VARIATION IN WOOD COLOR AND PRIMER DESIGN OF 4CL COLOR-CODING GENE ON NYAMPLUNG (Calophyllum inophyllum L.)

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Submission date: 14-Mar-2021 08:03AM (UTC-0700)
Submission ID: 1532555353
File name: SYAHIDAH2121-222020PCBMB7491_Wood_Color.pdf (225.12K)
Word count: 3121
Character count: 16037

Plant Cell Biotechnology and Molecular Biology 21(21&22):80-87; 2020 ISSN: 0972-2025

VARIATION IN WOOD COLOR AND PRIMER DESIGN OF 4CL COLOR-CODING GENE ON NYAMPLUNG (*Calophyllum inophyllum* L.)

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Received: 28 May 2020
Accepted: 03 August 2020
Published: 19 August 2020

Original Research Article

ABSTRACT

Indonesia is a habitat of many wood species having various patterns and colors. Nyamplung is a tree which has several variations in its heartwood color due to its extractive contents. This research aimed to determine variation in heartwood color of Nyamplung and design specific primer of the 4CL gene, which can amplify the DNA of Nyamplung. The samples used in the study had different heartwood color level and were collected from Bojo village, Barru and Jennae village, Soppeng in South Sulawesi, Indonesia. The wood color assay was determined based on the CIEL*a*b Color System, and molecular data were analyzed by electrophoregrams. The result showed that both samples had different level of wood color that could be observed with the naked eye. Meanwhile, primer design at first stage generated 12 primer combinations which were able to amplify Nyamplung DNAs, whereas, the second one generated five primer combinations. Therefore, The findings suggest performing an advanced examination on developing dominant and/or co-dominant markers that can be used as molecular markers for wood color on heartwood of Nyamplung.

Keywords: Wood color; primer design; gene-coding; nyamplung; *Calophyllum inophyllum* L.

INTRODUCTION

Indonesia is a habitat of many tree species including timber-producing trees. The produced timbers are not only strong and durable but also have various patterns, colors, and fragrances. The bright color of the timber can be obtained from Teak and *Gonystylus bancanus* Kurz and timbers having dark color are from Ebony and Dalbergia latifolia Roxb [1]. Moreover, those having unique fragrances are from Agarwood and Sandalwood.

Wood color is also a criteria requirement in selection for daily-use trees along with its durability and strength. Heartwood color is generally darker than sapwood, but it has more variations in color than sapwood [1]. Variation in heartwood color can also occur in a species, for instance, Nyamplungs' heartwood color.

Nyamplung (*Calophyllum inophyllum* L.) has various heartwoods' colors from brown-pink, brown-red, and brown-orange up to purple-red. Its sapwood color is pale brown to pinkish yellow-brown. The difference between its heartwood and sapwood can be easily identified. Wood texture is moderate rough to rough. The fiber is not either twisted or combined. The wood surface is generally smooth and glossy, meanwhile, radical side is quite rough [2,3].

The extractive compound in heartwood is a controlling factor on wood color variation. This compound can be yellow, red, brown, and some of them are colorless/transparent. One of the genes that controls the synthesis of this compound is 4CL gene. This gene produces an enzyme, which becomes precursor in the biosynthesis of lignin, suberin, as well as flavonoid [4,5].

4CL sequence variation causes color changes in specific plant organ. In Panicum virgatum, it changes stem color to brown. This gene also controls color changes in leaf margin and sclerenchyma in mutant sorghum. Deletion in 4CL1 gene changes wood color from orange to brown on Populus [6,7,8].

Variation in 4CL gene sequence controlling phenotype changes can be detected using molecular markers. Here, we conducted an initial stage for this approach by designing 4CL specific primer that can amplify Nyamplung DNA. This study aimed to determine the color level of Nyamplungs' heartwoods and generate 4CL specific primer for amplifying Nyamplung DNA. The amplification products will be applied for developing dominant and co-dominant markers as heartwood color detector of Nyamplung.

PLANT MATERIALS AND METHOD

Color Level Assay

The color level assay was carried out at the Laboratory of Food Science and Technology Department, Faculty of Agricultural Engineering, Bogor Agricultural University, Bogor, Indonesia. The selected trees used as samples were trees possessing different level of wood color (Fig. 1). Determination of wood color was based on parameters of wood color using CIEL*a*b Color System [9].

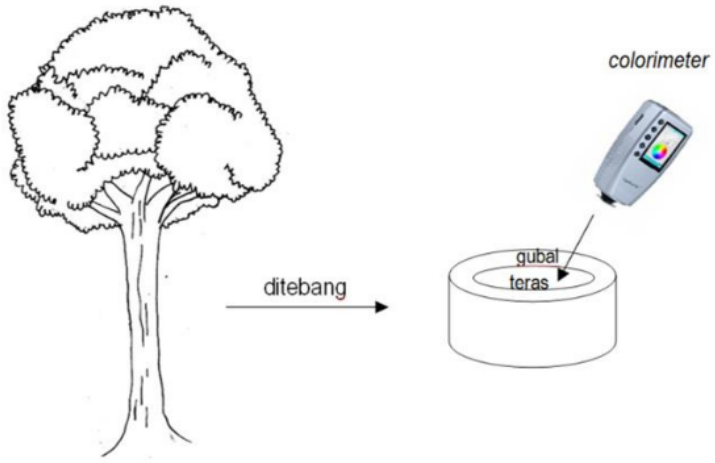

Fig. 1. Color level assay

Sample Collection and DNA Extraction

Sample employed in this present study was fresh, non-infected, and intermediate-leaf-age, which collected as many as five leaves for each tree. Each sample was collected at different altitudes; sample A was from lowland area (7 m above sea level/asl), and sample B from highland (105 m asl). DNA extraction was performed using DNeasy ® Tissue Kit (Qiagen) with modification [10]. The DNAs were then stored at -200°C freezer until processing.

Specific Primer Design of 4CL Gene

Designing of primer was done twice (first stage and second stage). The first one used gene sequences from six plants originated from several families and species, and the second one was conducted using five plants from different species but still in the same family, Malpighiales. Specific primers of 4Cl gene were designed based on coding-gene sequences that derived from the National Center for Biotechnology Information (NCBI). The sequences were complete gene sequence (gene, complete cds).

DNA Amplification Using Specific Primer of 4CL Genes and Sequencing

Nyamplung DNA were amplified using the designed primers with PCR Thermocycler (sensoquest). Amplification procedure was performed according to the following steps: A cycle of initial denaturation at 950°C for 5 mins, 35 cycles of denaturation at 94°C for 1 min, primer annealing at specific temperature of each primer for 1 min and extension for 72°C at 1 min, and a cycle of final extension at 72°C for 10 mins. The amplified products were then stored at the -200°C freezer until separation process using horizontal electrophoresis. The primers producing DNA amplified products were then used in DNA sequencing step.

Data Analysis

Table 1 presents the determination of wood color that was conducted based on wood color parameters using CIEL*a*b Color System (L*, a*, dan b*) [9].

Table 1. Wood color parameters based on CIEL*a*b* *Color System*

Parameters of wood color		
L*	**a***	**b***
Shows the brightness level of color	(+) Red (-) Green	(+) Yellow (-) Blue

The results were then calculated using CIELAB (L1,a1,b1) and (L2, a2, b2) with the following formula (ISO:12647-1, 2013):

$$\Delta E = \sqrt{\left(L_2^* - L_1^*\right)^2 + \left(a_2^* - a_1^*\right)^2 + \left(b_2^* - b_1^*\right)^2}\,.$$

Table 2. The effect of wood color value (ΔE) on human eyes

Wood color value (ΔE)	The effect on human eyes
<0.2	Invisible
0.2-1.0	Very low
1.0-3.0	Low
3.0-6.0	Moderate
>6.0	High

Meanwhile, molecular data used for DNA sequencing were in the form of DNA amplification bands using the designed primers. The analysis was done by observing the presence of bands on electrophoregram agarose.

RESULTS AND DISCUSSION

Level of Wood Color on Heartwood

Table 3 presents color parameters on two samples (A and B) which had positive (+) values. L* and a* values of A were higher than sample B, whereas b* of B was higher than A. A which was from lowland area had redder heartwood color than B which tended to have yellower heartwood (table 1). Variation on wood color is presumed due to different altitude where the samples were grown. In addition to number and type of extractive compound, provenance is also a factor that influences wood color. Plant genetic and type, extractive compound, and silviculture practice are factors that affect wood color [11,9].

The calculation of ΔE on both samples was 4.1, and it was categorized as moderate (Table 2).

Table 3. Parameters of wood color on heartwood of Nyamplung based on CIEL*a*b* system

No.	Parameter of wood color	Sample A	Sample B
1	L*	57,13	56,79
2	a*	+13,24	+11,13
3	b*	+22,27	+25,78

4CL Gene-specific Primer Design

Primer design of 4CL gene successfully generated ten forward and 12 reverse primers. The first designs obtained four forward and seven reverse primers, and the total combination was 28 primer pairs. That of the second design generated six forward and five reverse primers (30 combination primer pairs). Primer sequences from the first and second designs are described in Tables 4 and 5.

Amplification of 4CL Specific Primer on Nyamplung DNA

Sixteen combination primers from the first design and five primer pairs from the second design could amplify Nyamplung DNA. Size of PCR products amplified using those primers ranged from 250 bp to 450 bp (the first design) and 700 bp to 800 bp (the second design). Primer size of first and second design primers were 23 and 18 bases, respectively. Even though the first design had more bases than the second ones, those primes were still optimal for primer size. Sasmito et al. [12], an ideal primer size is ranged from 18 to 30 bases depending on the combination of genome nucleotides. On White Birch (Betula platyphylla), Hao et al., [13] had designed 18 to 24-SSR primers for analyzing the genetic diversity of this species.

Table 4. The first primer design

No	Primer Name	Sequence	Tm (°C)
		Forward	
1	M4CL1F	TGT GTG TCT TGC CTA TGT TCC AT	56.6
2	M4CL2F	TGT GTG TCT TGC CTA TGT TTC AT	54.7
3	M4CL3F	TGT GTG TGT TGC CTA TGT TCC AT	57
4	M4CL4F	TGT GTG TGT TGC CTA TGT TTC AT	55.2
		Reverse	
1	M4CL1R	GCA ACC TGA AAT CCT TTG TAC TT	53.7
2	M4CL2R	GCA ACC TGA AAC CCT TTG TAC TT	55.8
3	M4CL3R	GCA ACC TGA AAC CCT TTA TAC TT	53.3
4	M4CL4R	GCA ACC TGG AAT CCT TTG TAC TT	55.5
5	M4CL5R	GCA ACC TGG AAT CCT TTA TAC TT	52.9
6	M4CL6R	GCA ACC TGG AAC CCT TTG TAC TT	57.6
7	M4CL7R	GCA ACC TGG AAC CCT TTA TAC TT	55.1

Table 5. The second primer design

No	Primer name	Sequence	Tm
		Forward	
1	F1M4CL	CTT TGT GTG TTG CCG ATG	52.1
2	F2M4CL	CTG TGT GTC TTG CCG ATG	53.8
3	F3M4CL	TTT TGT GTC CTG CCG ATG	53.1
4	F4M4CL	TTA TGT GTG CTG CCG ATG	52.9
5	F5M4CL	TTG TGT GTC CTG CCG ATG	55.4
6	F6M4CL	ACC ACA GGG TTG CCA AAG	55.8
		Reverse	
1	R1M4CL	AGC AAC CTG GAA CCC TTT	54.3
2	R2M4CL	TGC AAC CTG GAA CCC TTT	54.6
3	R3M4CL	AGC AGG AGC AAC CTG AAA	54.2
4	R4M4CL	AGC TGG AGC AAC CTG AAA	54.2
5	R5M4CL	AGC AGG TGC AAC CTG GAA	57.2

Table 6. Primer pairs of the first design that could amplify Nyamplung DNA

No	Primer Name	Combination of forward and reverse primers	Annealing temperature (°C)	Amplified band
1	Primer 1	M4CL1F + M4CL1R	-	Multiple and unclear bands/could not be used
2	primer 2	M4CL2F + M4CL1R	-	Multiple and unclear bands/could not be used
3	primer 3	M4CL2F + M4CL3R	55	single and clear band
4	primer 4	M4CL4F + M4CL3R	53	single and clear band
5	primer 5	M4CL4F + M4CL5R	54	single and clear band
6	Primer 6	M4CL1F + M4CL3R	-	Multiple and unclear bands/could not be used
7	Primer 10	M4CL1F + M4CL4R	-	Multiple and unclear bands/could not be used
8	primer 11	M4CL3F+ M4CL7R	56	single and clear band
9	primer 18	M4CL2F + M4CL2R	56,4	single and clear band
10	primer 19	M4CL4F + M4CL4R	57,3	single and clear band
11	primer 20	M4CL3F + M4CL1R	50.9	single and clear band
12	primer 21	M4CL1F + M4CL2R	54	single and clear band
13	primer 22	M4CL3F + M4CL2R	55	single and clear band
14	primer 23	M4CL4F + M4CL6R	53	single and clear band
15	primer 24	M4CL3F + M4CL6R	55.1	single and clear band
16	primer 25	M4CL2F + M4CL5R	52.5	single and clear band

Twelve of 16 primers which could amplify the DNAs were used in the sequencing process. Primers produced clear and bright bands (Table 6). Moreover, four primers were also able to amplify the DNAs, but they generated non-specific and smear bands, and consequently might misinterpret the sequencing results (Figs. 2 and 3).

We performed twice primer designs in this study. The first one was carried out using 4CL gene sequences from six individuals belong to different families. It was done because the 12 selected primers could not be able to produce any DNA band/PCR product during DNA preparation for sequencing. The failure of the process was assumed due to degraded DNA and primer. The DNA and primer degradation can be caused by unstable electricity for quite a long time in the laboratory, and in the consequent, some of the laboratory tools broke down, such as freezer where the DNAs stored. Every 2°C increase in temperature will result in an increased DNA degradation, and 1 to 40 bases will be degraded at 25°C [14]. Liu et al., [15] reported DNA will be completely degraded if it is stored at room temperature for nine days.

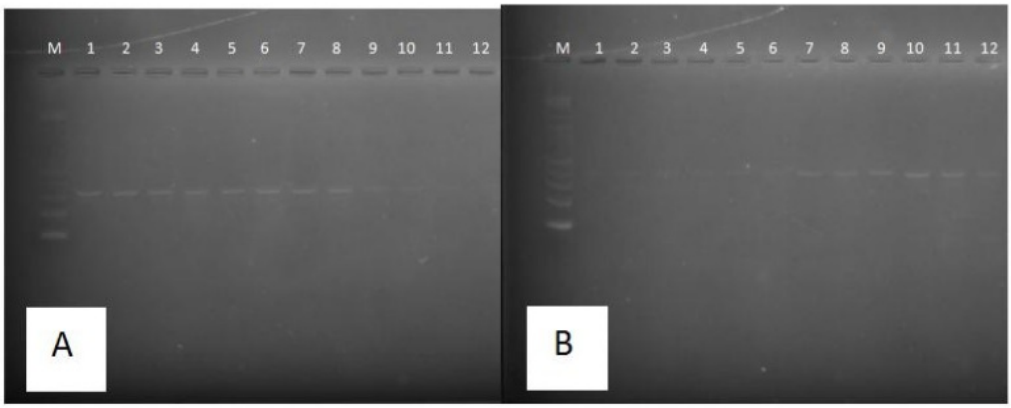

Fig. 2. Electrophoregrams of DNA amplification using Primer 19 and 25. Notes: A = Primer 19, and B = Primer 25

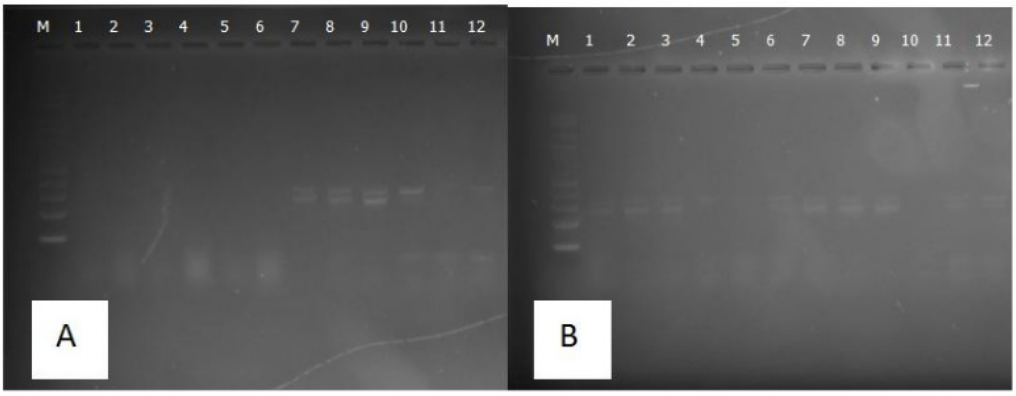

Fig. 3. Electrophoregram of DNA amplification using primer 1 and 10. Notes: A = Primer 1, and B = Primer 10

Table 7. The second primer design that could amplify Nyamplung DNA

No	Primer Name	Combination of forward and reverse primer	Annealing temperature (°C)	Band
1	Primer 16	M4CL4F + M4CL1R	51.7	single and clear band
2	primer 18	M4CL4F + M4CL3R	51.7	single and clear band
3	primer 19	M4CL4F + M4CL4R	49.5	single and clear band
4	primer 23	M4CL5F + M4CL3R	51.4	single and clear band
5	primer 30	M4CL6F + M4CL5R	49.8	single and clear band

Five primers from the second design could amplify the DNAs (Table 7). The produced bands were clear, but thinner than the first ones. The primer number was also less than the first design due to low concentration of the DNAs [16].

Low DNA concentration induces non-optimal DNA amplification and produces thin DNA band. Moreover, it generally causes failure in the amplification process (no PCR product) [17]. We failed to extract high concentration of DNAs because of plant materials used in this study were repeatedly thawed. Henry [18] stated the thawing process on plant material induces DNA degradation in the plant tissues. Moreover, it was by dysfunction of the freezer as DNA storage due to power failure.

CONCLUSION

It is concluded that color level assay on both evaluated heartwoods based on CIEL*a*b* system exhibited different color levels that could be visually detected with the naked eye. The first design successfully generated 12 primer combinations amplifying Nyamplung DNA. The second primer design was able to produce five combinations for amplifying the DNAs.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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