Rooting American chestnut cuttings with a slow-release auxin conjugate

Submitted to:

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FOR:

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Date Submitted:

August 30 , 2024

A. Project title:

Rooting American Chestnut Cuttings with a Slow-Release Auxin Conjugate

B. Summary:

An efficient rooted cutting system will allow rapid and clonal propagation of American chestnut, accelerate the breeding process, and facilitate germplasm conservation. However, the species is notoriously recalcitrant. This project will build on the current achievements and aim to enhance American chestnut cuttings' rooting rate and adventitious root number, with a slow-release auxin conjugate, 4-chlorophenoxyacetic acid–l-tryptophan-OMe, 1q. This indole-3-butyric acid (IBA) conjugate has been recently found to be effective in promoting ARs in recalcitrant plants, including apple and argan (Roth et al. 2024) by prolonged auxin signaling due to initial fast uptake and slow release and clearance of the free auxin 4-chlorophenoxyacetic acid. A chemist at Clemson University, Daniel Whitehead, has successfully synthesized this compound for us. Our initial experiments show promising results. Our goal is to develop an easy-to-apply and efficient rooted cutting system for the heritage tree species.

C. Principal Investigators (PI) and Institutional Affiliation

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Phone: 864-656-2414

D. During of project:

Twelve months from the time funding is received.

E. Total amount requested

We are requesting $10,000 from TACF.

The unrecovered overhead amount ($5,250) will serve as the matching fund, because TACF has a policy to not pay overhead (F&A – Facilities and Administration) charges– see budget.

F. Short and long-term goals of the project

Short-term Goal (12 months)

- Improve the rooting system, with a focus on the potential synergistic effects between indole-3-butyric acid (IBA) and a slow-release auxin conjugate (4-chlorophenoxyacetic acid–l-tryptophan-OMe, 1q) (we will refer this compound as 1q in the remaining of the proposal).
- Determine the expression level of significant AR genes upon auxin induction.

Long-term Goal:

- To establish an easy-to-apply vegetative propagation system that is efficient in rooting cuttings of American chestnut and its hybrids. Impacts of cutting age, collection season, and genotypes, as well as growth performance, will be studied,
- Unravel the physiological and molecular mechanisms of rooting induction in American chestnut cuttings.
- Train the next generation of the workforce.
- Work with chestnut breeders and restoration project staff to disseminate the techniques we develop.

G. Narrative (no more than five (5) pages)

1). American chestnut cuttings are notoriously difficult to form adventitious roots (ARs).

While clonal propagation by rooting cuttings is the most commonly used method to fix and maintain desired genotypes in many woody species, this approach has been proved extremely difficult for the American chestnut tree. This is due to the fact that American chestnut cuttings are notoriously recalcitrant to adventitious root (AR) formation. This has hindered the efforts in restoration, diversity conservation, and research of the species. There is an urgent need for debottlenecking and establishing an easy-to-apply vegetative propagation system that is efficient in rooting American chestnut cuttings.

2). Rooting chestnut cuttings with auxin induction is feasible albeit difficult (current results).

A). We have achieved rooting with chestnut cuttings with 2 hours of 100 ppm 1-Naphthaleneacetic acid (NAA) treatment (Fig. 1). With the current method, it takes at least two months for ARs to emerge, and often time, calli are formed. Occasionally, ARs emerge from calli but are thin and short (Fig. 2). Due to the long period of time required for AR formation, leaves can become brown or rotten (Fig. 1), negatively affecting the quality of the rooted cuttings. As a result, not all rooted cuttings survive after transplanting.

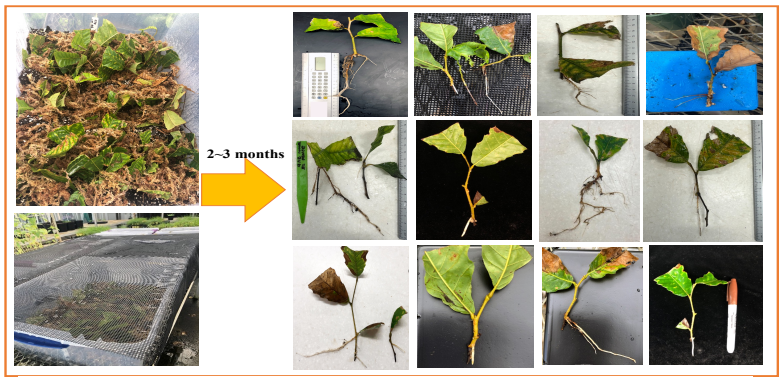

Fig. 1. Rooting chestnut cuttings is feasible with auxin induction (two hours of 100 ppm NAA treatment), including AC cuttings.

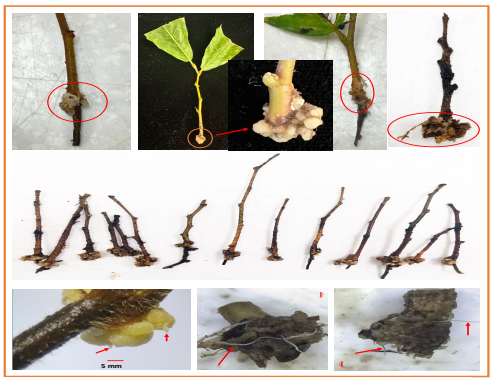

Fig. 2. Calli are formed on chestnut cuttings. ARs emerged from calli are thin and short.

B). Histology shows an enclosed sclerenchyma ring and a more condensed vascular region. In the cross sections of American chestnut cuttings, we noticed a closed sclerenchyma ring (Fig. 3A). In easy-to-root weeping willow and poplar *'Ogy'*, sclerenchymas are discontinuous. Because AR primordia need to force apart the sclerenchyma before ARs can emerge, the closed sclerenchyma structure could prevent the outgrowth of ARs. Also, the cells in the vascular cambium region seem less condensed in the easy-to-root cuttings, suggesting these cells are more actively dividing. American chestnut ARs are initiated in the vascular region, and AR's vascular is connected with the one in the cutting (Fig. 4).

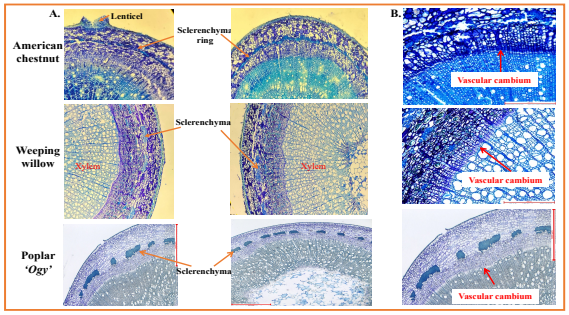

Fig. 3. Examples of cross sections of AC, weeping willow, and poplar *'Ogy'* cuttings.

C). American chestnut cuttings have a lower level of indole-3-acetic acid (IAA). As reported in Lu et al. (2023), we found that the endogenous hormone profile in American chestnut cuttings is not in favor of AR induction. Compared to easy-to-root poplar, levels of known AR-inhibiting hormones, i.e. cytokinin (CK), an inactive form of IAA amino acid conjugate aspartate (IAA-ASP), abscisic acid (ABA), jasmonic acid (JA), JA-IlE J(isoleucine), oxylipin 12-oxo-phytodienoic acid (OPDA), and salicylic acid (SA), were significantly higher in American chestnut cuttings (Fig. 5A). In contrast, the IAA level was significantly lower in American chestnut stems. Furthermore, the American chestnut had a different hormone distribution profile between leaves and stems than the poplar (Fig. 5B). For instance, the poplar leaf and stem contained a similar level of IAA (1.2), while the American chestnut was 0.4. Also, the American chestnut's cytokinin stem/leaf ratio was approximately 30 times higher than that of poplar.

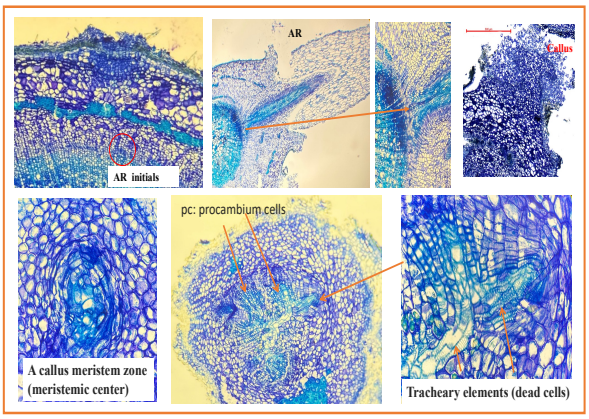

Fig. 4. Cross sections of AC cuttings, showing AR initial cells, AR, and callus structure.

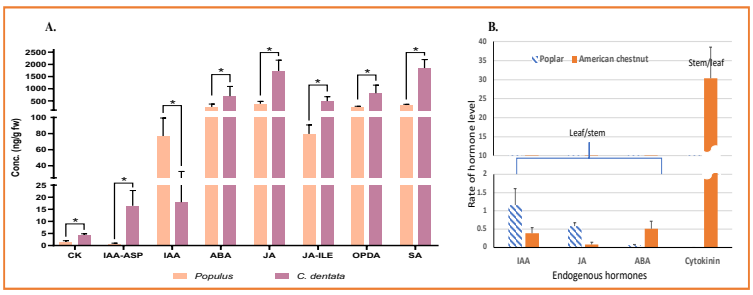

Fig. 5. Levels of endogenous hormones in AC and *'Ogy'* cuttings (A) and ratios between leaf and stem (B). IAA: indole-3-acetic acid; IAA-ASP: IAA amino acid conjugate aspartate; JA: jasmonic acid; JA-IlE: JA- Isoleucine; CK (cytokinin): t-Zeatin, c-Zeatin, dihydrozeatin (DHZ), and trans-zeatin riboside (t-ZR); ABA: abscisic acid; OPDA: oxylipin 12-oxo-phytodienoic acid, a biosynthetic precursor of JA; SA: salicylic acid.

D). American chestnut cuttings show different profiles of metabolites from poplar and willow. In primary metabolites (Fig. 6), we found that willow cuttings generally showed a low accumulation of organic acids, amino acids, amino alcohol, and sugar acid, and poplar had a high accumulation of amino acids, amino alcohol, and sugar acid. American chestnut had the highest level of citric acid, quinic acid, ascorbic acid, and gallic acid, while poplar contained the highest content of malic acid, furmaric acid, and shikimic acid. For amino acid, L-threonine, 4-aminobutyric acid, and L-glutamic acid were lowest in American chestnut. For sugar alcohol, iso-erythritol, arabitol, and mannitol were highest in willow, and inositols were highest in American chestnut. American chestnut and willow had the highest accumulation of 11 sugars, and poplar had five. Among the known compounds of flavonoids, phenolic acid derivatives, and organic acids that have been demonstrated or suggested to have a positive relationship with rooting in published studies, American chestnut had six that were expressed at a significantly lower level, while poplar contained seven species-specific ones (Table 1). These data indicate that American chestnut may have a metabolite profile that is not favorable to rooting.

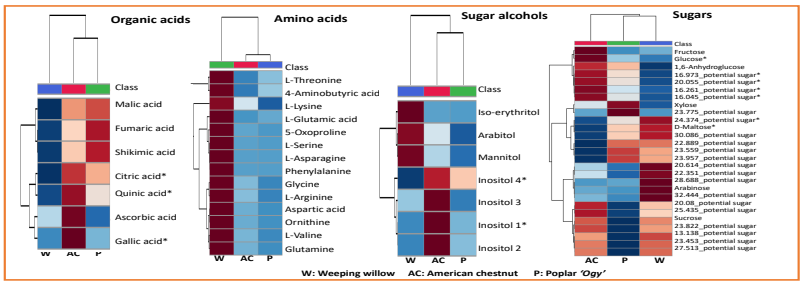

Fig. 6. Clustering results of known primary metabolites shown as heatmaps. * indicates important features identified by PLS-DA.

Table 1. Numbers of AR-promoting secondary compounds in poplar *'Ogy'* and American chestnut (AC)

Class	Type	Upregulated in AC	Down-regulated in AC	Poplar specific isoform	AC specific
Flavonoid	Quercetin & glucosides	0	2	2	0
	Kaempferol	0	1	2	0
	Catechin	0	0	0	0
	Luteolin & glucosides	0	1	2	0
Phenolic acid derivative	Syringic acid & glucosides	0	1	0	0
	Chlorogenic acid	0	0	2	0
	Caffeic acid			7	
Organic acid	Jasmonates	0	1	0	0
Sum		0	6	15	0

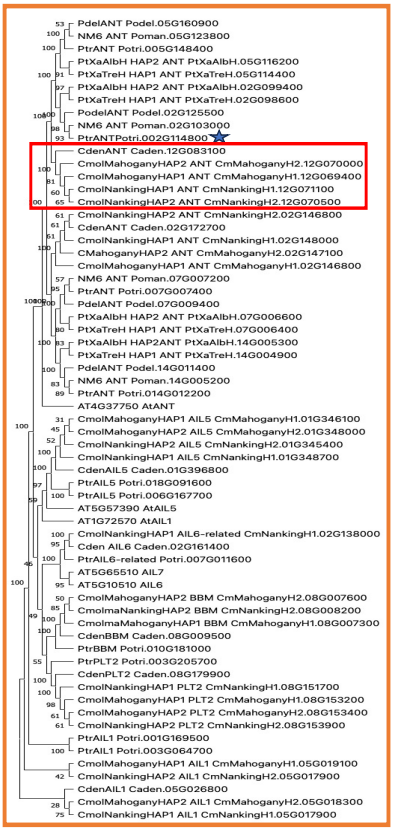

Fig. 7. A maximum likelihood phylogenetic tree of ANT amino acid sequences from *Castanea* (chestnut), *Populus*, and *Arabidopsis*. Sequences were retrieved from sequenced genomes in Phytozome 13. The star indicates the poplar *ANT* gene that has been found to promote ARs.

E). American chestnut *AINTEGUMENTA* (*ANT*) homolog contains fewer auxin *cis*-elements. *AINTEGUMENTA* (*ANT*) encodes a transcription factor that directly regulates genes involved in auxin signaling and cell proliferation (Mizukami et al. 2000). Overexpression of the poplar *ANT* led to an increased number of ARs, whereas RNA interference-mediated down-expression of its expression caused a delay in adventitious root formation. Also, the poplar *ANT* rapidly responded to auxin induction (Rigal et al. 2012). We found that *Populus* species contain two copies of *ANT*, while the chestnut species have one copy (Fig. 7), and the number of auxin *cis* elements in poplar *ANT* promoters was 4 to 7 times that of chestnuts (Table 2). It is worth pointing out that 717-1B4, a poplar hybrid *P. tremula x P. alba*, cannot form ARs as easily as *'Ogy'* without auxin induction. However, 717-1B4 cuttings root within 10 days upon auxin induction, and multiple ARs are formed by 15 days (Fig. 8). We suspect that chestnut cuttings' basal *ANT* expression level is low and cannot respond rapidly to auxin induction, contributing to chestnut cuttings' less efficient response to auxin induction (at least two months for ARs to emerge, and usually only 1-3 ARs are formed, Fig. 1).

Table 2. Numbers of hormone signal motifs present in the 3kb-promoter of *AINTEGUMENTA* (*ANT*) genes in *Populus* and *Castanea* species.

		Hormones	Auxin	Abscisic acid	Ethylene	Gibberellin	Salicylic acid	Jasmonic acid	Cytokinin
Populus (poplar)	Podel.02G125500	133	13	22	3	41	6	5	1
	Podel.05G160900	129	20	14	3	52	12	0	6
	NM6_Poman.05G123800	145	14	17	6	60	14	0	3
	NM6_Poman.02G103000	121	16	21	2	38	5	3	1
	717-1B4_HAP1_PtXaTreH.02G0	113	14	17	7	34	7	1	0
	717-1B4_HAP1_PtXaTreH.05G1	119	23	15	5	39	11	1	0
	717-1B4_HAP2_PtXaAlbH.02G0	116	12	19	4	32	6	4	3
	717-1B4_HAP2_PtXaAlbH.05G1	129	14	11	4	54	16	3	3
	Potri.005G148400	132	14	20	2	42	5	2	1
	Potri.002G114800	123	13	11	5	56	15	2	4
Castanea (chestnut)	CmolMahoganyHAP2	93	3	8	4	38	7	3	3
	CmolMahoganyHAP1	93	3	8	4	38	7	3	3
	CmolNankingHAP1	92	3	8	4	38	7	3	3
	CmolNankingHAP2	92	3	8	4	38	7	3	3
	Caden.12G083100	97	3	9	4	40	6	1	2

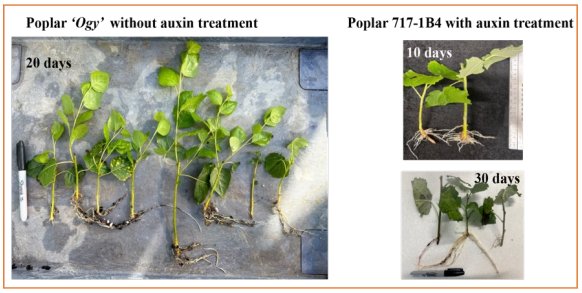

Fig. 8. Multiple ARs are formed on poplar '*Ogy*' and 717-1B4 cuttings.

F). Initial results with 1q induction. In our initial trials, we treated AC and American and Japanese hybrid cuttings with IBA and 1q. We obtained ARs in cuttings by the 40th or 43rd day, and by the 60th day, an average of 6.8 ARs per cutting was achieved with an average root length of 15.0 cm and (Fig. 9). This is a very promising improvement.

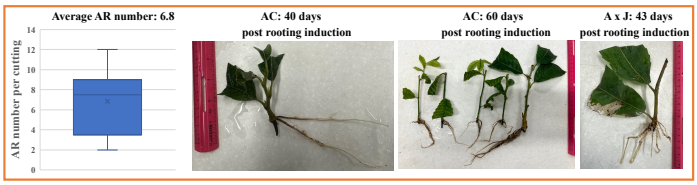

Fig. 9. Preliminary rooting results with 3,000 ppm IBA and 25 µM 1q. The basal part of the chestnut cuttings was dipped into the combined solution for one minute.

3). Ongoing projects.

Currently, we are conducting bulk analyses of AR formation in AC cutting, which include four timepoints to cover the period up to the formation of the root primordia (induction, initiation, and extension/elongation phases). Our results will provide valuable information and lay the foundation for studies of gene regulation in chestnut species.

4). Objectives of the current proposal.

While we have obtained adventitious roots from cuttings with auxin induction, more efforts are needed to improve the rooting rate and root number per cutting, as well as understand adventitious root formation in American chestnut cuttings at the physiological and molecular levels. In this one-year project, we aim to achieve the following two specific objectives:

- Improve the rooting system, with a focus on the potential effects of 1q.
- Determine the expression level of significant AR genes upon auxin induction.

5). Approaches and methods.

A. Rooting experiments with 1q.

Current-year's semi-lignified AC twigs are to be cut into segments that are 10-15 cm in length, containing at least two buds and 2~3 leaves. The potting substrate will be a mix of Canadian sphagnum peat moss, bark, perlite, and vermiculite (Sungro, Fafard 3b). Wet sphagnum mosses are to be used to partly cover the leaves of cuttings (Fig. 1). The cuttings are put in a transparent box covered with a screen. A temperature-controlled (22-23 °C) mist room will be employed (mist is 14 minutes off and 6 seconds on). The basal part of the AC cuttings will be dipped into the hormone solutions. Based on the initial results (Fig. 8), we will test the following combination:

- 3000 ppm IBA+25 µM 1q: treatment times of 1, 10, and 30 minutes;
- 6000 ppm IBA+50 µM 1q: treatment times of 1, 10, and 30 minutes.

There will be at least 10 cuttings per treatment. The experiments will be conducted at least three times, depending on the availability of twigs. Rooting rate, root length, and root number will

be recorded. The data will be processed using SPSS v. 23.0 software (SPSS Inc., IL, USA). One-way analysis of variance is to be performed to identify statistically significant differences among treatments, followed by Duncan's multiple range test at $P < 0.05$.

B. Gene expression analysis with RT-qPCR (Reverse Transcription Quantitative Polymerase Chain Reaction).

The slow response to auxin induction may contribute to the low AR number and long period in rooting chestnut cuttings, as suggested by the limited number of auxin-responsive elements in *ANT* homologs. The chestnut *ANT* homologs contain only ~1/5 of auxin-responsive *cis*-elements found in Populus species (Table 2). The poplars are known for their rapid and efficient response to auxin induction (Chen et al. 2013). We will examine the promoters of the significant genes identified by the ongoing RNA sequencing for auxin-responsive *cis*-elements and assess gene expression upon auxin induction. Comparisons will be made with *Populus* species.

Promoter sequences (3kb) will be retrieved from Phytozome, which contains five chestnut reference genomes, including AC, and five *Populu*s genomes. We will use the online program A Database of Plant *Cis*-acting Regulatory DNA Elements (New PLACE) for the motif analysis. For gene expression post-auxin induction, we will collect the basal part of the cuttings at 0, 12, 24, 48, and 72 hours for RNA extraction and subsequent RT-qPCR analysis. Primer design, RT-qPCR reaction setup, and relative expression analysis will be performed according to our previous reports (Xu et al. 2013a; 2013b). Four independent qPCR experiments per gene will be included for each cDNA sample. Several house-keeping genes such as ACT, TUA, UBQ, and 18S, will be evaluated for their stability, and the one with a CT value closest to that of target genes will be selected as the internal controls for normalization. The $2^{-\Delta\Delta CT}$ method will be used for the calculation of the relative expression level of target genes.

6). Potential pitfalls.

American chestnut cuttings are notoriously recalcitrant to rooting. However, we have succeeded in rooting AC cuttings. The auxin conjugate 1q has shown promising results. We are confident that we will be able to further improve AC's rooting efficacy through the research described in this proposal. The main potential obstacle can be getting adequate AC cuttings for the experiments. We will continue to reach out to AC breeders. There are also AC plants in our Clemson greenhouse that can provide materials for year-round use.

7). Literature Cited.

Chen Y et al. (2013) DR5 as a reporter system to study auxin response in *Populu*s. Plant Cell Rep 32:453–463.

Lu X et al. (2024) Integrating histology and phytohormone/metabolite profiling to understand rooting in yellow camellia cuttings. Plant Sci 346:112160.

Rigal A et al. (2012) The AINTEGUMENTA LIKE1 homeotic transcription factor PtAIL1 controls the formation of adventitious root primordia in poplar. Plant Physiol 160(4):1996–2006.

Roth, O et al. (2024) Slow release of a synthetic auxin induces formation of adventitious roots in recalcitrant woody plants. Nature Biotech 24:1–2.

Xu Y et al. (2013b) Wood chemistry analysis and expression profiling of a poplar clone expressing a tyrosine-rich peptide. Plant Cell Rep 32: 1827–21841.

Xu Y et al. (2013a) LtuCAD1 is a cinnamyl alcohol dehydrogenase ortholog involved in lignin biosynthesis in *Liriodendron tulipifera* L., a basal angiosperm timber species. Plant Mol Biology Rep 31:1089–1099.

C. Timeline (December 2023 – November 2024)

Research Work	1st quarter	2nd quarter	3rd quarter	4th quarter
Rooting with 1q with cuttings from greenhouse (winter) (Obj#1)				
Rooting with 1q with spring cuttings (Obj#1)				
Rooting with 1q with summer cuttings (Obj#1)				
Promoter motif analysis (Obj#2)				
RT-qPCR (Obj#2)				
Data analysis & interpretation				
Present results and manuscript drafting				

D. How results will be measured and reported

Rooting rate, root length, and root number will be recorded and compared. Results will be presented at one meeting and reported to TACF. One manuscript will be drafted.

E. Breakdown of how and when funds will be spent

Items	Amount $
Hourly Salaries (WAGES)	$6,000
Fringe	$522
Greenhouse rental	$2,000
Materials and Supplies	$1,478
Total	$10,000

Budget Justification:

- Hourly Salaries (WAGES) $6,000: for graduate students working on the project.
- Fringe $522: is calculated at the rate of 8.7%
- Greenhouse rental $2,000: covers pots, potting mix, water, labels, fertilizers, and electricity
- Materials and Supplies $1,478: covers DNA and RNA extraction kits, cDNA synthesis and qPCR reagents, shipping fees, liquid nitrogen, gloves etc.

Total request from TACF: $10,000. The fund will be needed during the project period (one year).

- Matching funds provided by Clemson University ($5,250)

TACF has a policy not to pay overhead (F&A – Facilities and Administration) charges since its grants are relatively small. Therefore, this amount ($5,250) (calculated at a rate of 52.5%) is regarded as part of matching funds from Clemson University.

F. Brief Curriculum Vitae (CV) of Haiying Liang- Clemson University

a) Professional Preparation:

Beijing Forestry University, China	Forestry	B.Sc.	1990
Beijing Forestry University, China	Plant Biology	M.S.	1993
College of Environmental Science &Forestry State University of New York	Plant Science & Biotechnology	Ph.D.	2000
College of Environmental Science & Forestry State University of New York	Plant Science & Biotechnology	Postdoc	2000~2004
The Pennsylvania State University	Plant functional Genomics	Postdoc	2004~2006

b) Appointments:

07/2012—present: Associate professor, Clemson University, Clemson, SC
09/2006—06/2012: Assistant Professor, Clemson University, Clemson, SC.
04/2004—08/2006: Postdoctoral Fellow, The Pennsylvania State University, State College, PA.
08/2000—03/2004: Postdoctoral Fellow, College of Environmental Science and Forestry, State University of New York, Syracuse, NY.
07/1993—07/1996: Instructor, Beijing Forestry University, Beijing, China.

c) Publications in the last four years:

1. (2024) LeBoldus JM, Lynch SC, Newhouse A, Søndreli KL, Newcombe G, Bennett PI, Muchero W, Chen J-G, Busby PE, Gordon M, **Liang H**. Biotechnology and genomic approaches to mitigating disease impacts on forest health. Annual Review of Phytopathology. In Press.
2. (2024) Wang S, Li B, Ma H, Shen J, Liu Y, **Liang H**, Li Y, Song C, Guo L, Hou X. PomiR396g-5p/PoACO1 module regulates the response of tree peony to drought stress through reactive oxygen species pathway. Industrial Crops and Products. 221:119323.
3. (2024) Lu X, Chen X, Liu J, Zheng M, **Liang H**. Integrating Histology and Phytohormone/Metabolite Profiling to Understanding Rooting in Yellow Camellia Cuttings. Plant Science. 346:112160.
4. (2023) Lu X, Cuarto M, **Liang H**. Histology of adventitious root formation and phytohormone analysis of American Chestnut Cuttings. Journal of Environmental Horticulture. 41(3):80-6.
5. (2022) L Guo, J Shen, C Zhang, Q Guo, **H Liang**, X Hou. Characterization and bioinformatics analysis of ptc-miR396g-5p in response to drought stress of *Paeonia ostii*. Non-coding RNA Research. 7(3):150-158.
6. (2022) C Shen, Q Li, Y An, Y Zhou, Y Zhang, F He, L Chen, C Liu, W Mao, X Wang, **H Liang**, Yin W, Xia X. The transcription factor GNC optimizes nitrogen use efficiency and growth by driving the expression of nitrate uptake and assimilation genes in poplar. Journal of Experimental Botany. May 8.
7. (2022) O Gailing, M Staton, SE Schlarbaum, MV Coggeshall, J Romero-Severson, **H Liang** & J E Carlson. Progress and prospects of population genomics of North American hardwoods. In: Population Genomics. Springer, Cham.

8. (2021) B Lei, CJ Frost, T Xu, JR Herr, JE Carlson, **H Liang.** Poplar allene oxide synthase 1 gene promoter drives rapid and localized expression by wounding. Biotechnology Journal International. 25(5):16-28.
9. (2021) W Lu, E Wang, W Zhou, Y Li, X Song, J Wang, M Ren, D Yang, S Huo, Y Zhao, **H Liang**. Morpho-histology, endogenous hormone dynamics, and transcriptome profiling in Dacrydium Pectinatum during male cone development. Forests. 12(11):1598.
10. (2021) X Wei, S Wu, X Liang, K Wang, Y Li, B Li, J Mac, **H Liang**. Paclobutrazol modulates endogenous level of phytohormones in inducing early flowering in *C. tamdaoensis*, a golden Camellia species. HortScience. 56(10):1258-1262.
11. (2021) KX Li, L K iu, Y Chen, X Huang, W Liang, B Li, Y Shen, **H Liang**. Comprehensive transcriptome and metabolome analysis of *Lithocarpus polystachyus* leaf revealed key genes in flavonoid biosynthesis pathways. Journal of the American Society for Horticultural Science. 1(aop):1-11.
12. (2020) S Li, **H Liang**, L Tao, L Xiong; W Liang, Z Shi, Z Zhao. Transcriptome sequencing and differential expression analysis reveal molecular mechanisms for starch accumulation in chestnut. Forests. 11:388.
13. (2020) Y-Y Xia, D-X Wang, B-Q Hao, Z-P Jiang, G-C Chen, **H Liang**. Nitrogen fertilizer mitigates water loss and restores pigment composition in *camellia oleifera*, an oilseed crop. Journal of Soil and Plant Biology. 2020:106-112.

d) Courses taught:

1. GEN/BCHM8100 (Principles of Molecular Biology) for graduate and undergraduate students
2. GEN/BCHM8000 (Special Topics on Plant Genetics) for graduate and undergraduate students
2. GEN3000 (Fundamental Genetics) for undergraduate students
3. GEN3020 (Molecular & General Genetics) for undergraduate students
4. GEN 4930 (Undergraduate Senior Seminar)

e) Synergistic Activities:

1. Membership & Outreach Officer, North and South Carolina Chapter, The American Chestnut Foundation (since 2024).
2. Serve in the University's Graduate and Undergraduate Integrity Hearing Boards since 2015.
3. Chair or serve in the Clemson University Genetics and Biochemistry Department's Graduate Committee since 2017 and PhD program assessment committee; Chaired the Clemson University Genetics and Biochemistry Department's curriculum committee for three years.
4. Program Committee member for the 2013 Southern Forest Tree Improvement Conference and recipient of a USDA conference award for a career development workshop and supporting students and postdoctoral fellows to attend the conference.
5. A science fair judge for local and region schools for more than 10 years. A frequent manuscript reviewer for various journals, including Plant Physiology, Tree Physiology, BMC Genomics, Plant Cell, Tissue and Organ Culture, PLoS ONE, and Horticulture Research. Served as an *ad hoc* proposal reviewer and review panel member for USDA and NSF.

G. A Conflict of Interest or Commitment (COI or COC) statement.

There is no known COI or COC regarding this project.