

**Molecular Mechanisms of Reproduction and Wood Formation in Willow**

**Annual Progress Report  
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### **Project Summary**

A number of tasks were undertaken in an effort to enhance biomass production through understanding molecular mechanisms involved in reproduction and wood formation in willow. The primary objective of the research is to create a pedigree of basket willow (*S. viminalis* L.) so that we may build upon our previous work in a sex determination system in that species. A female clone of *S. viminalis* (78090) from Sweden is known to exhibit two molecular markers linked to and flanking a locus involved in sex determination in the species. This clone was established in the willow breeding program at SUNY ESF in May, and is expected to produce flowers this winter. We assessed male *S. viminalis* clones as potential pollen parents for the proposed pedigree, and found one male clone--SV5--that lacks at least one of the markers related to gender that is present in 78090, as determined by Sequence Characterized Amplified Region (SCAR) analysis. These markers were also assessed in non-*S. viminalis* willow species and found to be more significantly associated with male gender rather than with femaleness as in *S. viminalis*. The second marker is characterized by a lack of non-repeatability in its amplification profiles, and for this reason was re-isolated and re-sequenced from genomic DNA of clone 78090. Sequence information was 92-99% identical among a total of four clones sequenced. We propose to design primers that amplify a smaller region of this marker in order to increase consistency and accuracy of amplification in future experiments.

The second focus of this research involves the phenomenon of tortuosity, or spiral growth along the branches and petioles, in corkscrew willow, *S. matsudana* 'Tortuosa.' We have made crosses between female corkscrew willow and male *S. fragilis*, a species that does not exhibit tortuosity, in order to look at segregation of the trait in the progeny. Three of twelve crosses between three *S. matsudana* females (SM1,2 and 3) and an *S. fragilis* male clone (99010) were successful and produced a total of 122 progeny. Progeny were evaluated for degree of tortuosity but did not exhibit statistically significant expected segregation ratios.

## Objectives

The overall objectives of the research are to enhance biomass production through understanding molecular mechanisms involved in reproduction and wood formation in willow: The first objective is to identify the genetic mechanism involved in sex determination in certain genetic backgrounds of basket willow (*Salix viminalis*). The second is to determine the molecular mechanism controlling twisted growth form (tortuosity) in corkscrew willow (*S. matsudana* ‘Tortuosa’).

### Objective 1-Identification of a Gender Determination Locus in *S. viminalis*

Through segregation analysis and testing of RAPD primers, we have derived two SCAR markers (SCAR 354<sub>520</sub> and SCAR AE08<sub>1100</sub>) flanking a locus involved in sex determination in three pedigrees of *S. viminalis* that share a common maternal parent. Since the original pedigrees from Sweden no longer exist, and in order to more accurately estimate linkage of the SCAR markers to a gender determination locus, we need to construct additional and larger pedigrees. These pedigrees will be constructed by crossing the Swedish *S. viminalis* clone 78090 that served as the mother in the previously tested pedigrees to one or more male *S. viminalis* clones available through collaboration with the willow breeding program at SUNY-ESF. **Task 1:** To provide a segregating F1 population for further linkage analysis. **Task 2:** To apply marker-assisted selection criteria to predict phenotypes of progeny obtained in breeding work. **Task 3:** To verify the presence of the markers in the new pedigrees and develop additional markers that more closely flank the region of interest. **Task 4:** To construct a genomic DNA or BAC library of the female parent and sequence the region within the flanking markers to find a genetic locus influencing gender in this species.

We are also interested in determining if the markers related to gender in *S. viminalis* are applicable to other species, since the ultimate goal is to understand mechanisms of sex determination in the genus and in the family Salicaceae, which includes *Salix* and *Populus*. **Task 5:** To test markers associated with a sex determination locus in *S. viminalis* in other *Salix* species.

### Objective 2-Identification of Genetic Control of Spiral Growth in *S. matsudana* ‘Tortuosa’

Corkscrew willow exhibits an unusual phenomenon—tortuosity, or spiral growth along the branches and petioles—that is common to only a few species of woody plants. We hypothesize that the phenotype exhibited is due to a single gene trait, and that uncovering the molecular mechanism controlling this trait that could be fundamental in understanding and control of wood formation in trees. We have made crosses between female corkscrew willow and male *S. fragilis*, a related species that does not exhibit tortuosity, in order to look at segregation of the trait in the progeny. **Task 1:** To obtain interspecific F1 hybrid progeny segregating for tortuosity and collect phenotypic data associated with growth and development. **Task 2:** To find molecular marker (RAPD, SSR, and AFLP) associations between the phenotype of the progeny and genetic loci associated with tortuosity.

## Accomplishments

### Objective 1- Identification of a Gender Determination Locus in *S. viminalis*

**Task 1:** To provide a segregating F1 population for further linkage analysis. **Accomplishment:** In May we sent dormant plant material from the female *S. viminalis* clone 78090 to SUNY. Cuttings were planted in the field and by October at least one ramet had produced flower buds. In November we tested two male *S. viminalis* clones in cultivation at SUNY for the presence of the SCAR markers. Clone 78101 lacks SCAR AE08<sub>1100</sub> and SV5 lacks SCAR 354<sub>520</sub>. **Conclusions:** Since SCAR 354<sub>520</sub> is the more closely linked of the two markers to femaleness in *S. viminalis*, if flowers are limiting in the female clone, it would be best to develop a pedigree using SV5 as the male parent.

**Task 2:** To apply marker-assisted selection criteria to experimental design of families obtained. **Fulfillment of Task 1 is necessary for the initiation of this Task. See Renewal Proposal.**

**Task 3:** To verify the presence of the markers in the new pedigrees and develop additional markers that more closely flank the region of interest. **Accomplishment:** SCAR AE08<sub>1100</sub> did not amplify as expected in the original pedigrees, and has since been recloned from the original RAPD marker. Two species of clones have been sequenced in order to verify the previous cloning results. **Conclusions:** SCAR primer sequences may need to be modified in order to generate a smaller fragment than we have attempted previously in order to circumvent amplification of a ~75 bp A-T rich region within the marker sequence that may be affecting the efficiency of the marker in linkage analysis.

**Task 4:** To construct a genomic DNA or BAC library of the female parent and sequence the region within the flanking markers to find a genetic locus influencing gender in this species. **Accomplishment:** We have experimented with both BAC and bacteriophage lambda library methods, and have produced a partial library using the latter method. **Conclusions:** At the present time, library construction using bacteriophage lambda seems the more likely method of success in obtaining stable, size-restricted high molecular weight DNA ; however, we have decided to delay further construction until we are certain which pedigree will best suit our research goals.

**Task 5:** To test markers associated with sex determination locus in *S. viminalis* on other *Salix* species. **Accomplishment:** Forty-one clonal accessions representing 6 species were tested with SCAR 354<sub>520</sub> and SCAR AE08<sub>1100</sub>. **Conclusions:** Although there was a significant association of the marker occurrence with gender in *Salix* species surveyed, it was predominantly associated with maleness in the tested clones.

## **Objective 2- Identification of Genetic Control of Spiral Growth in *S. matsudana* ‘Tortuosa’**

**Task 1:** To obtain interspecific F1 progeny segregating for tortuosity and collect phenotypic data associated with growth and development of hybrid seedlings. **Accomplishment:** In March, we collected ~25 cuttings from four flowering *S. matsudana* ‘Tortuosa’ female trees in Oak Ridge, extracted leaf tissue for DNA analysis and sent the cuttings to SUNY for pollination by a non-corkscrew male. DNA fingerprinting analysis using RAPD markers suggests that the four female trees represent ramets of the same clonal species. Three of twelve crosses between *S. matsudana* females and an *S. fragilis* male clone (99010) were successful and produced a total of 122 progeny. Progeny were evaluated for degree of tortuosity but did not exhibit statistically significant expected segregation ratios. **Conclusions:** There is currently not enough data to determine the adequacy of the test since the number of progeny is small and the rating system used is rather crude. We need to obtain more progeny and refine our phenotypic scoring mechanism in order to obtain useful segregation data on the traits associated with tortuosity.

**Task 2:** To find molecular marker (RAPDs, SSR, and AFLP) associations between the phenotype of the progeny and genetic loci associated with tortuosity. **Fulfillment of Task 1 is necessary for the initiation of this Task. See Renewal Proposal.**

## Introduction

The mechanisms of sexual differentiation in plants are almost overwhelmingly diverse. Most models of sex determination are based on dioecious annuals like *Silene* or *Humulus*, both of which possess heteromorphic sex chromosomes. Although these species represent useful models for understanding the control of gender in a broad sense, the lability of sex determination mechanisms in plants without sex chromosomes suggests that such models may prove to be incorrect or misleading when applied to the majority of dioecious plants. The isolation of a gene or genes involved in gender determination in a species without sex chromosomes (the majority of plants) may contribute to a greater understanding of how sexual differentiation occurs.

A combination of quantitative genetics modeling and molecular genetics assays lead us to identify two rare DNA markers closely associated with sex determination in *Salix viminalis* L. (basket willow). Using these markers as an indirect indicator of gender, we have been successful in identifying female progeny resulting from specific crosses. Both markers are physically linked to a putative sex determination locus in willow, as predicted by a quantitative genetics model, and may be used to identify the locus.

In order to increase efficiency in feedstock production in biomass-based fuels, it is important to understand the genetic mechanisms of traits that may contribute to increased productivity, such as reproduction and structural growth. In dioecious woody energy crops of the family Salicaceae (hybrid poplar and willow), sex may have a large affect on biomass production. For instance, in *Populus* short-rotation energy plantations, male clones typically achieve higher dry weight yields than female clones. However, it is not possible to determine gender until the trees are reproductively mature, which may be anywhere from 5-20 years. This also hinders progress in advanced generation breeding schemes, particularly when all superior parental selections or all progeny turn out to be composed of one gender.

Wood properties can also have a large impact on biomass. Secondary growth is the primary characteristic differentiating trees from herbaceous plants and is important to long-term carbon storage. Therefore, understanding the mechanisms involved in wood formation would be beneficial for customizing feedstocks.

**The Environmental Sciences Division at Oak Ridge National Laboratory is the primary location for the laboratory portion of this research. Breeding work is being conducted at the College of Environmental Science and Forestry at the State University of New York-Syracuse campus.**

Researchers involved in this project include the following:

G.A. Tuskan, Principal Investigator	Senior Staff Member	ESD, ORNL, Oak Ridge, TN
L.E. Gunter	Research Associate	ESD, ORNL, Oak Ridge, TN
R.F. Kopp	Graduate Research Asst.	ESF, SUNY, Syracuse, NY

## Materials and Methods

### Objective 1-Identification of a Gender Determination Locus in *S. viminalis*

**Task 1:** Produce a segregating F1 population for further linkage analysis.

Branches and rooted stems of the female *S. viminalis* 78090 parent that had been grown in the greenhouse at ORNL were sent to SUNY in May. The cuttings were planted in their greenhouse under mist, and the rooted plants were transferred to the SUNY-ESF research station at Tully, NY. In November, SUNY sent us cuttings of two male *S. viminalis* clones (78101 and SV5) that are maintained at their site. The dormant cuttings were potted in soil and placed in the greenhouse under 16 h days. A subset of the cuttings was placed on tissue culture media supplemented with growth hormones BA (1.0 mg/L) and NAA (0.01 mg/L) to induce bud break. 100-160 mg of leaf tissue was harvested from the cuttings in tissue culture for DNA extraction using a CTAB genomic DNA extraction protocol. Clones were tested for presence/absence of SCAR markers using the protocol described in Task 5 below.

**Task 3:** Verify the presence of the markers in the new pedigrees and develop additional markers that more closely flank the region of interest.

Five ng of total genomic DNA extracted from the female *S. viminalis* parent 78090 was amplified with RAPD primer AE08 (Operon Technologies). A band migrating to approximately 1.5 kb previously observed to be present in the female parent and progeny but not the male parent or progeny was excised from an agarose gel, purified, diluted, reamplified and repurified to obtain a single amplification product. This marker was subsequently reamplified and labelled with digoxigenin d-UTP and used as a probe to a Southern Blot of male and female individuals to confirm homology with the RAPD marker. The amplification product was then ligated to a TA cloning vector (Invitrogen), transformed into *E. coli* and grown on selective media. Colonies containing the insert as determined by blue/ white selection were amplified on selective media, harvested, and plasmid DNA containing the insert was purified for restriction digest analysis. Digestion with *Rsa* I revealed the presence of two ligation products, representing two clone species. Both clones were sequenced on an ABI 377 DNA Sequencer using fluorescent dye terminator technology (Applied Biosystems) and compared to information obtained from previously sequenced clones.

**Task 4:** Construct a genomic DNA or BAC library of the female parent and sequence the region within the flanking markers to find a genetic locus influencing gender in this species.

We had previously tested several nuclear DNA isolation methods that were developed for other woody species. These methods incorporated polyphenolic absorbants, oxidation inhibitors or antioxidants, or phenoloxidase inhibitors. None of these procedures were consistently effective in circumventing problems associated with polyphenolic oxidases. Total recovery of DNA remains at less than 10 ug per 1 g of tissue. After isolation, nuclei were lysed directly (without being embedded in agarose), extracted with phenol:chloroform:isoamyl alcohol, precipitated with

ethanol, washed, dried, resuspended in TE and incubated for 12-24 h at room temperature. After quantitation, partial digestion conditions using three enzymes (*Bam* HI, *Mbo* I, and *Sau* 3AI) were tested on 10 µg of DNA. Optimally sized fragments (15-23 kb) for insertion into a lambda vector were found under conditions using .005-.01 units of *Mbo* I. Per µg of DNA. After digestion, sticky ends of the DNA produced by restriction digest were partially filled in to prevent self-ligation. Ligation of the insert to the Lambda Fix II/*Xho* I digested vector (Stratagene) was carried out at 4° C overnight, and the phage were packaged prior to transformation into *E. coli* strain XLI-Blue. Cells were plated on LB agar overnight and recombinant phage was observed, although packaging efficiency is much lower than in the test insert, which produces approximately  $5 \times 10^6$  plaque forming units (pfu) per µg of vector. Low packaging efficiencies are usually a consequence of low insert concentration. We are currently working to optimize yield from digestion, ligation and packaging efficiencies.

**Task 5:** Test markers associated with sex determination locus in *S. viminalis* on other *Salix* species.

Willow DNA samples were extracted using a CTAB protocol from young tissue collected from SUNY plantations in May 1998, and diluted to 10 ng/ul in water for use in PCR reactions. Genotypes represented 41 clones and six species (five within Subgenus *Caprisalix*). Each reaction contained 50mM KCl, 10mM Tris-HCl pH 8.0, 15mM MgCl<sub>2</sub>, 200 uM dNTPs, 1µg BSA, 50 ng each SCAR primer (forward and reverse) and 10 ng genomic DNA. Reaction conditions included 30 cycles of 94° C 1 min, 55° C 1 min, and 72° C 2 min, initiated with a 5 min 94° C denaturation step, and terminated with a 5 min 72° C polymerization step. Amplification products were electrophoresed on 1.5% agarose/ 0.5X TBE/0.1 µg/ml ethidium bromide, visualized under UV light, and scored for presence/absence of the marker.

## **Objective 2-Identification of Genetic Control of Spiral Growth in *S. matsudana* ‘Tortuosa’**

**Task 1:** Obtain interspecific F1 progeny segregating for tortuosity and collect phenotypic data associated with growth and development of hybrid seedlings.

Corkscrew willow cuttings were obtained from four individuals in Oak Ridge, TN in March 1999. Trees were designated SM1-SM4, and branches bearing female flowers were sent to SUNY. Leaf buds were collected for tissue analysis at ORNL. Tissue samples from the male *S. fragilis* clone 99010 used in pollination were sent from SUNY for analysis. DNA was extracted from bud and leaf tissue following a CTAB protocol and amplified with four RAPD primers that produced 17 bands. These bands were scored across all individuals to determine if they represented ramets of the same clone or different clone species.

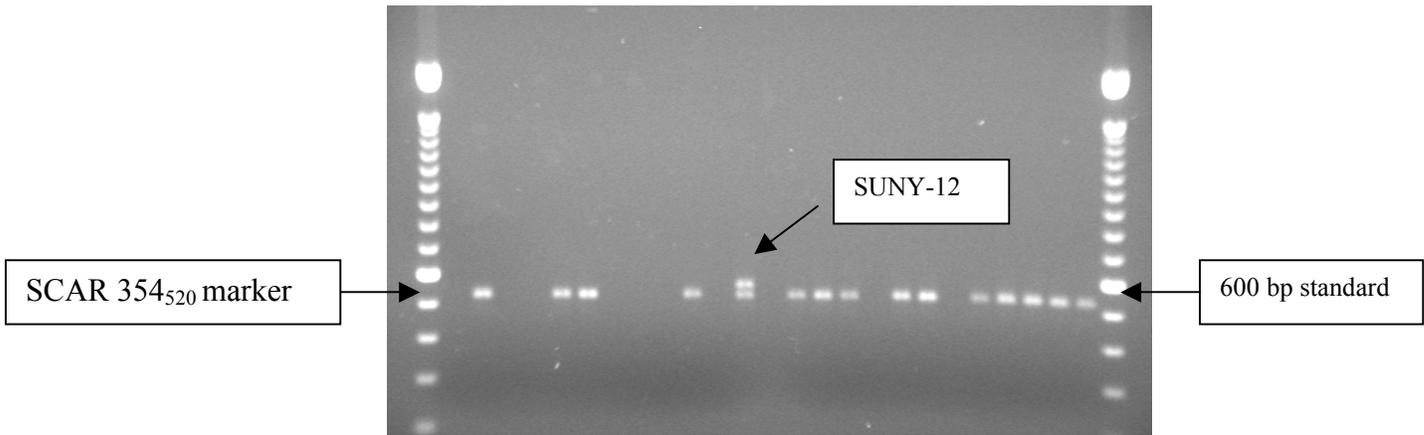
Controlled crosses were set up between SM1, SM2 and SM3 and 99010, resulting in families 99271, 99270, and 99272, respectively. Approximately 130 seedling were obtained from famliy 99270, but fewer than 20 seedlings were obtained from the other two families. Seedlings were germinated in April, grown in the greenhouse under mist conditions, and transplanted to the field in August. Prior to outplanting, seedlings were scored on a scale of 1-3 for tortuosity.



This second marker associated with gender in three families of *S. viminalis* has been somewhat problematic since its discovery. First of all, the entire marker sequence is somewhat large, close to 1.2 kb, and did not segregate as expected in the progeny which contained the original RAPD marker. This may be a function of template integrity rather than marker integrity.

When the two SCAR markers were tested on other species in the willow breeding program at SUNY, two things were apparent: First, SCAR 354<sub>520</sub> amplified in several willow species other than *S. viminalis*. Secondly, although the marker was associated with gender in these other species, it was more frequently associated with male clones (observed: 17/24), rather than with females (observed: 6/17) as it is in *S. viminalis* (Table 1; Figure 2). This is a significant observation since it suggests that the mechanism may be different, but that the same locus may still be involved in gender determination in other *Salix* spp. We had previously observed that the markers only occurred consistently in certain genetic backgrounds; therefore, we were not too surprised by these results. Interestingly, in the one *S. discolor* clone tested, SCAR 354<sub>520</sub> marker was present as a doublet (520/600 bp). This clone is a sterile male. We would eventually like to expand our analysis to examine more clones of *S. dasyclados*, which is in the same section (Section Viminalis) as *S. viminalis*.

**Figure 2.** SUNY genotypes 1-24 exhibiting presence or absence of SCAR 354<sub>520</sub> marker. A 100 bp molecular weight standard flanks the reaction lanes. SUNY-12, a male *S. discolor* sterile clone, exhibits a doublet.



**Table 1.** Results of scoring *Salix* spp. with marker SCAR 354<sub>520</sub>. Sample numbers were assigned to prevent bias in scoring. Gender information was not known at the time of testing. X=cross; N= native accession.

sample	354 scar	comments	sex	species	clone	notes
suny-44	0		F	DASYCLAD	SV1	X
suny-12	11	520/600	M	DISCOLOR	S365	X/STERILE
suny-31	11	520/600	M	DISCOLOR	S365	X/STERILE
suny-1	0		F	ERIO	95064	N
suny-4	0		F	ERIO	95331	N

suny-8	0		F	ERIO	95060	N
suny-16	0		F	ERIO	96305	N
suny-17	1		F	ERIO	95306	N
suny-19	0		F	ERIO	95311	N
suny-5	1		M	ERIO	95333	N
suny-15	1		M	ERIO	95024	N
suny-20	1		M	ERIO	95054	N
suny-22	1		M	ERIO	95061	N
suny-26	0		M	ERIO	95022	N
suny-32	1		M	ERIO	95316	N
suny-33	0		M	ERIO	95019	N
suny-23	1		F	ERIO	95040	N/STERILE?
suny-21	1		F	ERIO	S557	X
suny-24	1		F	ERIO	S599	X
suny-30	1		F	ERIO	S566	X
suny-38	0		F	ERIO	S34	X
suny-40	0		F	ERIO	S652	X
suny-41	1		F	ERIO	S25	X
suny-2	1		M	ERIO	S287	X
suny-3	0		M	ERIO	S71	X
suny-6	1		M	ERIO	S546	X
suny-10	1		M	ERIO	S646	X
suny-25	1		M	ERIO	S185	X
suny-28	1		M	ERIO	S19	X
suny-29	1		M	ERIO	S287	X
suny-14	1		M	INTXERIO	S301	X/STERILE
suny-7	0		F	PUR	94006	N
suny-11	0		F	PUR	94003	N
suny-18	1		M	PUR	95049	N
suny-27	1		M	PUR	94001	N
suny-36	0		M	PUR	94002	N
suny-43	1		M	PUR	B194	N
suny-37	0		M	PUR	FC186	N/94002
suny-39	0		F	PUR	FC190	N/94006
suny-9	0		M	PUR	PUR12	X
suny-13	1		M	PUR	PUR34	X
suny-34	0		F	UDENSIS	SX61	X
suny-35	1	750 bp	M	UDENSIS	SX64	X
suny-42	0		M	UDENSIS	SX67	X

**Table 1.** continued

Construction of a genomic DNA or BAC library of the 78090 will be essential if this female is used as the maternal parent in our pedigree. We have made some advances towards this goal, but have not spent a great degree of effort this year. If the crosses made at SUNY this winter are successful, we will work towards making additional crosses in the coming year.

## Objective 2-Identification of Genetic Control of Spiral Growth in *S. matsudana* ‘Tortuosa’

The corkscrew willow hybrid progeny grew well in the field trial this summer. Since the numbers of progeny was small, however, it is likely that we will collect flowering branches again this spring and send to SUNY in order to increase family sizes. The system we are currently using to quantify tortuosity is not very informative, and there are several environmental factors that could be influencing the degree of twisting in the stem. Table 2 shows the rating system used for the phenotyping.

**Table 2.** Segregation analysis of tortuosity in F1 progeny of a *S. matsudana* ‘Tortuosa’ X *S. fragilis* interspecific cross. 1=straight like the *S. fragilis* male parent ; 2=intermediate; 3=twisted like the *S. matsudana* ‘Tortuosa’ female parent.

Family	Sample Size	1	2	3
99270	98	31	48	19
99271	5	1	3	1
99272	19	8	7	4

A Chi square test was used to determine the probability that family 99270 fit a 1:2:1 ratio. The *P* value was 0.22, suggesting that the fit was not very good. However, it is unlikely that we can conclude much from this data for at least two reasons: First, our sample size is small. Second, our rating system is crude. The degree of tortuosity observed may be related to differences in microclimate in the greenhouse, so it is possible that seedlings rated as 2 and 3 are really no different from each other (Figure 3). Conversely, maybe the rating system is not sensitive enough and there is continuous variation. Recent work on inheritance of twisted growth form in contorted hazelnut, an ornamental tree that is also characterized by twisted trunk and branches, suggests that tortosity may be controlled by a single recessive gene, at least in that species.

**Figure 3.** *S. matsudana* ‘Tortuosa’ X *S. fragilis* hybrid F1 progeny seedlings exhibiting various degrees of stem twisting in the greenhouse at SUNY.



### List of Publications

L.E. Gunter, G.T. Roberts, K. Lee, F.W. Larimer, and G.A Tuskan. SCAR Markers Linked to Sex Determination in Basket Willow (*Salix viminalis* L.) (in preparation).

### Financial Summary for Willow Gender Project

	FYTD Amount
MATERIAL AND MATERIAL BURDEN (Materials and Supplies)	5,060
SUBCONTRACTS AND SUBCONTRACT ADMINISTRATION (student hourly)	1,137
EFFORT AND ORG BURDEN (Gunter, 0.3 FTE)	43,735
SITE OVERHEAD	17,663
TOTAL	67,595