAAACCGCTC
CCTTGTGTGCCTCTAAGCTTAATCAAGCTTA

WIMP 66 - PUTATIVE IDENTITY - UNKNOWN

PRIMER COMBINATION

5′ -AAGCT$_{11}$G- 3′ and 5′ -AAGCTTAGAGGCA- 3′

>Wimp66
AAGCTTAGAGGCAGAAAGGGAATTCCAGGCTCTGGTCAGTTTAGAGGGTG
ATTATCTTGGACCGGTGTCTTAAAGTTCTACCTTGCACTCAGCTGGAGTA
TCATATCTTCTGAAGTTTTCTAATCAAGTTAGTTTAACTGCCAGCTTGTT
GGTTGTTTCTGTAGTATTGTATATCCTTGTGCCACTCTACTCCTTTTTGT
GACTGTTGTCTTGGAGTTCTTGCAACTTCAGTGATTGCTAGACTATATGT
TTAGCAACTTGATGTTTCCTTCTGTATGCAGATGTTGACAAATAGTATAA
TTGATTCGAGGACATCTCATCTTTTGTCAAAAAAAAAAAG

WIMP 67 - PUTATIVE IDENTITY - UNKNOWN

PRIMER COMBINATION

5′ -AAGCT$_{11}$C- 3′ and 5′ -AAGCTTACCAGGT- 3′

>Wimp67
TCAACCTTATTGATAGCGGTCAAANTCAANGGGGGGGCNCGGNTTCCAAT
TCCCCNTATTNGNTTTTTTTTCNNNNCTCTCGGGGGGCNTTTANAACNTT
TTANTGGGGAAACCGGNGNTCCAATTTTTAGNCTNGGGGTTCCCNTTTCN
CTNGGTTTTTNAAAAGNCNCATTTNCTTCCAAATTGGCGCCGATGGGAAG
GAATNACCTAATTNTNTNTAANCNNTATTTGNTTGNCTTTTANNAGNGAANG
AACCTTNACAAAAAAAAAA

WIMP 68 - PUTATIVE IDENTITY -DEHYDRATION RESPONSE PROTEIN

PRIMER COMBINATION

5′ -AAGCT$_{11}$C- 3′ and 5′ -AAGCTTACCAGGT- 3′

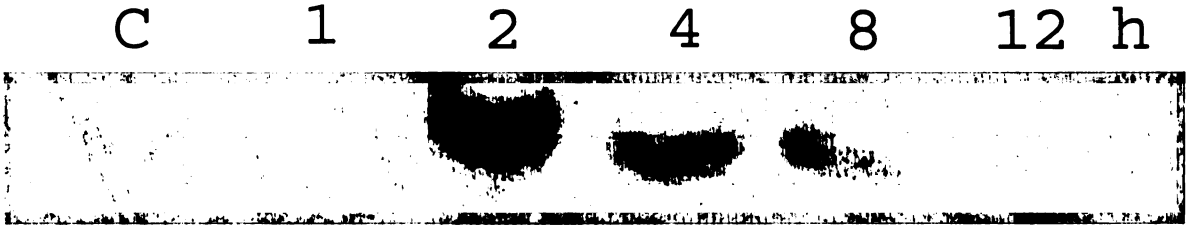
>Wimp68
TTTTTTTTTTTCGATTAACAAAATGCTCAAGTAGGCCCTTTGTGCTGGTT
TACTCTTCTCATGGAGGTCTCTCTTTTCACATTACATGCTCATGTGGATA
GGAAAATAGCAACAGAAGGAGATGTTATCTAATAATAAACACCGTGATTC
ACTGAATCTATAGCAAGCTTTATATTATACATAACCTTTGTAGTGGGTTT
TAGATGTATTATTATGAAAACCGAGTAGATATATAAGCATACAACATTCC
AATTTCAGCTACCGTGGACCGAAGAGAAACGAAATGACTGGGATCCANAC
TCCTAGTTGGGAAGCCACACAACATGATCCTGAAGAANGAAATGGCAGAC
TGGTACGGTTCCTGGCTTAACATTGANCACCTGGTAAGCTT

BLASTx homology

>gi|1172874|sp|Q08298|RD22_ARATH DEHYDRATION-RESPONSIVE PROTEIN RD22
PRECURSOR >gi|479589|pir||S34823 dehydration-induced protein RD22 -
Score = 43.4 bits (100), Expect = 0.001

Query: < 389 AYQVXNVKPGTVPVCHFXLQDHVVW 315
A++V VKPGTVPVCHF + HVVW
Sbjct: 365 AFKVLKVKPGTVPVCHFLPETHVVW 389

NORTHERN HYBRIDIZATION



Band 68

WIMP 69 - PUTATIVE IDENTITY - UNKNOWN

PRIMER COMBINATION

5′ -AAGCT$_{11}$C- 3′ and 5′ -AAGCTTACCAGGT- 3′

>Wimp69
AAGCTTACCAGGTTAAATAAAGGTGTTCTCAACTAGCCTACCTGGTTAAA
TAAAGGTGTTCTCAACTGGCCTACCTGGTTAAATAAAGGTGTTCTCAACT
GGCCTACCTGGTTAAATAAAGGTGTTCTCAACTGGCCTACCTGGTTAAAT
AAAGGTGTTCTCAACTAGCCTACCTGGTTAAATAAAGGTGTTCTCAACTG
GCCTACCTGGTTAAATAAANGTGTTCTCAATTAGCCTACCTGNTTAAATA
AAGGTGTTCTCATCTANCCTACCTGNTTNAAATAAAAGGTGTTCTCAACT
AACCCTACCTGNTTTAAATAAAGGTGTTCTCCAACTAACCTACCT

WIMP 70 - PUTATIVE IDENTITY - UNKNOWN

PRIMER COMBINATION

5′ -AAGCT$_{11}$C- 3′ and 5′ -AAGCTTACCAGGT- 3′

>Wimp70
AAGCTTACCAGGTCATGCCATCATTTATTTGTACAAAGATGCACGCACAA
GTGCGCTTCCTGTGAATTTGACTCCTTCAAAAAGACAGGATAGCAATAAG
GTCAAACATTGTTATTGATATTGAGTTTGAAACTGATCTTGCAGTTTCCT
GTAGAAGCCAACAGTACCCGATGAAATATGCTGTTGTTTCATGTAAAATT
GTTATTATCATAACTAATAAGGCATTTTTCTTCTCCGAAAAAAAAAAAA

WIMP 71 - PUTATIVE IDENTITY - UNKNOWN

PRIMER COMBINATION

5′ -AAGCT$_{11}$C- 3′ and 5′ -AAGCTTACCAGGT- 3′

>Wimp71
TACCAGGTCCAGACATAGTAAGGATTGACAGACTGGGAGCATGTCGGTTG
TGCTTAGATTACTGGGAAACTACATTCCAGCTGTAGTGTGAACCAACACC

AGATATTTAAATAAAATGCCCGTCTTTCTTGTGTTGGCAGAACTTCTTTC
AGGGTTGTTTGGATATAGCATGTCAAGAAAATTTTAGTATTTTATCTTTT
AATAAGACAATATAGTGTGAAAAAAAAAAA

WIMP 72 - PUTATIVE IDENTITY - UNKNOWN

PRIMER COMBINATION

5′ -AAGCT$_{11}$C- 3′ and 5′ -AAGCTTACCAGGT- 3′

>Wimp72
TTTTTTTTTTTCCATCCAAAAGATAGGCATCGAATCATCCCAAGTAGTTC
AAGCTTACAAAAGGATCAAAAAGGCAAATTATAAACTGGCAAAGACAACA
ACCAAGTTTCACAAGACAAATTAAAGGAAGAGTGAACATTCCAACAAAAC
CACCTGGAAAAGCCACCAACACCACCTGGTAAGCTTAATCAAGCTT

WIMP 73 - PUTATIVE IDENTITY - UNKNOWN

PRIMER COMBINATION

5′ -AAGCT$_{11}$C- 3′ and 5′ -AAGCTTACCAGGT- 3′

>Wimp73
AAGCTTACCAGGTGCCAGATCAATGCTTATTATGAAGGGCATTTAGCTTT
GTTTGAGTTCATATTTCTCCTCTTATTGTTTTGCAGAGTTTTTGTTTCAG
TCTCTGTGGTTAAATAGACTGATGACTTTTGGGAAAAAAAAAAAG

WIMP 74 - PUTATIVE IDENTITY - UNKNOWN

PRIMER COMBINATION

5′ -AAGCT$_{11}$A- 3′ and 5′ -AAGCTTACCAGGT- 3′

>Wimp 74
ACAACAATCATTTANCATTTCTATANCCAAAATTCACATNAATNCCAGTC
CAANAAGGTTTCTGAAGATAGATTAAAGTGTATAAAGGGGAANAANACNC

ATTTCAGAGCTCGAGGGACANGTCNANCTTGTNAATNCGACCATCTTNAT
CNGTCNNCNNGTGATCNTTGCCTATCNCCCTACCNTNTCCACNGTCTGCC
GGAGACACTNTCTNCTNNTCCCCCCCTTNTTNACCCTTNCNACACTGGCC
A

WIMP 75 - PUTATIVE IDENTITY - UNKNOWN

PRIMER COMBINATION

5′ -AAGCT$_{11}$A- 3′ and 5′ -AAGCTTACCAGGT- 3′

>Wimp75
TTTTTTTTTTTACGACAAGGAAAGGGGGAGCAGAAGACCTCGAATTACTT
TATTTACCGTTTAAAAAAGCATTGCAGATGATAATTGGTAGAGACATGGC
ATCGTTATAGTTTCTGTGAAGTCGGCATTGCTAGAGAAACTCCACCACCA
CCTGGTAAGCTTAATCAAGCTT

WIMP 76 - PUTATIVE IDENTITY - UNKNOWN

PRIMER COMBINATION

5′ -AAGCT$_{11}$A- 3′ and 5′ -AAGCTTAGAGGCA- 3′

>Wimp76
TTTTTTTTTTTAACTCGCAACATCATATATTAAACTAAAATGCCAGTGCT
TGAGGACTTAAAGCACCTCATGGATAATACATAGTAAACATAGATAGATT
CAATCTGAAAATATAATTAAACATTACTGCGTATCTTGCTTATCCTGCCT
CTAAGCTTAATCAAGCTT

WIMP 77 - PUTATIVE IDENTITY - OSMOTIN LIKE PROTEIN

PRIMER COMBINATION

5′ -AAGCT$_{11}$A- 3′ and 5′ -AAGCTTATCGCTC- 3′

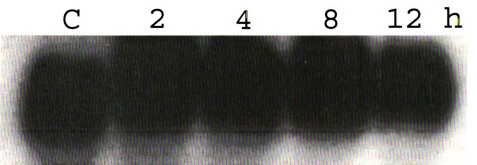
>Wimp77
CTTTTTTTTTTTAGCAAGACGAAAGCATCTAAAATTAAGATTTGATTAAA
CAGAAACCCCAAACATGACATTCCCATACACACAACATAAAGACCAGAAG
ATTATGACATTATCAATCTTTCCTAATATTCCAAGCTGGGCCAGAGAGAG
AATTAGGATAGATCATACAAAAGACCAAAACATAGATACGATATTCATTC
TTCGACTTTGCTACTTACAGACTCTTTTAGTGACAAAAGATAACTTTAAG
TTCACGTGGCGAAGAACACTCATGCGTGAGCGATAAGCTTAATCAAGCTT

BLASTx homology

>gi|2501182|sp|Q41350|OLP1_LYCES OSMOTIN-LIKE PROTEIN PRECURSOR
>gi|2129934|pir||JC5237 Score = 41.4 bits (95), Expect = 0.003

Query: < 289 SLSLTHECSSPRELKVIFCH 230
S SL HECSSPRELKVIFCH
Sbjct: 233 SPSLMHECSSPRELKVIFCH 252

NORTHERN HYBRIDIZATION



Band 77

WIMP 78 - SEQUENCED TWICE POOR SEQUENCE

PRIMER COMBINATION

5′ -AAGCT$_{11}$A- 3′ and 5′ -AAGCTTATCGCTC- 3′

WIMP 79 - PUTATIVE IDENTITY - ESTERASE

PRIMER COMBINATION

5′ -AAGCT$_{11}$G- 3′ and 5′ -AAGCTTTCTCTGG- 3′

>Wimp79
TTTTTTTTTTTGGAGGGTGCCGTTGGGGACGGGAAGAGGAAGTACAAAAG
TTACTCCAGATCACCATCACGACATAGTTACCACATTATGTATGGATTAC
TGCAGACTTAAAGTTTAAGAGCATGAGCATGGTGGTGAATGTGGTCATCG
ATGAAAGTGGCAATGAAAAAGTAGGAGTGATCATAACCAGGTTGTAATCG
CATCAGAACTGAAACATTTGCACTCCTGCATGCCTCTTCAAACTTGTTTG
GCAGCAGCTGATCATGCAAGAATTTATCCTCATCTCCCTGATCAATTAAA
ATGGTAGCAGATACATCATGGACCTTTGACACCAGAGAAAGCTTAATCAA
GCTT

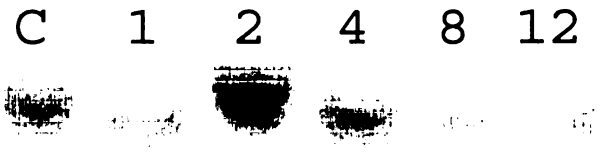
BLASTx homology

>gi|6984138|gb|AAF34769.1|AF227624_1 (AF227624) putative esterase D
[Euphorbia esula] Score = 129 bits (320), Expect = 2e-29

Query: < 338
SLVSKVHDVSATILIDQGDEDKFLHDQLLPNKFEEACRSANVSVLMRLQPGYDHSYFFIA 159
SLVSK HDVSATILIDQG +DKFLH+QL+P KFEEACR ANV +L+R PGYDHSYFFI+
SLVSKFHDVSATILIDQGGDDKFLHEQLMPGKFEEACRLANVPLLLRTHPGYDHSYFFIS 115
Sbjct: 56

Query: < 158 TFIDDHIHHHAHALKL 111
              TFIDDHI HH AL L
Sbjct: 116 TFIDDHIRHHVQALNL 131

NORTHERN HYBRIDIZATION



Band 79

WIMP 80 PUTATIVE IDENTITY - UNKNOWN

PRIMER COMBINATION

5′ -AAGCT$_{11}$A- 3′ and 5′ -AAGCTTATCGCTC- 3′

>Wimp80
AAGCTTTCTCTGGGGATCAACTGACTTCTGCTAAAGAAAAAGCGAGCAAC
TGGTTGCATATGAAATGTTACCATAGAAAGGAGTTATGCATCTGGCCACA
CTTTCAGTGCGACAAGGGTCCGTTTGTTAACAAACGCCACTTAGTGAGAA
GTTCTATATCGGGCCACGCCTGTTGGACTGATCGTTTTGTGCCTATATTT
AATTGATGAGGATTTCATTTTGTTACTCTAATCTTCACGCTATTTTGTTA
CCCTTATTCTTGTTACTTGTGATGCCCTGTCCCAAAAAAAAAAA

WIMP 81 - PUTATIVE IDENTITY - UNKNOWN

PRIMER COMBINATION

5′ -AAGCT$_{11}$G- 3′ and 5′ -AAGCTTGTTGTGC- 3′

>Wimp81
TCGCAGGAATCGATTAAGCTTGTTGTGCAGTTAAATATCATGTACAATGT
AAATTAAATAATAATAATAATAACAGCACTCTATCTCCTTTATTGTTTAT
CCAAAAAAAAAAAG

WIMP 82 - PUTATIVE IDENTITY - UNKNOWN

PRIMER COMBINATION

5′ -AAGCT$_{11}$G- 3′ and 5′ -AAGCTTTTGATCC- 3′

>Wimp82
AAGCTTTTGATCCTGTAGTAGCGTGTAAGTCCTTTCAAATTGTTCTTTTT
TCTACTCACAGTGGGGAATAAGTCAGGCTTCAATGGCTAAGTTATCACTT
TAGAGTATGTTTTATTTGTTTTTTCATGTCTTATTGTTTTAGCCTGTGTT
ATTTCTTTCTTTCTTGCCTTCTTGTATACGGTTTGTGATCTGTATTTTGT
GGTANATACTGTAGATCCATGGAAAACGTTGTTATTGTCATCGACCAGAA
GATTTTGGCCTANTAAAATAGAATTCTTGTGTGCAAAAAAAAAAAG

WIMP 83 - PUTATIVE IDENTITY - UNKNOWN

PRIMER COMBINATION

5′ -AAGCT$_{11}$G- 3′ and 5′ -AAGCTTTTGATCC- 3′

>Wimp83
CTTTTTTTTTTTGGGACGATAAAGCAAGGGTATTTGTGCCATACAAGTTT
AAGAAATTATCTCATACAACATGGAGGAGATGAACAGTATCATTTCTAGC
GGCCAAATACTTTGGGATTGGGATCAAAAGCTTAATCAAGCTT

WIMP 84 - PUTATIVE IDENTITY - UNKNOWN

PRIMER COMBINATION

5′ -AAGCT$_{11}$C- 3′and 5′ -AAGCTTTCTCTGG- 3′

>Wimp84
CACGTATGTAAATTTACACAGAACAACTCTGAAAAGATACAACATAGATG
AGCATCGACCGAAGAGAAAGGTCTGCAACATTTGGAAAAGAAATGAGGAA
AAAATTACAACATACAAGGAAACCAAAACTCTACAAACCATCTAAAGCAC
AATTAGAAGATGTTGTTATACTGACTGGTGAGAGAATCCCTGANAATTGA
ATAAAGACGTAAAGCGAGTACTGTCNAAAGTTTTCCCCTCCTACCNTGAA
TTTAAAACNCAAAAATTNTTGTCNNNTGCCAAAAAAACC

WIMP 85 - PUTATIVE IDENTITY - SINA PROTEIN

PRIMER COMBINATION

5' -AAGCT$_{11}$C- 3'and 5' -AAGCTTTCTCTGG- 3'

>Wimp85
AAGCTTTCTCTGGTGGGGATAGGAANGAGCTGAAGCTAANAGTTACCGGG
AGGATATGGAAGGAACAACAAAATCCANAAACTGGAGTGTGTATACCCAA
CCTTTGTAGCTAAGGCAAAGTGATTTTGAAGACATTTACACTATTTGTGT
GTTTGGGACTGTTGCGTGTTGTCCCAACCTCTTCCACCCTTCTCTCACAT
GCTGTAATGGATTGAATTAATGAAATTCCCCATTGTTTTTTTTTTAAATA
AAATTGTATAATAAACAGCCGTTCTTCCCGAAAAAAAAAAAA

BLASTx homology

>gi|9759183|dbj|BAB09798.1| (AB013388) developmental protein SINA (seven in absentia) [Arabidopsis thaliana]
Score = 63.6 bits (152), Expect = 6e-10

```
Query:  > 6   FSGGDRXELKLXVTGRIWKEQQNPXTGVCIPNLCS 110
              FSGGD+ ELKL VTGRIWKEQQNP +GVCI ++CS
Sbjct:    228 FSGGDKKELKLRVTGRIWKEQQNPDSGVCITSMCS 262
```

NORTHERN HYBRIDIZATION

C C 2 4 8 12 h

Band 85

WIMP 86 - PUTATIVE IDENTITY -UNKNOWN

PRIMER COMBINATION

5′ -AAGCT$_{11}$C- 3′and 5′ -AAGCTTTCTCTGG- 3′

>Wimp86
CTTTTTTTTTTTAGAAACACGGAATGTACTAGTATTATTCTAATTTGTAT
TTGTGGGGAATGGCAATTCAACACTATCAATCAAAAACGAAAATGAAAAG
CAACATGAGCCTTCCAGCTGCAAATTGGGCCTACATGCGAAATGATTTTA
CCAGAGAAAGCTTAATCAAGCTT

WIMP 87 - PUTATIVE IDENTITY - UNKNOWN

PRIMER COMBINATION

5′ -AAGCT$_{11}$A- 3′and 5′ -AAGCTTTTGATCC- 3′

>Wimp87
AAGCTTGTTGTGCGAAAGTAATGGAAGGATGAAAGAGAGTGCCATTGAAT
AATATCATCCAGAAAAATGTTTAATGGCCTGTTTACAAGGCCAAAATGCT
ATCAATCAGTATCTGTCTTGTTTAAAAACGTGTATATGCATTATATTGTG
ACCAGTTCAAAGAGAGTAGCTGGATAATTAAATCTGCTGGCAAAACATGA
GCGCGGATGCCCTCGCCCCCTCCCTGTATTTTGTACTTTGTTTAAATTTA
AACACATTTTAATTTTAAATTGTTTCCTCCTAAAAAAAAAAA

WIMP 88 NO REAMPLIFICATION - NOT CLONED

WIMP 89 - PUTATIVE IDENTITY - UNKNOWN

PRIMER COMBINATION

5′ -AAGCT$_{11}$A- 3′ and 5′ -AAGCTTTCCTGGA- 3′

>Wimp89
TTTTTTTTTTTACAAGGAGAATGAGAATTATTATAGAAATGCAGACCTTA
ACTGTACAGAACATTAATTCAAGCTGTCCAGAGAAAGTATAAATTGTATT
ATACAATTACAACCAAGGAATGGCTATAATGAAACATCAATTTTCTTTAT
TCTTTGAGGGGCAACTTTTTTATTTTGGGTTGTCGGTAAATATCATTAAT
CCAGAGAAAGCTTAATCAAGCTT

WIMP 90 - PUTATIVE IDENTITY - UNKNOWN

PRIMER COMBINATION

5′ -AAGCT$_{11}$A- 3′ and 5′ -AAGCTTTCCTGGA- 3′

>Wimp90
CTTTTTTTTTTTAGAAACACGGAATGTACTAGTATTATTCTAATTTGTAT
TTGTGGGGAATGGCAATTCAACACTATCAATCAAAAACGAAAATGAAAAG
CAACATGAGCCTTCCAGCTGCAAATTGGGCCTACATGCGAAATGATTTTA
CCAGAGAAAGCTTAATCAAGCTTAANTCCCGCCCGTNANAAAAATTGGGC
NATTATGGCCCCCC

WIMP 91 - PUTATIVE IDENTITY - UNKNOWN

PRIMER COMBINATION

5′ -AAGCT$_{11}$A- 3′and 5′ -AAGCTTTTGATCC- 3′

WIMP 92 - PUTATIVE IDENTITY - ASCORBATE PEROXIDASE

PRIMER COMBINATION

5′ -AAGCT$_{11}$G- 3′ and 5′ -AAGCTTGGCTATG- 3′

>Wimp92
AAGCTTGGCTATGAAACAGAAGATACGAGCAGAGTATAAAGCAATTGGTG
GAAGCCCAGATAAGCCTCTCCAGTCTTAACTATTTTCTAAATATCATGAT
CACGATTGCTGTTCTTGCATTCTTAACATATCTTCTTGGAAATTATGTTT
GTTTTTGAGGATTGTTATAAAATAGTGTTGTTTCTACCCAAAAAAAAAAA

BLASTx homology

>gi|7484621|pir||T12282 L-ascorbate peroxidase (EC 1.11.1.11) precursor - common ice plant Score = 35.6 bits (80), Expect(2) = 1e-08

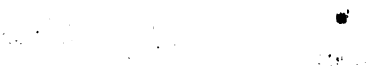
```
Query:  > 8   AMKQKIRAEYKAIGGSPDKPLQS*LF 85
              +M+QKIRAEY+  GGSP+ PL +  F
Sbjct:    385 SMRQKIRAEYEGFGGSPNNPLPTNYF 410

 Score = 42.6 bits (98), Expect(2) = 1e-08

Query:  > 78  NYFLNIMITIAVLAFLTYLLGNY 146
              NYFLNIMI +AVLA LTYL GNY
Sbjct:    408 NYFLNIMIVVAVLAVLTYLTGNY 430
```

NORTHERN HYBRIDIZATION

C 2 4 8 12 h



Band 92

WIMP 93 - PUTATIVE IDENTITY - UNKNOWN

PRIMER COMBINATION

5′ -AAGCT$_{11}$G- 3′ and 5′ -AAGCTTGGCTATG- 3′

>Wimp93
TTTTTTTTTTTGGAAACAAAGGACCAAACAACGTGGAAATACCTTCCAAC
CATGATTATACAAAGACGATGTCTGACCAATACAGTATGCAACATCATTG
AGCTTAAGAGCTAAACAGATCTTCATTTCCTATAGCTCACTACTTGATAT
ACTGAGAAAGCCACCTAAAACCCTCTCCATAGCCAAGCTTAATCAAGCTT

WIMP 94 - SEQUENCED TWICE - POOR SEQUENCE

WIMP 95 - PUTATIVE IDENTITY - RECEPTOR KINASE

PRIMER COMBINATION

5′ -AAGCT$_{11}$G- 3′ and 5′ -AAGCTTGGCTATG- 3′

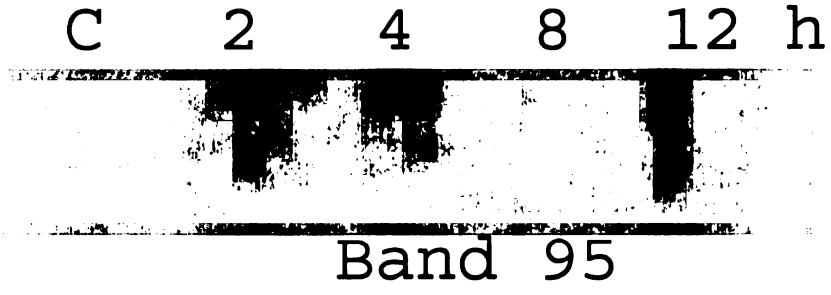
>Wimp95
TTTTTTTTTTTGGTAAGTTGCTACTAAAACATCTCGATAGACTTGAAACT
AGCTGACCCGAGATTTTTATACGGGCCGGGTGATACCTCAGTCTTCTCCT
CCAAGACCCGTTTAGGCCCTCTTCTCCTCCTATTCCAGATCTTATAACCT
CCATAGCCAAGCTTAATCAAGCTT

BLASTx homology

>gi|11358818|pir||T48397 S-receptor kinase-like protein - Arabidopsis thaliana Score = 52.3 bits (123), Expect = 7e-07

```
Query:  < 170 MIKLGYGGYKIWNRRRRGPKRVLEEKTEVSPGPYKNLGSASFKSIEM 30
              M+ + Y G++ W R     KRVLEE   +SPGPYKNLGS SF S+EM
Sbjct:    437 MVAMVYVGFRNWRRE----KRVLEEDNGLSPGPYKNLGSDSFNSVEM 479
```

NORTHERN HYBRIDIZATION



WIMP 96 - PUTATIVE IDENTITY - SELENIUM BINDING PROTEIN

PRIMER COMBINATION

5′ -AAGCT$_{11}$G- 3′and 5′ -AAGCTTCACTAGC- 3′

>Wimp96
AAGCTTCACTAGCCCATGAGATGAGATACCCAGGAGGTGACTGCACATCA
GATATATGGATCTAAGCATCCTGGATGGTTTGGCAGTGAGGGGTTACCTG
AGAACAGACCTTAGGAAGCAATAATATCAATGTGTAAATTGCAATGGCAT
GTTGTGGCACTTACTCTTTGATGGATGAGTAACTTCGTTCCTTTTAATGC
TTTAATCCATCGTTTAATATCAAATGATAATTGCAGTTTCATAATCAACA
TAAACTTATGCCAAAAAAAAAAA

BLASTx homology

>gi|6094242|sp|O23264|SBP_ARATH PUTATIVE SELENIUM-BINDING PROTEIN
>gi|7488183|pir||D71401 Score = 47.3 bits (110), Expect = 4e-05

Query: > 6 SLAHEMRYPGGDCTSDIWI 62
SLAHEMRYPGGDCTSDIWI
Sbjct: 472 SLAHEMRYPGGDCTSDIWI 490

NORTHERN HYBRIDIZATION

C 1 2 4 8 12 h

Band 96

WIMP 97 - SEQUENCED TWICE - POOR SEQUENCE

WIMP 98 - PUTATIVE IDENTITY - UNKNOWN

WIMP 99 - PUTATIVE IDENTITY - UNKNOWN

PRIMER COMBINATION

5' -AAGCT$_{11}$G- 3'and 5' -AAGCTTCACTAGC- 3'

>Wimp99
CTNGCNTNCCTNAGTTCCTGAGTNCTAGAGCGANCGANNAANCTTTTGGG
NTCTGGACTAACNTACCTCGTAATAATATACGCTTATCCATATGTCACNN
NANNTNTGCTNATGANNAGNTACACATANCTTATTTAANTAANAACTNCA
ANANCAGTGCATGGTGGTGGAGTAAGGANTCAGAAAGNTANCATGGNAAT
CTNCAAGAGTNCGAGCTNCAATCCNGTGAATCTNANTCAANCTTANNGAT
NCNGTCCACC

WIMP 100 - PUTATIVE IDENTITY - UNKNOWN

PRIMER COMBINATION

5' -AAGCT$_{11}$G- 3'and 5' -AAGCTTCACTAGC- 3'

>Wimp100
TTTTTTTTTTTGGTGCAGGAGGAGGTAATGCTGGAGCTTTATGTGGAGGA
GCTTTTGGTTTTGGTACTCCAGTATGGTGTTGGTGATGGCTGCTGCCATG
GCTTTTCCCTTTCTTCTTGGCTAGTGAAGCTTAATCAAGCTTATCGATAC
CGTCGACC

WIMP 101 - PUTATIVE IDENTITY - UNKNOWN

PRIMER COMBINATION

5′ -AAGCT$_{11}$G- 3′ and 5′ -AAGCTTCACTAGC- 3′

>Wimp101
TCGATTAAGCTTCACTAGCCATGATTACCCAATGCCCGATGGACAGTGGA
CAAATACATGCATTGATACAGGTAGTGGCACACTACACCATTCTCCGTTT
CTTTTCCCAAAAAAAAAAAG

WIMP 102 - PUTATIVE IDENTITY - UNKNOWN

PRIMER COMBINATION

5′ -AAGCT$_{11}$C- 3′ and 5′ -AAGCTTCACTAGC- 3′

>Wimp102
TTCGATTAAGCTTCACTAGCCACTCGAGGAAACAAATCTTCCAAGTTTGC
TAAACTAGTCATTCTGGTATATAGCATCGACTTGCTGGGAATATGCATGT
TGGGTGCAACTGATTTGATATATTTCAGATAATGATCACGGTTTTCGTTT
TGGGAAAAAAAAAAAG

WIMP 103 - PUTATIVE IDENTITY - UNKNOWN

POOR SEQUENCE

WIMP 104 - PUTATIVE IDENTITY - UNKNOWN

POOR SEQUENCE

WIMP 105 - PUTATIVE IDENTITY - FLAGELLIN

PRIMER COMBINATION

5′ -AAGCT$_{11}$G- 3′ AND 5′ -AAGCTTACGATGC- 3′

>WIMP105
TTCCTATGGCTTTNACAGTAAACACCAACGTAGCATCGTTGAACGTCCAG
AAGAACCTGGGTCGNGCCTCCNTNGCTCTTTCGACCTCGATGACTCGTCT
GTCCTCCGGTCTGAAAATCAACAGCGCTAAAGACGACGCTGCCGGCCTGC
AAATCGCTACCAAGATCACTTNGCAGATCCGTGGCCAGACAATGGCGATC
AAAAAC

WIMP 106 - PUTATIVE IDENTITY - UNKNOWN

PRIMER COMBINATION

5′ -AAGCT$_{11}$G- 3′ AND 5′ -AAGCTTACGATGC- 3′

>WIMP106
TTTTTTTTTTTGATGAATTTCAATGTATATACATGTCAAGTGAACTGAAG
AACCTGGAAAGTCTTATCGAGCCTCAGGCAGGCTAATTGTCAATTCGTTG
TTAGAATATATAGTTGATGTGTTGTGAAAGGCAACAAAATTGCTAGATCT
CCGACGACTAAGACTAGAGAATGCAGGTTCTTTTGGAGAAGGAAGTGATG
CATTGTCATTGCTCAACATCTGAACCACTAATGGCATCGTAAGCTTAATC
AAGCTT

WIMP 107 - PUTATIVE IDENTITY - UNKNOWN

PRIMER COMBINATION

5′ -AAGCT$_{11}$G- 3′ AND 5′ -AAGCTTACGATGC- 3′

>WIMP107
TGCAGGAATTCGATTAAGCTTACGATGCCATTTTAATCGGCAAATCTCTG
CCATATGTTGTTATCTGAGAGGAATTTAAGCATGGTTGCTCGACGTTGTT
TATGTTCACAGTTTGATATTATACCAATCAAATTACAATAGGATTGTACT
TCCCAAAAAAAAAAA

WIMP 108 - PUTATIVE IDENTITY - METAL-BINDING PROTEIN

PRIMER COMBINATION

5′ -AAGCT$_{11}$G- 3′ AND 5′ -AAGCTTAGCAGCA- 3′

>WIMP108
AAGCTTAGCAGCAGCAGCACCAGCACCAGCTGAGGAAACTACAGAAGAAA
CCACGGTGGTAGAGCTCAGGAAAATGGATTTTTATAATTATTATTCACCA
ACAAGATATGAGCACTATTCACCACCTCCTCAGATCTTCAGTGACGAGAA
CCCCAATGCATGTTCTGTTATGTAAGACAGTTTTGAAAATGAAGGGCAAG
ATGGGATGGGAAAAAATAATTACGGGCGATAAATTTGCTGCTTATAAATA
ATATATATATATATATATATCCTGCTTCCATGTGCAAAAAAAAAAAG

BLASTx homology

>gi|6469127|emb|CAB61745.1| (AJ275311) farnesylated protein [Cicer arietinum] Score = 52.3 bits (123), Expect = 2e-06

```
Query:  > 44  EETTVVELRKMDFYNYYSPTRYEHYSP-------PPQIFSDENPNACSVM 172
              EET VVE++K ++Y  Y    + +  P       PPQIFSDENPNACSVM
Sbjct:    52  EETKVVEMKKNEYYYKYGTEVFAYPDPAYPLQAYPPQIFSDENPNACSVM 101
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NORTHERN HYBRIDIZATION

C 2 4 8 12 h

Band 108

WIMP 109 - PUTATIVE IDENTITY - UNKNOWN

PRIMER COMBINATION

5′ -AAGCT$_{11}$G- 3′ AND 5′ -AAGCTTAGCAGCA- 3′

>WIMP109
CTTTTTTTTTTTGGCAAAAATACCGT
CGATAATAAAGTAGTAAATCACCTTAGAAAACATCCTTAAGCATCCAGAT
ACCATAAAATTAGACAAAAACATAAAATGAGATCTTTCGGAAACTTTAAA
AAAACCCAAAAGAGCAACAATTTCTCAATCATTCAGAAATCTCAACATCA
CCATCAGTAATCTCCTGCTGCTAAGCTTAATCAAGCTT

WIMP 110 - PUTATIVE IDENTITY - UNKNOWN

PRIMER COMBINATION

5′ -AAGCT$_{11}$G- 3′ AND 5′ -AAGCTTAGCAGCA- 3′

>WIMP110
CTTTTTTTTTTTGGGCTGAAATGGCAGGAATTGCAATCAAATGTTGCTCA
CAGATCCCCCACTTAATTATATATTGTGTTGTAGAGGAGAAAAAATAAAA
ACACAAATGGCAGCATGACATGCACAAAGAGGGTTTTGCTGTTTTTGTGC
GTCATTCTGGCTGCTGCTGCTGCTGCTAAGCTTATCAAGCTT

WIMP 111 - PUTATIVE IDENTITY - UNKNOWN

PRIMER COMBINATION

5′ -AAGCT$_{11}$G- 3′ AND 5′ -AAGCTTAGCAGCA- 3′

>Wimp111
TTTTTTTTTTTGGAAAGAGACTAAAACTTTGATTCAAACATTTGGGACAA
AATTTAGACTAGAATATGATGATAAATACAACTAAGATTCCTAAAATTAC
ACTGATTAATTAATATAGCAAAAACAAAACTCACACCCCTAATTATACAC
CAATTCTGCTGCTAAGCTTAATCAAGCTT

WIMP 112 - PUTATIVE IDENTITY - UNKNOWN

PRIMER COMBINATION

5′ -AAGCT$_{11}$G- 3′ AND 5′ -AAGCTTAGCAGCA- 3′

>Wimp112
TCGATTAAGCTTAGCAGCAGATATATGTACAGTATTTTTGTTTCCAAATG
GGGATTGGATGGTAGGTCGTGCTATGGCTTTACTGTTTGTTTAGTTGTCA
TTGTTACCCTAATTGTTTATTATTCAAGGTCTACTGTTTCTTCCAAAAAA
AAAAA

WIMP 113 - PUTATIVE IDENTITY - HYPOTHETICAL PROTEIN

PRIMER COMBINATION

5′ -AAGCT$_{11}$G- 3′ and 5′ -AAGCTTCGTACGT- 3′

>Wimp113
AAGCTTCGTACGTTCAGAGGAAGATGATATGGATACATCCTAGGAGCAAA
GGCAAGTGAAGCGTTTTTATGATAAGCTTTTAAAGAGCATCCTTTGTGAA
TGGATGTTCTTCAATGGTCTGAACAGTATTGTTACTCCTACATCTGAAAT
TCACTTCAATATACCGTGAACTTAAACTTTTTGAAGACAATGCATAGCTG
ACATGTCACTCTACAACATTGGCAAGGTGTGTGGTTTCCCATTGCATAAA
GCTGTTTAATCTCAGCTGTAATGACTTGAAATGGTCACTCTGATGATCTT
AAGAGCGTATTTCTTTGTTTCCCCAAAAAAAAAAA

WIMP 114 - PUTATIVE IDENTITY - RIBOSOMAL PROTEIN 40S

PRIMER COMBINATION

5′ -AAGCT$_{11}$G- 3′ and 5′ -AAGCTTCGTACGT- 3′

>Wimp114
TTTTTTTTTTTGGGGATTCTAAAACTTCAAAACTTGAATGGAAAATTAT
TAAAATCTTGGACATAACGAAATTTCAAAATATAATAGCTAAGTAATAGA
TTCTTGTTCGGGCAGAGAGCGAAAAGAACATGGTTCACGAAAATGAGAGC
AGTTGGCCACTTTCTTGCACTAACAATGTTTAAAGGACAAAAACAAAACT
CTAATGCAGTTACTGCTGGCGGAGTTCAGTCTTCTTCTTTTGCCAAAGCC
TGTCCACGGCACTGTCAGCATCACCCTGGGCACGTACGAAGCTTAATCAA
GCTT

WIMP 115 - PUTATIVE IDENTITY UNKNOWN

PRIMER COMBINATION

5′ -AAGCT$_{11}$G- 3′ and 5′ -AAGCTTCGTACGT- 3′

>Wimp115
TTTTTTTTTTTGAACATACACAAAGAATCCTATTCTTGATCTGAATCTCG
AGTTCTTGGATTTTAATCTTATTGAACAAATTAATCAAAGCTTGATCAGC
TTCCTTTTAACACCTCCAAACGTTGAAGAATCGCAAATAACCAAGTAGTA
GTCTTTTACTTGTGATCATGATGAGCTTGATTTGATGACAGTACGTACGA
AGCTTAATCAAGCTT

WIMP 116 - PUTATIVE IDENTITY - RIBOSOMAL PROTEIN 40S

PRIMER COMBINATION

5′ -AAGCT$_{11}$G- 3′ AND 5′ -AAGCTTAGCAGCA- 3′

>Wimp116
TGAAAATGAGAGNAATTGGTCANTTTCTTGCACTAACAATGTTTATAGGA
CANAAACAAAACTCTAATGCANTTACTGCTGGCGGAGTNNATTCTTCTTC
TTTCGCCANAGCCTGTCCANNGCACNGTCAGCATCACCCTGNGCACGTAC
GAAGCNTAATCAANCTTATCGATACNG

WIMP 117 - PUTATIVE IDENTITY - UNKNOWN

PRIMER COMBINATION

5′ -AAGCT$_{11}$G- 3′ AND 5′ -AAGCTTAGCAGCA- 3′

>Wimp117
TTTTTTTTTTTGGAATAAAACACTCAACAGCTATATATGACAGCCAATT
AAGACAACAACTAACAATAGATAGGTGATTATTACACAAGCCAGCCAATC
GGGGGTTAAGATTTTTGCTTCTTGTCCATTACAAATGTCTAGATCTTTGA
TTTCTATATACGTACGAAGCTTAATCAAGCTTCNAAAAGAGGAAAANCAA
GNACGGCCNGGCNGCTTTTCNTCCCCCTTTCNCCCCCCCCCNTNATCCCCT
AGGNCTNTNTGTTTNCGGAATTCGAACCCCTTTTTTTNCNNTCCATTTNCC
CC

WIMP 118

PRIMER COMBINATION

5′ -AAGCT$_{11}$G- 3′ AND 5′ -AAGCTTAGCAGCA- 3′

>Wimp118
TGCAGGAATTCGATTAAGCTTCGTACGTGCTAAATTCTAGCTGATCAACT
TGTGCTGTATTAAAGACTCGGATGATATCCTGAATCCATCAAGGCCTGTT
ATATTACATACGTTTTTTCCAAAAAAAAAAA

WIMP 119

PRIMER COMBINATION

5′ -AAGCT$_{11}$C- 3′ and 5′ -AAGCTTACGATGC- 3′

>Wimp119
TTTTTTTTTTCAACCATGATTATACAAAGACGATGTCTGACCAATACAG
TATGCAACATCATTCAGCTTAAGAGCTAAACAGATCTTCATTTCCTATAG
CTCACTACTTGATATACTGAGAAAGCCACCTAAAACCCTCTCCATAGCCC
ATTTTGCGGACAATGCTACACATGAATACCTCAAGGGGACGGACATTTGA
GTCGACCAAGTTCACCTTACCCTTGCCAGTGGTGAAGTTGGTGAGACCCA
GGTTGTAACGCAACTCATCTTCCGAGGCAGCATATGGGATATCAATCTTG
TTGCCCAAAACAAGAAA

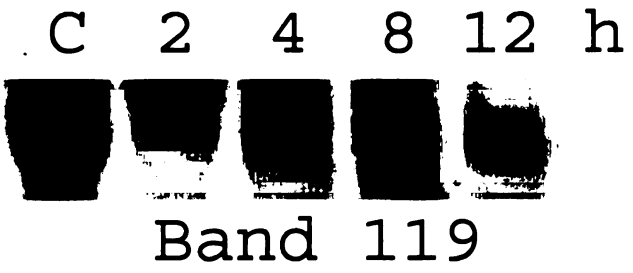
BLASTx homology

>gi|3334323|sp|O04834|SARA_ARATH GTP-BINDING PROTEIN SAR1A
Score = 139 bits (347), Expect = 9e-33

Query: < 317
FLVLGNKIDIPYAASEDELRYNLGLTNFTTGKGKVNLVDSNVRPLEVFMCSIVRKMGYGE 138
FL+LGNKIDIPYAASEDELRY+LGL+NFTTGKGKVNLVDSNVRPLEVFMCSIVRKMGYGE
FLILGNKIDIPYAASEDELRYHLGLSNFTTGKGKVNLTDSNVRPLEVFMCSIVRKMGYGE 183

Sbjct: 124

Query: < 137 GFRWLSQYIK 108
GF+W+SQYIK
Sbjct: 184 GFKWVSQYIK 193



Band 119

WIMP 120 PUTATIVE IDENTITY - UNKNOWN

PRIMER COMBINATION

5′ -AAGCT$_{11}$C- 3′ and 5′ -AAGCTTACGATGC- 3′

>Wimp120
TTCGATTAAGCTTCACTAGCCACTCGAGGAAACAAATCTTCCAAGTTTGCTAAACTAGT
CATTCTGGTATATAGCATCGACTTGCTGGGAATATGCATGTTGGGTGCAACTGATTTGA
TATATTTCAGATAATGATCACGGTTTTCGTTTTGGGAAAAAAAAAAAG

WIMP 121 - PUTATIVE IDENTITY - UNKNOWN

PRIMER COMBINATION

5′ -AAGCT$_{11}$C- 3′ and 5′ -AAGCTTACGATGC- 3′

>Wimp121
TTTNCTCTTTCGACCTCGATGACTCGTCTGTCCTCCGGTCTGAAAATCAA
CAGCGCTAAAGACGACGCTGCCGGCCTGCAAATCGCTACCAAGATCACTT
NGCAGATCCGTGGCCAGACAATGGCGATCAAAAACGCCAACGACGGTATG
TCCCTGGCGCAAACCGCTGAAGGCGCACTGCAAGAGTCGACCAACATTNT
GNAGCTGTATGCGTGAACTGGCTGNCCAGTCGCGAAACGACAGCNACAGT
GCCACCGACCGTGAAGCGCTGAACAAAGAATTNATCATGGCTTTAACAGT
AAACACNAACGTAGCATNNATG

WIMP 122

PRIMER COMBINATION

5′ -AAGCT$_{11}$C- 3′ and 5′ -AAGCTTACGATGC- 3′

>Wimp122
TAGCAGCAGCAGCATAACTGGCATTGACACCCATCCAATGTCCAATGATA
ACAATATTGCAACCGGTACACTTAGCAGCAAAGGCATGATTGTCAATGGC
TGCAATGAGGTTGTCAAGCAAGAAGTGCTGAAGAACACTTGATGCATTTC
TAGAGCACGGAAAGTAAAGATGTAATTTCATTATTTTAGATGGGGTTTCA
TTTTTCAAGCTGAGGCAGATAA

WIMP 123

PRIMER COMBINATION

5′ -AAGCT$_{11}$C- 3′ and 5′ -AAGCTTACGATGC- 3′

>Wimp123
AAGCTTAGCAGCAGAAGCTTTGGAAGACATCACTGCTCTCTTCTATGATG
AAGAGCGCAATGAGATCTATACAGGCAATAGGCTTGGTCTAGTTCATGTG
TGGTCTAACTGATTTTTTGACAAATCCTTGTTTGCTTAAGGTTGTTAATG
TTTAATCAGATGGTTGAATGAGCATGCTGCCTGGATGTGAGAGTCCCCTG
A

WIMP 124 - PUTATIVE IDENTITY - UNKNOWN

PRIMER COMBINATION

5′ -AAGCT$_{11}$C- 3′ and 5′ -AAGCTTACGATGC- 3′

>Wimp124
TTTNCTCTTTCGACCTCGATGACTCGTCTGTCCTCCGGTCTGAAAATCAA
CAGCGCTAAAGACGACGCTGCCGGCCTGCAAATCGCTACCAAGATCACTT
NGCAGATCCGTGGCCAGACAATGGCGATCAAAAACGCCAACGACGGTATG
TCCCTGGCGCAAACCGCTGAAGGCGCACTGCAAGAGTCGACCAACATTNT
GNAGCTGTATGCGTGAACTGGCTGNCCAGTCGCGAAACGACAGCNACAGT
GCCACCGACCGTGAAGCGCTGAACAAAGAATTNATCATGGCTTTAACAGT
AAACACNAACGTAGCATNNATGAACGTTCAGAAGAACCTGGGNCGCGCCT
CCGACGCTTNTTTCGACCTCNATGACTCGCTGTTCTCCGGTCTGAAAATC
AACNAGCCCTTCAAGACNACGCNTNTCGGTCTGCAANTTGCTACCCAAGA
CACTTTGCAGATNCGTGGCCAGACAAATGGCGANTCAAAAA

WIMP 125 - PUTATIVE IDENTITY - UNKNOWN

PRIMER COMBINATION

5′ -AAGCT$_{11}$C- 3′ and 5′ -AAGCTTAGCAGCA- 3′

>Wimp125
TAGCAGCAGCAGCATAACTGGCATTGACACCCATCCAATGTCCAATGATA
ACAATATTGCAACCGGTACACTTAGCAGCAAAGGCATGATTGTCAATGGC
TGCAATGAGGTTGTCAAGCAAGAAGTGCTGAAGAACACTTGATGCATTTC
TAGAGCACGGAAAGTAAAGATGTAATTTCATTATTTTAGATGGGGTTTCA
TTTTTCAAGCTGAGGCAGATAA

WIMP 126 - PUTATIVE IDENTITY - HYPOTHETICAL PROTEIN

PRIMER COMBINATION

5′ -AAGCT$_{11}$C- 3′ and 5′ -AAGCTTAGCAGCA- 3′

>Wimp126
AAGCTTAGCAGCAGAAGCTTTGGAAGACATCACTGCTCTCTTCTATGATG
AAGAGCGCAATGAGATCTATACAGGCAATAGGCTTGGTCTAGTTCATGTG
TGGTCTAACTGATTTTTTGACAAATCCTTGTTTGCTTAAGGTTGTTAATG
TTTAATCAGATGGTTGAATGAGCATGCTGCCTGGATGTGAGAGTCCCCTG
AAACTGTAACATTTACCATACTTGTACCATGTCATATTTCTTTTTGGTTT
TGGAAATTGATTTCCTTGTGTCTGTTTCCCTCGAAAAAAAAAAA

WIMP 127 - PUTATIVE IDENTITY - UNKNOWN

PRIMER COMBINATION

5′ -AAGCT$_{11}$C- 3′ and 5′ -AAGCTTAGCAGCA- 3′

>Wimp127
AAGCTTAGCAGCAGCAACTTCAACAGCAGCAGGAGCAACAGCAGCAGCAA
CAAGGTGGGGATTTGAAAATGAGAGGATCATTGACTTCTTCAAGCCAGAA
AGATAATGGATCTGAGGCTAATTCCTCTTCATCAAAGGATTGAAAATGAG
AAGGCAGCTACTTAATGTTCTCTACTGTTGTCTACATCCTAGTTGCAATC
TGTTGTCACATAGGATATTTTCCCTTCCTTTTATCTCCAGTCCTTCTAAT
ATACATTACCGAACATTTCTACCGAAAAAAAAAAAGC

WIMP 128

PRIMER COMBINATION

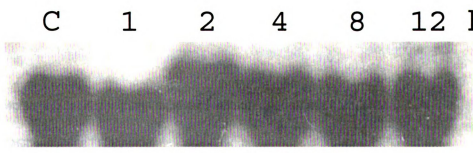
5′ -AAGCT$_{11}$C- 3′ and 5′ -AAGCTTAGCAGCA- 3′

>
Wimp128
TTTTTTTTTTTCGATACACAGCAAAACTAACGAAGATATTAAATTATAAA
TAAGAGGACAAAAGGAAGAAAAAGAAAGGTCCATCATAATCCGATCTCTC
TTACACACAAACAAACAAATAGAGACAGGGGGGGTGGATAAAGACAAGCT
CTTTTCATTTAATTTGTGGGGTGGCTACGGAAAGATCCCAAAGCCTTAAT
GGCTCCAGCTCTCAGAATGTACTGGTGGTATGCAGCTGCTGCTAAGCTTA
ATCAAGCTT

BLASTx homology

>gi|8071628|gb|AAF71820.1|AF141900_1 (AF141900) putative aquaporin
Score = 55.0 bits (130), Expect = 2e-07

Query: < 248 SLAAAAYHQYILRAGAIKALGSFRSHPTN 162
+LAAAAYHQYILRA AIKALGSFRS+PTN
Sbjct: 251 ALAAAAYHQYILRAAAIKALGSFRSNPTN 279



Band 128

WIMP 129 - PUTATIVE IDENTITY - UNKNOWN

PRIMER COMBINATION

5′ -AAGCT$_{11}$C- 3′ and 5′ -AAGCTTAGCAGCA- 3′

>Wimp129
TTTTTTTTTTTCAATGAAACGCAGGTGGTAATGGAAAGCTATAAGATAAT
AATATATTTGGAAATTACAATAAAGAAAAGAAAAGAGTGTGATATCGTCT
TTCTGCTGCTAAGCTTAATCAAGCTT

WIMP 130 - PUTATIVE IDENTITY - RUBISCO ACTIVASE

PRIMER COMBINATION

5′ -AAGCT$_{11}$C- 3′ and 5′ -AAGCTTCGTACGT- 3′

>Wimp130
TTTTTTTTTTTCAAAAAATACCAATAGAAGAGATTATTCAATAATATACT
CAATTGACTATAAAAAAGAAGTCAGCTGTTATATATACCTTGATATTAGG
CAGGCTCATGAAGTTCTTGGAGATGTGAACAACAAGCTTGTCCATGAAAG
CAGGAGCAATGTAGAAACCATCCATGTTGTTGTCCAAGTTGTACCTAAAG
CACAGTCACCAAGTCATGTTCATAATCAAACTTGTCCACAACTTCAGACG
AAATGGAAATCATAATTCATAAGTATTAGCTAAACAGCGAGAATAATCTA
CAATAATTCCCATATATAAAAGTATGGTACTTACGTACGAAGCTATCAAG
CTT

WIMP 131 PUTATIVE IDENTITY - UNKNOWN

PRIMER COMBINATION

5′ -AAGCT$_{11}$C- 3′ and 5′ -AAGCTTCGTACGT- 3′

>Wimp131
TTTTTTTTTTTCAAGTCAATTTGCTGTCACGCACACATGCAATATAGATT
ATAATTGCAACGTGACAATGGTGTGTAACATCAAGCCAATTCAACAAAAC
TTGTTCTATTTTAATTCGGCTCAACTATACAACGATCATCTATTTGGCCT
TTACAGAGTATTTTGCTCGCAAAGCAAGACCTAATCCAATACGTACGAAG
CTTAATCAAGCTT

WIMP 132 - PUTATIVE IDENTITY - UNKNOWN

PRIMER COMBINATION

5' -AAGCT$_{11}$A- 3'and 5' -AAGCTTACGATGC- 3'

>Wimp132
AAGACCAACTTTGTCTTTGAATTCTCATAGATCAGTCATATTAAATATGA
AAAGTTAAGCACAGCTTAGACATGATAAAAACAAGGCACAGTTAGAACAA
GACAGCCTTCTACATTGCAATAGCACCCGTCACTTTCTGGTGACAAGGGT
TAGCCTGCAACTATTTAAGCCTACATGTAGATATCTACAGTATATGTGTC
TGAGTACTTGTCAACCTGTATCTGCATCGTAAGCTTAATCAAGCTTATCG
ATACCGTCGACC

WIMP 133 SEQUENCED TWICE - POOR SEQUENCE

WIMP 134 - PUTATIVE IDENTITY - UNKNOWN

PRIMER COMBINATION

5' -AAGCT$_{11}$A- 3'and 5' -AAGCTTACGATGC- 3'

>Wimp134
AAGCTTACGATGCTGTAGATTTTCTGTTCTGAGAATTTGATTGGATTGTA
ACAAATTTTCCTTTGAATTATAGAGATCCACCTCTTCGGTTTTCTAAAAA
AAAAAG

WIMP 135 - PUTATIVE IDENTITY - UNKNOWN

PRIMER COMBINATION

5' -AAGCT$_{11}$G- 3'and 5' -AAGCTTGGTGAAC- 3'

>Wimp135
AAGCTTGGTGAACANAANATATTATTTTGCCAAGTATATTANTTNTAAAA
NGTCGTNGTCTCTGGCNTANTATCANTNACTNTACCATCCAANGCNNANT
ANNCNAGNTAGCCTCATATGTATTTAATANCANTACTGGGACCATTGTNT
ANCTGCGAAATCAGTACCTGATNATGATTTCTAACTTTATTTCAAAAAAA
AAAA

WIMP 136

PRIMER COMBINATION

5' -AAGCT$_{11}$G- 3'and 5' -AAGCTTGGTGAAC- 3'

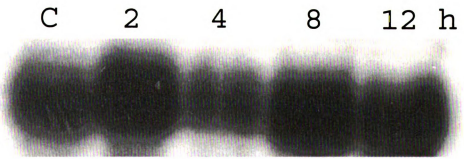
>Wimp136
TTTTTTTTTTTGGGGAAGAATGGTATTTGCGAAGACCGGAACAGAAAAGC
TAATGATAATTGAATAGTTGGACCTAATCCTTATACTTCATCTCAGACAT
CAGAAAGCCAGGCATCTCAAAGGAGCCTGAGAACTGTTCAACATCAGCCT
TGAGGGCCTCGATTTCCTTATTGTTCACCAAGCTTAATCAAGCTT

BLASTx homology

>gi|7433539|pir||B71400 glycine hydroxymethyltransferase (EC 2.1.2.1) - Arabidopsis thaliana Score = 63.2 bits (151), Expect = 4e-10

```
Query:  < 181 MVNNKEIEALKADVEQFSGSFEMPGFLMSEMKYKD 77
              +VNNK+++ LKADVE+FS S+EMPGFLMSEMKYKD
Sbjct:    437 LVNNKDLDQLKADVEKFSASYEMPGFLMSEMKYKD 471
```

NORTHERN HYBRIDIZATION



Band 136

WIMP 137 - PUTATIVE IDENTITY - UNKNOWN

PRIMER COMBINATION

5′ -AAGCT$_{11}$G- 3′ and 5′ -AAGCTTCCTGCAA- 3′

>Wimp137
NTNNATTCNANTAANCTTCCTGCAAACATTCTAGANGATGCTGACTGCGC
GGATNTCGATCTCCTGAAACCCACCCATTGAATGGTTGAAATATCTTGGA
ANTCGCCCACGTTACNNGCGTGGGTNNTTCAANTNNCTAGGAGCGANATN
NGGNGGATGNTCANTATNGTTTTTGCTATNCNGNANCNNGCCTTNCTGGG
CACCTGTCTNATGTCATCGTGACCGTCCGGTTCAGGGCNGGNAGNGTTNC
CCCNACCANTTCCNGNGANCATCAGNTGANTNTCCC

WIMP 138 - PUTATIVE IDENTITY - UNKNOWN

PRIMER COMBINATION

5′ -AAGCT$_{11}$G- 3′ and 5′ -AAGCTTCCTGCAA- 3′

>Wimp138
AAGCTTCCTGCAAGGAAAAGAGTATGTGCAAGCTTTTGTTAGCAAGGACG
ACAGTGCTCGTGCTGTTGATGCTGCTGCATGAAATCTGCTTTCGCTGGTG
TAAATCAAACTGAAATATTAGCATGATCATATTTTCAAAATAAAATACCC
TGTCTTGGAAAACAGAATTTAAAACCTTCCTAACTGTGATAACTATATCT
ATAACCTATATGGCTGCTTGTGCTTGTGCCAAAAAAAAAAA

WIMP 139

PRIMER COMBINATION

5′ -AAGCT$_{11}$G- 3′ and 5′ -AAGCTTCCTGCAA- 3′

>Wimp139
GGTAATCCAGGTTCAGGTGTGCTCACTAATCTACTCTTAAGCATAACAAT
AGAATATGATAACTTAGGTGTAAACCTTAGCAAGTGGTGTATCTTCTATT
CTTGGTTTCATTCCAAAAAAAAAAA

WIMP 140 - PUTATIVE IDENTITY - UNKNOWN

PRIMER COMBINATION

5′ -AAGCT$_{11}$G- 3′ and 5′ -AAGCTTCCTGCAA- 3′

>Wimp140
AAGCTTCCTGCAAGTTGGTGGGTGATTGCCCGTTGTAATAATGTATGGGG
TCTTAGAATTGGAGTGAGGTTGTAATCATTTCATAAGAAAACATTTTGTA
AAGAGTGTTTCTGCCTTCTCTGCCTCCAAAAAAAAAAA

WIMP 141 - PUTATIVE IDENTITY - UNKNOWN

PRIMER COMBINATION

5′ -AAGCT$_{11}$C- 3′ and 5′ -AAGCTTGGTGAAC- 3′

>Wimp141
CTTTTTTTTTTTCGACAGCTAAAAACCAGTAACAAATCAATAACCAAGC
TCCCAACTTTCCTGAGCTTCAGAATCAGCTTTAGCTTATCCTGGCAAAGA
TTAAAAAAAGAAACTACAACAAATATGACATCTCCTACACGGCAGTTGTC
AGTATTCAGTACCCATACACTACTCAATTTAGTTCACCAAGCTTAATCAA
GCTTAAAACTTGNACCCCCTCAAGGGGAAAACCCTTTTNGGGNAAAGCCC
CTNNTAAANCCCCCACCNTTTTTGGGGGAGGNGCNNACCCNTTCGANCCG
GGGCCCCTTATTNTGGGACGGNNTNCAAAGGGAAAAAANGGGCGGGCCGN
TTTTNTCCCCTTCNCCCCCCTANACCCCCGGCNTGGNNTTCGGAATTNNA
ACCCTTTTTTTAAACCNTTTNCCCNAAAACCCNANNCCNTTNAAGGANAA
TTNATCCCNCCNCCCTGGGGNTCTTTTNCCANCC

APPENDIX B

RESULTS OF

DATABASE SEARCHES

			APPENDIX B -		
		DATABASE SEARCHES	**ALL WIMP SEQUENCES**		
	Wimp Number	BLASTx - nr	BLASTn dbEST	BLASTn - nr	*Populus* DB
1	1	No hits	3e-09 Rice panicle	7e-07 Drosophila	No hits
2	2 (twice) poor seq				
3	3	N.S.	2e-09 Barley leaf library	None significant	No hits
4	4	No hits	None significant	None significant	No hits
5	5	3e-19 cytochrome f	3e-71 tomato P. susceptible	4e-81 A.thaliana comp.genome	No hits
6	6	1e-09 put.kinesin like protein	2e-10 Rice panicle	None significant	No hits
7	7	No hits	None significant	None significant	No hits
8	8	No hits	None significant	None significant	No hits
9	9	N.S.	7e-10 Six day cotton fiber	4e-10 A.thaliana BAC	No hits
10	10	No hits	6e-10 Moss library	None significant	No hits
11	11	No hits	4e-10 Hordeum vulgare leaf	None significant	No hits
12	12 (twice) poor seq				
13	13	N.S.	2e-9 Sorghum pathogen ind	None significant	No hits
14	14	N.S.	None significant	None significant	No hits
15	15	No hits	N.S.	N.S.	No hits
16	16	N.S.	2e-10 wild tomato	N.S.	No hits

17	17	N.S.	N.S.	N.S.	No hits
18	18	5e-9 H.prot A.thaliana	3e-12 Wheat endosperm	N.S.	No hits
19	19	N.S.	2e-10 Pinus taeda clone	N.S.	No hits
20	20	N.S.	2e-9 Sorghum Pathogen induced	N.S.	No hits
21	21	N.S.	N.S.	N.S.	2e-15 A049p80u
22	22	N.S.	7e-78 Roots A.thaliana	7e-78 18S P.tremuloid.	No hits
23	23	3e-05 Oenethera h.	4e-88 A.thaliana	3e-92 A.thaliana	No hits
24	24	4e-10 Enolase phosphatase A.thaliana	1e-15 Glycine max drought ind.	N.S.	No hits
25	25 No signal				
26	26 (twice) poor seq				
27	27	N.S.	5e-11 tomato elicit.fruit	N.S.	No hits
28	28	No hits	N.S.	N.S.	No hits
29	29 never reamplifie d				
30	30	2e-10 unknown prot A.thaliana O.sativa	5e-39 Gossypium cotton fiber	N.S.	No hits
31	31	No hits	N.S.	N.S.	No hits
32	32	No hits	N.S.	N.S.	No hits
33	33	No hits	6e-10 Ice plant NaCl	N.S.	No hits
34	34	N.S.	7e-10 tomato nutrient def. Roots	N.S.	No hits
35	35	N.S.	N.S.	N.S.	No hits
36	36	2e-29 amino peptidase	7e-51 Glycine max induced	3e-28 A.thaliana	No hits
37	37	N.S.	N.S.	N.S.	No hits
38	38	N.S.	N.S.	N.S.	No hits

	39	39 Never existed			
	40	40 Never reamplifie d			
41		41	N.S.	N.S.	No hits
42		42	N.S.	N.S.	No hits
43		43	N.S.	N.S.	No hits
44		44	N.S.	N.S.	No hits
45		45	N.S.	N.S.	No hits
46		46	No hits	N.S.	No hits
47		47	N.S.	N.S.	No hits
	47	47A	N.S.	N.S.	No hits
48		48	N.S.	N.S.	No hits
	49	49	9e-56 ribosomal protein	e-113 Hybrid aspen	N.S.	e-113 A063P45U Rib. assoc.prot.
50		50	N.S.	1e-49 drought Medicago s.	8e-56 18S	No hits
51		51	No hits	N.S.	N.S.	No hits
52		52	No hits	N.S.	N.S.	No hits
	53	53 (twice)	N.S.	6e-11 P.taeda inclined xylem	N.S.	No hits
54		54	N.S.	N.S.	N.S.	No hits
	55	55	N.S.	7e-10 Medicago innoculated	N.S.	No hits
	56	56/57	No hits	N.S.	N.S.	No hits
58		58	No hits	N.S.	N.S.	No hits
	58	58A	No hits	N.S.	N.S.	No hits
	59	59	8e-08 Beta- glucosidase	3e-15 Oryza gr.shoot	N.S.	No hits
	59	59A	N.S.	N.S.	N.S.	No hits
	59	59B	No hits	2e-09 P taeda compression wood incl.	N.S.	No hits

60	60	N.S.	N.S.	N.S.	No hits
61	61	9e-08 Glycine/ proline rich prot. A. thaliana CorA alfalfa drought/cold	2e-32 Sorghum bicolor drought stressed	4e-34 18S	No hits
62	62	1e-06 A.thaliana subtilisin like prot. P.abies antifreeze like protein	3e-09 Cold stressed winter rye	N.S.	No hits
63	63	N.S.	2e-09 Ice plant NaCl	N.S.	No hits
64	64	No hits	N.S.	N.S.	No hits
65	65	N.S.	N.S.	N.S.	No hits
66	66	N.S.	2e-35 Hybrid aspen RNA Pol II	N.S.	2e-35 A050P02U
67	67	No hits	N.S.	N.S.	No hits
68	68	1e-04 Vigna BURP domain Dehydration responsive	2e-17 Soybean A.thaliana dehyd.resp.	N.S.	No hits
69	69	N.S.	N.S.	N.S.	No hits
70	70	N.S.	3e-09 Medicago	N.S.	No hits
71	71	N.S.	2e-56 Hybrid aspen	N.S.	2e-56 A039P43U
72	72	N.S.	N.S.	N.S.	No hits
73	73	No hits	N.S.	N.S.	No hits
74	74	No hits	N.S.	N.S.	No hits
75	75	N.S.	6e-10 Glycine max	N.S.	No hits
76	76	No hits	N.S.	N.S.	No hits
77	77	1e-25 Osmotin like protein tomato	1e-36 Hybrid aspen	1e-12 Benincasa hispida osmotin	e-36 B007P13U1
78	78 (twice) poor seq				
79	79	2e-29 esterase Euphorbia	1e-31 Medicago innoculated	4e-37 esterase Euphorbia	No hits
80	80	N.S.	N.S.	N.S.	No hits
81	81	No hits	N.S.	N.S.	No hits
82	82	N.S.	N.S.	N.S.	No hits

83	83	No hits	N.S.	N.S.	No hits
84	84	N.S.	N.S.	N.S.	No hits
85	85	6e-10 A. thaliana development. (SINA prot)	8e-35 Grapefruit	7e-20 SINA zinc finger Gossypium	No hits
86	86	No hits	N.S.	N.S.	No hits
87	87	No hits	2e-0 Glycine max clone	N.S.	No hits
88	88 Not cloned				
89	89	No hits	3e-18 Poplar xylem 3e-10 drought Medicago t.	N.S.	7e-12 est.397 Poplar xylem turgor response
90	90	N.S.	N.S.	N.S.	No hits
91	91	No hits	N.S.	N.S.	No hits
92	92	9e-08 Ascorbate peroxidase	5e-32 Gly. Max	2e-22 A.thal. Asc. peroxidase	No hits
93	93	N.S.	9e-12 tomato mix elicitor	4e-14 A. thaliana BAC	No hits
94	94 (twice) poor sequence				
95	95	7e-07 A.thaliana receptor kinase	1e-09 Medicago t. elicited culture	2e-15 A.thaliana clone	No hits
96	96	6e-05 A.thaliana selenium binding	2e-19 Medicago developing stem	8e-07 A.thaliana DNA	No hits
97	97 (twice) poor seq.				
98	98	N.S.	N.S.	N.S.	No hits
99	99	No hits	N.S.	N.S.	No hits
100	100	N.S.	N.S.	N.S.	No hits
101	101	No hits	N.S.	N.S.	No hits
102	102	No hits	N.S.	N.S.	No hits

103	103(twice) poor seq.				
104	104 (twice) poor seq.				
105	105 contam?	6e-25 flagellin P.putida	N.S.	2e-41 flagellin P.putida	No hits
106	106	N.S.	8e-10 P.taeda xylem inclined	N.S.	No hits
107	107	No hits	N.S.	N.S.	No hits
108	108	7e-05 A.thaliana metal-binding prot	6e-13 Ice plant expr. Lib.	1e-12 A.thaliana genomic	No hits
109	109	N.S.	5e-21 Hybrid aspen lib	N.S.	5e-21 A039P77U 60S Ribos. prot.
110	110	N.S.	1e-08 Glycine max	N.S.	No hits
111	111	No hits	N.S.	N.S.	No hits
112	112	N.S.	N.S.	N.S.	No hits
113	113	1e-04 A.thaliana put. Prot.	2e-17 Glycine max clone	N.S.	No hits
114	114	1e-05 A.thaliana 40S	2e-23 Gossypium a. lib.	7e-17 Z.mays 40S	No hits
115	115	N.S.	N.S.	N.S.	No hits
116	116	8e-04 Oryza 40S		1e-14 Z.mays 40S	No hits
117	117	No hits	N.S.	N.S.	No hits
118	118	N.S.	N.S.	N.S.	No hits
119	119	9e-33 A.thaliana SAR I	Many	4e-40 Nicotiana GTP bind/SAR	4e-23 A057P38U
120	120	No hits	N.S.	N.S.	No hits
121	121 (contam)?	7e-30 flagellin P.putida	N.S.	8e-66 flagellin P.putida	No hits
122	122	N.S.	N.S.	N.S.	No hits
123	123	N.S.	N.S.	N.S.	No hits

124	124 (contam)?	9e-30 flagellin P.putida	N.S.	1e-65 flagellin P.putida	No hits
125	125	N.S.	N.S.	N.S.	N.S.
126	126	1e-11 A.thaliana hyp. prot.	1e-23 A.thaliana	N.S.	N.S.
127	127	N.S.	N.S.	N.S.	No hits
128	128	2e-07 aquaporin	7e-38 Hybrid aspen	9e-16 Picea mariana put.aquapor.	7e-38 A092P63U
129	129	No hits	N.S.	N.S.	No hits
130	130	3e-14 Gossypium h. Rubisco activase	4e-19 Glycine max Rubisco activase	3e-41 A.thaliana Rubisco activase	No hits
131	131	N.S.	N.S.	N.S.	No hits
132	132	No hits	N.S.	N.S.	A023P29U
133	133 (twice) poor seq				
134	134	No hits	N.S.	N.S.	No hits
135	135	No hits	N.S.	N.S.	No hits
136	136	4e-10 A.thaliana hydroxymethltransferase	1e-20 Lotus japonicus cDNA clone	3e-18 A.thaliana DNA	No hits
137	137	No hits	N.S.	N.S.	No hits
138	138	No hits	N.S.	N.S.	No hits
139	139	No hits	N.S.	N.S.	No hits
140	140	No hits	2e-18 Medicago	N.S.	No hits
141	141	N.S.	N.S.	N.S.	No hits