Original Paper

Relationship Between Seed Coat Color and Cytokinin Concentration in Efficiently Regenerating Leaf Lettuce Shoots: In Vitro Experimental Study

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Abstract

Background: Lettuce (*Lactuca sativa* L.) is an economically important leafy vegetable that is cultivated worldwide. Advances in plant biotechnology have enabled the development of transgenic and transplastomic lettuce lines with specific agronomic traits that produce pharmaceutical proteins and biological compounds. Plant regeneration efficiency is a critical and highly cultivar-dependent step in plant genetic transformation. No morphological markers have been identified that predict the regeneration ability or cytokinin requirement of lettuce cultivars, hindering the establishment of efficient regeneration systems.

Objective: This study aimed to optimize the direct shoot regeneration efficiency of leaf lettuce cultivars and identify a morphological trait that predicts the optimal cytokinin concentration for each cultivar.

Methods: The direct shoot regeneration of two cultivars (Chima-sanchi and Chirimen-chisya) was tested on media containing various concentrations of the cytokinin 6-benzylaminopurine (BAP). Four additional cultivars with different seed coat colors were analyzed to determine the relationship between seed coat color and the optimal BAP concentration. Statistical significance was evaluated using the Student *t* test, with significance set at $P<.01$.

Results: The highest regeneration efficiencies in Chima-sanchi (80.5%, SE 3.0%; 103 of 128 explants) and Chirimen-chisya (50%, SE 4.4%; 64 of 128 explants) were obtained with 0.05 and 0.5 mg/L BAP, respectively. Therefore, the optimal BAP concentration differed significantly between the cultivars ($P<.01$). The seed coat color and the optimal BAP concentration required for efficient direct shoot regeneration were strongly correlated among the six cultivars.

Conclusions: Seed coat color is a useful morphological marker for predicting the optimal BAP concentration required for efficient direct shoot regeneration in leaf lettuce cultivars. These findings contribute to optimizing lettuce shoot regeneration systems for specific cultivars.

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Keywords: leaf lettuce; shoot regeneration efficiency; 6-benzylaminopurine; seed coat color; CIELAB color scale; flavonoid; BAP

Introduction

Lettuce (*Lactuca sativa* L.) is a major vegetable crop cultivated worldwide that belongs to the *Asteraceae* family. The total world production of lettuce and chicory has increased 1.3-fold in the 20 years since 2005 according to the Food and Agriculture Organization of the United Nations [1]. Asia produced 18.1 million tons of lettuce in 2023, which was 64.4% of global production; Japan produced 0.6 million tons of lettuce, ranking seventh highest in the world [1]. Lettuce is a dietary source of vitamins and minerals [2]. Thus, lettuce cultivars with increased yield and resistance to biotic and abiotic stresses have been developed using conventional breeding methods [3].

Transgenic and transplastomic lettuce lines with specific agronomic traits that accumulate pharmaceutical proteins and biocompounds have been developed using transformation procedures mediated by particle bombardment and *Agrobacterium* [3]. The major lettuce varieties worldwide include leaf, crisphead, butterhead, and romaine lettuce [3]. Leaf lettuce varieties have wrinkled leaves with frilly edges and no head; their fresh shoots are heavier than those of butterhead varieties under light-emitting diode lighting [4]. Therefore, leaf lettuce varieties are more suitable for indoor growth. Moreover, leaf lettuce contains more β-carotene, a precursor to vitamin A, and more lutein per dry weight than either crisphead or butterhead lettuce [5].

Plant tissue cultures have been widely used in plant breeding and industrial applications, such as for propagating virus-free plants, producing valuable compounds, and producing somaclonal variations [6]. The shoot regeneration efficiency of most plant species is highly dependent on the explant sources, the basal salt mixtures, sugars, and plant growth regulators [7]. Combining the cytokinin 6-benzylaminopurine (BAP) and the auxin 1-naphthaleneacetic acid (NAA) effectively regenerates lettuce shoots, but the optimal combination differs among cultivars [8-10]. Optimizing plant tissue culture parameters is labor-intensive and time-consuming because culturing plant tissues is a slow process. The molecular mechanisms regulating shoot regeneration in lettuce have been examined [11]. The effects of auxins and cytokinins on lettuce regeneration have been studied: the response is mostly cultivar-specific [8-10], and no reliable morphological marker has been identified to predict regeneration ability. For example, Bull and Michelmore [11] molecularly characterized the genetic and regulatory mechanisms underlying regenerative competence in lettuce, but how visible traits relate to hormonal responsiveness was not examined. Certain visible traits may reflect the regenerative capacity, as demonstrated in *Cymbidium* [12]; however, these morphological cues have not been explored in lettuce. We found that seed coat color strongly correlates with the cytokinin requirements for efficient shoot regeneration in leaf lettuce cultivars. Seed coat color is a simple, nondestructive morphological marker that can be used to accelerate the optimization of regeneration systems for genetic transformation. Therefore, this study aimed to evaluate whether seed coat color can reliably predict the cytokinin concentration required for efficient direct shoot regeneration across multiple leaf lettuce cultivars.

Methods

Plant Materials and Growth Conditions

Six leaf lettuce cultivars were used in this study: Chima-sanchi and Chirimen-chisya (purchased from Tohoku Seed), Red fire and Green wave (purchased from Takii Seed), and Fringe green and Shiki-beni (purchased from Sakata Seed). Chima-sanchi, Red fire, and Shiki-beni are white seed cultivars; Chirimen-chisya, Green wave, and Fringe green are brown seed cultivars . The seeds were stored in a constant humidity chamber (SD-302-01, Toyo Living) at 25 °C and a relative humidity of 0%-1% until sowing. Seeds were surface-sterilized via immersion in 70% ethanol for 1 minute. The seeds were treated with 20% commercial bleach (Kao) containing 6% sodium hypochlorite, resulting in a final NaOCl concentration of 1.2%, for 15 minutes. The seeds were then rinsed 3 times with sterile distilled water. The sterilized seeds were placed on a germination medium containing half-strength Murashige and Skoog medium (2.3 g/L, Wako Pure Chemical Industries) [13] supplemented with 10 g/L sucrose and 2.5 g/L Phytagel (Sigma-Aldrich) in Petri dishes with a diameter of 9 cm. The pH of the medium was adjusted to 5.8 with 1N KOH and 1N HCl. The medium was then autoclaved at 120 °C and 0.1 MPa for 20 minutes. Seeds were germinated in an environmentally controlled growth chamber (LPH-411S, NK systems) fitted with fluorescent light (FLR40SW/M/36, NEC) at a photosynthetic photon flux density of 300 µmol photons/m^2/s under continuous white light conditions at 20 °C. All experiments were conducted at Takasaki University of Health and Welfare, Takasaki City, Gunma Prefecture, Japan (36.33°N, 139.00°E) between September 2021 and September 2022, in a humid subtropical climate (Köppen climate classification: Cfa).

Media Composition for Shoot Regeneration

Shoot regeneration efficiency was examined using a medium supplemented with different basal salt mixtures, sugars, and concentrations of BAP and NAA (Nacalai Tesque) following a previously described method [14] (Table 1). NAA was dissolved in a 10 mM NaOH solution and BAP was dissolved in a 10 mM HCl solution before either was added to the culture media. All media contained 0.5 g/L polyvinylpyrrolidone (Nacalai Tesque) and 2.5 g/L Phytagel (Sigma-Aldrich) at pH 5.8. The medium was sterilized via autoclaving at 121 °C for 20 minutes. Cotyledons from 7-day-old seedlings were used as explants and placed on the medium in Petri dishes with a diameter of 9 cm. Each treatment included 16 explants that were cultured per dish, with 8 dishes per treatment, for a total of 128 explants. The explants were maintained for 4 weeks under continuous white light conditions (photosynthetic photon flux density=300 µmol photons/m^2/s) at 25 °C and transferred to fresh medium every 2 weeks.

Table 1. Media composition and growth regulators for shoot regeneration of lettuce.

Medium	Basal salt mix	Sugar	BAP[a] (mg/L)	NAA[b] (mg/L)
M1	1 × MS[c]	3% sucrose	0.5	0.1
M2	1 × MS	3% sucrose	0.05	0.01
M3	1 × MS	3% sucrose	0.05	0.1
M4	1 × MS	3% sucrose	0.05	1
M5	1 × MS	3% sucrose	0.5	0.01
M6	1 × MS	3% sucrose	0.5	1
M7	1 × MS	3% sucrose	5	0.01
M8	1 × MS	3% sucrose	5	0.1
M9	1 × MS	3% sucrose	5	1
M10	1/2 × MS	3% sucrose	0.5	0.1
M11	1 × B5	3% sucrose	0.5	0.1
M12	1 × MS	6% sucrose	0.5	0.1
M13	1 × MS	1.5% glucose	0.5	0.1

[a]BAP: 6-benzylaminopurine.
[b]NAA: 1-naphthaleneacetic acid.
[c]MS: Murashige and Skoog.

Seed Coat Color Measurement

The color parameters of the seeds from each cultivar were measured with an SD 7000 spectrophotometer (Nippon Denshoku Industries) using the CIELAB L^*, a^*, and b^* color scale. The L^* axis represents the degree of brightness ranging from black (L^*=0) to white (L^*=100). The a^* and b^* axes represent redness (positive number) to greenness (negative number) and yellowness (positive number) to blueness (negative number), respectively [15].

Total Flavonoid Content Analysis

The total flavonoid content was analyzed according to a previously described method, with some modifications [16]. A 50 μg aliquot of seeds was homogenized in 0.5 mL of 80% methanol with 5.0 mm stainless beads (Biomedical Science) at 1100 rotations per minute for 45 seconds using a Shake Master (Biomedical Science). The homogenized solutions were incubated for 15 minutes at 70 °C, then centrifuged at 10,000 × g for 10 minutes at 4 °C. The resulting supernatants were incubated at 60 °C, and the dried pellets were dissolved in 20 μL of 80% methanol. The extracts were spotted on a 5 × 5 cm TLC Silica gel 60 F_{254} plate (Merck). For staining, the blots were sprayed with a methanolic solution containing 1% diphenylboric acid 2-aminoethylester (DPBA, Tokyo Chemical Industry), then sprayed with a methanolic solution containing 5% PEG 4000 (Nacalai Tesque). The fluorescence was visualized using an iBright CL1000 imaging system (Thermo Fisher Scientific).

Statistical Analyses

All statistical analyses were performed using EZR software [17], a free graphical interface for R that is widely used for standard biostatistical analyses. Significance was determined using a Student t test for two-group comparisons or a one-way ANOVA followed by a Tukey test for multiple group comparisons. The statistical significance was set at P<.01 for Student t tests and P<.05 for one-way ANOVA. All values are expressed as means and SE.

Results

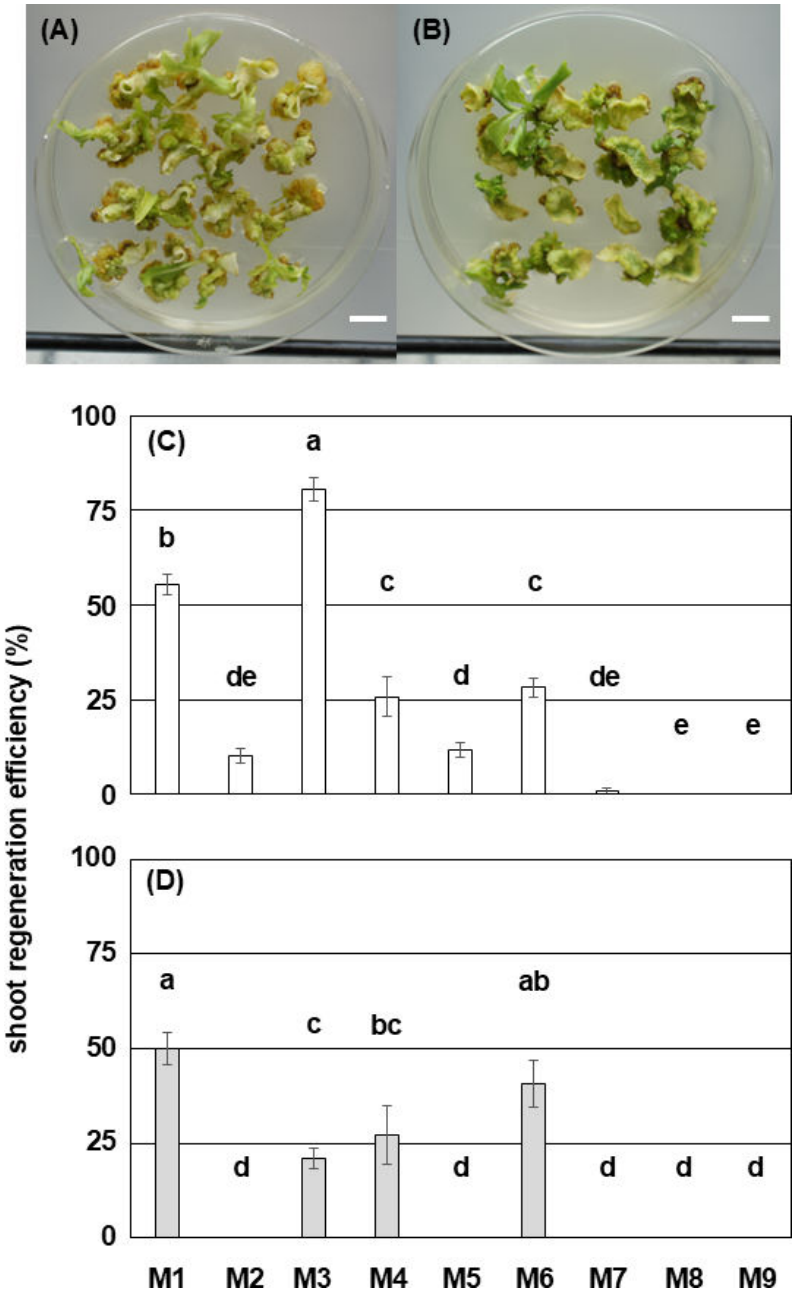
In this study, we found that seed coat color is strongly associated with the cytokinin requirement for efficient direct shoot regeneration across 6 leaf lettuce cultivars. White seed cultivars exhibited their highest regeneration efficiency at low BAP concentrations (0.05 mg/L), whereas brown seed cultivars required higher BAP levels (0.5 mg/L) to achieve comparable regeneration. Seed brightness (L*) and yellowness (b*) positively correlated with the M3/M1 ratio, supporting seed coat color as a predictive morphological marker.

Effects of Medium Composition on Shoot Regeneration

BAP and NAA are commonly used as plant growth regulators for regenerating lettuce shoots [8,10,14,18]. We examined the effects of different concentrations of BAP and NAA on the efficiency of shoot regeneration in Chima-sanchi and Chirimen-chisya cultivars (Figure 1). We used different concentrations of BAP with 0.1 mg/L NAA (M1, M3, and M8). The shoot regeneration efficiency was highest in Chima-sanchi (Figure 1C) and Chirimen-chisya (Figure 1D), with 0.05 mg/L BAP (M3) and 0.5 mg/L BAP (M1), respectively. We then tested different concentrations of NAA with 0.5 mg/L BAP (M1, M5, and M6). The efficiency was highest in Chima-sanchi using 0.1 mg/L NAA (M1) (Figure 1C). The Chirimen-chisya shoot regeneration efficiency did not differ between the 0.1 mg/L (M1) and 1 mg/L (M6) NAA treatments (Figure 1D). The shoots of both cultivars weakly regenerated when treated with 5 mg/L BAP (M7-9; Figure 1). The BAP concentration strongly and cultivar-dependently influenced the shoot regeneration efficiency, whereas the

basal salt mixture and sugar composition did not (Multimedia Appendices 1 and 2).

Figure 1. Shoot regeneration from cotyledon segments of (A) Chima-sanchi and (B) Chirimen-chisya on medium M1 after 4 weeks of culture. Bar=1 cm. Effects of different concentrations of BAP and NAA on shoot regeneration from cotyledon segments of (C) Chima-sanchi and (D) Chirimen-chisya after 4 weeks of culture (n = 16 explants per dish × 8 dishes). All media were supplemented with 1 × MS, 30 g/L sucrose, and 0.5 g/L polyvinylpyrrolidone. Different letters indicate statistically significant differences (one-way ANOVA followed by Tukey test, $P<.05$). BAP: 6-benzylaminopurine; MS: Murashige and Skoog; NAA: 1-naphthaleneacetic acid.



Seed Coat Color Phenotype and Relationship With Cytokinin Requirement

The seed coat color is a key phenotypic trait in many crops such as lettuce [19-21]. The Chima-sanchi seeds were lighter, redder, and yellower than those of Chirimen-chisya (Figure 2). The ratio of shoot regeneration efficiency under 0.05 mg/L BAP to that under 0.5 mg/L BAP (M3/M1) positively correlated with seed brightness (L*) and yellowness (b*) in all 6 cultivars (r=0.834 and 0.722, respectively; Figure 3B and D; Multimedia Appendix 3). The brown-seeded cultivars contained more flavonoids than the white-seeded cultivars (Multimedia Appendix 4). The regeneration efficiency of the white-seeded types was higher than that of the brown-seeded types, indicating that flavonoid accumulation negatively modulates cytokinin responsiveness.

Figure 2. (A) Seed samples of Chima-sanchi (left) and Chirimen-chisya (right). Average values of CIE (B) L*, (C) a*, (D) b* color coordinates of seed coat color in the cultivars (n=5). Horizontal bars within the box indicate the median value of the data, and the outer vertical bars represent the maximum and minimum values of the data. **P<.01 (Student t test).

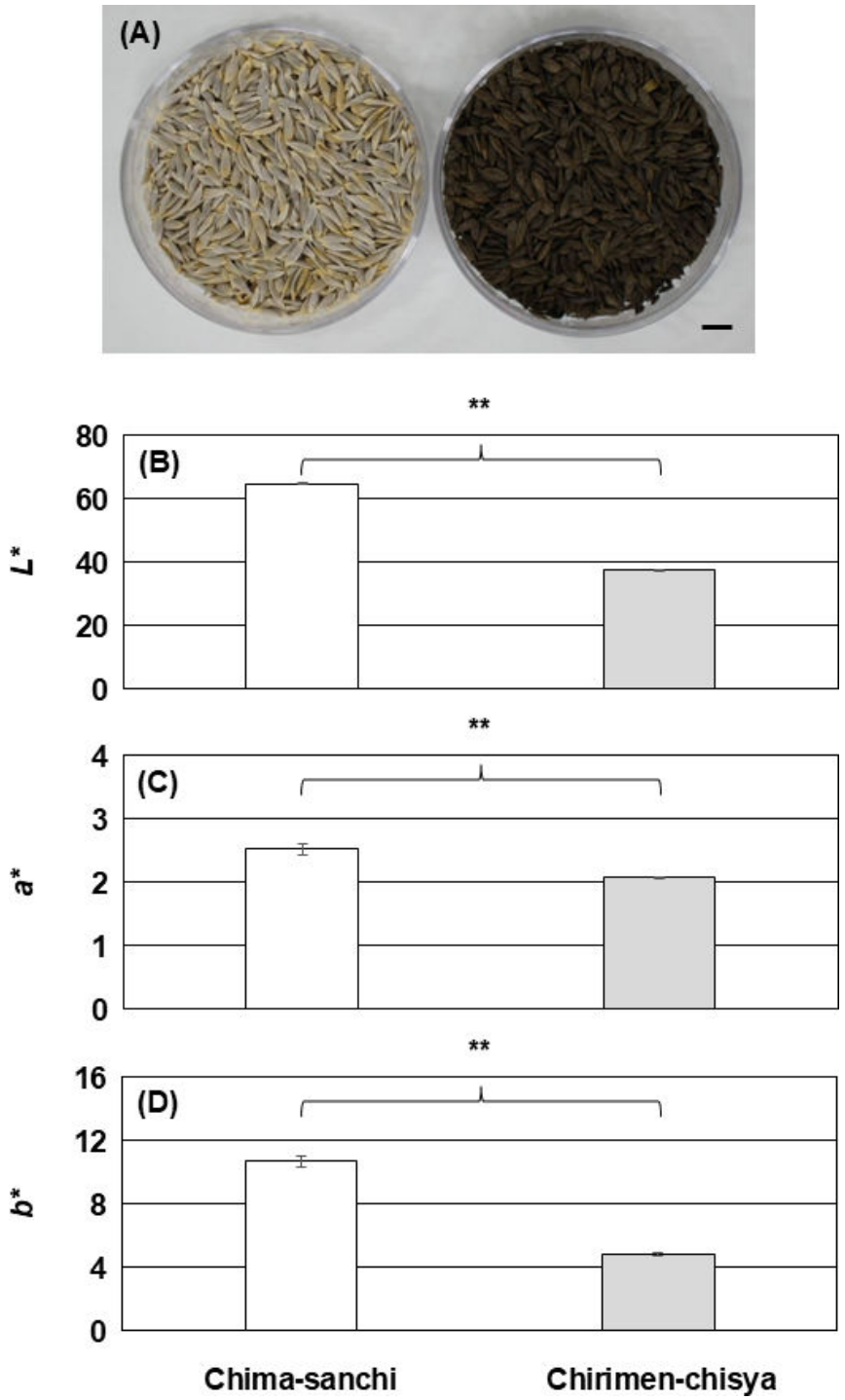
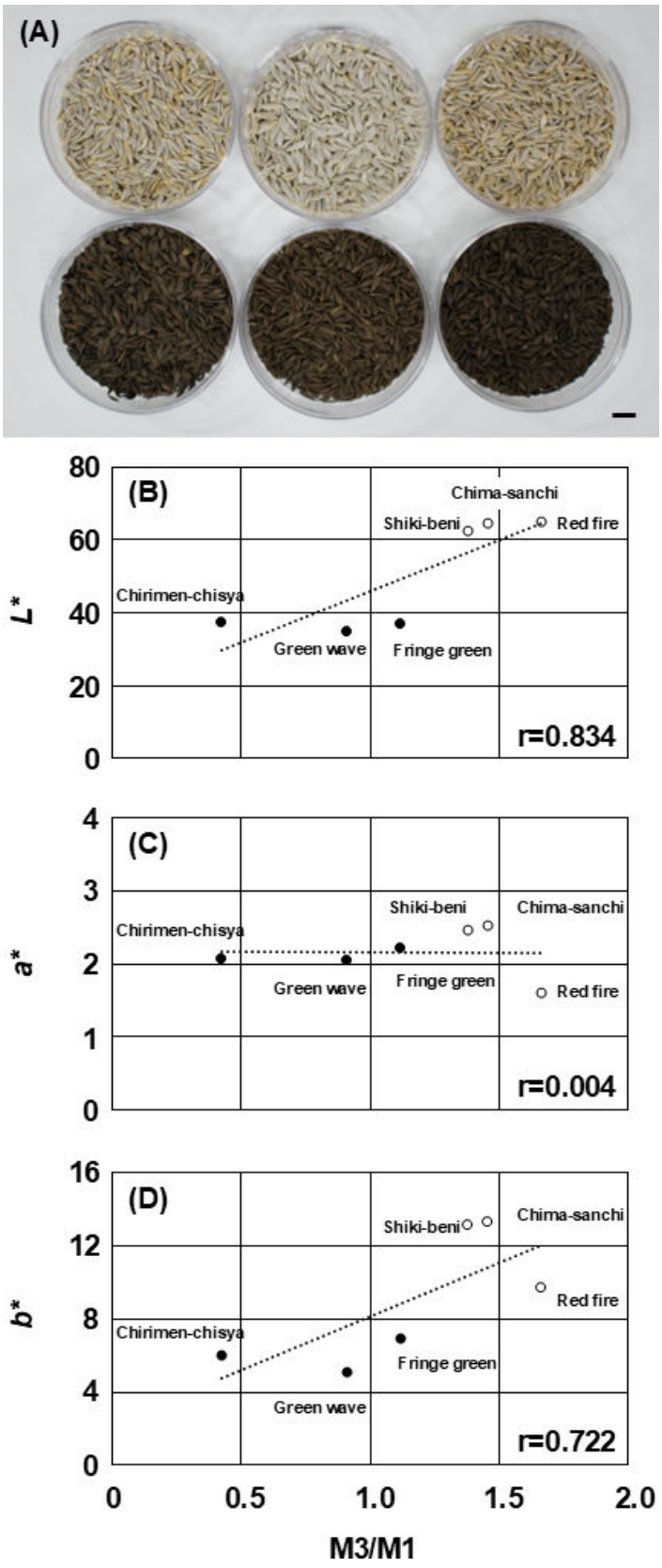


Figure 3. (A) Samples of 6 lettuce cultivars. Genotype names are as follows, clockwise from the upper left: Chima-sanchi, Red fire, Shiki-beni, Green wave, Fringe green, and Chirimen-chisya. Bar=1.0 cm. Correlations between values of CIE (B) L*, (C) a*, (D) b* color coordinates of seed coat color (n=5) and ratio of shoot regeneration efficiency using M3 to the efficiency using M1 (M3/M1) in the 6 cultivars (n = 16 explants per dish × 8 dishes) are shown.



Relationship Between Flavonoid Biosynthesis and Shoot Regeneration Capacity

Flavonoids are secondary metabolites that play multiple roles in auxin transport, oxidative stress tolerance, and cell division [22]. In *Arabidopsis*, *transparent testa* (*tt*) mutants are defective in enzymes or regulatory factors involved in the flavonoid biosynthetic pathway. These mutants have a pale seed coat and show altered responses to phytohormones [23]. The *Arabidopsis tt4* mutant is deficient in chalcone synthase, a key enzyme in the flavonoid biosynthetic pathway. The *Arabidopsis tt4* mutant has a pale yellow seed coat and markedly reduced shoot regeneration efficiency compared with the wild type [24]. This finding suggests that certain downstream flavonoid metabolites produced after chalcone synthase catalysis are required for efficiently regenerating shoots in *Arabidopsis*. The brown seed cultivars contained higher levels of flavonoids than the white seed cultivars in this study (Multimedia Appendix 4); however, the shoot regeneration efficiencies of the brown seed cultivars were considerably lower (Figure 3). These patterns imply that although flavonoid metabolism may influence regenerative competence, the mechanisms underlying this metabolism differ between lettuce and *Arabidopsis*. However, the strong correlation we observed between seed coat color and the optimal BAP concentration provides a practical morphological marker for predicting the cytokinin requirements of leaf lettuce.

Discussion

Principal Findings

Seed coat color is a simple morphological marker for predicting the most appropriate cytokinin concentration required for efficiently regenerating shoots regardless of lettuce cultivar. This approach can be quickly and easily used for optimizing regeneration conditions, thereby increasing the efficiency of transgenic and genome-edited lettuce production. However, this study was limited to 6 leaf lettuce cultivars, which were tested under controlled laboratory conditions. Further validation with additional genotypes and under different environmental conditions is necessary. This marker-based method is more cost- and labor-efficient for laboratories; however, scaling up tissue culture systems for industrial use remains challenging because maintaining aseptic conditions, controlling the environment, and performing manual subculture steps substantially increases labor and energy costs. Further technical improvements and validation studies are required before genotype-specific protocols can be applied to large-scale propagation or transformation systems. However, integrating visible traits, such as seed coat color, into regeneration and transformation workflows could help balance the overall costs and benefits of cultivar-specific plant biotechnologies and support the broader use of lettuce as a bioproduction platform.

Conclusion

Seed coat color is strongly correlated with the cytokinin required for efficiently regenerating shoots in leaf lettuce. The maximum shoot regeneration occurred with 0.05 and 0.5 mg/L BAP for white and brown seed cultivars, respectively. Pigmentation-related metabolites, possibly flavonoids, may modulate cytokinin responsiveness during the regeneration process. This simple visual marker can accelerate the optimization of regeneration systems, reduce experimental costs, and facilitate cultivar-specific transformation. Our findings directly support the development of lettuce cultivars with specific traits, such as enhanced yield, stress tolerance, and nutritional value, by enabling the rapid establishment of efficient regeneration and transformation protocols. Additionally, understanding the interplay between flavonoid metabolism and cytokinin signaling may provide new options for engineering regenerative competence in other horticultural crops. Our findings not only clarify the physiological basis of genotype-dependent regeneration but also provide a practical framework that connects the fundamental knowledge of plant regeneration mechanisms to their application in lettuce breeding and commercial production.

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Data Availability

All data are available from the corresponding author upon reasonable request.

Authors' Contributions

MK and TY conceived, designed, and supervised the project. MK performed all experiments. TY provided some important suggestions. MK wrote the paper. TY critically reviewed and approved of the final version.

Conflicts of Interest

None declared.

Multimedia Appendix 1

Effects of different basal media on shoot regeneration from cotyledon segments of (A) Chima-sanchi and (B) Chirimen-chisya after 4 weeks of culture (n = 16 explants per dish × 8 dishes). All media were supplemented with 30 g/L sucrose, 0.5 mg/L 6-benzylaminopurine, 0.1 mg/L 1-naphthaleneacetic acid, and 500 mg/L polyvinylpyrrolidone. Different letters indicate statistically significant differences (one-way ANOVA followed by a Tukey test, $P<.05$).
[PNG File (Portable Network Graphics File), 21 KB-Multimedia Appendix 1]

Multimedia Appendix 2

Effects of different sugar concentrations and types on shoot regeneration from cotyledon segments of (A) Chima-sanchi and (B) Chirimen-chisya after 4 weeks of culture (n = 16 explants per dish × 8 dishes). All media were supplemented with 1 × Murashige and Skoog, 0.5 mg/L 6-benzylaminopurine, 0.1 mg/L 1-naphthaleneacetic acid, and 500 mg/L polyvinylpyrrolidone. Different letters indicate statistically significant differences (one-way ANOVA followed by a Tukey test, $P<.05$).
[PNG File (Portable Network Graphics File), 21 KB-Multimedia Appendix 2]

Multimedia Appendix 3

Shoot regeneration efficiency using M1 and M3 and CIELAB values of seed coat color in 6 lettuce cultivars. 1: w=white seed cultivar, b=brown seed cultivar. 2: L* corresponding to the brightness, a* to the red/green coordinates, and b* to the yellow/blue coordinates.
[PNG File (Portable Network Graphics File), 33 KB-Multimedia Appendix 3]

Multimedia Appendix 4

The flavonoid content of methanolic extracts of seeds in 6 lettuce cultivars. Bar=0.5 cm.
[PNG File (Portable Network Graphics File), 119 KB-Multimedia Appendix 4]

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Abbreviations

BAP: 6-benzylaminopurine
NAA: 1-naphthaleneacetic acid
***tt*:** *transparent testa*

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