# STRATEGIES TO IDENTIFY GENES INVOLVED IN FOREST TREE DEFENSE

**John M. Davis**[1] **& Susan D. Lawrence**[2]

School of Forest Resources and Conservation, and Program in Plant Molecular and Cellular Biology
University of Florida, Gainesville, FL 32611–0410, U.S.A.
[1] Corresponding author
[2] Current Address: Plant Science Center, Cornell University, Ithaca, NY 14853, U.S.A

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## ABSTRACT

As advances in molecular cloning and gene transfer techniques are being made in forest trees, it is important to consider what genes are desirable to identify and clone in trees, and how the functional roles of those genes can be defined. Recent progress in identifying and cloning genes that regulate defense responses in herbaceous annuals has begun to reveal, as well as dissect, the regulatory complexity of defense responses. Many genes involved in defense can be cloned from tree genomes based on their sequence or functional similarity to genes from herbaceous plants. Testing the presumed functions of these genes in trees can involve established techniques such as antisense methods. Genes from herbaceous annuals, genetic mapping, and genetic transformation techniques are valuable tools for improving our understanding of the genetics of forest tree interactions with other organisms.

**Key words:** antisense, disease resistance, gene regulation, signal transduction, wound response

## WHY STUDY GENES INVOLVED IN TREE DEFENSE?

The identification and functional analysis of genes and signaling molecules that are involved in regulating plant defense are currently areas of active research (STASKAWICZ *et al.* 1995). The reasons for studying genes involved in tree defense range from practical to fundamental. On the practical side, wood is a valuable raw material, and trees are an important ecological and recreational resource in the United States and other countries. The ability to alter genes through genetic engineering could increase the precision of, and reduce the time required for, improvement of disease and pest resistance (RIEMENSCHNEIDER *et al.* 1987). Manipulation of wound-induced signaling pathways in transgenic trees may help to minimize losses due to herbivory, or to optimize local wound-repair processes in order to exclude opportunistic pathogens that enter wounds and diminish wood quality and/or tree survival.

On the fundamental side, forest trees are continually exposed to many different pathogens during a lifetime that spans decades, in contrast to annual species. Our understanding of how forest trees recognize and respond to fungal pathogens, fungal symbionts, and herbivorous arthropods in natural systems would be greatly enhanced if the genes involved in promoting or limiting the interactions could be identified.

In this article, we discuss recent progress in the identification of genes and molecules involved in defense responses and disease resistance in herbaceous annuals. Because many biochemical processes are likely to be conserved in herbaceous annuals and trees, we suggest how related genes from forest trees could be cloned, mapped and functionally tested to define their roles in trees. Genes from herbaceous annuals provide a reservoir of information and materials that can be used to promote a better understanding of forest tree interactions with other organisms.

## GENETIC CONTROL AND REGULATORY COMPLEXITY IN PLANT DEFENSE

The genes and signaling molecules described in this section are involved in plant defense. The simplicity of the term "defense" belies its potential complexity, however. For example, one consequence of defense can be resistance to a pathogen (disease resistance). Disease resistance is often associated with a localized cell death at the site of infection, known as the hypersensitive response (HR). The HR is in turn associated with the localized and systemic transcription of genes encoding defense proteins including hydrolytic enzymes and other "pathogenesis-related" proteins that are capable of inhibiting pathogen growth or survival (rev. by KEEN & DAWSON 1992; DIXON *et al.* 1994; MEHDY 1994). Mechanical wounding also induces local and systemic transcription of genes encoding defense proteins but the genes induced by pathogens and wounding are not necessarily the same, because some are induced only by pathogens and not wounding, and *vice versa*. This is consistent with the idea that the signals generated in

response to pathogens differ from wound-induced signals (FARMER & RYAN 1992), with distinctly different selective pressures operating on the signaling pathways during evolution. Important goals are to identify the cellular players involved in specific defense responses, define the order in which key events occur, and determine which components function to couple more than one signaling pathway together.

Several plant disease resistance genes whose function is required for "gene-for-gene" interactions with bacterial (MARTIN *et al.* 1993; MINDRINOS *et al.* 1994; BENT *et al.* 1994), fungal (JONES *et al.* 1994) or viral (WHITHAM *et al.* 1994) pathogens have recently been cloned and sequenced. The products of these resistance genes are generally predicted to function early in a signal transduction pathway, in the initial interaction between plant and pathogen. Consistent with this expectation, the proteins predicted from the DNA sequence of the resistance genes have characteristics of signal transduction pathway components, including kinase and/or nucleotide binding domains, and potential interaction with other proteins *via* leucine-rich repeats (STASKAWICZ *et al.* 1995). Further studies are required to determine how these proteins interact directly or indirectly with products of pathogen avirulence genes, and with other components that are involved in transducing pathogen-induced signals.

Certain genes, when mutated, cause the plant to exhibit disease phenotypes in the absence of any pathogen and therefore may be involved in as-yet--undefined steps of pathogen perception, or the regulation of a genetically programmed resistance response (NEUFFER & CALVERT 1975; GREENBERG & AUSUBEL 1993; GREENBERG *et al.* 1994; DIETRICH *et al.* 1994). Other mutants cannot implement an effective defense response due to a reduction in the quantity of one or more of the gene products that accumulate in association with the HR (CAO *et al.* 1994; MAHER *et al.* 1994; GLAZEBROOK & AUSUBEL 1994). Conversely, mutants that constitutively express genes that are usually transcribed only in the presence of a pathogen (BOWLING *et al.* 1994) may define genes whose products normally repress signaling. If homologs of such genes function similarly in trees, they can be cloned and used as tools to better understand and manipulate defense responses in forest trees.

A variety of different signaling molecules are thought to play important roles in plant defense, including active oxygen species (such as peroxide), salicylic acid, and jasmonic acid. Any one of these molecules can affect activity of the others. Active oxygen is thought to play an early and important role in triggering and orchestrating the HR (APOSTOL *et al.* 1989; LEVINE *et al.* 1994), and can promote peroxidation of lipids in the plasmamembrane (MEHDY 1994). Lipid peroxidation, in turn, is thought to create intracellular precursors for synthesis of jasmonic acid (ROGERS *et al.* 1988; MULLER *et al.* 1993; MEHDY 1994), which induces expression of genes involved in accumulation of antifungal compounds (MULLER *et al.* 1993). Salicylic acid also increases in plants in association with the HR, and is necessary for the onset of systemic acquired resistance, a physiologically induced and non-specific form of disease resistance that occurs after exposure of plants to pathogens that induce an HR (RYALS *et al.* 1994; VERNOOIJ *et al.* 1994). The coordinate accumulation of jasmonate and salicylate might suggest they act in an additive or synergistic fashion to induce common or complementary suites of defense genes. However, regulation of the response to mechanical wounding illustrates this is not always the case. Whereas jasmonic acid is thought to be a signal molecule required for accumulation of proteinase inhibitors (anti-herbivore defense proteins) in response to wounding, salicylate strongly inhibits the wound-induced accumulation of proteinase inhibitors, and is thought to do so by blocking activity of an enzyme required for jasmonic acid synthesis (PENA-CORTES *et al.* 1993). Salicylate is therefore an example of a compound that can act as either an inducer (acquired resistance) or inhibitor (wound responses) of systemic defenses. The complex interactions of potential defense signaling molecules such as active oxygen, jasmonate and salicylate will be better understood as mutants in perception and/or synthesis of these compounds are identified.

Mechanical wound signaling has begun to be dissected genetically, making use of antisense methods (antisense sequences can form mRNA hybrids and reduce protein abundance *in vivo*; ECKER & DAVIS 1986; VAN DER KROL 1988) and mutants generated by chemical means. Systemin is a phloem- mobile oligopeptide that appears to transduce wound signals systemically throughout tomato plants (MCGURL *et al.* 1992, 1994). Plants that express an antisense systemin gene are impaired in systemic transcription of proteinase inhibitor genes (MCGURL *et al.* 1992). In contrast, transgenic plants that overproduce systemin constitutively produce proteinase inhibitors (MCGURL *et al.* 1994) and polyphenol oxidase (CONSTABEL *et al.* 1995) and are protected from insect herbivory. Although trees accumulate defense proteins systemically after wounding (PARSONS *et al.* 1989; DAVIS *et al.* 1991), genes related to systemin have been found only in tomato and potato plants (MCGURL *et al.* 1992) and therefore systemin-like genes are unlikely to be cloned in trees based on sequence similarity. Lipid peroxidation products such as jasmonic acid appear to act as signaling compounds along with systemin in the wound signaling pathway (FARMER & RYAN 1992), suggesting that genes involved in jasmonate perception (STASWICK *et*

*al.* 1992; FEYS *et al.* 1994) or synthesis (discussed in DIXON *et al.* 1994; MEHDY 1994) may be involved in the transduction of wound signals. Two tomato mutants lacked systemic, but not local accumulation of proteinase inhibitors in response to wounding, suggesting local and systemic responses are controlled by different genes (LIGHTNER *et al.* 1993). Antisense methods were used to eliminate a putative transcription factor that could bind *in vitro* to a *cis*-element required for wound-inducible expression of peroxidase; loss of wound-induced transcription of the peroxidase gene confirmed a role for this DNA-binding protein in wound-induced transcription (KAWAOKA *et al.* 1994).

## ANTISENSE METHODS AS TOOLS TO TEST GENE FUNCTION

The examples presented in the previous section show the potential complexity of defense responses, but illustrate that the complexity can be dissected and the defense response can be better understood when attention is restricted to a specific defense response. Mutants are particularly informative in defining genes that act at key regulatory steps. Conventional strategies for creating and identifying such mutants in *Arabidopsis* and maize are not commonplace in forestry. However, there are "reverse genetic" strategies (beginning with a gene and working toward the phenotype; BERG 1991) for disrupting gene function in plants, and antisense methods have been used to fulfill this purpose. The roles that individual genes play in tomato fruit ripening are better understood because of antisense analysis (GRAY *et al.* 1992, 1994). Gene products that have been disrupted include those involved in carotenoid synthesis (BIRD *et al.* 1991), ethylene synthesis (HAMILTON *et al.* 1990; OELLER *et al.* 1991), and cell wall softening (pectinase, TIEMAN *et al.* 1992; polygalacturonase, SMITH *et al.* 1988; WATSON *et al.* 1994). The FlavrSavr™ tomato that is offered commercially from Calgene contains an antisense polygalacturonase gene. Antisense has been used to perturb photosynthesis and carbon metabolism (rev. by SONNEWALD & WILLMITZER 1992; SONNEWALD *et al.* 1994), nitrate assimilation (VAUCHERET *et al.* 1992), floral organ identity (PNEULI *et al.* 1994) and pollen germination (MUSCHIETTI *et al.* 1994). Antisense plants altered for specific steps of lignin biosynthesis, including cinnamyl alcohol dehydrogenase (HALPIN *et al.* 1994) and an O- methyltransferase (DWIVEDI *et al.* 1994) indicate that lignin composition and lignin quantity can be directly manipulated in this manner.

The low efficiency of transformation and regeneration methods for many forest tree species (ELLIS 1995) could severely restrict the use of antisense methods for trees in the near future. Antisense analysis can require a large number of independent transgenic plants; for example, the variable degree of target protein loss among a large number of plants revealed a 'graded series' of expression in ripening fruits (GRAY *et al.* 1994). In addition, the effects of regulatory genes may be difficult to interpret due to their potentially pleiotropic effects, and the roles of individual genes in multigene families may be difficult to define. The potential limitations of antisense methods may be avoidable in some cases, and some examples from the animal literature help to illustrate this point.

The difficulty of regenerating transgenic trees should not preclude antisense experiments in trees. In animal systems, antisense oligonucleotides (ASO's) are often used to disrupt expression of specific genes (WALDER & WALDER 1988; SCHWAB *et al.* 1994; WAGNER 1994). ASO's were successfully used to transiently but effectively disrupt synthesis of an apparent transcription factor whose function was critical for neural crest formation in vertebrate embryos (NIETO *et al.* 1994), as well as protooncogene products in developing embryos, and oncogene products in carcinomas (BEAUPARLANT *et al.* 1994; PEREZ *et al.* 1994; YAMAUCHI *et al.* 1994). ASO's have been used to disrupt gene expression in pollen (ESTRUCH *et al.* 1994), but their use is not widespread in plants. ASO's are worthy of consideration as a transient antisense method, particularly in tissues that are specialized for solute uptake such as pollen (ESTRUCH *et al.* 1994), seeds, cells adjacent to vasculature, or even cell suspension cultures.

Also in animal systems, cell lines are manipulated by antisense methods to dissect signal transduction pathways. The Toll and IL-1 receptors from *Drosophila* and mammals, respectively, are at the entry point of a pathway that is induced by viral pathogens and other acute stresses including active oxygen. In fact, the IL-1 receptor shows sequence similarity to a viral resistance gene in tobacco (WHITHAM *et al.* 1994). In the animal system, a cytoplasmic transcription factor (NF-kappaB), when bound by an inhibitor protein, is inactive. Receptor activation causes phosphorylation of the inhibitor protein, which releases the transcription factor to enter the nucleus and activate transcription of specific genes (KERR *et al.* 1993). As predicted by this model, reducing the abundance of the transcription factor by antisense methods eliminated receptor-induced gene transcription, which in turn eliminated the cellular responses that normally resulted from expression of those genes (HIGGINS *et al.* 1993; KITAJIMA *et al.* 1992; PEREZ *et al.* 1994). Also as predicted, reduction of the inhibitor protein by antisense RNA de-repressed the pathway, causing constitutive transcription and cellular response (BEAUPARLANT *et al.* 1994). This illustrates that signal transduction is an interplay between positive-acting and negative-acting factors (BOWLER &

CHUA 1994) that can be dissected with antisense methods. This suggests signaling pathway components may be conserved in animals and plants, and that a number of these genes could be cloned in trees and manipulated by antisense methods in cell lines.

The presence of complex multigene families in trees may not preclude disruption of individual members of the family. A family of proteins that is a component of virtually all signaling pathways in higher eu-karyotes are GTPases (BOURNE *et al.* 1990, 1991; BOGUSKI & MCCORMICK 1993). Specific GTPases called G-proteins are activated by receptors at the cell surface and transduce that information into the cell by altering the activity of one or more effectors, such as enzymes (-thereby promoting or diminishing activity of the enzyme) or ion channels (thereby opening or closing the ion channel). Antisense methods have been used to reduce the abundance of individual G-protein subunits in transgenic mammalian cell lines, and as a consequence specific cellular responses were eliminated (WANG *et al.* 1992; WATKINS *et al.* 1992; KLEUSS *et al.* 1993; ALBERT & MORRIS 1994; GOETZL *et al.* 1994). This indicates the potential to systematically distinguish signaling roles of closely related G-proteins (*i.e.*, multigene family members) by observing individual responses that are disrupted when any one member is eliminated from the cell line by antisense methods.

The point of this section is not to defend the use of antisense methods to the exclusion of any other method for disrupting gene function, but rather is intended to point out that in some cases perceived barriers to experiments in trees may be circumvented by using techniques common in animal systems. The overriding issue remains that we know little about the genes that regulate defense responses in trees, and a combination of antisense methods and simplified experimental systems such as cell cultures could provide insights into the identity and function of those genes.

## IDENTIFICATION OF GENES AND TESTS OF GENE FUNCTION

Due to the long generation intervals, large genome sizes, and/or dioecy (which makes self-pollination impossible) of most forest trees, they are normally considered to be recalcitrant to the approaches used to identify and clone mutant genes in model systems. However, a number of approaches can now be envisioned to clone genes in trees that were not possible a few years ago.

A growing number of genes involved in controlling disease resistance and other aspects of plant defense have been cloned in herbaceous plants. Techniques to clone related genes by hybridization or PCR with degenerate oligonucleotides should permit many of these genes to be isolated from tree genomes. Evidence for a possible role of such genes can be obtained by testing for tight linkage between the gene and loci involved in disease resistance (DEVEY *et al.* 1995, WILCOX 1995) or other phenotypes that involve a defense response.

The use of *Arabidopsis* as a surrogate genome for tree transgenes could create access to important signal transduction pathways in trees. In the context of defense, there are many similarities between the early responses of gymnosperms and angiosperms to general elicitors, including the appearance of an oxidative burst, calcium uptake and kinase activity (SCHWACKE & HAGER1992; DIXON *et al.* 1994; MEHDY 1994). Later responses in both systems include induction of hydrolytic enzymes and genes in the phenylpropanoid pathway (KEEN & DAWSON 1992; POPP *et al.*, manuscript submitted). A gymnosperm gene appears to be transcriptionally inducible by general elicitors in tobacco (H. WU and J. DAVIS, unpublished data), suggesting at least part of the signaling pathway is conserved. If gymnosperm promoters are transcriptionally inducible by general elicitors in *Arabidopsis*, then transgenic *Arabidopsis* lines with tree promoter-reporter fusions could be subjected to mutagenesis, and mutants that show altered transgene regulation would be potentially impaired in a signal transduction pathway essential for proper regulation (BOWLING *et al.* 1994; CAO *et al.* 1994; DIXON *et al.* 1994). Mutant genes identified in this manner could be cloned from *Arabidopsis*, and then cloned from trees based on sequence similarity. Once any component of the signaling pathway is identified, other components with which they interact can be pursued, for example through the use of the yeast two-hybrid system (FIELDS & STERNGLANZ 1994; SATO *et al.* 1994). Of course, the functional relevance of such genes to tree defense must be experimentally established in trees, and this could be accomplished using methods such as antisense techniques.

Disease resistance genes have recently been mapped in trees (DEVEY *et al.* 1995; WILCOX 1995). It is reasonable to hypothesize that these resistance genes, like their herbaceous plant counterparts, encode components of signal transduction pathways. As discussed in the previous sections of this review, a key function of those pathways is to activate or repress the activity or production of one or more enzymes that directly affect pathogen growth and/or survival (STASKAWICZ *et al.* 1995). Families known to segregate for these resistance genes are valuable tools to identify those defense enzymes, determine which enzyme activities are differentially regulated in response to specific pathogen races, and to determine the plant and fungal components that interact with the resistance gene product. Such

studies are required to establish the biochemical functions of resistance genes in forest trees.

## OPPORTUNITIES

In this article, we described a broad array of genes and molecules that are involved in regulating defense responses in plants. Mutagenized inbred lines have been invaluable for the identification of genes that carry out known, or previously unknown, regulatory steps in defense. These mutants serve to illuminate steps that, if studied using nongenetic means, may fail to identify defense components encoded by the plant genome. For example, the identification of mutants with constitutive defense responses (BOWLING *et al.* 1994) indicates there are genes whose products normally repress pathogen-induced signaling. Certain mutants show necrotic lesions that continue to expand, even in the absence of a pathogen, and indicate the presence of genes whose products normally act to localize cell death associated with the HR (DIETRICH *et al.* 1994).

There are also examples of antisense approaches to disrupt expression of genes potentially involved in defense. Systemin was originally purified from tomato leaves, and was initially proposed to be a mobile wound signal due to its potency as an inducer of proteinase inhibitors. Antisense methods were invaluable to demonstrate the direct involvement of systemin in systemic wound-induced transcription of proteinase inhibitor genes (MCGURL *et al.* 1992). The important message from these studies is that mutants provide the required framework for modeling a number of possible ways in which related genes and gene products may function in trees. The cloned genes themselves provide the tools to test those models, because using the genes in antisense experiments can simulate the effects of mutations at those loci.

Contributions from a variety of forest research approaches are needed to fully integrate these new technologies. Identification of genes that are likely to be involved in defense is an important area to which molecular biologists can contribute. Functional testing of genes through genetic engineering is an area in which tree tissue culture experts are needed. Identification of the relevant loci through genetic and physical mapping is an important area to which forest geneticists and breeders can contribute. Interpretation of how these genes contribute to defense phenotypes in a variety of genetic backgrounds requires the expertise of physiologists, pathologists and entomologists. Integration of research efforts will substantially enhance our fundamental understanding of the biological interactions that occur between trees and other organisms in forest ecosystems, and expand opportunities for effective and environmentally sound control of diseases and pests in intensively managed plantations.

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