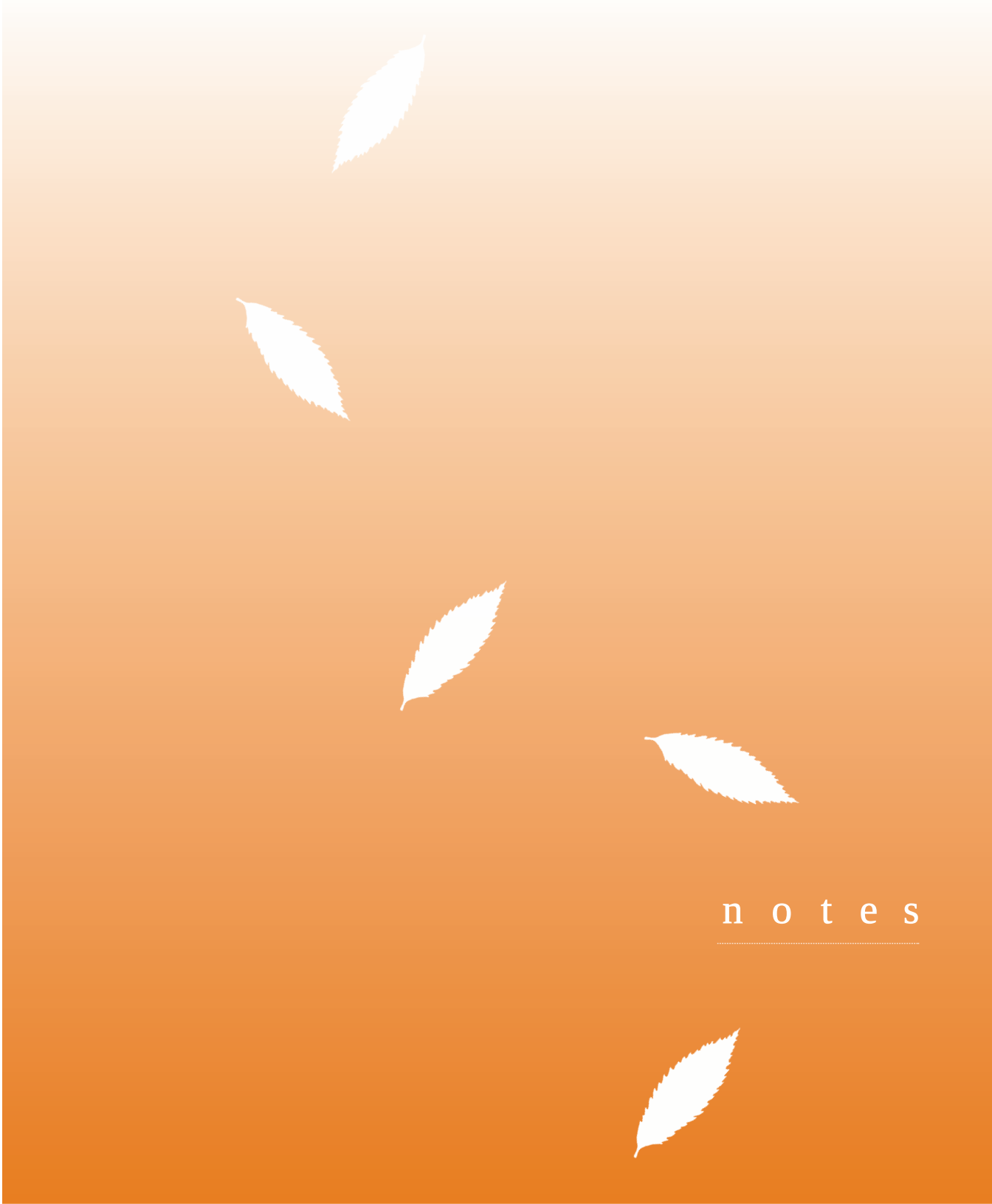
Vol.XII, #2 Autumn 1998

Notes

Memories

Science and Natural History

BLANK PAGE



notes

BLANK PAGE

FROM THE EDITOR

Autumn 1998

With this issue of The Journal, as with all of them, we hope to offer you a sampling of the many ways the American chestnut has touched and still touches people's lives. And so we range widely, both in time and as to geography, and from the simplest of natural history questions to the most esoteric.

Volunteers for the National Big Trees Register, for example, ask the straightforward question: where is the biggest American chestnut in my state, and just how big is it? But then follows a good deal of sleuthing, a passel of paperwork, and on occasion (as in Kentucky) an early morning drive to check whether the Big One survived a tornado. (It did.) You'll find the results of their efforts in our list of the largest native-range American chestnuts - the current "chestnut champs."

Fifty-two years ago, noted chestnut breeder and horticulturist Arthur Graves thought he was nudging closer to a solution to the chestnut blight with his work with Asian / American hybrids of many combinations. He shared his optimism with the general public in a 1946 article in Yankee magazine, an article we reprint here. We know now that Dr. Graves' work was largely misdirected, although it yielded at least one good result: the 'Graves' hybrid, a tree we use extensively in the breeding program at our TACF Meadowview research farms. You'll see it mentioned in Dr. Fred Hebard's annual update on the growing chestnut collection at the farms. Dr. Hebard also gives us an idea of what our scientists do when not caring for the trees. (They study them, from any of a number of perspectives.)

Dr. Emily Russell contributes an interview to this issue of The Journal that causes us to look way back. Have you wondered how American chestnut survived glaciation? why it was confined to the eastern part of the continent? how it was moved from place to place? why it was at one time a dominant species? Dr. Russell, a historical ecologist, suggests some answers to those questions.

Dr. Hongwen Huang's work on native chestnut diversity also treats to some extent the effects of glaciation. If chestnut retreated to climatically safe havens during the last ice age, those havens should be reflected in today's tree populations. Dr. Huang's findings regarding the

locations of genetic hot spots could prove immensely useful as we begin to incorporate American chestnuts from throughout the native range in our breeding program.

Finally, Drs. Charles Maynard, Zizhuo Xing, Sharon Bickel, and William Powell contribute a summary of their work to date on gene transfer technology and on micropropagating chestnuts. Much of their research has been devoted to discovering compounds present in other organisms that, when introduced into American chestnut, could confer blight resistance. Because it is difficult to predict the wider effects these compounds might have, the research team has spent a good deal of effort on discovering and designing safety mechanisms for them. You'll read in their article about acceptable levels of damage to cell walls and molecular switches that go on only when blight is present. You'll also read about novel plant propagation techniques, including a sandwich-like growing medium.

We hope you enjoy this issue and that you'll let us know what you think about it and about the items it reports on. We're always glad to hear from our members!

Shelly Stiles, Editor

MEADOWVIEW NOTES 1997-1998

by Dr. Fred V. Hebard, TACF staff pathologist

The year 1997 began inauspiciously. A refrigerator froze up in January, killing many nuts and damaging others. (We have installed alarm systems in the refrigerators to help prevent such an occurrence in the future. In addition, we have expanded our refrigerator capacity to accommodate the larger volumes of nuts we are harvesting.) An unusually wet spring hampered seedling emergence by flooding some of the planting sites, which killed the nuts. (Out of 3275 planted seed, only 1,706 emerged, about 50%. Usually, we get over 80% emergence.) And prolonged cool wet weather also promoted damping off.

At pollination time, we placed 510 fewer bags than in 1996. The bag count was down because a late spring frost killed flower primordia on many trees. (For instance, in 1996 we were able to place 928 bags on second backcross trees. In 1997 we placed only 452 bags on such trees.) Additionally, there were no male flowers on several promising first and second backcross trees, so we were unable to cross them onto American chestnut female flowers. Nonetheless, the 1997 harvest was a good one, second only to 1996's record 5,979 nuts. Because the bags contained more burs in 1997 (11,727) than in 1996 (11,431), the total nut harvest did not drop much.

Among breeding crosses, we harvested 2,818 third backcross nuts from two sources of resistance, 291 second backcross nuts from three sources of resistance, 355 first backcross nuts from three sources of resistance, and 979 first hybrid nuts from 12 sources of resistance. Many of the first hybrid nuts were produced for research purposes rather than to expand our breeding stock. Our current holdings, including 1997 nuts planted in 1998, are shown in Table 1. Changes from 1997 to 1998 are illustrated in Table 2. The provenances of the 1997 harvest are summarized in Table 3.

Blight Resistance

We've learned a little more about our sources of resistance. 'Nanking' Chinese chestnut appears to be homozygous for resistance as the variance of resistance metrics of its first hybrids with American chestnut is comparable to those of the parental types. Some first backcross progeny of

'Nanking' have markedly smaller cankers than their F1 parent. The homozygosity of several other Chinese chestnut sources of blight resistance was indicated in 1989 by the homogeneity of canker phenotype within F1 families (there was heterogeneity between families). The 'Graves' first backcross also appears to have a complete set of the genes for blight resistance since its second backcross progeny have mean canker sizes a bit smaller than those on first backcross progeny derived from the same source of blight resistance as the 'Graves' tree (which is the 'Mahogany' Chinese chestnut). Canker sizes on second backcross progeny of the 'Clapper' first backcross are a bit smaller yet than those on second backcross progeny of the 'Graves' tree.

Most F2 and BC1F2 plants which showed high levels of blight resistance in 1993, when we began innoculating with the blight fungus, continue to fare well; the resistance is holding up. Test crosses of some of these plants to American chestnut were evaluated for blight resistance this year. No clear evidence emerged that any of the parents were homozygous for blight resistance, and several clearly were not homozygous for blight resistance. We will follow up on these findings in much more detail in 1998.

Since two blight resistance genes from Chinese chestnut appear sufficient to confer high levels of resistance, it isn't possible to detect more resistance genes phenotypically without test crosses. The diversity of resistance genes in Chinese chestnut is being investigated by making crosses of Chinese chestnut, by making crosses of backcross progeny derived from different sources of resistance, and by comparing the location of genes for blight resistance on molecular genetic maps of progenies derived from different sources of blight resistance.

Selection for American Type

As well as selecting for resistance to blight, we also select among backcross trees for individuals with American traits. Among morphological traits, we select for progeny without simple hairs on the interveinal regions of abaxial leaf surfaces and with 1) sparse, long, simple hairs on abaxial midribs and secondary veins; 2) hairless twigs; 3) red stem color; 4) small stipules; 5) small, dense lenticels; and 6) cylindrical, pointed buds. We select against dwarf progeny, progeny that form thick bark layers early, and male-sterile progeny. We select for progeny which do not break bud

Table 1

Type and Number of Chestnut Trees and Planted Nuts at the Meadowview Research Farms in April 1998, with the Number of Sources of Blight Resistance and the Number of American Chestnut Lines in the Breeding Stock

	Number of		
	Nuts or Trees	**Sources of Resistance**	**American Lines***
Type of Tree			
American	1125		53
Chinese	394	30	
Chinese x American: F_1	508	20	57
American x (Chinese x American): BC_1	796	11	37
American x [American x (Chinese x American)]: BC_2	2558	11	55
American x {American x [American x (Chinese x American)]}: BC_3	3001	2	61
(Chinese x American) x (Chinese x American): F_2	311	3	4
[(Ch x Am) x (Ch x Am)] x [(Ch x Am) x (Ch x Am)]:F_3	9	1	1
[Amer x (Chin x Amer)] x [Amer x (Chin x Amer)]: BC_1-F_2	464	2	2
{Am x [Am x (Ch x Am)]} x {Am x [Am x (Ch x Am)]}:BC_2-F_2	476	1	1
Chinese x (Chinese x American): Chinese BC_1	145		
Chinese x [American x (Chinese x American)]	44		
Japanese	3	2	
American x Japanese: F_1	6	4	4
(American x Japanese) x American: BC_1	5	1	1
Castanea sequinii	48	1	
Chinese x Castanea pumila: F_1	8		
Large, Surviving American x American: F_1	274	9	10
(Large, Surviving American x American) x American: BC_1	271	2	7
Large, Surviving American x Large, Surviving American: I_1	297	6	6
Irradiated American	48	3	3
Other	27		
Total	**10,818**		

* The number of lines varied depending on the source of resistance. We will have to make additional crosses in some lines to achieve the desired number of 75 progeny per generation within a line. In keeping with past practice, the number of lines for each source of resistance are added separately; thus, progeny from two sources of resistance with the same American parents would be counted as two lines rather than one line (this occurs rarely).

Table 2

Changes between 1997 and 1998 in the Number of Chestnut Trees and Planted Nuts of Different Types at the Meadowview Research Farms, Including Changes in the Number of Sources of Blight Resistance and the Number of American Chestnut Lines in the Breeding Stock

	Increase or Decrease* in Number of		
	Nuts or Trees	Sources of Resistance	American Lines
Type of Tree			
American	84		14
Chinese	2	-12	
Chinese x American: F_1	-304	1	-3
American x (Chinese x American): BC_1	213	2	8
American x [American x (Chinese x American)]: BC_2	-311	5	8
American x {American x [American x (Chinese x American)]}: BC_3	785	0	22
(Chinese x American) x (Chinese x American): F_2	27	0	0
[Ch x Am) x (Ch x Am)] x [Ch x Am) x (Ch x Am)]:F_3	0	0	0
[Amer x (Chin x Amer)] x [Amer x (Chin x Amer)]: BC_1-F_2	4	0	0
{Am x [Am x (Ch x Am)]} x {Am x [Am x (Ch x Am)]}:BC_2-F_2	0	0	0
Chinese x (Chinese x American): Chinese BC_1	0		
Chinese x [American x (Chinese x American)]	0		
Japanese	-1	-1	
American x Japanese: F_1	5	3	3
(American x Japanese) x American: BC_1	0	0	0
Castanea sequinii	0	-2	
Chinese x Castanea pumila: F_1	6		
Large, Surviving American	-1	-1	-1
Large, Surviving American x American: F_1	-57	0	0
(Large, Surviving American x American) x American: BC_1	271	2	7
Large, Surviving American x Large, Surviving American: I_1	255	2	2
Irradiated American	0	0	0
Other	-5		
Total	**973**		

* The decrease in F1 trees reflects lack of emergence of nuts planted in 1997. The decrease of BC2 trees reflects roguing of trees with inadequete levels of blight resistance. The increases in BC1 and BC3 trees are due to further breeding of those, minus lack of emergence. The increases in Large, Surviving American chestnut trees of various types are due to further breeding.

before the first frost-free date (about May 15-17 in Meadowview). This latter trait is critical as early bud break leads to freeze-killing of the emerged bud, which leads in turn to clusters of branches derived from buds immediately below the dead terminal bud. The branch clusters give very poor tree form. Additionally, death of the terminal bud can severely impair flowering and fruiting during the year of bud death.

The selection for appropriately timed bud emergence must be done on a regional basis and is a strong argument for establishing regional breeding centers. In 1997, we initiated a test planting at Meadowview of American chestnut trees from various parts of the country. That test will help us determine whether trees from different regions of the country vary in their time of bud emergence. Preliminary data reported in the Journal of Heredity (85:440-446, 1994) suggest the occurrence of such regional variation. In several sets of backcross progeny derived from two Chinese chestnut trees, 'Nanking' and 'Mahogany,' the time of bud emergence was strongly associated with one linkage group on a molecular map. There was no linkage to blight resistance in one of the progenies.

We would like to investigate additional macroscopic traits. Among these are branch angle and branch size, which may be important factors in tolerance of heavy snow loads. Another easily scored trait is the time leaves turn from green to yellow in the fall (so that it occurs prior to leaf-killing frosts); this could be important to mineral nutrient conservation by the tree.

Resistance Instability

There is always the possibility that the blight fungus will evolve to overcome blight resistance, although the blight resistance of Chinese chestnut appears stable in the U.S. (There are no documented cases of American-type cankers killing Chinese chestnut.) This could be investigated more thoroughly by surveying for canker severity on Chinese chestnut, followed by isolation and performance of pathogenicity tests on Chinese chestnut. We are beginning to investigate resistance stability in China by planting grafted U.S. cultivars there. The blight fungus is more diverse in China than in the U.S., so one would expect the blight resistance of U.S. cultivars of Chinse chestnut to be overcome more easily in China than in the U.S.

TABLE 3

The American Chestnut Foundation 1997 Nut Harvest from Controlled Pollinations and Selected Open Pollinations

Type Type	Female Parent	Pollen Parent	Pollinated			Unpollinated Checks			Number of American Chestnut Lines*
			nuts	bags	burs	nuts	bags	burs	
BC_1	American	72-211 F_1	58	104	214	4	8	22	3
BC_1	American	Meiling F_1	30	89	214	2	7	18	2
BC_1	American	Nanking F_1	186	370	987	16	29	82	12
BC_1	Nanking F_1	American	81	53	115	0	8	17	4
BC_1-F_2	Nanking BC_1	Nanking BC_1	14	42	93	0	4	9	2
BC_1-F_2	S.Lot R1T10 BC_1	S.Lot R1T10 BC_1	14	23	37	0	3	7	2
BC_2	Mahogany BC_1	American	47	15	25	0	2	4	2
BC_2	American	Nanking BC_1	166	204	508	2	16	32	4
BC_2	Nanking BC_1	American	11	51	114	0	5	13	2
BC_2	American	S.Lot R1T10 BC_1	67	72	146	0	4	6	2
BC_2-F_2	Clapper BC_2	Clapper BC_2	67	77	202	0	7	17	2
BC_2-F_2	Mahogany BC_2	Clapper BC_2	133	99	435	0	11	43	2
BC_2-F_2	Mahogany BC_2	Mahogany BC_2	16	52	116	0	5	11	1
BC_2-F_2	Clapper BC_2	open pollinated	1001			open pollinated			
BC_3	American	Clapper BC_2	1431	875	2204	9	68	155	35
BC_3	Clapper BC_2	American	757	373	979	4	37	87	10
BC_3	American	Mahogany BC_2	417	497	1009	9	40	72	22
BC_3	MahoganyBC_2	American	213	79	542	0	7	36	3
Chinese	72-211	Orrin	3	20	22	0	0	0	
Chinese	Meiling	72-211	110	66	133	0	4	10	
Chinese	Orrin	Meiling	116	60	203	4	5	10	
F_1	65-18	American	11	6	6	0	1	1	1
F_1	American	65-18	59	82	313	0	4	12	1

Nut Type	Female Parent	Pollen Parent	Pollinated			Unpollinated Checks			Number of American Chestnut Lines*
			nuts	bags	burs	nuts	bags	burs	
F_1	American	72-211	58	78	263	1	7	23	1
F_1	American	Kuling	85	65	158	0	8	21	4
F_1	American	Meiling	96	49	210	0	5	17	1
F_1	American	Nanking	169	42	149	1	8	27	4
F_1	American	Orrin	50	83	250	8	6	21	1
F_1	American	P11	239	104	117	12	15	10	1
F_1	American	P13	4	50	83	0	5	16	1
F_1	American	P17	0	12	42	0	2	4	1
F_1	American	FP 7284	12	65	186	0	4	7	1
F_1	Meiling	American	60	43	153	0	5	16	1
F_1	FP 7284	American	38	47	79	1	3	7	1
F_1	American	European	98	68	115	5	4	8	1
$LSBC_1$	American	Gault F_1	67	117	293	3	6	11	2
$LSBC_1$	American	Scientists' Cliff F_1	497	245	538	6	26	49	9
$LSBC_1$	Gault F_1	American	18	5	13	0	1	0	1
LSF_2	Gault F_1	Gault F_1	182	67	257	0	6	21	1
LSF_2	Scientists' Cliff F_1	Scientists' Cliff F_1	73	33	65	0	3	5	1
complex	American	Meiling x Nanking F_1	72	58	139	2	6	9	
Total Controlled Pollinations			**5,825**	**4.540**	**11,727**	**89**	**395**	**936**	

*The number of American lines for this table is restricted to the number of American chestnut trees that were direct parents, not grand parents, of progeny.

Number of Backcrosses

One of the moost important questions we ask is How many cycles of backcrossing are needed to recover the American type? Our current program assumes this can be done with three backcrosses. The only full answer to this question would come from planting out highly blight resistant trees at various stages of backcrossing and determining how they grow over a full rotation of 30 to 50 years. Because of the long testing period, it might be more efficient to set out the third backcross generation and meanwhile advance aggressively to the sixth backcross generation. The recurrent parent has always been recovered after six backcrosses in other plants. We expect that will occur also in American chestnut.

CHESTNUT CHAMPS

The American Forestry Association (also known as American Forests) began keeping track of the nation's biggest trees in 1940. Now its National Big Trees Register maintains records on more than 800 tree species in all the states.

Whether a tree is a really Big Tree depends on c+h+1 / 4s (translation: circumference in inches plus height in feet plus one quarter its average crown spread in feet).

So, for example, an American chestnut with a circumference of 235 inches (that's a diameter of a little more than six feet), a height of 106 feet, and an average crown spread of 101 feet would score 366 points. An American chestnut with c=247 inches, h=86 feet, and s=111 feet would score 361 points. Each would stand as the current national American Chestnut Co-champion. Both are in Washington state.

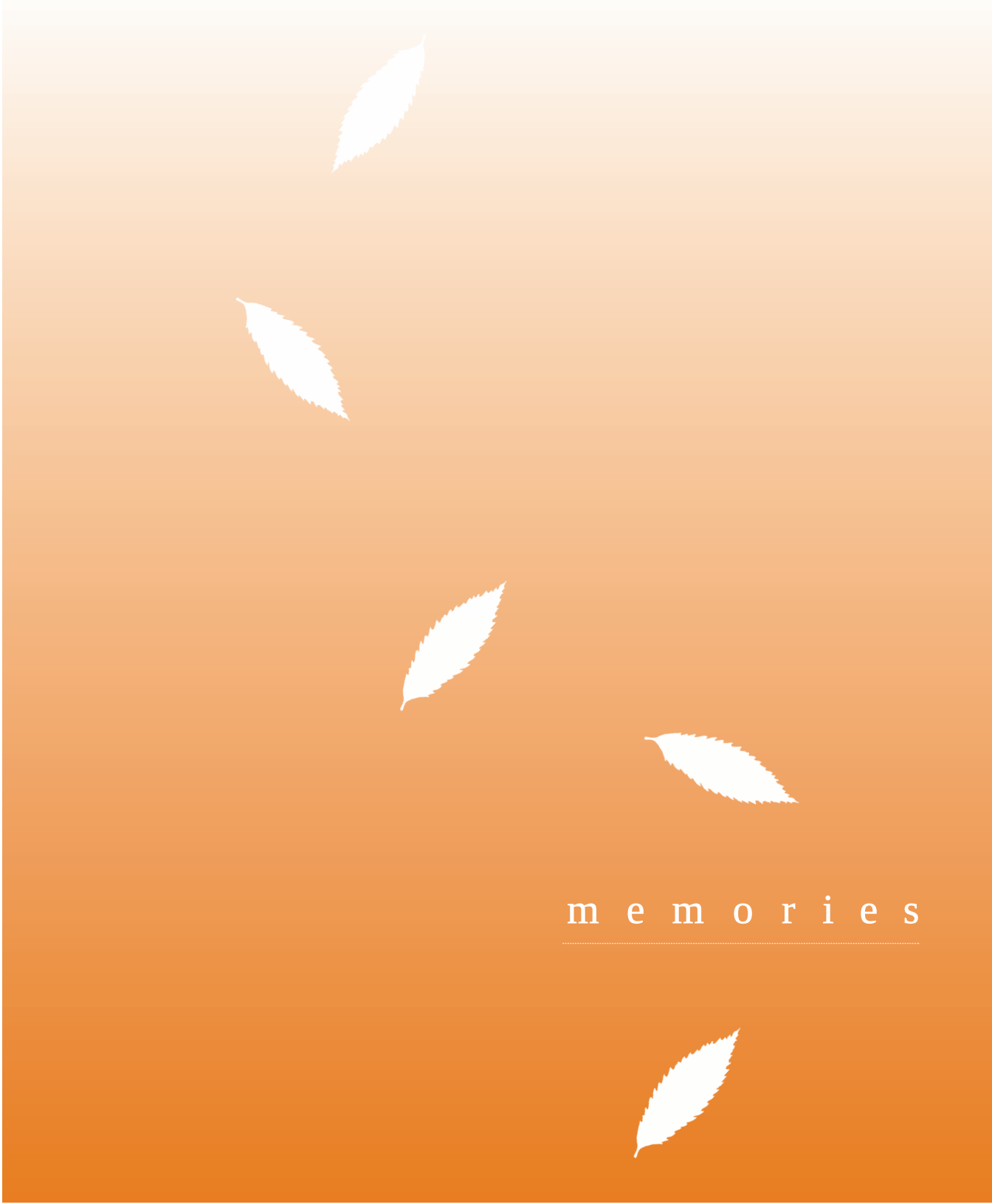
Back east, in and near the native range of the tree, the state Champion Chestnuts are:

State and town or county	Circumference (in inches)	Height (feet)	Average Spread (feet)	Score
Alabama, Chilton County	44	49	101.5	118
Connecticut, Madison	53	70	38	133
Delaware, Dover	121	51	51	184
Georgia, Ray City	165	45	52	226
Illinois, Mt. Carroll	120	70	47	201.75
Indiana	Champ died 1997; no new nominee			
Kentucky, Columbia	118	55	10	183
Maine, Orono	87	44	38	141
Maryland, Calvert County	87	75	36	171
Massachusetts, Royalston	41	65	29	113
Michigan, Grand Traverse County	208	64	80	292
Mississippi, Smith Cty	128	44	50.5	185
New Hampshire, Sandwich	49	80	36	138
New Jersey, Pittstown	50	68	42	129
New York, Chataqua County	73	85	12	170
North Carolina, Wilkes County	94	76	57	184
Ohio	Champ died recently; no new nominee			
Pennsylvania	Champ died; no new nominee			
Rhode Island, Foster	26	39	29	72

State and town or county	Circumference (in inches)	Height (feet)	Average Spread (feet)	Score
South Carolina, Pickins County	45	79	37	133 (in 1981)
Tennessee, Sumner County	55	66	30	129
Vermont, Berlin	67	88	38	165
Virginia, Amherst	122	53	62	191
West Virginia, Mineral County	277	50	20	83
Wisconsin, Hamilton	136	76	68	229

The Big Tree program is run entirely by volunteers, from the local folks who find and nominate trees through the state coordinators who compile and rank the nominations to the national big tree coordinator at the American Forests office in Washington, D.C. If you'd like to nominate a Big American chestnut, the Bennington office can put you in touch with your Big Tree coordinator - who is hoping to see your state's current record fall!

Kentucky's chestnut champ was photographed shortly after a tornado blew through, destroying a house on one side of it and a garage on the other. (Storm debris still hangs from one of its limbs.)


memories

MEMORIES

Dr. Arthur Graves, a professor at the Yale School of Forestry early in the century and a curator at the Brooklyn Botanic Garden from 1921 to 1947, instituted one of the country's earliest and, eventually, one of its largest chestnut breeding programs. The many hybrids he and his students produced were planted on his property in Hamden, Connecticut, later deeded to the State of Connecticut, and now managed by the Connecticut Agricultural Experiment Station. This article, which summarizes many years' work by Dr. Graves, appeared in the September 1946 issue of ***Yankee Magazine*** *("A Good Deal on Every Page"). We reprint it with* ***Yankee****'s permission.*

Making New Chestnut Trees

by Arthur Harmount Graves
(Curator, Brooklyn Botanic Garden)

For those unacquainted with the chestnut history of the last 50 years may I say briefly that the American chestnut, growing naturally from northern New England southward in the Appalachians to Alabama, was attacked some time, apparently, during the 90's by a deadly parasitic fungus introduced into this country evidently on imported Japanese or Chinese trees. For a time the miserable stowaway worked unnoticed, but was at length (1904) discovered on native chestnut in the New York Zoological Park in New York City. The subsequent history of the progress of the disease through the chestnut forests of the country would require more words than you would care to read; the main point is that now, after 50 or 60 years, our fine old American chestnut tree has all but disappeared from our forests. Only scattered shoots, with a short lease of life, remain, and, so it is reported, a few isolated large trees in the high mountains of the Southern Appalachians.

What does this mean to the American people? Many things, but if we speak in terms of money it means a loss of millions of dollars' worth of valuable lumber; and when we consider the loss for all time in the future the figures become astronomical. For the principal value of the chestnut was in its timber - long-lasting and not easily subject to decay. Its tall straight trunks were invaluable for telegraph and telephone poles. Many an old New England farmhouse can proudly display its sills and con-

struction timbers made of chestnut. The bark was precious for its tannic acid content used in tanning leather.

And the nuts! In comparison with its timber value their worth was slight indeed, but there are still many of us living who remember with delight those joyous occasions when we went "chestnutting" on crisp October days, in the "woods" or under isolated trees in the open, and gathered the shining brown nuts, sweet to eat raw and delicious when roasted, with a little salt added. Those were happy days!

What are we doing about it? After many attempts in other directions we decided that the only way to outwit the parasite was by tree breeding - that is, by producing a kind of chestnut tree that the parasite didn't like. Fortunately for us, the Japanese and Chinese chestnuts resist the attacks of the parasite often so effectively that certain individuals of those species, especially the Chinese, are practically immune. However, these Asian chestnuts are generally low-growing, comparatively bushy trees, and cannot replace the tall American chestnut as timber producing trees. Therefore we conceived the idea of breeding these low-growing, disease resistant Asiatics with the tall, susceptible American, in the hope that in some of the offspring the desirable characters, namely the tall, erect growth of the American parent and the disease resistance of the Asiatic parent would be combined. This breeding was begun in 1930, and since that time we have continued the work at each flowering season, namely, in the latter part of June and early July. The Division of Forest Pathology, United States Department of Agriculture, had started similar work at about the same time, and has been cooperating with us through the years.

In the beginning the Japanese species was the only Asiatic available to us for breeding, and the result of the combination of American and Japanese chestnuts was at first most encouraging. The Japanese-American hybrids showed great vigor and rapid growth - some grew four feet in a year, which is much in excess of the rate of growth of the American chestnut. This was an expression of the phenomenon of "hybrid vigor," well known to all geneticists. In addition, the Japanese-American hybrids in most cases possessed the erect habit of the American parent - that is, with a straight central trunk, not branchy and bushy like the Japanese. But unfortunately, they are still susceptible to the blight disease, although not nearly as much as is the American parent. In other words, to use genetic terms, the American parent is *dominant* in the hybrid.

But, as Dr. D. F. Jones, Geneticist of the Connecticut Agricultural Experiment Station, who is also cooperating with us, says, "The disease resistance is in these hybrids. If we put in resistance at the beginning, it must be still there (although 'recessive') and continued breeding should eventually bring it out."

In this connection, another problem now faces us: at the age of seven years or so, these Japanese-American hybrids, "taking after" their American forbears, began to get the blight and threatened to leave this world for the next, so that further breeding would have been impossible. Here Nature stepped in and gave us a lift. For at the bases of the diseased Japanese-American hybrids, or below lesions caused by the blight fungus, numerous shoots began to appear, which, by the way, is a well known symptom of the disease. I believed that if we could graft the tops of these shoots *above* the blighted area we could "bridge" it so that the communication of living bark above and roots below could be restored; and that if this could be done effectively we could save the lives of our Japanese-American hybrids and continue breeding them. I am glad to say that this method of grafting (or more correctly "inarching") has been absolutely successful so that we still have our Japanese-American hybrids, and have continued breeding with them up to and including the present year.

I have said that at the beginning the Japanese chestnut was the only Asiatic kind available to us for breeding. But I soon found that the Chinese species, some fine seedlings of which were given us in 1929 by the Division of Forest Pathology, United States Department of Agriculture, was by far the more disease resistant of any species, and crossing this with the American chestnut began in 1934. In 1938 we began crossing our Japanese-American hybrids with the Chinese, and this combination seems to date by far the best. Inoculation tests have shown that individuals with this pedigree in most cases do not take the disease.

However, in the Chinese Japanese-American hybrids the Chinese parent, usually a spreading tree, not suitable for timber, is dominant, and this year we are adding the American species to the combination to give it a more erect character. In a few cases, indeed, the Chinese Japanese-Americans do show an erect habit. And this year, through the kindness of Mr. Michael Evans of Greenville, Delaware, and Professor Maurice A. Blake of the New Jersey Agricultural Experiment Station, I have received

a supply of pollen from tall, erect Chinese individuals, which we have applied to our most desirable hybrids.

As far as the production of nuts is concerned, the Chinese species seems to me perfectly satisfactory. Most individuals - there are exceptions - seem to be perfectly disease resistant, and we find that care, i.e., a little cultivation, pruning and fertilizing, increases resistance to the blight. The nuts are large and sweet. The nuts of the Japanese - often larger than those of the Chinese - have usually a somewhat bitter taste, although this tends to disappear on boiling.

On our plantation on the Sleeping Giant Mountain at Hamden, Connecticut, we have had nearly 400 bagged flowering branches this summer representing 72 combinations in which different trees are involved. We have there and at the plantation at the White Foundation at Litchfield, Connecticut, as well as at the Yale Forest in Tolland and Windham Counties, Connecticut, two of our cooperators, about 1,000 hybrids plus about 1,000 more trees of straight species, representing nearly every kind of chestnut known - from Europe, Northern and Southern United States, and Asia. This year the project is receiving the support of the Connecticut Geological and Natural History Survey as well as that of the Division of Forest Pathology, U. S. Department of Agriculture, and, of course, of the Brooklyn Botanic Garden.

How can you help us? In two ways. First: by sending pollen of the American chestnut. It is getting scarce. Roots of chestnuts resist the disease more than do the trunks and branches, so that when a tree dies above there is time for shoots to arise from the base, and sometimes these grow old enough to bear flowers before being laid low by the disease. But these flowering shoots are getting less and less frequent.

Second: if you find nuts borne on any of these native shoots we shall be glad to receive them, and will plant them and label the resulting trees with the name of the finder and the locality. But the nuts (usually ripe about the first week in October) should not be allowed to dry out, for drying kills the embryo so that the nuts will not germinate. They should be wrapped in sphagnum or peat moss, moist cotton, or something of the sort, so that they will not dry out in transit, and mailed to me at the Brooklyn Botanic Garden, 1000 Washington Avenue, Brooklyn 25, New York.

Editor's note: Please remember that Dr. Graves' request is more than 50 years old. Don't send nuts!

BLANK PAGE

science and natural history

THE HISTORICAL ECOLOGY OF AMERICAN CHESTNUT

An interview with Dr. Emily W. B. Russell, historical ecologist and research associate professor, Department of Geological Sciences, Rutgers University, Newark, New Jersey.

A note from the editor:
I was fascinated by Dr. Russell's 1986 paper in the Bulletin of the Torrey Botanical Club, "Pre-blight distribution of Castanea dentata (Marsh.) Borkh.," and by several references to American chestnut in her 1997 book, ***People and the Land through Time: Linking Ecology and History*** *(published by Yale University Press). Reading her work raised any number of additional questions on how chestnut came to occupy its former place in the North American landscape, questions Dr. Russell very kindly agreed to answer. Although she is, as she put it, "particularly interested in the residual impact of past human disturbance on plant communities," we started at the beginning, with the glaciers.*

Editor: Where were American chestnut glacial refugia located? *(Refugia are those places to which plants and animals retreated during the advance of the glaciers, and from which they migrated as the glaciers retreated.)* And in what directions and at what rates did chestnut spread from those refugia as the glaciers retreated?

Dr. Russell: I haven't studied this question myself, but the work of Thompson Webb III at Brown University, Hazel and Paul Delcourt at the University of Tennessee, and Margaret Davis at the University of Minnesota establishes pretty well that chestnut refugia were located in uplands in the southeastern United States.

So as the glaciers retreated, chestnut migrated generally northeast along the Appalachians. And it's interesting that while oak and maple pollen records show huge, wide fronts heading north, chestnut pollen records show a much narrower front, shaped something like a finger.

The chestnut front moved sporadically and slowly until about 8,000 years ago. Between 8,000 and 4,000 years ago the pace picked up and the front moved as far north as Pennsylvania. Then it sat there until about 2,000 years ago, when it migrated north to occupy its present range.

Editor: Why was chestnut confined to the eastern United States when many of its associated species were so much more widespread ?
Dr. Russell: Climate was the major control. But habitat change, and by that I mean especially soil development, may have been a reason.

As you probably know, chestnut is a calciphobe: it dislikes limey soils, soils high in calcium. As you move westward from the Appalachians, soils generally contain more calcium. In fact, soil scientists used to call eastern soils "pedalfers" - from the word roots for "soil," "aluminum," and "iron." Western soils - which would certainly include soils on the far side of the forest / prairie boundary - were called "pedocals," from the roots for, again, "soil," and "calcium." (I'm not sure just what the "o" stands for.)

Author Dr. Emily Russell

Editor: So that's why it's an eastern species. But why was chestnut *dominant* in the Appalachians? What explains "dominance" anyway?
Dr. Russell: Dominance is a result of having a slight edge in competition. And a major part of chestnut's edge was its ability to sprout. The species only became particularly abundant after European settlement, in response to cutting. Its dominance was most likely an artifact of logging.

Editor: Many people, including members of TACF, have written that in the heart of its range before the arrival of chestnut blight, one in every four hardwoods was an American chestnut. So these trees were sprouts?
Dr. Russell: There were certainly forests containing chestnut that were never logged. But the "sprout hardwoods" that chestnut dominated were, yes, an artifact of logging.

Editor: But why did it evolve an ability to sprout in the absence of logging pressure? And why would a tree that produces such copious seed also reproduce so well vegetatively?

Dr. Russell: Well, they're two different adaptations. The nuts are a reproductive adaptation. The sprouting is an adaptation to disturbance - maybe fire, maybe hurricanes, wind and ice storms.

Editor: How were chestnut seeds dispersed? How did the species colonize the Appalachians?
Dr. Russell: Blue jays and passenger pigeons probably dispersed the nuts. And probably other animals too. The bur is certainly "meant" to stick in the fur of animals.

Editor: What if chestnut blight had never arrived. What would our chestnut forests be like?
Dr. Russell: We can't answer your question with any confidence, but an even more interesting question, to me, is how the loss of chestnut has affected our current forests. When we study these forests we just assume that chestnut is simply absent, succession has replaced it. We do not know whether the absence has changed the habitats of other species in any significant way.

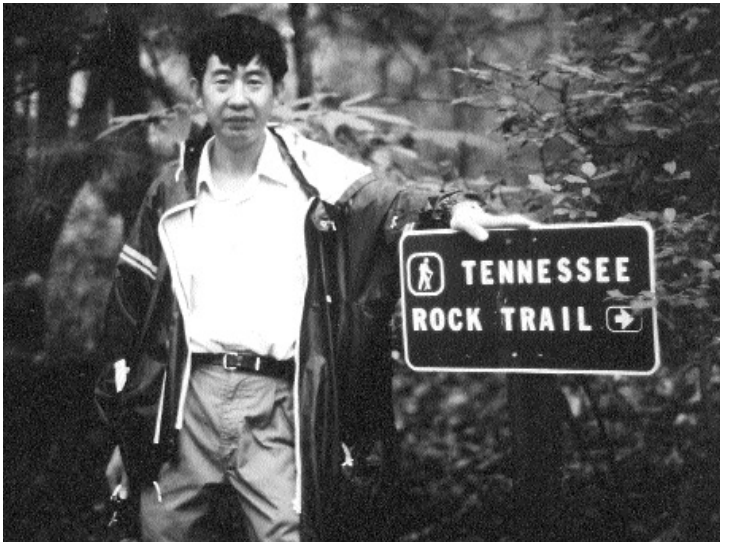
RESTORING THE AMERICAN CHESTNUT TO ITS NATIVE RANGE:

Genetic Variation in the American Chestnut and Selection Strategies for Recurrent Parents

by Dr. Hongwen Huang, Professor, Wuhan Institute of Botany, The Chinese Academy of Sciences, and Joint Research Scientist, member of the Science Cabinet of The American Chestnut Foundation

Introduction

The American chestnut [*Castanea dentata* (Marsh.) Borkh.] was once a dominant species in the eastern deciduous forest before the chestnut blight [caused by *Cryphonectria parasitica* (Murrill) Barr] arrived on the North American continent near the beginning of this century (Davis, 1981). The disease spread rapidly, and reduced the entire species to a minor understory shrub within 50 years. Prolific stump sprouting has enabled the American chestnut to persist over most of its native range. The gene pool of the species still exists, but it could face serious erosion as old root systems fail to produce sprouts and perish.



Author Dr. Hongwen Huang

Beginning in the 1920s, considerable breeding efforts were carried out by the U.S. Department of Agriculture (USDA) in an attempt to save the American chestnut. Unfortunately, those breeding programs failed to produce a desirable timber-type American chestnut with blight resistance (Burnham et al., 1986) and they were abandoned in the 1960s.

In the early 1980s, Burnham (1981, 1982) critically reviewed previous work and concluded that the traditional backcross method, used successfully in crop breeding, offered a more promising approach to the problem than methods employed earlier. Burnham et al. (1986) proposed

a backcross breeding program designed to introgress the blight resistance of the Chinese chestnut into American chestnut. By repeated backcrossing of selected resistant American x Chinese hybrids to the American chestnut, one could reconstruct the American chestnut genome (along with its desirable timber qualities) with the addition of blight resistance from the Chinese chestnut. Burnham's program for restoration of the American chestnut is based on two crucial assumptions: 1) blight resistance in the Chinese chestnut is heritable and at least partially dominant (Clapper, 1952); and 2) blight resistance is under oligogenic control (presumably two genes, see Burnham, 1981). Kubisiak et al. (1997) produced a genetic linkage map for *Castanea* species using an F_2 population derived from an interspecific cross between the American and Chinese chestnut, and detected three chromosomal regions that likely play roles in conditioning resistance to *C. parasitica* ($p<0.001$). An aggressive backcross breeding program led by The American Chestnut Foundation (TACF) has recently produced BC_3 populations (Hebard, 1996).

Allard (1960) outlined principles for selecting recurrent parents in backcross breeding programs. However, the program for chestnut is unique in plant breeding history. Although introgression of two or three genes conferring blight resistance is theoretically straightforward, compared to crop breeding it will be far more complicated to apply backcross breeding to a forest tree with a broad natural range and with the ultimate goal of restoring the species to its original native range. A well designed strategy for capturing as much genetic variation as possible from the recurrent parents becomes an important factor that affects the recovery of the gene pool of the American chestnut, its adaptability and desirable timber qualities, and, ultimately, will influence the success of the program.

Allard (1960) suggested that when applying backcross methods to an unimproved species, the genetic variation of the recurrent parent should be sampled by using many individuals from different source populations: if the species being improved has a wide geographic range, a program should be considered for each major region. Unfortunately, information on genetic diversity and geographic variation in American chestnut populations has been previously unavailable. To fill this important gap in our knowledge of American chestnut, during 1994 and 1995 twelve populations of *C. dentata* were sampled from across the native range, from Alabama north to central New York. Each population consisted of sprouts from at least 30 trees

(remnants of the original blight-killed trees), except the population from Connecticut where only 11 trees were sampled. The samples were subjected to both allozyme (12 populations) and random amplified polymorphic DNA, RAPD (four populations) analyses. Intra- and inter-population genetic statistics were calculated, based on allele frequencies of 20 isozyme and 22 RAPD gene loci, by using the BIOSYS-1 program (Swofford and Selander, 1981) and PC-SAS for Windows (SAS, 1989).

In this paper, I will discuss the degree and patterns of distribution of genetic diversity in American chestnut. Based on the results of my genetic analyses (see also Huang et al., 1994), I will also suggest some conservation and selection strategies for restoring this species as a forest tree throughout its native range.

Genetic diversity in the American chestnut

Genetic variability in American chestnut populations was found to be lower than that in any other chestnut species. Averaged across populations, the [1]percentage of polymorphic loci (P), the [2]mean number of alleles per locus (A), the [3]effective number of alleles per locus (A_e), and the [4]expected heterozygosity (H_e) are 53.5, 1.67, 1.19 and 0.161, respectively (Table 1).

The underlying genetic control of blight resistance is still poorly understood. Furthermore, the genetic vulnerability of the American chestnut, resulting in its elimination as a predominant canopy species, has received inadequate attention. To date, no attempt has been made to investigate a possible link between levels of population genetic diversity and genetic vulnerability in this species. Comparisons with related species may provide us some insight into the part genetic diversity plays in genetic vulnerability to pathogens.

Within the genus *Castanea*, much higher genetic diversity has been reported for the Chinese chestnut, European chestnut and even a locally endemic chestnut species, Seguin chestnut, as compared to the American chestnut (Table 1). Oak-chestnut forest (*Quercus-Castanea*) was a key component of the deciduous forests of eastern North American for the 8,000 years since the last glacial maximum (Davis, 1983). In a recent study of five populations of two red oak species (*Quercus*) in Michigan, Hokanson et al. (1993) reported higher genetic diversity in red oak species than that found in the American chestnut (Table 1). It is possi-

Table 1

Comparison of levels and distribution of genetic variability of chestnut species in genus Castanea *and oak species (at population level)* [a]

Species	P	A	A_e	H_O	H_e	H_T	H_s	G_{st}	F_{IS}	F_{IT}	F_{ST}
American chestnut [b]	53.5	1.67	1.19	0.187	0.161	0.214	0.196	0.087	- 0.129	- 0.016	0.108
seguin chestnut	68.4	1.74	1.25	0.218	0.203	0.297					
European chestnut [c]	76.9		1.47	0.256	0.317	0.291	0.262	0.095	- 0.03	0.06	0.10
Chinese chestnut [d]	85.4	2.06	1.49	0.334	0.328	0.348	0.325	0.093	- 0.06	- 0.008	0.106
Red oaks [e]	63.5	2.8	1.70	0.228	0.301				0.15	0.183	0.041

[a] P: percentage of polymorphic loci (95% criterion); A: mean number of alleles per locus; A_e: effective number of alleles per locus; H_O: observed heterozygosity; H_e: Hardy-Weinberg expected heterozygosity; H_T: total genetic diversity; H_s: genetic diversity within population; G_{st}: relative magnitude of genetic differentiation among populations(Nei, 1987); F_{IS}: fixation index of individuals within populations; F_{IT}: fixation index with respect to the total population; F_{ST}: proportion of genetic differentiation (Wright, 1978).

[b] P, A, A_e, H_O, H_e were calculated as the average of 12 American chestnut populations within its native range and one population outside the native range (Huang et al., 1994; 1996). H_T, H_s, G_{st} , F_{IS} , F_{IT} and F_{ST} are based on 12 American chestnut populations in its native range.

[c] P, A, A_e, H_O, H_e, H_T, H_s and G_{st} were calculated as the average from different regions of European chestnut populations (Villain et al., 1991); F_{IS}, F_{IT} and F_{ST} were calculated as the average of 18 European chestnut from Italy (Pigliucci et al., 1990)

[d] Average of available data (Huang et al., 1994 and Huang's unpublished data)

[e] From Hokanson et al. (1993)

ble that its narrow genetic base (as compared to congener species and closely related *Quercus* species) may have contributed to the demise of the American chestnut. Given that American chestnut likely has the lowest genetic diversity in the genus *Castanea*, the introduction of the chestnut blight on the North American continent was probably a trigger event for the devastation that followed, combining the impact of uniformity of blight susceptibility and lack of sufficient levels of genetic diversity to adapt to and survive the resulting environmental challenges.

In contrast, although the European chestnut suffered severe damages as a result of infection by the chestnut blight fungus during the 1930s, it is likely that sufficient levels of genetic diversity in this species played an important role in allowing the species to survive and recover.

Distribution and geographic patterns of genetic diversity in the American chestnut

The proportion of the genetic diversity found among American chestnut populations Gst (0.087) and F_{ST} (0.108) (Table 1) was much lower than averages reported for species with a wide geographic range (0.210), for species with any seed dispersal mechanisms (0.124-0.277), for species with similar modes of reproduction (0.213-0.225), or for temperate species (0.246). It was similar to other long-lived woody perennials (0.076), wind outcrossing species (0.099) and late successional species (0.101) (Hamrick and Godt, 1989). Low levels of differentiation among chestnut populations are typical throughout the genus. The American chestnut had almost the same level of genetic diversity among populations as its congener species, the Chinese chestnut and European chestnut (Table 1). Based on an average across all isozyme loci, the American chestnut appears to harbor most of its genetic diversity within populations ($\pm$90%). However, differences in Gst and F_{ST} were observed from locus to locus, Gst ranging from 0.000 to 0.259 and F_{ST} ranging from 0.026 to 0.393 for the 14 polymorphic loci.

Observed heterozygosity in 10 of 12 populations was higher than heterozygosity under Hardy-Weinberg expectation, which indicates a trend that excessive heterozygotes are increasing in remnant populations of *C. dentata*. The increase in heterozygosity in remnants of wild *C. dentata* is more likely attributable to epidemic pressure of the chestnut blight or adaptation to changed eastern deciduous forests. However, expected genotype frequencies at all loci and in all populations conform existence of Hardy-Weinberg equilibrium except *Prx-3* and *Acp-3*, which have significantly excessive heterozygotes in eight and four populations, respectively (data not shown). Average gene heterozygosity (He) was significantly different among the 12 populations within the native range. The highest (H_e=0.181$\pm$0.046) was found in east-central Alabama (Macon County) and the lowest (H_e=0.089$\pm$0.033) was found in southern Appalachia (Block Rock Mountain, Georgia) (Figure 1). Moderately higher levels of average gene heterozygosity were also observed in the northern Appalachian region (H_e = 0.167$\pm$0.042 at Troy, New York and H_e= 0.172$\pm$0.044 at Essex, Connecticut). The average gene heterozygosities found in central Appalachian populations were intermediate to those of the southern and northern populations. This geographic pattern

of genetic diversity would not be expected under a model of migration from a single refugium. It is also contradictory to the general notion that in eastern North America, populations at the margins of a species' native range, particularly northern populations, maintain less genetic diversity than centrally located or southerly populations (Critchfield, 1984; Waller et al., 1987; Godt and Hamrick, 1993).

It has been well documented that many plant species were forced southward to refugia in Gulf Coastal regions and Florida during the Wisconsin glacial maximum 18,000-20,000 years ago, and migrated northward after glacial retreat (Davis, 1981, 1983; Pielou, 1991). However, the migration route for *Castanea* on the North American continent remains obscure as its fossil record is poor.

Davis (1976) proposed that *Castanea* may have survived on the Atlantic continental shelf, or at least may have used the shelf to migrate from its eastern refuge to the west after the glacier retreated. Her hypotheses implied multiple refugia and migration routes. However, later reports by Davis (1981, 1983), primarily based on limited palynological data (Delcourt, 1980), hypothesized a south-north migration route for *Castanea*.

Based on my findings, I suggest that at least two refugia for *Castanea* existed near the close of the Wisconsin glaciation: one located in southern Alabama, the other on the Atlantic continental shelf east of North Carolina or Virginia. Evidence supporting this hypothesis includes a lack of spatial patterning of most allelic frequencies for both the isozyme and RAPD markers along the Appalachian axis. For the European oak, *Quercus petraea* (Matt.) Liebl., Zanetto (1995) demonstrated concordant correlations between allelic frequencies and longitude for seven of eight loci examined, which was then related to a putative, post-glacial migration pathway. In our study, only two of 14 polymorphic loci showed clinal trends along the Appalachian axis (Figure 2). It seems more likely that the clinal patterns reported for these two loci resulted from selective forces manifest across a geographical or ecological gradient along the Appalachian Mountains. That the American chestnut is the most cold hardy species in the genus *Castanea* (Rutter et al., 1990) and that it was historically associated with boreal species in southern Maine suggest that it may have survived on a continental shelf refugium.

A strong association was found between genetic distance and geographic distance, particularly among populations along the Appalachian

axis (MI population excluded). Genetic distance and geographic distance were negatively correlated ($r=-0.7077$, $P<0.01$), suggesting limited gene flow and possible geographical isolation among remnant American chestnut populations. This contention is reinforced by UPGMA (unweighted pair-group method using arithmetic average) of allozyme and RAPD genetic distances (Roger's distance) and principal component analysis. It is clear that the distinction can be made between the southernmost population (AL), the south central Appalachian populations (GA, NC, Great Smoky Mts, OH, VA-1, VA-2), north central Appalachian populations (PA-1, PA-2) and northern Appalachian populations (NY, CT).

The importance of regional and local alleles should not be overlooked when a conservation plan or breeding program is considered for restoring the American chestnut. For instance, based on visual inspection of 38 allele frequencies of the 14 polymorphic isozyme loci, *Mdh-2a* and *Pgi- 2a* are unique alleles only detected in Alabama and Great Smoky Mountains populations, respectively; *Pgm-a* is only associated with northern populations from Pennsylvania to New York; and *Prx-3c* is unique to Pennsylvania populations. Similar, unique allelic distributions can also be found for other alleles, such as *Pgi-2b* and *Prx-1b*.

Conservation and breeding considerations for the restoration of the American chestnut

Relatively high levels of genetic diversity in the southernmost populations in Alabama, presumably related to glacial refugia, indicate that conservation efforts should consider such populations a focal point for capturing much of the American chestnut's genetic variation. And quickly: relict chestnut populations in that region are particularly vulnerable and the number of remnant populations is declining rapidly due to logging disturbance and large-scale reforestation to pine species by the pulp and paper industry. In central Alabama, many relict populations are disappearing and resprouting stumps seem to be declining (Huang, personal observation). The State of Kentucky has classified the American chestnut as an endangered species (Kentucky State Nature Preserves Commission, 1996). Relict populations throughout the south need to be extensively surveyed and evaluated so that appropriate conservation strategies can be implemented.

Horticulturists and plant breeders have traditionally used only a few genotypes as recurrent parents in backcross programs for cultivar improve-

ment. To date, this has been the case in the American chestnut backcross breeding program. TACF has used only a few genotypes (approximately 14 lines) that are mostly growing in the vicinity of Meadowview, Virginia (Hebard, 1996). Progenies derived from these lines will more than likely be only locally adapted, and might not be expected to do well in other parts of the native range. The final BC_3-F_2 or BC_4-F_2 generations with such a restricted genetic base may suffer founder effect and lack sufficient genetic variation to survive and to adapt to the eastern deciduous forest.

Therefore, I suggest that at least three to four regional backcrossing breeding programs encompassing the southern, central and northern Appalachian regions should be established to recapture as much genetic diversity as possible. Regional programs should focus on local and regional alleles as well as locally unique morphological types and ecotypes, particularly in terms of tree form, growth rate, timber quality and many other quantitative characters. Fifty to 100 genotypes sampled from well represented regional populations need be incorporated in BC families as early as BC_1. Plant breeders should also be aware of the limitations of results based on presumably neutral molecular markers (isozyme, DNA) in representing quantitative variation of adaptive characters sensitive to natural selection. It is equally important to sample and select as many different morphological, ecological and physiological types as recurrent parents.

Secondly, although the use of "foreign" genetic sources to restore extirpated or declining populations is still under debate in conservation circles, there is little doubt that the Chinese chestnut must be used in the breeding program for the restoration of the American chestnut, as it is, to date, the best known source of blight resistance. The Chinese chestnut has also been shown to be the most genetically diverse species in the genus *Castanea* (Huang et al., 1994). One important question that still needs to be answered is: what is the optimal number of Chinese genotypes that needs to be included in the breeding program to provide sufficient levels of genetic diversity and blight resistance, without risking the capture of inferior genetic backgrounds (which may take many generations to sort out by natural selection)? Only a limited number of the Chinese chestnut genotypes available in the U. S. have been examined for blight resistance and cold hardiness (Rutter et al., 1990). Less than ten blight resistant Chinese chestnuts are being used as donor parents in

the TACF breeding program (Hebard, 1996). To address this question, more information is needed on population structure and patterns of genetic diversity in Chinese chestnut. A cooperative research effort is needed to survey variation in levels of blight resistance of the Chinese chestnut in its native range, so that the most highly resistant individuals encompassing all possible major and minor modifying genes conferring blight resistance (Burnham et al., 1986; Kubisiak et al., 1997) are incorporated into the program.

Finally, the ultimate success of the breeding program will depend upon the survivability and adaptation of BC_3 or BC_3-F_2 populations in natural forest settings and the reestablishment of resistant American chestnuts by seed propagation throughout the native range. Evolutionary processes driven by natural selection will take their course on such populations, and genetic diversity is a vital part of these processes. Sufficient genetic diversity and maintenance of such genetic diversity must be monitored and evaluated in each subsequent generation. Field tests across the native range, combined with close monitoring of genetic diversity, should be carried out as early as BC_2 generations. Together they should provide

Figure 1 Mean gene diversity (He) in American chestnut populations across its native range

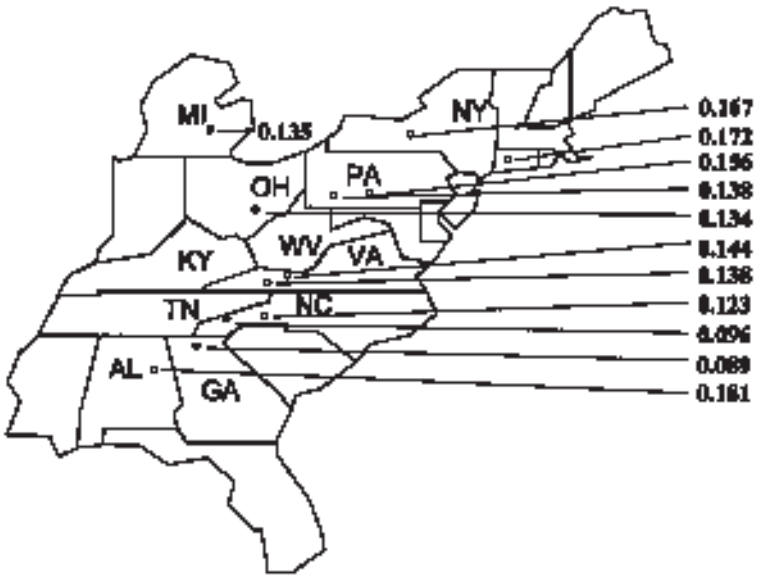
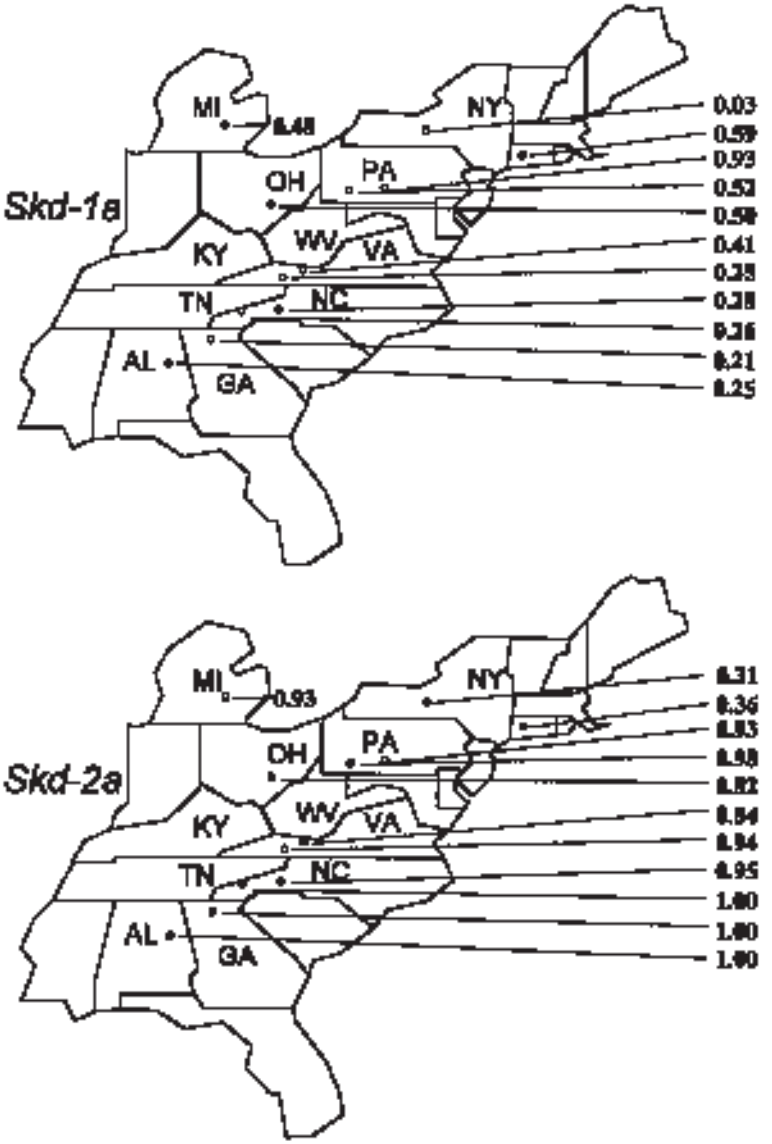


Figure 2 Allelic frequency of SKD in American chestnut populations



us with a clearer understanding of how the genetic base is associated with successful colonization and could also be used to direct and improve the breeding program.

Acknowledgments

I would like to thank the USDA-ARS and the National Science Foundation of China (NSFC) for partial support of this work, and Fenny Dane and Tom Kubisiak for all their comments and suggestions. I would also like to thank Joe Norton, Bill Carey, Dan James, Fred Hebard, Ann Leffel, Greg Miller, Bill Nash, Phillip Gordon, John Gordon, Scott Schlarbaum, Bill McDonald, Herbert Darling, Frank Munzer, Anthony Lampros and John Herrington for help with field sampling.

References

Allard, R. W. 1960. *Principles of plant breeding*. Wiley, New York, NY.

Burnham, C. R. 1981. Blight-resistant American chestnut: there's hope. *Plant Disease* 65: 459-460.

—— 1982. Breeding for chestnut blight resistance. *Nutshell* 35: 8-9.

Burnham, C. R., P. A. Rutter, and D. W. French. 1986. Breeding blight resistant American chestnuts. *Plant Breeding* Rev. 4: 347-397.

Clapper, R. B. 1952. Relative blight resistance of some chestnut species and hybrids. *J. Forestry* 50: 453-455.

Critchfield, W. B. 1984. Impact of Pleistocene on the genetic structure of North American conifers, p. 70-118. In: R. M. Lanner (eds.). *Proceedings of the Eighth North American Forest Biology Workshop*, Utah State University, Logan, UT.

Davis, M. B. 1976. Pleistocene biogeography of temperate deciduous forest. *Geoscience and Man* 13: 13-26.

Davis, M. B. 1981. Quaternary history and the stability of forest communities, p. 133-153. In: D.C. West, H.H. Shugart, and D. B. Botkin (eds.). *Forest succession - concepts and application*. Springer-Verlag, New York, NY.

Davis, M. B. 1983. Quaternary history of deciduous forests of eastern North American and Europe. *Ann. Missouri Bot. Gard.* 70: 550-563.

Delcourt, P. A., H. R. Delcourt, R.C. Brister, and L.E. Lackey. 1980. Quaternary vegetation history of the Mississippi embayment.

Quaternary Research 13: 111-132.

Godt, M. J. W., and J. L. Hamrick. 1993. Genetic diversity and population structure in *Tradescantia hirsuticaulis* (Commelinaceae). *Amer. J. Bot.* 80: 959-966.

Hamrick, J.L., and M.J.W. Godt. 1989. Allozyme diversity in plant species, p. 43-63. In: A. H. D. Brown, M. T. Clegg, A. L. Kahler and B. S. Weir (eds.). *Plant population genetics, breeding and genetic resources*. Sinauer Associates, Inc. Publishers, Sunderland MA.

Hebard, F. V. 1996. Notes from Meadowview. *J. American Chestnut Foundation* 10: 8-12.

Hokanson, S. C., J. C. Isebrands, R. J. Jensen, and J. F. Hancock. 1993. Isozyme variation in oak f the Apostle Islands in Wisconsin: genetic structure and levels of inbreeding in *Quercus rubra* and *Q. ellipsoidalis* (Fagaceae) *Amer. J. Bot.* 80: 1349-1357.

Huang, H., F. Dane, and J. D. Norton. 1994. Allozyme diversity in Chinese, Seguin and American chestnut (*Castanea* spp.) *Theor. Appl. Genet.* 88: 981-985.

Huang H., F. Dane, and T. L. Kubisiak. 1996. Allozyme and RAPD analysis of the geographic variation and genetic diversity in natural populations of the American chestnut. *HortScience.* 31:591.

Kentucky State Nature Preserves Commission. 1996. Rare and extirpated plants and animals of Kentucky. *Transactions of the KY. Acad. Sci.* 57: 69-91

Kubisiak, T. L., F. V. Hebard, C. D. Nelson, J. Zhang, R. Bernatzky, H. Huang, S. L. Anagnostakis, and R. L. Doudrick. 1997. Mapping resistance to blight in interspecific cross in the genus *Castanea* using morphological, isozyme, RFLP, and RAPD markers. *Phytopathology* (in press).

Pielou, E. C. 1991. *After the ice age: the return of life to glaciated North America.* The University of Chicago Press, Chicago, IL.

Pigliucci, M., S. Benedettelli, and F. Villani. 1990. Spatial patterns of genetic variability in Italian chestnut (*Castanea sativa*). *Can. J. Bot.* 68: 1962-1967.

Rutter, P. A., J. A. Payne, and G. Miller. 1990. Chestnut, p. 761-788. In: J. N. Moore, and J. R. Ballington, Jr. (eds.). *Genetic resources for temperate fruit and nut crops*. The International Society for Horticultural Science, Wageningen, The Netherlands.

SAS. 1989. *SAS/STAT user's guide, release 6.03*. SAS Institute Inc., Cary, NC, USA.

Swofford, D.L., and R.B. Selander. 1981. BIOSYS-1: a FORTRAN program for the comprehensive analysis of electrophoretic data in population genetics and systematics. *J. Hered.* 72: 281-283.

Villani, F., M. Pigliucci, S. Benedettelli, and M. Cherubini. 1991. Genetic differentiation among Turkish chestnut (Castanea sativa Mill.) populations. *Heredity* 66: 131-136.

Waller, D. M., D. M. O'Malley, and S. D. Gawler. 1987. Genetic variation in the extreme endemic *Pedicularis furbishiae* (Scrophulariaceae). *Conservation Biology* 6: 131-134.

Zanetto, A., and A. Kremer. 1995. Geographical structure of gene diversity in *Quercus petraea* (Matt.) Liebl. 1. Monolocus patterns of variation. *Heredity* 75: 506-517.

ENDNOTES

[1] Percentage of polymorphic loci is defined as $P=(k/n) \times 100\%$, in which k is the number of polymorphic loci (95% criterion); n is the total number of loci tested.

[2] Mean number of alleles per locus is defined as $A=\_A_i / n$, in which A_i is the number of alleles of ith locus; n is the total number of loci tested.

[3] Effective number of alleles per locus is defined as $A_e = 1 / \_(q_j)^2$, in which q_j is allele frequency of jth allele.

[4] Expected heterozygosity, also called gene diversity, is a common measure of gene diversity. It is defined as $H_i = 1 - \_(q_j)^2$, in which q_j is homozygote frequency of jth allele; $H_e = \_H_i / n$, in which H_i is the expected heterozygosity of ith locus; n is the number of loci tested.

USING GENETIC ENGINEERING TO HELP SAVE THE AMERICAN CHESTNUT: A PROGRESS REPORT

by Charles Maynard, Zizhuo Xing, Sharon Bickel, William Powell
Professor, Research Associate, Laboratory Technician,
and Associate Professor, respectively, State University of
New York College of Environmental Science and Forestry
Syracuse, NY, USA

ACKNOWLEDGEMENTS

This work was supported by grants from the New York State chapter of The American Chestnut Foundation; the New York Center for Forestry Research and Development (979401-07); and the International Society of Arboriculture. We wish to especially thank the following individuals: Herb Darling, Stan Wirsig, Arlene Wirsig, Phil Rutter, Chip Levey, and Bob Blackmon.

INTRODUCTION

In this paper we describe the New York State American Chestnut Research and Restoration Project conducted at the College of Environmental Science and Forestry in Syracuse, New York between approximately June, 1988 through June 1998. We will attempt to summarize the approaches we have taken and what we have accomplished, provide some background on our techniques, and finally, describe what steps still need to be completed.

The goal of the project is to produce blight resistant American chestnut trees using genetic engineering. In order to accomplish this goal, we have identified six critical steps:

1) Identify and design one or more gene constructs containing multiple resistance genes and their promoters;
2) Improve tissue culture techniques for genetic transformation, regeneration, and propagation of chestnut plants;
3) "Transform" (or transfer) the resistance gene constructs into American chestnut cells and regenerate whole plants from these cells;
4) Confirm gene transformation and expression using molecular biology techniques;

5) Perform blight resistance tests and other safety tests required for engineered organisms;
6) Identify the most resistant chestnut clones and mass-produce them for commercial release.

The American Chestnut Research and Restoration Project started out as two independent projects in the mid-1980s. Dr. Powell began studying some of the molecular aspects of the chestnut blight fungus, *Cryphonectria parasitica*, during his graduate research project under the direction of Dr. Van Alfen at Utah State University (Hansen et al.1985, Powell and Van Alfen 1987 a and b, Gobbi et al. 1990). In 1989 Dr. Powell moved to Syracuse and continued his study of the fungus (Rizwana and Powell 1992, 1995, Powell 1995).

Dr. Maynard first began working on American chestnut in 1988 with a small grant from The American Chestnut Foundation to study pollen storage and handling techniques (Maynard 1988, Maynard 1991a, de Niella and Maynard 1993). During this time, Dr. Maynard was also initiating tissue culture research with chestnut (Maynard et al. 1993).

For nearly a decade, the authors have been collaborating on these two parallel paths — Dr. Powell and his graduate students designing, collecting and testing potential blight resistance genes (Powell et al.1995, Catranis et al. 1995, Powell and Maynard 1997); Dr. Maynard, his graduate students, and postdoctoral assistant developing tissue culture methods for putting those genes into cells of American chestnut (Maynard 1991b, Xing et al. 1997b), regenerating those cells into whole plants (Xing et al.1996, 1997a, 1998a), and hardening those plants off so that they can be reestablished in the field (Xing et al. 1998b).

The major milestones we have achieved include:

- Designed nearly 50 antimicrobial peptides and examined them using computer models. Fifteen of these designs were synthesized and tested *in vitro*. Three peptide designs that demonstrated high levels of inhibition to *C. parasitica* growth and very low hemolytic activity were chosen for further work.
- Designed and constructed plant genes encoding antimicrobial peptides under the control of either constitutive or wound-inducible promoters.
- Identified two other genes coding for the enzymes oxalate oxidase and chitinase, which will be used in various combinations with the antimicrobial peptides.

- Refined and extended the somatic embryogenesis techniques first reported by Merkle et al. (1991) to the point where we have ten somatic embryo-derived plants growing in the field.
- Developed an *Agrobacterium* transformation technique using embryogenic cultures.
- Produced more than a dozen transgenic cell lines.
- Regenerated shoots (but not yet whole plants) from two cell lines containing one of the first putative blight resistance gene constructs to be tested.

We are now examining the resulting shoots for stability of gene expression. If they are stable, they will be rooted and acclimatized. Blight-resistance tests and field trials could begin as early as the summer of 1999.

Building genes from the ground up

For most of this century, plant breeders have been developing new and improved varieties of our staple foods by making crosses between existing varieties and then searching among the offspring for new combinations of desirable traits. Although incredibly successful, this approach is limited to recombining genes already present in the gene pools of the crop species being improved or of a few closely related species.

Over the last few decades this limitation has been overcome. With the development of genetic engineering techniques, interesting genes from virtually any organism can be isolated, studied, modified for improved function, and transferred into crop species. Currently, 15% of the U.S. corn crop, 30% of the soybean crop, and more than half of the production of cotton comes from genetically engineered plants.

The approach we have taken for developing blight resistant American chestnut trees is to look far outside the *Castanea* genus for interesting genes, and then, rather than transfer those genes, to study the chemical properties of the gene products and then build new genes optimized for expression and function in chestnut.

The first gene products we tested for their ability to convey blight resistance were small, antimicrobial peptides. Over the past ten years there has been an extensive amount of research in many laboratories on antimicrobial peptides which are naturally produced in most plants and animals. Most of the research has been in the pharmaceutical area where efforts are underway to develop these peptides or synthetic

analogs for treating stomach ulcer infections, tumors, and for enhancing wound healing (Maloy and Kari 1995, review). Significant progress has also been made in developing these types of peptides for use in plant pathology (Rao,1995, review). Our use of antimicrobial peptides in developing blight resistance genes is, therefore, in the mainstream of current research.

In selecting genes to convey blight resistance, it is critically important that the gene product is effective at inhibiting fungal growth but has no toxicity to the plant or to animals consuming the plant. For example, one of the gene products we plan to use, the enzyme oxalate oxidase, is generally recognized as safe because it is already produced in wheat and therefore is already consumed by animals and humans. The synthetic peptide design we will test, on the other hand, does not have this history of consumption and therefore must be studied in more detail to ensure its safety.

One advantage to building a new gene product such as a small antimicrobial peptide is that we can design in certain safety features. In our peptide designs, we have incorporated amino acid sequences that are easily recognized by several mammalian digestive enzymes, thereby ensuring quick inactivation of the peptides in the digestive tract. Inactivation with one such enzyme, trypsin, has been confirmed with *in vitro* tests (Powell et al. 1995). We have also manipulated certain physical features, such as the peptide's hydrophobicity, to minimize any activity to mammalian cell membranes. This feature has been tested in hemolytic assays in which we use human red blood cells to test the peptide's ability to disrupt the cell membrane. (Red blood cells were chosen because they are among the more fragile cells in the body and it is easy to track any leakage of hemoglobin from them.) In our assays, we compared our peptide's lytic ability to a buffer control with no activity and to a 0.1% detergent solution which can lyse red blood cells. We tested the peptides at a concentration more than 25 times that needed to inhibit the growth of *C. parasitica*, and found no significant difference in their ability to lyse red blood cells compared to the inactive buffer control. We believe these two safety features in the design, along with controlled expression (see below), will make antimicrobial peptides useful in the development of a blight resistant American chestnut tree.

In addition to the antimicrobial peptide genes discussed above, we are also collaborating with other molecular biologists in assembling other

genes and plan to test them alone and in combination in American chestnut. We are in various stages of preparing or testing genes that code for:

- An oxalate oxidase enzyme: This gene originates from wheat which expresses the gene during seed germination and during fungal infection (Hurkman and Tanaka 1996). The gene we will use was obtained from Dr. Randy Allen's laboratory at Texas Tech University, where he has shown it enhances fungal resistance in tobacco plants (Zaghmout et al. 1997). An interesting aspect of this gene product is that it breaks down oxalic acid into CO_2 and H_2O_2. Oxalate (or oxalic acid) is thought to be one of the virulence factors used by *C. parasitica* to lower the pH at the canker margin to plant-toxic levels (McCarroll and Thor 1978, Havir and Anagnostakis1983). Therefore, the proper expression of this gene might help inactivate this fungal weapon. In addition, H_2O_2 has been associated with signaling plant cells to produce indigenous plant defense products.
- A chitinase enzyme: Chitinase genes are natural defense genes found in all plants. The chitinase gene we are interested in will come from a *Trichoderma* fungus, fungi which live off other fungi. We want to try this chitinase gene because of its high activity at degrading the chitin in fungal cell walls and because it is stable at the low pHs which can be found at the blight canker margin. We are obtaining this gene from Dr. Gay Harman at Cornell University, NY.

In the final transgenic American chestnut tree, we hope to have two or three different genes, each of which can convey blight resistance by a completely different mechanism. By combining up to three mechanisms in a single tree, we hope to extend resistance longevity. It is extremely unlikely that the blight will be able to simultaneously overcome all three mechanisms.

Promoter regions

Commonly, when people think of a gene, they think only about the part that codes for a product and results in an observable trait such as hair or eye color. This part of a gene is called the "coding region" because it contains the genetic code for a specific gene product. We have described above the primary gene products we are developing as blight resistance genes. As important as the coding region of a gene, however, is the "promoter region" that controls when and where a gene gets expressed.

A rough analogy can be drawn between promoter regions and electrical switches. Some electric switches are very simple, turning an appliance either on or off. Other switches may control a whole room full of equipment. More sophisticated switches may contain timers or motion, smoke, or heat detectors which allow these devices to "perceive" their environment and respond appropriately.

Gene promoter regions serve the same sorts of functions in living organisms. Promoters have been identified that turn genes on or off in response to heat or cold, light intensity, light color, or insect feeding. Another group of promoters turns on or off genes at different stages of development. Some genes are expressed only during embryo development, or, in deciduous trees, only during leaf senescence. Other promoters are tissue specific, allowing genes to turn on or off only in certain tissues of a plant or animal.

In designing genes for blight resistance, we are interested in wound-inducible promoters that express genes in wounded tissues, and cambium-specific promoters that express genes only in cambial tissues where *C. parasitica* attacks. Either type of promoter or, ideally, a combination of the two, will allow us to target the expression of antimicrobial peptides and enzymes where they have the greatest chance of helping the chestnut withstand attack from the blight. This places less of a resource drain on the plant by keeping the gene products out of tissues where they are not needed or not wanted. Presently, we are using a wound-inducible promoter isolated from poplar (Hollick and Gordon 1993). We are initiating a search for a cambium-specific and / or wound-specific promoter from American chestnut.

Tissue culture

The other major component of the American Chestnut Research and Restoration Project has been to develop tissue culture techniques. Once the new genes were identified and optimized for expression, we knew it was going to be necessary to deliver them into American chestnut cells and to regenerate whole plants from those cells. Moreover, once blight resistant genotypes are identified, there will be a need for a micropropagation system to propagate them rapidly for large-scale field testing and eventually for commercial and restoration planting.

Previous studies had shown some success in regeneration through micropropagation (Keys and Cech 1982, Read et al. 1985, Serres et al.

1990, Maynard et al. 1993) and initiation of somatic embryos in American chestnut (Merkle et al. 1991). These studies also uncovered numerous problems in both micropropagation and embryogenesis, such as low rooting rate, shoot tip necrosis during rooting, low conversion of somatic embryos into whole plants, and difficulty in acclimation of tissue cultured plants to greenhouse or field conditions. Over the last ten years of research on American chestnut tissue culture, we have addressed many of these problems.

Somatic embryogenesis

The ideal tissue for genetic transformation is one that is proliferating rapidly and is capable of regenerating whole plants from single cells. In the natural life cycle of most plant species, the only stage where a whole plant regenerates from a single cell is from a fertilized egg, so many researchers have used developing embryos as a source of tissue for embryogenesis experiments. (In order to distinguish between the original fertilized egg-derived embryo and the new embryos developing from it, the term "somatic embryo" was coined.)

Other researchers have reported somatic embryogenesis techniques for other chestnut species (Vieitez 1995). However, to our knowledge, the first researcher to use somatic embryogenesis with American chestnut was Dr. Scott Merkle (Merkle et al. 1991). He and his graduate students were successful in extracting immature embryos from developing nuts and establishing them in a tissue culture medium. They were also able to reverse the development of embryos so that many hundreds or even thousands of new embryos could be produced from a single fertilized egg cell (Merkle et al. 1991, Carraway et al. 1994).

We have regenerated whole plants through somatic embryogenesis of American chestnut (Xing et al. 1996,1998a). A total of 18 embryogenic cell lines were initiated between 1995 and 1997 (Table 1) using the method described by Merkle and co-workers (Merkle et al. 1991). All cell lines have retained embryogenic capacity through August 1998 when observations ended. We formulated development, maturation, and germination media, which enabled us to obtain fully developed somatic embryos and regenerate whole plants from these embryos (Figure 1A and 1B). Approximately 3% of the embryos could be grown directly into whole plants. An additional 6% of the embryos produced shoots but no

TABLE 1

Embryogenic Cell Lines Established from 1995 through 1997

Genotype	Pollination	Ovules donator [1]
1995		
'Wishing Well 1'	open	J.R. Ellis and J.D. Donowick
'Wishing Well 3'	open	J.R. Ellis and J.D. Donowick
'Pond 1-1'	open	J.R. Ellis and J.D. Donowick
'Pond 1-2'	open	J.R. Ellis and J.D. Donowick
'Pond 2'	open	J.R. Ellis and J.D. Donowick
1996		
'Moss 4 x Moss 3 #1'	semi-control2	H.F. Darling
'Moss 4 x Moss 3 #4'	semi-control	H.F. Darling
'Nagel 1 x Zoar #2'	semi-control	H.F. Darling
'Nagel 1 x Zoar #4'	semi-control	H.F. Darling
'Nagel 1 x Zoar #9'	semi-control	H.F. Darling
'Moss 3 x Moss 4'	semi-control	H.F. Darling
'WIR 516 x Nagel 1'	semi-control	H.F. Darling
30015 #1	open	C.R. Hibben
30015 #2	open	C.R. Hibben
30015 #3	open	C.R. Hibben
'RFW'	open	R.F. Wiltse
1997		
'Nagel 1 x Nagel 2'	semi-control	H.F. Darling
30031	open	C.R. Hibben

1) Bur collections were made by members of the New York State Chapter of the American Chestnut Foundation. Names of trees are from their records.
2) "Semi-control pollination" was performed by placing a single pollen source in proximity to the female flowers. No other pollen source was obvious, but female flowers were not bagged.

roots. However, using the micropropagation technique described below, an additional 6% could be rooted and grown into whole plants.

Micropropagation

Micropropagation is used to produce many genetically identical copies of a particular plant. It is very similar to conventional rooting of cuttings in cold frames or mist beds. The only important difference is that it takes place in a nutrient medium within a sterile test tube or other small container and uses much smaller pieces of plant tissue (hence the prefix 'micro') for proliferation and rooting of tiny shoots (2 to 3 cm tall). A standard micropropagation procedure consists of initiation, multiplication, rooting, and acclimation stages. A commercial-scale micropropagation facility may produce thousands to millions of plants per year from a single shoot.

Fortunately, many people have grown American chestnut and other chestnut species *in vitro*, so media and techniques were well developed to get tissues clean, growing well, and proliferating in a sterile environment on artificial medium in a test tube or petri dish (Hebard and Kaufman 1976, Keys and Cech 1982, McPheeters et al. 1980, Read et al. 1985, Serres et al. 1990). Within a few years, we had established *in vitro* about a dozen clones from mature trees and seedlings and were studying ways to root them.

Rooting turned out to be vastly more difficult than establishing and multiplying. Dr. Paul Read and others had reported on a rooting procedure (Read et al. 1985) which we attempted to repeat. However, in our hands the resulting rooted plantlets were difficult to acclimatize because of weak shoot growth.

We improved rooting and the vigor of the resulting plantlets by adding pre- and post-rooting stages (Xing et al. 1997a). The additional two stages were designed to prevent shoot tip necroses by use of a shoot-elongation medium. The new formulation was based on Woody Plant Medium salts and Nitsch and Nitsch vitamins plus 2-[N-Morpholino] ethanesulfonic acid (MES) to buffer pH and polyvinylpyrrolidone (PVP, MW 40,000) to absorb phenolic exudates. The medium also contained a low concentration of cytokinin (0.89 MM BA) but omitted auxin, because auxin has been found to cause shoot tip necrosis in chestnut (Veitze et al. 1989).

Split-wounding of shoots also stimulated rooting. Individual shoots harvested from the pre-rooting stage were vertically split at the base to approximately 2 mm through the pith, dipped in 5 or 10 mM indolebutyric acid (IBA) for 1 minute, and rooted in half-strength Murashige and

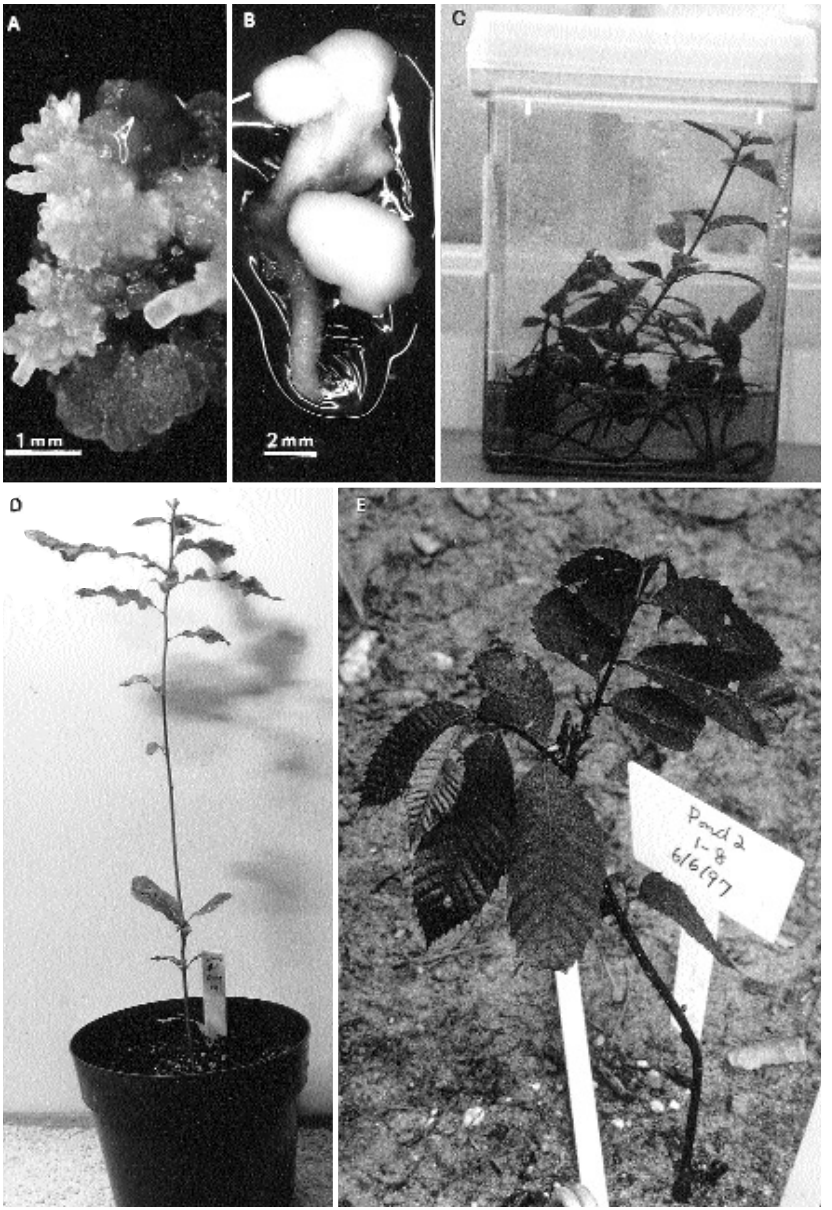


Figure 1 Tissue culture of American chestnut. (A) embryogenic tissues maintained on an embryo initiation medium for two and a half years. (B) germination of a somatic embryo. (C) plant regeneration through rooting of microcuttings. (D) a fully acclimatized plant. (E) a plant grown in the field for 11 months.

TABLE 2

Rooting of American Chestnut

Genotypes	Number of shoots	Rooting %
'B'ville'	62	71
'Iowa #2'	48	73
'VDW'	30	57
'Pond 1-1'	114	40
'Pond 1-2'	129	43
'Pond 2'	140	48
Total	**523**	**50**

Skoog basal medium plus 0.2 g / l charcoal for two weeks. Rooted plantlets were then transferred back to the shoot-elongation medium and grown for three weeks, allowing growth of both roots and shoots. A total of 523 shoots from six genotypes have been tested (Table 2), including shoots from one mature tree ('B'ville'), two one-year-old seedlings ('Iowa #2' and 'VDW'), and three somatic embryos ('Pond 1-1', 'Pond 1-2', and 'Pond 2'). The average rooting rate was approximately 50%. The plantlets resumed shoot growth by either recovery of apical buds or breaking of axillary buds to replace dead apical buds (Figure 1C).

Acclimation

After a successful rooting protocol had been developed, the next major hurdle was to acclimatize these rooted plantlets so that they could be established in the field. We first successfully acclimatized tissue-cultured chestnut plants in 1997 (Xing et al. 1998b). The novel aspect of this procedure was the use of a "sandwich" treatment. The "sandwich" contained a layer of potting mix (1 perlite: 1 vermiculite: 1 sand, v / v / v) at the bottom, a layer of shoot-elongation medium in the middle, and a thin layer of potting mix on the top. To our knowledge, this is the first time anyone has combined a standard plant potting mix and a tissue culture medium. The roots of plantlets were inserted into the medium layer. Root growth gradually transferred from the gel matrix (shoot-elongation medium) to the soil-like matrix (potting mix) during 4 weeks of aseptic growth in the

"sandwich." Plantlets were then transferred to pots in a growth chamber and covered with clear polypropylene boxes. After growing completely covered for two weeks and under a cover raised 3 mm above the potting mix for one week, plants were then grown uncovered in the growth chamber for an additional eight weeks (Figure 1D). Fifty plants survived, for an average survival rate of 29%. The height of surviving plants increased an average of three-fold during acclimation (from 4.7 to 15.8 cm).

We planted the biggest 12 surviving plants in the field in June and September 1997. Six were planted at the SUNY College of Environmental Science and Forestry Lafayette Road Experiment Station in Syracuse. The other six were planted on properties near Buffalo owned by Mr. Herbert Darling, President of the New York State chapter of The American Chestnut Foundation. Four of the six trees planted at the Lafayette Road Experiment Station survived through August 1998, when observations ended (Figure 1E). Twenty-two other plants were planted in the field in the New York City, Syracuse, and Buffalo areas during April to June 1998.

Transformation

Gene transfer or genetic transformation in plants is a three-step process. First, a small piece of DNA containing the genes of interest must be transferred into the nucleus of a plant cell. Second, it must be incorporated directly into one of the chromosomes. Third, that cell must be regenerated into a whole plant.

Once we had actively growing somatic embryo cultures, we began developing methods to transfer genes into these rapidly dividing cell lines. To our knowledge, the first reported attempt to transform American chestnut was in 1994 (Carraway et al. 1994). The researchers used particle bombardment (the gene gun) to produce transgenic calli, but no somatic embryos were reported. Our attempts to use particle bombardment were also unsuccessful and, after two attempts, we switched to using *Agrobacterium*-transformation. We have had more success with this technique (Xing et al. 1997b).

Agrobacterium tumefaciens is a soil-inhabiting bacterium that causes the disease crown gall on a wide range of plant species. There are any number of pathogenic bacteria and fungi, but *A. tumefaciens*, and a few close relatives, are unique in their mode of attack. *Agrobacterium* is a natural genetic engineer. *Agrobacterium* cells adhere to plants and inject short pieces of DNA into the plant cell. These small pieces of DNA pass into the nucleus and incorporate themselves into the plant's chromosomes where they are

duplicated and passed along every time the cell divides. The wild-type strains of *Agrobacterium* inject genes that cause plants to loose control of cell division and form galls. The strains used as vectors for plant genetic transformation are "disarmed" strains which have had the disease-causing genes deleted and replaced with other genes of interest to researchers.

By 1991 we had demonstrated that *Agrobacterium* transformation could be effectively used on American chestnut (Maynard, 1991b). The first genes we transferred in were simple markers that would allow us to identify approximately how many cells had been transformed.

In 1996 we began using both marker genes and one of the potential blight resistance genes developed by Dr. Powell. The transformation procedure was based on the somatic embryogenesis system described previously (Xing et al. 1997b). The first putative blight resistance gene to be tested encodes the antimicrobial peptide "ESF12" under the control of a poplar wound-inducible promoter (Powell et al. 1995, Powell and Maynard 1997). Since this gene was linked with an antibiotic resistance gene, the antibiotic resistant cell lines should consist of transformed cells containing the putative blight resistance gene. A total of 19 cell lines from independent *Agrobacterium*-mediated transformation events have survived on the selective medium containing antibiotics for at least 18 months. The non-transformed controls died within 4 months on the same medium. We are attempting to regenerate whole plants from these cell lines and will conduct molecular biological assays to confirm transformation and evaluate gene expression. We hope to have transformed American chestnut plants available within the next two years for field testing and pathological assays. Other putative blight resistance genes encoding oxalate oxidase and chitinase are also being constructed and evaluated for use in American chestnut.

Future activities

Thanks in large part to the sponsorship of the New York State chapter of The American Chestnut Foundation, we have made a great deal of progress in using genetic engineering techniques to develop a blight resistant American chestnut. We do, however, have a long way to go. The milestones we see in the immediate future are:

- Regenerate whole transgenic plants and test for stable expression of a gene coding for the "ESF 12" antimicrobial peptide.

- Isolate stem and/or wound-specific promoters from our American chestnut genomic library.
- Construct more plant vectors containing various combinations of the three genes to be tested driven by different promoters.
- Develop and test transformants with other potential resistance genes and combinations of genes.
- Screen the transgenic plants for blight resistance in greenhouse inoculation trials.
- Begin field tests of those clones that show resistance in the greenhouse trials.
- Transform at least 20 different genotypes of American chestnut to produce a population large enough for field testing.

Citations

Carraway, D.T., H.D. Wilde, and S.A. Merkle. 1994. Somatic embryogenesis and gene transfer in American Chestnut. J. *American Chestnut Foundation* 8:29-33.

Catranis, C.M., C.A. Maynard, and W.A. Powell. 1995. Antimicrobial peptides: design and application. *Phytopathology* 86:S26.

de Niella, P.R. and C.A. Maynard. 1993. Storage of chestnut and willow pollen. Pp. 171-180 In: Proceedings of the *Second Northern Forest Genetics Association Conference*. July 29-30, 1993. St. Paul, Minnesota.

Gobbi, E., Y. Wang, R.M. Martin, W.A. Powell, and N.K. Van Alfen. 1990. Mitochondrial DNA of *Cryphonectria parasitica*: lack of migration between vegetatively compatible strains. *Mol. Plant-Microbe Interact.* 3:66-71.

Hansen, D.R., N.K. Van Alfen, K. Gillis, and W.A. Powell. 1985. Naked dsRNA associated with hypovirulence of *Endothia parasitica* is packaged in fungal vesicles. *J. Gen. Virol.* 66:2605-2614.

Havir, E.A. and S.L. Anagnostakis, 1983. Oxalate production by virulent but not by hypovirulent strains of *Endothia parasitica*. *Physiol. Plant Pathol.* 23:369-376.

Hebard, F.V. and P.B. Kaufman. 1976. Studies of chestnut in vitro. *Plant Physiol.* 57 (Supplement 5):88.

Hollick, J.B., and M.P. Gordon. 1993. A poplar tree proteinase inhibitor-like gene promoter is responsive to wounding in transgenic tobacco. *Plant Molecular Biology* 22:561-572.

Hurkman, W.J. and C.K. Tanaka. 1996. Germin gene expression is induced in wheat leaves by powdery mildew infection. Plant Physiol. 111:735-739.
Keys, R.N. and F.C. Cech. 1982. Propagation of American chestnut in vitro, p. 106-110. In: H.C. Smith and W.L. MacDonald (eds). *Proc. U.S. Dept. Agri. For. Serv. Amer. Chestnut Coop. Mtg.*

Maloy, W.E., and Kari, U.P. 1995. Structure-activity studies on magainins and other host defense peptides. *Biopolymers* 37:105-122.

Maynard, C.A. 1988 (Unpublished Research Project) Title: Developing Pollen Collection, Handling, and Storage Techniques for American Chestnut (*Castanea dentata*). Agency: The American Chestnut Foundation. Duration: 7 months.

Maynard, C.A. 1991a. Chestnut pollen collection and handling. *J. American Chestnut Foundation* 5:101-106.

Maynard, C.A. 1991b. Using PCR to confirm Agrobacterium transformation of American chestnut (*Castanea dentata*). Abstract No. 927, In: *Program and Abstracts of the Third International Congress of Plant Molecular Biology: Molecular Biology of Plant Growth and Development.* Tucson, Arizona. October 6-11, 1991.

Maynard, C., M. Satchwell, and H. Rieckermann. 1993. Micropropagation of American chestnut (*Castanea dentata* (Marsh.) Borkh.): rooting and acclimatization. Pp. 161-170 In: *Proceedings of the Second Northern Forest Genetics Association Conference.* July 29-30, 1993. St. Paul, Minnesota.

McCarroll, D. R., and E. Thor. 1978. The role of oxalic acid in the pathogenesis of *Endothia parasitica*. Pages 60-63 in: *Proc. Am. Chestnut Symp.* W. L. MacDonald, F. C. Cech, J. Luchok, and H. C. Smith, eds. West Virginia University Books, Morgantown. 122 p.

McPheeters, K., et al. 1980. Culture of chestnut (*Castanea spp.*) in vitro. *HortSci.* 15 (3):417-418.

Merkle S.A., A.T. Wiecko, and B.A. Watson-Pauley. 1991. Somatic embryogenesis in American chestnut. *Can. J. For. Res.* 21:1698-1701.

Powell, W.A. 1995. Mycelial death and vegetative incompatibility of *Cryphonectria parasitica* monitored with pH indicators. *Mycologia* 87:738-741.

Powell, W.A. and N.K. Van Alfen. 1987a. Differential accumulation of poly(A)+ RNA between virulent and dsRNA-induced hypovirulent strains of *Cryphonectria* (*Endothia) parasitica*. *Mol. Cell. Biol.* 7:3688-3693.

Powell, W.A. and N.K. Van Alfen. 1987b. Two non-homologous viruses of *Cryphonectria* (*Endothia*) *parasitica* reduced the accumulation of specific virulence associated polypeptides. *J. Bact.* 169:5324-5326.

Powell, W., C. Catranas, and C.A. Maynard. 1995. Synthetic antimicrobial peptide design. *Mol. Plant-Microbe Interact.* 8:792-794.

Powell, W.A. and C.A. Maynard. 1997. Designing small antimicrobial peptides and their encoding genes. pp. 165-172 (Chapter 22) In: N.B. Klopfenstein, Y.W. Chun, M.S. Kim, and M. R. Ahuja eds. M.C. Dillon, R.C. Carman, and L.G. Eskew, tech. eds., *Micropropagation*, *Genetic Engineering*, and *Molecular Biology of Populus*. Gen. Tech. Rep. RM-GTR-297. Ft. Collins, CO: U.S. Department of Agriculture, Forest Service, Rocky Mountain Forest and Range Experiment Station. 326 p.

Rao, A.G. 1995. Antimicrobial peptides. *Mol. Plant-Microbe Interact.* 8:6-13.

Read, P., M.A. Hosier, Y. Qiguang. 1985. Tissue culture of chestnuts. *Annual Report of The Northern Nut Growers Association* 76:142-145.

Rizwana, R. and W.A. Powell. 1992. Ultraviolet light-induced instability of vegetative compatibility groups of *Cryphonectria parasitica*. *Phytopathology* 82:1206-1211.

Rizwana, R. and W.A. Powell. 1995. Ultraviolet light-induced heterokaryon formation and parasexuality in *Cryphonectria parasitica*. *Experimental Mycology* 19:48-60.

Serres, R., P. Read, W. Hackett, and P. Nissen. 1990. Rooting of American chestnut microcuttings. *J. Environ. Hort.* 8:86-88.

Vieitez, A.M., et al. 1989. Prevention of shoot-tip necrosis in shoot cultures of chestnut and oak. *Scientia Horticulturae* 41:151-159.

Vieitez, F.J. 1995. Somatic embryogenesis in chestnut. In: *Somatic Embryogenesis in Woody Plants*. Jain, S., P. Gupta and R. Newton. Netherlands, Kluwer Academic Publishers 2:375-407.

Xing, Z., W. A. Powell, and C. A. Maynard. 1996. Mature somatic embryos of American chestnut from ovule culture. *In Vitro Cell. Dev. Biol.* 32A:70-71.

Xing, Z., M. Satchwell, W.A. Powell and C.A. Maynard. 1997a. Micropropagation of American chestnut: increasing rooting rate and preventing shoot-tip necrosis. *In Vitro Cell. Dev. Biol.* (33:43-48).

Xing, Z., W.A. Powell, and C.A. Maynard. 1997b. Using silicon carbide fibers to enhance Agrobacterium-mediated transformation of American chestnut. *In Vitro Cell. Dev. Biol.* 33A: 63.

Xing, Z., W.A. Powell, and C.A. Maynard. 1998a. Development and germination of American chestnut somatic embryos. *Plant Cell, Tissue and Organ Culture* (in review).

Xing, Z., W. A. Powell, and C. A. Maynard. 1998b. Acclimatization and field establishment of tissue-cultured American chestnut plants. *In Vitro Cell. Dev. Bio.* (in review).

Zaghmout, O.F., P.D. Dang and R.D. Allen. 1997. Expression of oxalate oxidase in transgenic plants provides resistance to oxalic acid and oxilate-producing fungi. *Plant Physiology. Supplement to Plant Physiology* 114:227.