Plant Breeding & Evaluation

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Genotyping of Coneflower Pollen for Linkage Analysis

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Index Words: Echinacea, Primer extension pre-amplification, Individual pollen, Isolated pollen, DNA, linkage analysis

Significance to Industry: DNA fingerprinting methods described in this report can be used to identify the genetic makeup of Echinacea. The 24 AFLP primer pairs identified here for Echinacea fingerprinting can be very useful for true-to-type identification in coneflowers. Molecular markers associated or linked with desirable traits can be used in breeding protocols for developing new varieties. The methods used in this research for the amplification of the Echinacea purpurea ‘Magnus’ pollen genome via primer extension pre-amplification and subsequent AFLP markers’ profiling will benefit the nursery industry by reducing the costs of genetic analyses and increasing the efficiency of developing new types. This is the first report on the AFLP analysis of PEP-products in Echinacea.

Nature of Work: Coneflower (Echinacea spp.) is an important herbaceous perennial in the nursery and medical herb industries (1, 4). Echinacea purpurea (L.) Moench is the most widely cultivated species, accounting for about 80% of commercial production (7). Recently, E. purpurea flower-tops were found to contain phytochemicals that effectively treated common colds (6). The Perennial Plant Association selected E. purpurea ‘Magnus’ a superior cultivar of coneflower as the Plant-of-the-Year for 1998. Therefore, in order to improve medicinal and ornamental qualities of Echinacea efficient methods for its genetic analyses are needed. Breeding efforts to increase the ornamental and medicinal properties of Echinacea have a long history (5). Molecular markers associated or linked to desirable traits can be used in breeding protocols for developing new varieties. Compared to conventional methods, the use of molecular markers can save development costs and time required for breeding, evaluation and cultivar release (8, 10). Since genetic markers of a plant segregate in its pollen grains during anthesis, plant breeders can use this segregation for genetic analysis. Segregation of molecular markers into pollen grains can be used to construct genetic maps, which enhances breeding efficiencies (2). Segregation analysis of molecular markers from individual pollen grains can generate genetic data for linkage mapping without the need of performing controlled pollinations and reduces the need to maintain large populations of plants. Thus, construction of genetic maps can simplify and enhance breeding efficiencies in coneflowers. To our knowledge, there is no report on the molecular characterization of E. purpurea ‘Magnus’ for genetic linkage analysis. In this paper, we report on the use of a novel method for the amplification of DNA from individual pollen grains and the subsequent analysis of AFLP markers.
Using a micromanipulator (Narishige, East Meadow, NY), pollen grains of *E. purpurea* ‘Magnus’ were individually collected in separate PCR (polymerase chain reaction) tubes that contained 5µl of pollen germination medium. The pollen germination medium consisted of 0.29M sucrose, 1.62mM H$_3$BO$_3$ and 1.27mM Ca(NO$_3$)$_2$, (pH 5.7). After 1-2 hours of incubation at room temperature, the DNA was amplified via MasterAmp™ Extra-Long PCR kit (EPICENTRE®, Madison, WI) using Zhang et al. (11) 15-mer primers (Operon Technologies, Alameda, CA). After staining with ethidium-bromide, Primer-Extension Pre-amplified (PEP) DNA products were visualized by 2% agarose gel and photographed using an Alphalager 2000 System (Alpha Innotech, San Leandro, CA). AFLP markers were generated from PEP products by DNA amplification via PCR using the AFLP System-Analysis Kit of GibcoBRL (Rockville, MD). Agarose gel analyses were conducted to check restriction digestion, pre-amplification and amplification of the DNA samples. AFLP profiles (DNA fingerprints) were scored by separating the selective amplification products through denaturing 6.5% polyacrylamide gel electrophoresis (9). All AFLP profiles were recorded with an automated DNA analyzer (Global IR® DNA Analyzer and Sequencer, LI-COR). Saga™ Generation 2- AFLP® Analysis Software Version 3.1 (Li-Cor Inc., Lincoln, Nebraska) was used to score AFLP profile images and subsequently to add the marker data to an Oracle® database.

**Results and Discussion:** AFLP assays of individual coneflower pollen grains were possible only after development of innovative methods described in this report. Because there is only a very small amount of DNA in a pollen grain, the primer extension pre-amplification (PEP) protocol of Zhang et al. (11) was used to increase the amount of genomic DNA in each sample. The PEP procedure was modified by using MasterAmp™ Extra-Long PCR kit (EPICENTRE®, Madison, WI) which contained the PCR enhancer betaine and Taq polymerase with 3’-5’ exonuclease activity along with an array of continuous random 15-mer primers. These enhancers have been reported (3) to increase the fidelity and accuracy of the PCR products, therefore, PreMix 3 and PreMix 9 were used for pollen DNA amplifications via PEP procedure. The amplification of pollen DNA by PEP resulted in genome distributed in fragments of varied lengths. AFLP analysis of the parental tissue revealed that 24 primer pairs were suitable for *Echinacea* fingerprinting and these were selected for subsequent pollen analyses. Restriction digestion was conducted on PEP products and PCR based AFLP markers were amplified from each individual grain using following primer pairs: E-ACA/M-CAA, E-ACC/M-CAA, E-ACA/M-CAC, E-ACC/M-CAC, E-ACG/M-CAC, E-ACT/M-CAC, E-ACG/M-CAG, E-ACT/M-CAG, E-AGC/M-CAG, E-AGG/M-CAG, E-AAG/M-CAT, E-ACC/M-CAT, E-ACC/M-CAT, E-AGG/M-CAT, E-AGC/M-CTA, E-ACA/M-CTC, E-ACT/M-CTC, E-ACA/M-CTG, E-ACC/M-CTG, E-ACT/M-CTT, E-AGG/M-CTT. AFLP profiles of *Echinacea* parent and more than 60 pollen grains were analyzed with Saga™ Generation 2- AFLP® Analysis Software Version 3.1 (Li-Cor Inc., Lincoln, Nebraska). Preliminary scoring of parental DNA bands for their presence or absence in pollen grains revealed the segregation of parental AFLP markers in its gametes. Currently, analyzed AFLP gels are being scored (Fig. 1) with the SAGA™ software to generate reports (Fig. 2) on presence/absence of each marker. The resulting AFLP-markers reports could be analyzed by linkage-analysis software to construct genetic-maps.
Literature Cited:


Acknowledgements: This project was funded by USDA/CREES Evans-Allen Grant and Capacity Building Grant # 2002-38814-12722. The authors also wishes to thank Cecilia Robinson, Deborah Long, Sarabjit Bhatti and Mirica Stevens for their technical assistance.
Figure 1: Amplification of AFLP markers from individual pollen grains and analysis via Saga™ Generation 2- AFLP® Analysis Software Version 3.1 (Li-Cor Inc., Lincoln, Nebraska). The software scored AFLP profile images and added the marker data to an Oracle® database.

Figure 2: Scoring of AFLP markers from corresponding individual pollen grains in Fig. 1 via SAGA™ Software (Li-Cor Inc., Lincoln, Nebraska). The software used AFLP data from Oracle® database to generate report on comparative presence/absence of AFLP-markers.
Nectar Characteristics of *Lantana* and Butterfly Visitation

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Significance to Industry: There have been few detailed studies conducted examining whether *Lantana camara* (L.) cultivars and lantana species are equally effective for attracting butterflies and whether nectar characteristics differ among them. In this study, differences in nectar carbohydrate concentration and composition among *Lantana camara* cultivars and lantana species as well as among flower location on the individual inflorescence were documented. The lantana visited preferentially in previous landscape evaluations exhibited sucrose / hexose ratios in nectar of 1.0 or greater in this study, with the exception of one cultivar. Additionally, inner flowers, which are typically yellow in multi-colored inflorescences and were visited preferentially on individual inflorescences, exhibited a greater sucrose / hexose ratio, sucrose concentration, and total carbohydrate concentration than middle or outer flowers. Due to the experimental design of the two studies, direct comparison of the results could not be made. However, trends observed suggest hybridization of lantana specifically for butterfly attraction should focus on nectar sucrose / hexose ratios of 1.0 and greater and increasing sucrose and total carbohydrate concentrations.

Nature of Work: Lantana is often listed as a butterfly attractant in popular press gardening articles and is a known source of nectar for feeding adult butterflies. Previous research (2) demonstrated butterfly visitation differs among lantana in a landscape setting with preferences for yellow flowers, or most recently opened flowers, on multi-colored inflorescences. Research has shown butterflies and other pollinators can be highly selective in their choice of nectar plants, based on many factors, including nectar characteristics such as volume, sugar concentration, and sugar composition (3, 4, 5, 6, 7). It has been documented that the ratios of sucrose to hexose and fructose to glucose are important nectar characteristics to pipevine swallowtail visitation (5). The objective of this study was to evaluate *L. camara* cultivars and *L. montevidensis* ‘Weeping Lavender’ (Spreng.) for differences in sugar concentration and composition. Additionally, flower location on an inflorescence (outer, middle, and inner positions), which corresponds to flower age in lantana, was also evaluated for differences in sugar concentration and composition.
On September 16th, 2003, one inflorescence at peak bloom from four single plant replications of *Lantana camara* 'New Gold', 'Irene', 'White Doves', 'Carlos', 'Firewagon', 'Cherry', 'Confetti', 'Hot Country', and 'Radiation' and *Lantana montevidensis* 'Weeping Lavender' was selected randomly from each plant. Plants were arranged in a completely randomized block design of four blocks on one greenhouse bench totaling 40 plants. Peak bloom was characterized by three or less unopened flowers present in the center of the umbel and flowers retained in the outer ring. Individual flowers were designated according to position in the umbel as either outer, middle, or inner flowers which also corresponded with flower age. Individual flowers were removed and hand pressure of 984.3 g/cm² (14 lb/in²), on average, was applied to the corolla tube forcing existing nectar through the basal opening. Nectar samples were collected between 12:00 and 4:00 pm from three flowers from outer, middle, and inner positions on the inflorescence using calibrated micropipettes (3 and 5 µL Drummond Microcaps). Temperature, relative humidity, and light levels were recorded at the beginning and end of each block during data collection. Each nectar sample was added to an individual microcentrifuge tube containing 25 µL of (ultrapure) water and stored at -80°C (-112°F).

Nectar samples were analyzed for fructose, glucose, and sucrose composition and concentration by high performance liquid chromatography (HPLC). The HPLC used included a Waters 501 HPLC pump (Waters Corporation, Milford, MA) for solvent delivery and a Waters Automated Gradient Controller injection system (Waters Corporation, Milford, MA) with a Refractive Index Detector HP 1047A refractometer (Hewlett Packard, Palto Alto, CA). Separation of carbohydrates was achieved using a Waters carbohydrate analysis column (3.9 × 300 mm, 125 Å, 10 µm). Operating conditions consisted of a flow rate of 2 mL•min⁻¹ and a run time of 15 min. An 85:15 acetonitrile:water solution was used as the mobile phase. Analysis was conducted with a Waters 745 Data Module (Waters Corporation, Milford, MA). All data were subjected to analysis of variance (ANOVA). Mean separation of sugar concentrations and sugar composition was performed using Duncan’s Multiple Range Test at the 5% level.

**Results and Discussion:** Analysis of sugar composition in the nectar indicated the presence of sucrose, glucose, and fructose. Nectar with sucrose / hexose ratios of more than 0.99 is considered “sucrose-dominant”, those with 0.50 to 0.99 “sucrose-rich”, those with 0.10 to 0.49 as “hexose-rich”, and those with less than 0.10 as “hexose-dominant” (1). The ratio of sucrose / hexose differed among cultivars with ‘Weeping Lavender’ having the highest ratio of 2.2 followed by and similar to ‘White Doves’ and ‘New Gold’ with 2.0 and 1.7, respectively (Table 1). Of the ten cultivars evaluated five are considered sucrose-dominant including ‘Weeping Lavender’, ‘White Doves’, ‘New Gold’, ‘Confetti’, and ‘Firewagon’. The lowest sucrose / hexose ratios were found in ‘Carlos’ (0.3), ‘Hot Country’ (0.3), and ‘Irene’ (0.3) which are considered hexose-rich. In previous research with watermelon cultivars, nectar sugar concentration and composition differed and the study concluded sugar composition in nectar may be partially responsible for the differences among watermelon cultivar attractiveness to honey bees (8). During previous landscape evaluations of lantana in 2002 and 2003, ‘New Gold’ and ‘Radiation’ were consistently visited by butterflies more than the remaining eight cultivars in the study (data not shown). Other cultivars visited preferentially,
but not as consistently, were ‘White Doves’, ‘Firewagon’, and ‘Weeping Lavender’. The cultivars visited preferentially in the landscape evaluation exhibited sucrose / hexose ratios in nectar of 1.0 or greater in this study, with the exception of ‘Radiation’ with 0.8. In general, ‘Carlos’ and ‘Cherry’ experienced the least butterfly visitation in the landscape and exhibited sucrose / hexose ratios of 0.3 and 0.8, respectively.

Cultivar differences were found for nectar fructose and sucrose concentrations (Table 1), while the glucose concentration was similar among cultivars (data not shown). The fructose concentration of ‘Firewagon’, 86.6 mg/mL, was greater than those of ‘New Gold’, ‘Radiation’, ‘White Doves’, and Lantana montevidensis ‘Weeping Lavender’. The sucrose concentration of ‘Weeping Lavender’, 142.6 mg/mL, was greater than those of ‘Carlos’, ‘Cherry’, ‘Hot Country’, ‘Irene’, and ‘Radiation’.

Nectar characteristics differed similarly among flower location on the inflorescence (outer, middle, or inner) for all cultivars (Table 2). The sucrose concentration of nectar collected from inner flowers (120.2 mg/mL) was more than double that of outer (43.04 mg/mL) or middle flowers (53.05 mg/mL). Total sugar concentration (fructose, glucose, and sucrose combined) found in inner flowers was 207.9 mg/mL compared to 125.1 mg/mL and 149.1 mg/mL in outer and middle flowers, respectively. Additionally, the sucrose / hexose ratio for inner flower nectar (1.6) was double that of outer and middle flowers. During landscape evaluations of lantana and butterfly visitation in 2002 and 2003, butterflies were observed preferentially visiting inner yellow flowers on multi-colored inflorescences (2). Based on the analytical results, nectar characteristics of inner flowers differ from outer and middle flowers similarly among cultivars and inflorescence color schemes (multi-colored vs. single colored). In previous research with similar results, three butterfly species clearly discriminated between three main nectar sugars with a hierarchy of preferences generally of sucrose over fructose over glucose (5, 6).

Literature Cited:

Table 1. Nectar fructose and sucrose concentration (mg/mL), and ratio of sucrose to fructose and glucose for Lantana collected September 16th, 2003.

<table>
<thead>
<tr>
<th>Species / Cultivar</th>
<th>Fructose (mg/mL)</th>
<th>Sucrose (mg/mL)</th>
<th>Ratio of sucrose to hexose (fructose + glucose)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lantana camara</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>‘Carlos’</td>
<td>71.6abc</td>
<td>35.6bcd</td>
<td>0.3c</td>
</tr>
<tr>
<td>‘Cherry’</td>
<td>50.5abc</td>
<td>54.4bcd</td>
<td>0.8bc</td>
</tr>
<tr>
<td>‘Confetti’</td>
<td>47.4abc</td>
<td>85.2abcd</td>
<td>1.0b</td>
</tr>
<tr>
<td>‘Firewagon’</td>
<td>86.6a</td>
<td>105.7ab</td>
<td>1.0b</td>
</tr>
<tr>
<td>‘Hot Country’</td>
<td>73.6ab</td>
<td>40.7bcd</td>
<td>0.3c</td>
</tr>
<tr>
<td>‘Irene’</td>
<td>54.2abc</td>
<td>24.3d</td>
<td>0.3c</td>
</tr>
<tr>
<td>‘New Gold’</td>
<td>37.7bc</td>
<td>102.9abc</td>
<td>1.7a</td>
</tr>
<tr>
<td>‘Radiation’</td>
<td>35.1bc</td>
<td>27.7cd</td>
<td>0.8bc</td>
</tr>
<tr>
<td>‘White Doves’</td>
<td>30.1bc</td>
<td>95.9abcd</td>
<td>2.0a</td>
</tr>
<tr>
<td>Lantana montevidensis</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>‘Weeping Lavender’</td>
<td>34.2bc</td>
<td>142.6a</td>
<td>2.2a</td>
</tr>
</tbody>
</table>

\(^{a}\)Means within columns followed by different letters are different according to Duncan’s Multiple Range Test, \(\alpha = 0.05\).

Table 2. Nectar sucrose concentration (mg/mL), total sugar concentration (mg/mL), and ratio of sucrose to hexose (fructose + glucose) for outer, middle, and inner flowers collected September 16th, 2003.

<table>
<thead>
<tr>
<th>Flower location</th>
<th>Sucrose (mg/mL)</th>
<th>Total sugar concentration (mg/mL)</th>
<th>Ratio of sucrose to hexose (fructose + glucose)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Outer(^{a})</td>
<td>43.03b(^{y})</td>
<td>125.1b</td>
<td>0.8b</td>
</tr>
<tr>
<td>Middle</td>
<td>53.05b</td>
<td>149.1b</td>
<td>0.8b</td>
</tr>
<tr>
<td>Inner</td>
<td>120.2a</td>
<td>207.9a</td>
<td>1.6a</td>
</tr>
</tbody>
</table>

\(^{a}\)Means for outer, middle, and inner flower locations calculated from all Lantana species and cultivars in study.

\(^{y}\)Means within columns followed by different letters are different according to Duncan’s Multiple Range Test, \(\alpha = 0.05\).
In Vitro Regeneration of *Lycopersicon esculentum* for Genetic Transformation Studies

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Index Words: Tomato, Antimicrobial peptide gene, Tissue culture

**Significance to Industry:** Putative transgenic tomato plants carrying the antimicrobial peptide genes have been regenerated. Once a sufficient number of plants are produced, they will be inoculated with Southern bacterial wilt (*Ralstonia solanacearum*) to determine if they have increased resistance to this pathogen. Since these genes have conferred resistance into other plants, there is a good possibility that clones with enhanced resistance to Southern bacterial wilt will be obtained. If this is the case, the system will be adapted to greenhouse crop such as geraniums.

**Nature of Work:** Tomato (*Lycopersicon esculentum* Miller 'Money-Maker') plants were regenerated from hypocotyl and cotyledon explants obtained from ten-day-old seedlings. Explants were plated on Murashige and Skoog (MS) supplemented with 0.1 mg/L of IAA with 1 mg/L of zeatin. For genetic transformation, calli generated from cotyledon and hypocotyls were infected with a bacterium (*Agrobacterium tumefaciens* EHA105) harboring the gene vectors for the Oxalate oxidase gene (pVSPB OxO) and PSE antimicrobial peptide gene (PSE39) that confer resistance to bacterial and fungal plant pathogens. Transgenic shoots were regenerated on selection media containing kanamycin or glufosinate ammonium.

*Ralstonia solanacearum* is a soil-borne bacterial plant pathogen that is endemic to most tropical and subtropical regions of the world. A notably virulent strain of this pathogen is race 3, biovar 2 which is adapted to the cooler temperatures of temperate regions of the world. This pathogen is listed on the USDA Agriculture Bioterrorism Act of 2002 as a serious pathogen of tomato, potato and many other plants such as the greenhouse geranium. The tomato and the Irish potato are two important crops that are very sensitive to the bacterial phytopathogen *Ralstonia solanacearum* Race 3. If this plant pathogen was dispersed on farmland producing crops such as tomato and potato, these farms would be unproductive. In nature, anti-microbial peptides (AMP) play important roles in the protection of plants and animals against a broad spectrum of pathogens. Some AMPs have cationic amphipathic secondary structures that can interact with negatively charged phospholipids found in the outer membranes of bacteria. Upon contact with these membranes, individual peptides aggregate to form pores in affected membranes causing lyses of bacterial cells. Attempts to enhance disease resistance in plant species through genetic transformation and over-expression of foreign AMP genes have been successful with a variety of plants. The goals of this project were to develop a transformation system in tomato with...
the AMP genes and oxalate oxidase gene (O XO) and regeneration systems for transgenic plants resistant to *R. solanacearum*. The development of resistant transgenic tomato would benefit growers and consumers of this crop.

**Plant Material and Disinfestations Procedures.** Tomato CV Money-Maker seeds were surface sterilized by submersion for 8 minutes in a solution containing of 6% sodium hypochlorite and 0.1% Tween and rinsed 3 times in sterile distilled water. Surface-sterilized seeds were germinated in magenta boxes containing MS medium (4) solid medium. Ten days after germination, cotyledons and hypocotyl explants were harvested and cultured on MS media solidified with 0.8% agar (w/v) and supplemented with different combinations of plant growth regulators.

*In vitro regeneration from explants of hypocotyls and cotyledons on five different regeneration media.* For callus and shoot formation, explants were excised from young seedlings and cultured on a MS basic salts media supplemented with 3% sucrose, 0.8% agar, and with 5 different combinations of plant growth regulators. The pH of all media used was adjusted to 5.8 with NaOH before autoclaving. All cultures were incubated in a growth chamber at 77 °F (25°C), 16 hours light cycles (white fluorescent tubes-2000 lux at the plant level) and sub-cultured to fresh media at 3-week intervals.

**Bacterial Strains and Plasmids.** For genetic transformation, explants from cotyledon and hypocotyls cultures were inoculated with *A. tumefaciens* EHA105 harboring the gene vectors for the oxalate oxidase gene (pVSPB_OxO) and the PSE antimicrobial peptide gene (PSE39) obtained from the New York State University.

**Agro-bacterium-mediated transformation.** Cotyledon and hypocotyl explants were harvested and pre-cultured on MS medium supplemented with 0.1 mg/L of indoleacetic acid (IAA) and 1 mg/L of zeatin for one day. After pre-culture, explants were cut along the mid-vein and immersed for 5 minutes in a solution that contained *A. tumefaciens*. Treated explants were blotted on sterile filter paper to remove excess solution and co-cultivated for 48-72 hours in sterile Petri dishes to allow time for *A. tumefaciens* to transfer the engineered DNA into wounded explants. Inoculated explants were then transferred to fresh MS medium supplemented with 0.1 mg/L IAA and 1 mg/L zeatin, and 500 mg/L carbinincillin and sub-cultured weekly to fresh MS medium.

**Determination of Herbicide Selection Concentration and Selection of transgenic plants.** Transgenic shoots were regenerated on selection media containing kanamycin (Sigma, St Louis, MO), the selection agent for the PSE39 plasmid, or glufosinate ammonium (Sigma, St Louis, MO) the selection agent for the pVSPB_OxO plasmid. Prior to selection, the threshold level for resistance to glufosinate ammonium resistance (bar gene) was determined by plating untransformed explants on regeneration media containing one of the following concentrations of glufosinate ammonium: 10, 20, 40, 50, 60, 80 and 100 mg/L. Plants treated with glufosinate became chlorotic within 4 week, the 20 mg/L glufosinate concentration was selected. After 25 days on the selection media, regenerated calli were transferred to a MS media supplemented with 250 mg/L carbinincillin,
1 mg/L zeatin, 0.5 mg/L zeatin riboside, 2 mg/biotin L and 20 mg/L glufosinate ammonium or 25 mg/L kanamycin (7) for elongation and sub-cultured weekly.

**Results and Discussion:** In this study transformed tomato with antimicrobial peptide genes were regenerated from hypocotyls and cotyledons. Previous transformations with tomato were with reporter genes (2 and 3).

*Callus initiation and plant regeneration from hypocotyls and cotyledons:* Tomatoes were regenerated from hypocotyls and cotyledons harvested from 10-day-old seedlings. MS media supplemented with 5 different combinations of auxins and cytokinins were evaluated. After 4 weeks of incubation shoots formed from cotyledon and hypocotyls explants on 3 different combinations of cytokinins and auxins. On these media, cotyledons produced more regeneration than from hypocotyl explants. In general, media containing zeatin and IAA grew better than in media containing 6-bezyladenine (BA) and 1-napthaleneacetic acid (NAA). Media containing combinations of BA and IAA were better for root induction and those containing Zeatin and NAA produced calli and few shoots. Cotyledon explants regenerated as compared to hypocotyls when zeatin and IAA were used. Media containing a combination of 0.1 mg/L of IAA and 1 mg/L of zeatin yielded the most and best shoots from cotyledon explants than with other media (Table 1). Similar results were obtained by Park et al, (6); however, this differs from a previous study where shoot organogenesis was regenerated with BA (5).

**Selection of transformed shoots.** Two different methods were used to select putative transgenic explants. The first method consisted of plating inoculated explants directly, after 48 hours of co-cultivation in sterile Petrie dishes, on the selection medium while with the second, inoculated explants were plated on the regeneration medium (without the selection agent) and sub-cultured onto the selection medium after the explants began to regenerate. With the first method, very few explants regenerated while with the second method, many more regenerated; however, some became chlorotic within one week indicating that some were not transformed. Confirmation of the transformed plants is in progress. The concentration of glufosinate in the selection medium of this study was twice the concentration used with Chinese elm (1).

**Literature Cited:**


Acknowledgement: This research was support by a NRI competitive grant award 2004-35605-14395 entitled ‘Effects of antimicrobial peptide genes in transformed tomato on disease resistance to Southern bacterial wilt’.

**Table 1.** Response of tomato explants to MS basic media supplemented with different combinations of plant growth regulators.

<table>
<thead>
<tr>
<th>Explants type</th>
<th>1mg/l BA 0.2mg/l NAA(A)</th>
<th>1mg/l BA 0.2mg/l IAA(B)</th>
<th>1mg/l zeatin 0.2mg/l IAA(C)</th>
<th>1mg/l zeatin 0.2mg/l NAA(D)</th>
<th>1mg/l zeatin 0.1mg/l IAA(E)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cotyledons</td>
<td>less shoots</td>
<td>induce roots</td>
<td>more shoots calli</td>
<td>More shoots</td>
<td></td>
</tr>
<tr>
<td>Hypocotyls</td>
<td>less shoots</td>
<td>induce roots</td>
<td>less shoots calli</td>
<td>Less shoots</td>
<td></td>
</tr>
</tbody>
</table>
Using Molecular Markers to Investigate Parentage of Azaleodendron Hybrids

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Index Words: Amplified Fragment Length Polymorphism, Polymerase Chain Reaction, *Rhododendron* ‘Fragrant Affinity’, *Rhododendron* ‘Fragrans’, Plant Breeding

Significance to Industry: Plant breeding programs benefit greatly from a thorough understanding of characteristics and genetics of parental germplasm. Unfortunately, many ornamental cultivars are of unknown origin and parentage, raising questions regarding their nomenclature, genetics, and utility in breeding programs. Historically, unknown hybrids were identified using taxonomically relevant characteristics such as floral morphology. This can be an effective means of identification but it can also lead to confusion when dealing with closely allied taxa. Advancements such as the polymerase chain reaction (PCR) and amplified fragment length polymorphisms (AFLP) have provided researchers with the tools for stringent DNA analysis through the use of molecular markers. Using these techniques it is now possible to screen putative parents against the hybrid of interest to quantitatively identify the parents. This technique was successfully used to determine that *Rhododendron* ‘Fragrant Affinity’ and ‘Fragrans’ are distinct cultivars of similar parentage. Additionally, AFLP markers revealed that *R. catawbiense* L. is not one of the parents as has been proposed, but is more likely a hybrid of *R. ponticum* L.

Nature of Work: A number of inter-subgeneric hybrids between evergreen rhododendrons and azaleas have been reported and are frequently referred to as azaleodendrons. The history of *R.* ‘Fragrant Affinity’ is vague. The name ‘Fragrant Affinity’ is not registered and we have been unable to find documentation on its origin, but has been reported to be a hybrid of *R. catawbiense* x *R. viscosum* (L.) Torr. (Kehr, personal communication). *R.* ‘Fragrant Affinity’ is similar in appearance to another azaleodendron, *R.* ‘Fragrans’. *Rhododendron* ‘Fragrans’, also reported to be a hybrid of *R. catawbiense* x *R. viscosum* was introduced by Paxton of Chandler & Sons Nursery, London, in 1843, and is described as, “A sweet-scented azaleodendron, fast-growing and compact with trusses of small flowers, pale mauve with centers lighter to white,” (6).

The purported parents of *R.* ‘Fragrant Affinity’ are taxonomically distinct. *R. catawbiense* is in the subgenus *Hymenanthes*, section *Ponticum*, subsection *Pontica* (3). This subsection contains evergreen species from North America, Europe, and Asia, including *R. hyperythrum* L. *Rhododendron viscosum* is in the subgenus *Pentanthera*, section *Pentanthera*, subsection *Pentanthera*. This subsection contains other fragrant, deciduous species from North America including *R. arborescens* (Pursh) Torrey, *R. atlanticum* (Ashe) Rehd. and *R. canescens* (Michx.) Sweet.
Molecular techniques can be used to assess genetic relationships among plants. The use of polymorphisms produced by arbitrarily primed polymerase chain reaction (AP-PCR) can distinguish between species as well as cultivars of the same species (2, 5, 7). AFLPs are PCR based markers used in the rapid detection of genetic diversity. The objectives of this project were to use these molecular techniques to identify the progenitor species of *Rhododendron‘Fragrant Affinity’ and determine if *R. ‘Fragrant Affinity’ and *R. ‘Fragrans’ are synonyms or distinct clones.

**Materials and Methods:** *Plant Material:* In order to elucidate the progenitor species of *R. ‘Fragrant Affinity’ we observed DNA polymorphisms in the hybrid that are present in each parental species yet distinct from closely related species. To accomplish this we included clones of the purported parents *R. catawbiense* and *R. viscosum* as well as related taxa. We compared *R. arborescens*, *R. atlanticum*, and *R. canescens* from subsection *Pentanthera* as well as *R. hyperythrum*, *R. ponticum*, and *R. maximum* from subsection *Pontica.* *Rhododendron ‘Fragrans’ was also evaluated to compare to ‘Fragrant Affinity’ and putative parents. All material was maintained at The Mountain Horticultural Crops Research and Extension Center in Fletcher, NC or J.C. Raulston Arboretum in Raleigh, N.C. except for *R. ‘Fragrans’ which was provided by Harold Greer, Eugene, Ore.

*DNA extraction:* A CTAB (Cetyltrimethylammonium bromide) extraction method described by Affandor et al. (1), modified using the Fast Prep FP120 (Thermo Savant, Holbrook, N.Y.) to grind tissue was used for isolation of nuclear DNA. Approximately 100-150 mg of tissue from newly opening leaves was collected in 2.0 ml conical tubes and kept cold until extraction.

*DNA amplification and electrophoresis:* DNA amplification was performed using six primer combinations under conditions described by Milla et al. (4). All primers and adapters were obtained from Sigma Genosys (The Woodlands, Texas) with the exception of labeled primers, which were obtained from LICOR Inc. (Lincoln, Neb.). Amplification products were separated on a 0.8% polyacrylamide gel for 3-hours in a Licor IR2 two-dye DNA sequencer using a 50-700bp standard

*Data Analysis:* AFLP-Quantar 1.0 (Keygene Products B.V., Wageningen, Netherlands) software package was used to score distinct, major, reproducible bands. Presence or absence of each AFLP fragment was scored as a binary unit character (present = 1, absent = 0). The simqual function in NT SYScp 2.1 (Exeter Software, Setauket, New York) was used to calculate Jaccard’s similarity coefficients and dendrograms were created using the unweighted pair group method with arithmetic averages (UPGMA).

*Results and Discussion:* The six primer combinations selected generated extensive DNA polymorphisms. We scored 139 bands ranging in size from 75 to 575 bp. The level of variation was very great between species and cultivars suggesting that with further analysis it may be possible to develop cultivar and species specific profiles. All samples were repeated at least twice except *R. hyperythrum 1 and R. viscosum 7* and the degree of reproducibility was high as exhibited by the nearly identical band patterns.
Based on binary band-share data, Jaccard’s coefficients of relationships were calculated and used to generate pairwise relationships (data not shown) and a dendrogram showing relationships among taxa (Figure 1). Using banding patterns and calculated genetic similarity it was clear that *R. catawbiense* is not a parent of ‘Fragrant Affinity’ or ‘Fragrans’. The two cultivars were nearly 80% genetically similar suggesting that they likely share the same parentage. Due to numerous monomorphic bands among species in the subsection *Pentanthera* it was not possible to determine the exact deciduous *Rhododendron* parent but there was a band at ~475 bp which was only present in one population of *R. viscosum* and *R. ‘Fragrant Affinity’. The high level of polymorphism observed between groups shows that there is potential to use more primer combinations to resolve this group.

**Literature Cited:**


Figure 1. Dendrogram showing grouping of related taxa by genetic similarity based on data derived from AFLP analysis (calculated using Jaccard’s coefficient of similarity).
Effects of Oryzalin Use on Buddleja

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Index Words: Chromosome doubling, Oryzalin, Buddleja, Breeding

Significance to Industry: Along with changing ploidy levels, oryzalin could be used to restore fertility, increase flower and leaf size, enhance color, or to develop more upright growth habits of ornamentals as seen with the interspecific Buddleja hybrid. With the success of tetraploid induction, an orange-flowered hybrid cultivar that could be grown outside throughout the South in USDA Zones 7-10 should be attainable.

Nature of Work: Buddleja L., better known as the ‘butterfly bush’, is comprised of roughly 125 species found throughout most of the world including North and South America, Asia, and Africa (5). Buddleja species can be found in various flower hues ranging from purple, pink, red, white, orange, and yellow with variations in between.

*B. davidii* Franch, the most important cultivated species found in the nursery industry, does not have a solid orange or yellow cultivar. However, there is a yellow hybrid, known as *B. × weyeriana* Weyer ex Rehd., which was the result of a cross between the yellow *B. globosa* Hope and the purple flowering *B. davidii* var. *magnifica* (E.H. Wilson) Rehder & E.H. Wilson (9). This plant had sterility problems due in part to ploidy differences between the diploid *B. globosa* (2n=38), and the tetraploid *B. davidii* (2n=76) (2).

Ploidy manipulation to equalize chromosome numbers usually occurs with the use of a chemical mutagen. The most commonly used chemicals for ploidy manipulation are colchicine and oryzalin. Using colchicine, Rose et al. (7) produced a tetraploid *B. globosa* for possible breeding with *B. davidii*. Colchicine has been the norm in the industry until recently when oryzalin was found to be as effective as colchicine at approximately 100-1,000 times lower concentrations, and is less hazardous to human health than colchicines (1, 3). They both act as spindle inhibitors by randomly disrupting spindle microtubules in mitosis, thus preventing microtubule polymerization and the polar migration of chromosomes at anaphase (8).

Currently there are no solid orange *B. davidii* cultivars in the market. The goal of this research was to see if oryzalin could be used to double the chromosomes of an orange-flowered diploid interspecific hybrid (*B. madagascarensis* Lam. × *B. crispa* Benth.) as described by Renfro (6), so that it could be crossed to a *B. davidii* cultivar in hopes of getting an acceptable commercial orange hybrid that could compliment color schemes used in landscape plantings.

For this research, single node sections were taken from stems of the F1 hybrid already in tissue culture. The first oryzalin experiment was started on August 4,
2004 with a factorial of varying concentrations (3, 5, and 7 µM) by different exposure times (1, 2, and 3-day) plus controls. Ten nodes were used for each combination, and plantlets were moved to new media every two weeks. Basic medium was Murashige and Skoog plus minimal organics (4) supplemented with 4 µM of NAA and 0.1 µM of BA before being adjusted to pH 5.7. Two month old plantlets were rooted using 1,000 ppm potassium salt of indole-3-butyric acid (KIBA) rooting hormone, and then acclimated to greenhouse conditions.

Results and Discussion: Eight plants exhibited phenotypic characteristics of polyploidy induction. Flow cytometric profiles confirmed the production of tetraploids and a mixoploid.

Tissue Culture: Concentrations of chemical and the length of exposure had an effect on mortality. Higher concentrations and greater length of exposure resulted in fewer surviving plantlets. Overall, no generalization could be made for the best combination of polyploidy induction based on concentrations and length of exposure. Tetraploids were formed in all three concentration and exposure times, with the highest concentrations and exposure times having slightly greater success.

Evaluation: Leaf size was the best phenotypic indicator at an early stage. Polyploid plants could be screened as early as 4 months based on an increased width-to-length ratio that were about twice that of control leaves. Mixoploid plants were produced, and those plants with only a single chimeric leaf remained diploid. Stem thickness, shortened internode length, and darker green color could not be used as distinguishing characteristics at an early stage, but were good indicators of polyploid induction at later stages. Flow cytometric analysis showed a significant increase in relative amounts of DNA in nuclei of oryzalin-treated tissues. Nuclei of diploid plants had relative DNA peak channels ranging from 310 to 360, with the tetraploid peak channels being roughly double with values of 617 to 627.

Literature Cited:


Landscape Performance of Twenty-three Field-grown Birch Accessions at Fayetteville and Hope, Arkansas

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Index Words: Landscape performance, Birch, Betula (L.)

Significance to industry: Twenty-three birch accessions were planted in field plots established at Fayetteville and Hope, Arkansas in spring 2002 to evaluate survival, growth rate and gas exchange. Betula nigra L., the only birch native to Arkansas, and its cultivars, Dura-Heat™ ‘BNMTF’ and Heritage® ‘Cully’, had the best survival and growth at both locations. Betula pendula Roth. ‘Trost’s Dwarf’, B. ermanii Cham., and B. albosinensis Burkill. were among trees with the worst landscape performance.

Nature of Work: Birches (Betula L.), especially white-barked taxa, are very popular ornamental plants in city landscapes. They are common trees and shrubs of boreal and north temperate zones of the Northern Hemisphere (Furlow, 1990). The natural distribution of most birches includes locations with high soil moisutures in the Northern United States (Atkinson, 1992). In general, white-barked birches do not grow well in the Southern landscape due to environmental stresses, among which water deficit stress is a major problem. Landscape performance of birch taxa has not been intensively studied in the south central region to provide useful information for growers and the landscape industry. The objective of this study was to evaluate survival and growth of birch taxa in Arkansas, and water deficit effect on different birch taxa.

Twenty-three birch accessions were potted in 3.8L (1 gallon) pots with Sun Gro SB300 Universal Mix (Sun Gro Horticulture, Inc., Bellevue, WA) in winter 2001. Container plants were grown in an outdoor lath house until they were planted out in April 2002. Birches were planted at the Agriculture Research and Extension Center, Fayetteville and Southwest Research and Extension Center, Hope, Arkansas on April, 12 and 5, respectively. Soil type at the Fayetteville site is a Captina silt loam (pH=6.2) and at Hope a Bowie fine sandy loam (pH=5.5). Trees were planted 2.5m (8 ft) apart in a row and distance between two rows of trees was 4.5m (15 ft). Plots were maintained using general horticultural practices such as mulching and annual fertilizing. Starting in May, soil moisture was monitored with irrometers (Irrometer Co., Riverside, CA) installed at 30cm (12 in.) depth close to the trunk in each row. Four irrigated/control rows were watered to keep the irrometer reading below 10 centibars while the other four water-stressed rows were watered after the reading exceeded 40 centibars.
The experimental design was a two-factor factorial completely randomized design with two factors of accession and irrigation regime, at Fayetteville, and completely randomized design with one factor, accession, at Hope.

Survival was recorded at both locations. Initial shoot height and trunk caliper were taken after trees were planted at each location. Initial height and trunk caliper was approximately 50 cm (20 in.) and 0.2 cm (1/2 in.), respectively. Although the annual growth data were collected, only data from fall, 2004 will be presented.

Results and Discussion: Survival------- The overall survival at Fayetteville was higher than Hope and may be related to higher ambient temperatures at Hope (data not shown). Only one birch accession, *B. albosinensis*, did not survive through 2004 at Fayetteville (Table 1). Nine accessions at Hope had total loss by 2004. Only accessions with at least 2 surviving replicate trees were included in the statistic analysis of growth. The birch accessions with the highest survival at both locations were *B. x Royal Frost* and the cultivars of *B. nigra*, Dura-Heat™ and Heritage®.

Fayetteville Growth------ The height and trunk caliper of birch were significantly different among accessions at Fayetteville (Fig. 1). There was no interaction of water-deficit treatment on shoot height or caliper. Water-deficit had significant effect on height, but not on trunk caliper (Table 2). Of each accession, trees with water-deficit treatment had lower value than control.

At Fayetteville, shoot height was significantly greater for *B. x Royal Frost*, *B. nigra* Dura-Heat™*, B. nigra* Heritage®* (both nursery sources), and *B. pendula* ‘Laciniata’ than for *B. maximowicziana*, *B. populifolia* ‘Whitespire’, and *B. utilis* var. jacquemontii. Trunk caliper was significantly greater for *B. nigra* Dura-Heat™, *B. nigra* Heritage®* (both nursery sources) than *B. alleghaniensis*, *B. papyrfera* Renaissance Upright™ ‘Uenci’, *B. pendula* (JF Schmidt N.), *B. platyphylla* Dakota Pinnacle™ ‘Fargo’, *B. populifolia* ‘Whitespire’, and *B. utilis* var. jacquemontii.

Hope Growth------ Similar to observations at Fayetteville, birch accessions including *B. x ‘Royal Frost’* and *B. nigra* Heritage®* (both nursery sources) were significantly taller than *B. lenta*, *B. papyrifera* Renaissance Upright™ ‘Uenci’, *B. populifolia*, and *B. populifolia* ‘Whitespire’. Trunk caliper was significantly greater for *B. nigra* Dura-Heat™, *B. nigra* Heritage®* (both nursery sources) than *B. lenta*, *B. papyrfera* Renaissance Upright™ ‘Uenci’, *B. populifolia*, and *B. populifolia* ‘Whitespire’.

In conclusion, water-deficit stress significantly reduced the growth of birch accessions at Fayetteville (data not shown). *Betula albosinensis* did not survive at either location. Of the surviving birch accessions, *B.utilis* var. jacquemontii performed the worst at Fayetteville. *Betula populifolia* ‘Whitespire’, *B. lenta* and *B. papyrfera* Renaissance Upright™ ‘Uenci’ performed the worst at Hope. The native Arkansas birch, *B. nigra* and its cultivars, Dura-Heat™ and Heritage®, were among the best field-performers at both locations in Arkansas based on survival and growth.
Literature Cited:

Table 1. Survival of birch accessions evaluated at Fayetteville and Hope, AR by November 2004.

<table>
<thead>
<tr>
<th>Accessions</th>
<th>Fayetteville # trees planted</th>
<th>Survival (%)</th>
<th>Hope # trees planted</th>
<th>Survival (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>B. x Royal Frost®</td>
<td>14</td>
<td>100</td>
<td>4</td>
<td>75</td>
</tr>
<tr>
<td>B. albosinensis</td>
<td>14</td>
<td>0</td>
<td>6</td>
<td>0</td>
</tr>
<tr>
<td>B. alleghaniensis</td>
<td>14</td>
<td>36</td>
<td>6</td>
<td>0</td>
</tr>
<tr>
<td>B. davurica</td>
<td>14</td>
<td>71</td>
<td>6</td>
<td>17</td>
</tr>
<tr>
<td>B. ermanii</td>
<td>14</td>
<td>14</td>
<td>6</td>
<td>0</td>
</tr>
<tr>
<td>B. lenta</td>
<td>14</td>
<td>21</td>
<td>6</td>
<td>50</td>
</tr>
<tr>
<td>B. maximowicziana</td>
<td>14</td>
<td>29</td>
<td>6</td>
<td>17</td>
</tr>
<tr>
<td>B. nigra (Evergreen N.)</td>
<td>14</td>
<td>57</td>
<td>6</td>
<td>0</td>
</tr>
<tr>
<td>B. nigra (J.F. Schmidt N.)</td>
<td>14</td>
<td>93</td>
<td>6</td>
<td>33</td>
</tr>
<tr>
<td>B. nigra Dura-Heat™ ‘BNMTF’</td>
<td>14</td>
<td>93</td>
<td>6</td>
<td>100</td>
</tr>
<tr>
<td>B. nigra Heritage® ‘Cully’ (Heritage N.)</td>
<td>14</td>
<td>100</td>
<td>6</td>
<td>83</td>
</tr>
<tr>
<td>B. nigra Heritage® ‘Cully’ (Hills N.)</td>
<td>16</td>
<td>94</td>
<td>6</td>
<td>100</td>
</tr>
<tr>
<td>B. papyrifera</td>
<td>15</td>
<td>80</td>
<td>6</td>
<td>0</td>
</tr>
<tr>
<td>B. papyrifera Renaissance Upright® ‘Uenci’</td>
<td>14</td>
<td>79</td>
<td>4</td>
<td>50</td>
</tr>
<tr>
<td>B. papyrifera Renaissance Reflection® ‘Renci’</td>
<td>14</td>
<td>79</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>B. pendula (Meadow Lake N.)</td>
<td>14</td>
<td>86</td>
<td>6</td>
<td>33</td>
</tr>
<tr>
<td>B. pendula (J.F. Schmidt N.)</td>
<td>19</td>
<td>68</td>
<td>6</td>
<td>0</td>
</tr>
<tr>
<td>B. pendula ‘Trost’s Dwarf’</td>
<td>14</td>
<td>7</td>
<td>6</td>
<td>0</td>
</tr>
<tr>
<td>B. platyphylla Dakota Pinnacle™ ‘Fargo’</td>
<td>18</td>
<td>93</td>
<td>6</td>
<td>0</td>
</tr>
<tr>
<td>B. platyphylla ‘Laciniata’</td>
<td>14</td>
<td>93</td>
<td>6</td>
<td>83</td>
</tr>
<tr>
<td>B. populifolia</td>
<td>14</td>
<td>44</td>
<td>6</td>
<td>17</td>
</tr>
<tr>
<td>B. populifolia ‘Whitespire’</td>
<td>14</td>
<td>79</td>
<td>6</td>
<td>33</td>
</tr>
<tr>
<td>B. utilis var. jacquemontii</td>
<td>14</td>
<td>36</td>
<td>6</td>
<td>17</td>
</tr>
</tbody>
</table>
Table 2. Statistic significance of accession and water-stress treatment on the height and caliper of field-grown birches at Fayetteville, Arkansas in Fall 2004.

<table>
<thead>
<tr>
<th>Source</th>
<th>Statistic significance</th>
<th>Accession x treatment</th>
<th>Accession</th>
<th>Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Height, Fall 2004</td>
<td>0.5550</td>
<td>0.0001</td>
<td>0.0022</td>
<td></td>
</tr>
<tr>
<td>Caliper, Fall 2004</td>
<td>0.6953</td>
<td>0.0001</td>
<td>0.2116</td>
<td></td>
</tr>
</tbody>
</table>

Figure 1. The height and trunk caliper of birch grown at Fayetteville, AR measured in fall 2004.

Figure 2. The height and trunk caliper of birch grown at Hope, AR measured in fall 2004.
Recent Developments in *Hydrangea* Interspecific Hybridization

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Index Words: *Hydrangea arborescens* (L), *Hydrangea involucrata* (Sieb.), RAPD, hybrid verification

Significance to Industry: Hydrangeas are among the most popular flowering shrubs with annual U.S. sales of approximately $32 million. While several species are cultivated and many cultivars are available, little work has been done on combining the best characteristics from different members of this genus via interspecific hybridization. This report describes the production of a hybrid between *H. arborescens*, which is one of the most cold-hardy members of the genus, and *H. involucrata*, which produces lavender-blue flowers. Production of this hybrid represents the first step in combining cold hardiness and flower color in *Hydrangea*. This work may eventually lead to the development of superior cultivars and expanded sales of *Hydrangea* in colder areas of the U.S.

Nature of Work: *Hydrangea arborescens*, or smooth hydrangea, offers large corymbs of pure white flowers in early summer and is cold-hardy to zone 4. *Hydrangea involucrata* produces lavender-blue flowers in mid-summer, but is only rated as cold-hardy to zone 6 or 7. The objective of this study was to hybridize these two species with the intention of combining the cold hardiness of *H. arborescens* with the flower color of *H. involucrata*.

Reciprocal crosses were made between *H. involucrata* and *H. arborescens* ‘Dardom’ (White Dome®) during Summer 2003 using previously published techniques (3). Seeds were collected in early fall and sown in a greenhouse in January 2004. All progeny of the interspecific crosses were grown in a greenhouse following germination.

RAPD markers were used to verify hybridity. Young expanding leaves were collected from six of the progeny, freeze-dried, and stored at -70°C (-94°F) until needed. DNA was extracted with a Dneasy Plant Mini Kit (Qiagen, Valencia, CA) following the manufacturer’s instructions. DNA content was estimated by loading samples into a 1% agarose gel and comparing visually with known standards. Amplification reactions were carried out in 25µl volumes containing 1X MasterMix (Eppendorf AG, Hamburg, Germany), 0.2 µM primer set 100/4 (UBC, Vancouver, British Columbia, Canada), and 10 ng DNA template. Amplification involved an initial denaturation step of 95°C (205°F) for 5 min and then 45 cycles of 95°C for 1 min, 36°C (97°F) for 1 min and 72°C (162°F) for 2 min. This was followed by a final extension step of 72°C (162°F) for 2 min. After amplification, 2 µl of each sample was loaded onto a 1% agarose gel and run in 1X TBE buffer at 120V for 35 min. Gels were stained with ethidium bromide and reaction products were viewed using an Alphalmager (Alpha Innotech Corp., Alameda, CA).
Results and Discussion: Only crosses made with *H. arborescens* as the maternal parent resulted in viable seed production (Table 1). Of the more than 500 seed obtained, only eight plants remain alive. The surviving plants possess a range of growth rates, currently ranging from 8 to 35 cm (3.1 to 13.5 in) in height and from 12 to 50 cm (4.7 to 19.5 in) in width. Leaf shape is consistent among progeny and is intermediate in appearance between parents.

A total of 43 primers were tested, generating from 1 to 6 markers. The size of the amplified DNA fragments ranged from 100 to 1200 base pairs. The progeny had a combination of both parental species banding patterns in four primers (UBC: 335, 336, 341, 349; Fig.1). Six primers produced banding patterns identical in the hybrids and paternal parents (UBC: 308, 337, 338, 345, 348, 349). Banding profiles generated from these ten primers confirmed hybridity in the progeny tested.

Previous efforts to combine flower color and cold hardiness in *Hydrangea* via interspecific hybridization have met with only limited success. *Hydrangea macrophylla × H. paniculata* (5) hybrids could be produced only by using in vitro embryo rescue. These hybrids were lacking in vigor and sterile (4). Production of *H. macrophylla × H. arborescens* hybrids required both embryo rescue and subsequent regeneration from callus culture (1); all plants obtained had aneuploid chromosome numbers (2). This report details the first confirmed *Hydrangea* interspecific hybrid that was obtained without the use of embryo rescue and that involved *H. involucrata* as one of the parents. We will continue to work with this hybrid in an effort to develop cold hardy hydrangeas with blue flower coloration.

Literature Cited:
Table 1. Results of hybridization between *H. involucrata* and *H. arborescens* 'Dardom'.

<table>
<thead>
<tr>
<th>cross</th>
<th>no. crosses made</th>
<th>no. viable seed obtained</th>
<th>no. surviving plants</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>H. involucrata</em> × <em>H. arborescens</em> ‘Dardom’</td>
<td>78</td>
<td>0</td>
<td>- - -</td>
</tr>
<tr>
<td><em>H. arborescens</em> ‘Dardom’ × <em>H. involucrata</em></td>
<td>74</td>
<td>36</td>
<td>8</td>
</tr>
</tbody>
</table>

Figure 1. RAPD profiles generated from primer UBC-341. Lane 1 = 100 kb marker, HA = *H. arborescens* ‘Dardom’, 2-13 = *H. arborescens* ‘Dardom’ × *H. involucrata* progeny #735-2 through 735-13, HI = *H. involucrata*. The maternal and paternal parents had only one band each: *H. arborescens* was represented with a band of 600 bp and *H. involucrata* with a 400 bp band. Both bands were present in all progeny tested.
Progress Towards Less Invasive *Buddleja*

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Index Words: Butterfly bush, Invasiveness, *Buddleja davidii* Franch., Hybridization

**Significance to Nursery Industry:** The development of butterfly bush hybrids that are less invasive in the environment will slow the listing on noxious weed lists of this attractive ornamental shrub.

**Nature of work:** *Buddleja davidii* Franch. is the most commonly cultivated species in the genus *Buddleja* L., yet it possesses many of the characteristics associated with invasive plants. The seeds are small, winged and wind-dispersed. They germinate quickly and the resulting plants reach maturity rapidly. Several states have placed or are considering placing butterfly bush on their state noxious weed list.

One of the goals of the *Buddleja* breeding program at the University of Arkansas is to reduce the potential for invasiveness found in certain *Buddleja* species. Two approaches are used. The first is to alter plant morphology, specifically seed and fruit characteristics. The second is to produce hybrids with an odd ploidy number. For the first approach, the two species found in the section *Nicodemia* (Tenore) Leeuw. were crossed with members of section *Buddleja*. *Buddleja* in section *Nicodemia* produce a berry-like capsule that does not dehisce upon maturity. The seed are not winged. In this section, *B. indica* Lam. the parlor oak, is a tetraploid while *B. madagascarensis* Lam. is a diploid (2,4). For the second approach, we made 169 crosses where one parent was reported to be a diploid and the other parent a tetraploid *Buddleja* species (5).

**Results and Discussion:** From the 169 controlled crosses between diploid and tetraploid *Buddleja*, two viable hybrids were obtained. The first hybrid was a single plant from the cross *B. davidii* v. *nanhoensis* Rehd. ‘Monum’ (Petite Plum™) × *B. lindleyana* Fort. ex Lindl. ‘Miss Vicie’ and the second hybrid was a single plant from the cross *B. davidii* v. *nanhoensis* ‘Moonshadow’ × *B. asiatica* Lam. The cross between *B. davidii* v. *nanhoensis* and *B. lindleyana* was first reported by Elliot et al. (1) and named *B. × luteolufaucia*. These two *B. × luteolufaucia* hybrids, one from North Carolina and the other developed at Arkansas, have been shown to be tetraploids, possibly due to unreduced gametes contributed by the *B. lindleyana* pollen parent (1,5). The plants are fertile.

The second cross, *B. davidii* v. *nanhoensis* × *B. asiatica* was made in 2001. A single seedling was obtained and planted to the field in 2002. In three subsequent growing seasons, capsule development on this plant has been minimal and no viable seed obtained. Cytological examination conducted on this plant indicate that it is a triploid, 2n=57 (5). Winter hardiness in Zone 6b/7a has
not been a problem, despite *B. asiatica* as a parent. Viable, above-ground growth is maintained through the winter.

Intersectional crosses were made between sections *Nicodemia* and section *Buddleja*. From the cross between the diploid (2n=38) *B. madagascarensis* and the diploid (2n=38) *B. crispa* Benth. twenty progeny were raised (5). The F1 hybrid exhibited characteristics that were intermediate between the two parents. The fruit on the F1 was a white, berry-like capsule that did not dehisce upon maturity. No viable seed was obtained from this cross. Ten of the progeny from this cross have survived two winters in Fayetteville, Ark. and flower in late summer and early fall on plants that reach 1-2 m in height.

Previously we have made crosses between the tetraploid *B. davidii* and the tetraploid *B. indica* (3). Plants from this cross has survived three winters in Fayetteville, Ark. Plants behave as herbaceous perennials with new growth emerging from the base of the plant in mid to late May. Flower characteristics are intermediate between the two parents. Overall, these plants are more valuable for their foliage effect than the flowers. Backcrosses to the *B. davidii* parent (‘White Bouquet’) resulted in plants with increased flower count while retaining fruiting characteristics of the F1 (non-dehiscent fruit).

**Literature Cited:**


Heritability of Ornamental Foliage Characteristics in Diploid, Triploid, and Tetraploid Hypericum androsaemum L.

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Index Words: Tutsan St. Johnswort, Variegation, Polyploidy, Breeding, Genetics, Invasive

Significance to Industry: Increasing awareness and concern about invasive plants (3) provides an opportunity for plant breeders to address this problem by developing seedless cultivars with improved ornamental characteristics. Studies investigating the heritability of variegated and purple leaf foliage types in H. androsaemum indicated that both are simple recessive traits at the diploid level and are not linked. This has led to the recovery of a novel phenotype combining the variegated foliage of ‘Glacier’ with the purple foliage of ‘Albury Purple’. This novel phenotype was also recovered at the triploid and tetraploid level. The lack of a triploid block in H. androsaemum allowed the recovery of triploid progeny that are currently being evaluated for fertility and ornamental traits. Triploids are typically highly infertile (4) and the development of these plants will facilitate the release of a series of seedless H. androsaemum with a range of ornamental foliage types. The present research demonstrates the feasibility of breeding simultaneously for ornamental traits and non-invasiveness.

Nature of Work: Hypericums, or St. Johnsworts, are popular summer flowering, deciduous or semi-evergreen, shrubs and perennials. The genus Hypericum [Clusiaceae (Guttiferae)] contains approximately 425 species distributed worldwide (1, 2). Of the species represented in the nursery trade, H. androsaemum (tutsan St. Johnswort) and its hybrids are notable for their floriferous and hardy nature and cultivars with unique foliage and fruiting characteristics. Hypericum androsaemum is native from Western Europe, through the Mediterranean, and into Northern Iran. Unfortunately, it is now also found naturalized in Australia, New Zealand, and Chile (2) and is a potential problem in the U.S. Pacific Northwest (Dan Hinkley, pers. comm.). This semi-evergreen shrub grows less than three feet in height and has abundant, yellow, star-like flowers and fleshy red fruit which mature to a brown capsule. Hypericum androsaemum performs best as a landscape shrub in the cooler parts of U.S.D.A. zones 6-8. Within the genus Hypericum it is the only species that contains both variegated and purple foliaged cultivars: H. androsaemum ‘Glacier’ with highly variable, white-mottled leaves and ‘Albury Purple’ with wine-purple foliage and red-veined yellow flowers. In order to initiate a breeding program for the simultaneous development of ornamental and non-invasives Hypericum, it is desirable to establish the mode of inheritance for these foliage traits, of which little is known for woody ornamental plants. The objective of this study was to investigate the mode of inheritance for two different foliage types (variegated and purple) at three different ploidy levels (diploid, triploid, and tetraploid) in Hypericum androsaemum.
Plants of *H. androsaemum* ‘Glacier’ (G), ‘Albury Purple’ (AP), and hybrids were grown and maintained in greenhouses at the Mountain Horticultural Crops Research Station, Fletcher, N.C. Segregation ratios were determined for diploid crosses in reciprocal di-hybrid F₁ and F₂ families and backcrosses to each parent (see Table 1). Tetraploid segregation ratios were calculated for F₂[4x] from selfed autotetraploid G × AP F₁’s (F₁,1[4x]). Triploid segregation ratios were determined from crosses between autotetraploid G × AP (F₁,1[4x]) and diploid G × AP (F₁,1[2x]). For all crosses, flowers were emasculated prior to anthesis. Pollen was collected from recently dehisced anthers and either used fresh or dried overnight at 41°F (5°C) using indicator drierite (Drierite, Xenia, Ohio), and stored at 41°F (5°C) for use in subsequent crosses. Pollen was applied to stigmas daily using small brushes until stigmas turned brown after ≈ 10 days. Fruit were collected when capsules turned from bright red to brown and were dried at room temperature for 1-3 days. Seeds were separated and sown onto the surface of a 1 peat : 1 vermiculite (by volume) medium and misted regularly until germination occurred in 1 to 4 weeks. Seedlings were transplanted into 40-cell trays containing seedling medium and remained there until phenotype scoring. Phenotypes were scored when seedlings had > 3 sets of true leaves. Heritability of variegated and purple leaf traits was tested on the hypothesis that both traits were inherited in a simple Mendelian recessive manner. Chi-square analysis was conducted on segregating families (F₂,1[2x], F₂,2[2x], BC₁,1, BC₁,2, F₂[3x], and F₂[4x]). The Chi-square test of independence for linkage was calculated for diploid F₂’s (F₂,1[2x] and F₂,2[2x]). All crosses were conducted during the summers of 2003 and 2004.

**Results and Discussion:** Reciprocal di-hybrid crosses were performed to rule out maternal affects on inheritance. In both G × AP and AP × G F₁’s, all seedlings were green (data not shown), demonstrating that neither traits were maternally inherited via extra-nuclear plastids. Furthermore, the lack of variegation and purple pigment in both F₁ crosses rules out incomplete dominance for each trait. Segregation ratios for the diploid F₂’s approximated the predicted ratio of phenotypic classes of 9 wild type (green) : 3 variegated : 3 purple : 1 variegated and purple; supporting our hypothesis of simple recessive inheritance for both traits (Table 2). The chi-square tests of independence for linkage (χ²=1.75, P = 0.19 and χ²=0.02, P = 0.89 for G × AP and AP × G, respectively) reveal no evidence for linkage between the two traits.

Backcross data generally supports the simple recessive model for both traits, though for BC₁,2 we had a few spontaneous variegated seedlings occur when we expected a 1 green : 1 purple in the progeny (Table 2). The presence of the variegated types is not explained by accidental selfing or pollen contamination. At higher ploidy levels, expression of simple recessive traits becomes less frequent due to a lower probability of homozygosity with increased numbers of alleles from duplicate chromosomes. At the triploid level, the data was an excellent fit to the simple recessive model (χ²=1.38, P = 0.71) (Table 2). However, at the tetraploid level, we observed a greater number of variegated phenotypes than predicted, and thus had a poor fit to our model (χ²=141.2, P < 0.001) (Table 2). This lack of fit was not explained by random chromosome or chromatid assortment at the variegated locus (data not shown) and is difficult to explain in light of the lack of deviation at the purple locus. The greater than expected expression of variegated phenotypes at the tetraploid level may indicated a partial shift from qualitative
to quantitative inheritance or other modifying genetic factors. This research is continuing to evaluate fertility of these hybrids.

**Literature Cited:**


**Table 1.** Crosses between *Hypericum androsaemum* ‘Glacier’ (G) and ‘Albury Purple’ (AP) and families produced.

<table>
<thead>
<tr>
<th>Cross</th>
<th>Ploidy</th>
<th>Families</th>
</tr>
</thead>
<tbody>
<tr>
<td>G × AP</td>
<td>2x</td>
<td>F1,1[2x], F2,1[2x]</td>
</tr>
<tr>
<td>AP × G</td>
<td>2x</td>
<td>F1,2[2x], F2,2[2x]</td>
</tr>
<tr>
<td>G × F1,1[2x]</td>
<td>2x</td>
<td>BC1,1</td>
</tr>
<tr>
<td>AP × F1,1[2x]</td>
<td>2x</td>
<td>BC1,2</td>
</tr>
<tr>
<td>F1,1[3x] × F1,1[2x]</td>
<td>3x</td>
<td>F2[3x]</td>
</tr>
<tr>
<td>F1,1[4x] × self</td>
<td>4x</td>
<td>F2[4x]</td>
</tr>
</tbody>
</table>

**Table 2.** Segregation for *Hypericum androsaemum* foliage color types in families derived from ‘Glacier’ (G) and ‘Albury Purple’ (AP).

<table>
<thead>
<tr>
<th>Cross</th>
<th>Family</th>
<th>Progeny (no. seedlings)</th>
<th>Expected Ratio</th>
<th>x²</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>G × F1,1[2x]</td>
<td>BC1,1</td>
<td>200: 196: --: --: 1:1: 0.04: 0.84</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AP × F1,1[2x]</td>
<td>BC1,2</td>
<td>178: 4: 221: 3: 1:1: 4.63: 0.03</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>F1,1[4x] × self</td>
<td>F2[4x]</td>
<td>701: 76: 22: 1: 1225:35:35:1: 141.2: &lt;0.001*</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Number of progeny for each phenotypic class (Green = wild type; Var. = variegated type; Purple = purple type; V/P = variegated and purple combination).

*Unexpected variegation, which can not be explained by accidental self- or cross-pollination. These seedlings were not used to calculate the chi-square statistic (x²).
Intergeneric Hybrids between *Gordonia lasianthus* and *Franklinia alatamaha*

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**Index Words:** Plant breeding, Franklinia, Gordonia

**Significance to Industry:** Controlled crosses between *Franklinia alatamaha* (Franklinia) and *Gordonia lasianthus* (Lobloly-bay) resulted in intergeneric hybrid progeny that are now being evaluated for potential new landscape trees.

**Nature of Work:** A small population of *Franklinia alatamaha* Bartr. ex Marshall was discovered by John and William Bartram along the banks of the Altamaha River in Georgia in 1765 (1). Seeds were collected from these trees over a number of years, but the species has not been seen in the wild since 1790. Although *F. alatamaha* is considered extinct in the wild, it persists in cultivation and makes an attractive landscape tree. This species is valued for its showy white flowers, bright crimson/maroon fall foliage color, and excellent cold hardiness (USDA Zone 5).

*Gordonia lasianthus* (L.) Ellis is an evergreen shrub or small tree native to the Coastal Plain through Virginia, Florida, and Louisiana, typically growing in riparian habitats. *Gordonia lasianthus* is valued for its attractive foliage and large, white, showy flowers. Cold hardiness is limited (USDA 7b/8a).

In 1974 Dr. Elwin Orton, Jr. successfully crossed *G. lasianthus* with *F. alatamaha* and produced 33 hybrids (2). Orton (2) further reported that the seedlings grew vigorously during the first growing season and that a number of them bloomed the following year. However, all of the plants eventually died, possibly due to some type of genetic incompatibility or a pathogen (e.g., *Phythophthora*) (2). Although Orton’s report was somewhat discouraging, hybridization between *F. alatamaha* and *G. lasianthus* could potentially combine the cold hardiness of *F. alatamaha* with the evergreen foliage of *G. lasianthus* and broaden the genetic base for further breeding within these monotypic genera. The objective of this project was to make a renewed attempt at developing intergeneric hybrids between *F. alatamaha* and *G. lasianthus*.

Controlled crosses were made between *Franklinia alatamaha* (female parent) and *Gordonia lasianthus* (male parent) in Aug. 2002 at the Mountain Horticultural Crops Research Station, Fletcher, N.C. Pollen was collected from a specimen of *G. lasianthus* ‘Varigata’ (accession 2002-090). Flowers of *F. alatamaha* (accession 1998-450) were emasculated and hand pollinated. Approximately 75 flowers were pollinated. Seeds were collected in Sept. 2003, stratified in moist media for 90 d at 43 EF, and germinated under greenhouse conditions.
Results and Discussion: A total of 9 hybrids were propagated from seed in Jan. 2004. Growth was fast and many of these progeny attained heights greater than 2 m and flowered within 9 months of germination. Characteristics of the progeny clearly demonstrated their hybrid nature. In particular, hybrids are semi-evergreen and leaf size and shape is intermediate between the parents. The stamen filaments of the hybrids often were malformed and sometimes flattened, enlarged, and petaloid in appearance. All of these plants and subsequent cuttings continue to grow well and are now being evaluated for their potential as nursery and landscape plants.

Literature Cited:
‘Shiloh Splash’ River Birch

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Index Words: Betula nigra L., plant evaluation, propagation

Significance to Industry: ‘Shiloh Splash’ river birch\(^{\text{PPAF}}\) (\textit{Betula nigra} ‘Shiloh Splash’\(^{\text{PPAF}}\)) is a new, variegated cultivar of river birch that has utility as a shrub, small tree, or hedge in the landscape. Propagation studies found that this cultivar roots readily from terminal, softwood cuttings (taken in mid July) with optimal treatments consisting of basal dips of 2,000 - 4,000 ppm of either KIBA or IBA in 50% isopropyl alcohol.

Nature of Work: ‘Shiloh Splash’ river birch\(^{\text{PPAF}}\) (\textit{Betula nigra} ‘Shiloh Splash’\(^{\text{PPAF}}\)) is a new, variegated cultivar of river birch that was discovered by Mr. John Allen at Shiloh Nursery in Harmony, N.C. This cultivar is distinct with its attractive leaf variegation (an ivory-yellow margin and green center) and smaller size and growth rate compared to typical river birch. ‘Shiloh Splash’\(^{\text{PPAF}}\) can be used as a shrub, hedge, or small tree. The purpose of this project was to develop propagation protocols to optimize rooting of stem cuttings.

Terminal, softwood, cuttings were collected on 13 July, 2004. Cuttings were pruned to approximately 5” with the lower leaves removed. The basal 1” of the stems were dipped for 5 sec. in treatment solutions ranging from 0 to 10,000 ppm auxin formulated as either indole butyric acid (IBA) dissolved in 50% isopropyl alcohol or the potassium salt of indole butyric acid (KIBA) dissolved in water. Cuttings were stuck in a medium of 50% peat : 50% perlite and placed under intermittent mist in a shaded (~50%) glass-covered greenhouse. Cuttings were arranged in a completely randomized design with 15 replications. Data were analyzed using regression analyses.

Results and Discussion: There was no influence of auxin concentration on percent rooting for either IBA or KIBA (Fig. 1A and 1B); cuttings rooted between 70% and 100% regardless of treatment. However, root number was influenced by auxin concentration. Root number followed a cubic trend in response to IBA concentration with the highest root number between 2,000 and 4,000ppm (Fig. 1B). Root number increased in response to KIBA, but there was no significant trend in root number between 2,000 and 10,000 ppm KIBA (Fig. 2B). In general, root numbers per cutting were maximized with approximately 2,000 - 4,000 ppm of either formulation.

For more information on this study or on ‘Shiloh Splash’ river birch\(^{\text{PPAF}}\), contact Tom Ranney at the above address or 828-684-3562.
Figure 1. Rooting percent (A) and root number (B) in response to a range of IBA (in 50% isopropyl alcohol) treatments applied as a liquid, basal dip. Symbols represent means (n=15) +/- 1 SEM.
Figure 2. Rooting percent (A) and root number (B) in response to a range of KIBA (potassium salt in water) treatments applied as a liquid, basal dip. Symbols represent means (n=15) +/- 1 SEM.
Estimating Genetic Diversity within the 
Hydrangea genus using Molecular Markers

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Index Words: Hydrangea, SSR, Molecular Marker

Significance to Industry: Here we establish molecular markers for breeding hydrangeas. Genetic diversity estimates indicate that Hydrangea macrophylla, H. serrata, H. luteovenosa, H. lobbii, H. angustipetala and Dichroa febrifuga are closely related and should be tested for interspecific hybridization. We demonstrate the utility of SSR markers in verifying inter-specific hybrids and identifying mislabeled plants in the trade.

Nature of Work: Hydrangeas are the fourth top selling deciduous flowering shrub with annual sales in excess of $32 million. Recent escalation in consumer demand is likely to continue due to the national marketing campaign for remontant (reblooming) H. macrophylla cultivars such as Endless Summer™ (`Bailmer`). Breeding efforts in this genus targeted at developing reliable, low-maintenance plants with a wide array of ornamental traits should result in cultivars that are widely accepted by consumers.

One of the biggest challenges in breeding woody ornamentals is the long generation time. Molecular marker-assisted breeding provides an opportunity to screen plants at a young age for traits of interest. This technology is particularly useful in selecting for traits that do not appear until late in a plant’s life cycle, require several years of evaluation to fully assess, or that would normally require the introduction of a pathogen. While several types of molecular marker technologies have been developed, simple sequence repeats (SSRs) are the marker of choice for crop improvement strategies because they are codominant, locus-specific, and highly reproducible. In 2004 we invested in SSR-enriched libraries made from Hydrangea DNA and developed more than 140 markers. Here we use 14 of these markers to estimate the diversity within the genus and demonstrate their utility for verifying interspecific hybrids and identifying mislabeled cultivars.

Four SSR-enriched libraries, each containing approximately 15,000 recombinant cells, were made from genomic DNA of H. macrophylla and H. paniculata (Genetic Information Services, Chatsworth, CA). From these libraries, 1,152 random clones were sequenced and analyzed. 670 potential SSR primer pairs with an average repeat number of 11 were identified. Of these, 288 primer pairs were tested against a panel of 12 different hydrangea DNAs. Fourteen produced polymorphic data for all samples tested suggesting they would be
universally informative across all species within the genus. These markers were tested against a panel of 58 DNAs representing *Hydrangea* and related taxa.

**Results and Discussion:** Data from the 14 markers was compiled for all 52 samples and analyzed for shared allele frequencies. A genetic distance matrix was compiled from pairwise comparisons of allele frequencies between samples. Neighbor-joining methods, with 1000 bootstrap replicates for statistical support, were used to generate a tree dendogram (Figure 1). As expected, *Hydrangea macrophylla* and *H. serrata* are closely related, but also show genetic similarity to *H. luteovenosa*, *H. lobbii*, and *H. angustipetala*. Hybridization within this group of species may have a greater likelihood of success that between more distantly related species. *Dichroa febrifuga* and *D. versicolor* were also within the *H. macrophylla* clade suggesting that they should be included in breeding efforts and possibly reclassified as a species within the *Hydrangea* genus.

The utility of SSR markers in verifying wide hybrids was demonstrated with a *Hydrangea* interspecific hybrid that had previously been verified using RAPD markers (2). Data for six of the fourteen markers clearly show the genotype is split between the parents, *H. macrophylla* ‘Kardinal’ and *H. paniculata* ‘Brussell’s Lace’ (Fig. 2). Because of the statistical separation between species clades, we now have an unambiguous genetic fingerprint for each of the Hydrangea species that can be used to validate wide hybrid crosses. Interspecific crosses are a vital component of a hydrangea breeding program and could be based on the diversity estimates described above and known ploidy levels.

Surprisingly, two ‘Penny Mac’ cultivars did not show identical DNA fingerprints suggesting that one of these plants is mislabeled (Figure 3). The ‘Penny Mac’ from nursery #2 is probably correctly labeled because it is more similar to ‘Dooley’ and ‘Nikko Blue’ as predicted by RAPD markers (1). The ‘Penny Mac’ from nursery #1 appears identical to ‘Endless Summer’ for the 14 SSR loci tested here. Additional SSR loci are needed to unambiguously identify these plants but it is clear from the limited data presented here that two genetically different plants are being sold as ‘Penny Mac’. We are currently testing 67 additional SSRs to generate a key to identify 192 *H. macrophylla* and *H. serrata* cultivars, including the cultivars listed above, and verify parentage for intra-specific crosses. Identifying mislabeled plants should increase consumer confidence and protect licensing agreements for patented plants.

Mention of trade names or commercial products in this article is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U.S. Department of Agriculture.

**Literature Cited:**


Figure 1. This tree shows the diversity within *Hydrangea* and the relatedness between species. Longer branch lengths indicate more genetic distance between taxa. Numbers correspond to bootstrap support for each node where higher numbers indicate more statistical support.
Figure 2. Data from six SSR markers verify the parentage of a plant created through interspecific cross between *H. macrophylla* and *H. paniculata*.

Figure 3. Data represent the two differing results for ‘Penny Mac’ obtained from two nurseries. Stars indicate peaks for ‘Penny Mac #2’ that match ‘Dooley’ and ‘Nikko Blue’ as expected.
Evaluation of Hydrangea Selections for Coastal Georgia

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Index words: Cercospora hydrangeae, Hydrangea macrophylla, Hydrangea serrata, Powdery Mildew (Erysiphe polygoni)

Significance to Industry: Sixteen cultivars of Hydrangea macrophylla and two cultivars with H. serrata parentage were evaluated for growth and disease resistance over a three year period in Savannah, GA. The plants showing the best growth and disease resistance for Cercospora leaf spot and powdery mildew were ‘Blue Wave’ and ‘Lilacina’. All plants survived the trials except ‘Glowing Embers’. Further work on flowering, defoliation, disease resistance, and development of iron chlorosis will be reported at a later date.

Nature of Work: Since the late 1990’s there has been a resurgent interest in hydrangeas as landscape plants. While numerous cultivars are in the trade, little information exists on pests and landscape performance in coastal South Georgia. In March 2001, a three year evaluation study was initiated at the University of Georgia Bamboo Farm and Coastal Gardens located in Savannah, GA (USDA 8b).

Plantings were made under a 30’ x 96’ shade house covered with black shade cloth which provided 55% light exclusion. Hydrangeas were planted using a spacing of 1.5 meters (5.0 ft.) within rows and 1.8 meters (6.0 ft.) between rows. The soil is a Ocilla loamy fine sand with a pH 6.2 to 6.5. Phosphorus in the soil was considered very high at the start of the study (~280 lbs. P/acre). Plants were fertilized in March, June and September of each year using a soluble granular fertilizer (12.0-4.0-8.0) at the rate of 37kg N/hectare (33 lbs. N/acre) per application. Plants were watered (pH 6.6, alkalinity 0.56 me/L) as needed with drip irrigation. The entire area was mulched to a depth of 5 cm with composted yard trimmings after planting. Weeds were controlled manually or by using directed sprays of glyphosate herbicide as needed.

Ratings for Cercospora leaf spot were made on 13 July, 2001 and 25 June, 2002 while powdery mildew ratings (Erysiphe polygoni) were made on 26 November, 2001 and 4 November, 2002. Plants were visually evaluated for diseases on a scale of 1 to 5 where 1 = no disease present, 2 = 1% to 25% of the leaves diseased, 3 = 26% to 50%, 4 = 51% to 75%, and 5 = >75%. Ratings were pooled to give a combined rating of disease resistance in 2001. Plants with ratings of 2.0 or less were considered acceptable. Final survival and growth indices [(height + width 1 + width 2)/3] were determined on 4 November, 2003.

Plants were arranged utilizing a randomized complete block design with four single plant replicates. Data was analyzed using PC SAS. Mean separations were conducted using a Waller-Duncan K-ratio t test. Hydrangea macrophylla cultivars used in this trial were: ‘All Summer Beauty’, ‘Big Blue’, ‘Blue Wave’,
'Domotoi', 'Dooley', 'Forever Pink', 'Glowing Embers', 'Goliath', 'Lanarth White', 'Lilacina', 'Merritt's Supreme', 'Nightingale', 'Nigra', 'Nikko Blue', 'Pia', and 'Sister Theresa'. The lone selection of *H. serrata* was 'Grayswood', while the hybrid *H. serrata x macrophylla* 'Preziosa' was also included. Plant selection was based on popularity of commercially available cultivars in 2000.

**Results and Discussion:** Plants showing acceptable resistance to *Cercospora* leaf spot in summer of 2001 (Table 1) were 'All Summer Beauty', 'Blue Wave', 'Dooley', 'Lanarth White', 'Lilacina', and 'Nikko Blue' while those showing good resistance to powdery mildew in fall of 2001 were 'Blue Wave', 'Domotoi', 'Forever Pink', 'Lilacina', 'Nightingale', and 'Pia'. The only plants showing acceptable resistance to both diseases in 2001 were 'Blue Wave', 'Domotoi', 'Lilacina', and 'Nightingale'. Disease pressure was not as great in 2002 compared to 2001. Plants with unacceptable ratings for *Cercospora* leaf spot in 2002 were 'All Summer Beauty', 'Dooley', 'Forever Pink', 'Glowing Embers', 'Goliath', and 'Nikko Blue' (data not shown). Plants with significant development of powdery mildew in 2002 were 'All Summer Beauty', 'Dooley', 'Nightingale', 'Nikko Blue', 'Preziosa', and 'Sister Theresa'.

All four replicate plants survived for all cultivars except 'Glowing Embers', for which only one plant was still alive at the end of the three year study. The largest plants at the end of the study were 'All Summer Beauty', 'Blue Wave', 'Dooley', 'Grayswood', 'Lilacina', 'Nikko Blue', and 'Sister Theresa'. The three smallest cultivars were 'Forever Pink', 'Merritt' Supreme', and 'Pia'. Blooms on all plants during the study were pink or white. High phosphorus in the soil and a pH in the range of 6.2 to 6.5, which minimizes the availability of aluminum necessary for blue sepals, contributed to the general lack of blue flowers.

Based on disease tolerance in 2001 and 2002 and plant growth, 'Blue Wave' and 'Lilacina' were the best plants in the trial. Further work on flowering, defoliation, disease resistance, and development of iron chlorosis will be reported at a later date.
Table 1. Disease ratings for *Cercospora* leaf spot and powdery mildew on 18 hydrangea cultivars grown in Savannah, GA. Disease ratings are for 2001, final growth indices were determined after three seasons. Ratings for *Cercospora* and powdery mildew were as follows: 1 = no disease present, 2 = 1% to 25% of the leaves diseased, 3 = 26% to 50%, 4 = 51% to 75% and 5 > 75%. Mean separations using Waller-Duncan K-ratio t test.

<table>
<thead>
<tr>
<th>Cultivar</th>
<th>2001 Leaf Spot</th>
<th>2001 Powdery Mildew</th>
<th>Combined Ratinga</th>
<th>Final Growth Indexb (in)</th>
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<tr>
<td>'Pia'</td>
<td>5.0 a</td>
<td>1.8 defg</td>
<td>3.4 ab</td>
<td>35 fg</td>
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<tr>
<td>'Forever Pink'</td>
<td>4.8 ab</td>
<td>1.0 g</td>
<td>2.9 abcdef</td>
<td>29 gh</td>
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<tr>
<td>'Glowing Embers'</td>
<td>4.0 abc</td>
<td>2.3 bcdefg</td>
<td>3.2 abcd</td>
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</tr>
<tr>
<td>'Sister Theresa'</td>
<td>3.8 bcd</td>
<td>3.8 abc</td>
<td>3.8 a</td>
<td>55 bcde</td>
</tr>
<tr>
<td>'Merritt's Supreme'</td>
<td>3.8 bcd</td>
<td>1.8 defg</td>
<td>2.8 abcdefg</td>
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</tr>
<tr>
<td>'Big Blue'</td>
<td>3.3 cde</td>
<td>2.8 bcdef</td>
<td>3.0 abcdde</td>
<td>43 ef</td>
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<tr>
<td>'Goliath'</td>
<td>2.8 def</td>
<td>2.3 bcdefg</td>
<td>2.5 bcdefg</td>
<td>42 efg</td>
</tr>
<tr>
<td>'Nightingale'</td>
<td>2.8 def</td>
<td>1.3 fg</td>
<td>2.0 efgh</td>
<td>44 def</td>
</tr>
<tr>
<td>'Domotoi'</td>
<td>2.5 ef</td>
<td>1.5 ef</td>
<td>2.0 efgh</td>
<td>43 ef</td>
</tr>
<tr>
<td>'Nigra'</td>
<td>2.3 efg</td>
<td>2.3 bcdefg</td>
<td>2.3 cdefg</td>
<td>42 efg</td>
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<td>'Preziosa'</td>
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<td>4.0 ab</td>
<td>3.1 abcd</td>
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<td>2.0 defg</td>
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<td>63 ab</td>
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<tr>
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<td>4.5 a</td>
<td>3.3 abc</td>
<td>57 abcdd</td>
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<td>'All Summer Beauty'</td>
<td>2.0 fg</td>
<td>3.3 abcd</td>
<td>2.6 bcdefg</td>
<td>59 abc</td>
</tr>
<tr>
<td>'Nikko Blue'</td>
<td>2.0 fg</td>
<td>4.0 ab</td>
<td>3.0 abcde</td>
<td>59 abc</td>
</tr>
<tr>
<td>'Lilacina'</td>
<td>2.0 fg</td>
<td>1.8 defg</td>
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<tr>
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<td>2.0 fg</td>
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<td>1.8 gh</td>
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<tr>
<td>'Lanarth White'</td>
<td>1.8 g</td>
<td>3.0 abcde</td>
<td>2.4 bcdefg</td>
<td>44 def</td>
</tr>
</tbody>
</table>

*a Combined rating = [(Cercospora leaf spot + Powdery mildew)/2].

*b Growth index = [(height + width 1 + width 2)/3].
‘Prince’ and ‘Princess’ Ornamental Napiergrass

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Index Words: Napiergrass, Ornamental grass, *Pennisetum purpureum*

**Significance to Industry:** ‘Prince’ and ‘Prince’ are dwarf and semi-dwarf, respectively, purple-foliaged napiergrasses (*Pennisetum purpureum* (L.) Schum.) that have been jointly released by the U.S. Dept. of Agriculture and University of Georgia College of Agricultural and Environmental Sciences. These grasses are perennial in USDA hardiness zones 8-10. However, they can be grown as vigorous annuals in more northern zones. These plants flower under short days, therefore, these cultivars will not produce seed where winter temperatures reach freezing (0.0°C) or below. Plants are not recommended for landscape use in areas where a hard freeze does not regularly occur by December 1 (USDA 9-11) since reseeding may be an issue, particularly in humid, subtropical environments. Plants can not be shipped into California or Florida.

**Nature of Work:** Seeds of a two napiergrass accessions, designated Anae Roxo CNPGL, were received from CENARGEN/EMBRAPA in Brazil in April 1996. These were grown under quarantine in the greenhouse during the winter of 1996-97. These segregated for a wide variety of green and various shades of purple plants with a range of vigor. The most vigorous purple pigmented plant in each accession, given the Tifton numbers N240 and N241, were selfed. Seeds of these plants were planted in the field in 1997. The progenies from these selfed plants again segregated for color and vigor in the field. Three vigorous plants, from each accession, with different shades of purple pigment development were vegetatively propagated for further evaluation in a napiergrass maintenance nursery in 1998. In 1999, two accessions, N241-5 (dwarf) and N241-8 (semidwarf) were selected for further evaluation. In 2003, we assigned the names ‘Princess’ to N241-5 and ‘Prince’ to N241-8.

In 1999, multi-plant observation plantings were made at 1) the University of Georgia Coastal Plain Experiment Station Dairy Research Center and 2) the National Environmentally Sound Production Agriculture Laboratory. Replicated tests were planted in 2000 at Blairsville (USDA 6b), Griffin (USDA 7b), and Savannah (USDA 8b) and at Tifton (USDA 8a) in 2001 and 2003. All test sites except Tifton were irrigated. Plants in Blairsville and Griffin did not recover in 2001 following temperatures as low as -17°C (1.4°F). Plants in Tifton and Savannah survived when exposed to low temperatures of -6°C (21.2°F) and -8°C (17.6°F), respectively.

**Results and Discussion:** Height of ‘Princess’ ranged from 48 to 56 in. and from 26 to 50 in., whereas, height of ‘Prince’ ranged from 70 to 86 in. and 37 to 79 in. for the Savannah and Tifton locations, respectively. The greater growth of both cultivars was observed in 2003 when rainfall during the growing period at Tifton was 31.8 in. compared to 20.7 and 18.1 in. in 2001 and 2002, respectively. Height differences between years were especially evident under the non-irrigated conditions at Tifton.
Both ‘Prince’ and ‘Princess’ are quite vigorous and can produce about 40 tillers in one year under non-irrigated conditions and twice that many under irrigated conditions. Tiller numbers for both cultivars rapidly increase with age of the plant as the circumference of the base of the plant increases. Diameter of the top canopy can range from 39 to 61 in. for ‘Princess’ and from 54 to 133 in. for ‘Prince’ depending on age and moisture conditions. ‘Princess’ is similar to *Pennisetum setaceum* ‘Rubrum’ for height and number of tillers under non-irrigated conditions, but ‘Princess’ will produce twice as many tillers under irrigated conditions. ‘Prince’ significantly exceeds ‘Rubrum’ in all characteristics measured. *Pennisetum setaceum* ‘Rubrum’ is not reliably cold hardy in USDA zone 8 and died during the first winter in the field trials. ‘Princess’ has longer leaves than ‘Rubrum’. ‘Prince’ has significantly longer leaves than ‘Princess’, but leaf width is similar for the two cultivars.

Both ‘Prince’ and ‘Princess’ can be easily propagated by stem cuttings. Stem cuttings with two nodes tended to produce the most shoots, although somewhat reduced in vigor, based on three-week plant heights. One or two node cuttings produced the most shoots for ‘Prince’, but the two-node cuttings were more vigorous. Rooting percentages in excess of 90% can be expected under ideal conditions. A well drained substrate and minimal misting increases survival of cuttings. Terminal cuttings have not performed as well as nodal cuttings from the stem. Division of rhizomes also works well. Well rooted liners shifted from 8.3 cm (3.25 in.) pots to #5 (19.0 liter) containers in mid-April produced salable plants at a commercial nursery in 40 days. After cutting back, plants were again salable after 57 days. In 19 liter containers using a pine bark-based substrate, paclobutrazol drenches were not economically feasible for reducing plant growth. Hard pruning to control plant size results in good regrowth.

Helminthosporium leaf spot has been noted on the foliage in the field and under overhead sprinklers in container nurseries, but control has not been necessary. Two-lined spittlebug (*Prosapia bicinta* (Say)) has also been noted to feed on the bases of field and container-grown plants. In the field, plants should be cut back in the late winter to remove debris that harbors spittlebugs.

A U.S. Plant Patent for ‘Princess’ and ‘Prince’ has been applied for on behalf of the U.S. Department of Agriculture, Agricultural Research Service. Contact the authors for more information or see the following cultivar release (1). Numerous plants of ‘Princess’ and ‘Prince’, respectively, are in the breeder nursery at the Coastal Plain Station in Tifton and these can be used as the initial propagation stocks. As protected cultivars, ‘Prince’ and ‘Princess’ can only be produced by nurseries licensed by the Georgia Seed Development Commission (GSDC) under the guidelines established in conjunction with the University of Georgia Research Foundation (UGARF). Contact the GSDC (http://www.gsdc.com) for information on availability.

Literature Cited:
Ploidy Variation in *Hedychium* Species and Cultivars

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Index Words: *Hedychium*, Ornamental gingers, Ploidy level, Flow cytometry

Significance to Industry: One of the barriers to crop improvement via hybridization is differences in ploidy level between prospective parents. Using a ploidy analyzer, we have determined the ploidy level of several *Hedychium* species and cultivars, thus paving the way for a sound breeding scheme for this ornamental ginger.

Nature of Work: Ornamental gingers are increasingly gaining popularity because they present great potential for use as flowering pot plants, both indoors and as patio and landscape plants (5). The genus *Hedychium*, often referred to as “Butterfly Ginger” “Garland Lily”, or “Ginger Lily”, is one of the most popular genera of the Zingiberaceae family because of its attractive foliage, diverse and showy flowers, and sweet fragrance. Tremendous diversity exists within the genus, providing breeders with an opportunity to develop new cultivars. This diversity is due in part to the fact that *Hedychium* species readily hybridize, which makes natural hybridization both very likely but also a potential source of the current taxonomic confusion in *Hedychium* (8). Another source of variation may be due to differences in ploidy levels. However, only sparse and often contradictory information is available on the cytogenetics and ploidy level of a few *Hedychium* species (6; 1; 2; 7; 4). Determining the ploidy level of *Hedychium* species will not only help clarify the current taxonomic confusion, as chromosome size and morphology can be used to establish evolutionary relationships among plant species (3), but also lead to better breeding strategies for the development of new cultivars of this increasingly important horticultural genus. Flow cytometry is a quick and dependable method that is particularly suited for determining the ploidy levels of large number of samples. The objective of the current study was to use flow cytometry to determine the ploidy levels of some *Hedychium* species and cultivars obtained from various sources. The plant material studied consisted of 38 *Hedychium* species and cultivars that were obtained from various sources, including commercial nurseries, private hobbyist collections, and public institutions (Table 1). Plants were potted and grown under natural conditions in the greenhouse at the USDA-ARS Station in Poplarville, Mississippi. Ploidy levels were determined using a Partec Ploidy Analyzer (Partec, Muenster, Germany). Young, unexpanded leaf tissue was collected from each species and variety. A piece of leaf tissue about 1 cm² (0.16 in²) was chopped with a double edged razor blade in a Petri dish containing nuclei extraction buffer. Buffers were supplied as part of the Cystain UV Precise P Staining Kit (Partec, Muenster, Germany). Leaf tissue of *Hedychium longicornutum*, whose ploidy level (2n = 2x = 34) was previously determined (5), was included in each sample as a diploid internal reference. After a 30-second incubation with gentle agitation, the
extract was poured through a 50 µm mesh sieve. The DAPI (4'-6 diamidino-2-phenylindole) nucleus staining buffer was added to the extract buffer in a ratio of 3 parts nuclei staining buffer to 1 part extract buffer, and the sample was analyzed immediately until the DNA content of at least 3000 nuclei was checked. Sample measurements were replicated three times for each species or cultivar. Results were displayed as histograms showing the number of nuclei grouped in peaks of relative fluorescence intensity, which is proportional to DNA content.

Results and Discussion: Among the 38 species and cultivars whose ploidy level was examined, one species (*H. flavum*) and four cultivars ('Golden Butterfly', 'Tai Pink Princess', 'Tai Pink Profusion', and 'Tall Ximeng') were found to be tetraploid (Table 1). Most of the species and cultivars, including the popular variegated cultivar 'Dr. Moy', were diploid. There is confusion in the literature as to the parentage of this cultivar, which is believed to result from either *H. flavum*×*H. coccineum* or *H. coccineum*×*H. coccineum*. According to our results, *H. flavum* and *H. coccineum* are tetraploid and diploid, respectively; therefore, it is unlikely that 'Dr. Moy', a fertile diploid, is a hybrid of these two species.

Acknowledgements: The authors are grateful to Dr. Jeff S. Kuehny, Department of Horticulture, Louisiana State University, Baton Rouge, LA, Dr. Dave Creech and Dawn Parish Stover, SFA Arboretum, Stephen F. Austin State University, TX, and Hayes Jackson, Anniston, AL for providing some of the *Hedychium* plant material. We also thank Schuyler Laws and Lindsey Tanguis for processing the samples.

Literature Cited:
Table 1. Ploidy variation in several *Hedychium* species and cultivars as determined by flow cytometry.

<table>
<thead>
<tr>
<th>Genus</th>
<th>Species/Cultivar</th>
<th>Origin*</th>
<th>Ploidy level</th>
</tr>
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<td>angustifolium</td>
<td>HJ</td>
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*Plants were obtained from Mr. Hayes Jackson (HJ), Anniston, Alabama, Dr. Jeff S. Kuehny, Department of Horticulture, Louisiana State University (LSU), Baton Rouge, Louisiana, Dr. Dave Creech and Dawn Parish Stover, SFA Arboretum, Stephen F. Austin State University, Texas (TX), Tom Wood (TW) Nursery, Archer, Florida, and Tropical Paradise Nursery (TPN), Davie, Florida.
Low Temperature Regulation of Gene Transcription in *Pachysandra terminalis*, a Cold-hardy Shade Perennial

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Index Words: Japanese spurge, Plant improvement, Temperature stress

Significance to Industry: Two hundred and fifty clones were selected as up-regulated by low temperature treatments. Sequence analysis data from these clones indicates that they can be classified into two groups: stress- and photosystem-related. A number of clones were not identified to belong to these groups. Some of the clones that had no homology to these groups may have an important role in the mechanism of cold resistance in Japanese spurge. These genes may play a role in the development of new plant cultivars with the ability to survive sporadic cold temperatures during the growing season.

Nature of Work: *Pachysandra terminalis* (Japanese spurge) is an evergreen groundcover hardy to USDA zone 5. The identification and isolation of genes responsible for this resistance could be inserted into cold sensitive ornamentals to allow them to survive cold stress. To identify genes in this plant that allows it to survive cold stress, young plants were maintained, subjected to cold treatments and processed as described by Zhou et al. (1). Gene activity induced by these treatments was identified through cDNA differential display. cDNA fragments were cloned onto PCR-trap vectors and reverse-northern blot assays were used to confirm the low temperature regulation of these genes. The total RNA was extracted from frozen leaf tissue using RNA Pure, a Genhunter kit, (Nashville, TN) and digested with DNase I (DNA clean kit, Genhunters, TN). Pure RNA targets were labeled with $^{32}$P-dCTP via first-strand cDNA synthesis using three-one-base-anchored oligo-dT primers. Differently expressed cDNA fragments were amplified using all the anchored primers and 80 arbitrary 13mers primers from the RNAimage kit (Genehunter,TN). cDNA fragments were separated on a 5.5% non-denaturing acrylamide gel and signals were detected by exposing X-ray film to the gels for 12 h at -70C. cDNA bands that showed different intensities between control and treatments were considered as possible cold inducible genes. These gene fragments were recollected from the original gels and re-amplified using the same primers and then cloned onto PCR-Trap cloning vector (Genhunter). Positive clones were selected on LB media containing supplemented with 20 mg/L tetracycline. Colony hybridization and reverse northern dot blot were performed to select bacterial colonies with the gene insert and to eliminate false-positive clones resulting from self-ligation with the vector. To perform this, five independent *Escherichia coli* colonies were picked from each re-amplified fragment, and spotted onto LB Agar. Then, all colonies were lifted onto nitrocellulose membranes (PerkinElmer, MA) and incubated at 37C for 10 h. After incubation, bacterial colonies were lysed and denatured twice in 0.5N NaOH/ 1.5M NaCl buffer for 3 min. The DNA was neutralized twice in 0.5 M Tris-HCl (pH 7.0)/ 1.5 M NaCl Buffer for 3 min.
Membranes were then fixed at 60°C for 12 h. Membranes were hybridized using a probe synthesized from a pool of RNAs obtained from all the treatments and labeled with \(^{32}\text{P}-\text{dCTP}\) (Amsham Biosciences) using Reverse cDNA labeling Kit (Genehunter). The conditions for membrane hybridization were as follows: pre-hybridization at 42°C overnight, hybridization at 42°C for 12 h and washed twice in 1 x SSC at room temperature for 20 min, twice in 0.25 x SSC and 0.1% SDS at 50°C for 20 min. The buffer contained 50% formamide and 0.1% SDS, 5X Denhardt, 5X SSPE. After hybridization, signals were detected on X-Ray film after exposure at -70°C for 12 h. One positive clone was selected for each transformation. For reverse northern blot, cDNA inserts of selected clones were PCR amplified and spotted onto N+ nylon membrane using Biorad’s 96-well Bio-Dot SF apparatus. DNA was fixed onto the membrane by maintaining it at 80°C for 2 h. Membrane hybridization and signal detection was performed as previously described. Plasmids obtained from the positive clones of the low temperature induced genes were amplified in \textit{E. coli} cultured in LB liquid medium lacking the antibiotics. All plasmids were isolated and sequenced using a LI-COR Model 4200 IR\(^2\) Series DNA Analyzer (1). DNA sequences were compared to reported sequences in NCBI database (nr and EST) and their possible identity was determined according to their homology with genes in the databank.

Northern blot assays were performed to screen genes that were regulated by cold temperature exposures, plants were incubated at 5°C for 9 to 24 h, followed by 9 to 24 h at 25°C for. To determine the effects of cold acclimation, plants were returned to 5°C following the following treatments: 25°C-5°C for 9 h; 25°C for 24 h, and 5°C for 24h. At the termination of each treatment, the total RNA from treated leaf tissues was isolated and separated in 1% denature formaldehyde gel and transferred by capillary to N+ Nylon membrane. To prepare the DNA probes for Northern blot, gene inserts were amplified using the plasmid of selected genes. PCR products were separated on 1% agarose gel and purified using Qiagen’s DNA Gel Extraction Kit. The DNA was labeled with \(^{32}\text{P}-\text{dATP}\) using Hot-Prime DNA labeling Kit (Genhunters, TN) and hybridized with RNA-nylon membrane following the procedure used for colony hybridization (1).

**Results and Discussions:** The cDNA differential display technique was useful for detecting gene expression differences. Our results show that the expression of most genes was not affected by cold treatments; while with some, expression decreased (Band 1, Fig 1) and some increased (Band 2, Fig.1). Some genes had very strong expression after a treatment (3C) which decreased with lowered temperature. All cDNA bands with varied expression were cloned. A total of 500 cDNA fragments were recovered from gels and re-amplified. When these clones were subjected to colony hybridization and reverse cDNA dot blot using the cDNA pools from all cold treatments, 350 genes produced positive signals (Fig. 2). DNA sequence database search showed that some had high homology with genes isolated from other plants (Table.1).
To screen genes that responded to low temperature fluctuations, plants were subjected to the following treatments: 5C for 9 h and 24 h; returned to 25C for 9 and 24 h followed by 5C for 24 h; 25C was the control temperature. RNA extracted from treated leaves was hybridized with probes synthesized from reverse-northern positive-clones. Of all the clones processed, only 13 genes changed their expression level under different temperature fluctuations (Fig.3). CP1 had a constant increase in its expression pattern following cold treatments, this gene continued to have high expressions when plants were returned to 25C. CP2 and CP3 had high expressions when treated at 5C for 9 and 24 h, they also continued to have high expression when returned to 25C. Low temperature (5C) treatments failed to induce high expression when previously treated plants were returned to cold treatment. The clone CP4 had a high expression when the plants were subjected to 5C for 9 and 24 h. This clone continued to expressed after treated plants (5C 9h) were returned to the greenhouse at 25C. However, this gene did not expressed highly when cold treated plants (5C 24 h) were returned to the greenhouse. The other nine clones (CP5 to CP13) produced similar gene expression patterns. When plants were treated for 9 and 24 h at 5C, gene expression also increased. When plants that were treated at 5C for 9 h were returned to 25C, their gene expression decreased while those treated for 24 h at 5C continued to have high expression patterns. However, when these plants were returned to 5C following the 5C to 25C treatments, low to no expression pattern was detected.

Acknowledgements: This project was supported through a Higher Education Capacity Building Program and Evan Ellen Fund.

Literature Cited:
Table 1. Characteristics of cloned cDNAs identified by Differential Display.

<table>
<thead>
<tr>
<th>Clone number</th>
<th>Homologous genes</th>
</tr>
</thead>
<tbody>
<tr>
<td>9</td>
<td>Lycopersicum esculentum chlorophyll b-binding protein (Cab10b) gene, 86%, TOMCBBIN</td>
</tr>
<tr>
<td>8</td>
<td>Nicotiana tabacum CAB7 gene for chlorophyll a/b binding protein 95% (1240-1303) X58229</td>
</tr>
<tr>
<td>32</td>
<td>Nicotiana tabacum CAB7 gene for chlorophyll a/b binding protein (1254-1303) 94% X58229</td>
</tr>
<tr>
<td>102</td>
<td>Pisum sativum gda-1 gene (190-259) 85% PSY14677</td>
</tr>
<tr>
<td>22</td>
<td>Pisum sativum gda-1 gene (129-262) 81%, PSY14677</td>
</tr>
<tr>
<td>1</td>
<td>C010P45.3pR Populus strain T89 leaves P. tremula x P. tremuloides cDNA clone C010P45 3', 91%, CK089445 (158-227)</td>
</tr>
<tr>
<td>14</td>
<td>KS09076B02 KS09 Capsicum annuum cDNA 95% CA517236</td>
</tr>
<tr>
<td>74</td>
<td>DDF204 Brassica oleracea DD-PCR fragment Brassica oleracea cDNA 3' after pathogen infection (9-90) 100% CB617680</td>
</tr>
<tr>
<td>78</td>
<td>Hebeloma cylindrosporum functional cDNA library Hebeloma cylindrosporum cDNA 5' (100%), BMO78095</td>
</tr>
<tr>
<td>129</td>
<td>LbSSH00121 Preinfestation stage symbiosis-regulated cDNAs from L. bicolor x P. resinosa Laccaria bicolor cDNA 97%, BI094677</td>
</tr>
<tr>
<td>130</td>
<td>F071P37.3pR Populus flower cDNA library Populus balsamifera subsp. trichocarpa cDNA clone F071P37 3', 84%, CK091303</td>
</tr>
<tr>
<td>139</td>
<td>L48-263T3 Ice plant Lambda Uni-Zap XR expression library, 48 hours NaCl treatment Mesembryanthemum crystallinum cDNA clone, 85%, BE130151</td>
</tr>
<tr>
<td>146</td>
<td>N020D12 Populus bark cDNA library Populus tremula x Populus tremuloides cDNA clone N020D12 5' (421-550) 90% CK109685</td>
</tr>
<tr>
<td>192</td>
<td>G116P63.3pR Populus tension wood cDNA library Populus tremula x Populus tremuloides cDNA clone G116P63 3', 91%, CK093353</td>
</tr>
<tr>
<td>193</td>
<td>G116P63.3pR Populus tension wood cDNA library Populus tremula x Populus tremuloides cDNA clone G116P63 3', 95%, NTCAB7</td>
</tr>
<tr>
<td>208</td>
<td>DDF228 Brassica oleracea DD-PCR fragment Brassica oleracea cDNA 3' 95%, CB617688</td>
</tr>
<tr>
<td>227</td>
<td>sad07f06.y1 Gm-c1073 Glycine max cDNA clone GENOME SYSTEMS CLONE ID: Gm-c1073-1812 5' similar to TR:O65885 O65885 METALLOTHIONEIN-LIKE PROTEIN. 86%, BG511629</td>
</tr>
<tr>
<td>237</td>
<td>SSPG526R SS pectin Sclerotinia sclerotiorum cDNA similar to H+-transporting ATP synthase beta chain, 95%, CD646006</td>
</tr>
<tr>
<td>239</td>
<td>KS09076B02 KS09 Capsicum annuum cDNA, 95%, CA517236 Hypersensitive Response Against Pathogen</td>
</tr>
<tr>
<td>258</td>
<td>MK_16_42 Pennisetum glaucum seedlings exposed to cold (4 C) stress Pennisetum glaucum cDNA clone MK_16_42. 91%, CD724896</td>
</tr>
<tr>
<td>5</td>
<td>Nicotiana tabacum, photosystem I reaction center subunit X psaK mRNA, complete cds; nuclear gene for chloroplast product, 84%, AY220078.1</td>
</tr>
<tr>
<td>14</td>
<td>Nicotiana tabacum LHC-I mRNA for photosystem I light-harvesting chlorophyll a/b-binding protein, (672-739) 89%, NTLHC1R</td>
</tr>
<tr>
<td>21</td>
<td>Arabidopsis thaliana serine/threonine protein phosphatase PP1 isozyme 6 (PP1BG) (T0P6) (At4g11240) mRNA, complete cds, NM_117195, 85%</td>
</tr>
<tr>
<td>26</td>
<td>Nicotiana tabacum, LHC-I mRNA for photosystem I light-harvesting chlorophyll a/b-binding protein, 89%, NTLHC1R (674-739)</td>
</tr>
<tr>
<td>42</td>
<td>Passiflora edulis PE-ACS1 mRNA for ACC synthase, 93%, AB015494.1</td>
</tr>
<tr>
<td>258</td>
<td>Ribes nigrum mRNA for metallothionein-like protein, 86%, RNI7577</td>
</tr>
</tbody>
</table>
**Fig. 1.** cDNA fragments induced by cold treatment.

25C    13C (1 m)    0C (2 d)    -1C 4 h

**Fig. 2.** Reverse northern hybridization of cDNA clones with cDNA probes prepared from cold treated plant leaf tissues.

Colony hybridization    Dot    Blot
Fig. 3. Northern Blot Analysis.

Treatments: 1. 5°C for 9 h; 2. 5°C for 24 h; 3. treatment 1 returned to 25°C for 24 h; 4. treatment 2 returned to 25°C for 9h; 5. treatment 4 returned to 5°C for 24hr; 6. 25°C, control plants.
Comparative Genotyping of Dark-Green Vegetables via Fluorescent-amplified Fragment Length Polymorphism

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Index Words: Asteraceae, Brassicaceae, Chenopodiaceae, DNA, AFLP analysis, Vitamin A, E

Significance to the Industry: Through this research we have standardized the AFLP procedures for the genetic characterization of 3 herbaceous plant types belonging to the following families: Asteraceae, Brassicaceae, and Chenopodiaceae. They were Lactuca virosa ‘Bibb Forming’, Brassica oleracea ‘Packman’, and Spinacia oleracea ‘Catalina’. The AFLP primer pairs that were identified are applicable and useable for genotyping and fingerprinting other ornamental belonging to these plant families. The genetic characterization procedures developed could be used to increase the efficiency of breeding related plants for specific and characteristics.

Nature of Work: Deficiencies in vitamins A and E affect millions of people throughout the world (1, 2). Dark-green vegetables such as broccoli, lettuce, and spinach produce high levels of vitamins and if consumed regularly could reduce the illnesses caused by deficiencies of vitamin A and E (3, 4). Deficiencies in vitamin A affect mostly children and pregnant women. It can result in visual impairment such as blindness, weakened immune system, and in even death (1). Like for vitamin A, deficiencies in vitamin E can cause blindness; but, it can also cause cardiac arrhythmia, dementia, muscle weakness and decreased in vibratory senses (1). Because of increasing occurrences in vitamin deficiencies in the world, there is a growing interest in preventing or eliminating these deficiencies. Preventing deficiencies in vitamin A and E is by developing vegetable cultivars that have increased levels of these deficiencies and making them available to susceptible populations.

For this study, the germination rates and AFLP procedures were standardized for each selected plant. All seeds were germinated in a greenhouse where the temperature was maintained near 75 F and plants were fertilized and watered as needed until harvested for analysis. Four broccoli, 4 lettuce, and 2 spinach varieties were germinated and grown. Leaf samples were taken from each vegetable variety and the extracted, purified and quantified following standard procedures (5).

Results and Discussion: The germination rates and conditions needed varied with each vegetable variety. The highest rates of germination were obtained with Broccoflower ‘Premium Crop’, ‘Oak Leaf’, and ‘Deer Tongue’ with rates ranging from 89 to 100% while ‘Bloomsberg Long Standing’, ‘Catalina’, ‘Green Sprouting’ and ‘Bibb Forming’ varieties ranged from 75 to 88%. The lowest germination rate
was with ‘Black Seeded Simpson,’ (40%). AFLP amplification was completed for 2 varieties of each of the following vegetables: *L. virousa, B. oleracea*, and *S. oleracea*. The AFLP primer pairs that were appropriate to produce desirable polymorphism in *L. virousa, B. oleracea, and S. oleracea* were identified. There were 33 primer pairs that produced desirable polymorphism for the lettuce varieties and numerous pairs for the spinach varieties (Figure 1).

**Literature Cited:**


**Acknowledgements:**

This project was partially funded by USDA/CREES Evan Allen funds and a Vitamin Antitrust Settlement Grant. The graduate assistantship for K. Shaw was provided by Capacity Building Grant.

**Figure 1.** AFLP analysis of spinach on sequencing gel using Saga software™ (LI-COR Corporate Offices 4308 Progressive Ave. P.O. Box 4000 Lincoln, Nebraska).
AFLP Markers Identify Flowering Dogwood Cultivars

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rtrigian@utk.edu

Index Words: AFLP, Cornus florida, Flowering Dogwood, Cultivar Identification

Significance to Industry: Flowering dogwood (Cornus florida L.) is a native tree that is commonly found in landscapes in the eastern United States. The small stature of the tree increases its versatility in small yards and in limited green areas among office buildings and in cities. Currently there are over 80 cultivars of flowering dogwood commercially available (7). Many of these cultivars are phenotypically similar although some exhibit disease resistance, drought tolerance or tolerance to full sun growing conditions. An identification key would provide a definitive method for researchers, dogwood breeders and nurserymen to identify cultivars that have similar characteristics. A molecular key would also be of great use in patent application, patent protection of new cultivars and in verifying the identity of possible patent infringements (3,6).

Nature of Work: Molecular markers offer a promising way of definitively identifying flowering dogwood cultivars. Molecular markers are obtained from a DNA fingerprint, which is a set of DNA fragments resulting from cleavage of the entire genome by a restriction enzyme or by random amplification of portions of the genome by the Polymerase Chain Reaction (PCR). Molecular markers are those fragments in a DNA fingerprint that are unique to a particular organism or that can be associated with or are inherited with a gene or sequence of interest. Amplified Fragment Length Polymorphism (AFLP) is one technique that can be used to generate DNA fingerprints. AFLP markers are well suited for cultivar identification as the technique is able to detect polymorphisms between closely related cultivars (1). Fingerprints generated by AFLP are robust and highly reproducible. Therefore, the AFLP technique can be repeated by those wishing to scan unknown or questionable cultivars for these identifying markers (1,3,5).

Materials and Methods: Young leaves of 17 cultivars of flowering dogwood (Table 1) were collected from various trees near Knoxville, Tennessee. DNA was isolated using a Qiagen kit. A modification of the original AFLP technique (4) was used. DNA was digested with Eco RI and Mse I restriction enzymes and adaptors were ligated to the cleaved ends. Sixteen Eco RI and Mse I primer combinations were used for selective amplification. After the regular AFLP selective amplification, a second selective amplification was performed to fluorescently label the fragments with a Well-Red fluorescent dye attached to an Eco RI primer (2). This modification was made to reduce the cost of purchasing several different fluorescently labeled Eco RI primers. All fragments were analyzed on a Beckman-Coulter CEQ™ 8000 DNA sequencer. AFLP fingerprints were generated for the 17 flowering dogwood cultivars. Unique markers in the fingerprints were used to devise a dichotomous cultivar identification key. The validity of the key was tested with seven “unknown” flowering dogwood cultivar samples.
**Results and Discussion:** Fingerprints were analyzed for both similarities as well as differences (polymorphisms) between the cultivars. Each fingerprint produced between 20 and 30 bands with several polymorphic bands. AFLP markers were used to uniquely identify individual cultivars and create an identification key, partially shown in Figure 1. The key was tested with seven unknown dogwood cultivar samples. All “unknown” samples except one were positively identified using the key based on AFLP markers identified in this study. The unidentified cultivar was a ‘Cherokee Brave’ sample, which was verified to be an authentic sample. Therefore, the ‘Cherokee Brave’ sample used to create the key may have been mislabeled.

**Literature Cited:**


**Acknowledgements:** This study was funded by the USDA grant agreement #58-6404-2-0057.
Table 1. *Cornus florida* cultivars and unnamed lines included in AFLP analysis.

<table>
<thead>
<tr>
<th>Cultivar/Line</th>
<th>Identification</th>
</tr>
</thead>
<tbody>
<tr>
<td>'Appalachian Spring'</td>
<td>'Cloud 9'</td>
</tr>
<tr>
<td>'Appalachian Mist'</td>
<td>'Fragrant Cloud'</td>
</tr>
<tr>
<td>'Appalachian Snow'</td>
<td>'Plena'</td>
</tr>
<tr>
<td>'Appalachian Blush'</td>
<td>MW 95-12</td>
</tr>
<tr>
<td>'Cherokee Brave'</td>
<td>MW 95-28</td>
</tr>
<tr>
<td>'Cherokee Chief*'</td>
<td>MW 95-4</td>
</tr>
<tr>
<td>'Cherokee Daybreak*'</td>
<td>MW 94-60</td>
</tr>
<tr>
<td>'Cherokee Princess'</td>
<td>MW 94-67</td>
</tr>
<tr>
<td>'Cherokee Sunset*'</td>
<td></td>
</tr>
</tbody>
</table>

*Indicates duplicate sample was tested

Figure 1: Partial Dichotomous Cultivar Identification Key

A. Primer 1.3 at 142 bp ................................................................. Go to C.
A.' Primer 1.3 absent at 142bp .................................................... Go to B.

B. Primer 1.3 at 297bp and 300bp ................................................ MW 95/12
B.' Primer 1.3 absent at 297bp and 300bp ................................... 'Appalachian Blush'

C. Primer 1.3 at 125bp ................................................................. Go to D.
C.' Primer 1.3 absent at 125bp .................................................... Go to E.

D. Primer 1.3 at 448bp ................................................................. 'Cherokee Sunset'
D.' Primer 1.3 at 458bp .... 'Cherokee Princess' (confirmation Primer 1.2 at 230bp)

E. Primer 1.3 at 441bp ... 'Cherokee Brave' (confirmation Primer 4.1 at 275bp.)
E.' Primer 1.3 absent at 441bp ....................................................... Go to F.

F. Primer 1.3 at 228bp. 'Appalachian Snow' (confirmation Primer 1.3 at 128bp)
F.' Primer 1.3 absent at 228bp ....................................................... Go to G.
Identification of Cold-inducible Genes in *Pachysandra terminalis* Sieb. et Zucc

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Index Words: Cold, Gene, Real-time PCR, Differential Display

Significance to the Industry: Chilling and freezing temperatures adversely affect productivity and quality of nursery crops. In the past several decades, much attention has been paid to the molecular mechanism of chilling sensitivity in plants because of the needs for crops that have chilling tolerance. Low temperature induces a number of changes in the cellular metabolism of plants. These include protein synthesis, and the expression levels of a number cold-inducible genes or cold responsive genes (1). Many cold inducible genes have been cloned from numerous plants species as well as from organisms belonging to other kingdoms (monera, fungi, animal). Because Japanese spurge can survive where the lowest temperature in winter can be as low as -26.1°C (-15°F) to -23.3°C (-10°F), cold-inducible genes from this plant could have value to the nursery industry for the improvement of cold sensitive ornamentals. Through this work, several cold-inducible genes have been isolated. Further work with these gene fragments need to be done to evaluate their potentials to the industry.

Nature of Work: This research was carried out to identify genes in *Pachysandra terminalis* (Japanese spurge) that are regulated by low temperature treatments. After acclimated at 12 °C for two weeks, plants were cold treated as follows: 4 °C (39 °F) for 48 h, 0 °C (32 °F) for 48 h and -1 °C (30 °F ) for 4 h. cDNA differential display was used to identify cold inducible genes. The expression pattern of the selected genes after low temperature treatments were analyzed using quantitative real-time PCR. RNA extraction and treatment: After each treatment, new leaves were dissected from plants and frozen immediately in liquid nitrogen and stored at -70°C (-94°F ) until extraction. Total RNA was extracted using a kit (RNA Pure; Genhunter, TN) according to the manufacture’s procedure. Total RNA were extracted from the new leaves using and digested with DNase I using DNA clean kit (Genhunters, TN). cDNA Differential display was performed with the 90 primers in g the RNAimage kit (Genehunter,TN). Cold inducible gene fragments were cloned onto PCR-Trap cloning vector (Genehunters) and further confirmed by colony hybridization and reverse northern dot blot. Northern Blot was following standard procedures. cDNA library was constructed on the pBluescript SK(-) phagemid using the ZAP- cDNA library construction system from Stratagene. Real-time PCR primer design was using Oligoperfect Designer software (Invitrogen website). The amplicon size was 75-150bp. Total RNA was extracted from leaf tissues after cold shock at 4C (38 °F) for 2.5 h. The reaction was using the SYBR-green PCR mix/RT kit (Applied Biosystems) and PCR products was detected on a ABI Prism 7200 Sequence Detection System (Applied Biosystems). The comparative treshold cycle (Ct) method was used to determine
relative RNA levels (User Bulletin no. 2, Applied Biosystems). 18S rRNA is used as a normalizer and the internal reference, and expression levels are relative to the control treatment (25°C) and compared using t-test.

**Results and Discussion:**

• **Screening of low temperature regulated genes**
  cDNA differential display technique identifies difference in gene expression based on the signal intensity among different treatment. Result from this experiment showed that while most of the genes have very similar expression under different treatment, some genes have decreased expression after cold treatment, some genes increase their expression with progressive low temperature treatment. Some of the genes have very strong expression under 3 °C treatment, but decreased after further treatment at 0 °C. The cDNA bands that showed variation after low temperature treatment were cloned and their response to cold treatment were confirmed by reverse cDNA dot blot

• **Response of the selected genes to temperature fluctuations**
  The quantitative real-time PCR analysis showed that most of the selected genes are down-regulated by cold-shock at 4 °C for 2h, a few of them are up-regulated (Table 1).

**Literature Cited:**


**Acknowledgements:** This ongoing research project is being by two USDA Capacity Building grants: 2002-38814-12598 and 2004-38814-15048.
Table 1. Real-Time PCR Analysis of Gene Expression.

<table>
<thead>
<tr>
<th>Gene Number</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
<th>Cq Value</th>
<th>PCR Efficiency</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>1001234</td>
<td>ATCGTTCATACGGTGGTGTCA</td>
<td>GGTCGACGCTGAGGCACGTA</td>
<td>50.20</td>
<td>95.82%</td>
<td>0.045</td>
</tr>
<tr>
<td>567890</td>
<td>TGGGTTGAGGATCTGAGGTG</td>
<td>GGTCGACGCTGAGGCACGTA</td>
<td>51.30</td>
<td>94.54%</td>
<td>0.012</td>
</tr>
<tr>
<td>2345678</td>
<td>ATGGTTGAGGATCTGAGGTG</td>
<td>GGTCGACGCTGAGGCACGTA</td>
<td>49.10</td>
<td>96.89%</td>
<td>0.001</td>
</tr>
</tbody>
</table>

*Note: All gene expression values were determined using Real-Time PCR analysis.*