DOCTOR THESIS

SELECTION OF NON- FADING LINES FOR HIGH QUALITY CUT-FLOWER PRODUCTION IN *DAHLIA VARIABILIS 'NESSHO'* BY ELUCIDATING MECHANISMS OF LOW TEMPERATURE INDUCED FLOWER COLOR FADING

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MUTHAMIA Edna Kirumba

GRADUATE SCHOOL OF ENVIRONMENTAL AND LIFE SCIENCE
OKAYAMA UNIVERSITY

Declaration

I hereby declare that the research presented in this Ph.D. thesis entitled **'Selection of non- fading lines for high quality cut-flower production in *Dahlia variabilis 'Nessho'* by elucidating mechanisms of low temperature induced flower color fading'** is my own work and was carried out for the degree of Doctor of Philosophy in Agriculture under the guidance and supervision of Assoc. Prof Kitamura Yoshikuni (posthumous) and Prof Goto Tanjuro in the control of flowering laboratory, Department of Agriculture, Division of Agricultural and Life Science, Graduate School of Environmental and Life Science, Okayama University, Japan. All cited literature in the form of books, articles and websites are placed at the respective place in the text. This thesis has not been submitted to any other University for an award of any degree or conferment of a diploma or distinction.

Okayama, September 2024 **Muthamia Edna Kirumba**

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To God be all glory, Amen.

Dedication

This work is dedicated to my husband and best friend Moses Njire, my children Ryan Njire, Gabriella Woki and Ethan Mutugi, my parents, brothers and sisters and dear friends turned into family. Their earnest prayers of faith offered for me, words of encouragement, unwavering support enabled me pull through tough and challenging seasons. I made it through this academic pursuit because special people believed in me.

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List of abbreviations

3MT 3 Malonyl transferases

ANS anthocyanidin synthase

bHLH Basic helix loop helix

CHI chalcone-flavanone isomerase

CHS chalcone synthase (CHS)

CIE Commission Internationale de l´Eclairage.

DFR dihydroflavonol 4-reductase

DNA Deoxyribonucleic acid

F3H flavanone-3-hydroxylase

FNS Flavone synthase

FLS Flavonol synthase

HPLC High performance liquid chromatography

miRNA Micro RNA

NGS Next generation sequencing

PTGS post-transcriptional gene silencing

qPCR quantitative polymerase chain reaction

RNA ribonucleic acid

UFGT UDP- glycose: flavonoid 3-O-glycosyl transferase

Abstract of the thesis

Dahlia variabilis 'Nessho' is a cultivar that is popular because of its red color. However, during autumn and winter when the average minimum temperature falls below 10 ℃, the cultivar exhibits flower color fading in some plants. This phenomenon leads to reduction in quality and value of flowers produced during cold seasons in Japan. The extent of flower color fading varies in individual plants with some plants being highly sensitive while others are relatively low sensitivity to low temperature induced color fading. Relatively low temperature-sensitive plants are used as propagation materials for production of non-faded flowers. This selection method for propagation material takes a long time because selection of mother plants must be done in cold seasons when susceptibility color fading is apparent. Additionally, even if non-fading lines are selected for propagation, some vegetatively propagated plant from these lines still exhibit flower color fading at low temperatures. Therefore, seed company need to confirm the non-fading trait in the selected lines by observing flowering at low temperatures. A shorter selection method for non-fading lines is desirable for growers to produce high quality cut flowers throughout the year. Pigment accumulation in plants is a series of enzymatic-controlled reactions and is regulated by expression of genes that encode for the enzymes. In this study, the relationship between color fading and flavonoids gene expression profiles was evaluated.

In the first experiments, Nessho plants were classified according to their color fading index (CFI) when the minimum temperature was below 10 ℃. Forty-two plants received from Miyoshi company were assessed during flowering and classified into their respective CFIs in winter of 2021/2022. Flower color fading occurred from mid-November 2021 to end of February 2022 and the plants were classified into CFI 0 - CFI

4. Plants that exhibited low sensitivity (RL plants) to color fading were assigned CFI 0 and CFI 1, those that were highly sensitive to color fading (RH plants) were assigned CFI 3 and CFI 4 andCFI 2 plants were classified as intermediate plants. The color fading occurrence was random during the cold season with more plants exhibiting higher color fading indices (CFI3 and CFI 4) in January and February 2022 when the average minimum temperature was below 2 ℃. The lightness (L*) and chromatic components a* and b* of the Commission Internationale de l'Eclairage (CIE) L*a*b* of ray florets were measured by a color meter (TES-3250 color meter). The CIE L* a* b* color values were measured for the flowers exhibiting different flower fading indices. The L* and b* values increased with increase in fading and the a* values were lowest in non-faded flowers (CFI 0 and CFI 1).

In the second experiment, the relative expression profiles of flavonoids genes in 'Nessho' plants were evaluated. This was done to determine the potential gene responsible for color fading occurrence in Nessho cultivar. Red ray florets and faded orange ray florets were used as experimental materials. In dahlia, flower color intensity is regulated by the expression of structural genes in the anthocyanin synthesis pathway. In this study the structural genes analysed were *DvCHS1, DvCHS2, DvCHI, DvFNS, DvF3H, DvF3'H, DvDFR*, and *DvANS.* There was a significant difference (P 0.05) in relative expression of *DvFNS* between the red and the faded ray florets with an elevated expression in the faded florets. It therefore emerged that *DvFNS* maybe the gene responsible for color fading in 'Nessho' cultivar. The *DvFNS* expression in the in leaves was investigated to determine the possibility of temporal expression in other plant parts. It was observed that *DvFNS* expression was elevated in young unexpanded leaves and declined in mature expanded leaves. This confirmed the earlier observed temporal *DvFNS*

expression in ′Nessho′ and provided a possibility of using expression levels in leaves as a selection criterion for selection non-fading plants.

To investigate the relationship between *DvFNS* expression in young unexpanded leaves and color fading indices, sixty-nine ′Nessho′ plants were evaluated under controlled environmental conditions: acclimation condition 25/20 ℃ and 14 hours daylength; and inductive conditions 20/9 ℃ and 10 hours daylength. *DvFNS* expression after acclimation was compared to *DvFNS* expression after inductive conditions that mimic cold season conditions. The plants were later allowed to grow to flowering stage and the CFIs compared to relative *DvFNS* expression. Inductive conditions resulted in elevation of relative *D*V*FNS* expression in individual plants. The CFIs and *DvFNS* expression patterns in individual plants did not correlate. *DvFNS* expression in young unexpanded leaves of relatively highly sensitive plants to low temperature were compared with that of relatively low sensitive plants. There was significant difference ($P<0.005$) between *DvFNS* in RH and RL plants.

In the third experiment, the correlation between the suppression of *DvFNS* gene expression and the red color pigmentation in ′Nessho′ flowers was investigated. This was done to explain the high relative *DvFNS* expression levels in faded ray florets and the low expression levels in red ray florets. Unfaded red ray florets were used for extraction of small RNAs (microRNAs), which were sequenced using Next generation sequencing Novogene Japan-AlphaBio-2. The *DvFNS* coding region contained one intron of 1,150 bp (sense). Distinct antisense small RNAs were mapped to position 2,065–2,793 of the *DvFNS* genome. Genomic sequences of *DvFNS* in ‘Kokucho’ original were used as reference sequences. and the sequence was completely identical between ‘Kokucho’

original and the red ‘Nessho. These observations indicated that *DvFNS* was silenced post-transcriptionally by siRNAs in the red-colored flowers.

In the fourth experiment, the relationship between flower color and pigment accumulation in the ray florets and the leaves was investigated. This was done to determine the link between flower color and the amounts and types of pigments accumulated in plant tissues. High-performance liquid chromatography (HPLC) analysis was conducted on red and orange ray flowers and leaves of corresponding plants at four maturity stages (the youngest leaf being top on the apex and the oldest leaf at the bottom of the shoot). The detection wavelength was 350 nm and 530 nm for flavones and anthocyanidins respectively. There was a significant difference ($P<0.005$) between flavones accumulation in the leaves of RH and RL plants. The pigment content declined as the leaves matures, with the youngest leaves containing the highest amount.

In conclusion, selection of non-fading dahlia Nessho plants for high quality flower production is possible using *DvFNS* gene expression patterns in young unexpanded leaves. The use of the flavone content in young leaves is also a possible selection criterion that does not require visible flower color two approaches have the potential to reduce the selection time and improve flower productivity among producers.

CHAPTER I

General Introduction

1.1 Dahlia

Dahlias (*Dahlia variabilis*) are popular flowering bulb ornamental plants in the family Asteraceae with 35 species and more than twenty thousand known distinct cultivars all over the world (Gatt, 1998). They are indigenous to Mexico and Central America but are popularly produced in many parts of the world. Dahlia flowers are appreciated worldwide for their long-lasting majestic blooms which range from miniatures (<2.5 cm) to giant (>40 cm). They are easy to grow both in pots and on the ground The are extensively used for exhibition, garden display and home decoration as well as cut flowers. The cut flowers range from pompon forms, giant and miniature types. The vase-life of dahlia is short in most cultivars; four to five days at room temperature with several breeding activities focusing on flower longevity. Improved vase-life in hybrid cultivars make them moderately good garlands. The use of dahlia varies with the plant phenotype with some forms being suitable for use as bedding plants, pot plants and for cut flowers depending on the characteristics of the plant. Dahlia is a high value crop in many countries in the world but because they have long been grown for home gardens rather than commercial use, there are few reports aimed at improving useful traits (Onozaki & Azuma, 2019).

Dahlias are known for their diversity in flower shapes, sizes, and color due to the complexity of their genetic background; the polyploidy nature (Gatt, 1998) and possession of a large genomic variation. This contributes to varying general morphology from cultivar to cultivar. The presence of genetic variability is important for breeders to evolve varieties exhibiting novel characteristics. They are amongst the most diverse floral

form and colors of all popular garden flowers. Induced and spontaneous mutation in dahlia have continued to result in more diversity in flower colour, form, size and great improvement of dahlia for a variety of adoptive traits for overcoming biotic and abiotic stresses, improved vase life, fragrance.

Dahlia is commercially produced in many countries worldwide such as Mexico, Japan, France, South Africa, Italy, Germany and the United States. In the Netherlands bulb production area is significantly large covering approximately 400 ha (Priyanka et al., 2017). Dahlia germplasm has been collected and maintained by societies such as American Dahlia Society (ADS), USA; National Dahlia Society (NDS), UK; Dahlia Society of Australia and National Dahlia Society of New Zealand for breeding purpose (Behr and Debener, 2004).

Recent research in dahlia extends from optimizing production condition to improve quality and productivity (Abd-Elkader et al., 2020; Hamayl et al., 2016), breeding to improve the quality aspects such as appearance, flower color, type, size, and stem length and post-harvest life (Onozaki & Azuma, 2019) and evaluation of petal color and flavonoid biosynthesis pathway (Thill et al., 2012; Ohno et al., 2013, 2016, 2018; Lehnert & Walbot, 2014; Deguchi et al., 2015, 2016; Srivastava & Trivedi, 2022)

1.2 Flower color

Flower color is the most important trait in ornamental plants that plays a sensory role and determines the quality and value of flowers (Tanaka et al., 2008). Other than just a trait, color is the perceptual experience characterized by the interpretation of different wavelengths of light seen by eyes and processed by the brain of an individual (Garcia et al., 2020). The coloration on the flowers is determined by the types and amounts of pigments accumulated and this influenced by the internal or surface tissue structure of the petals ((Zhao et al., 2015). Flower color development is determined and regulated by both environmental and genetic factors. During the development of flower color, petals exposed to light absorbs it partially as it penetrates the pigment layer. The remaining light is reflected by the sponge tissues and passes back through the pigment layer. The reflected light is what is visible to the human eye as flower color (Zhao et al., 2015) The pigmentation components of showy flowers have been studied for over 150 years and carotenoids and flavonoids are the two primary types of substances that contribute to the color flowers (Maoka, 2020; Mekapogu et al., 2020).

Flavonoids are one of the major groups of specialized metabolites and is also the largest class of polyphenols with an estimate of more than 8,000 compounds in the plant kingdom (Wen et al., 2020)They determine flower colors and are greatly influenced by the cellular pH. Flavonoids are concentrated in the vacuoles of the flowers' epidermal cells; therefore, the pigments' absorption spectra are balanced by the pH of the vacuole (Stavenga et al., 2021). The role of flavonoids in flower pigmentation is through their diverse mechanisms and the key determinants of flower colors are anthocyanins, chalcones, aurones, and flavonols pigments (Wang et al., 2021). Variable spectral

absorption of anthocyanins results in a wide range of flower pigments which can confer red, blue, or purple colors to plant tissues. Flavonols exclusively virtually absorb in the UV and cause colors such as white and pale yellow (Stavenga et al., 2021). Flavones and flavonols are colorless flavonoids and less noticeable as the are masked by other pigments. They therefore act as co-pigments and confer rather pale colors in flowers such as yellow and orange (Wang et al., 2023).

Other than influencing the commercial value of ornamental plants, floral hue also plays a key role in attracting pollinators for reproduction, protection against photo-oxidative damage, and providing resistance to biotic and abiotic stresses (Kumar et al., 2023; Samanta & Das, 2011).

In dahlia, the color of the ray floret ranges from ivory, yellow, pink, red, orange, purple, black and bi-colors (McClaren 2009, Ohno et al., 2011a). The color of the ray florets is exclusively based on the accumulated flavonoids, mainly anthocyanins and biochemically related antho-chlors such as chalcones, aurones and their derivatives. Unlike in many other plants where the yellow and orange colors results from carotenoids, in dahlia these colors results from chalcones or as a function of co-pigmentation. In the dahlia black cultivars cyanidin- based anthocyanin accumulation is the major contributing factor, and this results in the reduction of Lightness (L*) and chroma (C*) (Deguchi et al., 2016). Different forms of anthocyanin contribute differently the lowering of L* and C* and this may influence their modification of the anthocyanidins.

In many ornamental plants there is a wide range of natural flower colors, however in some plants color is limited. Mechanisms underlying the wide variation within populations differ from those that give rise to fixed differences between species. This

makes research in flower color an important aspect in improving the production of ornamental quality in plants. Understanding how genetics, developmental and environmental differences shape phenotypes is important in selection of trait of interest such as color.

1.3 Pigment biosynthesis

Flavonoids biosynthesis is on a multi- level branched pathway that produces both the coloured and colourless compounds, and it is common to most plants (Davies & Schwinn, 2010). The biosynthesis pathways have been extensively studied in a number of plant species, in model species, the main backbone of the pathways is shared (Fig. 1). The main substrates for the biosynthesis of flavonoids are one molecule of coumarate-CoA and three molecules of malonyl-CoAand the biosynthetic reaction are catalysed by series of enzymes; chalcone synthase (CHS), chalcone-flavanone isomerase (CHI), flavanone-3-hydroxylase (F3H), dihydroflavonol 4-reductase (DFR), anthocyanidin synthase (ANS), and UDP-glycose: flavonoid 3-O-glycosyl transferase (UFGT) to produce various pigments. In dahlia anthocyanidins are formed by the consecutive action of enzymes CHS, CHI, F3H, DFR and ANS. The intermediates of anthocyanidin biosynthesis are Flavanones and 6'-hydroxychalcones whereas the formation of flavones, flavonols and 6'-deoxychalcones interferes with anthocyanidin formation. The pathway has branching points where enzymes such as flavone synthase (FNS), flavonol synthase (FLS) and CHS, respectively are responsible for using intermediates from the pathway leading to anthocyanidins.

In dahlia cultivars all enzymes involved in the flavonoid's biosynthesis pathway are detectable from crude enzyme preparations of cell cultures and flower petals except for ANS. In all dahlia cultivars the consistent low activity of FLS indicating that co-

pigmentation in dahlia is rather based on flavones and not flavonols (Halbwirth et al., 2008). Substrate competition at the branch points results in various flower colors. For example, an increased flavonol and flavone production; both of which are colorless pigments, can lead to a pigmentation loss due to insufficient substrate for the anthocyanin biosynthesis. In dahlia ′Kokucho′, a black cultivar and its purple mutant and other black cultivars, the variation in the flavonoids accumulation is partially explained by substrate competition at the naringenin- apigenin/luteolin and kaempferol branch (Deguchi et al., 2013; Thill et al., 2012). This implies that various factors can simultaneously contribute to flavonoid accumulation and flower color in plants. For instant in a chrysanthemum mutant cultivar, diverse flavonoids accumulated alongside dark-purple flower color due to down-regulation of F-box genes. This indicates that multiple factors influence flavonoid content and flower color(Jo et al., 2020) . In roses, genetic modification of the biosynthetic pathway and co-pigments can synergistically contribute to delphinidin accumulation and the resulting to the blue flower color (Katsumoto et al., 2007).

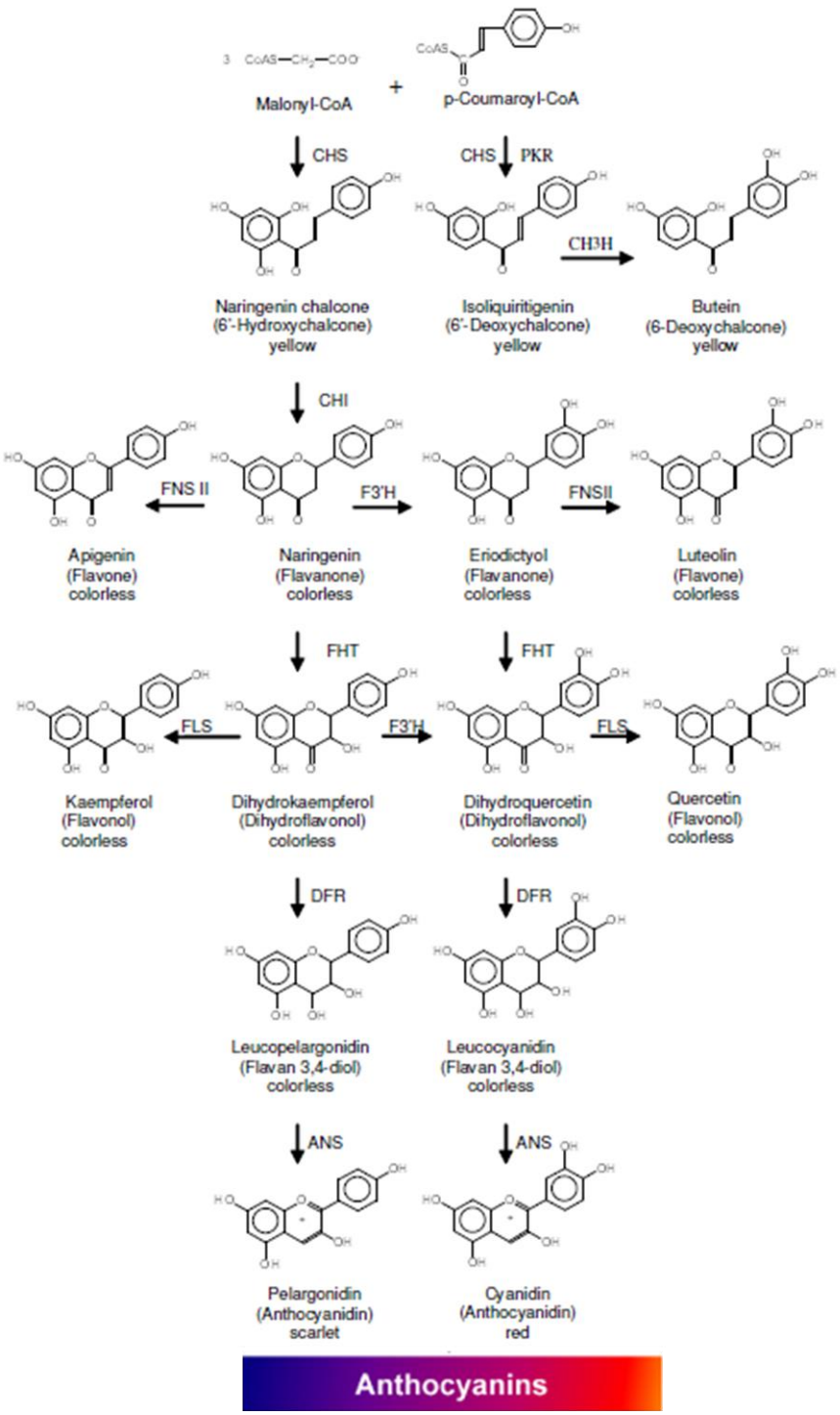

Figure 1.1: Flavonoid biosynthetic pathway in Dahlia variabilis

1.4 Role of core structural genes for flavonoid biosynthesis

The flavonoid biosynthesis pathway can be divided into three stages. The first stage is a common in the synthesis of many secondary metabolites. It involves the deamination of phenylalanine-by-phenylalanine ammonia-lyase into *trans*-cinnamic acid. The trans-cinnamic acid further produces *p*-coumaric acid by hydroxylation based on cinnamic acid 4-hydroxylase. Later, *p*-coumaric acid produces *p*-coumaroyl coumaric acid (CoA) by catalysis of the 4-coumaric acid: CoA ligase. The second phase starts with coumaroyl CoA and Malonyl co-A as substates to the synthesis of flavanonols. It is the key stage in the flavonoids metabolism with the first produce being naringenin. The main enzymes in stage of biosynthesis include chalcone synthase (CHS) and chalcone isomerase (CHI) encoded *CHS I, CHS II* and *CHI* genes respectively. Naringenin further produces dihydrokaempferol (DHK), which is an essential precursor substance for various anthocyanins *via* the catalysis of flavanone 3-hydroxylase (F3H). The third stage is the synthesis of flavones and synthesis and modification of different anthocyanins. Dihydroquercetin (DHQ) and dihydromyricetin (DHM) are formed upon hydroxylation in different loci of DHK by the flavonoid 3′-hydroxylase (F3′H) and flavanone 3′, 5′hydroxylase (F3′5′H). In addition, DHK, DHQ and DHM in the third stage are further reduced to various colorless anthocyanins by dihydroflavonol-4-reductase (DFR). The colorless anthocyanins are changed into colourful anthocyanins because of the catalysis by anthocyanidin synthase (ANS). Finally, more stable anthocyanins are formed through modifications, such as glycosylation of UDP-glucose: flavonoid-3-O-glucosyltransferase (UF3GT or 3GT), acylation of acyltransferase (AT) and methylation of methyltransferase (MT). These stable anthocyanins are transferred by glutathione S-transferase to the

vacuole where they accumulate as pigments (Zhao et al., 2020). As a type of major flavonoid pigment, anthocyanins control the pink-red, red, violet and blue colors of flowers.

In flowers pigment accumulation is a series of enzymatic reactions catalysed by enzymes in a stepwise manner. Each enzyme at every step on the pathway is encoded by a gene. Flower color genes can be divided broadly into structural genes and regulatory genes. The main structural genes that have been reported to cause pigmentation in flowers include CHS, F3H, F3′H, DFR, and ANS. These genes encoding chalcone synthase, chalcone isomerase, flavanone 3-hydroxylase; flavonoid 3'-hydroxylase, dihydroflavonol 4-reductase, and anthocyanidin synthase respectively have been cloned in various plants (Petroni and Tonelli 2011; Goswami et al. 2018; Zhou et al., 2024). In chrysanthemum, seven structural genes, such as CHS, F3H, F3′H, DFR, ANS, 3GT, and 3MT have been identified as key genes responsible for anthocyanin biosynthesis. In dIn herbaceous peony decline in the expression level of anthocyanin biosynthesis structural gene *PiDFR* cause flower color fading. Most of the structure genes such as CHS, CHI, F3H, F3'H, DFR, and ANS. In addition to the structural genes in the anthocyanin biosynthetic pathway, transcription factors also play important roles in flower color development through regulating the temporal and spatial expression of structural genes (Peng et al., 2021; Yin et al., 2021).

1.4 Flower Color Fading Occurrence

Flower color change is a common occurrence in many plant species (Weiss and Lamont 1997). The physiological mechanism of flower color change involves quantitative loss or gain of pigments such as anthocyanins, flavones, flavonols and carotenoids, the appearance of betalains and change in pH (Weiss 1992, Zhang et al., 2023). In many plant species the change in flower color progresses sequentially after the onset of the flowering process, however in others the color is maintained after flowering. Flower color change is of many forms; complete color loss, color fading, variegation, and bi-color occurrence. Color fading, a form of flower color change, is the reduction on the intensity. In the studies flower color, the regulation of anthocyanin biosynthesis has been widely elucidated, however little is understood about the regulation of flower color fading process. The color fading process may involve the rate of pigment biosynthesis, pigment transport, degradation, and their stability (Liu et al., 2018). Anthocyanin accumulation plays a major role in color intensity in many plant species and its stability is determined by factors such as its chemical structure, external environmental factors and the internal environment such as cell sap pH (Liu et al., 2022). In flowers, anthocyanin accumulation is generally constitutive and is determined by pollinator preference and the environment. In vegetative tissues flavonoids usually accumulate temporarily as an adoptive response to biotic and abiotic stressors (Hatier and Gould, 2009). Pigments such as flavones and flavonols act as co-pigments with anthocyanin and stabilize the colored structural forms and enhances the final color (Gomez-miguez et al., 2006).

Anthocyanins (derivatives of the 4′ -hydroxylated pelargonidin and the 3′ ,4′ -hydroxylated cyanidin) are responsible for the formation of red, magenta, and orange hues

in dahlia variabilis (Halbwirth et al. 2008 Nordstrom 1953, 1956a, 1956b; Walliser et al., 2021)

1.5 Problem statement and justification

With the recent exponential growth in the floriculture industry, quality flower production cannot be over emphasized. Flower color is one of the most important quality trait in ornamental that cannot be compromised on. Flower color is determined by many factors, one of which is the environmental conditions. Light and temperature play important roles in the synthesis and accumulation of pigments as well as the regulation of genes responsible for encoding enzymes involved in the various catalytic reactions. The effects of climate change are currently being experienced in the rapid changes of the environmental conditions and the fast adaptation of plant species to the changes. This influences the stability of phenotypic characteristics in plants such as color. Recent research in dahlia 'Nessho' has linked flower color fading to seasonal minimum temperature drop below 10°C in autumn and winter. The color fading occurrence has been linked to flavonoids accumulation as well as their gene expression patterns. During autumn and winter flower color fading correlates with relative *DvFNS* whereas during spring and summer the non-faded red flower production correlates with low *DvFNS* expression. It is therefore hypothesized that low *DVFNS* expression abolishes the competition for substrate in the anthocyanin biosynthetic pathway leading to more anthocyanin synthesis and accumulation. Therefore, during summer when *DvFNS* levels are low, there is more synthesis of anthocyanin which results in solid flower colors. In dahlia, there is a reported relationship between color expression in the ray florets and temporal flavonoids gene expression in the leaves. In this study we investigate the 'Nessho' plants response to low minimum temperature, gene expression patterns and the

relationship between minimum temperature, color fading occurrence, flavonoids gene expression and pigments accumulation. We also evaluated a short selection method for quality flower to optimize dahlia flowers production with stable color throughout the year without use of the visible flower color as a selection criterion.

1.6 Research objectives

The main aim of this study is to elucidate the mechanism for seasonal flower color change in dahlia 'Nessho' to produce high quality cut-flower that exhibit stable solid flower color throughout the year. The main objective is to effectively select non-fading plants for high quality cut flowers production throughout the year. To achieve this, we elucidated mechanisms underlying flower color change in dahlia 'Nessho', we evaluated the relationship between flower color fading and gene expression patterns and established optimum artificial climatic conditions that suppress *DvFNS* gene expression. In this study, we reported for the first time the relationship between climatic conditions and anthocyanin synthesis and the expression or suppression of *DvFNS* gene. Secondly, we successfully established a selection method for stable and unstable lines using *DvFNS* expression pattern in young unexpanded leaves. Finally, we illustrated the relationship between gene expression pattern and pigments accumulation in 'Nessho' plant materials (leaves and ray florets) of both solid red colored and color faded 'Nessho' in winter.

CHAPTER II

CLASSIFICATION OF DAHLIA NASSHO PLANTS ACCORDING TO THE COLOR FADING OCCURRENCE

2.1 Introduction

Dahlia plants possess a high level of variation in phenotypic character traits. The plant size, the flower shapes, forms and sizes and flower color are very diverse. Flower color is one of the most important quality trait in many ornamental plants and their variation greatly influence consumer preference and consequently commercial value (De Folter et al., 2015). While most flowering plant populations maintain uniformity in floral colors, some plants exhibit variation within a population either discretely (polymorphic) or continuous (Sapir et al., 2021). Flower color is determined by the presence and distribution of pigments mainly the flavonoids such as anthocyanins, flavones, flavonols and their derivatives. The accumulation of pigments is determined by factors such as physical factors (e.g temperature and light) physical factors such as mineral nutrition and plant hormones as well as external factors such as the presence of pollinators. Temperature is the major physical factor that affects flower color where extreme conditions affect flower color by altering anthocyanin accumulation and cellular structures of petal epidermal cells (Lai et al., 2011).In many plant species high temperatures lead to lighter flower colors due to reduced anthocyanin content while low temperatures result in darker flowers due to increased anthocyanin content (Stiles et al., 2007). Dahlia 'Nessho' flowers are originally produced because of their red color. However, they exhibit a color-fading characteristic during winter and autumn when the temperature falls below 10℃ and the degree of color fading has been indexed as color fading index 0 to 4 (CFI 0, CFI 1 CFI2 CFI3 and CFI4) (Okada et al., 2018).

Color fading makes quality flower color production challenging as it is not possible to select non-fading plants before the visible flower. The classification of dahlia plants according to their degree of flower fading is an important topic as it can help growers identify the optimal time for harvesting and improve the overall quality of their crops.

It is hypothesized that dahlia plants can be effectively classified based on their degree of flower fading, and that the minimum temperature determine the degree of fading. Some 'Nessho' plants however, are relatively low sensitive to color fading and hence produce the original dark red flowers even under low-temperature conditions. Such plants can be selected and propagated vegetatively for quality flower production throughout the year. In this study we screened 120 plants from a commercial propagation company for flower fading from November 2021 to March 2022. The degree of flower color fading varied in a single population throughout the flowering season. The plants were classified according to their level of flower fading from CFI 0 to CFI4. The L*a*b* Values of the also varied based on their degree of fading.

2.2 Physical assessment of the distribution color fading in flowers

2.2.1 Purpose

In this experiment the effects of average minimum temperature below than 10℃ on 'Nessho' flower color was investigated.

2.2.2 Materials and methods

120 Plantlets were received from a commercial dahlia producing company in September 2021. The plantlets were transplanted in 12 cm polythene pots and maintained in East greenhouse at Okayama University experimental field. Watering was done when the pot were dry (every one or two days) with OAT solution at 1/6 dilution. The plants

were pinched above the 2nd internode to allow new shoots to emerge. One or two shoots were maintained until flowering. Lateral shoots and suckers were removed for the stem to bear only one flower head. Support was provided by staking and training was done appropriately.

Flower color was observed from the bud formation stage until the flower head was fully open. The flowers were harvested above the first or second internode and the color fading index was recorded. Fully opened ray florets from both the faded (completely orange) and unfaded (completely red) flowers were sliced into thin section (approximately 60mm and observed under a light microscope. This was to assess the cellular features of the flower materials of different degree of flower fading.

In addition, the color intensities of dahlia 'Nessho' ray florets with different color fading index were measured using a colorimeter (CM-2300d, Konica Minolta, Tokyo, Japan) with a C/2° light source and a white background. In the colorimetric analysis, the CIE L*a*b* color space was employed using the indexes L*, a*, and b* to describe color. The L* index represents lightness, a* represents the red-green balance and b* index represents the yellow–blue balance. L* ranges from 0 (black) to 100 (white), with positive a* values indicating red and negative values indicating green, while positive b* values indicate yellow and negative values indicating blue. As shown in Fig 2.1, three different parts of the ray florets were measured. The average values of L*, a*, and b* were obtained. The hue angle (H°= arctangent (b*/a*)) and chroma (C = sqrt (a * a + b * b) were calculated according to the methods described by Voss 1992 and McGuire 1992.

Nessho plants were classified according to their degree of color fading in winter 2021/2022. Forty-two plants that flowered in that season were classified as relatively

highly sensitive plants, intermediate plants and relatively low sensitive plants based on the color fading index described by Okada et al., 2020; with the marketable quality being CFI 0 to CFI 2. Seasonal color fading occurrence was assessed in Winter 2021/2022, winter 2023/2023 and summer 2023. The distribution of colour fading in the three season was compared.

2.3 Results

2.3.1 Physical assessment of the distribution Color fading in flowers

Flower color fading in dahlia 'Nessho' started in Autumn as soon as the average minimum temperature fell below 10℃. Color fading continued until the end of winter. The color fading was influenced by low minimum temperature as shown by the onset of faded flower. The color fading occurrence however is an inherent plant characteristic. This is shown by the distribution of flowers exhibiting flower fading index one to four over the harvesting season. Some plants produce flowers with different color fading index (Fig 2.1 **h**) while other plants produce non-faded flower (CFI 1) even in winter (Fig 2.1 **a** and **b**).

Figure 2.1: The diversity in the degree of color fading among dahlia Nessho plant in a population. **a** and **b** are non-faded flowers (CFI 0), **c** is a flower with slightly faded disk florets (CFI 1), **h (ii)** is a flower exhibiting CFI 2, **f** and **h (i)** are flowers with CFI 3 and **d, e** and **g** are completely faded flowers (CFI 4).

The *L** values and b* values of the non-faded red flowers (CFI 0 and CFI 1) were the lowest and highest in the most faded flowers (CFI 4) (Tab 1). The non-faded red flowers (CFI 0 and CFI 1) had the highest *a** value with a mean of 63.98 and the most faded flowers (CFI 4) had the lowest a* value (mean of 50.17). All the L* a* and b* value of all the flower with varying CFIs are positive hence fall on the first quadrant of the CIEL color chart. In addition, the *C* value of the red group was highest followed by partially faded flowers. The *C* value was the lowest among the most faded flowers, CFI 4 (Fig 2.3). The *h* value gradually increases from the non-faded red flowers to the faded orange flowers.

Table 1: Relationship between the flower color fading indices (CFIs) and the corresponding CIELAB color space values (L* (lightness, a* (green to red) b* (blue to yellow), c (chroma) and h (hue).

Flower color fading Index	L*	a*	b*	C	h
CFI 0/CFI 1	25.37556	63.98	20.21222	67.16968	17.46083
CFI2	30.57	62.64	26.715	68.10889	23.09191
CFI 3	39.15	62.56667	42.19	75.69797	33.79941
CFI 4	41.38167	50.17	48.372	69.77142	44.11678

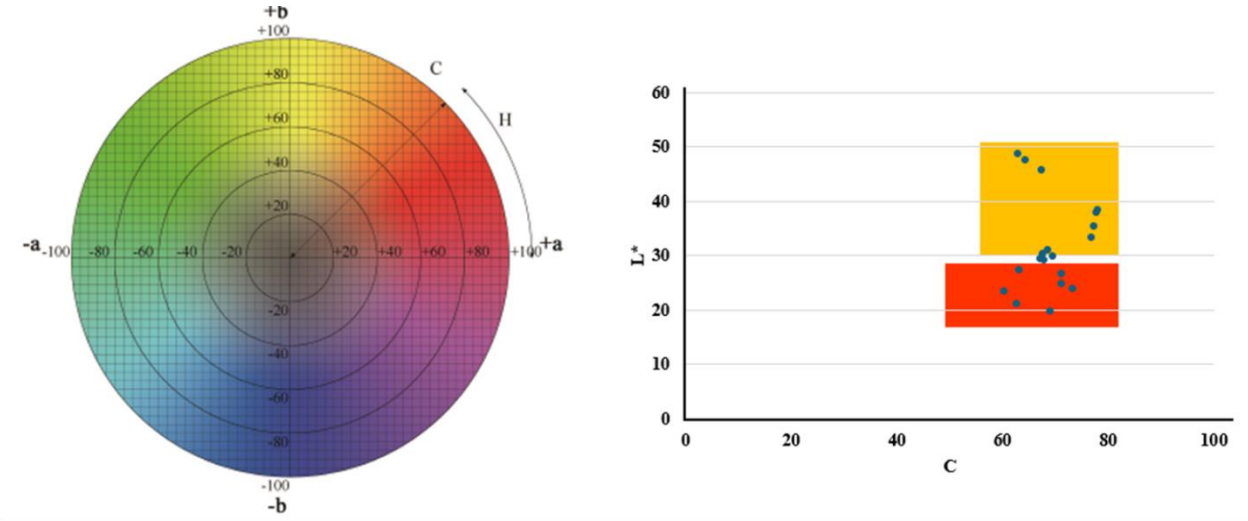

Figure 2.2: Distribution of L* (lightness) c* (chroma) and hue angel of red and orange faded petals of 'Nessho' on the CIELAB color space.

2.3.2 Relationship between epidermal cell and flower coloration

The cell structure and shapes of on both epidermal surfaces of the unfaded and the faded 'Nessho' flowers line were not distinctively different. However, the cells with red pigment have smoother round shapes compare to cells with lighter pigmentation. Cells with orange pigmentation appear more irregular in shape than the red pigmented cells and the cells with red pigments appeared round as compared to the faded pigmented cells (Fig 2.3).

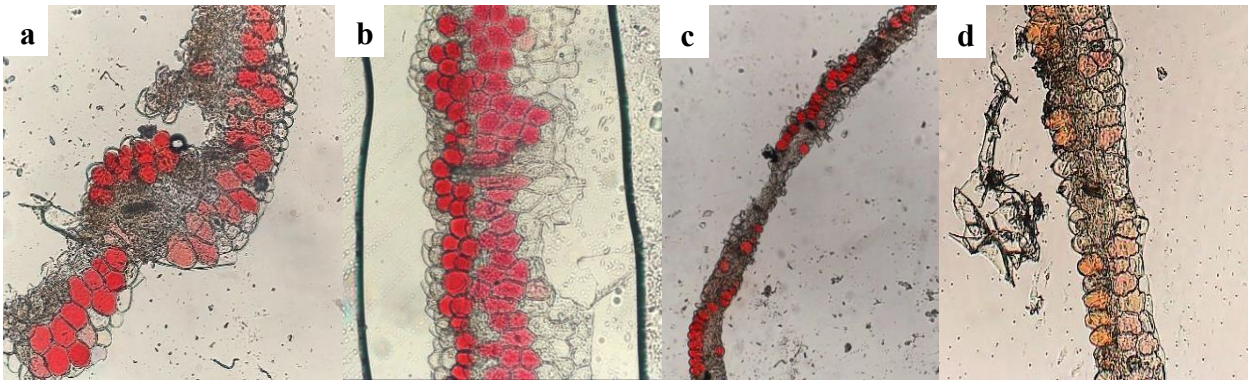

Figure 2.3: Epidermal cell structure of ray florets of 'Nessho'. **a**, **b** and **c** panels are red floret and **d** is an orange flower.

2.3.3: Seasonal flower fading occurrence in dahlia 'Nessho'

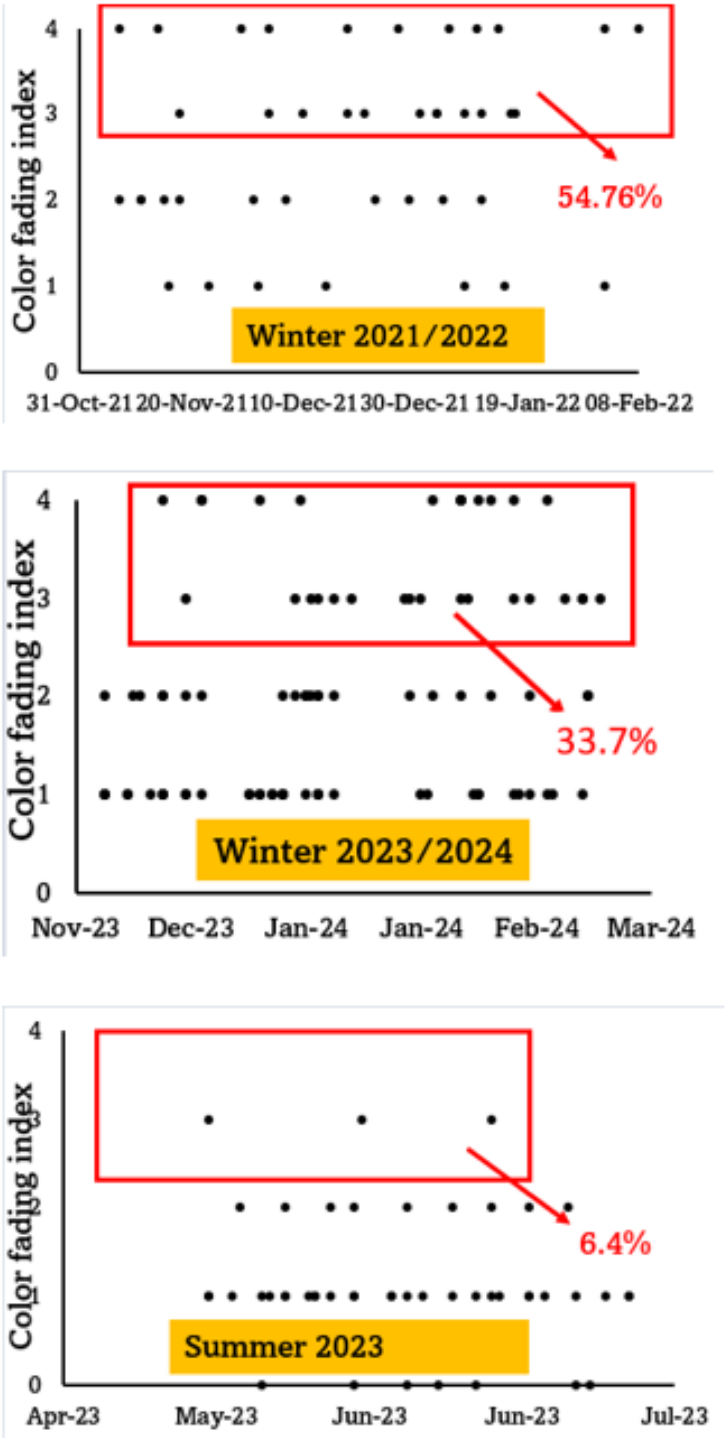

Figure 2.4 Flower color fading occurrence during the winter season (average minimum temperature <10°C) and summer (average minimum temperature > 23° C)

Color fading occurrence in 'Nessho' varies from season to season. The distribution of color fading when the average minimum temperature was 10℃ and below was higher in percentage than the distribution in summer; 54.7% and 33.7 % in winter 2021/2022 and 2023/2024 respectively. In summer 2023 there was 6.4% color fading occurrence and no CFI 4 among the flowering population. The severity in color fading increased with the decrease in average minimum temperature with most plants exhibiting CFI 2, CFI 3 and CFI 4 in December and January.

2.4 Discussion

Flower color, their intensity and pattern in plants is determined by the type of pigments and the amount in which the accumulate in plant tissues. Most of the pigments responsible for color in flowering plants belong to four major groups: chlorophylls, carotenoids, flavonoids and betalains, with flavonoids being the most diverse and widespread pigment group (Narbona et al., 2021). The flavonoids include the coloured anthocyanins, aurones and chalcones, as well as many flavonoid compounds such as the colorless flavones and flavonols. Flower color fading refers to the process where the color intensity of flowers transition to lighter shades. This phenotypic change of flower color is a widespread occurrence in flowers e.g. in petunia, lotus, *Linanthus parryae* and *Ipomoea nil* (Liu et al 2022, Guo et al 2020, , Morita et al 2018 and de Vlaming et al., 1983,) and it mainly results from the variation in pigment accumulation. Color fading in flowers takes different forms; for instance, a change in the specific type of pigment or in the ratio in which pigments mix can affect the resulting colour (hue), whereas changes in the concentration of the compounds would mainly affect the colour intensity. Flowers can exhibit from simple single colours to extremely complex color patterns. Color changing

in *D. variabilis* dark red cultivars results from the accumulation of more flavones and fewer anthocyanins (Deguchi et al., 2013; Thill et al., 2012). Similarly, the accumulation of more flavones instead of anthocyanins led to paler violet *Antirrhinum majus* flowers (Luo et al., 1991). Both anthocyanins and flavones are produced in the general flavonoid pathway, with flavones being a product of the branch pathway from chalcones.

The color fading indices in dahlia 'Nessho' (CFI 0 to CFI 4) reflect both the change hue and intensity as a result of co-pigmentation by these two flavonoids. This is reflected by the CIE L*a*b* color space values where the non-faded flowers (CFI 0 and CFI 1) have lower lightness L* and b* value as compared to faded flowers. The patterns of fading in CFI 2 and CFI 3 plants (Fig 2.1 panel f and g) show some faded and non-faded sections of flower. This complex pattern in coloring may be produced by the precise and orchestrated regulation of pigments biosynthesis in different parts of the petal (Narbona et al., 2021).

Most flowering plant species are characterised by having distinct flower colors that are stable within and among individuals regardless of the environment. However, in some species the flower colors vary across the landscape and or over time. In such cases petal colours may change over a flower's lifespan to become either darker or lighter or complete color change. In the case of, *Brunfelsia pauciflora* (yesterday-today-and-tomorrow) flower color change from dark purple to pure white progresses from early developmental stages and may caused by the active degradation of anthocyanin. In 'Nessho' the change in color is not a progressive change as the flower ages that may result from degradation of pigmentation. Rather, it may result from less common flower color polymorphisms that are caused by changes in anthocyanin composition (Sánchez-Cabrera

et al., 2021). This phenomenon causes flower colour variation among individuals within a population or among populations. The variation may be discrete, creating distinct colours or continuously gradual. Flower colour polymorphisms can be caused by changes in pigment concentration, leading to a gradient of flower colour intensity or total lack of pigments (Narbona et al., 2021)

Flowers acquire their characteristic hue due to pigment accumulation in combination with other chemical and physical factors. The physical factors include special localization of pigments and optical properties of petal epidermal cells. The shape of the cells that accumulate anthocyanin pigments influences their optical properties and thereby the colour that is perceived (Pfündel et al., 1998). In this way, various shapes of petal epidermal cells can also have an important impact on flower color. In 'Nessho' there is comparative no relationship between epidermal cell shape and L* value and thus an indication that cell shape is not involved in flower color intensity. Generally, conical cells increase the proportion of the incident light on epithelial cells, this improves the light absorption by pigments and leads to darkened flower color and improves on color saturation. Flat cells on the other hand can reflect more incident light resulting in lighter flower color. Flavonoids are water-soluble molecules and mainly accumulate in vacuoles; therefore, anthocyanins and other UV-absorbing flavonoids such as flavones, flavonols, flavanones and isoflavones may be mixed in the same vacuoles.

CHAPTER III

RELATIONSHIP BETWEEN FLOWER COLOR FADING DAHLIA VARIABILIS 'NESSHO' PLANTS AND FLAVONE SYNTHASE GENE (*DVFNS*) EXPRESSION PATTERNS

3.1 Introduction

Flower color is one of the most important quality traits in ornamental plants as it determines the commercial value of a flower. Flower color is the product of biochemical processes influenced by genetic, physiological, and environmental factors (Sapir et al., 2021). The color of the ray florets results from the accumulation of secondary metabolites such as flavonoids including anthocyanins and flavones. Deep color intensities result from higher pigment accumulation whereas pale colors result from lower pigment accumulation (Tanaka et al., 2008). White and yellow coloration in flowers results from an incomplete anthocyanin biosynthesis process. The red color results from accumulation of anthocyanins (Halbwirth et al., 2008). Flavonoid biosynthesis is a complex process that involves a multitude of well-characterized enzymatic and regulatory proteins (Li 2014) and their accumulation can be reflected in the gene expression. Characterization of spontaneous color change in flower mutants in *Petunia*, *Antirrhinum*, *Ipomoea*, and other ornamental plants demonstrates that there are roughly equal frequencies of both regulatory and functional mutations (Wessinger & Rausher, 2012) The expression of specific genes such as the structural genes and the regulatory genes and their transcription factors is directly linked to the biosynthesis and accumulation of flavonoids (Guan et al 2014, Wang et al., 2016, wang et al., 2023).

Dahlia (*Dahlia variabilis*) belongs to the family Asteraceae and the flowers vary in shape, size and color because of its polyploidy nature (Gatt, 1998). The variation in flower

color ranges from red, yellow, purple, pink, ivory, and black (McClaren 2009). The pigments that accumulate in the ray florets are mainly anthocyanins, flavones, buteins, and their derivatives (Ohno 2011a) hence floral color is based on the final pigment product of the anthocyanin biosynthetic pathway.

D. variabilis `Nessho` cultivar is a cultivar that is popular because of its solid red color. However, the cultivar has a unique characteristic where it exhibits flower fading during winter and spring even in plants that produce solid red flowers during summer and autumn. In a study comparing dahlia `Kokucho` a black cultivar and its purple mutant showed that suppression of *Dahlia variabilis* flavone synthase gene (*DvFNS*) abolishes the competition in the synthesis of anthocyanidin and flavone leading to higher accumulation of anthocyanins (Deguchi et al., 2013). In `Nessho` the flower color is also affected by the expression level of *DvFNS* in the whole plant (Okada et al., 2018). High temperature in autumn and summer suppress *DvFNS* expression favoring high anthocyanin accumulation hence the production of solid red color flowers. Low temperature in winter and spring induce *DvFNS* expression hence higher flavones synthesis and accumulation leading to faded flowers. Nevertheless, there are plant strains that can produce flowers with stable color through all the seasons. Such strains are desirable for constant production in Japan throughout the year. These strains of plants can be propagated asexually through cuttings for consistent quality production. Selection for non-fading strains however, takes a long time because plants have to be selected for solid red flower color during winter and spring seasons. A faster selection method is therefore desirable.

In dahlia `Nessho` cultivar there is also a correlation between *DvFNS* gene expression levels in young unexpanded leaves and flower color expression (Okada et al.,

2018). In the current study we sort to validate this correlation so as to use it as a selection method for non-fading strains. We compared *DvFNS* gene expression in young unexpanded leaves of relatively low sensitive and relatively high sensitive plants before and after *DvFNS* gene inductive conditions treatment.

3.2 Materials and Methods

3.2.1 Plant materials

D. variabilis `Nessho` mother plants were screened at Shinshu University, Japan (lat. 35°86'N and long. 137°93'E and grown at the Okayama University experimental field (lat. 34° 66'N and long.133°92'E). Approximately 10cm long shoots cutting were obtained from the mother plants and rooted in compacted block of soil-less media under mist conditions. Four weeks later all rooted cutting were re-potted into 12cm diameter pots on rock sand-based media. Fertigation was done on alternate days or when the media was dry.

3.2.2 Classification of plants

Plants maintained in greenhouse conditions were pinched during late summer (temperature between 23℃ to 28℃) and two shoot allowed to grow to flowering. Plants flowering in December 2021 and January 2022 (greenhouse conditions: average minimum temperature of 10℃ in December and 5℃ in January and supplemental lighting 16.00 to 22.00) were classified based on the color of flowers produced. A color fading index (CFI) of 0 to 4 (**Fig 3.1**) was used to classify the plants were 0 represented the unfaded flowers and 4 the extremely faded flowers. Plant with CFI value of 0 and 1 were classified as candidate low (CRL) sensitive to flower fading, CFI 2 as intermediate plants CFI 3 and CFI 4 as candidate relatively high (CRH) sensitive to flower fading. Eight

strains of CRL and a strain of three CRH plants were used in subsequent experiments for analysis of *DvFNS* gene expression.

Figure 3.1: `Nessho` flower color fading index (CFI). CFI 0 represent flowers that exhibit solid red color and CFI 4 represent flowers with extreme flower fading.

3.2.2 Expression analysis for flavonoid synthesis pathway genes.

Expression analysis of flavonoid synthesis pathway genes was conducted using real-time reverse transcription (RT)-PCR. Variegated ray florets (Fig 3.2) were used and red and orange sections sampled among the ray florets. Three red ray florets and four color-faded orange ray florets were analyzed as biological replicates. Central ray florets of red and color faded orange flowers at three developmental stages were also used for evaluation of late flavonoids biosynthetic gene after Naringenin. Total RNA (> 200 nucleotides) was extracted using NucleoSpin miRNA (Takara Bio Inc., Shiga, Japan), and purified with a high-salt solution for precipitation (Takara Bio Inc.) two times. The extracted total RNA was reverse-transcribed with ReverTra Ace® qPCR RT Master Mix with gDNA Remover (TOYOBO, Osaka, Japan), and 1 µL of RT product was used as a template for real-time RT-PCR. Real-time RT-PCR was performed with THUNDERBIRD® SYBR qPCR Mix (TOYOBO) according to the manufacturer's instructions using a Dice® Real-Time System Thermal Cycler (Takara Bio Inc.). The primers used were as reported by (Ohno et al., 2011b). The PCR program was set at 95°C for 1 min, followed by 40 cycles of 95°C for 15 s, 60°C for 1 min, and subsequent

dissociation steps. Expression profiles of *D. variabilis Chalcone synthaseI and II (DvCHSI* and *II), Chalcone isomerase (DvCHI), Anthocyanin synthase, DvANS, Dihydroflavonol 4-reductase*, *DvDFR*, *Flavone synthase DvFNS*, *Flavanone 3-hydroxylase*, *DvF3H*, and *Flavonoid 3'-hydroxylase DvF3'H* were analyzed. *DvActin* was used as an internal standard. Additionally, *DvFNS* expression was analyzed using ray florets that exhibited variegated coloration. Since 'Nessho' capitula often produce red and orange variegated ray florets, each red and orange area was separated from four distinct ray florets. Total RNA was extracted as mentioned above and used for the *DvFNS* expression analysis using the same primer sets and real-time RT-PCR conditions.

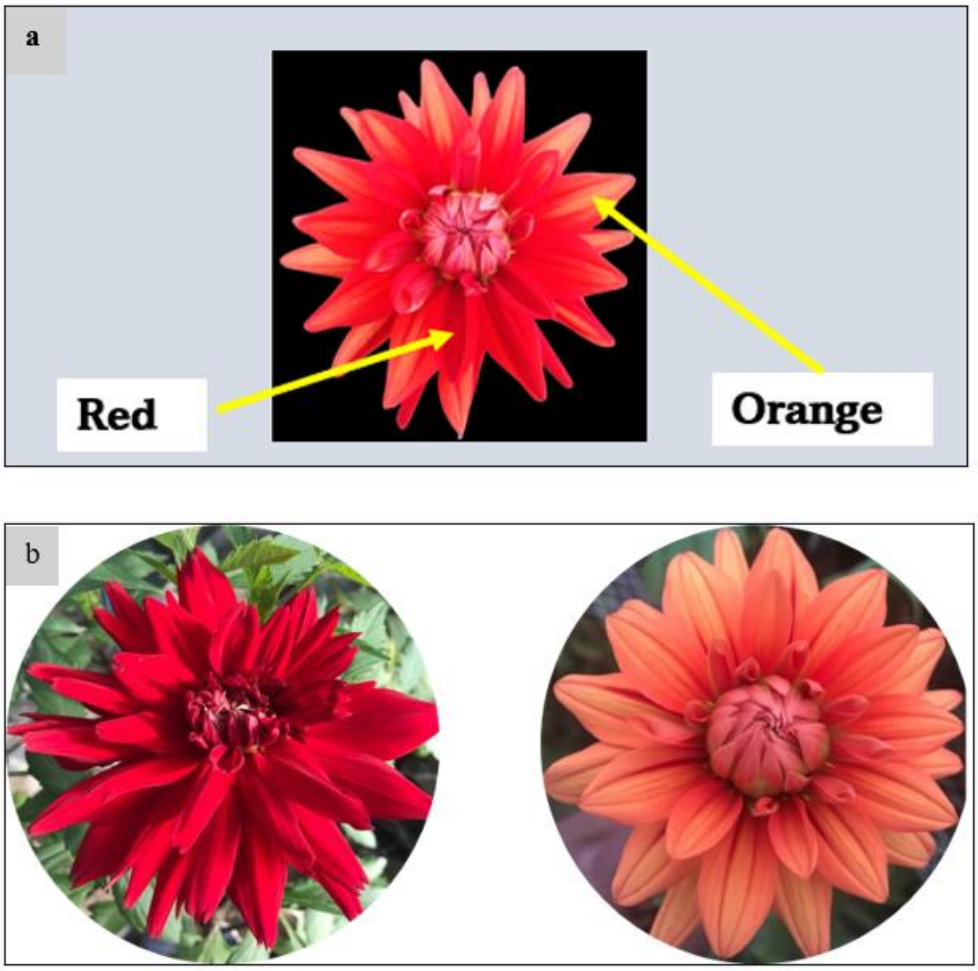

Figure 3.2: (a)Variegated flower used to obtain the red and the orange sections flavonoids structural genes expression analysis. (b) red and color faded flowers used for obtaining central ray florets at different developmental stages (10mm, 20mm, 30mm).

3.2.3Optimising of DvFNS expression conditions

3.2.3.1Optimum daylength treatment

Rooted cutting of relatively highly sensitive plants and relatively low sensitive plants were maintained in an incubator under acclimation conditions for fourteen days. Young unexpanded leaves were sampled in frozen in liquid nitrogen and later stored at -80°C until they were later used for RNA extraction. The conditions of the incubator were set to inductive conditions i.e. a combination of low temperature and four daylength conditions. The tested daylength conditions included long daylength 14:10 h, 10h daylength with a night-break from 20:00 to 24:00 hours, 10h daylength with a night-break from 22:00 to 02:00 hours, 10h daylength with a night-break at 01:00 to 05:00 hours and 10h daylength with a day extension from 04:00to 08:00 hours with incandescence bulbs. Fourteen days after inductive conditions, young unexpanded leaves sampled. Samples were immediately frozen in liquid nitrogen and stored at 80°C until they were later used for RNA extraction. The RNAs extracted were used for real-time qPCR gene expression analysis. The relative *DvFNS* expression levels were used to determine effects of day length on *DvFNS* expression in young unexpanded and the optimum daylength combination with low temperature the gene expression.

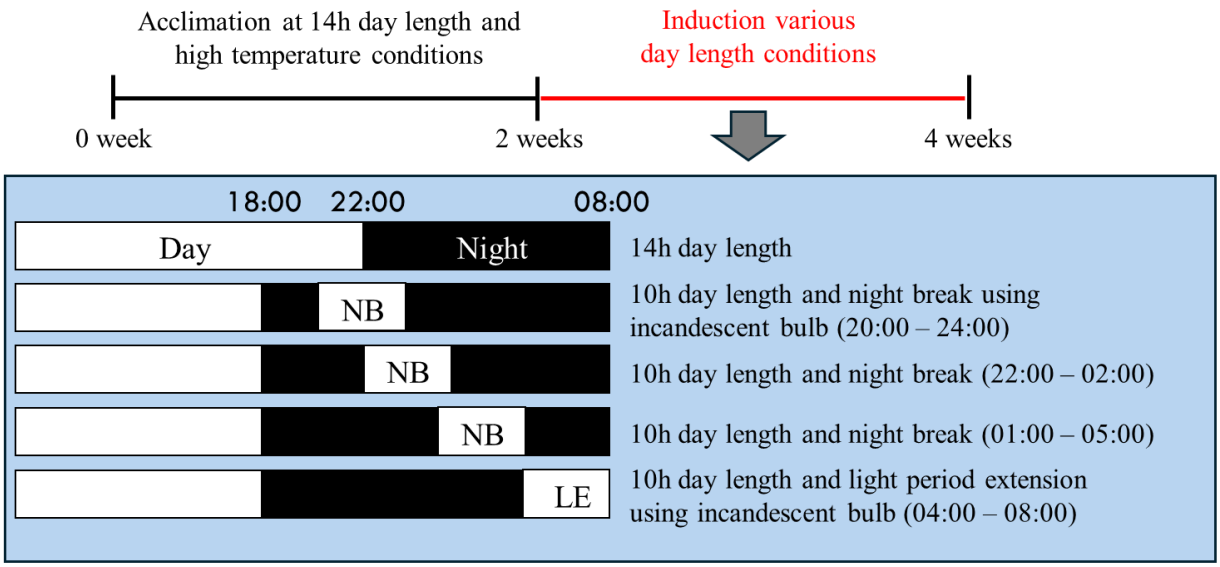

Figure 3.2: The analysed daylengths in combination with low temperature conditions. In all treatments, acclimation was conducted for two weeks before the indictive treatment.

3.2.3.2 DvFNS expression in leaves and its relationship with color fading occurrence in ray florets and identification of the optimum cutting size

To confirm whether the seasonal changes in *DvFNS* expression occur only in ray florets but not in leaves, we analyzed the *DvFNS* expression in leaves at different maturation stages. The optimum leaf maturation stage for *DvFNS* expression analysis was decided using leaf blades sampled from RH-sensitive plants in February, when they produced completely color-faded orange ray florets. Unexpanded leaves and leaves on the 1st to 4th internodes were sampled from four distinct plants as biological replicates. Total RNAs were extracted from the samples and relative *DvFNS* expression analyzed by real time qPCR using THUNDERBIRD® SYBR qPCR Mix (TOYOBO) according to the manufacturer's instructions using a StepOne™ Real-Time PCR System (Applied Biosystems, Foster City, CA, USA).

To determine the optimum cutting size for *DVFNS* expression in young unexpanded leaves, rooted cuttings of RH sensitive plants were re-potted into 12cm pots and maintained at the east greenhouse of the Okayama university experimental field until the plantlets attained 30cm height and pencil thick stems. Pinching was done above the first internode, above the second internode and above the fourth internode (Fig 3.3). Four to six plants were used for every treatment and the experiment replicated three times. Plants were first transferred to the incubator set at acclimation conditions for fourteen days. Young unexpanded leaves were sampled, and the incubator conditions changed to inductive conditions for fourteen days before the second sampling. The samples were used for total RNA extraction and real-time qPCR analysis to compare relative *DvFNS* expression before and after inductive conditions.

Figure 3.3: Dahlia plants pinched at different levels. (a)on the fourth internode (b) on the second internode (c) on the first internode.

3.2.4 Evaluation of DvFNS expression in 'Nessho' young unexpanded leaves

Eighty dahlia plantlets (approximately 25cm to 30cm in height) were pinched above the first internode and place in an incubator under controlled conditions to evaluate the change in *DvFNS* expression in young unexpanded leaves when the prevailing temperature was below 10 ℃. The conditions were first set at normal dahlia production conditions (acclimation conditions) for fourteen days to harmonize gene expression in all plants. The acclimation conditions were long daylengths of 14/10 hours and 25℃/20℃ day/ night temperatures. Sampling of young unexpanded leaves was done, and samples were immediately frozen in liquid nitrogen and stored at -80℃ prior to further analysis. The conditions of the incubator were changed to inductive condition (short day lengths 10/14 hours and 20/9℃ day/night) to mimic autumn

Some thirty-two plants that were not used incubator experiments were maintained under greenhouse condition until flowering in December to February 2022. Their flower color fading index were observed and recorded as described on fig.3.1. After the flowering season the plants were pruned back to allow growth of new lateral shoots. The plants were transferred to controlled incubator conditions for *DvFNS* relative expression assessment in young unexpanded leaves. The controlled acclimation and inductive conditions were as described above. The flower color fading index were compared with the relative *DvFNS* expression in young unexpanded leaves of the same plants as shown on fig 3.4.

In another experiment, eight strains of candidate relatively low sensitive (RL) plants and three strain of candidate relatively highly sensitive RH plants (approximately 20cm height with more than three internodes as on **Fig.2a**) were pinched on the second internode to allow for new shoots development (**Fig. 2b**). The pinched plants were put in

a growth chamber set at acclimation conditions of 25°C /20°C day and night temperature and 14 hours of day length to homogenize the gene express levels in all plants. Initial sampling was done 14 days after the onset of acclimation conditions treatment. The plants were then put again in the growth chamber set at inductive conditions of 20°C/9°C day and night temperatures and 10 hours of day length, mimicking winter conditions to induce *DvFNS* gene expression. Second sampling was done 14 days after the onset of inductive conditions treatment. All samples were used to obtain RNAs.

Figure 3.4: Plant materials used in the experiment; (a)`Nessho` plant with more than three internodes, (b) plant pinched above the 2nd internode, (c) young un-expanded leaf used for total RNA extraction**.**

Total RNA extraction and real-time qPCR

Young un-expanded leaves (**Fig. 2c**) of newly emerging shoots were collected, frozen in liquid nitrogen and stored at -80°C until when in use. Total RNA was extracted from approximately 100mg leaf samples using a modified CTAB- lithium chloride protocol. The integrity of the extracted RNA was checked using 1% agarose gel electrophoresis and the concentration determined using a Biodrop µLite+ spectrophotometer. The RNAs were reverse transcribed to obtain cDNAs which were further used as templates for real-time qPCR. Relative expression level of *DvFNS* were determined by real-time qPCR performed using Thermal Cycler Dice Real Time System (TaKaRa Bio Inc., Kyoto, Japan). *DvFNS* forward and reverse primers were used. Each reaction was performed using 1µL aliquot of 10µL cDNA solution derived from 0.5µg RNA. The qPCR thermal profile was as follows; initial polymerase activation at 95 ◦C for 1 minute; then 40 cycles at 95 ◦C for 15 seconds, 60 ◦C for 1 minute followed by a dissociation step. Single-target product amplification was checked using dissociation curves. *D. variabilis* actin gene (*DvActin*) was used as an internal standard. To standardize the data, the ratio between the relative expression level of the target gene *DvFNS* and the control gene (*DvActin*) was calculated for each sample. The experiments were replicated three times.

3.2.5 Analysis of DvFNS small RNAs

To determine whether the low expression of *DvFNS* in red Nessho flowers was due to post-transcriptional gene silencing (PTGS), *DvFNS* siRNA detection was performed using Next generation sequencing of small RNAs. Small RNAs were extracted from approximately 100 mg fresh red petals of unfaded Nessho using CTAB and miRNeasy mini kit RNA extraction protocol and sequenced by a 100-bp paired end method using an

Illumina HiSeq 2000 (Novogene, Japan-AlphaBio). Data was analysed using the National Institute of Genetics' supercomputer. Small RNAs (18-32 nt long) in the sample (original red 'Nessho') were mapped to the DvFNS genome of 'Kokucho' (mainly the second exon) with no mismatches allowed.

3.3 Results

3.3.1 Quantitative real-time RT-PCR analysis of flavonoid biosynthetic pathway genes

To determine the regulation of flavonoid biosynthesis genes in the 'Nessho' dahlia cultivar, the level of expression of major structural genes in the anthocyanin synthetic pathway was analyzed. The expression levels of anthocyanin genes *DvANS*, *DvDFR*, *DvF3H*, and *DvF3'H* did not differ between the color-faded orange ray florets and dark red ray florets.

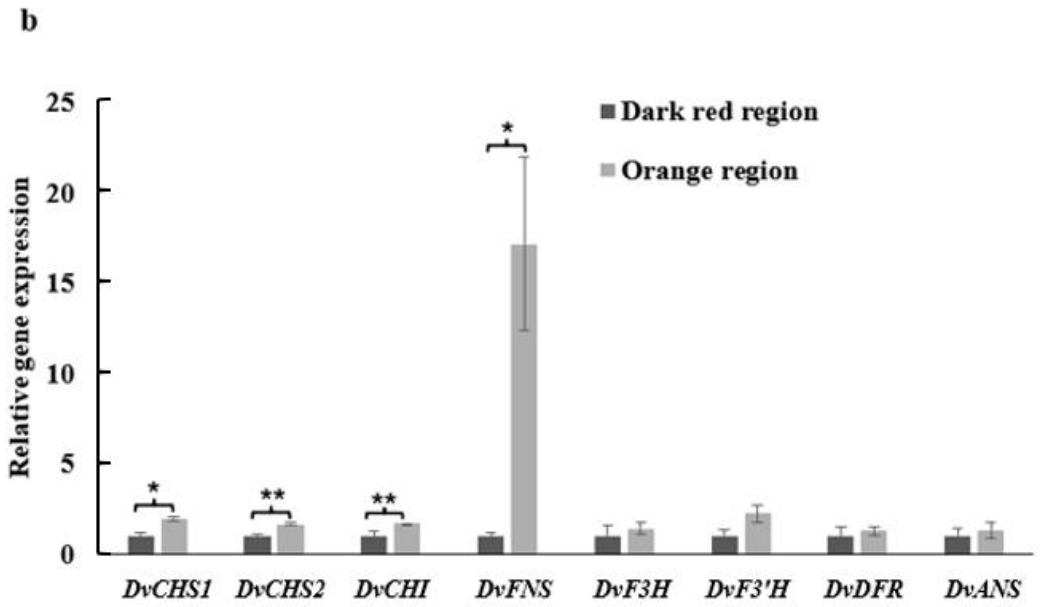

Figure 3.5: Gene expression profiles for structural genes; major structural genes are *DvCHS1, DvCHS2, DvCHI, DvFNS, DvF3H, DvF3'H, DvDFR*, and *DvANS* expressed in the red and orange regions of variegated ray florets. Vertical bars show ± SE of the means

(n = 3). * and ** indicate significant differences for *t*-test at 5% and 1% significance levels, respectively. Gene expression level in dark red region is set at a constant 1.

Comparison of the gene expression profiles for red and orange variegated ray florets showed that the expression level of *DvFNS* was significantly different between the red and the orange regions ($P < 0.05$). There were also small, but significant, differences in the expression of *DvCHS1, DvCHS2*, and *DvCHI.* The expression profiles of genes after the naringenin; the first major branch in the biosynthetic pathway show that only *DvFNS* was upregulated I the color faded ray florets. The expression of *DvFNS* progresses as the ray florets mature to full color (Fig 3.6).

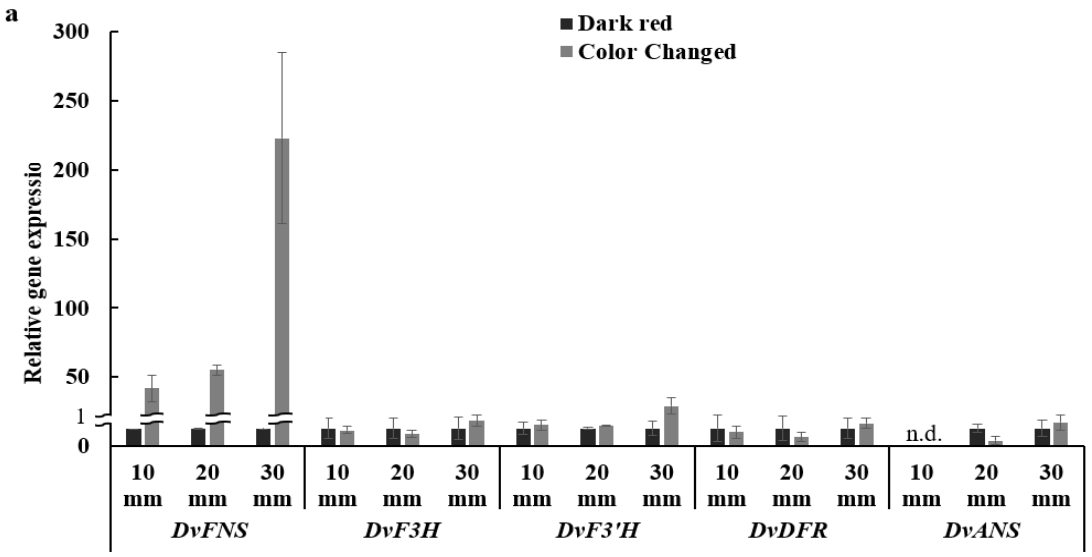

Figure 3.6: Gene expression profiles for five flavonoid biosynthesis genes (DvFNS, DvF3H, DvF3'H, DvDFR, and DvANS) in the anthocyanin biosynthetic pathway at three developmental stages (10 mm, 20 mm, and 30 mm indicate the length of ray florets) of dark red and orange flowers.

3.3.1 Optimum daylength treatment

Highly sensitive 'Nessho' plants screened at Shinshu University were used in this experiment. When the plants were treated with different light conditions and similar temperature conditions, there was significant difference in *DVFNS* relative expression at 14h daylength and daylength extension from 04.00 to 08.00 with incandescent bulb lighting. The greatest change in the expression after acclimation and inductive conditions was recorded in the day extension treatment. Under night break treatments (20.00 to 00.00, 22.00 to 02.00 and 01.00 to 05.00) the change in relative *DvFNS* expression after acclimation and after inductive conditions were not significant. The daylength extension treatment was considered the optimum combination with low temperature for inducing *DvFNS* expression. This was therefore used in the subsequent incubator experiments.

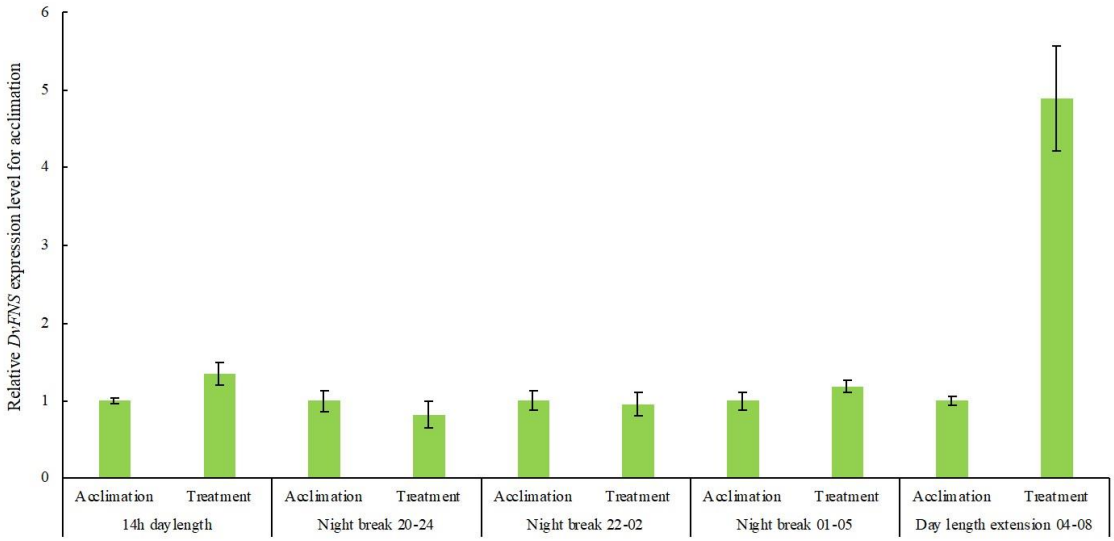

Figure 3.6: Relative *DvFNS* expression in highly sensitive 'Nessho' plants before and after inductive conditions under five different light conditions.

3.3.2 DvFNS expression in leaves and its relationship with color fading occurrence in ray florets and Identification of the optimum cutting size.

Leaves at five maturation stages; unexpanded leaves, leaves at the 1st, 2nd,3rd, and 4th nodes of RH-sensitive plants were used to evaluate whether *DvFNS* expression change occurred in the flower stage only or also in the leaves in response to minimum low-temperature. *DvFNS* expression levels were greatest in the young unexpanded leaves (Fig. 4a). The expression levels declined up to 5-fold and more in the leaves above the 1st, 2nd, 3rd, and 4th nodes.

The results of the relative *DvFNS* expression in 'Nessho' plants pinched at three different points before and after indictive condition show that plants pinched above the first internodes had the highest level of expression before and after inductive condition. Additionally, the highest change in *DvFNS* expression after inductive conditions was in the unexpanded leaves above the first node. There was no significant change at the second and forth nodes after inductive conditions treatment. Based on these results, unexpanded leaves above the first node were used for RNA extraction for real-time qPCR analysis in subsequent experiments.

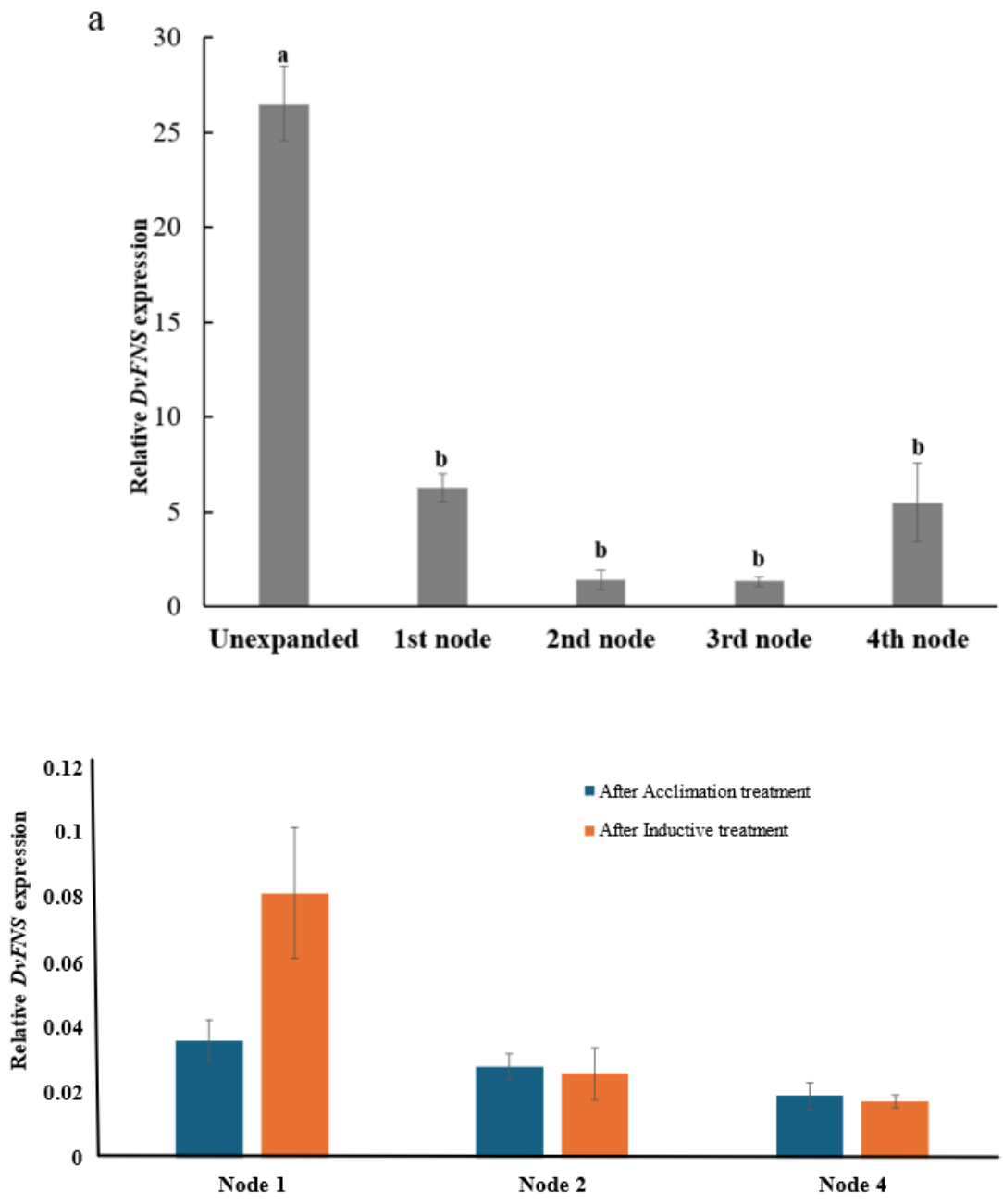

Figure 3.6: Relative DvFNS expression profiles in leave. (a) expression in leaves at five maturation stages (unexpanded leaves, leaf at node 1, node 2 node 3 and node 4), (b) expression in young unexpanded leave (above node 1, node 2 and above node 3).

The levels of relative *DvFNS* gene expression after acclimation conditions were relatively low with over 80% of the population exhibiting expressions of between 0.01 to

0.03. There was a change in *DvFNS* relative expression in all plants after inductive conditions (**Fig. 3.6a**). The extent of change in the relative expression varies from plant to plant with some plants exhibiting up to 5-fold change while others exhibiting change of less than 0.5-fold change.

There was a big variation in *DvFNS* expression from plant to plant in the same growth environment. The difference in expression after acclimation condition is an indicator of the heterogeneity of the dahlia population used for the experiment. This variance in relative expression after inductive condition is a factor of the degree of sensitivity to inductive conditions. Inductive low minimum temperature; 9°C induced a change in *DvFNS* relative expression in most plants in the population. This indicates that in dahlia 'Nessho' the prevailing environmental conditions (temperature and light condition) influence flavone gene expression.

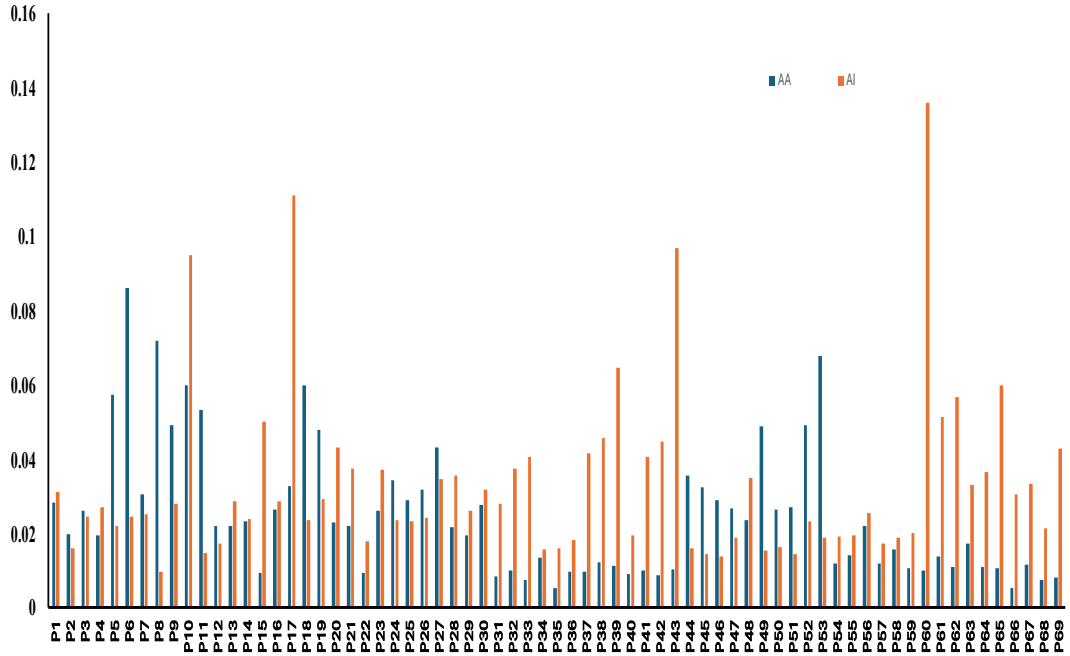

Figure 3.7: Relative DvFNS expression for 69 Nessho plants under controlled environment. (AA) after acclimation conditions (25/20ºC D/N 14/10 hours daylength) (AI) after inductive condiotins (20/9º C D/N and 10/14 hours daylenth).

In a different population where plants that flowered in winter 2021/2022 were later transferred to an incubator in controlled environmental conditions; acclimation conditions and indictive conditions as descried earlier, flower color fading indices were compared with the *DvFNS* expression profiles (Fig 3.8a). There was no pattern of expression profiles in plants in relation to their color fading indices for individual plants. Some in each classification; CFI 1 to CFI 4 had more than twice increase in gene expression. P25, a CFI 2 had the greatest change in gene expression after inductive treatment. P11, P13, P14 and P15 had less *DvFNS* expression after inductive treatment. The reason for this negative change is still unclear.

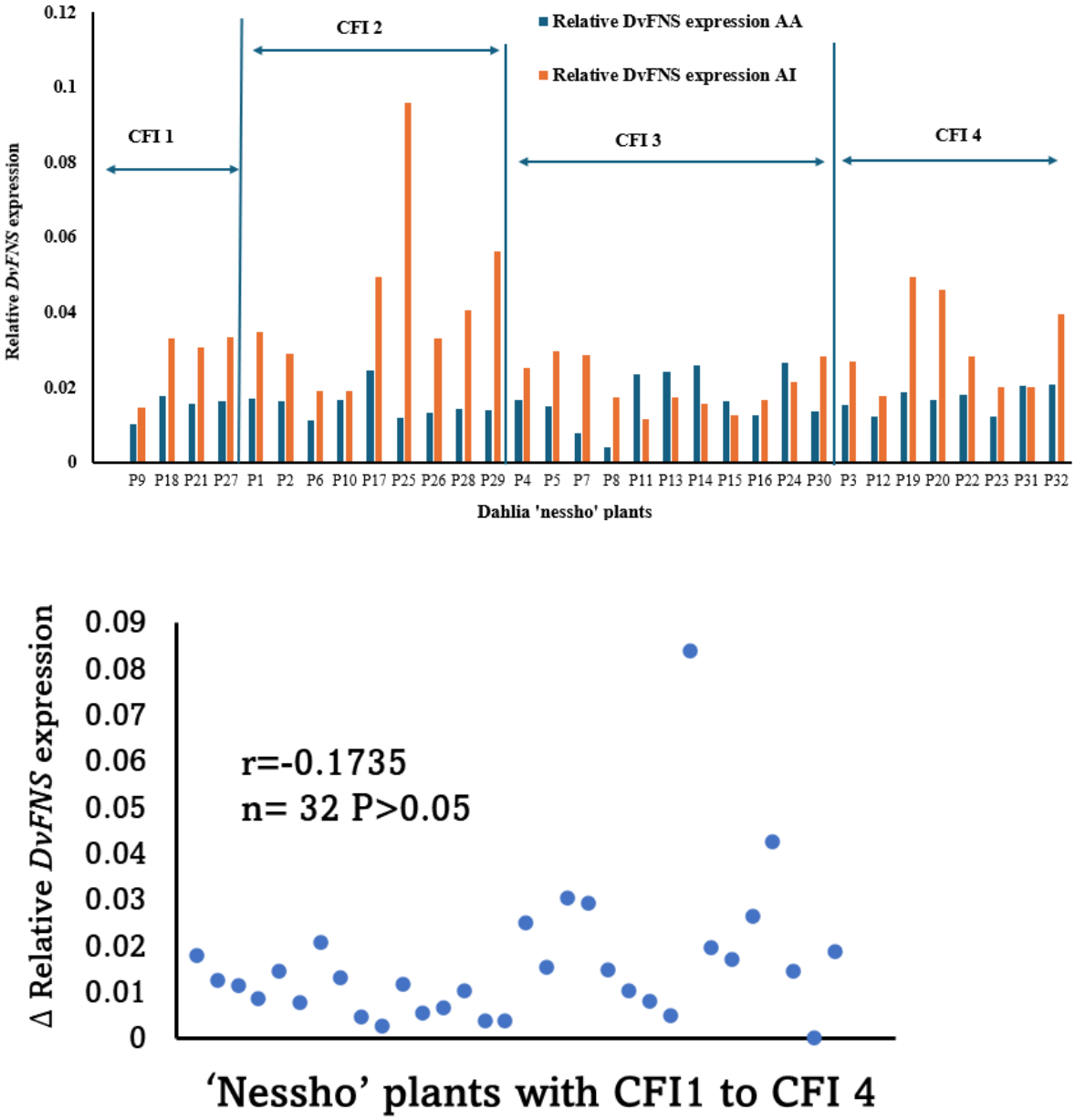

Figure 3.8 : (a) *DvFNS* expression in dahlia plants before and after inductive conditions and their respective color changing index (CFI 1 to CFI 4) (b) relationship between color fading and Δ in *DvFNS* expression after acclimation and after inductive treatment.

To clarify the relationship between highly sensitive plants and low sensitive plants in response to low minimum temperature treatment and *DvFNS* expression, eight RL lines and three RH lines were subjected to controlled acclimation conditions and Inductive conditions (as described previously). Four to six plants per line were used and the experiment replicated three times. Mean *DvFNS* relative expression were separated by Student t-test. All lines except RL3 and RL4 showed an elevated expression levels after inductive treatment.

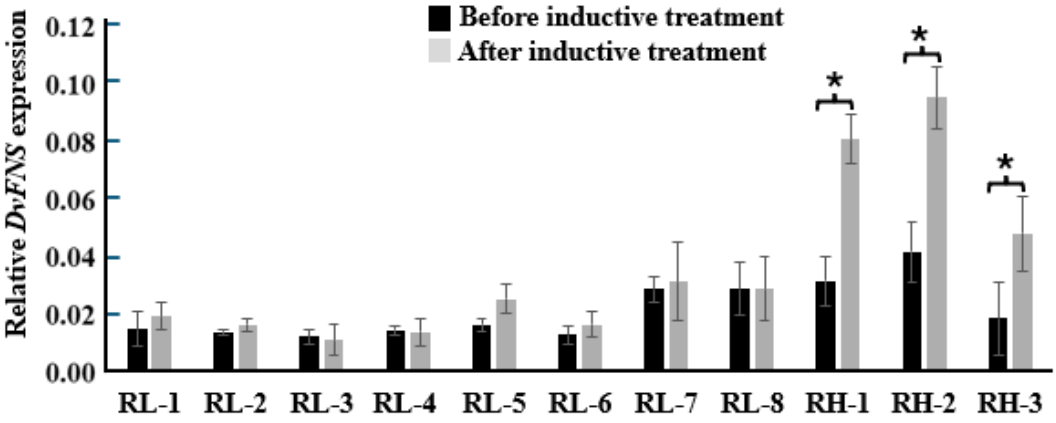

Figure 3.8: *DvFNS* expression for several RL- and RH-sensitive plants before and after inductive conditions (20℃/9℃ day and night temperatures and 10-hour day length. * indicates significant differences for the *t*-test at the 5% level. Vertical bars show ± SE of means (n = 4–8).

There was elevation in expression level after inductive conditions indicating the influence of environmental conditions on the gene expression pattern. In this experiment the relatively low sensitive strains; CRL1, CRL2, CRL5, CRL6, CRL7 and CRL8 showed a change of slightly above one-fold and CRL3 and CRL4 slightly less than one-fold in

relative *DvFNS* gene expression. However, the relatively highly sensitive strain CRH1 had a 2.5-fold change in *DvFNS* gene expression.

All the gene expression levels for RL strains before and after inductive treatment were not significant different (Fig 3.8). There was more than two-fold increase in *DvFNS* expression levels in the RH lines after inductive. A comparison between the change in *DvFNS* expression levels in young unexpanded leaves of RL- and RH-sensitive plants before and after inductive conditions indicated a significant difference at the 95% confidence level. The relative expression levels in RH- sensitive plants were higher than in RL-sensitive plants

3.3.3 Analysis of DvFNS small RNAs

To confirm that *DvFNS* was post-transcriptionally silenced, small RNAs were mapped to the DvFNS genome. As reference sequence, genomic sequences of DvFNS in 'Koku cho' original. The DvFNS coding region contained one intron of 1,150 bp, and the sequence was completely identical between 'Kokucho' original and the red 'Nessho'. The 21-nt mapped small RNAs were most mapped onto exon 2 (Fig. 3.6). The mapping pattern was identical to that of Kokucho as reported by Ohno et al., 2016 where *DvFNS* was said to be endogenously post-transcriptionally silenced in the black cultivars. These observations demonstrate that *DvFNS* in 'Nessho' was also silenced post transcriptionally by siRNAs in red unfaded lines.

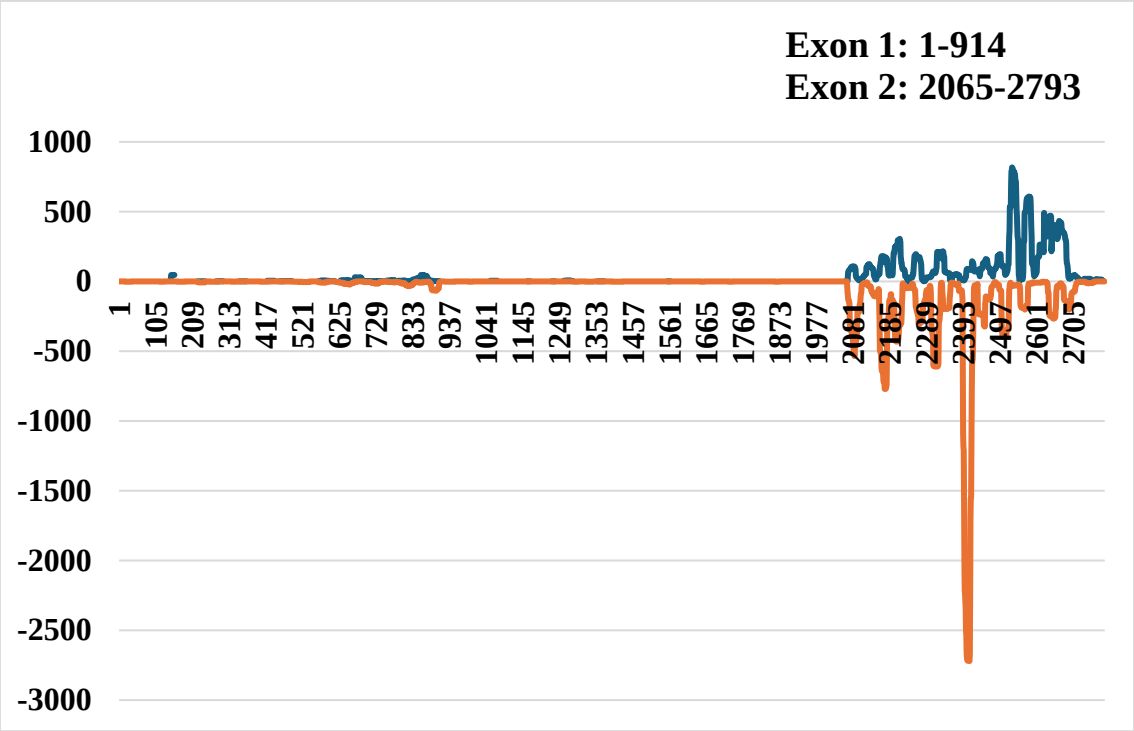

Figure 3.9: Mapping of small RNAs on DvFNS genome. Small RNAs (18–32 nt long) without mismatch to 'Kokucho' DvFNS DNA were mapped on either the sense strand (blue above the X-axis) or the antisense strand (orange below the X-axis)

3.4 Discussion

Flower color change in response to changes in environmental conditions, mainly temperature, light intensity and daylength is a common phenomenon reported in a variety of plants. Color intensity in plant organs is determined by the accumulation of flavonoids, particularly anthocyanins and flavones ((Harborne et al., 1990; Mizuno et al., 2015; Nordström & Swain, 1953; Tanaka et al., 2008). Prevailing environmental conditions influence flavonoid accumulation, as in the case of anthocyanin accumulation in *Arabidopsis thaliana* (Rowan et al., 2009). In many plant species, the accumulation of flavonoids in plant organs decreases under high temperature conditions. In grapes, high night temperatures reduced anthocyanin accumulation in the grape skin the quality of

berries (Mori et al., 2005). In the 'Nessho' red dahlia cultivar, low minimum temperature in winter results in orange flowers (Okada et al., 2020). Plants with higher FNSII gene expression yield paler flowers owing to a reduction of anthocyanins and increase in flavone accumulation because flavanones are precursors (common substrate) of anthocyanins and flavones. Color changing in *D. variabilis* dark red cultivars results from the accumulation of more flavones and fewer anthocyanins (Deguchi et al., 2013; Thill et al., 2012). Similarly, the accumulation of more flavones instead of anthocyanins led to paler violet *Antirrhinum majus* flowers (Luo et al., 1991). Both anthocyanins and flavones are produced in the general flavonoid pathway, with flavones being a product of the branch pathway from chalcones. Control of gene expression and enzymes encoding their activities are crucial to the balance at branch points (Davies et al., 2003). This is a result of substrate competition by the enzymatic activities at these branch points (Deguchi et al., 2015; Takos et al., 2006; Thill et al., 2012). When *DvFNS* was silenced in black dahlia cultivars, the competition between anthocyanin synthesis and flavone synthesis was disrupted leading to high accumulation of anthocyanins (Deguchi et al., 2013).

To verify the genes responsible for color change in the dahlia 'Nessho' cultivar, relative expression analysis was done for major structural genes of the flavonoid biosynthetic pathway, namely, *DvCHS1, DvCHS2, DvCHI*, *DvANS*, *DvFNS, DvFLS*, *DvDFR*, *DvF3H* and *DvF3'H* in red and orange ray florets. The expression profiles of all genes after the first main branch point on the biosynthesis pathway (at the naringenin stage) were not significantly different between the red and the orange ray florets except for *DvFNS*. This result suggests that *DvFNS* expression may be a major contributing factor to the red to orange color change of the 'Nessho' ray florets. The four-fold change in *DvFNS* expression levels in orange faded sections compared to red regions with

variegated ray florets further confirmed the possible role of this gene in the flower color change in 'Nessho'. Expression levels of anthocyanin structural genes (*DvDFR*, *DvF3H,* and *DvF3'H*) did not vary between the red and orange regions. This concurs with a report in *Gerbera hybrida,* where lines lacking *FNSII* expression and FNSII activity accumulated more anthocyanin and no flavones, leading to a notable increase in color intensity (Martens & Mithöfer, 2005). In a similar manner, the majority of black dahlia cultivars accumulate more anthocyanins due to low FNSII activity, as well as *FNSII* expression (Thill et al., 2012). In *Lonicera japonica* and *L. macranthoides,* higher expression levels of *FNSII* were consistent with more flavone accumulation in flowers and flower buds, respectively (Wu et al., 2016).

Having demonstrated that *DvFNS* is the likely gene responsible for the color change in 'Nessho' ray florets, we tried to evaluate whether the expression change occurred only in the ray florets or also in leaves. The results for five leaf developmental stages (Fig.3.6a) showed that *DvFNS* expression was elevated in the young unexpanded leaves as compared to older leaves on the 1^{st} – 4^{th} nodes in RH-sensitive plants. This indicates temporal expression of structural genes in the flavonoid biosynthetic pathway in this cultivar. This concurs with a report by Yuan et al., (2013), in which temporal expression profiles of some putative structural genes in various tissues (leaves, stems, stamen, pistil, and petals) revealed a strong correlation with the formation of the red pigment in tulip petals. Temporal expression of *CHS* genes in the dahlia bi-color cultivar 'yuino' was characterized by Ohno et al. (2011b) and was detected in all petal developmental stages except in the young stage, with lower expression in white flower parts than in red parts.

In ‘Nessho’ the corresponding *DvFNS* expression in young unexpanded leaves with color fading in winter may indicate temporal *DvFNS* expression in young leaf tissues that corresponds with flavone accumulation in flowers. As the leave mature, *DvFNS* expression levels decline. In *Gentiana triflora* ‘Maciry’ leaves there is a similar relationship where apigenin accumulation and *FNSII* expression correspond (Nakatsuka et al., 2005). The positive correlation between *DvFNS* expression and the color changing index CFI (Fig. 6a) agrees with the reports of Deguchi et al. (2013), Deguchi et al. (2015), and Thill et al. (2012) who found that in purple and red dahlia cultivars a decline in the expression of *FNSII* resulted in a deeper color in majority of the black dahlia cultivars and vice versa in the other colored cultivars such as white, yellow, orange, pink, and red.

The relative *DvFNS* expression in single plants under controlled incubator conditions do not correlate with the color fading during winter. Whereas the pathway leading to flavones and anthocyanins diverges downstream at the level of flavanones, the flux into the individual pathways depends not only on the presence or absence of enzymes but also of relative activities of enzymes competing for the same substrate (Walliser et al., 2022) and the substrate available. The negative correlations between CFI and average weekly min temperature and *DvFNS* expression in young unexpanded leaves further confirm the role of temperature in the expression of the *DvFNS* gene, accumulation of flavone and consequently flower color change in the ‘Nessho’ dahlia cultivar. Similarly, *Pohlia nutans* FNSI possesses hypothermic enzyme characteristics, retaining relatively high enzymatic activity in low-temperature environments in Antarctica. This led to high flavone accumulation in moss under low-temperature conditions (Wang et al., 2020). Light is the other environmental factor that has influence on the *DvFNS* expression and

flavone accumulation in Nessho. This is demonstrated by the effects of different daylength on the gene expression of in young unexpanded leaves.

Some ‘Nessho’ plants are more sensitive to color fading than others in similar environmental conditions. This is evidenced by the varying color-fading indices at the flowering stage and by the difference in gene expression at the young leaf stage (Fig. 3.8). Other factors may be involved in the difference in the expression of *DvFNS* in RL- and RH-sensitive cultivars at low-temperatures. In chrysanthemum high temperature regulation on the accumulation was affected at the transcription level (Wang et al., 2021). Similarly, in ‘Nessho’ miRNA analysis using NG sequencing reveal post transcription *DvFNS* gene silencing in red flowers that may be triggered by seasonal temperature changed. MicroRNAs (miRNAs) are endogenous non-coding RNAs with a length of 19–24 nucleotides and play key roles in regulating the development of plants by responding to environmental stimuli at the post-transcriptional level Tang and Chui 2017; Tang et al 2024; Aydinoglu and Lucas 2019). The flavonoid synthesis is predominantly regulated at the transcriptional level through the MYB−basic helix-loop-helix (bHLH)−WD40 (MBW) complex in to both environmental and developmental stimuli (Petridis et al., 2016). In ‘Nessho’ red flowers the *DvFNS* silencing is similar to that reported in darker Kokucho cultivars (Ohno et al., 2016) although the developmental regulation of flavonoid accumulation and the response of the flavonoid synthesis pathway to abiotic stress (particularly low temperature) remains unclear.

Flavones/flavonols and anthocyanins were found to be the main contributors to the coloration of “High Noon” and “Roufurong” petals, respectively. In dahlia `Kokucho` and its purple mutant experiment result by Deguchi et al., (2013), suppression of *DvFNS* resulted to more synthesis and accumulation of anthocyanins hence darker colored

flowers. In this experiment, inductive conditions mimic winter conditions and they induce *DvFNS* gene expression in young leaves of CRH strain plants. High expression on *DvFNS* pauses a competition for substrates for downstream anthocyanin biosynthesis related genes hence lower anthocyanin synthesis and accumulation in dahlia `Nessho` ray florets (Okada et al., 2018). This corresponds with results in a study by Thill et al., (2012) comparing flavones in red and rare black dahlia cultivars. They reported that relative expression of flavonoid pathway structural genes was largely comparable or sometimes lower in black cultivars than of in the red cultivars. This meant that the increase in anthocyanin formation in the majority of black cultivars was not as a result of induced anthocyanin pathway, but a promoted flux of flavanones intermediates into anthocyanin due to reduced flavone formation. This affects the color intensity in various cultivars. In the present study, we demonstrated that this relationship between flower color and *DvFNS* expression may be used for selection of the plantlets with stable flower color.

Environmental conditions such as temperature and light influence the accumulation of flavonoids especially anthocyanin. This is mainly because of the effects of the environmental factors on anthocyanin biosynthetic related genes (Guo et al., 2008 and Li et al., 2019). The expression of the genes for anthocyanin biosynthesis is affected by ambient temperature and the effects varies from species to species. In many flowers species pigmentation is suppressed by high temperature, and this is enhanced by low light intensity (Ichimura et al., 2021). In petunia, the gene expression for *CHS* is increased by exposure to low temperatures (Shvarts et al., 1997). In roses exposure to high temperature reduces the expression of *DFR* and *CHS* whereas in lilies it reduces the expression of *LhDFR*, *LhF3H*, and *LhCHS* genes. In strawberries, high temperature significantly upregulates the expression of PAL1, ANS, 3GT, CHS2, UA5, DF4R, CHI, UA3GT2, and

UA3GHT5, causing increase in anthocyanin (Zhang et al 2019). In this experiment, the inductive conditions increased the relative expression of *DvFNS* genes in the leaves of relatively highly sensitive strain of `Nessho`. This indicates that the effect of environmental factors on *DvFNS* expression can be applied to the selection of plants with favorable agronomic characteristic, stable flower color expression in this study.

The inductive effects of these condition on *DvFNS* gene expression in young unexpanded leaves of `Nessho` may correlates with the color fading of the ray florets. This is shown by high relative *DvFNS* expression levels in young unexpanded leaves of plants that were classified as relatively high sensitive based on their flower color in winter.

The spatial accumulation of pigments; specifically the flavonoids in flowers, is regulated by a MYB-bHLH-WD complex by targeting the anthocyanin biosynthetic pathway. The various transcript factors offer spatial and temporally specificity, regulatory and inhibitory functions to the flavonoids biosynthetic pathway. For instant, R2R3-MYBs in subgroup 6 (SG6) acts as primary activators of anthocyanin while R2R3-MYBs potentially activates or represses anthocyanin biosynthesis depending on the specific domains contained in their C-terminus (Albert et al., 2014).

CHAPTER IV

RELATIONSHIP BETWEEN DAHLIA 'NESSHO' FLOWER COLOR, FLAVONE GENE EXPRESSION AND FLANOIDS ACCUMULATION

Introduction

Flowers color diversity has evolved over time in many ecological settings. The diversity in petal colors in many plant species is a function of factors such as pollinator-mediated selection, plant abiotic environment and physiological and genetic factors. This diversity arises from specialized metabolic pathways that generate various pigments. Consequently, there is a link between flower color and floral pigment production and accumulation. In most flowering plants, Flower coloration is primarily determined by the accumulation of natural pigments which include flavonoids, carotenoids and betalains (Wang et al., 2022). Flavonoids play a key role in exhibiting a diverse array of colors ranging from orange, blue (through anthocyanins), and yellow (through chalcones, aurones, and flavonols). This makes anthocyanins, aurones, chalcones and flavonols the predominant pigment groups responsible for flower color (Tanaka et al., 2009). The various combinations and concentration of pigment are responsible for the wide range of flower colors. Numerous reports have explained flower color at the molecular level through the regulation of the flavonoid biosynthetic pathway (Zhao et al., 2024).

In *Canna glauca* differential metabolite analysis showed a significantly increase in abundance of flavonoid compounds in the petaloid staminodes of the 'Erebus' cultivar compared to the wild type. The cyanidin, pelargonidin, peonidin and their derivatives concentrations were higher Erebus cultivar whereas the wild type accumulated higher malvidins. The difference in the pigment contents accounted for the variation in color Zhao et al., 2024. The modulation of gene expression and enzymatic activities that drive

the flavonoid biosynthesis is essential in determining the types and quantities of flavonoids generated in different plant tissues. Enzymes catalyse specific reactions and give rise to specific pigmentation patterns such as stripes, spots, bi-colors, color fading and loss of color; ultimately determining the flower color and pattern in plants. The mechanism of modulation of these pigmentation patterns varies among plant species hence there exist no universal rule. The lability of anthocyanin pigmentation as a result of evolution, combined with its ecological and ornamental importance, has led to the widespread study of its biosynthesis and regulation hence has become a model for investigating trait evolution. For example, flower color breeding that allows the introduction of specific genes responsible for pigment production has enables the creation of certain traits in flowers such as blue species of roses and chrysanthemums (Davies & Schwinn, 2010; De Folter et al., 2015).

The diversity and changes in colors in many plant species depends on the chemical structure of the anthocyanin compound which includes the number of hydroxyl groups attached to the benzene ring, and the level of glycosylation and acylation (Marin-Recinos & Pucker, 2024). The interaction with co-pigments like flavonols and flavones is an important factor for the stabilization of anthocyanins in plants (Davies et al., 2003). All structural genes in the anthocyanin biosynthesis pathway must be functional and active to achieve anthocyanin pigmentation. Mutations in any regulator or enzyme encoding gene of the flavonoid biosynthesis can affect the coloration (Marin-Recinos & Pucker, 2024). Additionally, substrate competition at different branches of the flavonoid biosynthesis between the enzymes can impact anthocyanin synthesis and accumulation hence the color perceived(Rausher, 2008; Wessinger & Rausher, 2012).

Materials and methods

4.1 Plant materials

Dahlia 'Nessho' plants were re-classified based on the color fading index during the winter 2023/2024. In the 'Nessho' flower, the degree of fading can be determined at the flower bud stage before the flower opens (Fig. 4.1). During the flower opening stage, approximately 30 cm long ray (Fig. 4.2 a) florets from RH and RL plants were duplicated; one sample was for RNA extraction and the other for flavonoid extraction and analysis. Leaves were sampled from the same plants from which the ray florets were sampled. The leaves sampled were designated L1, L2, L3 and L4 based on their location on the flower stem. L1 was the topmost leaf (next to the flower head) and L4 was the lowest stem on the stem (Fig 4.2 c). The leaf sample was split in to two (a sample for flavonoids extraction and a sample for the other for RNA extraction). The sample for total RNA extraction was put in Liquid Nitrogen immediately whereas the samples for flavonoid extraction were weighed before freezing and storage. Approximately 100mg of the sample was used. This was equivalent to two or three ray florets depending on the size.

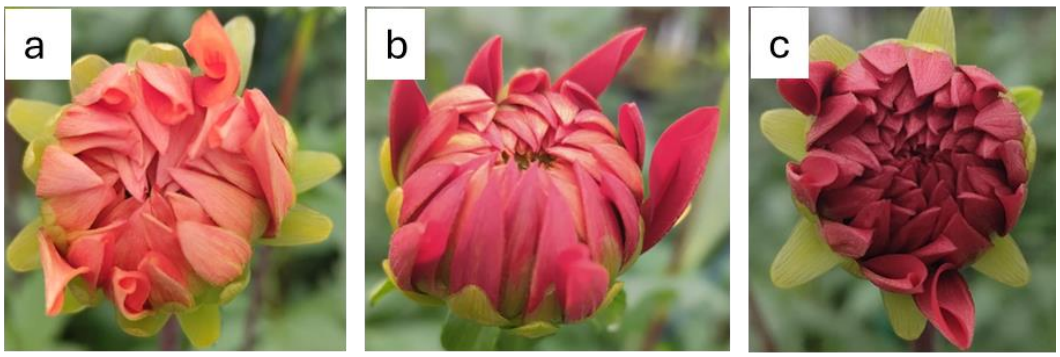

Figure 4.1: Nessho flowers exhibiting different degrees of fading before flower opening. (a) fully faded, (b) partially faded flower (c) non-faded flower.

4.2 Measurements of fresh flower color

The color of the fresh flowers for each degree of color fading (CFI 1 to CFI 4) were evaluated using their CIE L*a*b* chromaticity values recorded using TES-3250 color meter. Three fully opened ray florets (Fig 4.2 b) were placed overlapping each other on a white background and color measured.

Figure 4.2: 'Nessho' ray florets used for; (a) pigment extraction and (b) for CIE Lab chromaticity value measurement.

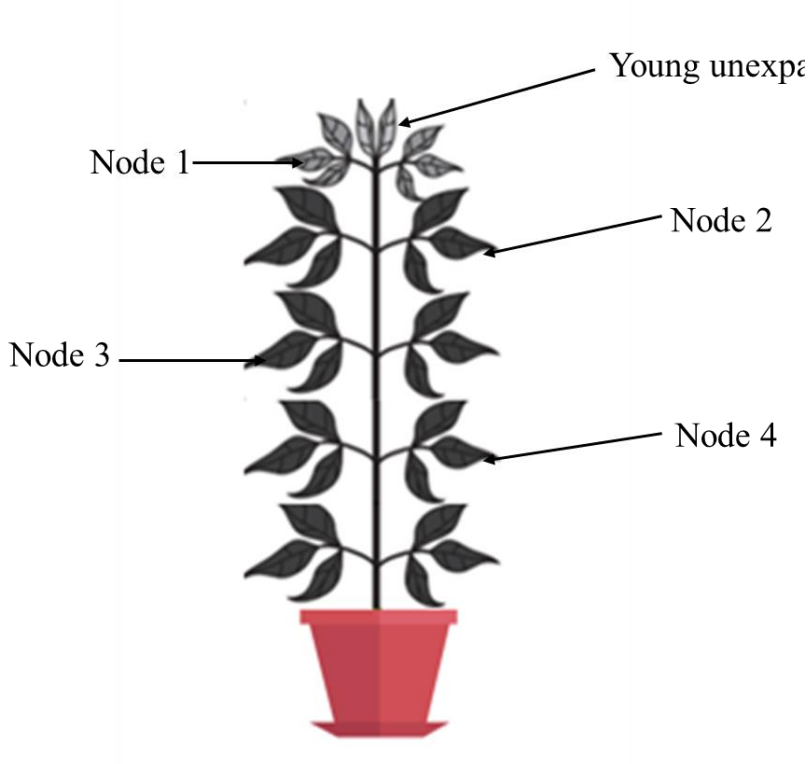

Figure 4.3: Leaves used for HPLC analysis of the pigments in the leaves of different maturity levels

4.3 Pigment extraction for HPLC

Approximately 100mg samples were crushed in liquid nitrogen using sterile mortar and pistil. 1ml aqueous methanol acetate solution (acetic acid: methanol: distilled water) in the ratio of 1:5:5 v/v was added to the sample and stirred until the sample dissolve. The dissolved sample was transferred into 1.5ml Eppendorf tubes and centrifuged at 14000rpm at 4°C for 15 minutes. The liquid phase was dispensed into petri dishes and air dried in a draft chamber. The dry samples were tightly sealed with parafilm and wrap with aluminium foil to avoid degradation by UV light and used for HPLC analysis at Kyoto university.

4.4 HPLC analysis

High-performance liquid chromatography (HPLC) was conducted at the Kyoto University. The samples were hydrolysed in a hydrolysis buffer (10% hydrochloric acid and methanol mixed in a ratio of 1:1 v/v) for two hours. A LC10A system, Shimadzu, Kyoto, Japan was used to separate hydrolysed pigments. The hydrolysed solutions were used as crude aglycones. HPLC was conducted on an HPLC system with a C18 column (Nihon Waters K.K., Tokyo, Japan), and peaks were detected with a photodiode array detector. The detection wavelength was 350 nm for flavones and 530 nm for anthocyanidins. Elutant A was 1.5% phosphate dissolved in water and elutant B was 1.5% phosphate, 20% acetic acid, and 25% acetonitrile dissolved in water. The analysis period for each sample was 45 min and comprised 0 min with 20%, 40 min with 85%, and 20% with elutant B at a flow rate of 1 ml min-1 at 40C.

Results

During winter 2023/24 dahlia ‘Nessho’ flowers growing in the East greenhouse of the Okayama University experimental area exhibited color fading as it was report in earlier cold seasons (winter 2021/22 and winter 20222/23). The range of flower color in a hundred harvested flowers harvested during that season was as shown in Fig. 2.1. The degree of color fading changed in individual plant indication a high level of instability within the population. Some plants that were classified as RL in winter 2021 exhibited high fading in winter 2023. HPLC analysis reveal a corelation between absorbance in the ray florets with that of top young leaves.

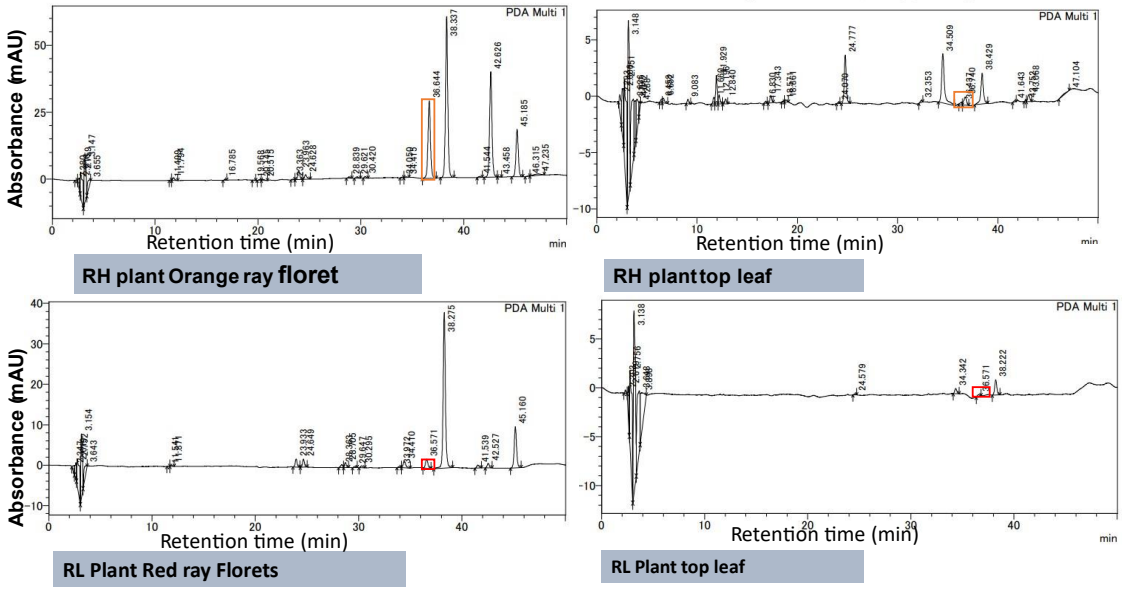

Figure 4.4: Chromatograms showing peaks obtained for the mixture of various phenolic compounds at 350 nm.

In this study, the orange ray florets of 'Nessho' accumulated more flavones than the red ray florets. The absorbance in the topmost leaf was higher in RH plants at 350nm was lower in RL plants (Fig 4.4). This shows that the flavones accumulation trend was similar in leaves as in the ray florets. In red flowered plants (RL) there is little to no absorbance in older leaves at 350nm (Fig 4.4). This indicates little or no accumulation of flavones in the older leaves of RL. There was absorbance in RH plant forth leaf (oldest leaf) however the area under the graph was very small indicating low flavone content and the leaf matures.

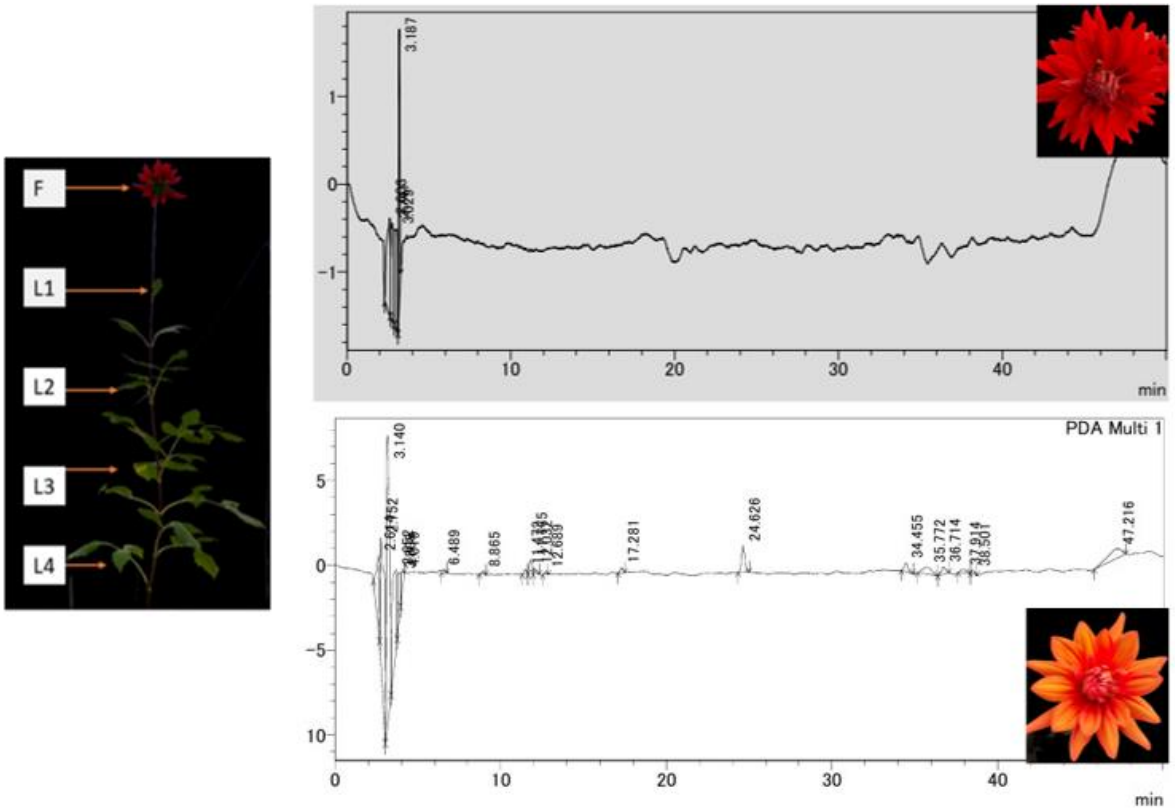

Figure 4.5: HPLC chromatograms at 350 nm (left) and 530 nm (right) for the leaves of RL plants (top) and RH plants (bottom).

In the ray florets of RH and RL plants there is absorbance at both 350nm and 530nm (Fig 4.5). This indicates accumulation of both anthocyanins and flavones in the ray florets. The co-pigmentation of the two flavonoids results in the color intensity of the ray florets. There was significant difference in the amount of flavone that accumulates in the young leaves of RH and RL plants (Fig 4.7) $P<0.005$.

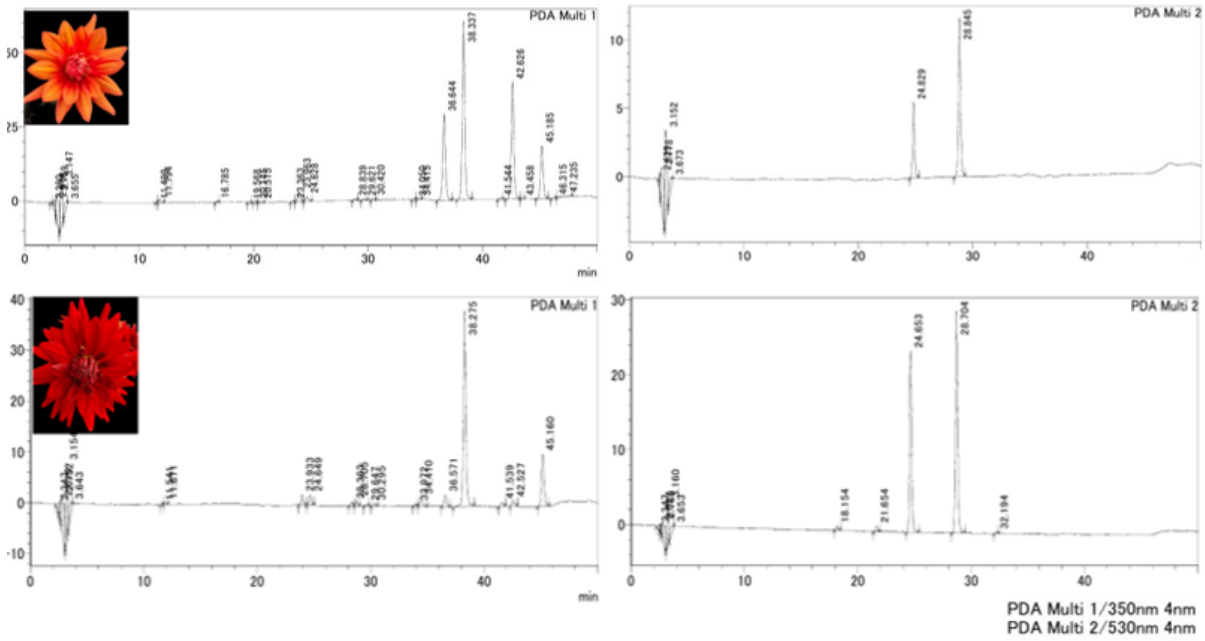

Figure 4.6: HPLC chromatograms at 350nm (left) and 530nm (right) in the ray florets of RH and RL plants.

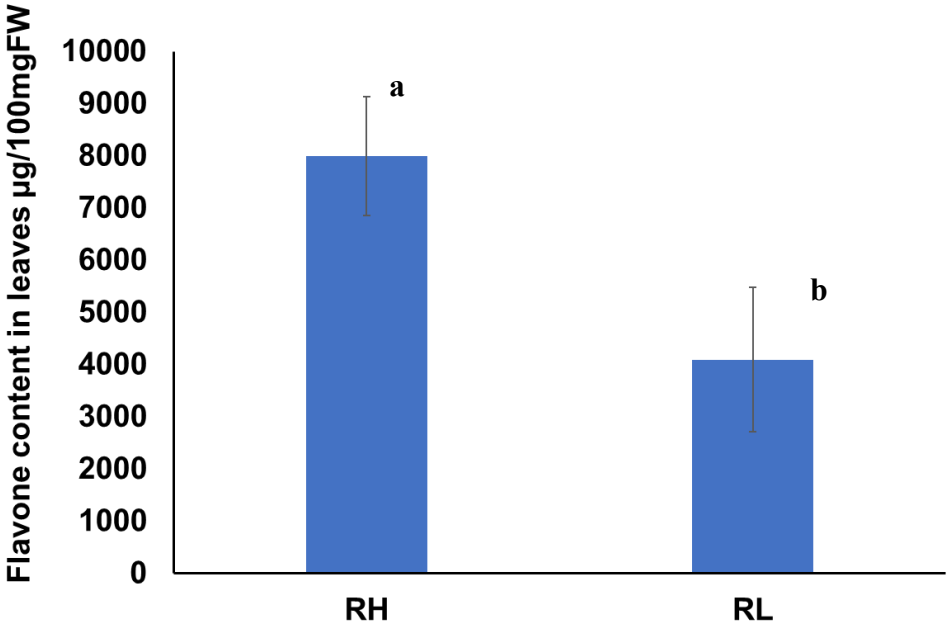

Figure 4.7: Flavone content in young leaves of Relatively high sensitive (RH) plants and relatively low sensitive (RL) plants. letters above the error bar indicate significant difference P<0.005.

Discussion

Flower color being one of the most important quality traits in ornamentals, requires high levels of stability. Higher flower stability over time like in Hungarian roses (Boronkay et al., 2009) is desirable and leads to more unform flower production and easier selection process for seed/ planting materials. However, many ornamental plants exhibit high levels of flower color instability as influenced by the prevailing environmental conditions. In the current face of rapid climate plant populations are responding with plastic and adoptive change in phenotypes such as color change (Parmesan, 2006). Across organisms, pigmentation phenotypes can strongly affect the response to temperature and light (Sullivan & Koski, 2021). Thus, pigmentation is a key selection target by altered abiotic environments associated with climate change. In flowering plants, the pigmentation can vary dramatically within a population with the variability being a key indicator of overall plant performance (Rausher, 2008). Flowers in which flower color plasticity/ variation is reported in response to change in environmental conditions include chrysanthemums, *Viola cornuta, Moricandia arvensis*, herbaceous peony, Eustoma (Fukuta & Nakayama, 2008), dahlia 'Yuino' (Ohno et al., 2016), Ipomoea tricolor (Yoshida et al., 2009) and Red-flowered elepidote rhododendrons exhibit color instability over time.

Flavonoid accumulation is important for flower color determination in many ornamental plants. For instance, in chrysanthemum, anthocyanins are crucial determinants of flower color, together with carotenoids(Kim et al., 2018). Anthocyanin and flavone co-pigmentation influences the flower color intensity with higher flavones resulting in paler flower colors. Flavone C-glucosides have shown strong co-pigmentation properties that lead to enhanced color (Mizuno et al., 2015; Yabuya et al.,

1997). Flavones also play important roles in plant–micro-organism interaction hence engineering flavone biosynthesis in plants might have a wider impact than flower colour modification. This could be evident in the temporal or spatial accumulation in other plant parts

In herbaceous peony (*P. lactiflora*) the flower color intensity varies with the content of flavonoids accumulation. The accumulation corelates with the developmental stages of the flower (Zhao et al., 2012). In dahlia 'Yuino' cultivar flower color and pigment content corelates with flavonoids content in the leaves (Ohno et al., 2016). In dahlia red and black cultivars darker colored flowers accumulated more anthocyanin and the lighter flowers accumulated more flavones (Deguchi et al., 2013). In 'Nessho' anthocyanins (pelargonidins and cyanidins) accumulates in higher amounts in red flowers while the color-changed orange ray florets accumulated more apigenin and luteolin, both of which are flavones. This finding corresponds with earlier reports on the dahlia 'Kokucho', including the original red and its purple mutants (Deguchi et al., 2015). There is a complex relationship between flavonoid pigment accumulation in flowers and leaves. This relationship is influenced by environmental factors and plant developmental stages. Flavonoids play crucial roles in both reproductive and vegetative tissues, serving protective and adaptive functions. In leaves, flavonoids, including anthocyanins and flavones, provide protection against UV radiation, pathogens, and other stressors. For instance, flavonoid accumulation in flowers and leaves of *Silene littorea*, vary in coloration and is influenced by environmental conditions(Del Valle et al., 2018). In chrysanthemum flavonoid pigment accumulation differs between flowers and leaves in response to light, with greater impact on anthocyanins in flowers(Hong et al., 2016). This

is similar to ‘Nessho’ where there is seasonal difference in accumulatio flavonoid in flowers and leaves with greater effect being in flowers.

CHAPTER V

GENERAL DISCUSSION, CONCLUSION AND RECCOMENDATIONS

Pigments biosynthesis and accumulation play a crucial role in the determination of color in many ornamental and other plant species. The pigments that primarily determine color include anthocyanins, flavonoids and their derivatives. The specific combination and concentration of pigments contribute to a wide range of flower color. Color expression is determined by a combination of factors; these include the type, quantity and stability of pigments, cell pH, co-pigmentation, and translocation of the pigments from the site of synthesis. Various environmental factors such as light and temperature can affect flower color development. In fruits, bagging treatments have been shown to inhibit the accumulation of anthocyanins and the expression of structural genes in the anthocyanin biosynthetic pathway. Upregulation of the specific genes is accompanied by elevation in anthocyanin accumulation when the fruits are debagged and exposed to sunlight (Wei et al., 2011). Temperature is another major environmental factor influencing anthocyanin accumulation in fruits and flowers. In many plants species high-temperature down-regulates the expression of genes involves in the anthocyanin biosynthesis hence reducing the synthesis and accumulation of anthocyanins(Li & Ahammed, 2023). High temperature also inhibits anthocyanin transport as well as the stability of anthocyanin biosynthetic enzymes, which eventually impacts anthocyanin synthesis (Mori et al., 2007). On the other hand, low temperature induces anthocyanin accumulation and up-regulates the transcript levels of PAL, C4H, 4CL, CHSs, CHI, F3H, F3′5′H, FLS, DFR, ANS and UFGT (Carmona et al., 2017). In dahlia ‘Nessho’ high temperature enhance anthocyanin accumulation and low temperature repress the synthesis and accumulation of anthocyanin hence the seasonal flower color fading.

Flower color fading is a complex phenomenon influenced by various biochemical and genetic factors. Recent studies have identified key mechanisms underlying this process, particularly focusing on flavonoids synthesis and degradation and phytohormonal changes. In rapeseed petals, a 60% decline in anthocyanin content correlated with the downregulation of biosynthetic genes and increased phytohormones like salicylic acid (Huang et al., 2024). Similarly, petal fading in Peony 'Coral Sunset' involves rapid decline in carotenoids and anthocyanins post-opening, with gene expression changes indicating inhibition of pigment synthesis and accelerated degradation mechanisms (Zhang et al., 2023). In lotus petals color fading is influenced by both reduced anthocyanin biosynthesis and active degradation processes as influenced by prevailing internal or external environment (Liu et al., 2022). Thus, besides the regulation of flavonoids via the biosynthesis pathway, the stability and degradation progress of flavonoids affect their total content in plants. The flower color fading in dahlia is determined by the synthesis and accumulation of flavones in the ray florets. The flavones act as co-pigments with anthocyanins leading to reduction in color intensity and increase in lightness. Flavone biosynthesis in ‘Nessho’ is regulated by *DvFNS* expression which encodes for the enzyme flavone synthase (FNS). *DvFNS* expression is regulated by environmental factors (temperature and light) at post transcription level. Higher activity of FNS competes for substates with ANS leading to higher flavones synthesis and accumulation at low minimum temperature (below 10℃). Color changing in hue from red to orange in winter is a result of varying ratio of anthocyanins and flavone pigments accumulation. Temperature influences color change/ fading in several plant. In chrysanthemums, high temperatures significantly affected color stability with certain

varieties exhibiting enhanced fading due to decreased enzymatic activity related to anthocyanin synthesis (Wang et al., 2024).

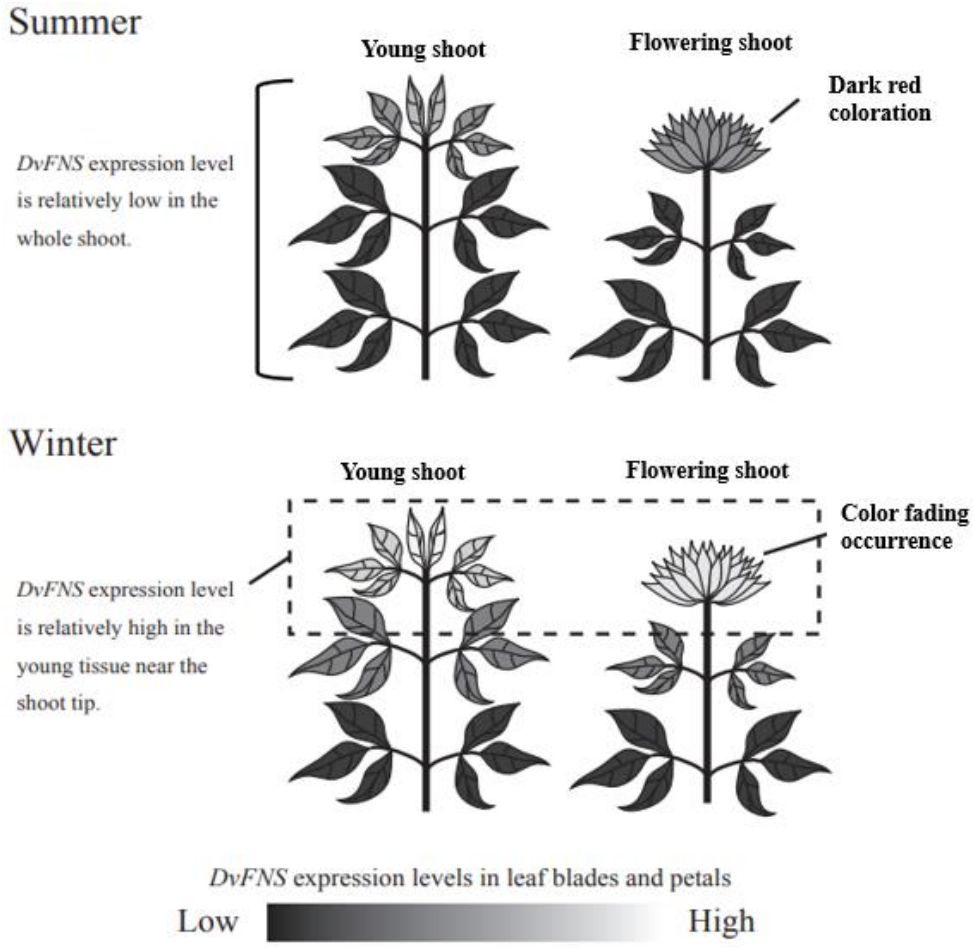

Figure 5.1: Schematic representation of the *DvFNS* expression in a RH sensitive plant in summer and winter.

To enhance the efficiency of quality flower production without waiting for flowering, researchers have explored various selection criteria that expedite the breeding process. These approaches are particularly crucial for geophytic herbaceous perennials, which typically have lengthy generation times and for plant species whose certain characteristics are enhanced by specific environmental conditions. Accelerate selection criteria such as use of growth metrics that are directly linked to flower quality such as

leaf number, stem elongation, and inflorescence height allows breeders to identify genotypes that will flower sooner, thus shortening the breeding cycle. Temporal gene expression also offers a promising approach for selecting flowering plants more efficiently, bypassing the need to wait for visible flowers. This method leverages the regulation of gene expression at specific developmental stages under specific environmental conditions, allowing for quicker assessments of genetic traits. In many plant species, there is relationship between flavonoid accumulation in leaves and flowers and the levels and types of flavonoids differ significantly between these organs. Flowers accumulate the highest levels flavonoids levels while leaves also accumulate significant amounts indicating a positive relationship between accumulation in various plant organs. This correlation In dahlia 'Nessho' there is a correlation between color fading occurrence, *DvFNS* expression, flavone content in both ray florets and in young leaves and prevailing environmental condition. The difference in *DvFNS* expression levels and flavones contents between the relatively highly sensitive and relatively low sensitive plants after inductive condition treatment and in winter respectively is an indication that the level of expression of this gene and flavone content in leaves can be used as a selection tool for non-fading strains. This selection method shorter compared with selection the visible flower color. Using this method selection will be faster and efficient. In the future, identifying the genetic differences between RL- and RH-sensitive plants that cause different *DvFNS* expression levels will lead to the development of genetically uniform cultivars and high-quality flower production.

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Appendices

RNA extraction protocol: Modified CTAB and LiCl protocol

A 100 mg portion of the plant material was taken and crushed with a mortar and pistil in liquid nitrogen. 1000 µL of prewarmed (at 65 °C) extraction buffer is added to the crushed plant material. . The crushed sample was transferred to 2 mL Eppendorf tubes, and 30 µL β-mercaptoethanol was added and vortexed for 1 min. Then, it was incubated at 65 °C in a water bath for 10 min and centrifuged at 14000 rpm for 5 min at room temperature. The supernatant (approximately 600–1000 µL) was taken to another Eppendorf tube and an equal volume of chloroform–isoamyl alcohol (24:1) was added. It was vortexed for 1 min and centrifuged at 14000 rpm for 10–15 min (at room temperature). Again, the supernatant was obtained (approximately 500–900 µL), to which half the amount of lithium chloride (4M) was added, and incubated overnight at 4 °C. The stored samples were centrifuged at 14000 rpm for 15 min at 4 °C. The supernatant was decanted, and the pellet was kept and washed with 500 µL of 70% ethanol at 14000 rpm for 15 min at 4 °C. Ethanol was carefully remove and the pellet air-dried using a vacuum centrifuge for 5 minutes. The pellet was resuspended in 30–50 µL TE buffer.

Electrophoretic Analyses of Isolated RNA

To check the integrity of RNA, 1 µL of total RNA mixed with 1 µL of gel loading dye was loaded in 1.5% agarose gel having 1 µL of 10 mg/mL ethidium bromide. The gel was made and run in 1X TAE [from a stock 50X TAE] at 100 V for 30 min and was observed in a UV Trans illuminator and photographed by AlphaDigiDocTM.

Spectrophotometric Analyses of Isolated RNA

The quantity and quality of isolated RNA were assessed by measuring the optical density (OD) at 260 and 280 nm. Purity (260/280 ratio) and contaminants (260/230 ratio) were checked using a Thermo Scientific, Nanodrop 2000c UV Spectrophotometer.

Small RNA extraction Protocol

CTAB and miRNeasy Mini Kit RNA extraction protocol

Composition of the extraction buffer:

100 mM Tris, pH 8.0,

2% (w/v) N-cetyl-N,N,N-trimethylammonium bromide (CTAB),

30 mM ethylenediaminetetraacetic acid (EDTA),

2 M NaCl,

2% (w/v) polyvinylpolypyrrolidone (PVPP),

0.05% (w/v) spermidine

2%(v/v) β-mercaptoethanol)

1. Prewarm the extraction buffet at 42 °C in a water bath
2. Aliquots of 1 ml RNA extraction buffer in 1.5ml tubes
3. Macerate plant tissue to a fine powder in liquid nitrogen with a mortar and pestle.
4. Add approximately 3–4 spatula tips (App100 mg) tissue to the 1 ml extraction buffer in tubes. Tightly fasten the tube caps and vortex to suspend the tissue in the extraction buffer.
5. Incubated the samples at 42 °C for at least 40 min with intermittent vortexing (6-7 time).
6. Centrifuge for 15 min at top speed (approximately 13 000 × *g*) at 4 °C.
7. Remove the nucleic acid-containing aqueous phase and put into a fresh 2 ml tube.
8. Add an equal volume of chloroform:iso-amyl alcohol, mix well by vortexing well and centrifuging for 15 min at 4 °C
9. Remove the final aqueous phase into a 1.5 ml tube and add modified extraction buffer (100 mM Tris, pH 8.0, 2% CTAB, 30 mM EDTA, 2 M NaCl) to a final volume of 1 ml if

the aqueous phase volume was 700 µl or more; or to a final volume of 800 µl if the volume of the aqueous phase was less than 700 µl.

10. After the addition of the buffer, add 10 M LiCl to a final concentration of 2 M. Allow RNA to precipitate at − 20 °C overnight.
11. After RNA precipitation, centrifuge the samples for 20 min at 13000rp at 4 °C to remove the aqueous phase without disturbing the RNA pellet.
12. Recover RNA pellet by dissolving in QIAzol Lysis reagent for miRNeasy Mini Kit isolation methods.
13. Proceed as per the miRNeasy Mini Kit manufacturer`s instructions

Flavonoids extraction protocol

1. Crush the sample in liquid Nitrogen using mortar and Pistil
2. Add 1ml of aqueous methanol acetate solution (acetic acid: Methanol: distilled water 1:5:4 v/v per 100mg of sample and stir occasionally until dissolved.
3. Transfer to a 1.5ml tube
4. Incubate overnight at 4 ºC
5. Centrifuge the solution at 14000rpm at 4 ºC for 15 minutes, the liquid phase is dispensed into a petri dish and dried in a film chamber.
6. Samples were sealed and covered with aluminium foil and kept in dark conditions at room temperature until HPLC analysis was done in Kyoto University.

HPLC analysis protocol

For pigment hydrolysis, extracted solutions were evaporated, redissolved in 2 ml of 20% hydrochloric acid, boiled, and used as crude aglycones. HPLC analysis was performed using an LC10A system (Shimadzu, Kyoto, Japan) with a C18 column (Nihon Waters K.K., Tokyo, Japan) maintained at 40 ºC and a photodiode array detector. The detection wavelength was 350 nm for flavones, 380 nm for chalcones, and 530 nm for anthocyanins. Elutant A was 1.5% phosphate dissolved in water and elutant B was 1.5% phosphate, 20% acetic acid, and 25% MeCN dissolved in water. Analysis was performed at a flow rate of 1 ml min 1 and column temperature of 40 ºC, using a mobile phase gradient starting at 20% B to 85% B over 40 min with 5 min re-equilibration at 20% B. As standards for the determination of flavonoids, commercially available naringenin, apigenin, and luteolin (Wako Pure Chemical Industries Ltd, Osaka, Japan) as well as HPLC-separated and HPLC-purified hydrolysed cyanidin, pelargonidin.