Inquiry: The University of Arkansas Undergraduate Research Journal

Volume 3 Article 16

Fall 2002

Characterization of Wound-Inducible Genes Encoding Enzymes for Terpenoid Biosynthesis in Medicago Truncatula

Mandy M. Cox
University of Arkansas, Fayetteville

CHARACTERIZATION OF WOUND-INDUCIBLE GENES ENCODING ENZYMES FOR TERPENOID BIOSYNTHESIS IN *MEDICAGO TRUNCATULA*

Mandy M. Cox
Department of Food Science
Dale Bumpers College of Agricultural, Food and Life Sciences

Faculty Mentor:
Kenneth L. Korth
Department of Plant Pathology

Abstract:

In addition to having numerous applications as food flavorings and pharmaceuticals, terpenoids are an important class of defensive compounds that can accumulate in plants after pathogen infection or injury by insects. Sequences of DNA encoding putative terpene synthases and an oxidosqualene synthase, isolated from insect-damaged Medicago truncatula *leaves, were selected from an expressed sequence tag (EST) database. The cDNA clones were used as radiolabeled probes to analyze gene expression in leaves treated with factors known to trigger a defense response in plants. Transcript levels for all of the genes examined increased in response to artificial wounding, insect herbivory, and methyl jasmonate (meJA) treatments, whereas salicylic acid (SA) and glucose oxidase (GOX) had no measurable effects on transcript levels. Furthermore, the genome of* M. truncatula *was analyzed via DNA blots for an estimation of the number of copies of enzyme isoforms and indicate that each of the enzymes examined is encoded by a single-copy gene or a small gene family. The results show that* M. truncatula *can serve as a valuable source for novel terpene synthase clones and potentially for strong wound-inducible regulatory elements.*

Introduction:

Plants have unique responses to specific physical or chemical stimuli and this can be manifested by induction of genes putatively involved in a defense system. An example of a plant response to herbivory is demonstrated when lepidopteran larvae feed on a plant and volatile compounds are produced and systemically released from leaves. These volatile compounds can attract parasitoid wasps that are natural enemies of the herbivorous insect. In some cases wasps lay eggs in the lepidopteran larvae, and when they hatch, the wasp larvae devour the caterpillar from within (Turlings *et al.*, 1995). Thus the plant is protected indirectly, through an induced mechanism, from further damage by the herbivore (Kessler and Baldwin, 2001).

One of the most abundant and common classes of induced volatile plant compounds released in response to insect herbivory is the terpenoids. For the induced production of volatile terpenoids, genes are possibly induced to express the proteins needed for volatile biosynthesis. The chemical pathways that lead to terpenoids begin with a five-carbon building block known as isopentyl diphosphate (IPP). Two independent biosynthetic pathways can produce IPP, the mevalonate (MVA) pathway localized in the cytosol and the 2*C*-methyl erythritol 4-phosphate (MEP) pathway found in plastids. The five-carbon IPP units bond together in a head-to-head, head-to-tail, or head-to-middle fashion to form an acyclic prenyl phosphate. Specificity of the terpenoid produced is determined by the activity of the terpene synthase enzymes that convert the acyclic prenyl phosphate. Mono- and di -terpenes (C_{10} and C_{20} compounds, respectively) are thought to originate via the MEP pathway, whereas sesqui-, tri- (C_{15} and C_{30}, respectively), and poly-terpenes are produced predominantly by the MVA pathway.

Terpenoids have a wide range of activities, and their applications range from flavorings and perfumes to pharmaceuticals. Individual sesquiterpenes have been shown to act as antimicrobial phytoalexins and as insect antifeedants. One form of triterpenes, known as saponins, have antifeedant and antifungal properties that aid in plant defense (Haralampidis *et al.*, 2001).

A plant may respond differently to mechanical damage than to actual insect damage. These differential responses may be due to insect-derived oral factors that are perceived by the plant. A protein component in the saliva of some insects, glucose oxidase (GOX), may trigger a response by the plant (Felton and Eichenseer, 1999). Plant defense responses may also be triggered by the perception of volatile compounds such as methyl jasmonate (meJA). Methyl jasmonate is produced constitutively in plants, but often increases in abundance as plants undergo stress. Plants release meJA, which serves as a signal to surrounding plants that an herbivore is feeding nearby. However, salicylic acid (SA), a key modulator of systemic acquired resistance (SAR) to pathogens, may interfere with the meJA-centered defense pathway (Felton *et al.*, 1999).

For insect herbivory experiments, *Spodoptera exigua*, the beet armyworm, was employed to feed on plants. This caterpillar can be a serious pest of many crops such as alfalfa, bean, broccoli, corn, cotton, soybean, and tomato to list a few. Gene isolation and expression studies used *Medicago truncatula*, an excellent model plant for genetic analyses. This self-fertilizing legume possesses a small diploid genome and is easy to transform. It also has a relatively short generation time, allowing for more rapid genetic studies. In addition, a large scale genomics project in *M. truncatula* is underway, including the sequencing of expressed mRNAs. To date, over 140,000 expressed sequence tags (ESTs) are available in a public database (http://www.tigr.org/tdb/mtgi/); this includes nearly 10,000 ESTs that were derived from *S. exigua*-injured *M. truncatula*.

To understand plant defense responses to insects, it is important to characterize the regulation of genes that encode the enzymes that produce terpenes and saponins. Based on sequence similarities with known genes from other plant species, we selected three putative terpene synthase cDNA clones and one putative ß-amyrin synthase (responsible for saponin biosynthesis) clone from the EST database. Accumulation of mRNA for each gene was measured in response to several types of wounding and treatment with meJA, SA, and GOX.

Materials and Methods:

Plant and insect maintenance:

M. truncatula, line A17, was grown under standard conditions in a growth chamber at 24°C with a 16:8 hour light:dark regime. Fertilizer was administered at two-week intervals. All treatments were carried out in a greenhouse and were started at 0900 hours. Eggs of *S. exigua* were obtained from the USDA Gast Rearing Lab (Starkville, Miss.). Larvae were maintained on an artificial diet under conditions at approximately 22°C.

DNA probes:

DNA probes were derived from a cDNA library of *M. truncatula* leaves that had been subjected to *S. exigua* herbivory. The clones were identified in a search of the *M. truncatula* EST database based on sequence similarity with characterized terpene and beta-amyrin synthases from other plant species. Four clones were chosen for analysis. The terpene synthase clones and their Genbank accession numbers, were A4 (accession no. BF639687); A7 (accession no. BF640252); and A10 (accession no. BE321953). The putative ß-amyrin synthase clone was designated B3 (accession no. BF642680).

Insect Treatment:

Insects were placed on plants and allowed to feed for 24 hours before samples were taken. Leaves that had been damaged by the insect were "local," and undamaged leaves on the same trifoliate as an insect-damaged leaf were "systemic." Artificial wounding was conducted by cutting leaves with scissors, and only the locally damaged leaves for this type of treatment were collected for sampling after 6 hours. Control samples came from leaves of undamaged plants.

Chemical treatments:

For all meJA treatments, intact plants were placed in 18-L glass chambers. Cotton swabs with volumes of 0.5 µL, 1.0 µL, and 2.0 µL meJA (calculated volatile concentration 0.125 µM, 0.25 µM, and 0.5 µM, respectively) were inserted into the soil next to each plant in separate chambers, and the open-end bottom of each chamber was covered with a layer of foil and cheesecloth. A control plant was placed in a glass chamber with no added meJA. The plant was removed from the chamber after 1 hour, and leaf samples were taken after 6 hours. A second experiment was conducted using the same technique but over an 18-hour time period.

Glucose oxidase was applied to leaves after wounding with a tracing wheel. As the wheel was rolled across the leaves, it punctured small holes in the plant, and 20 µL of 1.3 mg/mL GOX solution was pipetted onto the leaves. This level of GOX approximates the concentration measured in *Spodoptera* labial gland extracts (H. Eichenseer, personal communication). Local and systemic tissues were collected at 1 and 6 hours after the treatment. For testing the effects of SA, solutions of SA in water were sprayed on plants at 2 mM, 4 mM, and 8 mM. For control treatments, the solvent with no SA was used. Samples were taken at 1 and 6 hours after treatment.

Nucleic acid analysis:

Leaves were collected, immediately chilled in liquid nitrogen, and stored at -70°C until analysis. Total RNA was extracted using TriReagent (MRC, Inc. Cinncinnati, Ohio) and separated on 1% agarose formaldehyde gels. The RNA was transferred to nylon membranes and hybridized with radiolabeled probes (Church and Gilbert, 1984). Insert DNA from individual cDNA clones was amplified via polymerase chain reaction and radiolabeled with ^{32}P in random-primer reactions (Sambrook *et al.*, 1989).

M. truncatula genomic DNA was isolated according to Junghans and Mettzlaff (1990). DNA was digested in individual reactions with *Bam*HI, *Eco*RI, or *Hind*III overnight at 37°C. Cleaved genomic DNA was separated on a 1.0% agarose gel, denatured, and transferred to a nylon membrane (Sambrook *et al.*, 1989). Hybridizations were carried out as for RNA blots.

Results and Discussion:

EST clone selection:

A search of the *M. truncatula* EST database revealed the presence of at least three putative terpene synthase clones and one ß-amyrin synthase clone that were derived from insect-damaged leaves. Functional assignment of the clones was based on the presence of highly conserved sequence domains for each

type of enzyme. Terpene synthase clones were designated A4, A7, and A10. The A4 sequence is predicted to encode a plastid transit signal at its amino terminus, indicating that it probably encodes a mono- or di-terpene synthase. The A7 and A10 clones bear highest sequence similarity to known sesquiterpene synthase clones, whereas the B3 clone is highly similar to a characterized oxidosqualene synthase, namely ß-amyrin synthase. Consistent with their putative enzymatic functions, none of the A7, A10, or B3 sequences contains a predicted plastid transit sequence. Full-length cDNA clones for each sequence were obtained and utilized to characterize transcript accumulation and gene copy number.

Wounding induces transcript accumulation:

Measurement of RNA accumulation demonstrates that genes encoding terpene synthases and ß-amyrin synthase were induced by artificial- and insect-wounding. For most of the genes examined, the highest levels of transcripts are observed in leaves injured by insect herbivory (Fig. 1). For each gene, low levels of RNA were present in undamaged leaves. Artificial damage also caused an increase in transcript accumulation, but generally not to the same degree as insect damage. This was not the case for A10, in which the highest transcript levels were observed after artificial damage. For clones A7 and A10, two bands were consistently observed on RNA blots. This suggests there might be multiple forms of similar transcripts, derived from independent genes, that are cross-hybridizing on the membranes. For the A10 transcripts, the two bands were always observed at similar levels, suggesting that if they are derived from independent genes, these genes must be coordinately regulated. Transcripts for each gene were also induced in systemic tissues of insect-damaged plants, although not to the same level as in damaged leaves. The A7 transcript was consistently the most strongly induced of all the genes examined. Insect herbivory is known to often elicit a greater plant response than artificial damage, probably due to the differing types of wounding or the presence of elicitor compounds associated with the insect (Korth and Dixon, 1999; Walker-Simmons, *et al.*, 1984). The enzyme products of the genes examined here are predicted to be involved in the biosynthesis of defense compounds, so it is not surprising that transcripts accumulated to high levels following wounding. Probing for the constitutively present ribosomal RNA indicated that equivalent amounts of total RNA were present in each gel lane.

Gene induction by methyl jasmonate:

Treatment with meJA also led to transcript accumulation for each of the genes examined. Transcript levels were low in untreated samples, but RNA accumulation increased dramatically when plants were exposed to the lowest concentration of volatile meJA applied, 0.125 mM (Fig. 2). Levels of A4 transcripts increased with increasing levels of meJA, whereas transcripts for the other genes were somewhat lower with increasing levels of meJA. Temporal expression of transcript accumulation was tested for the terpene synthase clones after exposure to volatile 0.25 µM meJA (Fig. 3). The A4 transcripts were present to some degree even in untreated control samples in this experiment, but the RNA blot seemed to indicate that transcripts accumulated to higher levels between 2-6 hours after initial exposure to meJA. For A7 and A10, transcript levels clearly increased with time and returned to normal levels by 18 hours after the initial exposure. As in wound experiments, the A7 transcripts were the most abundant, and the A7 transcripts also were induced earlier than the other genes tested.

Glucose oxidase and salicylic acid treatments:

Glucose oxidase is the most abundant protein found in labial gland saliva of lepidopteran insect larvae (Eichenseer and Felton, 1999). This enzyme has been shown to affect plant responses to chewing caterpillars and to wounding when it is applied to a wound site (J. Bede and G. Felton, personal communications). Addition of GOX did not have any effect on transcript accumulation for any of the genes used in this study; levels of RNA in leaves treated with GOX did not differ significantly from those treated with water (data not shown).

In addition, treatment with SA ranging from 2-8 mM did not affect transcript accumulation (data not shown), therefore SA alone seems not to be directly involved in the regulation of these genes.

Enzymes encoded by small gene families:

Probing *M. truncatula* genomic DNA with the selected cDNAs revealed that this species contains low copy numbers of the genes examined. Banding patterns indicate that the terpene synthase sequences are present in one to three copies each, whereas sequences hybridizing to the B3 ß-amyrin synthase clone are present in three to four copies (Fig. 4). Although the genes examined here all encoded well-conserved protein domains, it is well established that enzymes similar in primary sequence can differ greatly in terms of the specific products that they synthesize (Bohlmann *et al.*, 1998). Therefore, independent genes that cross-hybridize on DNA blots might encode enzymes with different specificities. At the very least, our data indicate that there is a low degree of genetic redundancy for the sequences that we tested via genomic DNA blots. Knowledge of the copy number of these genes will be important if efforts are made to isolate genomic promoter sequences.

Conclusions:

The results from our experiments reveal that insect herbivory and chemical treatments can cause systemic gene responses. Systemic leaves of wounded plants accumulated transcripts for terpene and ß-amyrin synthases, showing that gene-induction signals are being transported through the plant. In wounded leaves, the highest levels of transcript accumulation were generally observed after insect herbivory. This result is indicative of the presence of specific insect-derived elicitors or a unique type of

damage during chewing by lepidopteran larvae as compared to mechanical wounding.

Treatments with meJA, a central modulator of wound responses in most plant species, showed that this plant hormone may regulate expression of defense genes in *M. truncatula*. Addition of volatile meJA to intact plants caused a rapid and transient accumulation of terpene synthase- and ß-amyrin synthase-encoding genes.

The addition of GOX and SA, compounds known to affect expression of some plant defense genes, did not affect accumulation of any of the genes examined in this study. Although GOX can repress levels of some plant-defense gene transcripts (J. Bede and K. Korth, unpublished data), we did not see any differences in transcript levels when comparing wounded leaves with and without added GOX.

Understanding the regulation of the genes described here might aid ultimately in manipulation of plant defense responses or in the biosynthesis of valuable terpenoid compounds. The role of these genes' products in defense is suggested by the strong and rapid induction of transcripts that occurred following insect herbivory. With the basic characterization reported here, targeted studies of the function of these genes in defense can be carried out. The enzymes that these genes encode, or the promoter sequences that control their regulation, might provide valuable tools in production of plants that are more insect-resistant. This work clearly demonstrates that *M. truncatula* can serve as a new source of novel and valuable genes encoding enzymes involved in plant defense and terpenoid biosynthesis.

Acknowledgements:

Thanks to Dr. Jacqueline Bede for technical instruction and helpful discussions and S. Karen Gomez for technical assistance. Thanks also to Cam Romund and Dr. John Clark for helpful comments on the manuscript, and Joe Clouse, Bob Gonzales, and Richard A. Dixon (Noble Foundation) for help in providing cDNA clones. This research was supported by the Arkansas Science & Technology Authority and a C. Roy Adair Internship awarded to M. M. Cox by the Department of Plant Pathology, University of Arkansas.

References:

Bohlmann, J., G. Meyer-Guaen, and R. Croteau. 1998. Plant terpenoid synthases: Molecular biology and phylogenetic analysis. *Proc. Natl. Acad. Sci. USA*. 95:4126-4133.

Church, G.M. and W. Gilbert. 1984. Genomic sequencing. *Proc. Natl. Acad. Sci. USA*. 81:1991-1995.

Felton, G.W. and H. Eichenseer. 1999. Herbivore saliva and its effects on plant defense Against herbivores and pathogens. In *Induced plant defenses against pathogens and herbivores*. Agrawal *et al.*, eds. American Phytopathogical Society Press. St. Paul, Minn.

Felton, G.W., K.L. Korth, J.L. Bi, S.V. Wesley, D.V. Huhman, M.C. Mathews, J.B. Murphy, C. Lamb, and R.A. Dixon. 1999. Inverse relationship between systemic resistance of plants to microorganisms and to insect herbivory. *Current Biology* 9: 317-320.

Haralampidis, K., G. Bryan, X. Qi, K. Papadopoulou, S. Bakht, R. Melton, and A. Osbourn. 2001. A new class of oxidosqualene cyclases directs synthesis of antimicrobial phytoprotectants in monocots. *Proc. Natl. Acad. Sci. USA* 98:13431-13436.

Junghans, H. and M. Metzlaff. 1990. A simple and rapid method for the preparation of total plant DNA. Biotechniques 8:176.

Kessler, A. and I.T. Baldwin. 2001. Defensive function of herbivore-induced plant volatile emissions in nature. *Science* 291:2141-2144.

Korth, K.L. and R.A. Dixon. 1997. Evidence for chewing-insect specific molecular events distinct from a general wound response in leaves. *Plant Physiol.* 115:1299-1305.

Sambrook J., E.F. Fritsch, and T. Maniatis. 1989. *Molecular cloning*. 2nd edition. Cold SpringHarbor Laboratory Press. New York, N.Y.

Turlings, T.C.J., Loughrin, J.H., McCall, P.J., R^se, U.S.R., Lewis, W.J. and Tumlinson, J.H. 1995. How caterpillar-damaged plants protect themselves by attracting parasitic wasps. *Proc. Natl. Acad. Sci. USA* 92:4169-4174.

Walker-Simmons, M., H. H^llander-Czytko, J.K. Andersen, C.A. Ryan. 1984. Wound signals in plants: a systemic plant wound signal alters plasma membrane intregrity. *Proc. Natl. Acad.* Sci. USA 81:3737-3741.

Mandy Cox

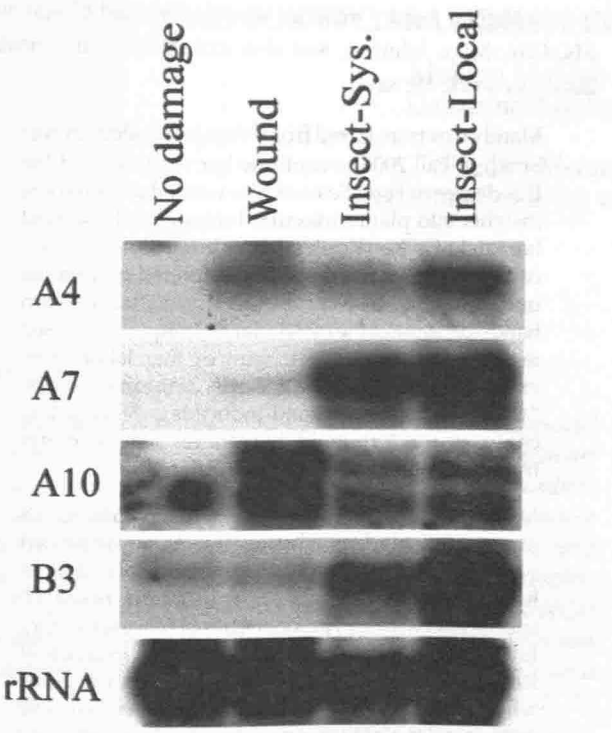

Figure 1. Transcript accumulation as indicated by RNA blots, in leaves following artificial damage with scissors ("wound"), or S. exigua herbivory. Leaves were collected at 6 hours after the initial damage. Membranes were hybridized with the indicated probes, and bands were visualized via autoradiography.

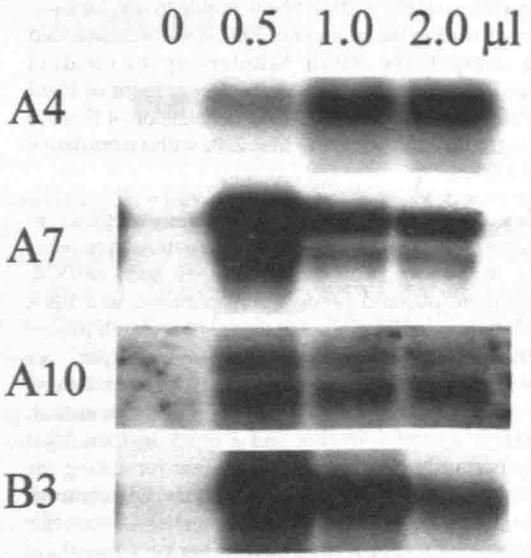

Figure 2. Transcript accumulation in leaves following exposure to differing levels of meJA. Leaves were collected 6 hours after intact plants were placed in a glass chamber with 0, 0.5 ml (0.125 mM), 1.0 ml (0.25 mM), or 2.0 ml (0.5 mM) meJA for 1 hour. Membranes were hybridized with the indicated probes, and bands were visualized via autoradiography.

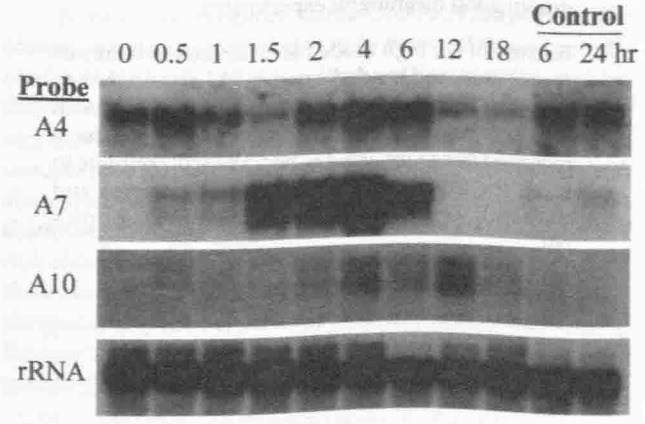

Figure 3. Transcript accumulation in leaves following exposure to 1.0 µl (0.25 µM) of meJA. Leaves were collected at the time indicated, after intact plants were placed in a glass chamber for 1 hour with meJA. Membranes were hybridized with the indicated probes, and bands were visualized via autoradiography.

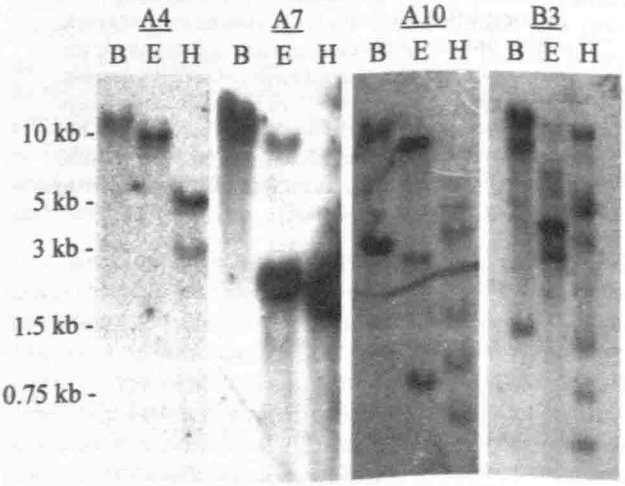

Figure 4. DNA blot analysis of terpene synthase and b-amyrin synthase clones. Genomic DNA from M. truncatula was digested with BamHI (B), EcoRI (E), or HindIII (H) and separated on a 1% agarose gel. Positions of DNA size markers are indicated at left. Identical membranes were hybridized with the indicated probes, and bands were visualized via autoradiography.

Faculty Comments:

Ms. Cox's faculty mentor, Ken Korth, had the following comments about Ms. Cox's work:

> Mandy Cox started working in my lab as a part-time employee early in 2001. She was able to continue her work during the summer of 2001 as she was awarded a competitive Adair Scholarship to conduct undergraduate research in the Department of Plant Pathology. Ms. Cox received a Bachelor of Science degree in Food Science in May 2002 with a cumulative 4.0 GPA.
>
> Ms. Cox came to my lab with little experience in laboratory work, but she quickly mastered a number of techniques in molecular biology such as PCR, bacterial plasmid preps, electrophoresis, and RNA blots. She initiated the independent research project that is described in the accompanying paper. Her work will comprise a major component of a peer-reviewed publication of which she will be a co-author. She is a careful worker and a quick learner. Most importantly, she has a strong talent for asking the right questions — a good sign that she will continue to succeed in academic (or any other) endeavors. She took a serious approach toward her coursework, as evidenced by her consistently being named to the Dean's and the Chancellor's Lists. By the time that Ms. Cox left my lab, she was among the best in the lab at performing several of the more challenging techniques that we use.
>
> Ms. Cox showed her dedication to her research project by completing the manuscript describing her work, which unfortunately can not fully communicate the time and effort that she put forth to make it a success. She worked well with everyone in the lab, and always impressed me with her respect for others and her maturity. I have high confidence in Ms. Cox's skills, intellect and potential, and it was truly a pleasure to have her working in our lab.

A second faculty member who has worked closely with Ms. Cox, Mike Johnson, was also extremely complimentary about her work. He said:

> Mandy Cox transferred from West Ark College in Fort Smith in Fall 2000 to continue her work toward the B.S. degree in Food Science. She wanted to gain some insights into plant molecular biology, and I steered her to take Dr. Ken Korth's Agricultural Biotechnology course. She then managed to get funded to do some undergraduate research in Dr. Korth's lab to learn hands-on some of the molecular techniques discussed in the above course. The fruit of that labor, with excellent guidance from Dr. Korth, is the manuscript, "Characterization of wound-inducible genes encoding enzymes for terpenoid biosynthesis in *Medicago truncatula*, which Ms. Cox has authored.
>
> My own independent observations of Ms. Cox's prowess as an undergraduate researcher are in concert with those of Dr. Korth. Ms. Cox has worked as an hourly researcher on an important research subcontract with the National Animal Disease Center/ Iowa State University to analyze the virulence of isolates of a bacterial pathogen, *Listeria monocytogenes*, which came from swine or turkey sources. My able colleague, Dr. Rama Nannapaneni has been coaching her on the techniques used, and we have high confidence in the results she has collected for us on this project. It is our intent that her name be included as a co-author on the refereed paper that will be submitted soon on this cooperative research project. She is a careful worker and asks lots of questions on the front end. This to me is the hallmark of a good researcher, learning to ask good questions so as to design good meaningful experiments.
>
> Because of her high academic achievement in formal course work and her dedicated work habits in the lab, I have invited Ms. Cox to do her M.S. degree research in my lab beginning Fall 2002. I feel fortunate to have recruited her to my lab. I expect she will continue to make the same kind of fine research progress and contributions as she has already made in Dr. Korth's lab.