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Association of the *cad-n1* allele with increased stem growth and wood density in full-sib families of loblolly pine

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Abstract Stem growth and wood density associated with a mutant null (*cad-n1*) allele were examined in three 15-year-old loblolly pine half-diallel tests established on two sites in the southern United States. In each half-diallel test, one or two *cad-n1* heterozygous parents were crossed with five unrelated wild-type parents to produce five or ten full-sib families. In all, 839 trees from 20 full-sib families in four genetic backgrounds (a *cad-n1* heterozygote × five unrelated trees) were sampled, genotyped at the *cad* locus, and assessed for growth and wood density traits. In a combined analysis of all four genetic backgrounds, we found evidence for effects of increased wood density associated with the *cad-n1* allele at age 15 years (p=0.03) and height growth at ages 6 (p=0.03) and 15 (p=0.005). There were differences in the *cad-n1* effects for the various growth and wood traits among the half-diallel tests. This variation may be due to either different genetic backgrounds among the parents of the different half-diallel tests or for different growing environments at the field sites. Even though the *cad-n1* effect on growth and wood density was significant across genetic backgrounds, the effect was variable among full-sib families within backgrounds. We speculate that certain wild-type alleles from second parents specifically interact with *cad-n1* producing large positive effects. In addition, pleiotropic effects on growth and wood density appear to be associated with the *cad-n1* allele. While substantial gains are possible through deployment of trees carrying *cad-n1*, these gains may be family-specific and should be verified for each cross through field-testing.

Keywords Cinnamyl alcohol dehydrogenase · Half-diallel mating · Null mutation · Heterozygotes · Stem growth · Wood density

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Introduction

Selection 7-56, owned by International Paper Company, is one of the best loblolly pine (*Pinus taeda L.*) parent trees in the North Carolina State University, Industry Cooperative Tree Improvement Program, producing progeny that are extremely fast-growing with excellent quality traits [7]. Selection 7-56 is also the only known natural carrier of a rare gene (*cad-n1*) [8, 9]. This allele encodes a two base adenosine insertion that results in a severe deficiency in the enzyme, cinnamyl alcohol dehydrogenase (CAD) [4], which catalyzes the last step in the biosynthesis of the major lignin precursors coniferyl alcohol. The extent to which this *cad-n1* allele affects the breeding value of 7-56 has been an important question in loblolly pine breeding programs. As a naturally occurring gene, the acceptance of *cad-n1* as a candidate for genetic improvement through breeding is expected to be higher than for a gene obtained from a different species introduced by genetic engineering. Before any use for marker-assisted breeding or genetic engineering can be considered, *cad-n1* must be well characterized and evaluated in harvest-age trees in a broad range of environments and in diverse genetic backgrounds.

Breeding with 7-56 produces three types of trees with respect to the *cad* locus. With self-pollination, *cad-n1* heterozygotes (*Cad/cad-n1*), wild-type homozygotes (*Cad/Cad*), and *cad-n1* homozygotes (*cad-n1/cad-n1*) are produced. With outcrossing, *cad-n1* heterozygotes and wild-type homozygotes are produced. The *cad-n1* homozygotes, originally characterized in this selfed family

(7-56×7-56), have a distinct brown wood color and have a nearly complete absence of CAD activity in developing xylem [4, 8, 9, 12]. Dimmel et al. [2] reported that homozygous *cad-n1* trees had poor growth after early stages and low pulp yields (due at least in part to inbreeding) compared to other genotypes, although they produced wood that was more easily delignified.

Wu et al. [17] reported that heterozygous *cad-n1* trees produced 14% more debarked wood volume at age 4 years in an open-pollinated progeny compared to wild-type trees. Previous studies comparing *cad-n1* heterozygotes with wild-type trees yielded inconsistent results on pulping and bleaching. In one study, kraft cooks of 4- and 6-year-old heterozygous trees resulted in kappa numbers (i.e., lignin contents) that were significantly lower than wild-type trees. In addition, significantly less energy was required (15 to 25% lower H-factor) to pulp to a given kappa number than for wild-type trees and the pulp of the heterozygotes was brighter and stronger [2]. On the contrary, Dimmel et al. [3] found no apparent differences in ease of delignification or pulp yield between heterozygous and wild-type trees that were 14 years old. In a much larger sample, effects associated with this *cad-n1* allele were found by comparing wood density and growth traits of *cad-n1* heterozygous trees with wild-type trees in a 10-year-old open-pollinated family experimentally growing under two levels of fertilization. The substitution of *cad-n1* for a wild-type allele (*Cad*) was associated with a significant effect on wood density, but not for growth traits [18].

Prior to the use of the *cad-n1* allele in tree improvement, it is necessary to estimate the direction and magnitude of the *cad-n1* effects on important traits at varying ages in different genetic backgrounds and field environments. In this paper, we extend previous findings to include three *cad-n1* heterozygous selections evaluated in three half-diallel test series on two field-test sites measured at two different ages. The objectives of this analysis are to: (1) quantify the association between *cad-n1* allele and tree growth and wood density in multiple genetic backgrounds, environments and ages; (2) estimate the *cad-n1* effects on growth and wood density; and (3) investigate the temporal stability of the *cad-n1* effect for these traits.

Material and methods

Plant material

Three selected *cad-nl* heterozygous parents (second-generation selections that are offsprings of 7-56) generating 20 full-sib families from three disconnected half-diallel progeny tests of loblolly pine were included in this study (Table 1). Each half-diallel consisted of six parents and 15 full-sib crosses without self-crosses and reciprocal crosses. In each test, only full-sib progenies produced by *cad-n1* heterozygote parents (selections) crossed with five unrelated, wild-type parents were sampled and measured for growth and wood density traits. The three half-diallel tests are numbered as series (S) 1, 2, and 3 and the three *cad-n1* heterozygote selections are A, B, and C. S1 included five crosses each for selections A and B, S2 included five crosses with selection C, and S3 included five crosses for selection A. Each half-diallel test was established in replicated trials at two sites, one in South Carolina and one in Georgia (Table 2). There are four field sites in each half-diallel test but only two sites were available for this study. Each site was planted in a randomized complete block design with six blocks in six-tree row plots for each cross [7], but only four blocks were used for sampling. Genetic background is defined by a particular heterozygous selection in a specific test series, resulting in four genetic backgrounds (Table 1).

Table 1 Parents, half-diallel test series, and genetic backgrounds sampled for analysis

cad-n1 selection	Diallel series	Genetic background	Wild-type parents
A	S1	A-S1	A1 A2 A3 A4 A5
A	S3	A-S3	A6 A7 A8 A9 A10
B	S1	B-S1	B1 B2 B3 B4 B5
C	S2	C-S2	C1 C2 C3 C4 C5

Growth measurements

At age 15 years, total tree height (HT, m) and diameter at breast height (DBH, cm) 1.3 m were measured in November 2003. In addition, all tests in each test series had growth measurements (HT, DBH) at age 6 years. Stem volume (VOL) was calculated using the equation of Shelton et al. [16]:

$$\mathrm{VOL} = 0.00748 + (0.0000353 \times \mathrm{DBH}^2 \times \mathrm{HT}) \qquad (1)$$

Genotyping and wood density measurements

Details of the genotyping and wood density measurement procedures are given in [18] but a brief description follows. Inner bark tissue was collected and DNA was isolated from each of the sampled trees (DNA Easy Kits, Qiagen, Inc., Valencia, CA, USA). Polymerase chain reaction (PCR) primers that flank the *cad-n1* specific mutation (2-bp insertion) [4] were used to amplify the *cad* locus from each DNA sample. PCR products were resolved and sized using capillary electrophoresis (ABI 3100 Genetic Analyzer, Applied Biosystems, Inc., Foster City, CA, USA). Trees were scored as either heterozygous mutants or homozygous wild type.

A 12-mm core was sampled from each tree at breast height for wood quality analyses. Cores were sectioned longitudinally to produce a strip approximately 2-mm

Table 2 Field-test locations and sampling characteristics for *cad-n1* heterozygous parents

cad-n1 selections	Diallel series	No. of sites	State	Year planted	No. of samples	No. of *cad-nl* heterozygotes	No. of wild types
A	1	2	Georgia	1988	225	114	119
A	3	2	South Carolina	1988	194	90	100
B	1	2	Georgia	1988	233	115	108
C	2	2	South Carolina	1988	192	100	93
Total		8			839	419	420

thick. The samples were conditioned to a uniform moisture content of 8% before they were scanned. Wood density was measured using X-ray densitometry. Each strip was scanned from pith to the bark on a QMS Tree Ring Analyzer (Model Qtrs-01x, Quintek Measurement Systems, Inc.). The last growth ring was excluded due to missing latewood on cores collected in midsummer. For each ring scanned, the following intraring wood density characteristics were determined: average ring density, earlywood density, latewood density, latewood percentage, and cambial age. Weighted average wood density traits were calculated by weighting ring mean density with total ring basal area, which approximates the average density of a disk sample of wood taken at breast height.

Statistical analysis

For the combined analysis of all four genetic backgrounds, the following linear model was used:

$$Y_{hijklm} = \mu + B_h + S_{i(h)} + R_{j(hi)} + M_{k(h)} + G_l + (GM)_{kl(h)} + (GS)_{il(h)} + (GR)_{jl(hi)} + (MS)_{ik(hi)} + (MR)_{jk(hi)} + e_{hijklm} \quad (2)$$

where Y_{hijklm} is the mth tree of the jth block within the ith site for the lth genotype of the kth mate of hth genetic background; μ is the overall mean; B_h is the effect of the hth genetic background; $T_{i(h)}$ is the effect of the ith site

Table 3 Least square means (±standard error) of *cad-n1* heterozygous (HZ) and wild-type (WT) trees in four genetic backgrounds

(a) Stem growth traits at ages 6 and 15 years

Genetic background	6 years						15 years					
	Height (m)		DBH (cm)		Volume (dm^3)		Height (m)		DBH (cm)		Volume (m^3)	
	WT	HZ	WT	HZ	WT	HZ	WT	HZ	WT	HZ	WT	HZ
A-S1[a]	6.0±0.1	6.1±0.1	9.4±0.2	9.6±0.2	28.0±0.9	28.6±0.9	17.5±0.1	17.8±0.1	18.4±0.3	18.8±0.3	0.23±0.01	0.24±0.01
A-S3	**6.9±0.1**	**7.2±0.1**	12.3±0.2	12.7±0.2	47.3±1.7	51.4±1.7	**19.1±0.2**	**20.1±0.2**	21.8±0.5	22.7±0.5	0.37±0.02	0.41±0.02
B-S1	5.9±0.1	5.8±0.1	9.5±0.2	9.2±0.2	28.2±0.8	27.1±0.8	16.9±0.2	16.9±0.2	18.3±0.3	18.0±0.3	0.22±0.01	0.21±0.01
C-S2	5.9±0.1	6.0±0.1	10.7±0.2	10.9±0.2	46.5±1.4	47.2±1.3	18.6±0.2	19.2±0.2	21.3±0.5	21.9±0.5	0.38±0.02	0.40±0.02
Estimated mean[b]	**6.4±0.1**	**6.6±0.1**	11.0±0.2	11.2±0.2	36.5±1.2	37.5±1.2	**18.6±0.2**	**19.1±0.2**	20.3±0.2	20.8±0.2	0.30±0.01	0.31±0.01

(b) Wood density traits at age 15 years

Genetic background	Weighted wood density (kg/m^3)		Weighted earlywood density (kg/m^3)		Weighted latewood density (kg/m^3)		Weighted latewood (%)	
	WT	HZ	WT	HZ	WT	HZ	WT	HZ
A-S1[a]	536±3.1	536±3.13	341±1.3	341±1.3	711±3.0	709±3.1	50±0.56	50±0.58
A-S3	**498±3.8**	**511±4.03**	344±1.7	348±1.8	658±3.2	665±3.4	**46±0.83**	**49±0.88**
B-S1	529±3.2	526±3.16	331±1.5	333±1.4	701±3.0	697±3.0	50±0.62	50±0.61
C-S2	487±3.6	492±3.49	339±1.5	341±1.5	657±2.9	660±3.0	43±0.72	44±0.69
Estimated mean[b]	**513±1.8**	**518±1.8**	342±1.0	341±1.0	688±1.6	690±1.6	**48±0.3**	**49±0.3**

p values ≤0.05 are in bold type
[a]From Eq. 3, estimated by Restricted Maximum Likelihood.
[b]From Eq. 2

Table 5 Significance levels (p values) for *cad* main effects and interactions from the separate analyses for each genetic background

(a) Stem growth traits (Height6, DBH6, Volume6) at ages 6 and (Height, DBH, Volume) at age 15

Genetic background	Effect	*df*	Den *df*	6 years			15 years		
				Height (m)	DBH (cm)	Volume (dm^3)	Height (m)	DBH (cm)	Volume (m^3)
A-S1	Site	1	6	0.181	0.734	0.559	0.431	0.000	0.000
	Mate	4	24	0.001	0.017	0.003	0.003	0.110	0.026
	Genotype	1	6	0.513	0.990	0.804	0.515	0.906	0.847
	S*G	1	6	0.292	0.526	0.550	0.113	0.154	0.167
	G*M	4	180	0.403	0.441	0.764	0.739	0.538	0.500
	T*M	4	24	0.072	0.018	0.010	0.670	0.697	0.476
	Random effect			Variance component (%)					
	Rep (S)			11.0	5.6	7.6	1.7	0.0	0.0
	Rep*G (S)			0.1	0.0	0.0	5.3	0.0	0.0
	Rep*M (S)			13.9	0.0	1.2	11.0	0.0	0.0
	Residual			75.0	94.4	91.1	82.1	100.0	100.0
A-S3	Site	1	6	0.496	0.075	0.501	0.001	0.002	0.000
	Mate	4	24	0.829	0.811	0.756	0.973	0.956	0.436
	Genotype	1	6	0.040	0.099	0.054	0.016	0.153	0.063
	S *G	1	6	0.998	0.215	0.173	0.716	0.385	0.153
	G*M	4	180	0.229	0.097	0.180	0.143	0.199	0.222
	T*M	4	24	0.172	0.002	0.004	0.143	0.003	0.001
	Random effect			Variance component (%)					
	Rep (S)			61.3	15.0	26.0	10.2	6.2	4.0
	Rep*G (S)			0.0	0.2	1.9	0.0	0.0	0.0
	Rep*M (S)			12.0	10.2	9.1	31.8	7.8	5.5
	Residual			26.6	74.7	62.9	58.0	86.0	90.5
B-S1	Site	1	6	0.016	0.024	0.053	0.019	0.016	0.029
	Mate	4	24	0.000	0.001	0.000	0.001	0.025	0.003
	Genotype	1	6	0.967	0.475	0.562	0.803	0.781	0.964
	S *G	1	6	0.978	0.772	0.844	0.377	0.933	0.883
	G*M	4	180	0.273	0.646	0.432	0.162	0.592	0.615
	T*M	4	24	0.007	0.019	0.013	0.251	0.214	0.064
	Random effect			Variance component (%)					
	Rep (S)			18.0	13.8	21.7	0.0	0.0	0.0
	Rep*G (S)			0.0	0.0	0.0	1.1	0.0	1.6
	Rep*M (S)			10.0	9.6	6.3	11.9	14.2	7.4
	Residual			72.0	76.6	72.0	87.0	85.8	91.0
C-S2	Site	1	6	0.000	0.000	0.000	0.000	0.000	0.000
	Mate	4	24	0.649	0.093	0.165	0.819	0.450	0.218
	Genotype	1	6	0.410	0.468	0.632	0.129	0.267	0.178
	S*G	1	6	0.494	0.729	0.757	0.628	0.861	0.673
	G*M	4	180	0.036	0.304	0.779	0.318	0.334	0.701
	T*M	4	24	0.217	0.421	0.307	0.324	0.091	0.103
	Random effect			Variance component (%)					
	Rep (S)			42.3	25.4	7.5	30.5	8.4	10.1
	Rep*G (S)			6.2	4.8	0.0	6.3	3.3	0.0
	Rep*M (S)			4.4	0.0	0.0	0.0	0.0	0.0
	Residual			47.2	69.8	92.5	63.2	88.3	89.9

within hth genetic background; $R_{j(hi)}$ is the effect of the jth block within the ith site and hth genetic background; $M_{k(h)}$ is the effect of kth mate within the hth genetic background; G_l is the effect of the lth genotype; $(GM)_{kl(h)}$ is the effect of the interaction of the kth mate and lth genotype within hth genetic background; $(GS)_{il(h)}$ is the effect of the interaction between lth genotype and the ith site within hth genetic background; $(GR)_{jk(hi)}$ is the effect of interaction between lth genotype within jth block within ith site; $(MS)_{ik(h)}$ is the interaction between the kth mate and the ith site within hth genetic background; $(MR)_{jk(hi)}$ is the interaction between the kth mate and the jth block within the ith site and hth genetic background; and e_{ijklm} is the residual random within plot error term $\sim NIDN\ (0, \sigma_e^2)$. The $R_{j(hi)}$, $(GR)_{jl(hi)}$, $(MR)_{jk(hi)}$, and e_{ijklm} are considered as random effects and the rest of terms as fixed effect. Standardized data was used for the combined analysis, which means each observation was divided by standard deviation of each field-test.

To evaluate the *cad-n1* allele effect in each genetic background, growth and wood density data were also analyzed separately for each background according to the following linear model:

$$Y_{ijklm} = \mu + S_i + R_{j(i)} + M_k + G_l + (GM)_{kl} + (GS)_{il} + (GR)_{jk(i)} + (MS)_{ik} + (MR)_{jk(i)} + e_{ijkl} \quad (3)$$

Table 4 Significance levels (p values) for *cad* main effects and interactions from the combined (over the four genetic backgrounds) analysis

(a) Stem growth traits at ages 6 and 15 years

Effect	df[a]	Den df[b]	6 years			15 years		
			Height (m)	DBH (cm)	Volume (dm^3)	Height (m)	DBH (cm)	Volume (m^3)
			Level of significance (p)					
Background	3	24	<0.001	<0.001	0.088	<0.001	<0.001	0.150
Site (B)	4	24	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Mate (B)	16	96	<0.001	<0.001	<0.001	<0.001	0.024	0.002
Genotype	1	24	0.031	0.211	0.206	0.005	0.138	0.087
B*genotype	3	24	0.253	0.283	0.133	0.179	0.572	0.472
Genotype*mate (B)	16	628	0.037	0.339	0.461	0.192	0.536	0.449
Site*genotype (B)	4	24	0.684	0.607	0.529	0.382	0.583	0.562
Site*mate (B)	16	96	0.011	0.000	0.000	0.139	0.002	0.002
Random effect			Variance component (%)					
Rep (B*Site)			41.2	19.5	23.9	13.2	4.8	6.6
Rep*mate (B*Site)			9.7	1.1	2.9	10.9	0	0
Rep*genotype (B*Site)			0.8	0.4	0	2.3	1.3	1.7
Residual			48.3	79	73.3	73.6	93.9	91.8

(b) Wood density traits at age 15 years

Effect	df[a]	Den df[b]	Weighted wood density (kg/m^3)	Weighted latewood (%)	Weighted earlywood density (kg/m^3)	Weighted latewood density (kg/m^3)
			Level of significance (p)			
Background	3	24	<0.000	<0.001	<0.001	<0.001
Site (B)	4	24	<0.000	<0.001	<0.001	<0.001
Mate (B)	16	96	0.004	0.004	<0.001	<0.001
Genotype	1	24	0.033	0.027	0.110	0.325
B*genotype	3	24	0.213	0.186	0.887	0.536
Genotype*mate (B)	16	628	0.043	0.046	0.150	0.198
Site*genotype (B)	4	24	0.268	0.188	0.947	0.582
Site*mate (B)	16	96	0.044	0.022	0.010	0.776
Random effect			Variance component (%)			
Rep (B*Site)			2.5	1.2	4.8	0.6
Rep*mate (B*Site)			0.0	0.0	2.3	3.6
Rep*genotype (B*Site)			0.0	0.0	3.5	0.7
Residual			97.5	98.8	89.4	95.1

p values were estimated from Eq. 2
[a]Degree of freedom of the numerator
[b]Degrees of freedom of the denominator

Table 5 (continued)

(b) Wood density traits at age 15

Genetic background	Effect	*df*	Den *df*	Wood density (kg/m^3)	Latewood (%)	Earlywood density (kg/m^3)	Latewood density (kg/m^3)
A-S1	Site	1	6	0.000	0.000	0.000	0.258
	Mate	4	24	0.216	0.242	0.706	0.001
	Genotype	1	6	0.733	0.476	0.844	0.992
	S*G	1	6	0.260	0.107	0.602	0.574
	G*M	4	180	0.428	0.203	0.331	0.590
	T*M	4	24	0.094	0.115	0.019	0.603
	Random effect			Variance component (%)			
	Rep (S)			3.6	0.0	0.0	5.1
	Rep*G (S)			0.0	0.2	5.6	0.7
	Rep*M (S)			0.0	0.0	0.9	0.0
	Residual			96.4	99.8	93.5	94.2
A-S3	Site	1	6	0.000	0.044	0.001	0.000
	Mate	4	24	0.391	0.695	0.043	0.121
	Genotype	1	6	0.026	0.023	0.261	0.177
	S*G	1	6	0.094	0.152	0.719	0.279
	G*M	4	180	0.035	0.081	0.105	0.335
	T*M	4	24	0.066	0.044	0.046	0.656
	Random effect			Variance component (%)			
	Rep (S)			0.0	0.0	0.0	0.0
	Rep*G (S)			0.0	0.0	0.0	0.0
	Rep*M (S)			5.0	0.0	0.0	17.2
	Residual			95.0	100.0	100.0	82.8
B-S1	Site	1	6	0.000	0.000	0.002	0.243
	Mate	4	24	0.015	0.052	0.012	0.001
	Genotype	1	6	0.801	0.976	0.317	0.826
	S*G	1	6	0.597	0.407	0.714	0.639
	G*M	4	180	0.261	0.464	0.319	0.024
	T*M	4	24	0.575	0.456	0.218	0.427
	Random effect			Variance component (%)			
	Rep (S)			0.0	1.5	6.1	0.0
	Rep*G (S)			0.0	0.0	3.0	0.9
	Rep*M (S)			0.8	0.0	8.2	0.0
	Residual			99.2	98.5	82.6	99.1
C-S2	Site	1	6	0.000	0.012	0.020	0.000
	Mate	4	24	0.043	0.009	0.000	0.211
	Genotype	1	6	0.515	0.514	0.475	0.569
	S*G	1	6	0.628	0.647	0.879	0.454
	G*M	4	180	0.164	0.087	0.233	0.926
	T*M	4	24	0.368	0.200	0.591	0.631
	Random effect			Variance component (%)			
	Rep (S)			4.5	3.8	17.8	0.0
	Rep*G (S)			2.4	4.2	0.0	0.0
	Rep*M (S)			0.0	0.0	0.6	4.3
	Residual			93.2	91.9	81.6	95.7

p values were estimated from Eq. 3

The statistical analyses were performed by using the SAS software package [14]. The genotypic effects (i.e., the difference between heterozygous *cad-n1* and wild-type trees) were estimated using PROC MIXED, and the ESTI MATE option was used to estimate the difference among the second parents in each genetic background. PROC MULTTEST provided *p* value adjustments of significance between heterozygous and wild-type trees in each 20 full-sib families using Bon (Bonferroni) option. The Bonferroni adjustment procedure concerns the question if, in the case of doing more

than one test in a particular study, the alpha level should be adjusted to consider the chance of making a Type I error. If adjusted p value exceeds 1, it is set to 1 [14].

Results

In the half-diallel progeny tests, trees within full-sib families were expected to segregate 1:1 for the two possible *cad* genotypes (*Cad/cad-n1* and *Cad/Cad*). In total, 839 trees were sampled from four genetic backgrounds; 419 were heterozygous *cad-n1* trees and 420 were wild types (Table 2). Chi-square analysis indicated no significant difference from the overall expected segregation and for each of the four genetic backgrounds separately (data not shown).

We estimated least square means of growth traits and weighted wood density traits for the two *cad* genotypes (Table 3a,b). In the combined analysis of the four genetic backgrounds, height, diameter, and volume at ages 6 and age 15 were higher for *cad-n1* heterozygotes than for wild-type trees and 2.4, 2.1, and 5.4% greater at age 15, respectively. But only the height at ages 6 (p=0.03) and age 15 (p=0.005) were there significant effects (Table 4a). In the combined analysis of wood density traits, weighted earlywood density and latewood density were about the same between the two *cad* genotypes (Table 3b). For weighted wood density and weighted latewood percentage, heterozygous trees were significantly higher by 1% (p=0.03) and 2.1% (p=0.03) than wild-type trees (Tables 3b and 4b).

We did not find interactions for growth traits of *cad* genotype × site and *cad* genotype × genetic background at either ages 6 or 15 or wood density at age 15 (Table 4a,b). We also did not find *cad* genotype × age interactions for any of the growth traits (results not shown). The differences in the growth traits between the two *cad* genotypes are consistent between ages 6 and 15 (Table 3a). Genetic background and test site and mate significantly affected all growth and wood density traits (Table 4a,b) except for volume affected by genetic background.

Although there were no genotypes by genetic background interactions, we analyzed each of the genetic backgrounds separately to determine if the magnitude of the *cad-n1* effect was similar in each background (Tables 3a,b and 5a,b). In the separate analyses of growth traits in each genetic background at age 15 (Table 3a), volumes of A-S1, C-S2, and A-S3 were 4.3, 5.3, and 10.8% higher for *cad-n1* heterozygotes than the wild types, respectively; however, B-S1 heterozygotes were 4.5% lower than the wild types. The effect of *cad* genotype was significant only for A in genetic background S3 (A-S3) on height both at ages 6 (p=0.04) and 15 (p=0.02) and marginally significant for volume at ages 6 (p=0.05) and 15 (p=0.06) (Table 5a). For wood density traits, there were large differences between A-S3 and the other three genetic backgrounds in the effect of *cad-n1* (Table 3b). In A-S3, weighted wood density and

Table 6 Least square means (±standard error) of *cad-n1* heterozygous (HZ) and wild-type (WT) trees in full-sib families in four genetic backgrounds and differences [Diff.=(HZ−WT)/HZ×100] in least square means between HZ and WT within full-sib families

Genetic background	Full-sib family	No. of trees		Weighted wood density (kg/m^3)					Volume (m^3)				
		WT	HZ	WT	HZ	Diff. (%)	p value	Bon	WT	HZ	Diff. (%)	p value	Bon
A-S1	A×A1	24	24	529±6.5	533±6.5	0.8	0.633	1	0.20±0.02	0.21±0.02	5.0	0.676	1
	A×A2	23	24	525±6.6	538±6.5	2.5	0.164	1	0.23±0.02	0.24±0.02	4.3	0.634	1
	A×A3	22	24	544±6.8	533±6.6	−2	0.244	1	0.28±0.02	0.25±0.02	−10.7	0.189	1
	A×A4	21	25	541±6.8	546±6.3	0.9	0.562	1	0.23±0.02	0.26±0.02	13.0	0.264	1
	A×A5	29	17	538±5.9	534±7.5	−0.7	0.663	1	0.22±0.02	0.21±0.02	−4.5	0.848	1
A-S3	A×A6	22	21	483±7.5	526±7.5	8.9	<0.001	0.002	0.29±0.04	0.43±0.04	48.3	0.005	0.11
	A×A7	24	16	491±7.1	502±8.5	2.2	0.280	1	0.37±0.03	0.38±0.04	2.7	0.801	1
	A×A8	16	23	503±8.6	522±7.1	3.8	0.078	1	0.36±0.04	0.46±0.03	27.8	0.060	1
	A×A9	17	18	506±8.2	504±8.1	−0.4	0.856	1	0.36±0.04	0.38±0.04	5.6	0.672	1
	A×A10	21	12	499±7.9	501±9.9	0.4	0.873	1	0.43±0.04	0.42±0.05	−2.3	0.948	1
B-S1	B×B1	25	23	532±6.4	544±6.8	2.3	0.205	1	0.25±0.02	0.25±0.02	0.0	0.820	1
	B×B2	23	23	539±6.6	523±6.6	−3	0.080	1	0.21±0.02	0.2±0.02	−4.8	0.699	1
	B×B3	22	23	519±6.7	526±6.6	1.3	0.435	1	0.17±0.02	0.21±0.02	23.5	0.194	1
	B×B4	20	22	514±7.0	514±6.7	0	0.930	1	0.19±0.02	0.19±0.02	0.0	0.827	1
	B×B5	18	24	532±7.4	534±6.4	0.4	0.856	1	0.26±0.02	0.24±0.02	−7.7	0.457	1
C-S2	C×C1	21	21	500±7.3	495±7.4	−1	0.617	1	0.43±0.03	0.41±0.03	−4.7	0.696	1
	C×C2	22	18	482±7.2	495±8.0	2.7	0.236	1	0.32±0.03	0.39±0.04	21.9	0.113	1
	C×C3	17	22	499±8.0	486±7.3	−2.6	0.185	1	0.39±0.04	0.42±0.03	7.7	0.538	1
	C×C4	16	21	484±8.2	501±7.4	3.5	0.110	1	0.35±0.04	0.38±0.03	8.6	0.570	1
	C×C5	17	18	471±8.1	479±7.7	1.7	0.452	1	0.37±0.04	0.42±0.04	13.5	0.307	1

p values and Bonferroni (Bon) adjusted p values were estimated using Eq. 3

weighted latewood percentage were 2.6 and 6.5% (p=0.03 and 0.02) higher for heterozygotes than wild types, respectively (Tables 3b and 5b), whereas no significant differences were found between heterozygous and wild-type trees for A-S1, B-S1, or C-S2 (Table 5b).

Overall, there was a significant genotype by mate interaction for height at age 6 years (p=0.04) and for weighted wood density (p=0.04) and latewood percent (p=0.05) at age 15 (Table 4a,b). Within individual backgrounds (Table 5a,b), the genotype by mate interaction was significant only for height (p=0.04) at age 6 in background C-S2 and for weighted wood density (p=0.04) at age 15 in background A-S3. The least square means for growth and wood density traits for all full-sib families in each genetic background are shown in Table 6. There are large differences between *cad* genotypes for volume and density among the full-sib families in the four genetic backgrounds. For 20 full-sib families, the volume and wood density were higher for heterozygous than wild-type trees (positive effects) in 12 and 13 full-sib families, respectively. The differences of these positive effects ranged from 2.7 to 48.3% and from 0.4 to 8.9% for volume and density, respectively, whereas for the negative effect, differences ranged from −0.4 to −10.7% and −0.4 to −3.0%, respectively. For one particular full-sib family (B×B2), wood density was 3.0% lower (p=0.08) for heterozygous compared to wild-type trees, whereas wood density was 8.9% (p<0.001) and 3.8% (p=0.08) higher for heterozygotes compared to wild-type trees for families A×A6 and A×A8, respectively. Heterozygotes also showed a significantly 48% higher volume growth for A×A6 (p=0.005) and marginally significant 28% volume growth for A×A8 (p=0.06) compared to their wild-type siblings. We did not find significantly lower volume growth for heterozygous trees compared to wild-type trees in any of the 20 full-sib families.

To test the chance of making a Type I error, Bonferroni correction was used. The alpha level of each full-sub family was adjusted downward to ensure that overall experiment-wise risk for a number of tests remains at 0.05. The correction indicated only wood density in full-sib A×A6 was significant (p=0.002), while in this same family volume (p=0.11) was nearly significant.

Figure 1 showed the average ring density traits of full-sib A×A6 plotted against cambium age. Ring density, latewood density, and latewood percentage increased from pith to bark as expected. The *cad* heterozygotes of full-sib family A×A6 had consistently higher ring density over the years at both field trials in test S3. Earlywood density plotted against age showed little variation and appeared to decrease with cambium age. Heterozygous trees had significantly higher ring density than wild-type trees throughout most years especially in the second field-test. The higher ring density for heterozygous trees was mostly associated with higher latewood and latewood percentage compared to wild-type trees at both field trials.

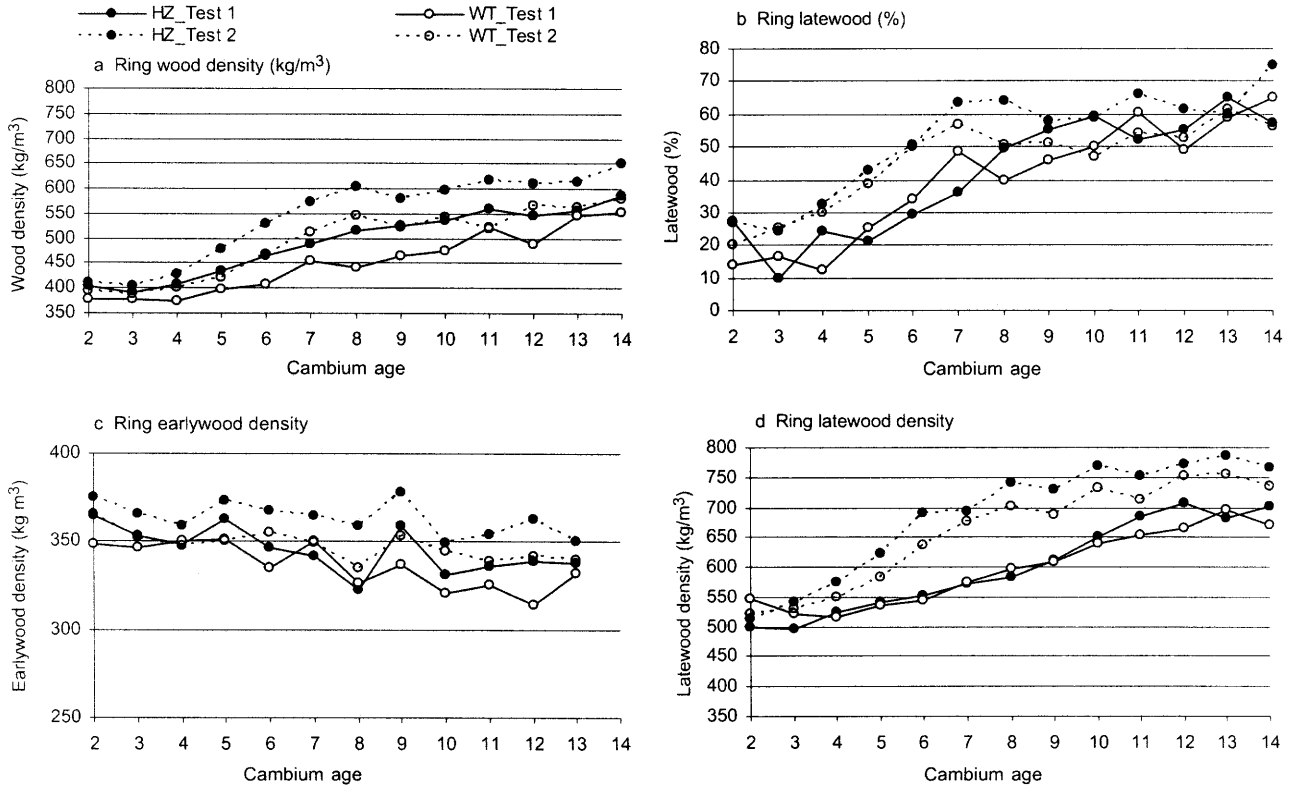

Fig. 1 Ring wood density (**a**), ring latewood percentage (**b**), ring earlywood density (**c**), and ring latewood density (**d**) for heterozygous (HZ) and wild-type (WT) trees of full-sib family A×A6 in half-diallel test S3

Discussion

Verification of the *cad-n1* effect is necessary to substantiate a biological basis for observed marker-trait associations, to provide precise estimates of the direction and magnitude of *cad-n1* effects and to predict *cad-n1* effects at various ages in various environments and genetic backgrounds. When all three half-diallel test series were considered in a combined analysis, significant differences in wood density (1%) and height at ages 6 (3%) and 15 (2.4%) years were found between *cad-n1* heterozygote and wild-type trees (Tables 3a,b and 4a,b). Although it was only marginally statistically significant, it appears that *cad-n1* was associated with the increase in volume of about 5% (p=0.09) (Tables 3a and 4a). We speculate that perhaps trees harboring the *cad-n1* allele may invest fewer resources into the production of monolignols, allowing reallocation of resource toward growth and wood density. Promotion of growth was also observed in transgenic poplar where the lignin biosynthetic enzyme 4-coumarate, coenzyme A ligase, was down-regulated [5]. Kirst et al. [6] found that the expression of the lignin-related genes was negatively correlated with diameter growth of a Eucalyptus's hybrid. Remington and O'Malley [13] found apparent overdominance at the *cad* locus for growth for both year 2 and 3, although overdominance was not statistically significant. Yu et al. [18] found that *cad-n1* heterozygous trees had greater wood density than wild-type trees but did not find an association of *cad-n1* with growth. Wu et al. [17] found that *cad-n1* heterozygous trees grow faster than their wild-type siblings in a single half-sib family. Possible explanations for this discrepancy include different genetic material used (heterozygous selections and second parents), environmental differences between field trials, and differing statistical power to detect small effects.

The segregation ratio for *cad-n1* mutant and wild-type alleles was consistent with the expected ratio of 1:1 [17, 18]. This suggests that *cad-n1* is not strongly deleterious for survival and adaptability through age 15 in our research planting. Remington and O'Malley [13] found that homozygotes for *cad-n1* showed no effect on survival of germination seedling after 3 years; however Dimmel et al. [2] did find homozygotes to significantly grow slower and less straight than wild-type trees at age 12 years. To date, the *cad-n1* allele has not been identified in any tree outside of the pedigree of tree 7-56.

Because 7-56 is one of the most valuable loblolly pine selections with extremely high breeding value for growth, it is reasonable to speculate that the *cad-n1* mutation may have a major effect on its breeding value. Table 7 shows breeding values for height at age 6 years (H6BV) of 30 second-generation offsprings of 7-56 trees selected in first-generation progeny tests [10]. These selections were made in crosses that included 7-56 as a parent and each cross had high breeding value for growth and quality traits. The selection criteria for the individual within each cross included the individual trees' phenotypes for height, diameter, resistance to fusiform rust [caused by *Cronartium quercuum* (Berk) Miyabe ex Shirai f. sp. *fusiforme*], and stem straightness [7]. Of the 30 offspring selections, only seven carried the *cad-n1* allele. Our expectation was that at least half the selections would have *cad-n1* because faster growth is associated with *cad-n1*. Based on the evaluation of progeny of these 30 selections (grandchildren of 7-56), the H6BV of the seven *cad-n1* heterozygotes ranged from −5.6 to 19.9 vs a range of 1.2 to 19.6 for the 23 wild-type trees. These results indicated that the breeding value for height at age 6 of the two *cad* genotypes is not significantly different.

It appears that the *cad-n1* allele does not have an overwhelming influence on the high breeding value of the growth of 7-56; other gene loci are likely involved. Quantitative traits such as growth and wood density in loblolly pine are assumed to be under polygenic control with relatively small effects coupled with environmental and epistatic interactions. Considering the complex nature of tree height and stem diameter, it is not surprising that *cad-n1* is not the only major allele associated with the high breeding value of 7-56.

In this study, we have extended studies on the effect of *cad-n1* allele over many growing seasons in multiple genetic backgrounds and field environments. We found large differences in the effect of the *cad-n1* allele among genetic backgrounds on growth and wood density, al-

Table 7 Breeding values for height at age 6 years (H6BV) of 30 second-generation selections that are progenies of 7-56

Heterozygote *cad-n1*		Wild type	
Selection	H6BV	Selection	H6BV
A	12.0	W1	16.7
B	−5.6	W2	15.6
C	0.4	W3	17.8
D	19.6	W4	19.6
E	19.9	W5	16.9
F	16.0	W6	7.4
G	17.5	W7	16.9
		W8	17.8
		W9	8.5
		W10	14.7
		W11	12.1
		W12	1.2
		W13	10.8
		W14	12.5
		W15	9.9
		W16	7.7
		W17	12.7
		W18	12.3
		W19	12.8
		W20	15.3
		W21	7.2
		W22	2.7
		W23	6.1
Average	11.4 ± 3.8		12.0 ±1.0

The H6BV figures are % height advantage compared with an unimproved checklot

though these comparisons were confounded by environmental differences at the test sites. The differences between the two *cad* genotypes on growth and wood density traits were not the same in each genetic background growing in a particular environment. However, we did not find *cad* genotype × genetic background interactions for any of the studied traits. Selection A in half-diallel S3 produced heterozygous trees that averaged 10.8% greater volume than wild-type trees, whereas in half-diallel S1, selection A heterozygous trees were only 4.3% greater than their wild-type siblings (Table 3a). This may be due to either different genetic backgrounds between half-diallel S1 (second parents A1–A5) and half-diallel S3 (A6–A10) or different growing environments (Georgia and South Carolina, respectively). The effect of *cad-n1* allele may be larger at better sites (S3) than poorer sites (S1).

Overall, there was a significant genotype by mate interaction for height at age 6 years and for weighted wood density and latewood percent at age 15 (Table 4a,b). The 10.8% volume (p=0.06) and 2.6% (p=0.03) wood density increases in genetic background A-S3 for *cad-n1* heterozygotes (Tables 3a,b and 5b) may be due to specific wild-type alleles at the same *Cad* locus or other loci that interact with the *cad-n1* allele. In particular, two second parents (A6 and A8) may have contributed certain genetic components that specifically interacted with *cad-n1* heterozygote (selection A) to produce the large positive effect on growth and wood density (Table 6). Further studies are needed to determine the genetic factors in these second parents that contribute to this interaction. In addition, there is evidence for the presence of pleiotrophy such that growth and density are associated with *cad-n1* allele but involve interactions with genes contributed from the second parents. Bradshaw and Stettler [1] reported that QTLs controlling basal stem area growth and sylleptic branch habit of poplar trees are probably controlled by the same genes.

Forest trees experience a variety of environmental conditions over their life spans. Long-lived trees also experience different developmental stages of growth (e.g., the change from juvenile to mature wood), which are most likely controlled by different sets of regulatory factors. Several QTL studies in forestry have examined the stability of QTLs over multiple growing seasons. Sewell et al. [15] indicated that the difference in the number of QTL between the single year and multiyear analyses suggest that possible genotype × environment interactions influence the temporal expression of different QTLs from year to year. A few of these studies repeatedly detected a subset of QTLs over time (e.g., [11]). In the present study, the effect of *cad-n1* allele on growth traits was consistent at ages 6 and 15 in the four genetic backgrounds (Tables 3a and 5a). There was no *cad* genotype × age interactions on growth and wood density (data not shown). These results are similar to our previous study examining *cad-n1* effects in a 10-year-old open-pollinated family experimentally growing under a two-level of fertilization [18]. The *cad-n1* effects on growth and wood density that are detectable over multiple growing seasons may be most valuable in large-scale breeding programs.

For full-sib family A×A6, we found the *cad-n1* allele associated with a significant increase in wood density through 15 years (Fig. 1). This increase can be attributed to higher earlywood density, latewood density, and a greater proportion of latewood in *cad-n1* heterozygotes. This result is similar to our previous study [18]. In the same half-sib family (selection A, open-pollinated), we found that differences in earlywood density and latewood percentage were consistent throughout tree development in both control and fertilized treatments, which resulted in high total wood density for heterozygous trees compared to wild-type trees up to age 10 years. The higher wood density was apparently due to the higher percentage of latewood in the heterozygotes.

Conclusion

The mechanism of high breeding value for the original founder of 7-56 is unknown but our data suggest it is not exclusively associated with the *cad-n1* allele. Both wood density and height growth traits were significantly higher for *cad-n1* heterozygotes than for their wild-type sibs. Although it was not statistically significant, the same trend was apparent for stem volume growth. Within genetic backgrounds, significant differences were limited to specific full-sib families, suggesting that alleles contributed by the second parents differentially affect the phenotype of *cad-n1* heterozygous trees. These alleles could be at the *cad* locus (i.e., variation in wild-type alleles) or elsewhere in the genome. The data also provide evidence of a pleiotrophic effect on growth and density because *cad-n1* has an effect on both of these traits. While substantial genetic gains in growth and wood density are possible through selection and deployment of trees carrying the *cad-n1* allele, these gains are family-specific and must be verified for each new cross through field-tests. In addition, further studies are needed to determine and evaluate the genetic factors that interact to cause variable phenotypic effects of *cad-n1* between full-sib families. Finally, it is clear that large, well-designed studies will be required to clearly define the main effects of the *cad-n1* allele and their interactions with other alleles and ultimately to determine the mechanisms of high breeding value in loblolly pine, especially for trees such as 7-56 and selection A.

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