

Short-Rotation Woody Crops Program

at

State University of New York
College of Environmental Science & Forestry

Biomass Power for Rural Development Technical Report:

GENETIC IMPROVEMENTS OF WILLOWS Interim Program Report

**Prepared for the United States Department of Energy
Under Cooperative Agreement No. DE-FC36-96GO10132**

Submitted to:

**Edward Neuhauser, Project Manager
Niagra Mohawk Power Corporation
Syracuse, New York**

by:

**R.F. Kopp, L.B. Smart, C.A. Maynard, T.A.
Volk, L.P. Abrahamson, and E.H. White**

March 2000

TABLE OF CONTENTS

EXECUTIVE SUMMARY	1
OBJECTIVES.....	2
PRESENT STATUS.....	2
<i>Objective 1</i>	2
<i>Objective 2</i>	7
FUTURE PROGRESS	9
APPLICATION TO COMMERCIALIZATION	9
LITERATURE CITED.....	9

LIST OF TABLES

TABLE 1. CONTROLLED POLLINATIONS COMPLETED DURING FEBRUARY 1998.....	4
TABLE 2. NUMBER CROSSES YIELDING VIABLE SEED OUT OF THE NUMBER OF CROSSES ATTEMPTED DURING FEBRUARY – MARCH, 1998, AND NUMBERS OF MALES AND FEMALES INVOLVED IN THE CROSSES.	5
TABLE 3. CONTROLLED CROSSES COMPLETED DURING FEBRUARY – MARCH, 1999 IN A GREENHOUSE.	6
TABLE 4. NUMBER CROSSES YIELDING VIABLE SEED OUT OF THE NUMBER OF CROSSES ATTEMPTED DURING FEBRUARY – MARCH, 1999, AND NUMBERS OF MALES AND FEMALES INVOLVED IN THE CROSSES.	7

LIST OF FIGURES

FIGURE 1. AFLP GEL OF <i>S. ERIOCEPHALA</i> PARENTS USED IN CONTROLLED MATINGS DURING 1998.	8
--	---

EXECUTIVE SUMMARY

The State University of New York College of Environmental Science and Forestry (SUNY-ESF) has been engaged in research geared towards developing a commercial bioenergy industry based on willows (*Salix*) in the Northeast United States since 1987. Genetic improvement of willows is critical for the long-term success of the industry and has been a central research topic since the program's inception. The goal of genetic improvement efforts is to develop willow clones with increased growth rate in multiple environments, straight stems, and resistance to insect and disease pests. Willow breeding in Europe resulted in yield increases of 20% after one breeding cycle (Larsson 1998). Genetics trials completed or in progress at SUNY-ESF include a genetic selection trial with 300 different willow clones replicated on four sites, 19 clone-site trials in 7 states, and collection of more than 75 willow clones from native stands in New York and Pennsylvania. These trials provided critical information for establishing a willow breeding program at SUNY-ESF.

Production of genetically improved willows through controlled breeding began at SUNY-ESF in 1998. Protocols for willow pollen collection and storage were developed concurrently with breeding efforts during 1998. Studies successfully identified a solvent (toluene) that could be used as an alternative to carbon tetrachloride for pollen collection, making controlled breeding safer for technicians and the environment while reducing the cost by an order of magnitude. Intra- and interspecific controlled pollinations with willows were completed during 1998, totaling 119 different parent combinations. Individual seedlings from successful crosses were propagated during winter 1998-1999, and then transplanted to an irrigated, fertilized cutting orchard during spring 1999. These trees grew well during 1999 and will be propagated during winter 1999-2000 for planting in field tests during spring 2000. These are the first new willow clones developed for commercial bioenergy production in the Northeast United States in nearly a decade. These clones will also be used in breeding, producing a second generation of bred seedlings. The fact that willows flower after only 2-3 years enables production of new generations in less than half the time as hybrid poplars

Breeding at SUNY-ESF completed during 1999 focused on hybridization with species combinations shown to be successful during 1998. A total of 74 different parent combinations were tested including 47 crosses between different species. The numbers of crosses within and between species that were successful were 16 and 21, respectively. Approximately 25 seedlings of successful crosses were planted in an irrigated observation trial during August 1999.

The most promising individuals produced in 1998 and 1999 will be propagated for testing on multiple sites in 2000 and 2001. Results of 1998 and 1999 breeding efforts provided critical basic genetics information, setting the stage for large genetic gains provided adequate additional funding is available for genetic improvement activities. Research funding under "Biomass Power for Rural Development" is not adequate to support future breeding, therefore additional funding is necessary to capitalize on breeding efforts to date.

Molecular fingerprinting using Amplified Fragment Length Polymorphism (AFLP™) technology was initiated on *S. eriocephala* used as parents in 1998 breeding. Fingerprints were obtained for all of the parents, and work on progeny is in progress. Protocol optimization is in progress to insure reproducibility. All the *S. eriocephala* progeny will be fingerprinted once the protocol is optimized. The goal of this study is to increase breeding efficiency by enabling favorable parent combinations to be identified prior to actually producing seedlings.

OBJECTIVES

1. Produce intra- (within species) and inter- specific (among species) hybrids between species that have shown promise in previous studies in New York and Ontario, and determine inheritance patterns of traits important to biomass production by genetic variance component estimation.
2. Molecular markers will be identified and mapped with the long term goal of identifying inheritance patterns of traits important to willow biomass production, thereby accelerating the rate of genetic improvement.

PRESENT STATUS

Objective 1

SUNY-ESF has been involved in genetic improvement of *Salix* by selection and breeding to improve biomass productivity since 1987. Genetic selection trials containing wild type and control pollinated F1 willows totaling 300 clones were planted in 1987 on three sites in Ontario, Canada and one site at Tully, NY. Approximately 20 willow clones were identified that were ranked in the top 10% for growth rate on all four sites. These clones were propagated for further testing.

Clones selected in genetic selection trials were planted in a total of 19 replicated site trials from 1993 to 1999 in seven states. The purpose of these trials was to observe performance of selected willow clones in a wide range of site condition, and identify problems that could occur in commercial-scale willow plantings.

Collections of approximately 75 wild-type willow clones from New York and Pennsylvania were completed during 1995 to expand the genetic base for future breeding efforts. Most of these clones were *Salix eriocephala* or *S. purpurea*, because these species performed well in site trials and could be found growing wild in the region. These clones were evaluated for form and growth rate, and the best ten are currently being propagated for site trials.

Traditional breeding with willow species known to be well adapted to the bioenergy production system developed by SUNY-ESF is the new focus of willow genetic improvement efforts at the college. Several willow species have been identified that have potential for bioenergy production including *S. dasyclados*, *S. eriocephala*, *S. miyabeana*, *S. purpurea*, and *S. sachalinensis*. Intra- and interspecific hybridization with these species is expected to yield willow clones with superior characteristics for bioenergy production.

Carefully chosen intra- and interspecific matings provide the possibility of rapid genetic improvement over many generations. Advantages of interspecific hybridization include the combination of desirable traits from different species, heterosis, and greater phenotypic stability in varied environments. Intraspecific crosses within the base breeding population may be useful for the long-term success of the breeding program.

A willow pollen collection and storage protocol was developed during 1998 based on a technique developed at SUNY-ESF (Maynard unpublished data). Pollen storage is desired so male and female flowers do not have to be synchronized for breeding. Organic solvents must be used for pollen extraction to remove sticky substances on the surface of pollen grains that interfere with pollen storage and use. Carbon tetrachloride was the solvent used in the original protocol because it was highly effective. However, carbon tetrachloride is dangerous to humans and the environment, so a study was

completed to identify an alternative solvent. Six solvents (acetonitrile, carbon tetrachloride, chloroform, dichloromethane, ethyl acetate, ethyl ether, and a no-solvent control) were tested using *in vitro* germination tests. Two willow clones (S646 and Sx64) were used in the test. All treatments were replicated three times. Significant ($p=0.009$) variation in pollen germination rates was observed among solvent treatments. Two solvents, toluene and ethyl acetate, showed potential for use in willow pollen extraction, but all solvents, including carbon tetrachloride, reduced germination compared with untreated pollen. Pollen germination averaged 25% in the untreated control compared with approximately 15% for the best three solvents (Kopp unpublished data).

A second test was initiated with carbon tetrachloride, toluene, ethyl acetate, and an untreated control to confirm the effectiveness of these solvents on eight willow clones. All treatments were replicated on three different days with freshly collected pollen. Solvents significantly ($P=0.0001$) affected germination rates, averaging 39, 21, 17, and 8% for the untreated control, toluene, carbon tetrachloride, and ethyl acetate, respectively. Clones differed significantly ($P=0.0001$) in pollen germination rates, ranging from 43% (clone 94002) to three percent (clone S646). Significant interactions were not observed. Toluene was selected as the solvent of choice because it had the least effect on willow pollen germination percentages and is inexpensive.

The total number of controlled crosses attempted during 1998 was 119, with 60 interspecific and 59 intraspecific crosses (Table 1). Several interspecific hybridization attempts were successful (Table 2). *S. dasyclados* clone SV1 successfully crossed with five different *S. eriocephala* males used as parents, and with one of the two *S. miyabeana* males. Viable seed was not obtained from any interspecific matings attempted with *S. purpurea* males. For of five *S. purpurea* x *miyabeana* hybridization attempts were successful. Viable seeds were produced from 38/49 and 6/10 of intraspecific matings attempted with *S. eriocephala* and *S. purppurea*, respectively.

Pollen viability was eliminated as a possible reason for failure of most crosses that failed based on the results of *in vitro* pollen germination tests. Crosses involving *S. purpurea* clones PUR12 and 94002 (15 crosses) all failed, including intraspecific crosses. Pollen from clones PUR12 and 94002 had normal and low viability percentages, respectively, in laboratory viability tests. A reason for intraspecific hybridization failures with these two clones, or any of the others that failed, was not determined.

Table 1. Controlled pollinations completed during February 1998.

Presence of “y” indicates viable seed was obtained, “n,#” indicates no viable seed was obtained and the number of times the cross was attempted. An empty cell indicates the cross was not attempted.

Male →	646 Se ¹	9506 1 Se	9531 6 Se	9501 9 Se	9502 4 Se	9502 2 Se	9505 4 Se	9533 3 Se	287 Se	Sx6 4 Sm ¹	Sx6 7 Sm	9504 9 Sp ¹	9400 1 Sp	9400 2 Sp	PUR1 2 Sp	9401 0 Sp
Female ↓																
9630 5 Se	y	y	y	Y	y				y	n,2						
S652 Se	y	n,2	y	Y	y	y										
S25 Se			y	Y	y	y	Y		y	n,2	n,2	n,1	n,2	n,2	n,1	n,2
9533 1 Se				Y	y	n,3	Y	n,3	y		n,2					
9504 0 Se	n,3				n,3	n,3	n,4	n,3		n,2	n,2	n,2		n,2		
9506 4 Se	y	y				y	Y	y	y	n,1			n,1	n,1		
9530 6 Se	n,3	y	n,1				Y	n,2	y							
9531 1 Se	y	y	y	Y				y	y	n,2	n,2	n,2	n,2	n,2		
Sx61 Ss ¹	n,2		n,1		n,1	n,1		n,1	Y	y	n,1			n,1	n,1	
9400 6 Sp	n,3	n,1	y		n,3	y			Y	y	y	y			n,2	y
9700 1 Sp	y					n,1					n,1	n,1		n,2		
SH3 Sp		n,2	n,2		n,2				Y	y	y	y		n,1	y	
SV1 Sd ¹	y	y	y		y			y	Y	n,2	n,1	n,1	n,2	n,2		
9506 0 Se	y					y				n,2				n,,2	n,1	

¹Sd = *Salix dasyclados*, Se = *S. eriocephala*, Sm = *S. miyabeana*, Sp = *S. purpurea*, and Ss = *S. sachalinensis*.

Table 2. Number crosses yielding viable seed out of the number of crosses attempted during February – March, 1998, and numbers of males and females involved in the crosses.

Cross (female x male)	# Successful/ # Attempted	# Females tested	# Males tested
<i>S. dasyclados</i> x <i>eriocephala</i>	3/5	1	5
<i>S. dasyclados</i> x <i>purpurea</i>	0/4	1	4
<i>S. dasyclados</i> x <i>miyabeana</i>	½	1	2
<i>S. eriocephala</i> x <i>eriocephala</i>	42/50	9	10
<i>S. eriocephala</i> x <i>purpurea</i>	0/14	4	5
<i>S. eriocephala</i> x <i>miyabeana</i>	0/10	7	2
<i>S. purpurea</i> x <i>eriocephala</i>	1/11	3	7
<i>S. purpurea</i> x <i>purpurea</i>	3/9	3	5
<i>S. purpurea</i> x <i>miyabeana</i>	1/6	3	2
<i>S. sachalinensis</i> x <i>eriocephala</i>	0/5	1	5
<i>S. sachalinensis</i> x <i>purpurea</i>	0/3	1	3
<i>S. sachalinensis</i> x <i>miyabeana</i>	2/2	1	2

Crosses completed during 1998 included a full-sib progeny test involving *S. eriocephala* (Table 1). Thirty-six full-sib F1 *S. eriocephala* families were produced using a 9 x 8 incomplete diallel mating design. Ten randomly selected seedlings from each family were propagated and planted in a replicated greenhouse study as dormant, unrooted, small (2 cm in length) cuttings. Nine ramets of every clone were planted. Time of budbreak, survival, number of stems, and height of plants were measured. These rooted cuttings were planted in an irrigated, fertilized cutting orchard during May, 1999, with 5-9 ramets of every clone. The trees grew vigorously during summer and fall 1999.

Controlled crosses with willows were completed during February – March 1999, with a large emphasis on interspecific hybridization. The total number of crosses attempted was 74, including 27 intraspecific and 47 interspecific matings (Table 3). Species used included *Salix dasyclados*, *S. eriocephala*, *S. miyabeana*, *S. purpurea*, *S. sachalinensis*, and *S. viminalis*. Most of the clones used as parents were selected for high biomass production and/or form after three or more years of growth in field plantings. Some of the crosses were ones that appeared promising during 1998 breeding efforts, but the majority of the crosses were never previously attempted. The number of successful crosses was 37 including 21 interspecific crosses (Table 4). At least 25 seedlings of each successful cross were planted in a greenhouse where they were grown for outplanting.

Seedlings produced during 1999 were planted in an irrigated observation trial during August 1999. *S. purpurea* x *miyabeana* hybrid seedlings grew particularly well. Rabbits damaged many of the trees during September 1999 but trees are expected to survive.

Table 3. Controlled crosses completed during February – March, 1999 in a greenhouse. All crosses were attempted once.

Female ↓	Male →												
	SX64 Sm ¹	SX67 Sm	S646 Se ¹	95019 Se	95022 Se	95316 Se	S287 Se	95049 Sp ¹	95042 Sp	95058 Sp	94002 Sp	94003 ¹ Sp	78101 Sv ¹
SV2 Sv	y	n	y	n				n	n				y
SV7 Sv	y	y	n	n				n					
95036 Se			n	y			n						
95060 Se				n			n						
95306 Se			y										
S652 Se			y										
96305 Se				y									
95026 Sp	y	y						y	y	n	n		n
94003 ² Sp	n	n						n	y		n	y	
94006 Sp	y	y			n				y	y		y	
95005 Sp	y	y						y	y		y		
SH3 Sp	n	n						n	n	n	y		
SX61 Ss ¹	y	y						n	n		n		n
SV1 Sd ¹	n		y		y	n							y
SV1-1 Sd x ?			y		y	n							
SV1-13 Sd x ?			n		y								
SV1-14 Sd x ?													
SV1-30 Sd x ?			y		n	y							
SV1-35 Sd x ?													
SV1-38 Sd x ?			n			n							
SV1-40 Sd x ?			y										
SV1-53 Sd x ?				n		n							

¹ Sd = *Salix dasyclados*, Sd x ? = open pollinated progeny of clone SV1, Se = *S. eriocephala*, Sm = *S. miyabeana*, Sp = *S. purpurea*, and Ss = *S. sachalinensis*.

² *S. Purpurea* clone 94003 produced both male and female flowers, so this clone was used as both a male and a female.

Table 4. Number crosses yielding viable seed out of the number of crosses attempted during February – March, 1999, and numbers of males and females involved in the crosses.

Cross (female x male)	# Successful/ # Attempted	# Females tested	# Males tested
<i>S. dasyclados</i> x <i>eriocephala</i>	2/3	1	3
<i>S. dasyclados</i> x <i>miyabeana</i>	0/1	1	1
<i>S. dasyclados</i> x <i>viminalis</i>	1/1	1	1
<i>S. dasyclados</i> progeny x <i>eriocephala</i>	6/13	8	4
<i>S. eriocephala</i> x <i>eriocephala</i>	4/8	5	5
<i>S. purpurea</i> x <i>eriocephala</i>	0/1	1	1
<i>S. purpurea</i> x <i>miyabeana</i>	6/10	5	2
<i>S. purpurea</i> x <i>purpurea</i>	11/18	5	5
<i>S. purpurea</i> x <i>viminalis</i>	0/1	1	1
<i>S. sachalinensis</i> x <i>miyabeana</i>	2/2	1	2
<i>S. sachalinensis</i> x <i>purpurea</i>	0/3	1	3
<i>S. sachalinensis</i> x <i>viminalis</i>	0/1	1	1
<i>S. viminalis</i> x <i>eriocephala</i>	1/4	2	2
<i>S. viminalis</i> x <i>miyabeana</i>	3/4	2	2
<i>S. viminalis</i> x <i>purpurea</i>	0/3	2	2
<i>S. viminalis</i> x <i>viminalis</i>	1/1	1	1

Objective 2

Molecular fingerprinting was initiated using Amplified Fragment Length Polymorphism (AFLP) and an automated DNA sequencer. Molecular fingerprints are valuable because, in addition to providing positive clone identification, they can be used to produce genetic maps that will enable seedlings with desired traits to be identified before being tested in the field. The AFLP system was selected for fingerprinting because it is reported to be highly reproducible and provides a large number of bands in each test. No prior molecular genetic information is required to use AFLP.

The AFLP protocol consists of six steps: DNA extraction, digesting the DNA with restriction endonucleases, ligating adapters to DNA fragments with complementary ends, amplifying specific fragments by polymerase chain reaction (PCR) (pre-selective amplification), amplifying a subset of the pre-selective products (selective amplification), and separating and detecting the fragments by polyacrylamide gel electrophoresis. Highly purified DNA is necessary for optimum results. PCR primers in selective amplification must be optimized to provide a large number of polymorphic bands. SUNY-ESF has an automated DNA sequencer that uses fluorescent tag to detect DNA fragments, rather than radioactivity as is often used with AFLP. The AFLP procedure had not previously been used at SUNY-ESF so a protocol had to be developed that was tailored to available equipment.

DNA was extracted from all parents used in 1998 breeding. DNA extraction was also completed for ten families (100 trees) of the *S. eriocephala* progeny produced during 1998 as part of the F1 progeny test described above. The DNA was quantified using a spectrophotometer and tested on agarose gels to determine its quality. Sixteen primer pairs were tested in selective amplification and one pair was

selected for further use because they provided a large number of polymorphic bands. Fingerprints were obtained from all parents of the 1998 F1 *S. eriocephala* progeny, and from 26 of the progeny clones (Figure 1). Problems were experienced reproducing fingerprints of some parents and progeny. The AFLP protocol will be optimized to increase fingerprint reproducibility and all samples will be tested again to confirm banding patterns are accurate.

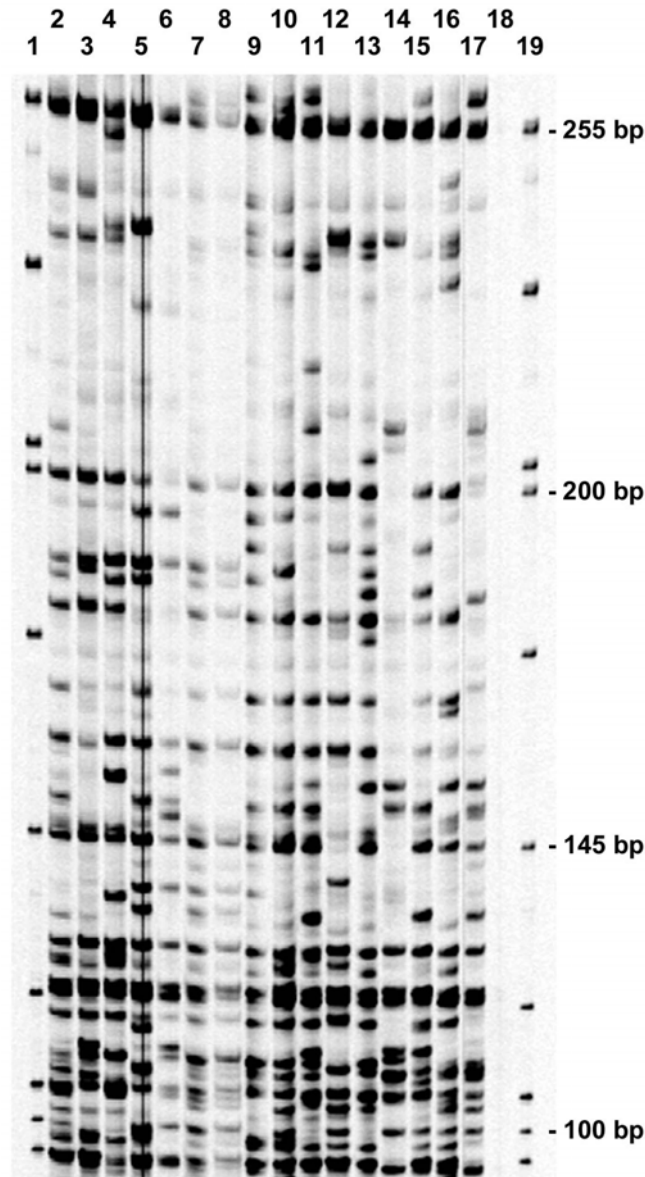


Figure 1. AFLP gel of *S. eriocephala* parents used in controlled matings during 1998. Lanes are as follows: 1) molecular weight standard, 2) clone 95316, 3) clone 95061, 4) clone S646, 5) clone 95019, 6) clone 95024, 7) clone 95022 [disregard due to error during sample loading], 8) clone 95022, 9) clone 95054, 10) clone S287, 11) clone 95331, 12) clone S652, 13) clone S25, 14) clone 96305, 15) clone 95064, 16) clone 95306, 17) clone 95311, 18) water control, and 19) molecular weight standard.

FUTURE PROGRESS

Statistical analyses will be completed on budbrak, survival, stem number, and height data collected during spring 1999 from F1 *S. eriocephala* progeny planted in a greenhouse study during January, 1999. Variance components will be estimated for calculating heritabilities. Flower-bearing shoot tips will be collected from F1 *S. eriocephala* progeny during winter 1999-2000 for breeding. Crosses will be completed to yield both outcrossed and inbred F2 progeny lines. Cuttings for clonal progeny tests with the *S. eriocephala* F1 progeny will be harvested during winter 1999-2000 from the cutting orchard established during 1999. Trials will be established on two sites with these cuttings during spring 2000. Both experiments will consist of four replications of four-tree linear row plots. Site preparation including contact herbicide application, plowing and discing will be completed. Spacing will be 0.6 by 1.1 m, which provides trees with approximately the same amount of space as they would have using the Swedish double-row spacing. Measurements on growth rate, form, and pest resistance will be completed annually and biomass will be measured three years after coppicing. These trials will enable estimates of additive and non-additive genetic variance components, general combining ability, specific combining ability, and genotype-by-environment interaction. Heritability estimates for any measurable traits will be available from these studies.

AFLP protocol optimization will be completed and fingerprints will be generated for all *S. eriocephala* parents and their F1 progeny that were created during 1998. Laboratory work and data interpretation are expected to be completed by April, and in June, 2000, respectively.

A proposal to develop a willow crop development center focusing on genetic improvement of willows was submitted for funding to the U.S. Department of Energy Oak Ridge National Laboratory. If funded, willow genetic improvement efforts will increase and include development of clones for the North-central United States. The project will be completed in cooperation with Dr. J.G. Isebrands at the USDA Forest Service Research Station, Rhinelander, WI.

APPLICATION TO COMMERCIALIZATION

Trees produced during 1998 and 1999 are being used in genetics studies and to steer future breeding efforts. Some of the trees produced during 1998 will be propagated for large-scale field testing, as will the best trees produced during 1999. These are the first new willow clones developed for commercial bioenergy production in the northeast United States in nearly a decade. Genetics information gained from crosses completed during 1998 and 1999 and molecular fingerprinting studies will increase the rate of genetic improvement, leading to willow clones that are better adapted to bioenergy production than currently available clones.

LITERATURE CITED

- Larsson, S. 1998. Genetic improvement of willow for short-rotation coppice. *Biomass and Bioenergy* 15(1): 23-26.
- Vos, P., R. Hogers, M. Bleeker, M. Reijans, T. Lee, M. vandeHornes, A. Frijters, J. Pot, J. Peleman, M. Huiper, and M. Zabeau. AFLP: A new technique for DNA fingerprinting. *Nucleic Acids Research* 23: 4407-4414.