Dissertation

Fruit Pigment Biogenesis in Raspberry Cultivars: Characterisation of Anthocyanin and Carotenoid Biosynthesis

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Abstract

Raspberry (*Rubus idaeus* L.) is a nutrient-rich fruit crop containing high yields of natural bioactive compounds, such as flavonoids and carotenoids, which are known to have potential health benefits in humans. Various colored raspberry fruits offer a unique possibility to study the genetics of pigment biosynthesis in this important soft fruit. Anthocyanidin synthase (*Ans*) catalyzes the conversion of colorless leucoanthocyanidins to colored anthocyanidins, a key step in biosynthesis of anthocyanins. The current study revealed that reduced anthocyanins in yellow raspberry ("Anne") were due to loss of function mutation or inactive *ans* allele. A 5-bp insertion (ans^{+5}) in the coding region creates a premature stop codon resulting in a truncated protein of 264 amino acids, compared to 414 amino acids of wild type ANS of red raspberry "Tulameen". Apparently, the mutated *ans* gene transcripts are suppressed as a secondary effect because of nonsense-mRNA mediated decay (NMD). Functional characterization and complementation of *Ans*/*ans* alleles *in planta* provide strong proof of inactive ANS protein of "Anne" as compared to the functional protein of "Tulameen". Further, molecular screening of various colored raspberries for *Ans/ans* alleles indicated that most of the yellow and orange fruiting raspberries contain various types of *ans* mutations that cause frameshifts and initiate premature stop codons leading to loss of function of the ANS proteins. In anthocyanin-free varieties, yellow/orange fruit pigmentation seems to exist as a net result of accumulation/degradation of specific carotenoids at ripe stage. The putative carotenoid pathway genes from *Rubus* "Anne" inserted in standard expression cassettes along with plasmids capable of generating different carotenoid precursors resulted in the successful characterization of the pathway genes via complementation in a bacterial host. It suggests that accumulation of *β*-branch carotenoids like *β*-carotene and xanthophylls (lutein) are the principal components that provide yellow coloration to anthocyanin-free raspberry fruits. Taken together, molecular and functional characterization of the carotenoid pathway genes helped to predict a preliminary pathway map for pigmentation in non-red (yellow, orange) fruiting raspberries.

Zusammenfassung

Die Himbeere (*Rubus idaeus* L.) ist eine Beerenfrucht mit hohen Gehalten an natürlichen, bioaktiven Verbindungen wie Flavonoiden und Carotinoiden, von denen bekannt ist, dass sie verschiedene förderliche Eigenschaften auf die menschliche Gesundheit haben. Himbeerfrüchte mit unterschiedlichen Farben bieten zudem eine einzigartige Möglichkeit, die Genetik der Pigmentbiosynthese in dieser wichtigen Frucht zu untersuchen. Die Anthocyanidinsynthase (ANS) katalysiert die Umwandlung von farblosen Leukoanthocyanidinen in die farbigen Anthocyanidine. Dies ist ein wichtiger Schritt in der Biosynthese der Anthocyane. Die aktuellen Untersuchungen ergaben, dass die fehlenden Anthocyane in der gelben Himbeersorte "Anne" auf einen Funktionsverlust der ANS bzw. ein inaktives ANS-Allel zurückzuführen sind. Eine 5-bp-Insertion (ans+5) im kodierenden Bereich führt dabei zu einem vorzeitigen Stopcodon, das wiederum zu einem verkürzten Protein von nur noch 264 Aminosäuren führt, verglichen mit 414 Aminosäuren der Wildtyp ANS aus der roten Himbeere "Tulameen". Es liegt nahe, dass die mutierten und genetisch veränderten Transkripte als Nebeneffekt des Nonsense-mRNA vermittelten Zerfalls (nonsense-mediated decay, kurz NMD) unterdrückt werden. Die funktionelle Charakterisierung von Ans/ans-Allelen mittels Komplementierung von Mutanten *in planta* lieferte einen eindeutigen Beweis für das inaktive ANS-Protein von "Anne" im Vergleich zum funktionellen von "Tulameen". Weiterhin zeigte ein Screening der Ans/ans-Allele in verschiedenfarbigen Himbeeren, dass die meisten gelben und orangefruchtigen Genotypen verschiedene Arten von Mutationen enthalten, die ebenfalls Frameshifts verursachen und damit vorzeitige Stopcodons initiieren, was vermutlich auch hier zu einem Funktionsverlust der Proteine führt. Bei anthocyanfreien Sorten ist die gelb-orange Fruchtfarbe auf die Akkumulation bzw. den Abbau bestimmter Carotinoide während der Reife der Beeren zurückzuführen. Die wichtigsten Carotinoid-Biosynthesegene von "Anne" wurden in Standard-Expressionskassetten kloniert, und zusammen mit entsprechenden Plasmiden, die in der Lage sind, verschiedene Carotinoid-Vorstufen zu synthetisieren, zur funktionellen Expression in Bakterien gebracht. Die nachgewiesene erfolgreiche Synthese der jeweiligen Produkte ist ein Indiz für die enzymatische Aktivität der jeweiligen Proteine. Die Akkumulation von β-Carotinoiden, wie β-Carotin und Xanthophylle (Lutein), deutet darauf hin, dass diese die Hauptkomponenten in den anthocyanfreien Himbeerfrüchten und verantwortlich für die gelbe Färbung sind. Diese molekulargenetische und funktionelle Charakterisierung der Gene des Carotinoidwegs hat es ermöglicht, ein vorläufiges Schema der Biosynthese bei nicht roten (gelb, orange) fruchtenden Himbeeren zu erstellen.

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1. INTRODUCTION

1.1. Raspberry – *Rubus idaeus* L.

Raspberries, belonging to the Rosaceae family, are commercially important berry fruits, particularly in Europe and North America. They belong to the genus *Rubus* which contains more than 700 species worldwide. Raspberries stand among the most popular berries and are often found growing as wild plants bearing only small fruits. Commercial raspberries are consumed as fresh fruits and/or as processed products in the form of jams, jellies, syrups, food supplements and wines (Kim and Padilla-Zakour, 2006; Pantelidis et al., 2007; Tokuşoğlu and Stoner, 2011). The domestic and international market value of raspberry is increasing almost every year, for example during 2015 to 2016 in Italy (2,238 to 2,245 tonnes), Europe (445,762 to 559,697 tonnes) and worldwide (676,447 to 841,899 tonnes) as indicated in Figure 1 that is equivalent to 24,5% increase in production globally (FAOSTAT, 2016).

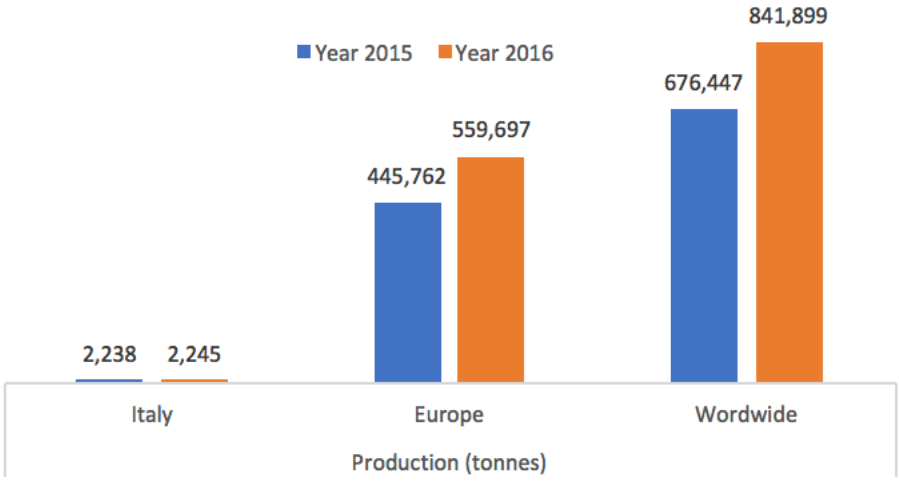


Figure 1: Production of raspberries in Italy, Europe and wordwide from 2015 to 2016 (FAOSTAT, 2016).

Raspberries are considered nutrient-rich fruit crops, being high in antioxidants, dietary fiber, ascorbic acid and having considerable amounts of manganese, folic acid, magnesium, copper, potassium and iron (Rao et al., 2010; Castilho Maro et al., 2013; Mejia-Meza et al., 2013; Bredsdorff et al., 2015; Kim et al., 2016). They also contain other key nutrients, such as β-carotene, thiamine, riboflavin, niacin, calcium, zinc, etc. (Balch CNC, 2003). In humans,

raspberry extracts are considered to facilitate in managing type II diabetes and hypertension, retarding the growth of cancer cells, reducing risks of cardiovascular and Alzheimer diseases, protecting skin damage caused by UV rays, reducing signs of aging, etc. (Cheplick et al., 2007; God et al., 2010; Burton-Freeman et al., 2016; Szymanowska et al., 2018). Raspberry ketone supplements are available which are considered to have positive effects for weight loss (Lopez et al., 2013). Further, consumption of berries is associated with a healthy lifestyle and there is increasing popularity and interest in natural bioactive compounds in the human diet.

1.2. Plant metabolites and pigments

Plants synthesize a variety of natural compounds, e.g. polyphenols and terpenoids, with certain benefits and advantages for the plant itself. There are two types of metabolites produced in plant cells, the primary metabolites and the secondary metabolites (nowadays also named specialised metabolites), as shown in Figure 2. Primary metabolites, such as building blocks for carbohydrates and proteins, are directly involved in basic physiological processes and metabolic reactions essential for growth, development and reproduction of plants (Schopfer, 1989; Plaxton and McManus, 2008).

In contrast, secondary metabolites, which are a characteristic feature of plants – there are 100,000 - 200,000 estimated compounds occurring in nature – play a key role in plant protection in response to biotic and abiotic stresses (Isah, 2019). They also contribute to many organoleptic characteristics as well as plant growth, development and adaptation to various environmental influences, but are not essential for the basic processes of life (Yang et al., 2018; Pott et al., 2019). Secondary metabolism is always linked to primary metabolism (Pott et al., 2019). Metabolism of phenylalanine offers an excellent example where carbon flow from photosynthesis to the biosynthesis of phenylpropanoids represents interconnection between primary and secondary metabolism (Pascual et al., 2016). As a primary metabolite phenylalanine is used as protein building block and phenylalanine-derived chemicals (e.g. phenylpropanoids) are crucial for plant growth, development and reproduction, but they (e.g. flavonoids, stilbenes) also induce secondary responses against various kinds of biotic and abiotic stresses. Secondary metabolites play an indispensable role by maintaining a balance between the plant and its surrounding environment. The major groups of secondary metabolites are alkaloids, polyketides, terpenoids and polyphenols (as presented in Figure 2). Here, important aspects of various plant metabolites in general as well as associated with raspberries will be described focussing especially on polyphenols and carotenoids, respectively.

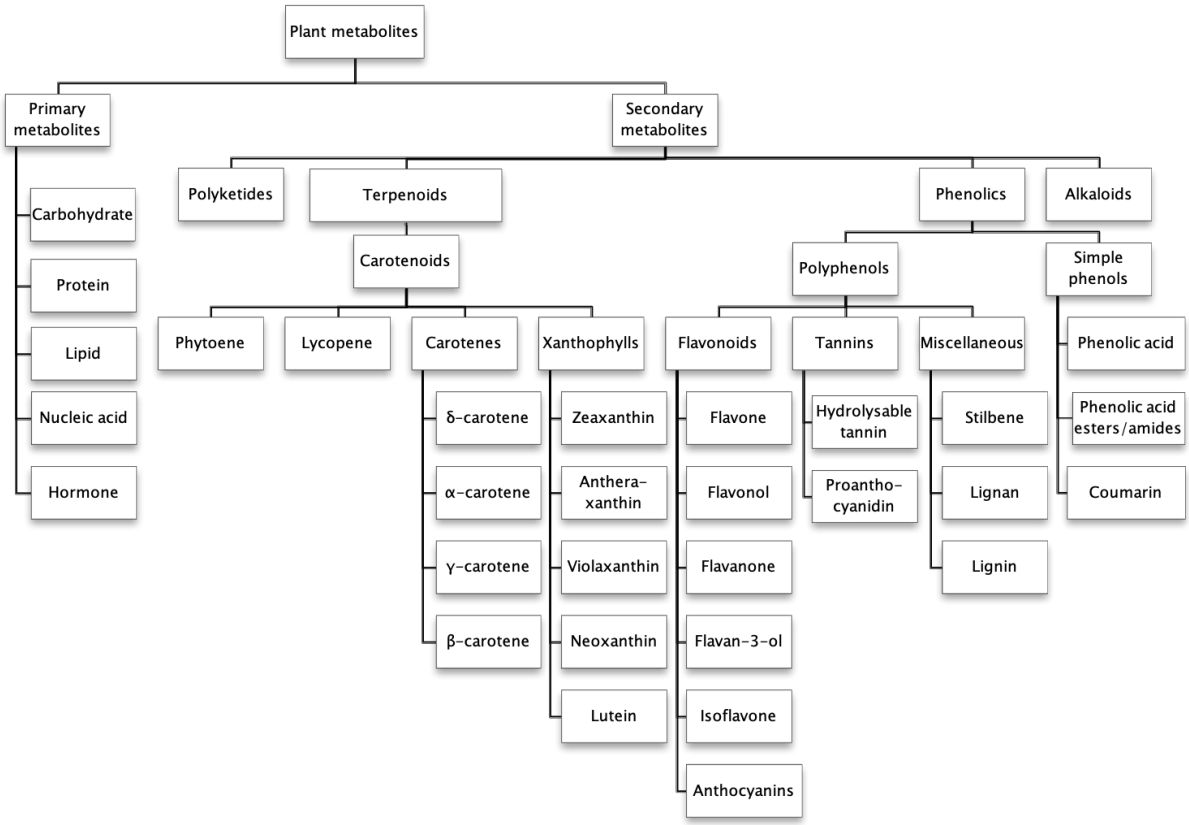


Figure 2: Major classes of metabolites in plants

Pigments, the colored substances, are widely distributed in nature from the simplest to more complex organisms. Chemically pigments can be categorized into four groups: natural, semi-synthetic, synthetic and inorganic. Mostly synthetic/semisynthetic pigments are used in food, fabric, paint and pharmaceutical industry as colorants and other purposes, for example, to enhance heat and light stability in paints and coatings (Muller, 2011). However, the interest and demand for natural pigments produced by living organisms, such as plants, cyanobacteria and algae have increased due to possible toxicity concerns of synthetic ones (Delgado-Vargas and Paredes-Lopez, 2002; Dufossé, 2016; Carle and Schweiggert, 2016). Pigments in nature make our environment colorful and beautiful. These pigments not only impart color to most flowers, fruits and seeds, but also play important biological and physiological functions in plants (Bradshaw and Schemske, 2003; Avalos and Carmen Limón, 2015; Sudhakar et al., 2016). They are also known to respond to many biotic and abiotic stresses in plants (Stafford, 1991; Pollastri and Tattini, 2011; Lee et al., 2016). Plant pigments impart a bright color to flowers, acting as a visual signal to attract pollinators to facilitate pollination (Bradshaw and Schemske, 2003). Development of fall color during leaf senescence avoids photooxidative damage and

helps in retrieving the nutrients in plants (Feild et al., 2001). Therefore, in the due response of pigments in biochemical reactions and converting chemical energy and inorganic forms to organic structures, they are considered as a basis of life. Among various fruit quality traits, pigmentation is one of the most important for tailored breeding and consumer choice. Various other traits like taste, texture, shelf life, aroma and nutritional aspects account together with pigmentation for the quality of fruits (van den Berg et al., 2000). Natural pigments can be grouped into four major groups: tetrapyrroles, tetraterpenoids, betalains and polyphenols/flavonoids including anthocyanins.

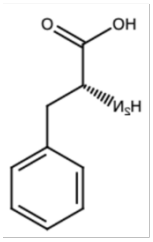
1.2.1 Polyphenols

Polyphenols are aromatic hydrocarbons containing hydroxyl group(s) produced mainly naturally by plants (Quideau et al., 2011). Chemical structures of some important polyphenols are shown in Figure 3. Among natural polyphenolic compounds, the major groups are flavonoids (e.g. anthocyanins, flavan-3-ols), benzoic acids (e.g. gallic acid), cinnamic acids (e.g. caffeic acid, ferulic acid), tannins (e.g. gallotannins, ellagitannins, proanthocyanidins), stilbenes, lignins and lignans (Hardman, 2014). To date, more than 10,000 plant-derived compounds with flavonoid structures have been identified in nature and they can be devided into different classes including flavonols, flavones, isoflavones, flavanones, anthocyanins and proanthocyanidins (Figure 2). They have diverse functions in plants and impart coloration in different plant tissues. Several flavonoids are known to function as protectant of cells against UV B-irradiation (Verdan et al., 2011; Falcone Ferreyra et al., 2012), have a role in nodulation and legume-rhizobium symbiosis (Subramanian et al., 2006; Zhang et al., 2009) and act in defense against infection-causing agents (Samac and Graham, 2007; Schenke et al., 2011). Further, they play important roles in plant production and fertility (Mahajan et al., 2011; Schenke et al., 2011), as genetic blocks in the flavonoid biosynthesis can result in male sterility in some plant species (van der Meer, 1992). In some cases, the bright yellow color of pollen is due to flavonoids, which act as attractants for pollinators (Zerback et al., 1989).

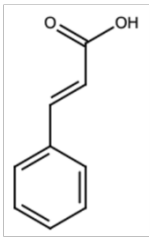
1.2.2 Anthocyanins – colored pigments in nature

Naturally occurring anthocyanins are water-soluble polyphenolic pigments found in vacuoles, belonging to the phytochemical class of flavonoids. Anthocyanins are the most widespread class of pigments in nature and they may appear as various even or mixed colored patterns of flowers (Chung et al., 2010; Luo et al., 2016; Sundaramoorthy et al., 2016), fruits (Saito et al.,

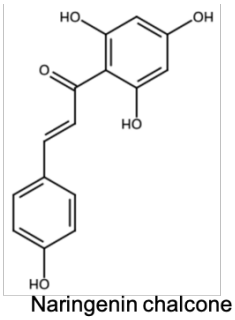
1999; Debes et al., 2011; Liu et al., 2013; Ben-Simhon et al., 2015) and other plant tissues (Gould et al., 2000; Kim et al., 2004, 2005; Zhou et al., 2010). The function of anthocyanins in plants is not fully understood. Their visual attraction, attract insects, birds and animals to facilitate in pollination and seed dispersals (Linhart, 2014). They have been assumed to have roles in protecting tissues from biotic and abiotic stresses, delaying senescence, assisting in photosynthetic machinery, delaying over-ripening, increasing shelf-life of fruits and acting as scavengers of reactive oxygen intermediates (Kumar et al., 2013; Zhang et al., 2013; Landi et al., 2015; Yousuf et al., 2016; Zhang et al., 2016; Yacout and Gaillard, 2017). Anthocyanins are often used as food colorants (Markakis, 2012; Carle and Schweiggert, 2016), but have also been considered to indicate beneficial effects in humans against cardiovascular and coronary heart diseases, cancer, aging, inflammation, obesity and neurodegenerative diseases (Ohrvall et al., 1996; de Ancos et al., 1999; Bowen-Forbes et al., 2010; Rao et al., 2010; Wallace, 2011; Yousuf et al., 2016).



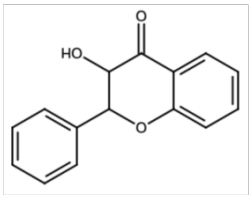
Phenylalanine



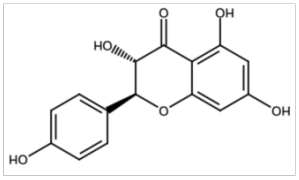
Cinnamic acid



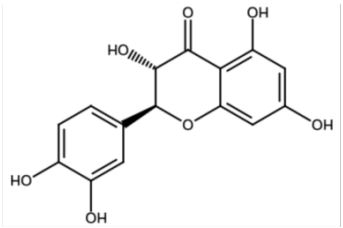
Naringenin chalcone



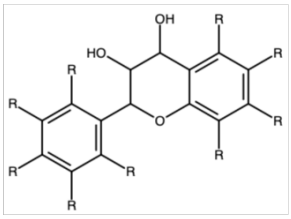
Dihydroflavonol



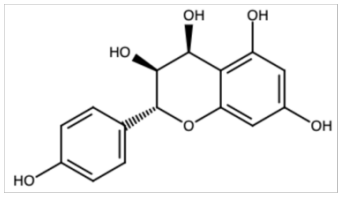
Dihydrokaempferol



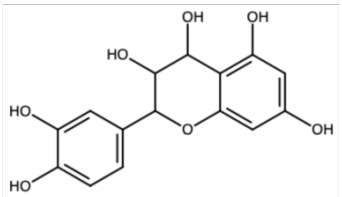
Dihydroquercetin



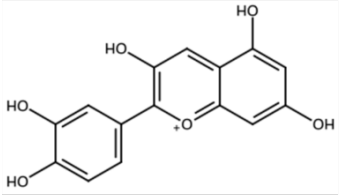
Leucoanthocyanidin



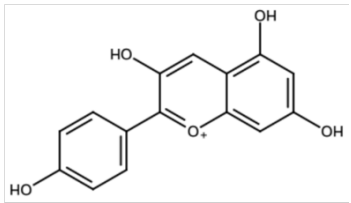
Leucopelargonidin



Leucocyanidin



Cyanidin



Pelargonidin

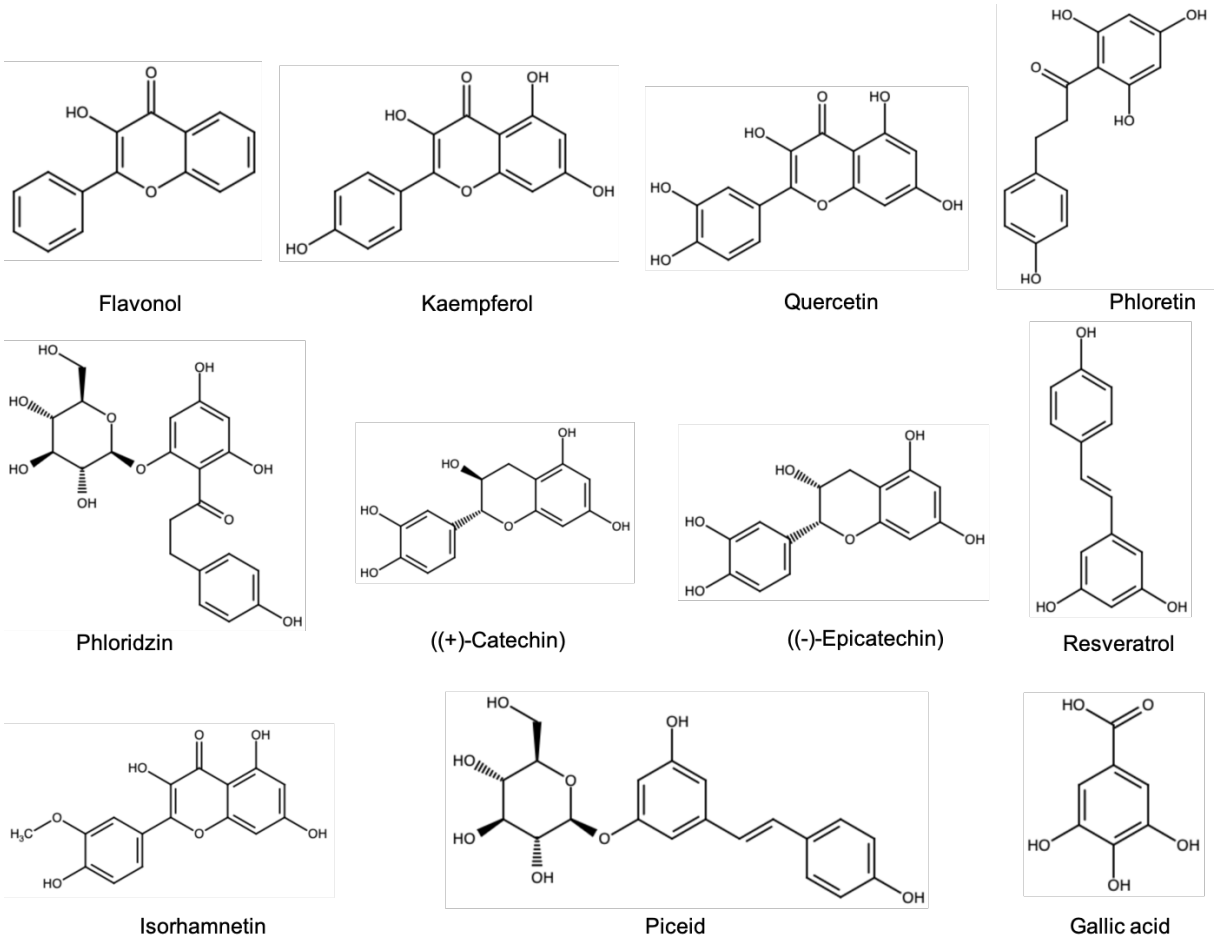


Figure 3: Chemical structures of different (poly)phenols

Raspberries are rich sources of various types of anthocyanins which are derivatives of cyanidin and pelargonidin. Among them cyanidin-3-*O*-sophoroside, cyanidin-3-*O*-rutinoside, cyanidin-3-*O*-glucoside and pelargonidin-3-*O*-sophoroside are the main components identified in different colored fruits so far (de Ancos et al., 199; Mullen et al., 2002; Pritts, 2003; Määttä-Riihinen et al., 2004). Cyanidin-3-*O*-sophoroside is known to be the most characteristic anthocyanin pigment of raspberry fruits (Rommel et al., 1990). However, the final composition of anthocyanins in fruits depends on many factors, such as variety/genotype, cultivation region, surrounding environment, etc. (Rommel et al., 1990; Boyles and Wrolstad, 1993). Black, purple and red fruiting varieties contain higher amounts of anthocyanins compared to orange ones (Wang et al., 2009; Dossett et al., 2010; 2011) which increase with the fruit ripening (Beekwilder et al., 2005). Raspberries with higher anthocyanin contents have been known to have potential health benefits in humans, e.g. antioxidant capacity and vasorelaxation properties

(Mullen et al., 2002; Bowen-Forbes et al., 2010). However, only few reports are available describing that yellow fruiting raspberries have a relatively higher impact on diseases like chronic diabetes or hypertension than the red fruiting raspberries (Cheplick et al., 2007; Zhang et al., 2010). Aside from health benefits, anthocyanins play an important role as an indicator of fruit quality and fresh fruit appearance (de Ancos et al., 1999; Espín et al., 2007). Therefore, a better understanding of genetic control and regulation of anthocyanin formation in raspberry and fruit ripening process is needed to accomplish an important association between beneficial compounds and their positive effects on human health.

1.2.3 Biosynthesis of flavonoids and anthocyanins

Genes encoding specific enzymes of the entire flavonoid/anthocyanin pathway have been well characterized in several plant species (Holton and Cornish, 1995; Broun, 2005; Lepiniec et al., 2006; Ferreyra et al., 2012; Huang et al., 2015). In general, structural and regulatory genes control the biosynthesis of these metabolites in flowers and fruits while color mutants of structural, regulatory or both groups of genes in combination have extensively been studied and characterized in several plant species, such as rose, grape, chrysanthemum, tomato, moth orchid, gerbera and pomegranate (Kobayashi et al., 2004; Butelli et al., 2008; Czemmel et al., 2009; Ma et al., 2009; Chung et al., 2010; Nishihara and Nakatsuka, 2011; Bashandy et al., 2015; Ben-Simhon et al., 2015). Modification of transcription of the genes of the flavonoid pathway can significantly influence the final synthesis and accumulation of specific compounds of the pathway. For example, downregulation of the anthocyanin branch can enhance the accumulation of flavones, flavonols, flavan-3-ols and/or isoflavones as more of the common precursors are available for these branches. All these metabolites may also affect coloration as they can act as co-pigments (Samac and Graham, 2007). Previous studies in Rosaceae and other genera have shown that the amount of anthocyanins is strongly associated with the level of expression of *Dfr* and *Ans* genes (Almeida et al., 2007; Chen et al., 2012; Rouholamin et al., 2015).

In the phenylpropanoid pathway, flavonoids are synthesized from phenylalanine, a common precursor for a variety of plant metabolites. Phenylalanine is converted through a number of enzymatic steps (as shown in Figure 4) into *p*-coumaroyl-CoA, which is the entry point for the flavonoid biosynthetic pathway. Genetic studies have shown the involvement of several genes, such as chalcone synthase (*Chs*), flavanone 3*β*-hydroxylase (*Fht*; synonym *F3h*), dihydroflavonol 4-reductase (*Dfr*), anthocyanidin synthase (*Ans*; syn. leucoanthocyanidin

dioxygenase, *Ldox*) and UDPG:flavonoid-glycosyltransferase (*Ufgt*), enabling the synthesis of anthocyanins in fruits of Rosaceae species (Manning, 1998; Moyano et al., 1998; Takos et al., 2006/3; Ravaglia et al., 2013). A general scheme has been drawn to illustrate the biosynthesis of flavonols, anthocyanins and proanthocyanidins in plants (as shown in Figure 4).

1.2.4 Biosynthesis of anthocyanin pigments in raspberry fruits

Fruit pigmentation in raspberries (*Rubus idaeus* L., Rosaceae) is a complex phenomenon where a range of color patterns from deep purple to yellow exists. It is supposed to be a complex trait involving not only the amount but also type of various pigments and co-pigments (Giusti et al., 1999; Castañeda-Ovando et al., 2009). Anthocyanins are considered to be the principal pigment components reflecting the red coloration in raspberry fruits (see also Section 1.2 paragraph 3; de Ancos et al., 1999; Carvalho et al., 2013a,b), but the biosynthetic pathway of anthocyanins in raspberry is not very well characterized. However, some efforts have been made in order to pinpoint and map the genes linked to the biosynthesis of raspberry anthocyanins (Kassim et al., 2009; McCallum et al., 2010).

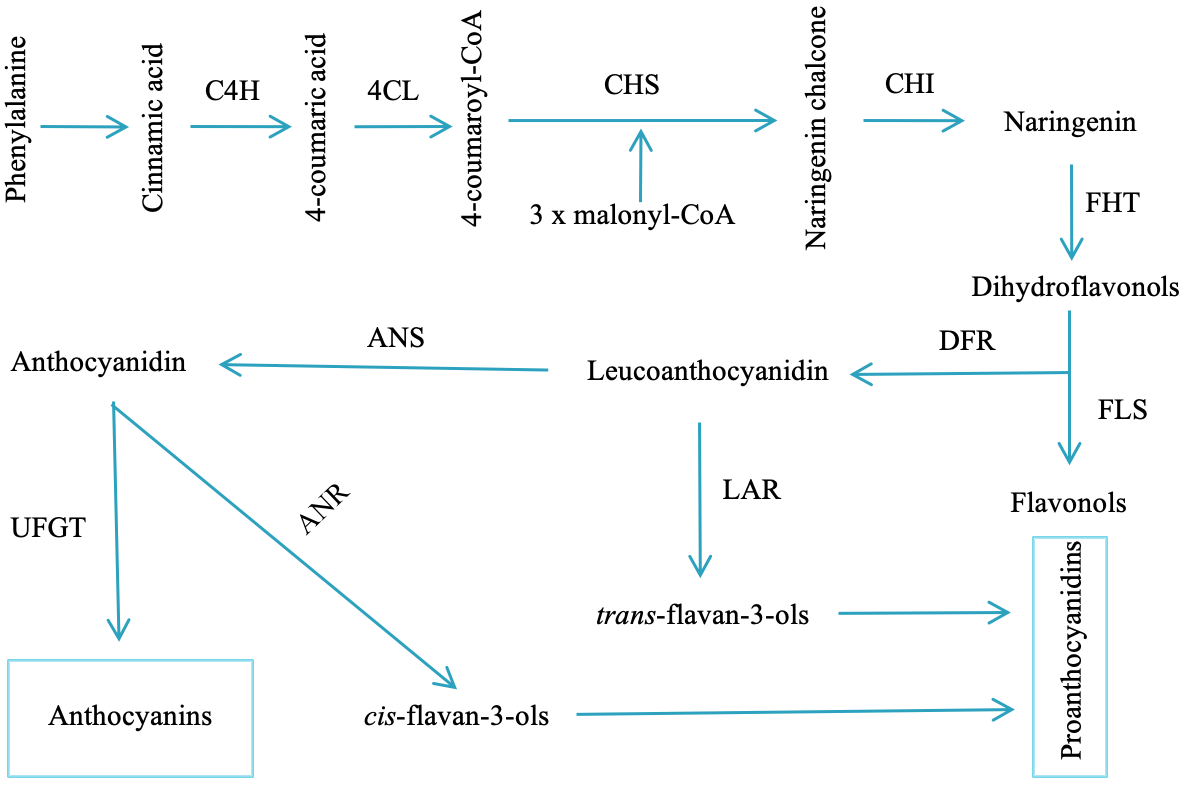


Figure 4: A general scheme indicating the anthocyanin and proanthocyanidin biosynthesis in the flavonoid pathway in plants; PAL, phenylalanine ammonia-lyase; C4H, cinnamate 4-hydroxylase; 4CL,

4-coumarate-CoA ligase; CHS, chalcone synthase, CHI, chalcone isomerase; FHT, flavanone 3*ß*-hydroxylase; DFR, dihydroflavonol 4-reductase; ANS, anthocyanidin synthase; UFGT, UDPG-flavonoid-glycosyltransferase; ANR, anthocyanidin reductase; LAR, leucoanthocyanidin reductase; FLS, flavonol synthase.

Previous studies carried out to understand the role of genes influencing fruit pigmentation patterns propose that a dominant allele of gene *T* plays a crucial role in regulating the synthesis of anthocyanins (Crane and Lawrence, 1931). In yellow raspberries, the anthocyanin synthesis is apparently reduced in all development stages (Määttä-Riihinen et al., 2004; Carvalho et al., 2013a). Literature gave some hypotheses of the origin of varieties with different colored fruits, such as purple, orange and yellow fruiting varieties. The purple fruiting raspberries originated from crosses between black and red fruiting varieties (Slate et al., 1963). Many yellow/orange varieties have originated from crosses of red x red/yellow fruiting genotypes or sports of other red varieties (see Table 1). It has been discussed that the homozygote recessive form (*tt*) of gene *T* might be responsible for the yellow phenotype (Crane and Lawrence, 1931). However, the involvement of other genes affecting the phenotype in the absence of the dominant allele *T* has not been excluded (Britton et al., 1959; Jennings and Carmichael, 1975; Macha, 1966).

Table 1: Origin of yellow and orange fruiting raspberries from cross of red x red/yellow parent lines or sports of other red varieties

Variety	Color	Parentage	Color
Anne	yellow	Amity x Glen Gerry	red x red
Lumina	yellow	Autumn Bliss x Tulameen	red x red
Alpen Gold	yellow	Polka x Tulameen	red x red
Autumn Amber	yellow	Polka x one of EMR earliest breeding line	red x red
Fall Gold	yellow	NH-R7 x (Taylor x R. Pungens var. Oldhamii)	red x (red x ?)
Zheltyi Gigant	yellow	Maroseika x Ivanovskaya	red x ?
Orange Marie	orange	Autumn Bliss x Fallgold	red x yellow
Valentina	orange	EM6225/11 x EM5588/81	red x ?
All Gold	yellow	Sport of Autumn Bliss	red
Golden Queen	yellow	Sport of Cuthbert (occidentalis)	red

Actually there are no convincing informations available concerning the genetic background of varieties with different phenotypes in *Rubus* and their origin during breeding processes. Some studies on metabolite relations among various genotypes have been carried out. They indicate that black and red raspberry fruit pigmentation owes to anthocyanins (Wang et al., 2009), but yellow and possibly orange fruits seem to lack the anthocyanins at all (Card, 1898; Jennings

and Carmichael, 1975; Carvalho et al., 2013a). However, the described composition of non-colored polyphenolic compounds between red and yellow raspberry fruits did not show any significant differences which might indicate the deviation of flavonoid metabolism into other directions (Carvalho et al., 2013a). A block in anthocyanin pathway is evident in yellow raspberries, even though there is no evidence in which step this genetic block might have occurred (Määttä-Riihinen et al., 2004; Carvalho et al., 2013a). Furthermore, it is possible that different yellow or orange varieties have blocks in different steps/genes of the anthocyanin biosynthetic pathway. As the knowledge of the molecular genetics is poor and still scanty for this species, more studies of biosynthetic genes of the anthocyanin pathway are indispensable to understand the mechanism controlling and regulating pigmentation in red, orange and yellow raspberry fruits.

1.3. Terpenoids

Terpenoids, sometimes known as isoprenoids, are a large and diverse class of naturally occurring organic chemicals derived from terpene units containing diverse functional groups. Some important examples of isoprenoids are carotenoids, chlorophylls and tocopherols. In plants, tocopherols facilitate the stabilization of the plant cell membranes, prevent cells from oxidative damage and help to maintain optimal growth in response to unfavorable conditions, such as extreme temperatures, salt stress and pollutants (Collin et al., 2008; Szarka et al., 2012). Tocopherols also play a vital role in humans, e.g. they prevent platelet aggregation, act as antioxidants (being source of vitamin E) and have the function as signaling molecules (Liu et al., 2003; Azzi et al., 2004; Zingg and Azzi, 2004).

1.3.1 Carotenoids - occurrence and function

Carotenoids, belonging to the tetraterpenoids, are a class of pigments that are produced by plants, algae and many bacteria. Carotenoids constitute a wide range of approximately 700 lipid-soluble compounds with 40 carbon units. They are naturally found in various plant parts, such as roots, leaves, flowers and fruits. Two branches of carotenoids are known: carotenes and xanthophylls. Xanthophylls differ from carotenes due to harboring one or more functional oxygen groups. Yellow to orange pigmentation in leaves in autumn is due to xanthophylls. In green tissues carotenoids are located in the chloroplasts, associated in carotenoid-protein-chlorophyll complexes and are involved in light harvesting and chlorophyll photoprotection (Ledford and Niyogi, 2005, Gruszecki, 2016; Young, 1993). In fruits, upon ripening, as the photosynthetic machinery is dismantled, chloroplasts are degraded. Chlorophylls and the

photosynthesis-associated carotenoids gradually decrease and completely disperse in fully ripenined fruits (Roca and Mínguez-Mosquera, 2001). In some fruits like tomato, a transition of carotenoids from chloroplast to chromoplast can be observed during fruit ripening (Egea et al., 2010; Egea et al., 2011).

In general, most of the carotenoids in fruits are esterified with fatty acids and are accumulated in ripened fruits ((Minguez-Mosquera et al., 1994) (Hornero-Méndez and Mínguez-Mosquera, 2000)). Some fruits (like mango or oranges) or flowers (marigold) are rich in hydroxyl-carotenoids (lutein, zeaxanthin, cryptoxanthin), where the hydroxyl groups of the xanthophylls can be partially or totally acylated with different fatty acids, resulting in a very complex carotenoid profile (Sowbhagya et al., 2004; Bunea et al., 2014). Carotenoids play an important role in plants, such as facilitation of pollination due to attracting colors and activation of defense systems by signaling for photo-oxidative stresses (Bartley and Scolnik, 1995; Demmig-Adams and Adams, 1996; Howitt and Pogson, 2006; Ramel et al., 2012). They are not only important in a variety of biological functions, such as photoprotection, but are also commercially utilized as food colorants, animal feed supplements and pharmaceutical and cosmetic compounds (Armstrong and Hearst, 1996; Schmidt-Dannert and Lee, 2009). Among naturally synthesized compounds, the role of carotenoids is considered to be important in determining fruit quality (van den Berg et al., 2000). Yellow, orange and red pigmentation have been described due to the accumulation of different carotenoids in different plant parts (Alquezar et al., 2008).

Carotenoids are not biosynthesized in animals and they need to take up these compounds as dietary ingredients in the form of fresh fruits and/or vegetables. In humans, carotenoids (e.g. β-carotene) act as precursors for vitamin A (Fraser, 2004; Mactier, 2005; Wolfe et al., 2008; Jean-Gilles et al., 2012). Most of the provitamin A (α- and β-carotene) which is converted partially to vitamin A, is taken through the dietary consumption of fruits, vegetables, eggs and dairy products (van Het Hof et al., 2000; Stahl and Sies, 2005; Schmid and Walther, 2013). They have beneficial effects against cardiovascular, macular degeneration and other chronic diseases, such as tumor formation, diabetes, etc. (Çekiç and Özgen, 2010; Yamagata, 2017; Mendiara and Perissinotti, 2017; Merhan, 2017). In spite of health beneficial effects of carotenoids their degraded derivatives, known as apocarotenoids, such as aromatic volatiles, attribute to the characteristic flavor, taste and fragrance of fruits (Lewinsohn et al., 2005 a,b).

1.3.2 Biosynthesis of terpenoids and carotenoids

Polymerization of five carbon (5-C) isoprene units constitutes a diverse group of the natural compounds known as isoprenoids (terpenoids). Isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP) are the basic building blocks of terpenoids which first combine in the formation of geranyl diphosphate (GPP). Depending on the number of 5-C isoprene units, compounds can be distinguished as hemiterpenes (1; 5-C), monoterpenes (2; 10-C), sesquiterpenes (3; 15-C), diterpenes (4; 20-C), sesterterpenes (5; 25-C), triterpenes (6; 30-C), sesquaterpenes (7; 35-C) and tetraterpenes (8; 40-C; carotenoids) (Ashour et al., 2010).

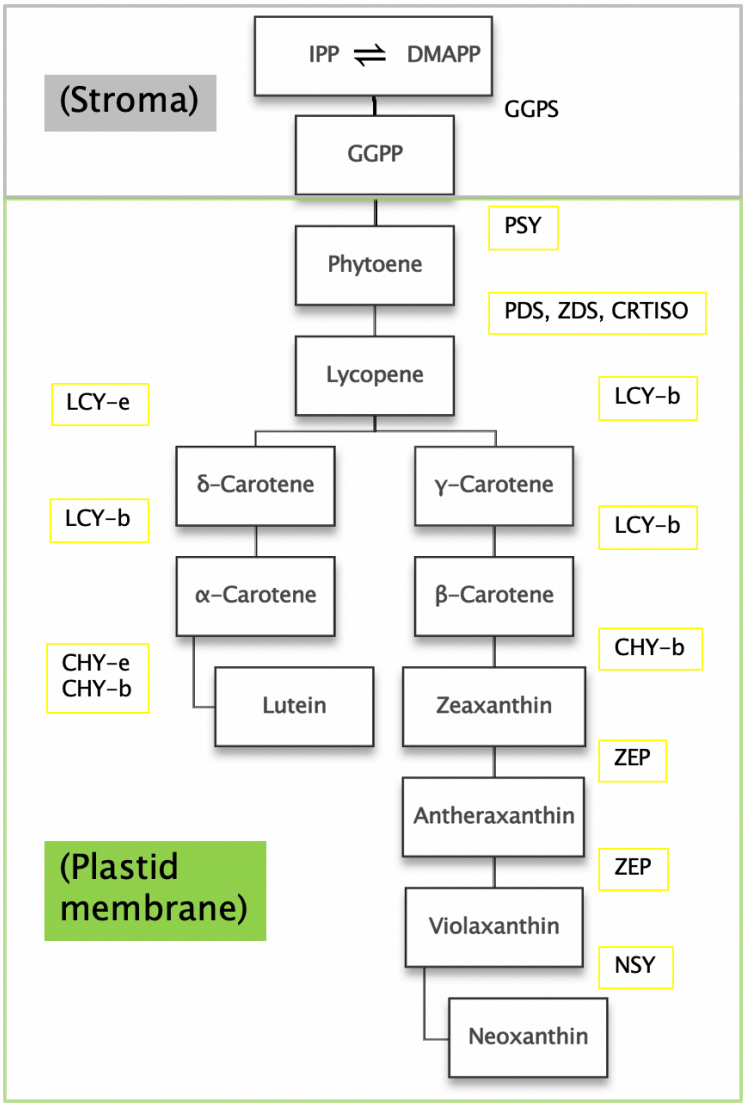


Figure 5: A general scheme of the carotenoid biosynthesis in plants; IPP, isopentenyl diphosphate; DMAPP, dimethylallyl diphosphate; GGPS, geranylgeranyl diphosphate synthase; PSY, phytoene synthase; PDS, phytoene desaturase; ZDS, ζ-carotene desaturase; CRTISO, carotenoid isomerase; LCY-b, lycopene *β*-ring cyclase; LCY-e, lycopene *ε*-ring cyclase; CHY-b, carotenoid *β*-ring 3-hydroxylase; CHY-e, carotenoid *ε*-ring 3-hydroxylase; ZEP, zeaxanthin epoxidase; and NSY, neoxanthin synthase.

Carotenoids are naturally synthesized in the photosynthetic machinery of cyanobacteria, algae and plants, and some fungi but not in mammals (Oliver and Palou, 2000). Carotenoid biosynthesis is localized in plastids and has been well characterized in many plant species (Britton, 1995; Britton et al., 1998; Cunningham and Gantt, 1998; Hirschberg, 2001; Welsch et al., 2010).

In a general, condensation of geranylgeranyl diphosphate (GGPP) units synthesizes phytoene, a primary product of the pathway, by the activity of phytoene synthase (PSY). In the next step, the colorless phytoene precursor is converted to lycopene via the ζ-carotene intermediate by the activity of phytoene desaturase (PDS), ζ-carotene desaturase (ZDS) and carotenoid isomerase (CRTISO). Here, a two-branch β-chain starts, where lycopene β-cyclase (LCY-b) catalyzes the formation of cyclic carotenoids, e.g. β-carotene, by the introduction of two beta-rings in one chain and lutein by the introduction of one β-ring and one ε-ring in the second chain to the acyclic lycopene precursor (Figure 5). Downstream in the metabolic pathway, β-carotene is transformed to cyclic carotenoids like zeaxanthin, antheraxanthin and violaxanthin by the activity of carotenoid β-ring 3-hydroxylase (CHY-b), zeaxanthin epoxidase (ZEP) and neoxanthin synthase (NSY), respectively. Carotenoid cleavage dioxygenases (CCDs) are known to form apocarotenoids, e.g. α-ionone and β-ionone, by breaking down the carotenoids, especially β-carotene, controlling the accumulation of β-carotene in plants.

1.3.3 Terpenoids and carotenoids in raspberries

Berries are among the important and rich sources of nutritive and bioactive compounds beneficial to human health (Jimenez-Garcia et al., 2013; Nile and Park, 2014). Raspberries contain high amounts of terpenoids, such as carotenoids, but also considerable amounts of tocopherols (Carvalho et al., 2013b). Carotenoids and tocopherols are the two most abundant groups of lipid-soluble compounds accumulated in the raspberry fruits as well as many other photosynthetic organisms (DellaPenna and Pogson, 2006; Carvalho et al., 2013b). The amounts of isoprenoids are different among raspberry varieties. The major carotenoids are lutein and its esters. Raspberries are also rich in apocarotenoids, also called norisoprenoids, particularly β-ionone, a degradation product of β-carotene. In yellow but also in red raspberries there are significant amounts of carotenoids that however decrease during ripening (Carvalho et al., 2013b). It is possible that the decrease of carotenoids during ripening results in the formation of norisoprenoids.

As mentioned before, pigmentation in plant tissues, especially in fruits, is a key quality trait that owes to various color patterns depending upon the accumulation/degradation of carotenoid pigments (Tadmor et al., 2005). Expression of carotenoid pathway genes correlates with the accumulation of carotenoids by the control of metabolic flux of respective precursors (Rodríguez-Villalón et al., 2009; Carvalho et al., 2016). Transcriptional regulation and natural genetic modifications have been known to alter the metabolic flux or causing pathway blocks and accumulating upstream carotenoids eventually leading to change in plant pigmentation (Welsch et al., 2010; Wolters et al., 2010). In the absence of anthocyanins, fruit pigmentation is considered in many raspberry varieties to be due to the accumulation of various yellow pigments (carotenoids) (Carvalho et al., 2013b). However, molecular and enzymatic understanding of biosynthesis and regulation of carotenoids during raspberry fruit development is still lacking. Even though chemical studies have been carried out to understand the accumulation of carotenoids in red and yellow raspberries (Carvalho et al., 2013b), more studies are needed at genetic level to understand the regulation of carotenoids and their role in imparting color to the raspberry fruits.

1.4. Aims of the study

As described before, two main pigments, anthocyanins and carotenoids, are important in contributing to the color of raspberry fruits. Non-red raspberries offer an opportunity to study and explore the reason behind the loss of red pigments, the anthocyanins. For this aim, it is of interest to investigate the anthocyanin pathway genes and to explore the genetic basis of yellow and orange fruiting varieties of *Rubus*. In absence of anthocyanins, carotenoids are considered to be the pigments imparting yellow to orange color patterns to the raspberry fruits (Card, 1898; Carvalho et al., 2013 a, b). One additional question that arises is where the yellow/orange color of raspberries comes from? Is it only due to an unmasking effect where yellow pigments (most likely carotenoids) are visible only because overlaying red pigments (anthocyanins) are missing, or is there a change in the composition of flavonoid/carotenoid pigments in due response to anthocyanin/carotenoid biosynthetic pathway mutants? The present thesis describes the key structural genes and putative protein functions involved in biosynthesis of raspberry flavonoids (anthocyanins) and carotenoids resulting in red, orange and yellow colored raspberry fruits. Omics approaches starting from metabolomics (polyphenols and carotenoids) to the transcriptomics pave the way for subsequent characterization at genomic level (structural and regulatory genes) were planned to improve the basic understanding of origin of various colored raspberries (Figure 6). To address the possible causes behind various pigmentation patterns in

raspberry and to improve the knowledge and understanding of their genetic control, herein, the following aims have been set:

a. Metabolic profiling in raspberry ripened fruits
b. Transcriptional analysis of flavonoid pathway genes during fruit development
c. Molecular and functional characterization of the "block" in flavonoid/anthocyanin pathway in yellow raspberry
d. Transcriptional analysis of carotenoid pathway genes during fruit development
e. Molecular and functional characterization of carotenoid pathway genes in *Rubus*
f. Integration of metabolic data with transcriptomic and genomic data to postulate a pathway map for pigmentation in yellow/orange raspberry fruits

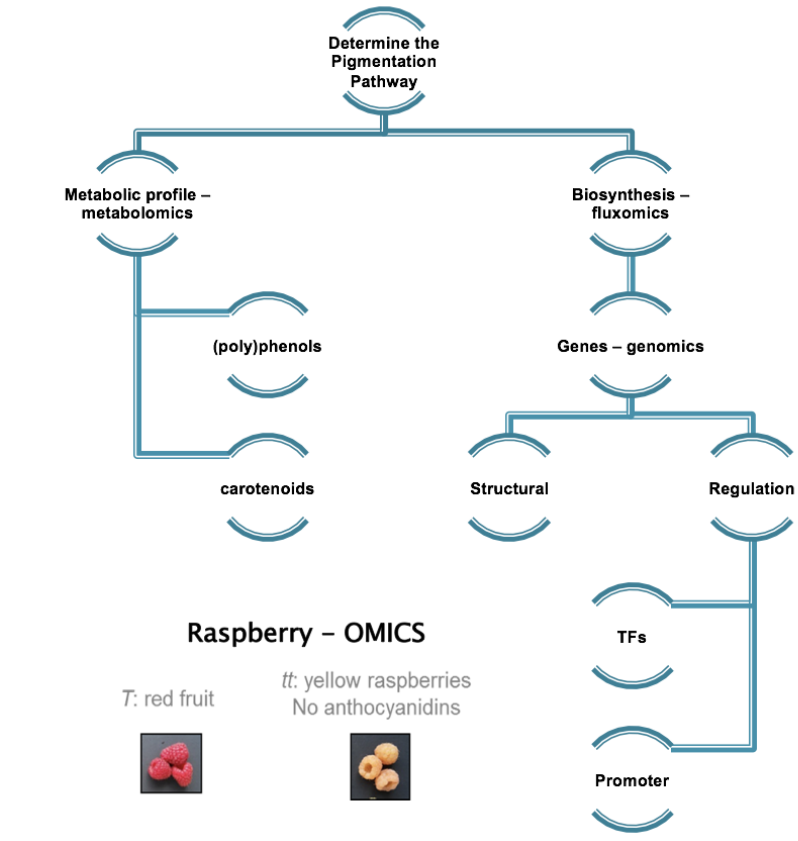


Figure 6: Raspberry - OMICS for the proposed study to analyze the anthocyanin biosynthesis block in yellow raspberries.

2. MATERIALS AND METHODS

2.1. Plant material

Fruits of raspberry varieties “Anne” and “Tulameen” were collected from 2 to 10 individual plants of each variety in 2013 and 2014. They were collected at different ripening stages from initial fruit formation (stage 0) to fully ripened fruits (stage 5) as shown in Figure 7. These fruit stages from “Anne” and “Tulameen” were named as A0 to A5 and T0 to T5, respectively. Ripened fruits (S5) of *Rubus* varieties, i.e. “Anne”, “Citria”, “All Gold”, “Juan de Metz”, “Fall Gold”, “Gelbe Antwerpener”, “Gelbe Siebenkugel”, “Gelbe Sugana”, “Golden Everest”, “Him13K39-8”, “Lumina”, “Zheltyi Gigant”, “Golden Queen”, “Orange Marie”, “Valentina”, “Tulameen”, “Pocahontas”, “Himbo Top”, “Meeker”, “Tayberry”, “Buckingham Tayberry” and “Black Jewel”) were collected in 2013 and 2014. Leaves of various varieties of *Rubus* (“Anne”, “Glen Garry”, “Amity”, “Tulameen”, “Nootka”, “Glen Prosen”, “All Gold”, “Fall Gold”, “Golden Queen”, “Gelbe Antwerpener”, “Gelbe Siebenkugel”, “Juan de Metz”, “Sugana Gold”, “Golden Everest”, “Lumina”, “Citria”, “Gelbe Sugana”, “Alpen Gold”, “Giallo Mutant”, “Him13K39-8”, “Herbert Gold”, “Valentina”, “Orange Marie”, “Heritage”, “Sugana Red”, “Autumn Bliss”, “Meeker”, “Pocahontas”, “Himbo Top”, “Tayberry”, “Buckingham Tayberry” and “Black Jewel”) and *Fragaria* (*F.* × *ananassa* and *F. vesca*) were collected in 2015. Source, fruit color and lineage informations on all the varieties under study are presented in Annex I. All samples were immediately frozen in liquid nitrogen and stored at -80°C until further use. Samples (fruits and leaves) from different plants were kept and analyzed separately.

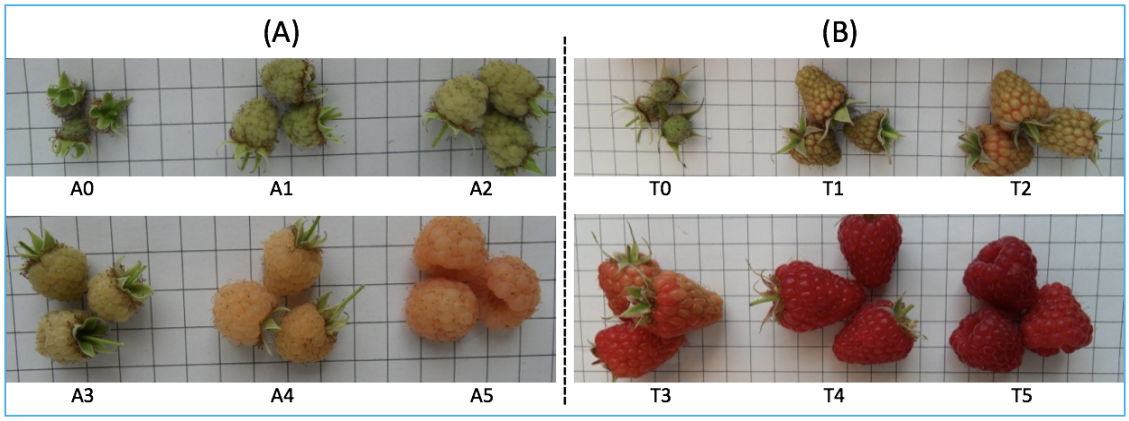


Figure 7: Different fruit ripening stages from initial fruit formation (stage 0) to fully ripened fruits (stage 5) where A0-A5 stand for “Anne” **(A)** and T0-T5 for “Tulameen” **(B)**, respectively.

2.2. Genome mining

The availability of a preliminary draft of the raspberry genome cv. "Heritage" helped to predict the homologous genes of *Rubus* in the present study. The *in-silico* search of the "Heritage" genome with candidate genes from *Fragaria*, *Pyrus* and *Malus* (GenBank Accession Numbers can be found in Annex III and XI) as templates enabled the identification/assembly of putative homologous genes of *Rubus*.

2.3. Primer design

Gene specific primers against full-length sequences including UTRs and/or coding regions were designed to enable gene cloning using PerlPrimer (Marshall, 2004) to enable PCR amplification. Primers for quantitative real-time PCR (qRT-PCR) were designed using Primer Express 3.0 (PE Corporation, Foster City, CA, USA). Primer sequences for the genes in the current study are listed in Annex II.

2.4. Genomic DNA extraction

Leaf samples stored at -80 °C were ground in liquid nitrogen using pre-cooled mortar and pestle. Genomic DNA was extracted from leaves of different varieties of *Rubus* and *Fragaria* according to instructions of NucleoSpin® Plant II kit (Macherey-Nagel, Germany).

2.5. RNA extraction and cDNA synthesis

Pre-cooled mortar and pestle were used to grind fruit samples in liquid nitrogen stored at -80 °C. Total RNA was extracted from the fruit powder by following the manufacturer's instructions of Spectrum Plant Total RNA kit (Sigma, Deisenhofen, Germany). Total RNA content and purity was assessed by Nanodrop 8000 (Thermo Scientific, USA) before proceeding to Reverse Transcription. RNA from independent fruits of each stage was reverse-transcribed using SuperScript® VILO™ cDNA Synthesis Kit (Invitrogen, The Netherlands).

2.6. Quantitative real-time PCR

Real-time PCR was carried out at different fruit developmental stages (S0-S5; analogous to the stages described in chapter 2.1) in triplicate using C1000^{TM} Thermal Cycler CFX ^{TM} (Bio-Rad Laboratories, Hercules, CA, USA) with the iQ^{TM} Syber® Green Supermix (Bio-Rad Laboratories). Amplification of different genes was efficiently determined and expression

levels were normalized to the constitutive expression of housekeeping genes as indicated in the sections 2.6.1 and 2.6.2. Real-time PCR was carried out by the following conditions: 98°C 5s, followed by 44 cycles at 98°C 5s, 58°C 5s, 60°C 5s, 76°C 10s. After a denaturation step at 98°C for 30 s the melting curve analysis was done increasing the temperature by 0.2°C, from 65 to 95°C, each 10s. The same instrument was used for copy number analysis using iQ™ Sybr® Green Supermix (Bio-Rad Laboratories, Hercules, CA) and probe-based analysis, but also for HRM analysis using the LightCycler 480 high-resolution melting master mix (Roche Diagnostics).

2.6.1 Flavonoid pathway genes

Primer sequences designed for the regulatory gene *MYB10* and for the *Rubus* structural genes *Chs*, *Fht*, *Dfr*, *Ans*, *Ufgt*, *Lar*, *Anr*, *Fls* and *Adh* genes (Annex II) were used to amplify the putative candidate genes. The expression levels of different genes were normalized to the constitutive expression of housekeeping gene alcohol dehydrogenase (*Adh*; GenBank Accession Number XM_004290519).

2.6.2 Carotenoid pathway genes

Gene-specific primers for qPCR designed for *Rubus* carotenoid pathway genes (*RiGgps*, *RiPsy*, *RiPds*, *RiLcy-b*, *RiLcy-e*, *RiChy-b*, *RiChy-e*, *RiZep*, *RiNsy*) and carotenoid cleavage dioxygenases (*RiCcd1.1*, *RiCcd1.2*, *RiCcd1.3*) are listed in Annex II. Normalization of carotenoid pathway genes was done with the constitutively expressed housekeeping genes (*Actin*, *Pap2* and *Sand*) of *Rubus*.

2.6.3 Copy number of Ans gene in Rubus

A quantitative PCR (qPCR) approach can be used to determine gene copy numbers if normalized to a single copy gene (Bustin, 2000; Solomon et al., 2008). For this purpose, full length "Anne" *Ans* gene cloned into pCR™4-TOPO® vector (pCR™4-1840) was used for generating a standard curve, following the instructions of Applied Biosystems (Life Technologies Corporation, 5791 Van Allen Way, Carlsbad, CA). The standard curve was created from the known mass of pCR™4-1840 plasmid and by running serial dilutions (D1-D5) as described in the protocol (https://isu.technion.ac.il/wp-content/uploads/2016/10/Creating_Standard_Curves.pdf). For this purpose, *Ans* target fragment was amplified by designing universal oligonucleotides ('RubUni-F' and 'RubUni-R';

Annex II) aimed at species with known (*F. vesca*, n=x=7; *F.* × *ananassa* Duch, n=4x=28) and unknown (*Rubus*, n=x=7) *Ans* copy number in comparison to the normalized plasmid reference. DNA amount as template was calculated from the known genome sizes of the species under study. Reactions were performed by following the program, 98°C 10 s (denaturation), 49°C 5 s and 51°C 5 s (annealing) 76°C 10 s (extension), 39 cycles from denaturation to extension, 98°C 30 s (final extension) and melting curve 65 °C to 95°C with 0.2°C. All the reactions were carried out with the same set of established conditions in biological and technical triplicates.

2.7. Cloning and subcloning

Bio-Rad thermal cycler (PTC 0200) was used for amplification of known sequences for cloning purpose. Platinum® *Taq* Polymerase High Fidelity (Invitrogen) was used to amplify products with 3' A overhangs to clone into pCR™4-TOPO® or pCR®2.1-TOPO® vector using TOPO® TA Cloning® Kit (Invitrogen). Platinum® *Pfx* DNA Polymerase (Invitrogen) was used to amplify blunt-end PCR products to clone into pJET1.2/blunt and pENTR™ D-TOPO® vector by following the instructions of CloneJET PCR Cloning Kit (Thermo Scientific) and pENTR™ Directional TOPO® Cloning Kit (Invitrogen), respectively. Coding regions of genes of interest cloned into pENTR™ D-TOPO® vector were subcloned to expression vector pDEST™17 and a binary vector pLEELA (Jakoby et al., 2004) by performing the LR recombination reaction (Gateway® cloning; Invitrogen). PCR reaction mixture for amplification of DNA fragments was prepared by adding dNTPs (0.3 MM), forward and reverse primer (0.3 mM each), 10 x PCR buffer (5 µl), $MgSO_4$ (1mM), polymerase (1.25 U), template (50 ng) and milli-Q water was added to make 25 µl volume. All PCR amplifications were performed by following the program, 94°C 150 s (1 cycle); 94°C for 30 s, 58°C for 45 s, 68°C for 120 s (34 cycles), 68°C 10 min (final extension) in Bio-Rad thermocycler. Cloning reactions were transformed into competent Top10 *E. coli* cells.

2.7.1 Cloning of Ans and Dfr genes

Gene-specific primer ‘Ans-utr-F’ and ‘Ans-utr-R’ (Annex II) were used to amplify and clone full-length *Ans* gene spanning the entire coding region and UTRs. The amplified *Ans* gene products from genomic DNA of both, “Anne” and “Tulameen”, were cloned into the pCR™4-TOPO® vector (as shown in Figure 8). The cloned *Ans* genes from “Anne” and “Tulameen” were named as pCR™4-1840 and pCR™4-1835, respectively. The *Ans* genes from genomic DNA of other *Rubus* varieties, such as “Glen Garry”, “Amity”, “Nootka”, “Glen Prosen”, “All

Gold", "Golden Queen", "Gelbe Antwerpener", "Gelbe Siebenkugel", "Golden Everest", "Sugana Gold", "Fall Gold", "Juan de Metz", "Lumina", "Him13K39-8", "Herbert Gold", "Valentina", "Orange Marie" and "Heritage" were cloned into pJET1.2/blunt vector. *Ans* transcripts of "Anne", "Tulameen", "Heritage", "Autumn Bliss" and "Sugana Red" were amplified using the primer set ('Ans-orf-F' & 'Ans-orf-R1') specific to the coding region. Further, *Ans* transcripts of "Anne" were also amplified using primer set 'Ans-orf-F' & 'Ans-orf-R2' as listed in Annex II. Coding regions of *Ans* gene of "Tulameen" and "Anne" were amplified and directionally cloned into pENTR™ D-TOPO® vector for further use as illustrated in Figure 9. Similarly, *Dfr* genes from both varieties, "Anne" and "Tulameen", were amplified and cloned into pENTR™ D-TOPO® vector using primers 'Dfr-orf-F' and 'Dfr-orf-R' (Annex II).

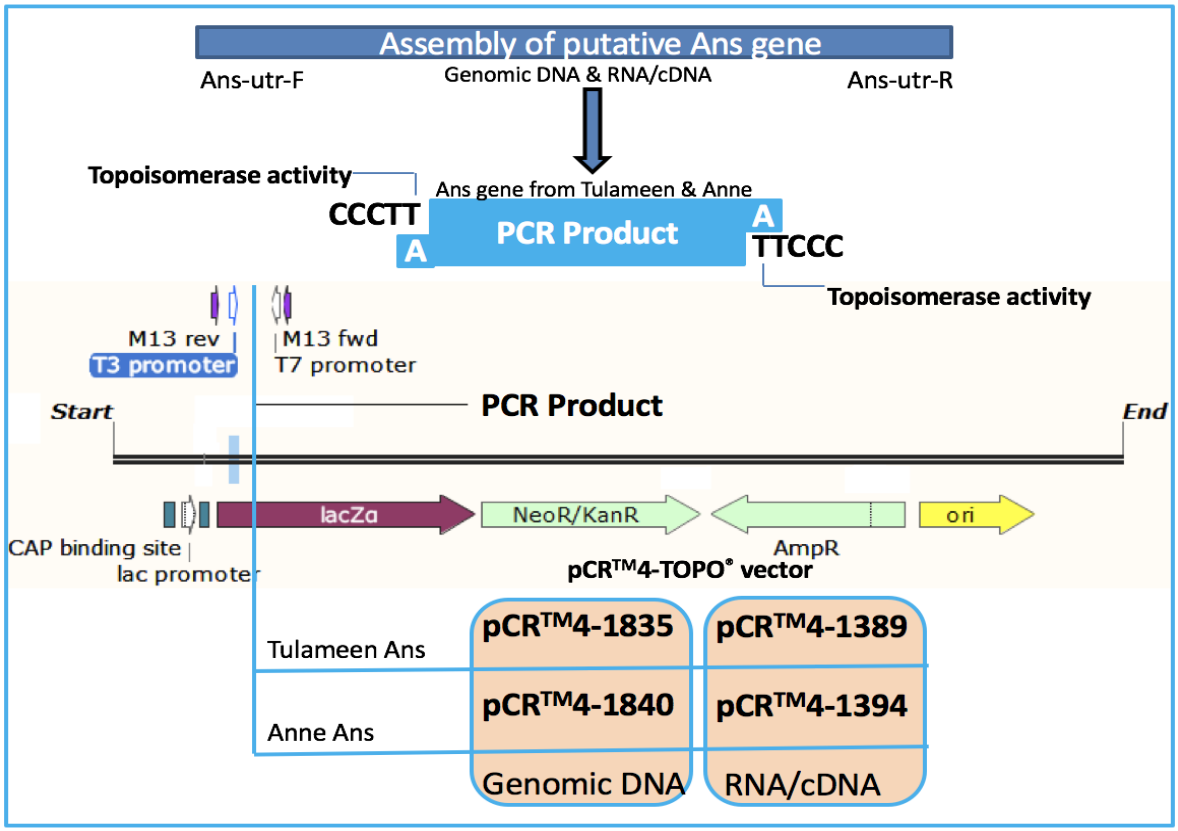


Figure 8: Cloning strategy of the assembled *Ans* gene from "Anne" and "Tulameen" into the pCR™4-TOPO® vector.

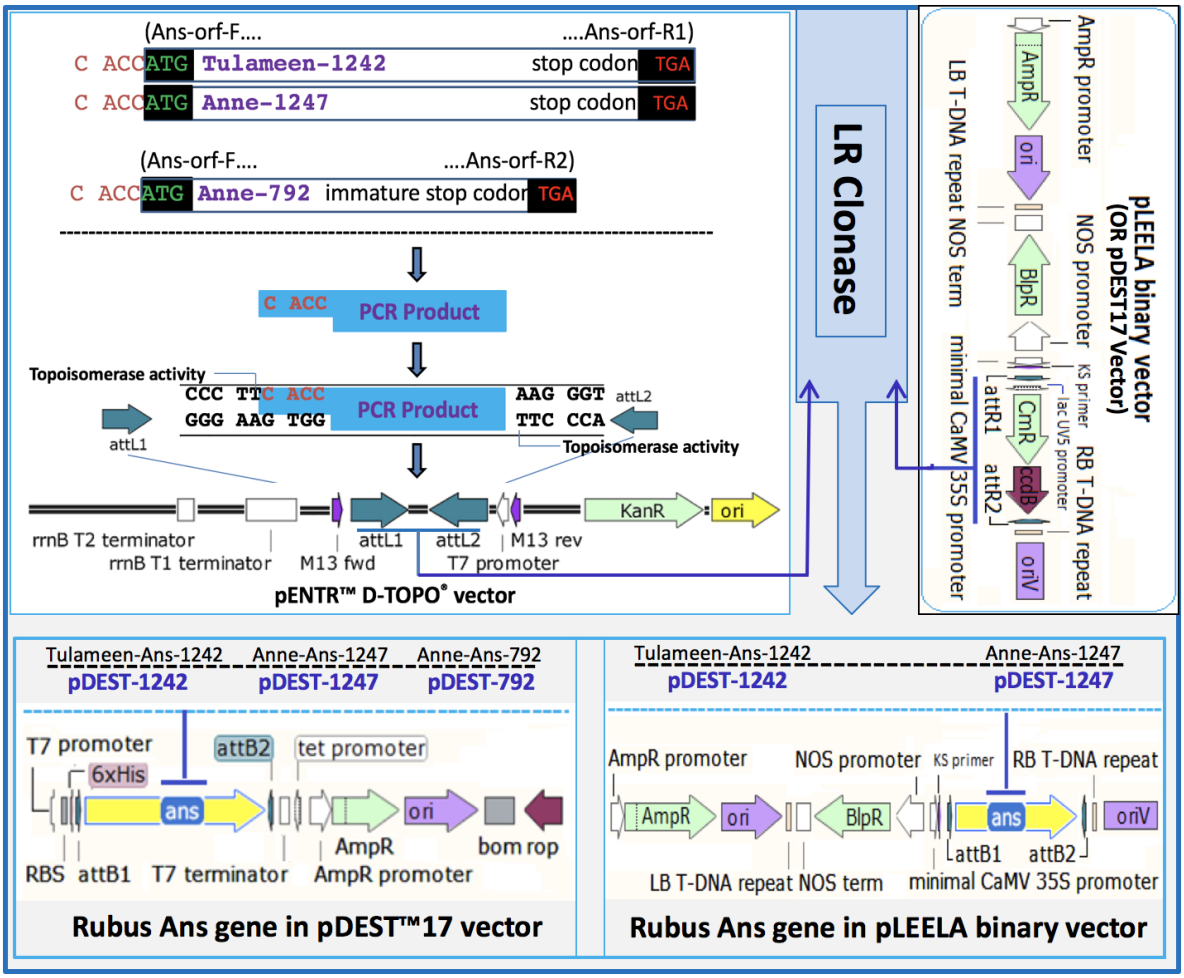


Figure 9: Cloning strategy of *Ans* gene versions from "Anne" and "Tulameen" varieties into the pENTR™ D-TOPO® vector and subsequently to expression vector pDEST™17 and binary vector pLEELA, respectively.

2.7.2 Cloning of carotenoids pathway genes

In silico search of *Rubus* draft genome sequence allowed the identification of putative homologous carotenoid pathway genes. The gene-specific primers were designed for genes, such as RiPsy-F & RiPsy-R, RiLyc-b-F & RiLyc-b-R, RiLyc-e-F & RiLyc-e-R and RiCcd1-F and RiCcd1-R as listed in Annex II. RNA/cDNA of "Anne" mixed in equal proportions from stage 1, stage 3 and stage 5 was used for efficient amplification of the carotenoid pathway genes. The amplified PCR products of *RiPsy* and *RiLcy-e* genes were cloned into the pCR®2.1-TOPO® vector (Invitrogen) and *RiLcy-b* and *RiCcd1* into pJET1.2/blunt vector, respectively (as illustrated in Figure 10). These cloned candidate genes were used as expression cassettes for their functional characterization via complementation in *E. coli* host. Carotenoid genes *RiChy-e* and *RiChy-b* from "Anne" were also cloned into a pJET1.2/blunt vector for sequencing.

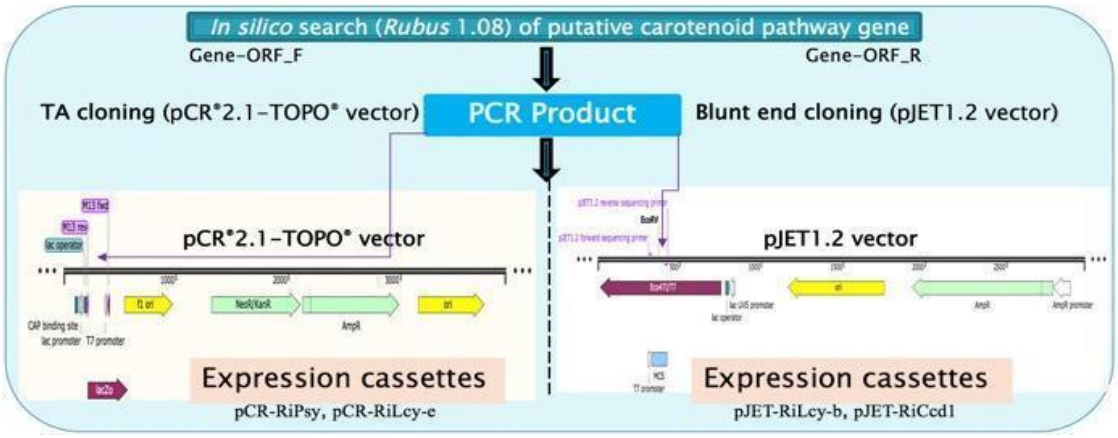


Figure 10: Cloning strategy of carotenoid pathway genes from "Anne" into pCR®2.1-TOPO® vector and pJET1.2/blunt vector.

2.8. Plasmid DNA extraction

A single colony of plasmid transformed Top10 *E. coli* cells was inoculated to 5 ml LB medium and incubated overnight at 37°C and 200 rpm. Out of 5 ml overnight culture, 1.5 - 2 ml culture was centrifuged at 12,000 x g for 1 min. The pellet was re-suspended in 200 µl of ice-cold solution "1" (100 µg/ml RNase A; 50 mM Tris-HCl, 10 mM EDTA pH 8.0). 200 µl of solution "2" (0.2 M NaOH, SDS 1%) was added to the suspension and was mixed by inverting the tubes 3-4 times. Immediately 200 µl of ice-cold solution "3" (3.0 M CH_3COONa, pH 5.5 with CH_3COOH) was added into the mixture and tubes were centrifuged at 12,000 x g for 1 min. The supernatant (about 600 µl) was collected into new tubes. 300 µl of phenol and 300 µl of chloroform : isoamyl alcohol 24 : 1 was added into the supernatant. Tubes were shaken and centrifuged at 12,000g for 5 min. Upper phase was collected without disturbing the interface into a new tube and was mixed with an equal volume of chloroform : isoamyl alcohol 24 : 1. After centrifugation at 12,000g for 5 min, the upper phase was re-collected into a new tube, without disturbing the interface and 2 - 2.5 volumes of absolute ethanol (EtOH) was added into the collected mixture/aqueous phase to precipitate the plasmid DNA. The mixture was centrifuged at 12,000g for 5 min and the pellet containing plasmid DNA was washed with 70% EtOH. Pellet was air dried and re-suspended in 100 µl mQ RNase free water.

2.9. Sequencing analysis

The nucleotide sequences of cloned genes were evaluated by Sanger sequencing (in house FEM sequencing platform). The obtained sequences were analyzed with "Vector NTi" software

package (Invitrogen). Multiple sequence alignment and sequence consensus were made using BioEdit (Hall, 1999) and molecular phylogenetic tree of the deduced amino acid sequences was clustered by unweighted pair group method with arithmetic mean (UPGMA) using Clustal Omega (EMBL-EBI).

2.10. Production of recombinant ANS protein

The *Ans* fragments from "Tulameen" (1242 bp) and "Anne" (792 bp & 1247 bp) cloned in pENTR™ D-TOPO® vector were subcloned into bacterial expression vector pDEST™17 (Figure 9). The resulting expression clones were named according to their expected fragments size as 'pDEST-792' 'pDEST-1242' and 'pDEST-1247', respectively. The constructed expression clones were introduced into *E. coli* RIPL strain and ANS protein was heterologously expressed by induction with 1 mM IPTG in LB medium containing 100 μg ml^{-1} carbenicillin at 28°C. Bacterial cells were harvested at 4°C, washed and re-suspended in sonication buffer (Qiagen Expression kit). After adding fresh 1 mM PMSF and 5 mM DTT, 1 mg ml^{-1} lysozyme was added by gently mixing the cells in sonication buffer. Cell disruption was done by performing sonication as 3x30 sec at 38% amplitude to obtain the protein extracts after centrifugation at 5,000g for 10 min at 4°C. Supernatants were collected in tubes kept on ice and remaining pellets inside tubes were dissolved in 300 μl denaturation buffer (50 mM Tris-HCl with pH 8.0 containing 8.0 M urea) for solubilization of bacterial inclusion body proteins. SDS-PAGE was run for ANS protein separations at 150 V for 45 min and stained with Coomassie dye. Precision plus protein ladder (Bio-Rad Laboratories) was run for comparison of protein bands obtained.

2.11. Complementation of anthocyanin and carotenoid genes

2.11.1 Complementation of Ans gene in Arabidopsis

The *Ans* genes from "Tulameen" (1242 bp) and "Anne" (1247 bp) cloned into pENTR™ D-TOPO® vector were subcloned into binary pLEELA vector and named as 'pLEELA-1242' and 'pLEELA-1247', respectively (as illustrated in Figure 9). Both binary vectors containing *Ans* gene driven by *2x35S* promoter harboring *Bar* gene (conferring resistance to the herbicide Basta 'phosphinothricin') were introduced into *A. thaliana ans* mutant line (*tt18-1*; *tds4*-2, anthocyanidin synthase, also called tannin-deficient synthesis) using floral dip technique (Clough and Bent, 1998) via *Agrobacterium tumefaciens* strain GV3101. The *A. thaliana* transformation was carried out by Dr. Ralf Stracke at Genome Research, Department of

Biology, Bielefeld University, Bielefeld, Germany. Seeds obtained were selected on half MS media containing kanamycin (40 µg ml^{-1}) and Basta (25 µg ml^{-1}) as selection markers (Murashige and Skoog, 1962). After two weeks, the selected plantlets were transferred to soil pots in greenhouse to obtain F1 progeny of seeds. Then, F2 progeny of seeds was obtained from F1 *A. thaliana* seeds to evaluate the phenotype. The *A. thaliana* mutants were grown in a growth chamber under the following conditions: 16/8 h light (100 µmol photons m^{-2} sec^{-1}), 70% humidity and 24 °C temperature. The transgenic status of *A. thaliana* lines obtained was evaluated by PCR amplification using 'Ans-orf-F' and 'Ans-orf-R' primers for *Ans* gene and 'Basta-F' and 'Basta-R' primers for *Bar* gene (Annex II). Wild-type (Col-0) and control (*tds4-2*) plants were also grown under the same conditions in the growth chamber.

To evaluate the anthocyanin accumulation in complemented mutant lines of *Arabidopsis*, sterilized seeds were grown in half MS liquid media containing 5% sucrose under shaking (50 rpm). Sucrose stress promotes anthocyanin accumulation in emerging seedlings. Seeds were also placed in media without sucrose as control. The anthocyanin-based phenotype was observed in 5 day-old seedlings and 2 week-old plantlets. Furthermore, these plantlets were shifted to the greenhouse for phenotype observation in 4 week-old plants, anthocyanin extraction and seed production.

2.11.2 Complementation of carotenoid genes in E. coli

Candidate genes of the biosynthetic pathway of *Rubus* cloned into expression cassettes were used to complement plasmids capable to generate different carotenoid precursors. The expression cassettes under ampicillin resistance containing carotenoid pathway genes together with plasmids under chloramphenicol resistance producing precursors were co-transformed in *DH5α* cells. A 3-ml culture was initiated to inoculate 50 ml culture at 0.5 OD by overnight induction with 1 mM IPTG in LB medium containing 33 µg ml^{-1} chloramphenicol and 100 µg ml^{-1} ampicillin at 37°C. Color of cultures was noticed, pelleted at 10°C and freeze-dried before chromatographic analysis.

2.12. *Rubus* screening for mutations in the *Ans* gene

2.12.1 Establishment of probe-based marker

Unlabeled oligonucleotides ('RubUni-F' and 'RubUni-R') and a 15 base pair TaqMan FAM dye-labeled probe (Annex II) were designed for detection of unique mutation in the *Ans* gene.

All samples including non-template control (NTC) were run in triplicates in a volume of 12.5 µl containing 6.25 µl iQ™ Multiplex (Bio-Rad Laboratories), 0.25 µM of each PCR primer, 0.5 µM of FAM-probe and 20 ng of genomic DNA or RNA/cDNA. Reactions were incubated in the thermal cycler for 3 min at 95°C (1 cycle) followed by 39 cycles consisting of 10 s at 95°C, 10 s at 51°C and 30 s at 72°C.

2.12.2 HRM Analysis

Sequence variations in different samples can be differentiated in unique peaks using High-Resolution Melting (HRM) Master Mix (Roche Diagnostics). The target fragments were amplified using HRM oligonucleotides (Annex II) to identify sequence variation in *Ans* amplicons of raspberry varieties; "Anne" (154 base pairs) and "Tulameen" (149 base pairs). The reaction mixture was prepared by means of 0.25 µM of each primer, 10 µl of LightCycler 480 HRM master mix, 0.25 µl of DMSO and 3.5 mM of $MgCl_2$ in a 20 µl volume. PCR amplifications were performed using about 5 ng of genomic DNA by applying the program as follows: 1 cycle of 2 min at 98 °C; 45 cycles of 5 s at 98 °C and 10 s at 51 °C; heated to 95 °C for 1 min and cooled to 70 °C for 1 min; melting curve 70 °C to 95 °C with 0.2 °C/min increment. A similar output of peaks can also be obtained using melting curve with iQ™ Cyber® Green to analyze homozygous or heterozygous nature of the mutation in "Anne".

2.12.3 CAPS marker analysis for Ans alleles

Cleaved amplified polymorphic sequence (CAPS) markers are a useful and simple technique that can be applied to discriminate PCR fragments for homozygous or heterozygous alleles through digestions (Konieczny and Ausubel, 1993). Sequencing analysis of *Ans* gene enabled to design CAPS marker based on HaeIII restriction site to distinguish the homozygous (*Ans*:*Ans*/*ans*:*ans*) or heterozygous (*Ans*/*ans*) alleles of *Ans* gene. Genomic DNA of yellow ("Anne") and red ("Tulameen") fruited varieties of raspberry was used as a template to amplify the fragments by PCR using CAPS-F and CAPS-R unique primers (listed in Annex II). PCR amplifications were performed by following the program, 98 °C for 30 s and 98 °C for 5 s (denaturation), 60 °C for 20 s (annealing) 72 °C 15 s (extension), 24 cycles from denaturation to extension, 72 °C for 160 s (final extension) using Q5® High-Fidelity DNA Polymerase (New England Biolabs) and Bio-Rad Thermal Cycler. The amplified PCR product was digested by HaeIII restriction enzyme (New England Biolabs, R0108). Digestion reaction was carried out with 5 µl PCR product and 1.5 µl (10 U/µl) HaeIII at 37°C for 2 hrs. The size of DNA fragments

was subsequently visualized On-Electrophoresis-Chip through Agilent 2100 Bioanalyzer by following the instructions of High Sensitivity DNA Kit (Agilent Technologies). Then, the marker was tested on parents of "Anne" ("Amity" x "Glen Garry") and "Tulameen" ("Nootka" x "Glen Prosen"). The CAPS analysis was further extended to various colored varieties, such as yellow fruiting "Citria", "Gelbe Sugana", "Golden Everest", "Lumina", "All Gold", "Alpnen Gold", "Giallo Mutant", "Golden Queen"; orange fruiting "Valentina"; red fruiting "Heritage", "Sugana Red", "Meeker", "Autumn Bliss", "Pocahontas", "Himbo Top"; purple fruiting "Tayberry", "Buckingham Tayberry" and black fruiting "Black Jewel" (Annex 1). The fragment separations were also done on 4.5% High-Resolution Agarose Gel (Sigma) by running samples at 150 V for 2-3 hours. Samples on agarose gel were run along with GeneRuler^{TM} 1 kb Plus DNA Ladder (#SM1331) and 50 bp DNA Ladder (#SM0371) (Thermo Scientific^{TM}).

2.12.4 Rubus sequencing for Ans mutations

The *Ans* gene from yellow, orange and red fruiting varieties (see under 2.1 and Annex I) was cloned and subjected to sequence analysis to evaluate more mutations/variations in the gene. These varieties include "Golden Queen", "All Gold", "Gelbe Antwerpener", "Golden Everest", "Giallo Mutant", "Gelbe Siebenkugel", "Sugana Gold", "Fall Gold", "Juan de Metz", "Lumina", "Him13K39-8", "Herbert Gold" bearing yellow fruits; "Orange Marie", "Valentina" bearing orange fruits; "Amity", "Glen Garry", "Heritage", "Glen Prosen", "Autumn Bliss", "Sugana Red", "Meeker", "Pocahontas", "Himbo Top" bearing red fruits; "Tayberry", "Buckingham Tayberry" bearing purple fruits and "Black Jewel" bearing black fruits (Annex I).

2.13. Extraction of compounds and analysis

2.13.1 Extraction of Rubus polyphenols

90-120 mg of the powder of ground fruit tissues was transferred to 1.5 ml Eppendorf tubes under liquid nitrogen. Polyphenolic compounds were extracted with 700 µl of 70% acetone containing 0.015% BHT. The mixture was centrifuged at 12,000g for 7 min to pellet the cell debris and to obtain the supernatant. The supernatant was collected into a new tube and pellet was re-suspended for the second extraction as described above. Both extractions were combined and filtered through 0.22 µm PVDF filter to carry out the chromatographic analysis as described earlier (Vrhovsek et al., 2012).

2.13.2 Anthocyanins from RiAns complemented A. thaliana

Anthocyanins were extracted in methanol-HCl (1% HCl) and subjected to HPLC analysis. The separation was accomplished under gradient conditions on a Nucleodur C18ec column (250/4; Macherey-Nagel, Düren, Germany) with solvent A 1% phosphoric acid in water and solvent B 1% phosphoric acid in acetonitrile. The gradient starts with 100% A to 50% A in 25 min, plateau of 3 min, up to 100% A in 7 min and final plateau of 5 min with a flow rate of 1 ml/min and monitored at 280 and 515 nm.

2.13.3 Carotenoids from E. coli complemented genes

Extraction of carotenoids was carried out by disrupting the pelleted freeze-dried cells by sonication of 15-20 min with 3-5 min intervals and by adding 375 µl methanol. 750 µl chloroform was added and vortexed for mixing before the mixture was chilled on ice for 20 min. 375 µl H_2O was added, mixed and centrifuged for 5 min at 12,000 g at room temperature. The centrifugation separated the mixture in three phases; the upper containing polar MeOH, the middle with disrupted cells and proteins and the lower non-polar (chloroform) which contains the carotenoids. These carotenoids containing phases were collected into the new Eppendorf tubes. The solvent was dried using speed vacuum centrifuge at low boiling point without light source for 30 min and stored at -20 °C until further analysis.

Dried carotenoids were re-suspended in 200 µl ethyl acetate and centrifuged at 12,000g for 10 min. The supernatant was collected in new tubes of which 30 µl was transferred to glass vials. 3 µl extract was injected to UPLC-PDA/MS system to ascertain the present carotenoids as previously described (Nogueira et al., 2013; Wehrens et al., 2013).

3. RESULTS

3.1. Polyphenols and flavonoid pathway genes

The study of polyphenol composition of yellow and red colored raspberries can show significant differences predicting the deviation of secondary metabolism into other directions. Further, structural and regulatory genes of the flavonoid biosynthetic pathway playing key roles in the biogenesis of different pigments including flavonols and anthocyanins in fruit tissues can predict the possible block step in the pathway. Various polyphenols in "Anne" and "Tulameen", and important findings of the flavonoid pathway genes in *Rubus* are described below in this section.

3.1.1 Polyphenols in "Anne" and "Tulameen"

The metabolic profiling of "Tulameen" and "Anne" indicates different concentrations of various polyphenolic metabolites in both varieties as presented in Figure 11. There are no particular differences among most of the compounds of various classes of metabolites studied between "Anne" and "Tulameen" except the known difference of anthocyanins between yellow and red cultivars. However, some important differences were noticed, such as protocatechuic acid, 3,5-diOH-benzoic acid (benzoic acid derivaties), procyanidin B2 and B4 (flavan-3-ol), naringenin-7-*O*-glucoside (flavanone) and quercetin-3,4-*O*-diglucoside (flavonol) were found higher in "Tulameen" than its counterpart "Anne". Similarly, some phenylpropanoids (chlorogenic acid, sinapyl alcohol) and stilbene (*t*-piceid) concentrations were also detected higher in "Tulameen" than "Anne". Some higher concentrations of metabolites were also found in "Anne" than "Tulameen", such as vanillic acid (benzoic acid derivative), naringenin (flavanone), taxifolin (syn. dihydroquercetin; dihydroflavonol) and quercetin-3-*O*-glucuronide (flavonol) and raspberry ketones (Figure 11). Although results indicate that concentrations of some metabolites are higher in "Tulameen" and some in "Anne", there is no specific class of compounds significantly different between these two varieties that may predict the blocked step of the flavonoid pathway in "Anne". Apparently, it indicates that the block is not in the main flavonoid pathway and proposes a downstream/late step of flavonoid pathway. Therefore, anthocyanin pathway step can be very important controlling/inhibiting the biosynthesis of anthocyanins in "Anne".

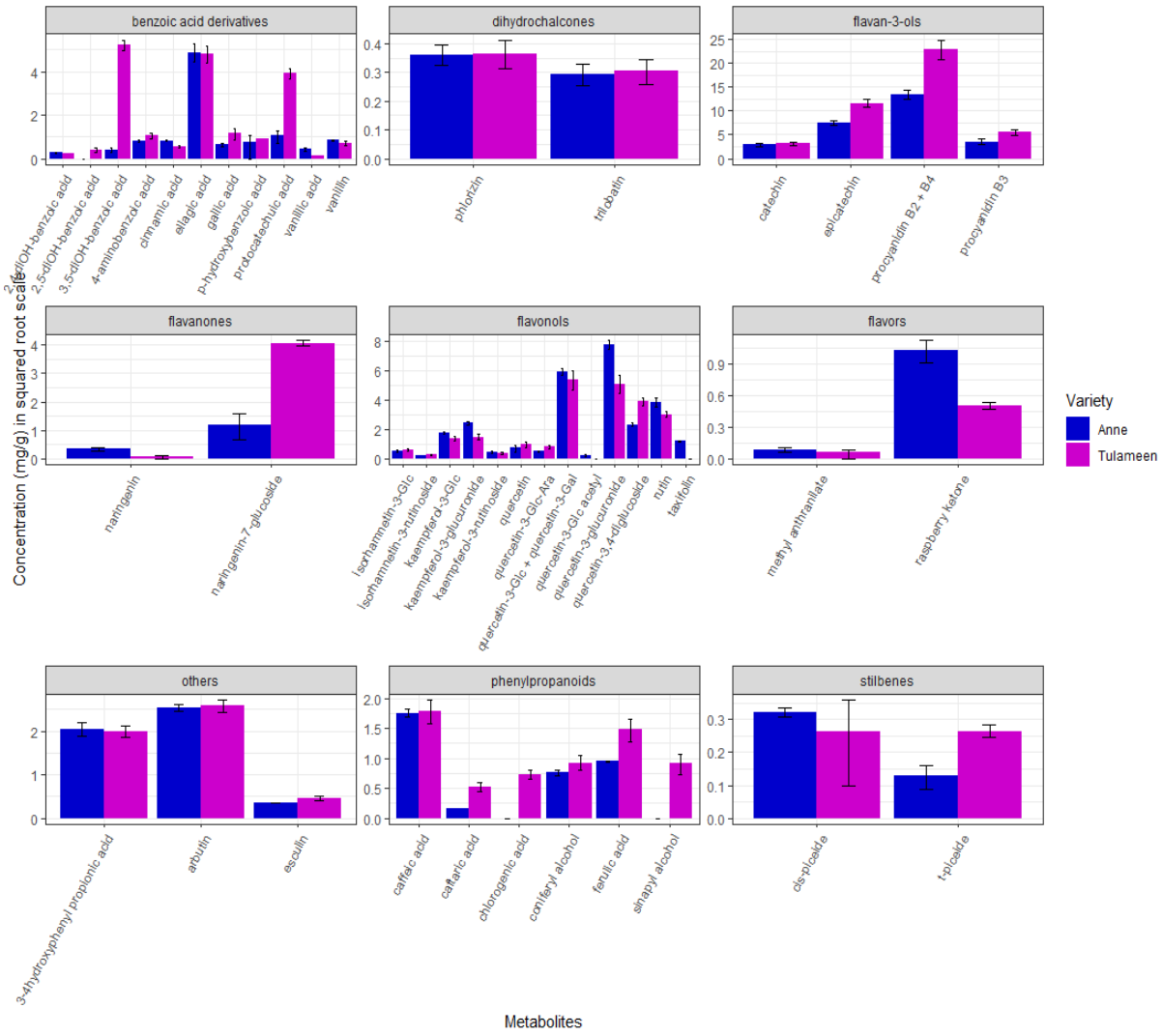


Figure 11: Concentrations (in square-root scale) of polyphenols present in yellow-fruiting "Anne" and red-fruiting "Tulameen" raspberries. Bar charts show the mean values of the metabolite concentrations in squared-root scale with standard deviation bars of 3 biological replicates.

3.1.2 In silico mining of flavonoid pathway genes of Rubus

In silico search of *Rubus* genome draft version 1.08 (cv "Heritage") and an in house 454 EST library of fruit stages of "Tulameen" (unpublished data) with flavonoid pathway genes of *Fragaria* species query enabled the identification of putative genes of *Rubus*. The following *Fragaria* genes were used to perform searches: *Chs*, *Fht*, *Dfr*, *Ans*, *Ufgt*, *Fls*, *Lar* and *Anr* (GenBank Accession Numbers AY997297, AY691919, AY695812, AY695817, AY575056, DQ087252, DQ087253 and DQ664193) (as listed in Annex III). The regulatory gene sequence of *MYB10* of *Fragaria* (GenBank Accession Number EU155162) was used to search for putative *Rubus MYB10* gene sequences using "Heritage" genome.

3.1.3 Transcriptional analysis of regulatory and structural genes

The biosynthesis of the flavonoid pathway related pigments is associated with transcriptional regulation of structural genes. The well-described transcription factor *MYB10* plays a key role in the initiation and regulation of the transcription of structural genes related to anthocyanin biosynthesis in Rosaceae plants, such as apple and strawberry. In view of understanding the role of *MYB10* in raspberry, expression analysis was carried out from all fruit stages (1-5) in both varieties "Anne" (yellow) and "Tulameen" (red). The expression level of *RiMYB10* gene increases as long as the fruit ripening proceeds from stage 1 to stage 5, both in "Anne" and "Tulameen". In both varieties, the minimum expression level was observed at fruit stage 1 and maximum expression at fruit stage 5 (Figure 12). Therefore, the demonstrated expression levels of transcription factor *MYB10* correlates with the fruit development and ripening in both varieties.

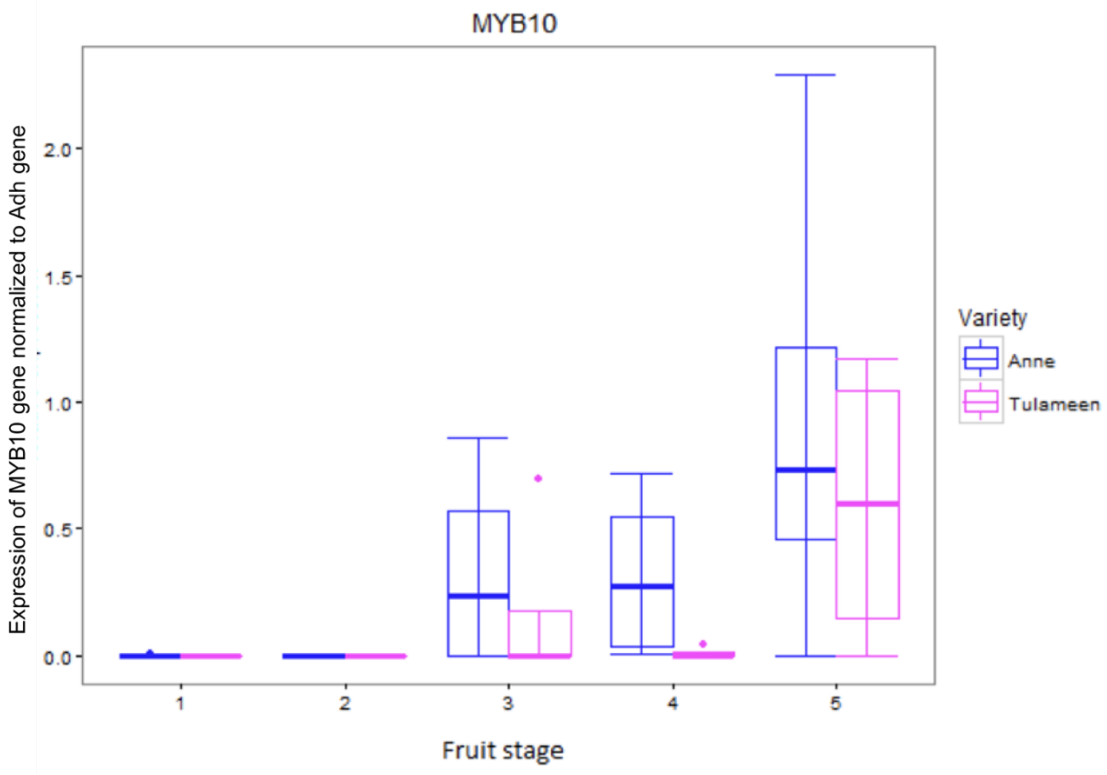


Figure 12: Expression analysis of transcription factor *MYB10* at fruit stages (1-5) in yellow variety "Anne" and red variety "Tulameen", respectively. Boxplots show the mean values of the expression level with standard deviation bars of 3 biological replicates. Data falling outside the lower quartile - higher quartile range are plotted as outliers of the data.

In raspberry fruits, pigments such as anthocyanins start to appear and become visible at fruit "turning" stage 3. Therefore, the expression profile of the flavonoid pathway genes at fruit development stages 3 and 4 was carried out in "Anne" and "Tulameen" as shown in Figure 13. In both varieties, upregulation of the anthocyanin pathway genes, such as *Chs*, *Fht*, *Dfr* and *Ufgt*, was observed from stage 3 to 4 at different levels. The *Chs* transcripts are not induced in "Anne" to the extent as in "Tulameen" from fruit development stage 3 to 4. The apparent RNA accumulation of *Ans* gene is coordinated with the other biosynthetic genes analyzed as it shows a dramatic increase from stage 3 to 4 in fruits of red variety "Tulameen". However, such increase in expression was not detected in yellow fruits of "Anne". Along with these anthocyanin pathway genes, the anthocyanidin reductase (*Anr*), a gene of a side branch of the flavonoid pathway, also shows the increase in the expression pattern of fruits of both varieties. The genes of the branching steps of flavonol pathway (*Fls*), as well as leucoanthocyanidin reductase (*Lar*) gene, shows a similar trend of increased expression levels in "Tulameen" from stage 3 to 4. However, transcript levels of *Lar* gene decreased from fruit development stage 3 to 4 in "Anne" but expression levels of *Fls* remained almost stable for both stages (Figure 13).

The expression profile shows that among all the anthocyanin pathway genes *Chs* and *Ans* genes are not upregulated onward fruit turning stage in "Anne" (Figure 13). However, identification of metabolites up-stream of chalcones (as indicated in Section 3.1.1) in "Anne" and "Tulameen" excludes the block at this step. Therefore, further analysis of the *Ans* gene was performed to ascertain the block at the genetic level. In addition to *Ans* gene, the role of *Dfr* gene has been well established in pigmentation hence its molecular analysis can be useful too.

3.1.4 Analysis of the Dfr sequence

Literature refers to DFR as the key enzyme in the anthocyanin biosynthesis during fruit development (Li et al., 2001; Tsuda et al., 2004; Miao et al., 2016). Therefore, sequence analysis of *Dfr* genes from "Anne" and "Tulameen" varieties was carried out. PCR amplification of *RiDfr* gene gave a 1032 bp amplicon encoding a polypeptide of 344 amino acids in both varieties "Anne" (GenBank Accession Number MF850337) and "Tulameen" (GenBank Accession Number MF850338). Comparison of DFR protein sequences showed 99% similarity with only three single amino acid polymorphisms (SAAPs) and none of them in or near conserved regions (Annex IV). "Tulameen" and "Anne" both presented 98% identity with the BLAST search of the *Rubus* draft genome of cv. "Heritage". Identity search with published DFR protein sequences from GenBank (NCBI) disclosed that both DFRs shared high

identities to DFR sequences of other members in the Rosaceae family, such as 91% to *Rosa* (Luo et al., 2016), 90% to *F. vesca* (Miosic et al., 2014), 88% to *Prunus* (Liu et al., 2013), 85% to *F.* × *ananassa* (Almeida et al., 2007), 85% to *Pyrus* (Yang et al., 2013) and 84% to *Malus* (Fischer et al., 2003). Molecular phylogenetic tree of the deduced amino acids shows that RiDFR sequences cluster within the other members of Rosaceae family (Annex V). Overall molecular analysis of *Dfr* genes from both varieties, "Anne" and "Tulameen" indicates that there is no obvious evidence of mutations resulting in inactive proteins and genetic pathway blocks at this level.

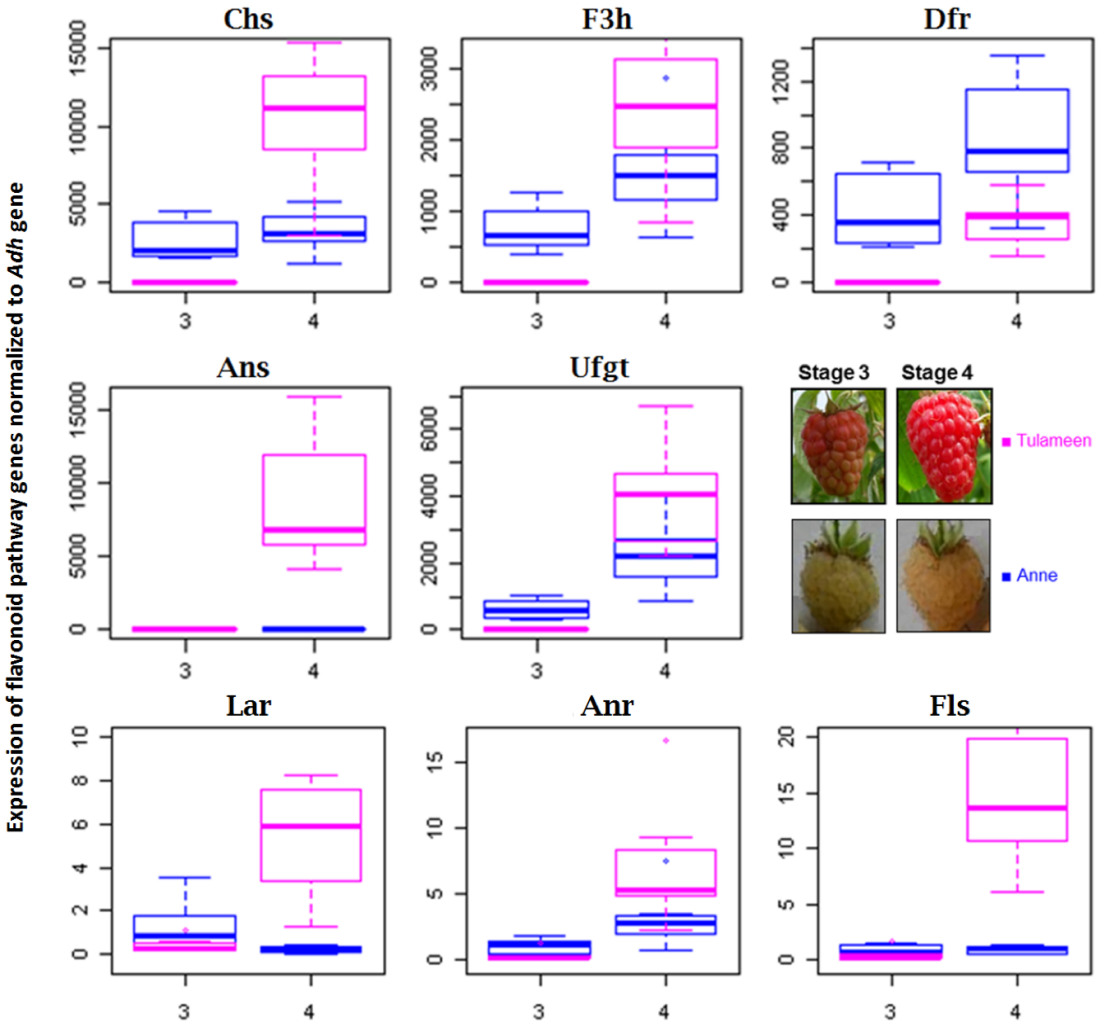


Figure 13: Expression analysis of flavonoid pathway genes *Chs*, chalcone synthase; *F3h*, flavanone 3*β*-hydroxylase; *Dfr*, dihydroflavonol 4-reductase; *Ans*, anthocyanidin synthase; *Ufgt*, UDPG-flavonoid-*O*-glycosyltransferase; *Anr*, anthocyanidin reductase; *Lar*, leucoanthocyanidin reductase and *Fls*, flavonol synthase at fruiting stage 3 and 4 in yellow variety "Anne" and red variety "Tulameen", respectively. The inset picture shows an example of a red and a yellow raspberry at the respective fruit ripening stages. Box plots show the mean values of the expression level with standard deviation bars of 3 biological replicates.

3.1.5 Cloning and genomic structure of Rubus Ans

Ans genes, spanning the full coding region, were cloned with the help of assembled contigs (as illustrated in Annex VI). Gene-specific primers were designed based on the sequence assembly for PCR amplification of a 1835 bp genomic *Ans* fragment from "Tulameen" that includes the entire coding region with one 446 bp intron, 118 bp of the 5'UTR and 29 bp of the 3'UTR (GenBank Accession Number KX950789). Subsequently, the same approach was used to amplify the respective *Ans* gene of "Anne" (GenBank Accession Number KX950788) resulting in an 1840 bp amplicon. The *Ans* fragments from genomic DNA of "Anne" and "Tulameen" cloned into the pCR™4-TOPO® vector were named pCR™4-1840 and pCR™4-1835, respectively (Figure 8). Direct comparison of these two genes revealed a 5 bp insertion (*GGCCT*) in the second exon at position 730 of the "Anne" gene (Figure 14). This insertion in the "Anne" gene (*ans*) was designated as ans^{+5}.

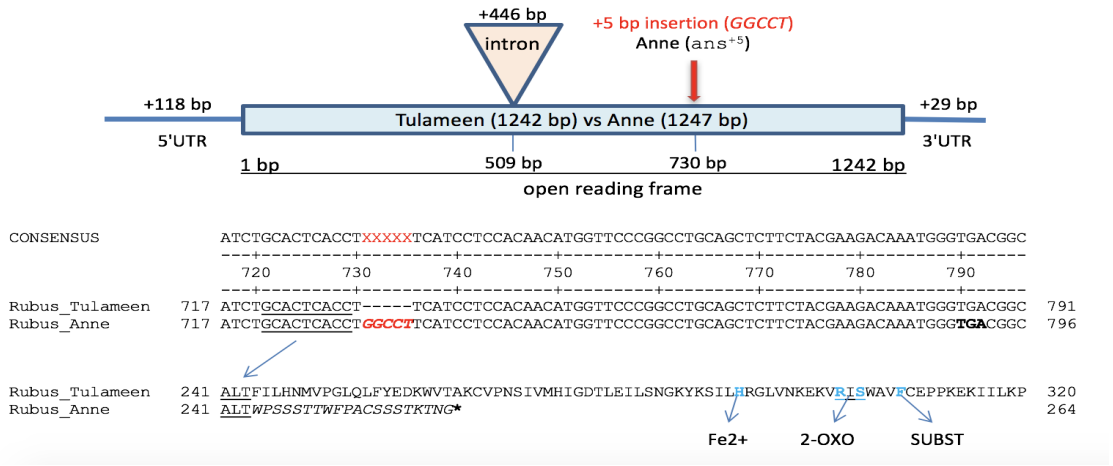


Figure 14: Analyses of anthocyanidin synthase genes from "Tulameen" and "Anne". *Ans* gene indicates a 5 bp insertion (ans^{+5}) in the second exon at position 730 bp of "Anne" causing a frameshift and thus leading to a truncated protein, missing conserved regions for substrate, co-substrate, and iron binding sites. The *Ans* fragment from "Anne" and "Tulameen" includes the entire coding region with one 446 bp intron and 118 bp of the 5'UTR and 29 bp of the 3'UTR.

3.1.6 Sequence analysis of Rubus Ans

A *RiAns* fragment of 1389 bp was obtained by PCR amplification using primers designed from the predicted sequence (3.1.4) using cDNA of "Tulameen" as a template. The fragment contained the 1242 bp open reading frame (ORF), encoding a polypeptide of 414 aa residues

and showing 92% similarity to the *F.* × *ananassa* ANS protein. The comparative sequence analysis of *Ans* fragments revealed the same ans^{+5} in "Anne" cDNA as found in genomic DNA sequence. This extra 5 bp sequence (*GGCCT*) creates a frame-shift in 'Anne-Ans' and results in a pre-mature stop codon after 20 aa from this mutation. As a result, a truncated ANS protein of only 264 aa was predicted instead of wild-type 414 aa protein. The smaller ANS protein in "Anne" lacks the necessary structural features required for substrate and co-substrate binding as indicated already above (Figure 14, Annex VII). This provides strong evidence for an inactive ANS protein and therefore a possible genetic block in the pathway in this step.

The nucleotide sequence alignment showed that the putative *Rubus* ANS grouped together with other known ANS sequences from Rosaceae as well as other plant families and is separated from other flavonoid pathway 2-ODDs, such as flavonol synthase and flavanone 3*β*-hydroxylase (Annex VII & VIII). Further, RiANS protein sequence of "Tulameen" showed high (88%) similarity to other plant species (*Malus* ANS, *Prunus* ANS and *Pyrus* ANS) of Rosaceae family (Annex VIII) as well as ANS from other plant genera, such as 81% to *Medicago* ANS (Pang et al., 2007); 81% to *Arabidopsis* ANS (Pelletier et al., 1997); 79% to *Petunia* ANS (Weiss et al., 1993); 78% to *Perilla* ANS (Saito et al., 1999); 75% to *Gynura* ANS (Shimizu et al., 2010); 73% to *Gerbera* ANS (Puzio et al., 2009) but comparatively less similarities to monocotyledon *Allium* ANS (62%; Kim et al., 2006) and *Oryza* ANS (52%; Reddy et al., 2007). It is obvious that most of the catalytical important residues are missing only in ANS of "Anne" in comparison to 2-ODD's as illustrated in Annex VII. Therefore, further analysis of *Ans* gene was performed to elucidate its role in red raspberries as compared to yellow ones.

3.1.7 Copy number analysis of Ans gene

Determination of *Ans* gene copies is desirable as more copies of *Ans* gene may exist and the genome of *Rubus* is not fully sequenced yet (89%; Ward et al., unpublished). The standard curve created, enabled the identification of *Ans* copy number in *Rubus* by comparing with the reference (as illustrated in Section 2.6.3). In order to estimate the copy number of *Ans* gene in *Rubus*, the genome of the diploid species *F. vesca* (2n=2x=14) with a single copy of *Ans* gene was exploited, and *F.* × *ananassa* Duch. (octaploid; 2n=8x=56) with four copies of *Ans* gene (Almeida et al., 2007) was used as an additional endorsement to the methodology. Mass of pCR^{TM}4-1840 plasmid (5795 bp) containing "Anne" *Ans* gene was calculated as 6.35e-18 g for determining the genomic complexity. Similarly, haploid genomes of *F. vesca* (n=x=7; 120 Mb)

and *F.* × *ananassa* Duch (n=4x=28; 349 Mb) and *Rubus* (n=x=7; approximately 150 Mb) were utilized and mass was calculated as 3.288e-13 g, 2.630e-13 g and 7.650e-13 g, respectively. Now, *Ans* target from *Rubus* and other reference species was put under comparative copy number analysis to the normalized single *Ans* copy (pCR™4-1840). Average numbers of *Ans* gene copies in *Rubus* varieties are closely correlated to the known *Ans* copies in *F. vesca* genome (as indicated in Figure 15). Melting curve analysis helped to prove the absence of any non-specific amplification. The corresponding ratio of *Ans* gene copies in haploid genomes of *F. vesca*, *F.* × *ananassa* and different raspberry varieties are given in Table 2. It is evident that copy number in haploid genome of all the tested *Rubus* varieties ("Anne", "Golden Queen" & "Heritage") is same as in haploid genome of *F. vesca* normalized to that of the single *Ans* copy gene (pCR™4-1840). Furthermore, haploid genomes of *F. vesca* and all the raspberry varieties present the same ratio (single copy of *Ans* gene) in comparison to the haploid genome of *F.* × *ananassa* reference with four *Ans* copies (Table 2, Figure 15). Finally, a single copy number of *Ans* gene in haploid *Rubus* genome is suggested.

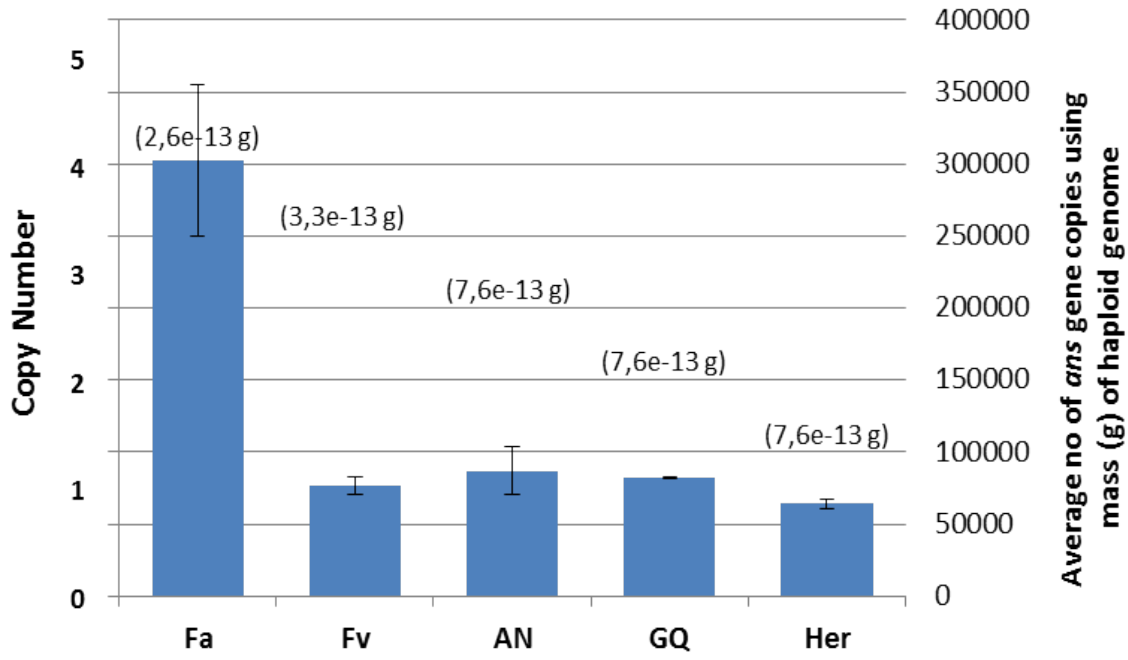


Figure 15: Bar chart showing *Ans* gene copy number in the analyzed haploid genomes. "Anne" (AN), "Golden Queen" (GQ), and "Heritage" (Her) normalized to that of the single copy *Ans* gene of *F. vesca* (Fv) and four *Ans* copies of *F.* × *ananassa* Duch. (Fa) presented with standard deviation bars of 3 biological replicates.

Table 2: Mean data values presented with standard deviations (SD) and corresponding ratio scored to give integer copy number of *Rubus ans* gene in "Anne", "Golden Queen", "Tulameen" and "Heritage" with respect to (w.r.t.) *F. vesca* and *F.* × *ananassa* references in haploid genome of the Rosaceae members.

Rosaceae members	Mean±SD	Ratio w.r.t F. vesca reference	Ratio w.r.t. F. × ananassa reference
F. × ananassa	302714±52332	-	4
F. vesca	76849±5970	1	-
Anne	87049±16857	1	1
Golden Queen	81970±655	1	1
Heritage	64137±3582	1	1

3.1.8 Heterologous expression in E. coli

Heterologous protein expression of *RiAns* was achieved by introducing the constructs with the different fragment versions ('pDEST-792' 'pDEST-1242' and 'pDEST-1247') into RIPL bacterial cells and IPTG driven induction. SDS-PAGE analysis showed that protein was not present in the soluble fraction (supernatant); however, most proteins were visible in insoluble fraction as presented in Figure 16. Both versions of "Anne" *Ans* gene (full length 1247 bp and ORF 792 bp) gave the same protein size, i.e 29.14 kDa. However, "Tulameen" version of *Ans* gene (ORF 1242 bp) gave a protein of the expected size of 45.77 kDa.

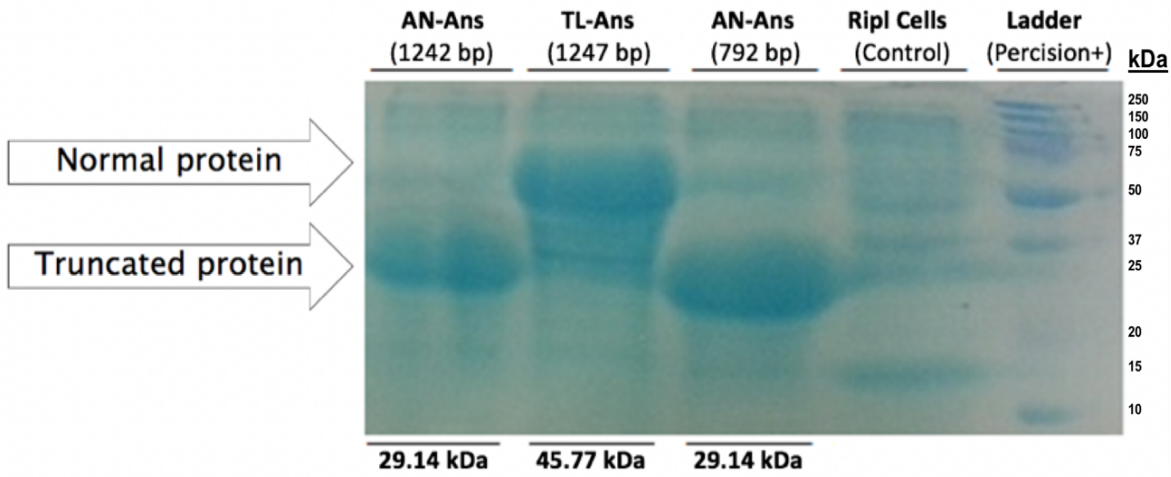


Figure 16: Protein expression in RIPL bacterial cells at 28 °C. Heterologous proteins from both versions of 'Anne-Ans' (AN-Ans) (full length 'pDEST-1247' and ORF 'pDEST-792') and 'Tulameen-Ans' (TL-Ans) (ORF 'pDEST-1242 bp').

3.1.9 Complementation of Arabidopsis ldox mutant

Arabidopsis transgenic lines 'ldox::35S:Ans_Anne' and 'ldox::35S:Ans_Tulameen' were obtained from *ldox* (i.e. *ans*) mutant of *A. thaliana* harboring *RiAns* coding sequences from "Anne" and "Tulameen", driven by the constitutive *CaMV35S* promoter with respect to the control *tds4-2* ('ldox:KO') line. DNA isolated from the transgenic lines of *Arabidopsis* was subjected to PCR amplification of *Bar* and *Ans* genes. PCR products were separated and fragment size was confirmed on agarose gels as expected (Figure 17A-B). In this experiment, to determine the activity of *Ans* gene from "Anne" and "Tulameen", 8 of 13 'ldox::35S:Ans_Tulameen' lines and 5 out of 6 'ldox::35S:Ans_Anne' lines were tested, respectively. One representative line is shown from all genotypes (Figure 18).

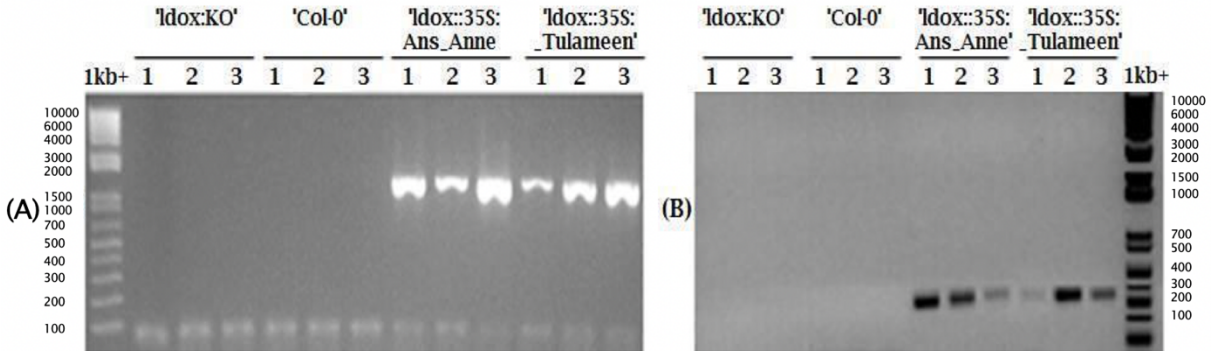


Figure 17: PCR analysis, detecting *RiAns* (A) and *Bar* (B) transgenes from "Anne" and "Tulameen", respectively, in independent transgenic *Arabidopsis* lines. 'Col-0' and 'ldox:KO' are non-transgenic *Arabidopsis* lines.

It is evident, as shown in Figure 18 that in response to sucrose stress induction, wild-type 'Col-0' showed anthocyanin accumulation in 5 day-old seedlings in contrary to the seedlings in sucrose-minus media. In either case, not taking into account the sucrose treatment, the control *tds4-2* line could not produce anthocyanins in its seedlings at all due to the block in the ANS step. However, in response to sucrose stress, in 'ldox::35S:Ans_Tulameen' lines, it could clearly be noticed that a wild-type-like anthocyanin phenotype in hypocotyl and cotyledons of 5 day-old seedlings was restored. On the other hand, it was observed that 'ldox::35S:Ans_Anne' did not complement the *tds4-2* phenotype and remain anthocyanin-less under sucrose stress (Figure 18A1-H1). Hence, 'ldox::35S:Ans_Anne' lines lack the anthocyanins.

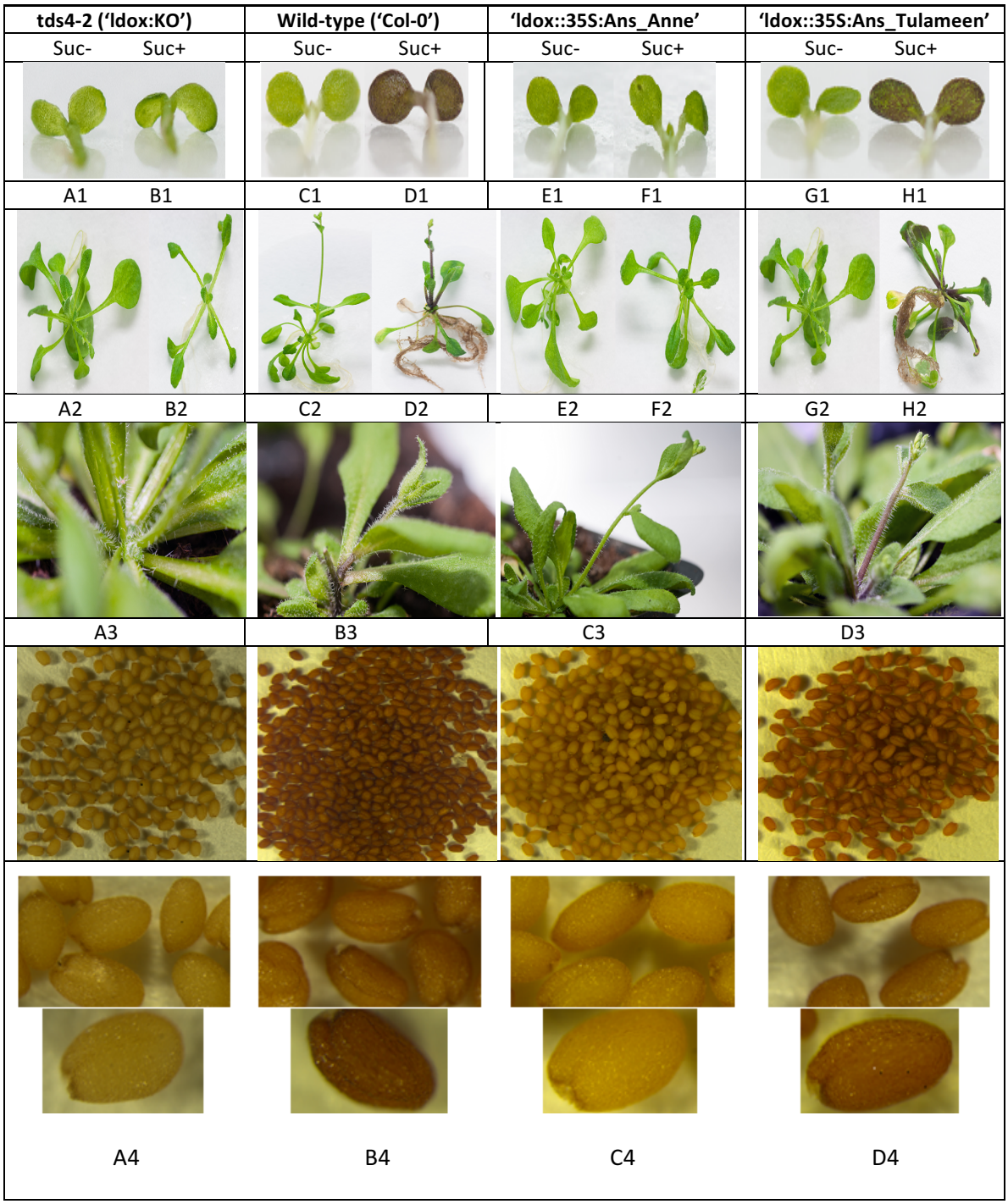


Figure 18: Phenotype of *Arabidopsis* transgenic lines in comparison to wild-type ('Col-0') and *ldox* knockout line (*tds4-2*; 'ldox:KO'). Control, wild-type, 'ldox::35S:Ans_Anne' and 'ldox::35S:Ans_Tulameen' seedlings grown in half MS without sucrose (Suc-), 5 day-old seedlings (A1, C1, E1, G1) and 2 week-old plantlets (A2, C2, E2, G2), respectively. Control, wild-type, 'Anne-Ans-KO' and 'Tulameen-Ans-KO' seedlings grown on half MS with 5% sucrose (Suc+), 5 day-old seedlings (B1, D1, F1, H1) and 2 week-old plantlets (B2, D2, F2, H2), respectively. Anthocyanin formation was also obtained in 4 week-old wild-type plants (B3) and 'Tulameen-Ans-KO' (D3) in comparison to control 'ldox:KO' (A3) and 'ldox::35S:Ans_Anne' (C3) lines. The seed coat color is associated with the presence (B4 & D4) or absence of proanthocyanidins (A4 & C4).

This result demonstrates the non-functional status of *Ans* gene in "Anne" in contrary to the functional gene in "Tulameen". None of the *A. thaliana* lines presents phenotype with anthocyanins accumulation in their 5 day-old seedlings in media without sucrose. In response to sucrose induction, the phenotype of 2 week-old plantlets in the growth chamber (Figure 18A2-H2) and 4 week-old plants in greenhouse remained unchanged, i.e. 'Col-0' and 'ldox::35S:Ans_Tulameen' lines showed presence of anthocyanins while 'ldox:KO' and 'ldox::35S:Ans_Anne' lines remained anthocyanin free (Figure 18A3-D3).

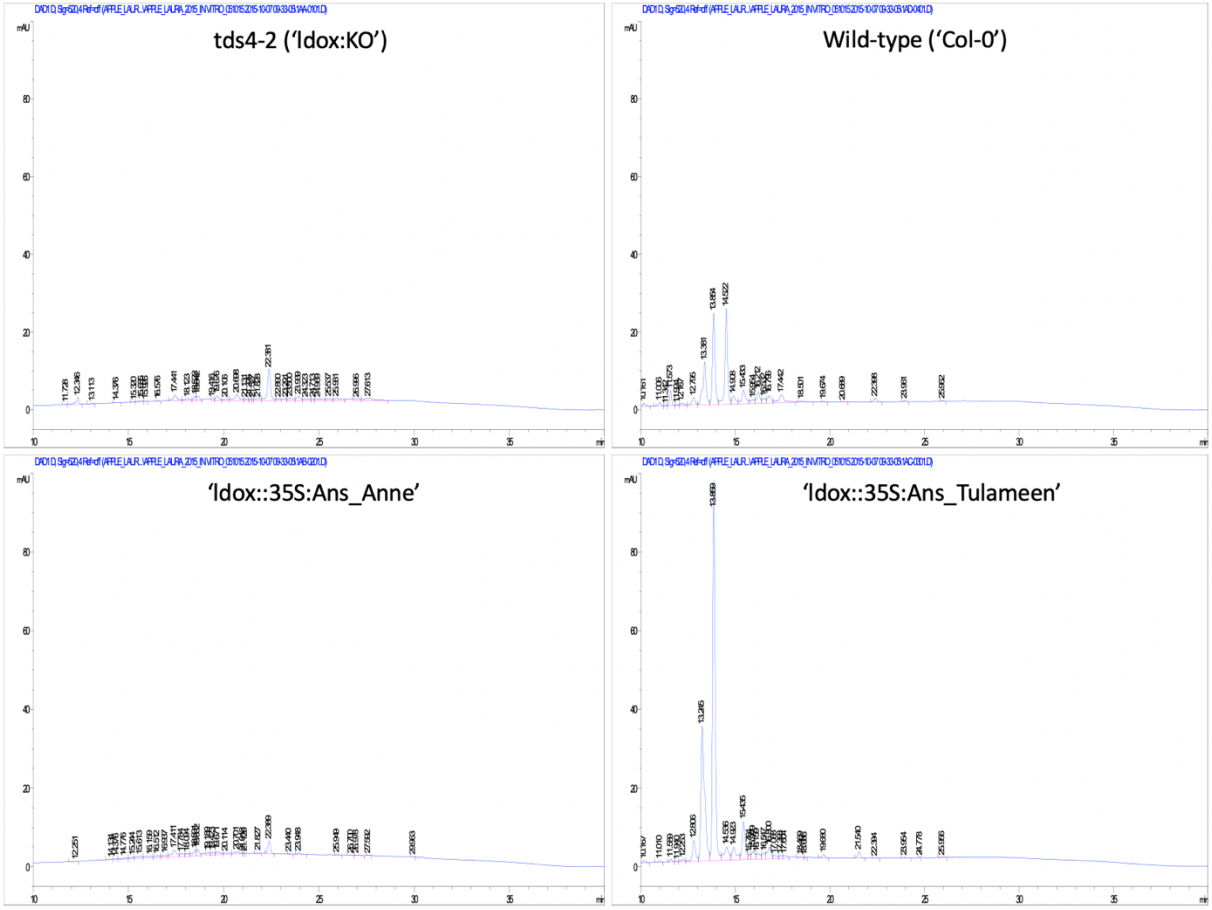


Figure 19: HPLC profile of anthocyanins (520 nm) obtained from extracts of 4 week-old *A. thaliana* transgenes for 'Anne-Ans' and 'Tulameen-Ans' in comparison to wild-type ('Col-0') and control *ldox* knockout line ('ldox:KO').

As expected from anthocyanin production in transgenic tissues, the lines of 'ldox::35S:Ans_Tulameen' showed the characteristic brown seed color of *A. thaliana* wild-type 'Col-0', whereas 'ldox::35S:Ans_Anne' had the same yellowish color as the 'ldox:KO' control line. Seed coat of 'ldox::35S:Ans_Anne' lines are yellow which is known to be comprised of

mostly flavonols as in control ‘ldox:KO’ line, while the brown seed coat color of ‘ldox::35S:Ans_Tulameen’ lines are associated with the presence of proanthocyanidins as found in the wild type (Figure 18A4-D4). The presence of proanthocyanidins in seed coat of ‘ldox::35S:Ans_Tulameen’ lines indicate the reinstated status of the *ldox* mutant of *A. thaliana*. Moreover, HPLC analysis from extracts of all ‘ldox::35S:Ans_Tulameen’ lines with wild type *A. thaliana* showed the presence and a similar pattern of anthocyanins while extracts of ‘ldox::35S:Ans_Anne’ and ‘ldox:KO’ lines did not exhibit any type of anthocyanin profile (Figure 19). Hence, in view of proposed *Ans* block in “Anne” functional characterization and complementation *in planta* provides additional strong proof of inactive ANS protein in “Anne” as compared to the functional protein in “Tulameen”.

3.1.10 Polyphenolic metabolites in various colored Rubus

The metabolic profile of yellow-fruiting “Anne” and red-fruiting “Tulameen” showed no variation in classes of metabolites studied, but only the concentrations (as indicated in Section 3.1.1). It is important to address the metabolic profiling in different colored raspberries including other yellow and orange colored raspberries because also other mutations can happen which could result in differences of polyphenolic metabolites or particular classes of compounds. Heat map, as shown in Figure 20, presents the concentrations of various phenolic metabolites in different colored varieties of *Rubus*. In all these varieties, 48 metabolites with different concentrations were identified. “Black Jewel” seems to be more concentrated in many metabolites, such as phloridzin, *t*-piceide, methyl anthranilate, kaempferol-3-*O*-glucuronide, quercetin-3-*O*-glucuronide, kaempferol-3-*O*-rutinoside, rutin (quercetin-3-*O*-rutinoside), anthranilic acid, etc., than other varieties in the current study. Concentrations of most of the metabolites in “Buckingham Tayberry” are higher than “Tayberry” while some of the metabolites are highly concentrated in “Golden Everest” and “Him 13K39-8” than other red and yellow fruiting raspberry varieties (Figure 20). It can be observed that cinnamic acid is distributed in low concentrations in red fruiting varieties but arbutin seems more concentrated in most of the yellow varieties. Furthermore, catechin and epicatechin based proanthocyanidins were also detected in all varieties. Procyanidins are randomly distributed among all varieties and seem higher in most of the red varieties. On the other hand, catechin and epicatechin were detected in lower concentrations in orange fruiting varieties “Valentina” and “Orange Marie”, red fruiting “Tayberry” and “Buckingham Tayberry” and black fruiting “Black Jewel”. Some varieties are abundant in specific compounds, such as “Him 13K39-8” in vanillin, caftaric acid and raspberry ketones, “Zheltyi Gigant” in sinapyl alcohol, “Golden Everest” in catechin. The

metabolic analysis overall didn't predict the absence of specific groups of polyphenols to indicate a possible block in the pathway of non-red varieties.

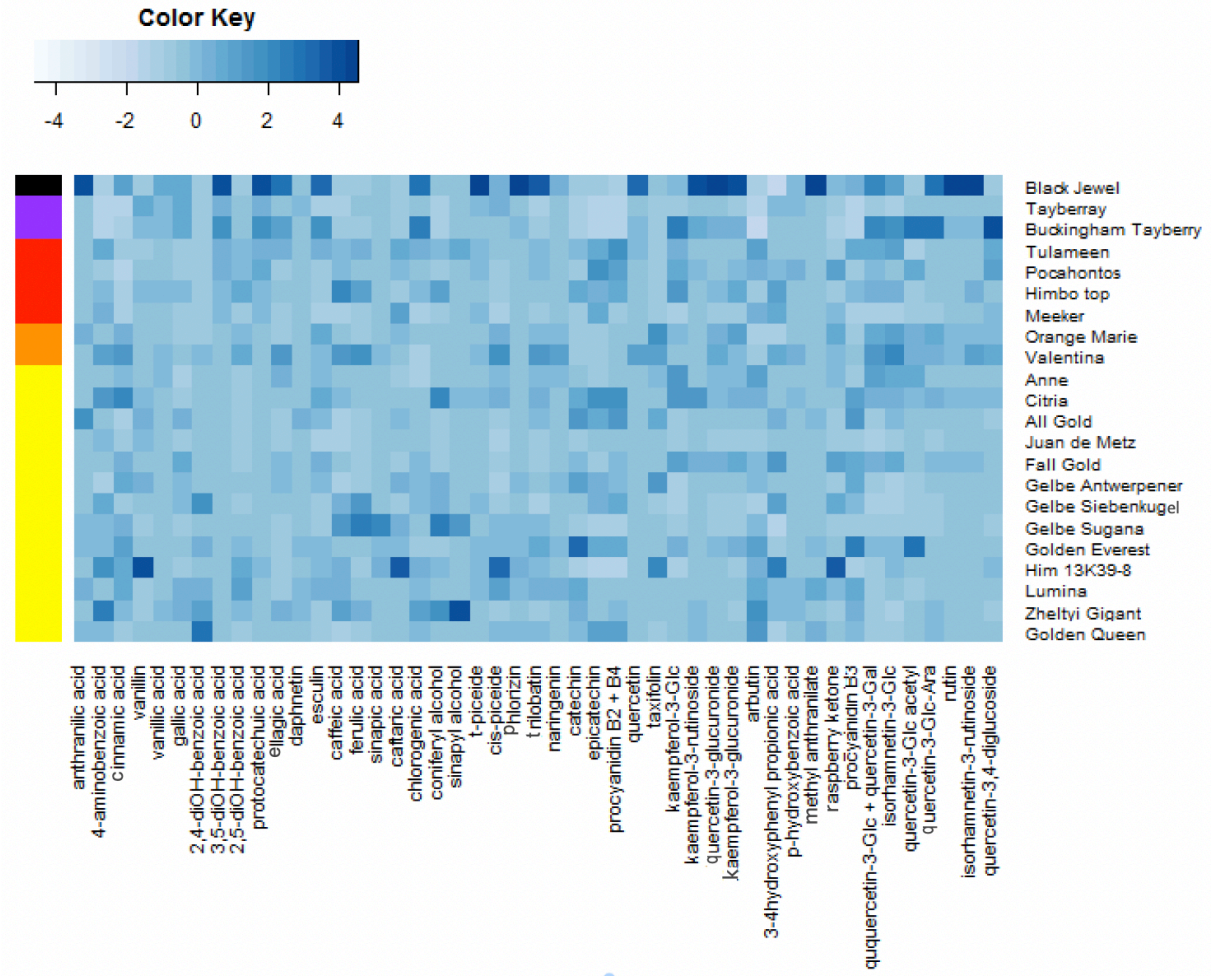


Figure 20: Heat map showing the concentrations of phenolic metabolites in a logarithmic scale of different raspberry varieties scaled from -4 to 4 level. Varieties are grouped according to the fruit color with respective color codes on the left such as black fruiting with black dark reddish-purple fruiting with purple color, red fruiting with red color, orange fruiting with an orange color and yellow fruiting varieties with yellow color.

Principal component analysis biplot (PCA biplot) shows how varieties are grouped according to various metabolites. Varieties with related compounds are clustered close to each other as presented in Figure 21. Most of the varieties lie in the closely related group of "Anne", "Citria", "Fall Gold", "Golden Everest", "Gelbe Antwerpener" etc. (yellow circle). Another related group can be observed as "Gelbe Siebenkugel", "Gelbe Sugana", "Golden Queen" and "Meeker" (red circle). However, yellow and red varieties share common characteristics in

context of concentrations of studied metabolites and can not be differentiated as indicated with yellow and red overlapping circles. Both orange fruiting varieties, "Valentina" and "Orange Marie", are also closely related as they lie near to each another (orange circle) separted from the previous once. Some varieties can be considered as separate groups, for example "Tayberry" and "Buckingham Tayberry" are different from other varieties (purple circle; Figure 21). Furthermore, "Zheltyi Gigant", and "Black Jewel" (black circle) does not belong to the previously described groups according to variation of metabolites. In view of PCA planes, varieties are distributed between both planes; however, some varieties like "Black Jewel", "Tayberry" and "Buckingham Tayberry" are separated from the common point of both planes (PC1 and PC2).

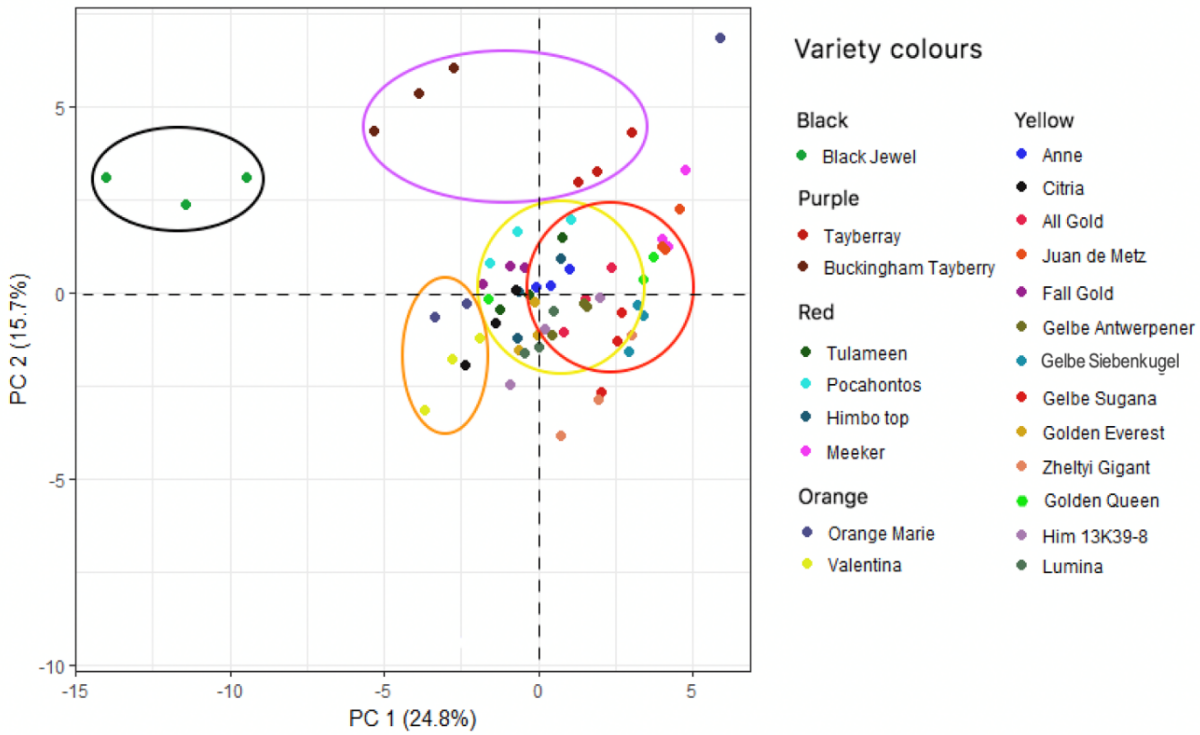


Figure 21: PCA biplot showing how raspberry varieties are grouped according to a variation of metabolites. Each variety is presented as different color code and grouped according to their fruit color. Each variety is presented with 3 samples and clustered nicely in PC. Varieties with more or less similar concentrations of compounds lie close to each other. Varieties are presented in PC1 x PC2 plane. Black, purple and orange circles represent the berry color of varieties. Yellow and red both circles have yellow and red fruiting varieties overlapped.

3.1.11 Development of DNA/RNA based probe marker for ans^{+5}

Germplasm collections of *Rubus* are available to breed and enhance their sustainable production at various platforms globally. It would be of great interest in breeding programs to enable screening of *Rubus* varieties and genotypes for Anne-like mutations inside *Ans* gene. Thus, a DNA/RNA based probe marker was developed to identify the presence of ans^{+5} mutation/insertion in other *Rubus* varieties. The probe developed was utilized to scrutinize the independent genomic DNA samples and also RNA/cDNA of different ripening stages of fruits of "Anne" and "Tulameen", respectively. As the FAM dye-labeled probe, containing 3 bp of ans^{+5} (*GGCCT*), is designed based on the above identified mutation in *Ans* gene, it will not be functional with the samples that are lacking the mutation. To validate the amplification, the same primer set was tested on genomic DNA template of "Anne" and "Tulameen" in a reaction mixture exempting the probe. Taking into account the probe specificity, full-length *Ans* gene from genomic DNA of both varieties cloned into pCR™4-TOPO® vector (pCR™4-1835 & pCR™4-1840) was also included in the analysis. It was observed from the analysis, as indicated in Figure 22A, that FAM probe was successfully applicable to 'Anne-Ans' (pCR™4-1840; violet circles) but not to 'Tulameen-Ans' (pCR™4-1835; pink squares on the baseline). Likewise, the fluorescent labeled probe applied on various independent genomic DNA samples showed the amplification for all the "Anne" samples (blue circles) but none of the "Tulameen" sample (pink square on the baseline). Despite it, not only "Anne" (blue lines) but all the "Tulameen" genomic DNA samples (pink lines) gave fluorescent signals without the probe (Figure 22A). The FAM dye-labeled probe applied on RNA/cDNA also detected the mutation from the fruit stages of "Anne" (blue lines) and none of the fruiting stage of "Tulameen" (pink) showed probe based signals (as shown in Figure 22B). NTC is also indicated along the baseline (green) like "Tulameen" samples (pink). It is evident from the analysis that the FAM probe is specific for mutation/insertion in "Anne" and does not work on genomic DNA or RNA/cDNA of "Tulameen" samples (Figure 22A-B).

3.1.12 Allelic discrimination in Rubus Ans by HRM Analysis

In HRM analysis, homozygous amplicon sequence with a gene-specific primer leads to a unique curve; however, a heterozygous sample gives different peaks. Red and green colors (Figure 23A) represent signal peaks of "Anne" and "Tulameen" *Ans* amplicons, respectively, by means of HRM analysis. The unique signal peaks for "Anne" and "Tulameen" amplicons demonstrate their homozygous status, i.e. *Ans* alleles for "Anne" are homozygous for 5 base pairs (*GGCCT*) mutation and *Ans* alleles for "Tulameen" are homozygous without any insertion in the amplified region. Analysis of quantitative PCR also showed a unique curve with the same set of

oligonucleotides confirming the homozygous status of *ans* and *Ans* alleles for "Anne" and "Tulameen", respectively. Both green peaks from left to right represent *Ans* amplicons from "Tulameen" and "Anne", respectively (Figure 23B). Thus, differences of "Anne" *Ans* amplicons to the "Tulameen" amplicons with unique peaks using both HRM and qPCR analysis suggests the homozygous nature of insertion/mutation in "Anne".

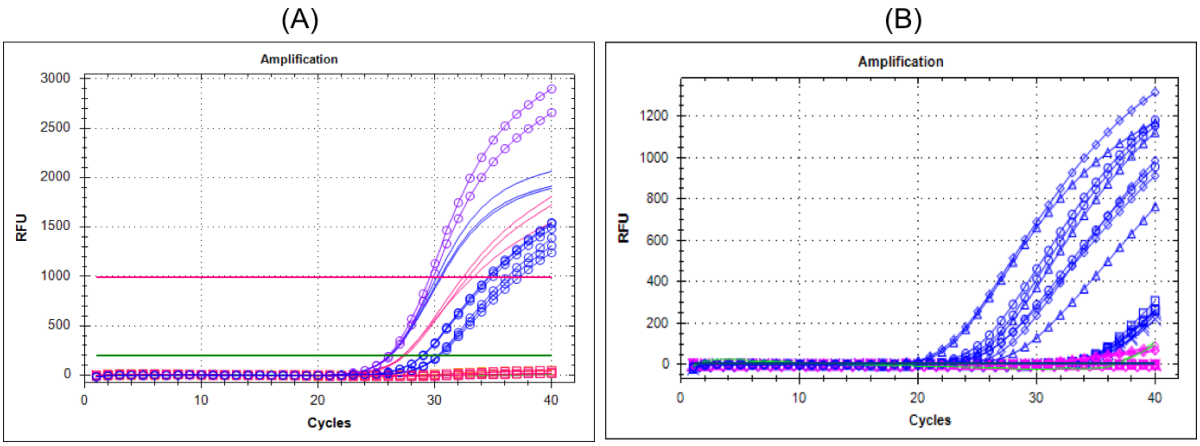


Figure 22: (A) Probe analysis on genomic DNA samples of "Anne" (blue) and "Tulameen" (pink) for the 5 bp insertion (ans^{+5}). Probe assay on both genomic DNA (blue circles), pCR^{TM}4-1840 (violet circles) of "Anne" and genomic DNA, pCR^{TM}4-1835 (pink squares) of "Tulameen". Genomic DNA samples amplified from "Anne" (blue lines) and "Tulameen" (pink lines) without probe in the reaction mix. **(B)** Probe analysis on different fruit stages of "Anne" (blue) and "Tulameen" (pink) for the 5 bp insertion (ans^{+5}). Transcripts from different fruit stages of "Anne" exhibits fluorescent signals (blue) but none of the "Tulameen" fruit stage (pink) as indicated by the diamond (Stage 1), cross (Stage 2), triangle (Stage 3), sequence (Stage 4) and circle (Stage 5). NTC is indicated along the baseline (green).

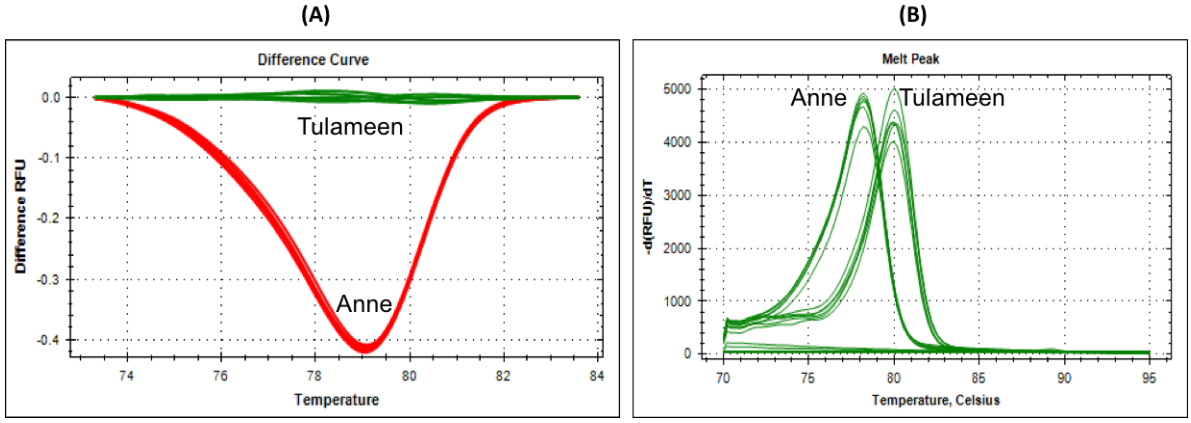


Figure 23: (A) Homozygous status of *Ans* gene in raspberries. The difference of *Ans* amplicons by means of HRM analysis. Red peek exhibits *Ans* amplicon from "Anne" and the straight green line represents *Ans* amplicon from Tulameen. **(B)** The difference of *Ans* amplicons using qPCR analysis with the same set of primers. Left peak represents *Ans* amplicons from "Tulameen" and right peak represent *Ans* amplicons from "Anne".

3.1.13 Screening of ans/Ans alleles by CAPS marker

CAPS, also known as PCR-RFLP markers, are locus oriented. The amplified genomic DNA fragments from the specific region of *Ans* gene spanning the 5 base pairs (*GGCCT*) insertion are subjected to HaeIII restriction enzyme digestion that cleaves the DNA at the positions where *GGCC* sequence is found. CAPS analysis with Electrophoresis-Chip (Figure 24A) shows that the fragment length of "Tulameen" samples is the same (lane 2, lane 4 & lane 6) irrespective of restriction enzyme digestion, whereas, restriction analysis of "Anne" samples generated short fragments (lane 3 & lane 5) in comparison to undigested sample (lane 1). The analysis indicates that the undigested sample (UcTL) and samples exposed to digestion (TLC1 & TLC2) from "Tulameen" show 192 bp fragments. On the other hand, "Anne", undigested sample (UcAN) shows 197 bp fragment while samples exposed to restriction enzyme digestion (ANC1 & ANC2) yielded two fragments of 152 and 45 bp size. The shorter fragment (45 bp) is not visible; however, the reduced main band (152 bp) is obvious (Figure 24A). So, the CAPS-marker used for identification of homozygous/heterozygous nature of *Ans* gene for 5 bp insertion in "Anne" exhibit its homozygous status, i.e. *ans* alleles for "Anne" are homozygous for 5 base pairs (*GGCCT*) mutation and *Ans* alleles for "Tulameen" are homozygous without the insertion.

In "Glen Garry" (GG), the red fruiting parent of "Anne", two different bands were detected without any digestion. However, on digestion and visualization on High-Resolution gel, it showed 4 bands, and apparently 3 alleles of *Ans* gene. Out of them, one allele was like 'Anne-Ans' and one like 'Tulameen-Ans' but the third allele looked different in size from the other two types (Figure 24B). In high concentration agarose (Figure 24B-D), the shorter fragment of 45 bp is also visible. Red fruiting "Amity" (AT) shows the same bands for *Ans* gene as "Tulameen" and its red fruit-bearing parents, "Nootka" (NK) and "Glen Prosen" (GP), irrespective of digestions (Figure 24B). Interestingly, an orange fruiting variety "Valentina" (Val) contains the same type of mutation like in "Anne" (AN) in homozygous status while yellow fruiting "Citria" (CT), "Alpen Gold" (Alp) and "Giallo Mutant" (GM) show 3 bands on digestion in comparison to 2 bands of undigested samples like "Glen Garry" (Figure 24C). Other yellow fruiting varieties like "Gelbe Sugana" (GSu), "Golden Everest" (GE), "Lumina" (Lum), "All Gold" (AG), "Golden Queen" (GQ); red fruiting "Heritage" (Her), "Autumn Bliss" (AB), "Sugana Red" (SR), "Meeker" (MK), "Pocahontas" (PH), "Himbo Top" (HT); purple fruiting "Tayberry" (TB), "Buckingham Tayberry" (BT) and black fruiting "Black Jewel" (BJ) all show single band like "Tulameen" regardless of digestions (Figure 24C-D).

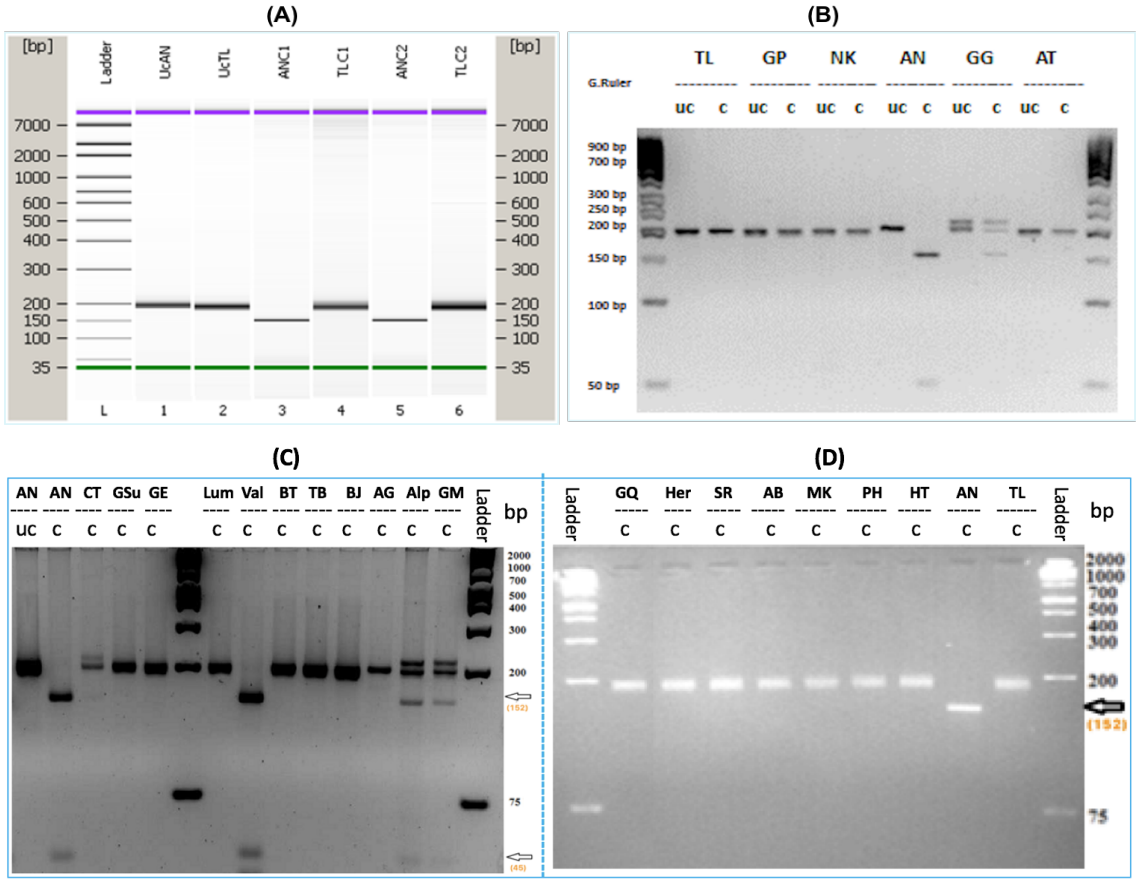


Figure 24: (A) CAPS analysis with Electrophoresis-Chip: Restriction analysis of two independent samples of genomic DNA amplified fragment by PCR for homozygous/heterozygous nature of mutation in the *Ans* gene. 1. UcAN (un-cut 'Anne-Ans'), 2. UcTL (un-cut 'Tulameen-Ans'), 3. ANC1 ('Anne-Ans' cut PCR-sample 1), 4. TLC1 ('Tulameen-Ans' cut PCR-sample 1), 5. ANC2 ('Anne-Ans' cut PCR-sample 2), 6. TLC2 ('Tulameen-Ans' cut PCR-sample 2). **(B, C, D)** CAPS analysis with High-Resolution gel. **(B)** Restriction analysis of *Ans* fragment from genomic DNA of independent parent samples of "Anne" and "Tulameen" (c) along with uncut (uc) samples as control. "Anne" shows 1 (uc) and 2 (c) bands, while "Glen Garry" (GG) shows 2 (uc) and 4 (c) bands, respectively. Other varieties including "Gelbe Sugana" (GSu), "Golden Everest" (GE), "Lumina" (Lum), "All Gold" (AG), "Golden Queen" (GQ); red fruiting "Heritage" (Her), "Autumn Bliss" (AB), "Sugana Red" (SR), "Meeker" (MK), "Pocahontas" (PH), "Himbo Top" (HT); purple fruiting "Tayberry" (TB), "Buckingham Tayberry" (BT) and black fruiting "Black Jewel" (BJ) show only single band irrespective of digestion. **(C)** "Valentina" (Val) shows Anne like mutation while "Citria" (CT), "Alpen Gold" (Alp) and "Giallo Mutant" (GM) show 3 bands (c) like "Glen Garry". **(C & D)** All other varieties, i.e. "Gelbe Sugana" (GSu), "Golden Everest" (GE), "Lumina" (Lum), "All Gold" (AG), "Golden Queen" (GQ), "Heritage" (Her), "Autumn Bliss" (AB), "Sugana Red" (SR), "Meeker" (MK), "Pocahontas" (PH), "Himbo Top" (HT), "Tayberry" (TB), "Buckingham Tayberry" (BT) and "Black Jewel" (BJ) shows single band like "Tulameen".

3.1.14 Other Ans mutations in yellow fruiting Rubus

In order to identify other possible mutations or variations in *Rubus*, the *Ans* genes or transcripts were amplified and sequenced from all the available yellow ("Golden Everest", "All Gold", "Gelbe Antwerpener", "Lumina", "Him13K39-8", "Herbert Gold", "Gelbe Siebenkugel",

"Juan de Metz", "Golden Queen", "Fall Gold", "Sugana Gold"), orange ("Valentina", "Orange Marie") and red ("Glen Garry", "Amity", "Glen Prosen", "Heritage" "Autumn Bliss", "Sugana Red") fruit-bearing varieties. GenBank Accession Numbers for all the *Ans* sequenced varieties are presented in Table 3. Multiple sequence alignment of *Ans* gene enabled to establish the sequence consensus. Sequence analysis shows high similarity for the *Ans* gene among all the varieties. An interesting aspect observed was that most of the yellow varieties contain different types of mutations including the earlier described ans^{+5} (*Rubus Ans* mutation type 1 (RAMT-1; *GGCCT*) in "Anne". RAMT-1 was also observed at the same position (730 bp) in red "Glen Garry" and yellow "Herbert Gold" and in orange fruiting "Valentina" (Figure 25).

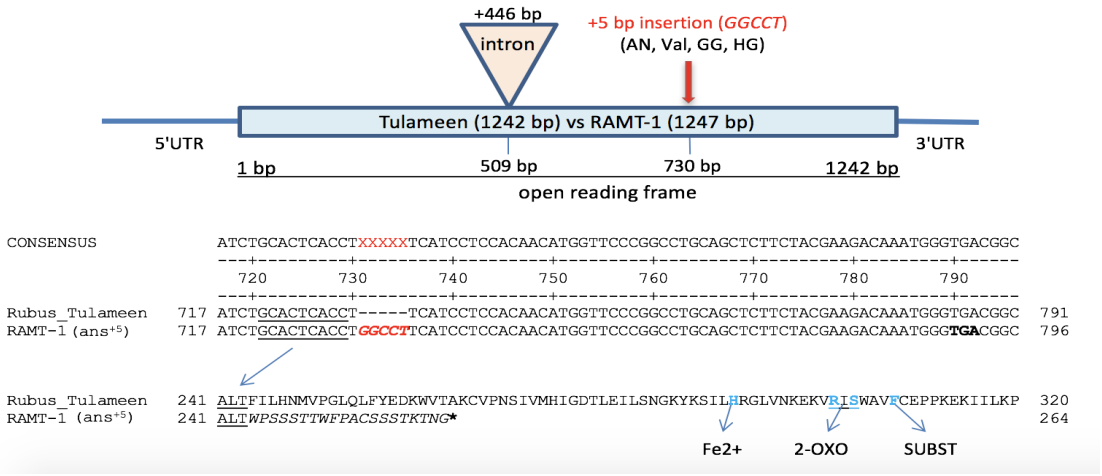


Figure 25: Analyses of anthocyanidin synthase genes from "Tulameen" and other varieties with *Rubus Ans* mutation type 1 (RAMT-1) indicate a 5 bp insertion (ans^{+5}) in the second exon at position 730 bp of "Anne" (AN), "Valentina" (Val), "Glen Garry" (GG) and "Herbert Gold" (HG) allele (*ans*) causing a frameshift and thus leading to a truncated protein in the yellow variety, missing conserved regions for substrate, co-substrate, and iron binding sites. The *Ans* fragment from "Tulameen" and varieties with RAMT-1 includes the entire coding region with one 446 bp intron.

However, sequencing analysis showed another type of 4 bp mutation (*ACGT*; ans^{+4}; RAMT-2) in the second exon at position 867 bp of many yellow fruiting varieties, such as "Golden Everest", "All Gold", "Gelbe Antwerpener", "Lumina", "Him13K39-8", "Herbert Gold" and an orange fruiting variety "Orange Marie". RAMT-2 also causes a frameshift like RAMT-1 and thus leads to a truncated protein (311 aa) in the varieties with missing conserved domains/residues for substrate, co-substrate and iron binding sites (F, RxS, H; Figure 26). One of the yellow fruiting varieties, "Juan de Metz", contains ans^{+4} but at a different position (526

bp) in the second exon causing also a frameshift and producing a short protein (178 aa) at C-terminus like the other yellow or orange varieties with ans^{+4} mutation (Figure 26).

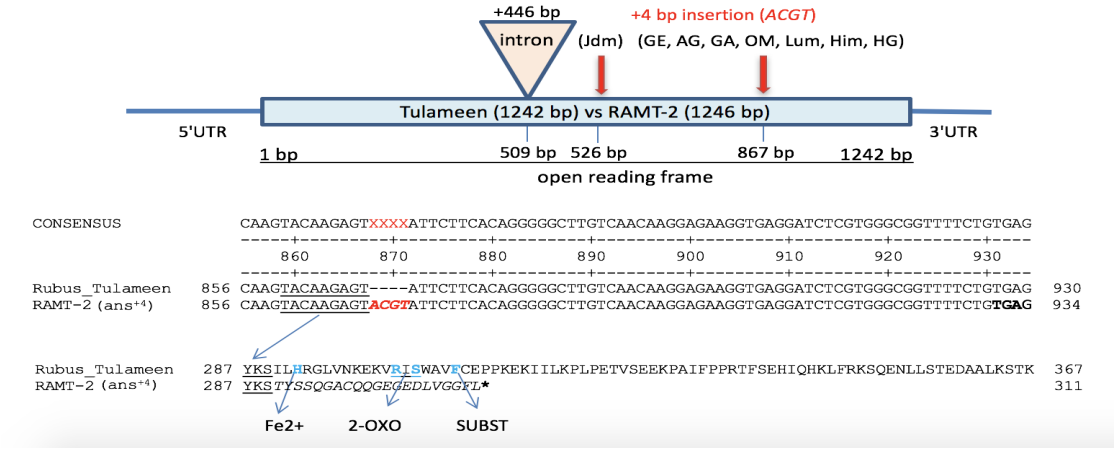


Figure 26: Analyses of anthocyanidin synthase genes from "Tulameen" and other varieties with *Rubus Ans* mutation type 2 (RAMT-2) indicate a 4 bp insertion (ans^{+4}) in the second exon at position 867 bp of "Golden Everest" (GE), "All Gold" (AG), "Gelbe Antwerpener" (GA), "Orange Marie" (OM), "Lumina" (Lum), "Him13K39-8" (Him), "Herbert Gold" (HG) and at position 526 bp of "Juan de Metz" (Jdm) allele (*ans*) causing a frameshift and thus leading to a truncated proteins in the yellow and orange varieties, missing conserved regions for substrate, co-substrate and iron binding sites. The *Ans* fragment from "Tulameen" and varieties with RAMT-2 includes the entire coding region with one 446 bp intron.

Some *Rubus* varieties also contain other distinct mutations in addition to RAMT-1 and RAMT-2 but in different *ans* alleles (as shown in Figure 27) to those which contain RAMT-1/RAMT-2. For example, one of the "Glen Garry" *ans* allele contains a 2 bp insertion (*GG*; ans^{+2}; RAMT-3) in the second exon at position 541 bp in addition to 5 bp (ans^{+5}; RAMT-1). Similarly, one of the "Herbert Gold" *ans* allele contains a 2 bp insertion (*AG*; ans^{+2}; RAMT-4) at position 597 bp in addition to both type of mutations (RAMT-1 and RAMT-2) in two different *ans* alleles. The important residues known for functional ANS proteins are missing based on allelic translation in the presence of RAMT-3 and RAMT-4, likewise, *Rubus ans* alleles with RAMT-1 and RAMT-2 as shown in Figure 27.

"Gelbe Siebenkugel" and "Sugana Gold" are the only varieties found to have a mutation in the first exon. "Gelbe Siebenkugel" contains a mutation/insertion of 8 bp (*TGCCCGAT*; ans^{+8}; RAMT-5) at position 504 bp of *Ans* gene predicting a truncated ANS protein of 183 aa. In the variety with RAMT-5, the conserved residues crucial for substrate, co-substrate, and iron binding sites are also missing (as illustrated in Figure 28).

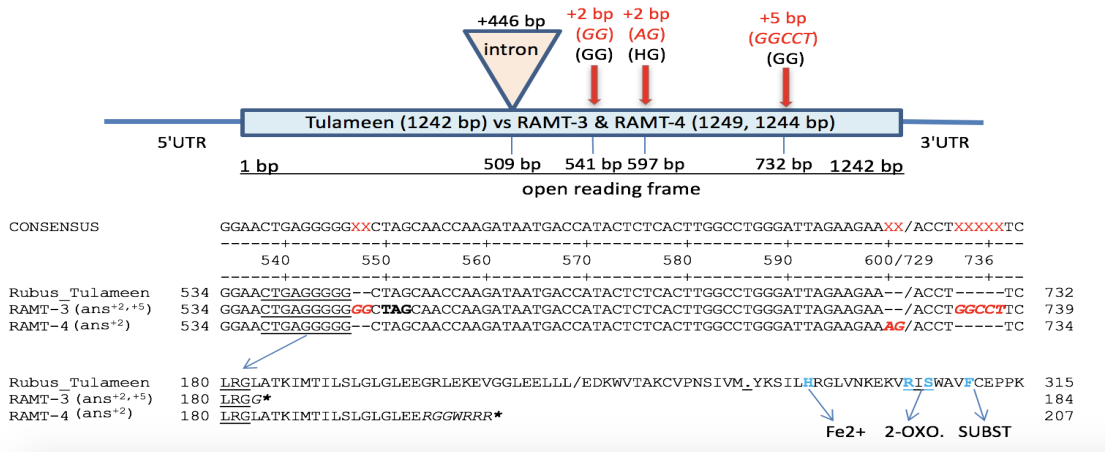


Figure 27: Analyses of anthocyanidin synthase genes from "Tulameen" and other varieties with *Rubus Ans* mutation type 3 (RAMT-3) indicate and 2 bp insertion at 541 bp in addition to RAMT-1 at 732 bp (ans$^{+2, +5}$) of "Glen Garry" (GG) allele. Analysis indicated another type of mutation (RAMT-4) of 2 bp insertion (ans^{+2}) in the second exon at position 597 bp of "Herbert Gold" (HG) causing a frameshift and thus leading to a truncated protein in both varieties, missing conserved regions for substrate, co-substrate and iron binding sites. The *Ans* fragment from "Tulameen" and varieties with RAMT-3 and RAMT-4 includes the entire coding region with one 446 bp intron. The position of stop codon in RAMT-4 is at 618 bp (not shown).

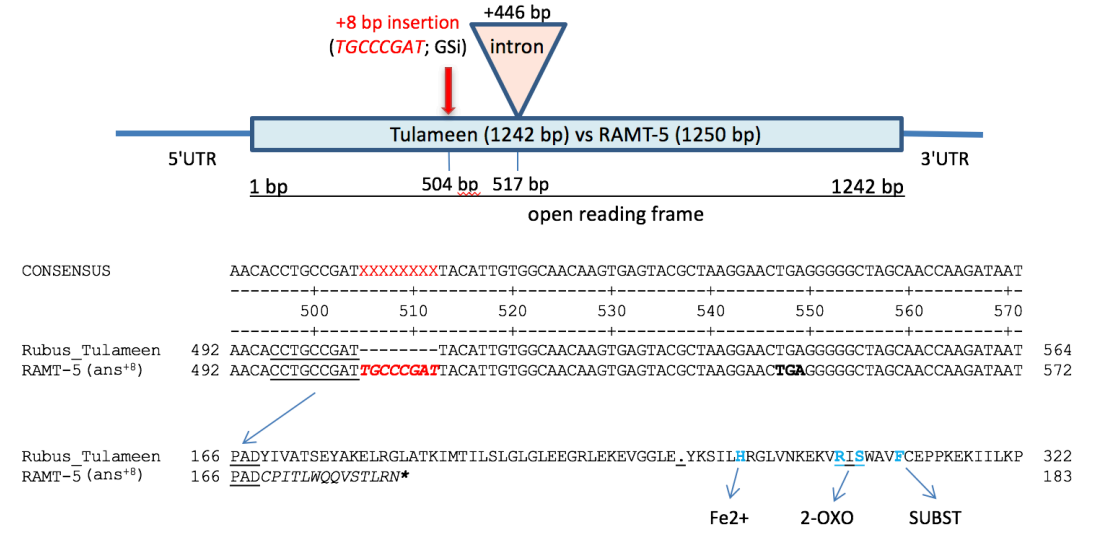


Figure 28: Analyses of anthocyanidin synthase genes from "Tulameen" and "Gelbe Siebenkugel" (GSi) with *Rubus Ans* mutation type 5 (RAMT-5) indicate an 8 bp insertion (ans^{+8}) in the first exon at position 504 bp of "GSi" (*ans*) allele causing a frameshift and thus leading to a truncated protein in the yellow variety, missing conserved regions for substrate, co-substrate, and iron binding sites. The *Ans* fragment from "Tulameen" and "GSi" includes the entire coding region with one 446 bp intron.

"Sugana Gold" contains an insertion of 4 bp (*AATT*; ans^{+4}; RAMT-6) at 41 bp position in the first exon, which caused a shorter protein (331 aa) at N-terminus (Figure 29). Sequencing analysis also revealed a 24 bp mutation (*ACTAAAGAAGCTGATCTCATCTCT*; ans^{+24}; RAMT-

7) in the second exon at position 1188 bp of some red fruiting ("Heritage" and "Amity") and yellow fruiting ("Juan de Metz") varieties. RAMT-7 did not cause a frameshift but lead to a 8 aa elongated protein in these varieties containing conserved regions for substrate, co-substrate and iron binding sites (Figure 30). In case of "Heritage" and "Amity" the protein predicted is longer (422 aa) but normal, however in yellow fruiting "Juan de Metz", it is short in size (178 aa) at C-terminus due to the presence of RAMT-2 in the same allele. The comparison of deduced amino acids of "Tulameen" ANS, elongated ANS of "Heritage" and "Amity" and truncated ANS of "Juan de Metz" together with other *Rubus* ANS is presented in Annex IX".

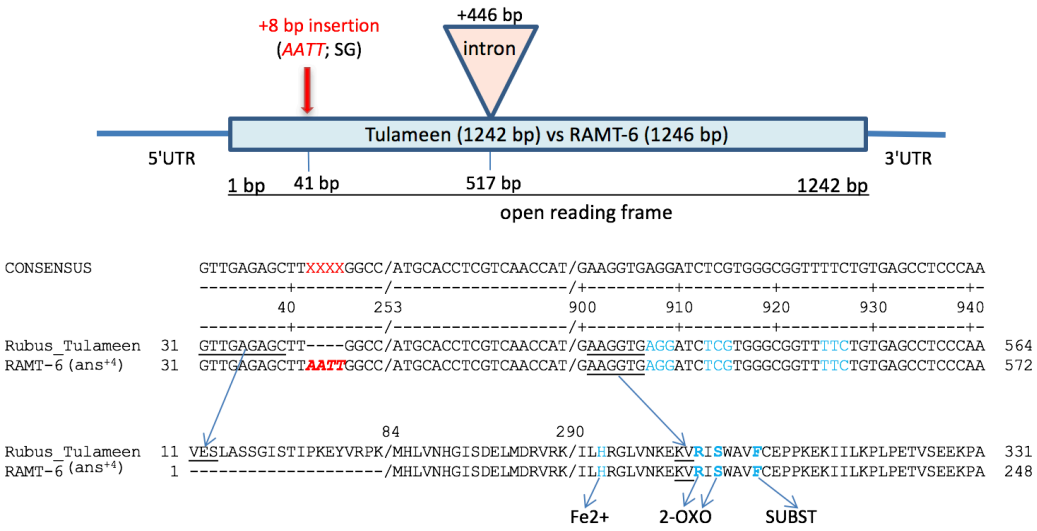


Figure 29: Analyses of anthocyanidin synthase genes from "Tulameen" and "Sugana Gold" (SG) with *Rubus Ans* mutation type 6 (RAMT-6) indicates the 4 bp insertion (ans^{+4}) in the first exon at position 41 bp of "Sugana Gold" (*ans*) allele leading to a short protein in the yellow variety, containing conserved regions for substrate, co-substrate and iron binding sites. The first spacer indicates a late start codon (1 aa) for ANS of "Sugana Gold" as compared to 84 aa long ANS in "Tulameen". The second spacer indicates the sequences containing conserved regions in both varieties. The *Ans* fragment from "Tulameen" and "Sugana Gold" includes the entire coding region with one 446 bp intron. The position of stop codon in "Sugana Gold" is same as in "Tulameen" (not shown).

The varieties with RAMT-1 to RAMT-7 contained a coding sequence (CDS) consensus of 792 bp, 933 bp, 552 bp, 621 bp, 549 bp, 1009 bp and 1266 bp encoding polypeptides of 264 aa, 311 aa, 184 aa, 207 aa, 183 aa, 331 aa and 422 aa residues, respectively (Table 3). Comparison of ANS protein sequences of most of the *Rubus* varieties showed 97% to 100% identity. However, "Gelbe Siebenkugel" showed only 93% similarity. Comparison of the deduced amino acids indicated the presence of SAAPs not only between yellow/orange and red varieties but also among the red varieties (Annex IX). The percentage values of the deduced amino acids of

RiANS of yellow and orange varieties as compared to the "Tulameen" reference is shown in Annex X via the molecular phylogenetic tree.

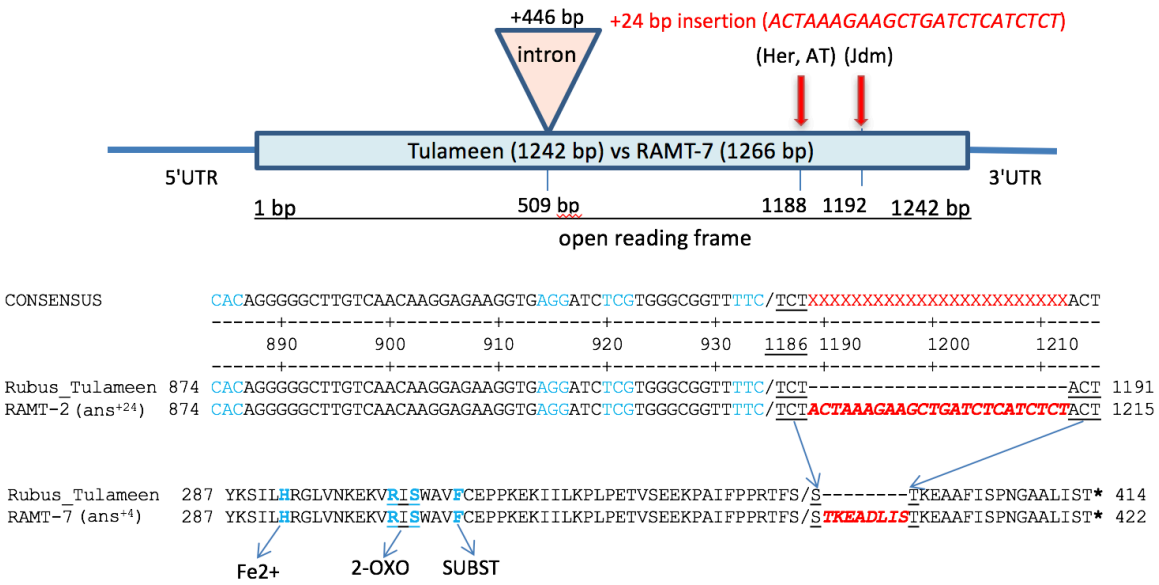


Figure 30: Analyses of anthocyanidin synthase genes from "Tulameen" and other varieties with *Rubus Ans* mutation type 7 (RAMT-7) indicate a 24 bp insertion (ans^{+4}) in the second exon at position 1188 bp of "Heritage" (Her) and "Amity" (AT), and 1192 bp of "Juan de Metz" (Jdm) alleles leading to an extended protein in the varieties, having conserved regions for substrate, co-substrate and iron binding sites. The *Ans* fragment from "Tulameen" and varieties with RAMT-7 includes the entire coding region with one 446 bp intron.

Sequencing analysis of *Rubus Ans* gene helped to predict that different type of *ans*/*Ans* alleles exists in *Rubus* based on mutation type RAMT-1 to RAMT-7. All RAMT-1 to RAMT-4, and RAMT-7 are present in the second exon, whereas, mutation types (RAMT-5; ans^{+8}, and RAMT-6; ans^{+4}) were found in the first exon in "Gelbe Siebenkugel" and "Sugana Gold", respectively. Most of the varieties producing yellow or orange fruits were found to have only one type of mutation (allele), apparently showing both alleles in homozygous nature; while some of them were found to have more alleles containing a different type of mutations. For example, "Herbert Gold" was found with 3 different types of mutations and alleles and red fruiting "Glen Garry" was observed to have 2 mutated alleles and one wild type allele. All the varieties with any kind of mutation (RAMT-1 to RAMT-6) produces yellow or orange colored fruits, apparently due to truncated or short ANS protein EXCEPT red fruiting "Glen Garry" (one of the "Anne" parents) whose 1 of the 3 alleles is also wild type (like "Tulameen"). However, there are two yellow fruiting varieties ("Golden Queen", "Fall Gold") whose sequenced allele predicts a

functional (wild type) protein having all the crucial elements needed for its activity (as pointed in Figure 14, Annex IX). Yellow fruiting "Sugana Gold" containing RAMT-6 also produces short ANS at N-terminus, however, it also contains necessary elements. Red fruiting varieties "Heritage" and "Amity" with RAMT-7, apparently do not affect conserved domains and predicts a bit longer protein at C-terminus. Remaining red fruiting varieties, such as "Glen Prosen", "Autumn Bliss" and "Sugana Red" do not contain any of the mutation types in *Ans* gene thus predicting the wild type (functional) proteins.

Table 3: Identification of different mutations in the alleles of anthocyanin synthase gene in *Rubus* and GenBank Accessions (GB Acc.) of cloned alleles from different *Rubus* varieties.

Variety	**Code**	**Fruit color**	**Mutation position**	**Mutation size (bp)**	**Mutation type**	**Protein seq**	**Protein nature**	***Rubus* GB Acc No**
Anne	AN	yellow	730	+5	RAMT-1	264 aa	truncated	KX950788
Lumina	Lum	yellow	867	+4	RAMT-2	311 aa	truncated	MG437166
All Gold	AG	yellow	867	+4	RAMT-2	311 aa	truncated	MG437165
Fall Gold	FG	yellow	---	---	---	414 aa	normal	MG437157
Sugana Gold	SG	yellow	41	+4	RAMT-6	331 aa	short	MG437156
Him13K39-8	Him	yellow	867	+4	RAMT-2	311 aa	truncated	MG437167
Juan de Metz	Jdm	yellow	526,1192	+4, +24	RAMT-2,7	335 aa	short	MG437170
Herbert Gold	HG	yellow	730	+5	RAMT-1	264 aa	truncated	MG437171
Herbert Gold	HG	yellow	867	+4	RAMT-2	311 aa	truncated	MG437172
Herbert Gold	HG	yellow	597	+2	RAMT-4	207 aa	truncated	GB Acc xx
Golden Queen	GQ	yellow	---	---	---	414 aa	normal	MG437169
Golden Everest	GE	yellow	867	+4	RAMT-2	311 aa	truncated	MG437164
Gelbe Siebenkugel	GSi	yellow	504	+8	RAMT-5	183 aa	truncated	MG437168
Gelbe Antwerpener	GA	yellow	867	+4	RAMT-2	311 aa	truncated	GB Acc xx
Valentina	Val	orange	730	+5	RAMT-1	264 aa	truncated	MG437159
Orange Marie	OM	orange	867	+4	RAMT-2	311 aa	truncated	MG437160
Glen Garry	GG	red	---	---	---	414 aa	normal	MG437161
Glen Garry	GG	red	730	+5	RAMT-1	264 aa	truncated	MG437162
Glen Garry	GG	red	541,732	+2, +5	RAMT-3,1	184 aa	short	MG437163
Amity	AT	red	1188	+24	RAMT-7	422 aa	normal	GB Acc xx
Heritage	Her	red	1188	+24	RAMT-7	422 aa	normal	MG437153
Tulameen	TL	red	---	---	---	414 aa	normal	KX950789
Glen Prosen	GP	red	---	---	---	414 aa	normal	MG437158
Sugana Red	SR	red	---	---	---	414 aa	normal	MG437155
Autumn Bliss	AB	red	---	---	---	414 aa	normal	MG437154

3.2. Carotenoids

Fruit development and ripening in raspberries is associated with biosynthesis of different amounts and patterns of carotenoids. Understanding the variation in gene expression, i.e. down-regulation or post-transcriptional silencing of genes, can be helpful in understanding the regulation or accumulation of carotenoids in fruits. In addition, the presence of carotenoid cleavage dioxygenases (*Ccds*), which on oxidative cleavage of carotenoids produce apocarotenoids, can also play an important role in controlling the accumulation of many carotenoids. Furthermore, molecular and functional analysis together with expression patterns of the carotenoid pathway genes can elucidate the accumulation and role of carotenoids in determining the pigmentation in raspberry fruits with different coloration.

3.2.1 In silico mining of carotenoid pathway genes of Rubus

The putative carotenoid pathway genes of *Rubus* were identified with the help of *in silico* searches of *Rubus* draft genome 1.08 (cv "Heritage") using *Fragaria*, *Pyrus* and *Malus* sequence queries. *RiGgps*, *RiPsy*, *RiPds*, *RiLcy-b*, *RiLcy-e* and *RiChy-b* were searched using GenBank Accession Numbers; XM_004300550, XM_004296142, XM_004296916, XM_004303559, XM_004287534 and XM_004308006 of *Fragaria*, respectively. *RiChy-e* was searched using GenBank Accession Number; XM_009377507 of *Pyrus*. *RiZep*, *RiNsy* and *RiCcds* were searched using GenBank Accession Numbers; XM_008340094, JN941557 and EU871633 of *Malus* (Annex XI). The retrieved sequences were used to design the primers for expression analysis as presented in Annex II.

3.2.2 Expression analysis of carotenoid pathway genes

The expression levels of the carotenoid pathway genes, i.e. *RiGgps*, *RiPsy*, *RiPds*, *RiLcy-b*, *RiLcy-e*, *RiChy-b*, *RiChy-e*, *RiZep*, *RiNsy* correlate to the production of various carotenoid pigments. Expression of most of the carotenoid pathway genes was assessed in a yellow ("Anne") and a red ("Tulameen") fruiting raspberry as presented in Figure 31. The fruit development stages in "Anne" and "Tulameen" have been described as A0-A5 and T0-T5, respectively, and in general for both varieties as S0-S5. Expression analysis of the *RiGgps* gene that preceedes in the carotenoid pathway, indicated the low expression level at early fruit development stages (A0, A1) in "Anne". Similar expression was observed in the early fruit stages of "Tulameen" except at T1; however, it increased dramatically at T4 and T5 as compared to "Anne". Following the *RiGgps* gene in the pathway, the expression level of

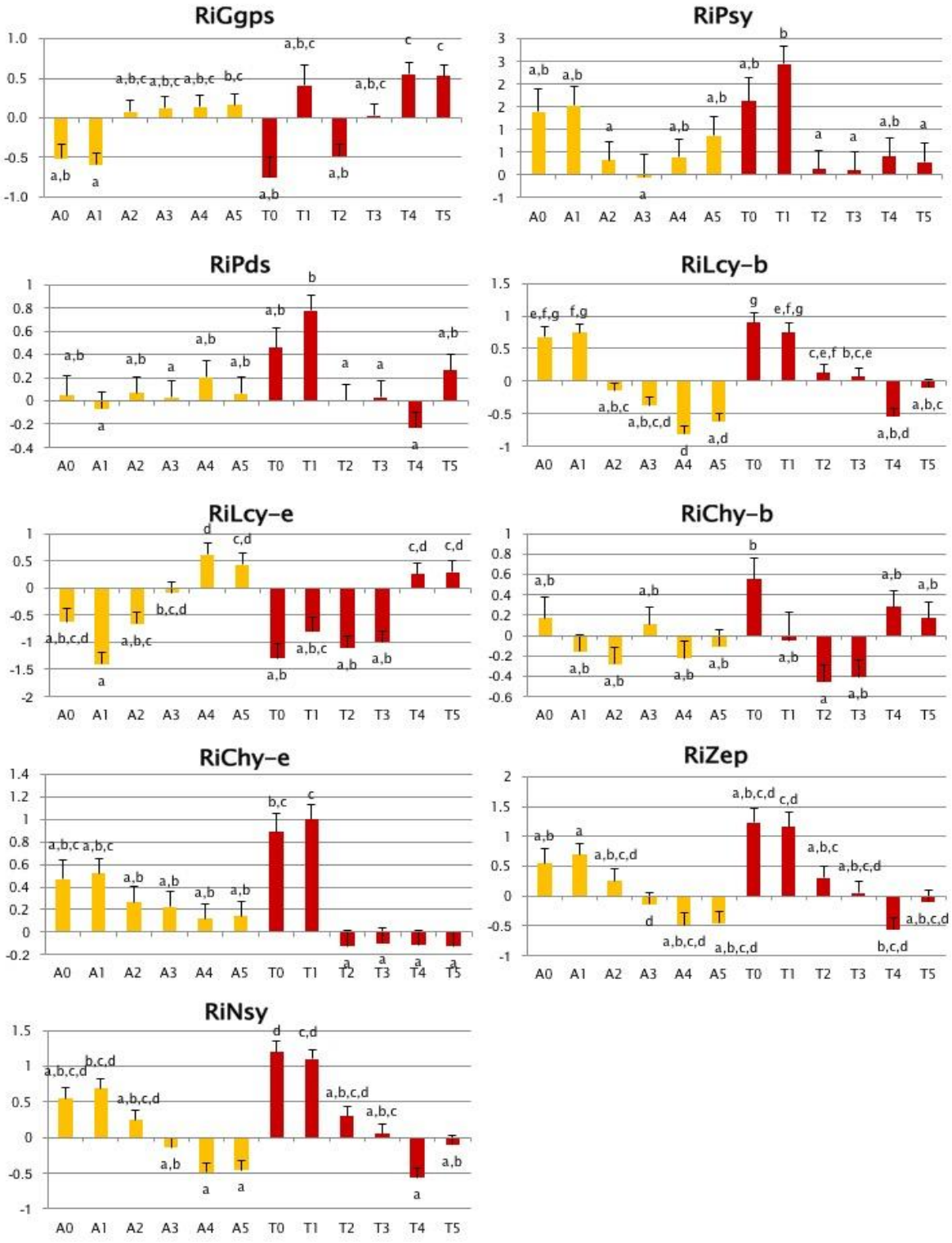


Figure 31: Expression analysis of carotenoid pathway genes; geranylgeranyl diphosphate synthase (*RiGgps*), phytoene synthase (*RiPsy*), phytoene desaturase (*RiPds*), lycopene *β*-ring cyclase (*RiLcy-b*), lycopene *ε*-ring cyclase (*RiLcy-e*), carotenoid *β*-ring 3-hydroxylase (*RiChy-b*), carotenoid *ε*-ring 3-hydroxylase (*RiChy-e*), zeaxanthin epoxidase (*RiZep*), neoxanthin synthase (*RiNsy*) during fruit development stages in yellow variety "Anne" (A1-A5) and red variety "Tulameen" (T1-T5), respectively. Data are presented as log values to have more comparable results between the higher (+ve) or lower (-ve) expressed genes normalized to the constitutive expression of housekeeping genes (*Actin*, *Pap2* and *Sand*) of *Rubus*.

RiPsy gene was found high in the early stages then after a decrease at S2 (A2, T2) and S3 (A3, T3), a relative increase was observed at later stages of "Anne" and "Tulameen". An overall similar pattern of expression of *RiPsy* gene was observed in both varieties. However, a discrete pattern of expression was obvious for *RiPds* gene as it is highly expressed at T0 and T1 and showed an adequate expression at A4 and T5, whereas all other stages show the very low expression level of *RiPds* gene in both varieties. The downstream *RiLcy-b* gene was highly expressed at early fruit stages (S0 and S1). In contrast, high expression for *RiLcy-e* was observed at later stages (S4 and S5) of both varieties (Figure 31). Significant downregulation of *RiLcy-b* was seen starting from S2 in all stages of both varieties and a low expression level of *RiLcy-e* was noticed in early fruit stages up to fruit turning stage (S3). The relatively high expression level of *RiChy-b* and *RiChy-e* was observed at early fruit stage (S0) then *RiChy-b* transcripts decreased up to A2 and T3. Later, an increase in *RiChy-b* expression was observed only at fruit turning stage (A3) in "Anne" and in ripe fruits (T4, T5) of "Tulameen". A decreasing trend of *RiChy-e* transcripts was found from unripe to ripe berries in "Anne". However, a sudden downregulation was obvious from T2 to T5. At early fruit development stages (S0 and S1), *RiZep* and *RiNsy* showed expression level almost double in "Tulameen" than "Anne" but decreased at later stages especially at S4 of both varieties (Figure 31). It looks that there is a continuous decrease in *RiZep* and *RiNsy* transcripts from unripe to ripe fruits of both the varieties. Overall it does not seem that expression of any of the pathway genes is thoroughly blocked or downregulated in any fruit development stage. However, a differential expression pattern found at various fruit development stages indicates the differential regulation and accumulation of different carotenoids in unripe and ripe fruits of raspberry.

3.2.3 Expression analysis of Ccd genes

Ccd genes play an important role of determining the accumulation of carotenoid pigments and production of volatile organic compounds (VOCs) in plants, especially in ripe fruits. Expression analysis of *Rubus Ccds* (*RiCcd1.1*, *RiCcd1.2*, and *RiCcd1.3*) was carried out in both varieties, "Anne" and "Tulameen". Among three different *Ccds*, it was observed that transcripts increased from A0 to A1 then decreased at A2 and seemed to diminish all the *Ccds* in the later fruit development stages (Figure 32). However, the expression pattern was found different in "Tulameen", which was highest at T0 and then significantly decreased from T1 to T5 in a continuous manner. An overall similar type of expression trend was observed in both varieties for all the *Ccds* in the current study, i.e. higher at early fruit development stages and significantly decreased in later stages, as shown in Figure 32. However, expression analysis of

the carotenoid and *Ccd* genes do not indicate that genes are necessarily functional too. Hence, further molecular and functional characterization can be helpful to understand their potential role in more detail.

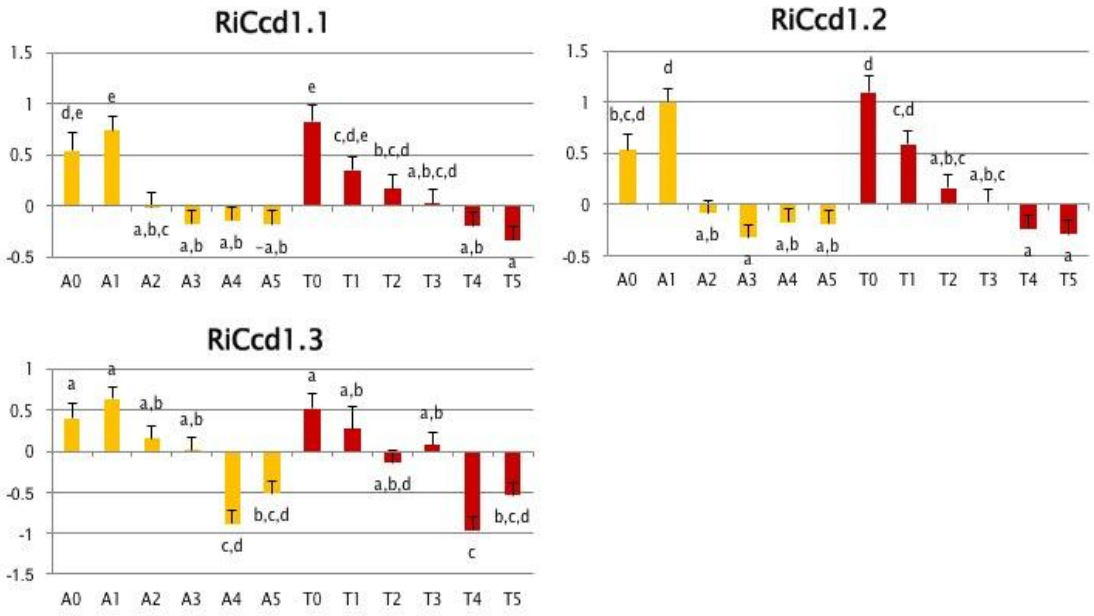


Figure 32: Expression analysis of carotenoid cleavage dioxygenases (*RiCCds*) during fruit development stages in yellow variety "Anne" (A1-A5) and red variety "Tulameen" (T1-T5), respectively. Data are presented as log valuesto have more comparable results between the higher (+ve) or lower (-ve) expressed genes normalized to the constitutive expression of housekeeping genes (*Actin*, *Pap2* and *Sand*) of *Rubus*.

3.2.4 Cloning of carotenoid pathway genes

Gene-specific primers were designed from the coding region of retrieved sequences (*Psy*, *Lcy-b*, *Lcy-e*, *Chy-b*, *and Chy-e*) and enabled their full-length amplification from yellow fruiting raspberry "Anne". GenBank Accession Numbers of the cloned genes with deduced amino acids were submitted to NCBI as listed in Annex XI. Phylogenetic analysis of deduced amino acids showed the percentage identities between *Rubus* "Anne" and published sequences from other species (Annex XII-XVII). Detailed sequence analysis of the important carotenoid pathway genes is described here.

Sequence analysis of RiPsy gene

The amplification of candidate *RiPsy* gene resulted in 1002 bp amplicon encoding a polypeptide of 333 amino acids in "Anne" (GB Acc. MF850339) with a calculated mass of 38.49 kDa. It contains DXXXD domains (DELVD and DVGED) including conserved aspartate residues necessary for its functional activity (López-Emparán et al., 2014). The protein sequence was found exactly homologous to *Psy* of *Rubus* draft "Heritage". Phylogenetic analysis revealed that *Citrus*, *Capsicum*, and *Arabidopsis* have a high percentage of homologies with RiPSY than other species, such as *Solanum*, *Narcissus* and *Pantoea* as shown in Annex XII.

Sequence analysis of cyclases

The amplified PCR product of the ORF of *RiLcy-b* gene (1494 bp) predicted a polypeptide of 497 amino acids (GB Acc. MF850340) with a calculated mass of 55.93 kDa. The analysis indicated that it contained *β*-cyclase motifs and conserved regions in C and N terminus (Hugueney et al., 1995; Alquézar et al., 2009). The phylogenetic tree showed a higher ratio of RiLCY-b protein sequence identities to the other species, such as *Vitis*, *Solanum*, *Nicotiana*, *Capsicum*, *Arabidopsis* as presented in Annex XIII. Similarly, the ORF of *RiLcy-e* contained 1629 bp, encoding 542 amino acid residues (GB Acc. MF850341) with a calculated mass of 60.18 kDa. It also contained the essential conserved domains for functional activity of LCY-e (Cunningham et al., 1996). Protein sequences are identical to the *Rubus* genome draft and show high identities to the other published LCY-e, such as *Coffea*, *Adonis*, *Arabidopsis*, *Solanum* as shown in phylogenetic analysis (Annex XIV).

Sequence analysis of hydroxylases

Using primers designed from the predicted coding sequence of *Rubus* genome draft, a 903 bp amplicon of *RiChy-b* was obtained from "Anne" by PCR amplification. The amplicon encoded 300 amino acids (GB Acc. MF850342) with a mass of 33.69 kDa. Sequence analysis indicated the presence of important conserved CHY-b domains (e.g. HDGLVHKRFP) needed for its functional activity (Linden, 1999). Sequence analysis and the constructed phylogenetic tree revealed high identity of RiCHY-b to the *Rubus* genome draft and to other plant species, such as *Capsicum*, *Coffea*, *Arabidopsis*, *Adonis* as presented in Annex XV. The retrieved sequences from *Rubus* draft enabled also to clone a *RiChy-e* fragment of 1668 bp encoding a polypeptide of 555 amino acids (GB Acc. MF850343) with a calculated mass of 61.63 kDa. It shows the same sequence as the CHY-e of *Rubus* genome draft. All known essential elements, e.g.

cleavage site of putative chloroplast-targeting sequence, a transmembrane domain, conserved cytochrome P450 molecular oxygen binding pocket, conserved threonine (Thr) and cysteine (Cys) motifs for the activity of this protein could be identified (Tian et al., 2004). Phylogenetic analysis also indicates high identity to not only Rosaceae members, such as *Fragaria*, *Prunus*, *Malus*, *Pyrus* but also to other published sequences of CHY-e as shown in Annex XVI.

Sequence analysis of RiCcd1 gene

An ORF of 750 bp of *Ccd1* named as *Ccd1.1* was obtained by PCR amplification. It encoded a protein sequence of 249 amino acids (GB Acc. MF850344) which contains two of four conserved histidine (H) residues, and one of two glutamates (E) and an aspartate (D). The calculated mass of CCD1.1 polypeptide sequences is 28.24 kDa. Phylogenetic tree of deduced amino acids indicated the clustering of CCD1.1 together with CCD1 of Rosaceae members and other plant species, such as *Rosa*, *Cucumis*, *Coffea*, *Vitis* as compared to 9-*cis*-epoxycarotenoid dioxygenases (NCEDs) and other CCDs as presented in Annex XVII. Overall sequence analysis of the important genes studied, indicated the identical protein sequence as obtained from the *Rubus* genome draft.

3.2.5 Functional analysis by complementation

Genes of the yellow fruiting raspberry “Anne” were selected to functionally characterize the carotenoid pathway genes at the biochemical level. Sequences of carotenoid pathway genes derived from *Rubus* genome draft (“Heritage”) enabled to clone the ORFs of carotenoid pathway genes as cDNA in pCR2.1 and pJET1.2 vectors as expression cassettes as described above (Section 2.7.2). The genes (*RiPsy*, *RiLcy-b*, *RiLcy-e*, and *Ccd1.1*) were cloned into these expression cassettes and were named as pCR-RiPsy, pCR-RiLcy-b, pJET-RiLcy-e and pJET-RiCcd1.1, accordingly. Co-transformation of expression cassettes of *Rubus* carotenoid genes and plasmids containing bacterial carotenoid genes capable of generating different precursors was carried out as given in Table 4. Co-transformation of expression cassettes and precursor plasmids (as indicated in Table 5) resulted in a visible color change in the *E. coli* host (Figure 33), indicating functional complementation. Upon the detection of visual color, analysis of carotenoids was carried out to ascertain the specific carotenoids formed in the cultures. In the case of carotenoid cleavage enzymes, the visible screen was the reduction in color compared to the precursor line (Figure 34). The detailed complementation analysis is given below.

Complementation of RiPsy gene

The bacterial gene cassette geranylgeranyl diphosphate synthase (*Ggps*) in pACCRT-E plasmid is producing geranylgeranyl diphosphate (GGPP), the precursor for PSY. The co-transformation of pACCRT-E and expression cassette (pCR-RiPsy) containing *Rubus* ORF of *Psy* resulted the in production of colorless phytoene. The plasmid pACCRT-EB harboring *Psy* from *Erwinia uredovora* and able to produce phytoene too was included in the analysis as a positive control of phytoene synthesis. The presence of the complementation product (phytoene) was confirmed by the spectrum as shown in Figure 33a when compared with the pACCRT-EB producing phytoene (Figure 33b). It provides the functional proof of *Rubus* phytoene synthase.

Table 4: List of plasmids containing bacterial carotenoid genes producing precursors and expression cassettes of *Rubus* carotenoid genes for complementation.

Precursor plasmid	Precursor (Gene cassette)	Expression cassette	Complementation product
pACCRT-E (Chamovitz et al., 1992)	GGPP (crtE)	pCR-RiPsy	Phytoene
pACCRT-EB (Misawa et al., 1995)	Phytoene (crtE + crtB)	---	---
pACCRT-EIB (Cunningham et al., 1993)	Lycopene (crtE + crtI + crtB)	pJET-RiLcy-b	*β*-carotene
pACCRT-EIB (Cunningham et al., 1993)	Lycopene (crtE + crtI + crtB)	pJET-RiLcy-e	*δ*-carotene
pACCRT-EIB (Cunningham et al., 1993)	Lycopene (crtE + crtI + crtB)	pCR-RiCcd1	Lycopene decay
pACCRT-EIBY (Misawa et al., 1995)	*β*-carotene (crtE+crtI+crtB+crtY)	---	---

Table 5: Accumulation of carotenoids in *E. coli* cells after complementation with candidate genes of carotenoid pathway from *Rubus*.

Precursor plasmid /+ Expression cassette	Carotenoids detected			
pACCRT-EB	Phytoene	---	---	---
pACCRT-E + pCR-RiPsy	Phytoene	---	---	---
pACCRT-EIB	---	Lycopene	---	---
pACCRT-EIB + pJET-RiLcy-b	---	Lycopene	*β*-carotene	---
pACCRT-EIB + pJET-RiLcy-e	---	Lycopene	---	*δ*-carotene
pACCRT-EIB + pCR-RiCcd1.1	---	Lycopene	---	---
pACCRT-EIBY	---	---	*β*-carotene	---

Complementation of RiLcy-b gene

To test the functional activity of *RiLcy-b* gene encoding lycopene *β*-ring cyclase, co-transformation of pJET-RiLcy-b (the expression cassette containing *Rubus* ORF) and pACCRT-EIB (producing lycopene precursor) showed a clear color change as compared to the precursor line (Figure 33). The precursor line gave reddish coloration based on lycopene accumulation as evident from pellet and solvent, while the complemented line showed yellowish pellet and subsequently also yellow coloration of solvent due to the formed carotenoid (Figure 33c, 33e). Upon detection of visual color, the UPLC spectrum confirmed the synthesis of *β*-carotene (Figure 33c) along with remaining lycopene as indicated in Figure 33e.

The activity of lycopene *β*-cyclase can be inhibited with the treatment of the bleaching herbicide 2-(4-chlorophenylthio) triethylamine (CPTA) as a specific inhibitor for the cyclization of lycopene. Thus, bacterial cultures containing both plasmids (pACCRT-EIB + pJET-RiLcy-b) were treated with various CPTA concentrations (0, 5, 10, 25 and 50 µM). The production of *β*-carotene completely ceased at 25 µM CPTA proving the functional lycopene *β*-cyclase from "Anne". To narrow down the optimal concentration of inhibition, bacterial cultures were treated with 0, 1.5, 3.0, 4.5, 6.0, 7.5, 9.0, 10.5, 12.0 and 13.5 µM CPTA and it was observed that *β*-carotene production was completely inhibited at 10.5 µM CPTA.

Complementation of RiLcy-e gene

Co-transformation of *Rubus Lcy-e* gene encoding lycopene ε-ring cyclase in the expression cassette pJET-Lcy-e together with gene cassette (pACCRT-EIB) producing lycopene as precursor resulted in a visible color change from red to light yellow in the *E. coli* host, indicating the functional assignment (Figure 33d, 33e). Upon detection of visual color, UPLC-PDA/MS system ascertained the presence of expected *δ*-carotene (Figure 33d) along with unconsumed lycopene (Figure 33e). The effect of CPTA herbicide was also observed by applying various concentrations (0-15 µM) to the suspension cultures of *E. coli* containing both expression plasmids (pACCRT-EIB + pJET-RiLcy-e). Minimum δ-carotene production was observed at 12.0 µM. Complete inhibition of *RiLcy-e* activity was observed at 13.5 µM CPTA.

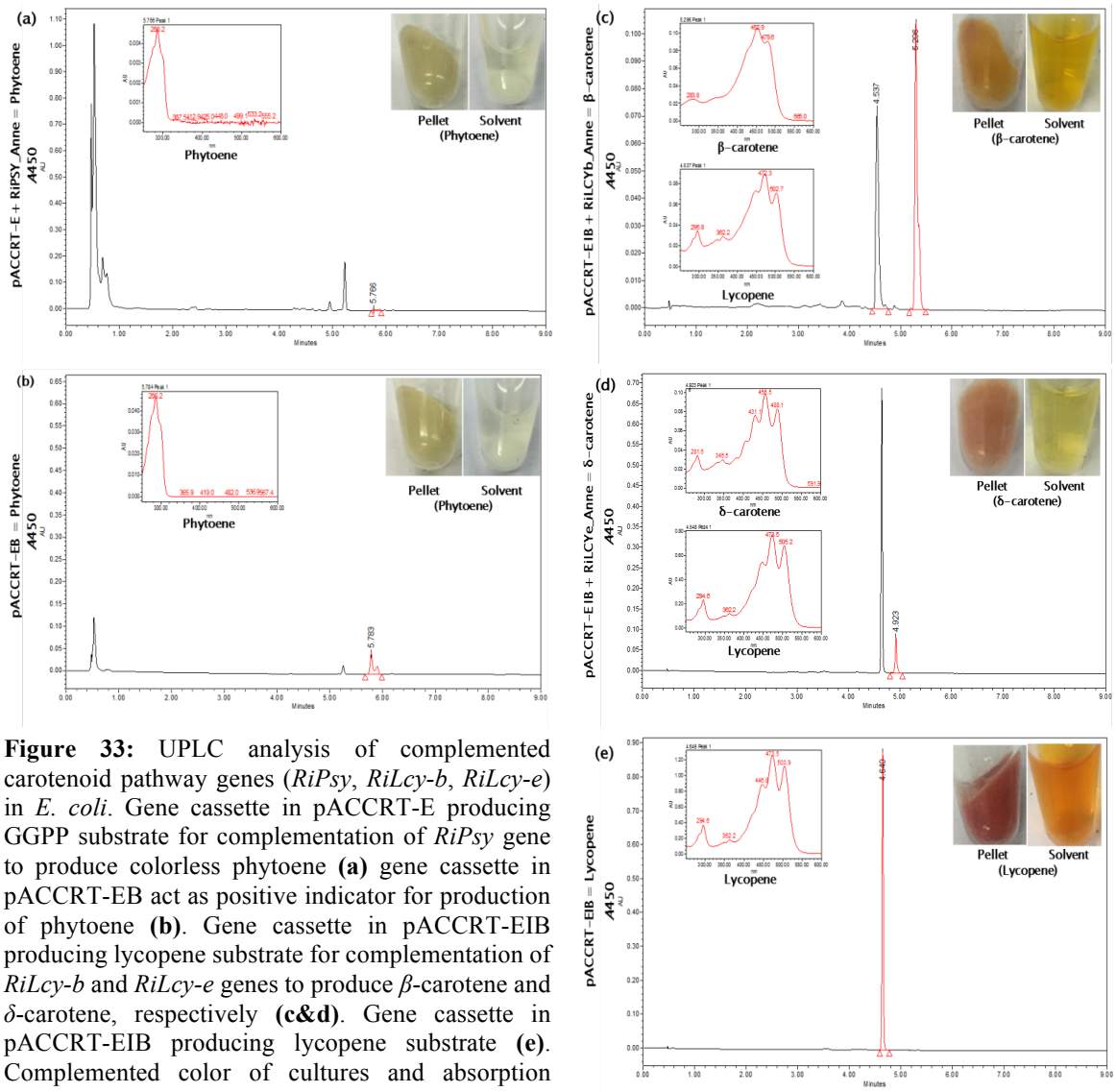


Figure 33: UPLC analysis of complemented carotenoid pathway genes (*RiPsy*, *RiLcy-b*, *RiLcy-e*) in *E. coli*. Gene cassette in pACCRT-E producing GGPP substrate for complementation of *RiPsy* gene to produce colorless phytoene **(a)** gene cassette in pACCRT-EB act as positive indicator for production of phytoene **(b)**. Gene cassette in pACCRT-EIB producing lycopene substrate for complementation of *RiLcy-b* and *RiLcy-e* genes to produce *β*-carotene and *δ*-carotene, respectively **(c&d)**. Gene cassette in pACCRT-EIB producing lycopene substrate **(e)**. Complemented color of cultures and absorption spectra of extracted carotenoids show the corresponding peaks.

Complementation of RiCcd1.1 gene

Raspberries are also rich in nor-isoprenoids, particularly *β*-ionone, a degradation product of *β*-carotene via *Ccds*. As *Ccd* genes were identified in *Rubus*, it is possible that the decrease in carotenoids and formation of nor-isoprenoids during ripening is due to the activity of *RiCcd* genes. To determine the functional role of *RiCcd1* gene, co-transformation of pCR-Ccd1 cassette together with pACCRT-EIB resulted in significantly reduced colony color as compared to the individual pACCRT-EIB line in reddish color (Figure 34a, b). By the activity of *RiCcd1*, lycopene was degraded into smaller units losing its reddish color. A similar color change was

observed in the solvent. UPLC-PDA/MS analysis system showed an almost 5-fold reduction of lycopene pigments as compared to the precursor line used as control (Figure 34b).

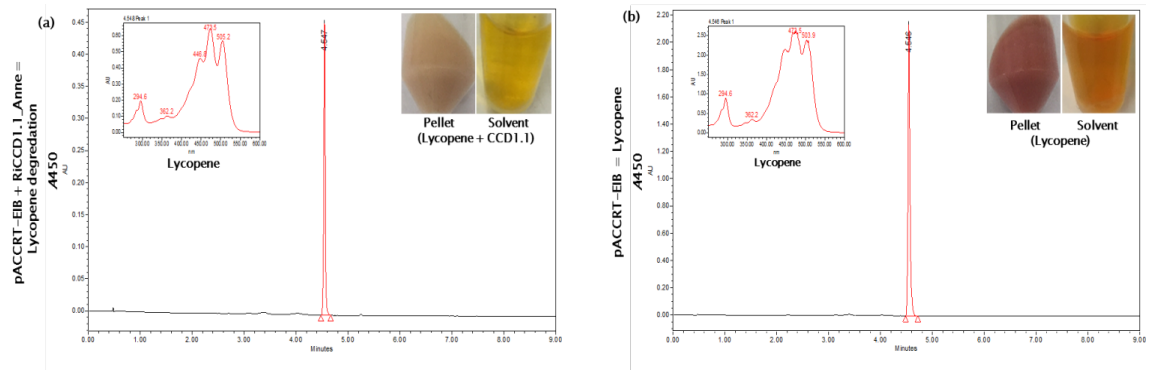


Figure 34: UPLC analysis of complemented *RiCcd1.1* in *E. coli* strain containing pACCRT-EIB cassette producing lycopene as a substrate to degrade it. **(a)** The color change of complemented cultures and absorption spectra with significantly reduced amounts of extracted carotenoids (lycopene) show the corresponding function of *RiCcd1.1*. **(b)** pACCRT-EIB plasmid act as a positive indicator to compare the decrease in the concentration of lycopene due to its degradation with the activity of *RiCcd1.1*.

The results of the expression analysis of most of the carotenoid pathway genes (*Ggps*, *Psy*, *Pds*, *Lcy-b*, *Lcy-e*, *Chy-b*, *Chy-e*, *Zep*, *Nsy*) exhibit that there is no reduction of transcripts of any gene at all the stages under study, which associates with the synthesis of various carotenoid pigments in "Anne". The molecular analysis of important carotenoid pathway genes, i.e. *Psy*, *Lcy-e*, *Lcy-b*, *Chy-e*, and *Chy-b*, and also *Ccd1* indicates that there is no mutation in the exploited genes of "Anne". Moreover, the functional characterization of the important carotenoid genes (*Psy*, *Lcy-e*, *Lcy-b*) indicates the regulation and accumulation of carotenoids at different fruit development stages of raspberry. Furthermore, the activity of *Ccd* plays a role in determination of accumulation/degradation of carotenoids, which control pigmentation and fruit quality traits of raspberry fruits.

4. DISCUSSION

The current study provides important information and new details in view of exploring the genetic mechanism controlling the biosynthesis of important fruit pigments, such as anthocyanins and carotenoids in raspberries. Pigmentation is an important fruit quality trait as it is associated with their ripening and nutritional value of the fruits. Pigmentation in raspberry fruits of different colored varieties owes to the accumulation of anthocyanins and carotenoids. The red pigmentation in raspberries is due to the presence of relatively high amounts of anthocyanins. Berries with yellow or orange coloration, which have been described already more than a century ago (Card, 1898), are most probably based on anthocyanin pathway mutants making the present carotenoids becoming visible. So far the available knowledge indicates that homozygous recessive alleles *tt* of gene *T* are considered to play a fundamental role in determining the yellow color of raspberry fruits (Crane and Lawrence, 1931; Jennings and Carmichael, 1975). However, knowledge of the related gene locus is lacking. In the present work, the most sold red fruiting raspberry "Tulameen" was included for comparative study with the important pale yellow fruiting variety "Anne" to elucidate the phenomenon involved in fruit pigmentation. Red raspberry "Tulameen" was selected from a cross of "Nootka" x "Glen Prosen", both bearing red fruits (Daubeny and Anderson, 1991). Yellow variety "Anne" has been selected from a controlled cross of two red fruiting varieties, "Amity" x "Glen Gerry" (Swartz et al., 1998); however, the authors did not provide any details on the inheritance of the yellow pigmented fruits. So far, there is no clear evidence what kind of genetic factor or factors control the pigmentation in red, orange and yellow fruiting raspberries. Thus, considering prior knowledge of understanding of pathway steps, in the present study it has been focussed on the metabolic profile and mainly on the genetic factors controlling the biosynthesis of key pigments including anthocyanins and carotenoids in raspberries.

4.1. Polyphenols and analysis of anthocyanin pathway genes

4.1.1 Polyphenolic metabolites in "Anne" and "Tulameen"

The present study assesses the metabolite profiling of yellow-fruiting variety "Anne" and red-fruiting variety "Tulameen". The analysis indicates that most of the flavonoids and other phenolics, such as flavonol rutinosides, *p*-coumaric acid, ferulic acid, caffeic acid, vanillin and *cis*-piceid were detected in "Tulameen" and "Anne". Some important compounds, such as

chlorogenic acid, sinapyl alcohol, protocatechuic acid, procyanidin B2 and B4, naringenin-7-*O*-glucoside, quercetin-3,4-*O*-diglucoside and *t*-piceid vary in their amount and are much higher but taxifolin, naringenin, quercetin-3-*O*-glucuronide, vanillic acid and raspberry ketones are lower in "Tulameen" than in "Anne". However, there is no clear difference in the metabolites of "Tulameen" and "Anne" with respect to absence or presence of a class of compounds. Similarly, previous findings indicate no different class of polyphenols between red and yellow fruiting raspberries except anthocyanins (Määttä-Riihinen et al., 2004). In addition to anthocyanins, procyanidin B1 was the only compound detected in "Tulameen" but not in "Anne" (Carvalho et al., 2013a). Biochemical analysis of "Tulameen" and "Anne" gave no clear evidence where a putative block in the anthocyanin pathway might have occurred, as no intermediate compounds or class of compounds were significantly accumulated in the turning stages (fruiting stage 3 to 4) of yellow fruiting raspberries. Thus, flavonoid pathway seems functional and suggests that the block in "Anne" must be quite late and close to the core anthocyanin pathway steps. The study of these steps in addition to flavonoids pathway at genetic level can be helpful to understand the putative step for the block of anthocyanins in yellow colored raspberries.

4.1.2 Regulation of gene expression

Association of regulatory gene *MYB10* with the anthocyanin pathway has been described in the Rosaceae family (Espley et al., 2007; Lin-Wang et al., 2010). A variation in the expression level of *MYB10* gene has been reported to cause altered anthocyanin pigmentation in apple and strawberry (Kotepong et al., 2011; Medina-Puche et al., 2014). As shown in Figure 11 (page 29), the expression of *MYB10* was found in various fruit stages of both varieties "Anne" and "Tulameen" and seems to correlate with the fruit ripening and at least in red fruits with pigmentation, too. These findings from expression analysis indicate that apparently, the regulatory gene *MYB10* is not associated with reduction/adsence of anthocyanins in yellow raspberry "Anne".

Varieties belonging to *Rubus* genus usually accumulate anthocyanins in fruits, but also to some extent in the vegetative plant parts while yellow varieties do not (Clark, 2004). The anthocyanin accumulation in raspberry fruits becomes visible from the ripening stage 3 to 4 (Figure 7; page 16). Therefore, expression analysis of the anthocyanin biosynthetic genes was carried out at these developmental stages. As indicated in Figure 13 (page 32), almost all structural anthocyanin pathway genes show a significant increase in their expression; however, this was

not the case for *Chs* and *Ans* in yellow fruits of "Anne". The identification of chalcones and other upstream flavonoids excludes the block at this level, but it suggests that the block is late in the pathway. The *Ans* transcripts were apparently reduced in "Anne" as compared to "Tulameen" (page 32). Similar profiles with a reduced *Ans* expression among the anthocyanin pathway genes were found in mock strawberry and pomegranate, leading to white fruiting, anthocyanin-free phenotypes (Debes et al., 2011; Zhao et al., 2015). In contrast, an unchanged expression of *Ans* was observed in the *Arabidopsis* loss-of-function mutant *transparent testa 17* (*tt17*), where a single nucleotide polymorphism (SNP) was found to result in an inactive protein and transparent testa seed phenotype (Appelhagen et al., 2011). However, in most of the cases, for example in white fruited mock strawberry and yellow onion bulb, no expression was observed of *Ans* and *Dfr* genes in comparison to highly expressed genes in their red phenotypes (Kim et al., 2004 a, b; Debes et al., 2011). Similarly, a recent study is in line with these findings which revealed almost no expression in non-functional *Pg-ldox* (*Pg-ans*) mutant, where an insertion in coding sequence resulted in lack of *Pg-ldox* transcripts and red to white phenotypic fruit character in pomegranates (Ben-Simhon et al., 2015). Thus, reduced *Ans* transcripts suggest a block at *Ans* level in "Anne". In accordance to this hypothesis, accumulation of flavonols and flavonol-*O*-glycosides was observed in both, red and yellow raspberry fruits (Määttä-Riihinen et al., 2004; Carvalho et al., 2013a). At the branching step in the flavonoid/flavonol pathway, the *Fls* gene, which is usually expressed at early stages, was also down-regulated from stage 3 to 4 in "Anne" and detection of similar flavonols as present in "Tulameen" corresponds to *Fls* functionality at the biochemical level also in "Anne". Another explanation can be that both, *Dfr* and *Fls* genes, are expressed where the level of *Dfr* expression is higher than of *Fls* gene and their products compete for their common substrates for the production of flavonols and anthocyanins, respectively. The same mechanism marked as differential gene expression between *Fls* and *Dfr* genes was observed directing the biosynthesis of flavonols and anthocyanins in white and red flowers of different plant species (Luo et al., 2016). However, the branching FLS step does not directly interfere with the anthocyanin pathway. In addition to *Ans* gene, analysis of *Dfr* gene is also important because mutations in *Dfr* gene irrespective of its expression have been discussed, resulting in altered color phenotypes (Inagaki et al., 1999; Itoh et al., 2002; Kazama et al., 2012; Bashandy et al., 2015). Even though *Dfr* transcripts increase with the fruit development in both varieties under study, still it is necessary to address this important gene - at least at the molecular level - to exclude the possible mutational impact that may account for altered raspberry fruit phenotype.

4.1.3 Molecular analysis of the Dfr gene

Several studies have shown that *Dfr* gene plays an important role in the biosynthesis of anthocyanin pigments during fruit development in *F.* × *ananassa*, a relatively close species among Rosaceae family (Moyano et al., 1998; Li et al., 2001). In *Rubus*, relatively higher traces of taxifolin (syn. dihydroquercetin) were observed in yellow varieties than red ones (Carvalho et al., 2013a). Reports are available describing the accumulation of higher amounts of taxifolin in *Dfr* mutants (Nyegaard Kristiansen et al., 1991). Therefore, the *Dfr* gene was studied at the genetic level to include or exclude the possibility of a block at this level. The molecular study of entire *Dfr* gene from both *Rubus* varieties "Tulameen" and "Anne" presents high identity (99%) with three SAAPs. However, two of them match to the sequences of other Rosaceae species (as shown in Annex IV) and none of them was detected among the amino acid residues strictly conserved in the *Dfr* gene family. Accumulation of flavonols and flavonol-*O*-glycosides was observed in both red and yellow raspberry fruits (Carvalho et al., 2013a, Määttä-Riihinen et al., 2004, Carvalho et al., 2013a). This observation is also in agreement with the detection of flavan-3-ols in yellow raspberries (Carvalho et al., 2013a). The presence of flavan-3-ols together with the sequence analysis might suggest a block downstream/late step of *Dfr* gene especially the downregulated *Ans* gene.

4.1.4 Molecular analysis of Ans/ans alleles

The gene encoding ANS protein, a member of 2-ODD family, has been reported for some Rosaceae members and other plant species but not from *Rubus* yet. Among all 2-ODD's the catalytic domains are characterized by highly conserved residues, such as histidine (His), arginine (Arg), serine (Ser) and phenylalanine (Phe) as shown in Annex VII. The His residues are required for ferrous-iron coordination, and Arg and Ser residues are needed for binding site of 2-oxoglutarate and Phe residue is known for binding to the substrate (Saito et al., 1999; Koehntop et al., 2005; Clifton et al., 2006; Gebhardt et al., 2007; Cheng et al., 2014). Molecular analysis of *Ans* gene at genomic and mRNA/cDNA level indicates a 5 bp insertion (GGCCT; ans^{+5}) in "Anne" *ans* alleles (Figure 14; page 33). The *Ans* gene of "Anne" and "Tulameen" contains a 446 bp intron (Figure 14). The intron comprises consensus 'GT' and 'AG' sequences at the 5' and 3' ends, respectively. This genomic structure, containing a single intron, is similar to those found in *F.* × *ananassa*, *F. vesca*, *A. cepa*, *T. cacao*, *A. thaliana*, *P. avium* and *P. hybrida* (Weiss et al., 1993; Rosati et al., 1999; Deng and Davis, 2001; Almeida et al., 2007; Liu et al., 2013; Shen et al., 2014; Kim et al., 2015). The *Rubus* protein sequence is

approximately 93% identical to *F. vesca* ANS, which is highly similar to other functionally characterized plant ANSs. For example, it is 83%, 78%, and 73% identical to ANS from *T. cacao*, *P. frutescens* and *G. hybrida*, respectively (Saito et al., 1999; Puzio et al., 2009; Liu et al., 2013). The known conserved domains were found in the deduced amino acid sequence of ANS from red fruiting raspberry "Tulameen". However, the ans^{+5} in *Ans* gene of "Anne" led to a pre-mature stop codon 20 aa downstream the insertion and the loss of the conserved His residue (iron binding site) and the RxS motif involved in 2-oxoglutarate binding. It is proposed that the presence of pre-mature stop codons led to the reduction of accumulation of mRNA transcripts. Thus, this reduction or failure of induction of *Ans* transcripts in "Anne" might be due to the secondary effect of a nonsense mutation in *Ans* gene. Such a mechanism, known as nonsense-mediated mRNA decay (NMD), has been reported in several plants (Schwartz et al., 2006; Wu et al., 2007) where pre-mature termination codons (PTCs) lead to NMD and degradation of the mRNA apparently to inhibit the accumulation of nonsense (inactive) proteins. Furthermore, it has been described that as long the PTCs are present at distance from 3' mRNA termini they act as substrates (*cis*-acting elements) to activate NMD mechanism (Kertesz et al., 2006; Schwartz et al., 2006; Hori and Watanabe, 2007), hence *ans* PTC in "Anne" which is 513 bp distant from native 3'UTR, strongly supports this phenomenon. Moreover, heterologous protein expression in *E. coli* provides strong evidence that "Anne" ANS protein is truncated in size compared to "Tulameen" ANS as presented in Figure 16 (page 36). This mutation in *RiAns* of "Anne" results in loss of function/red pigmentation and provides the genetic basis for yellow raspberry "Anne".

4.1.5 Complexity of Ans gene

As the available genome draft of *Rubus* is not completed yet and therefore it could not be excluded that more than one copy of *Ans* gene is present per haploid genome, although the screen gave strong support for single copy gene. Therefore, a quantitative PCR approach was adopted to determine the complexity of the *Ans* gene as it was applied earlier to determine gene copy number in filamentous fungi (Solomon et al., 2008). The analysis indicated that there is indeed only a single copy of the *Ans* gene in the haploid genome of *R. idaeus* (as presented in Figure 15; page 35), which is in agreement with the BLAST search of the *Rubus* genome draft ("Heritage"). The presence of a small gene family of the *Ans* genes has been reported for some species of Rosaceae such as, peach and octaploid strawberry. Peach and octaploid strawberry (*F.* × *ananassa*) haploid genome encodes two and four copies of *Ans* gene, respectively

(Almeida et al., 2007; Ravaglia et al., 2013). However, only one gene copy has been described in haploid genome of diploid strawberry (*F. vesca*) and apple (Takos et al., 2006/3).

4.1.6 Functional characterization of Ans gene

Studying gene functions through genetic transformation in raspberry is difficult due to its limited regeneration and transformation efficiencies (Utermark and Karlovsky, 2008). Therefore, *A. thaliana* was used as an alternative system to study the function of *Ans* gene from raspberry. Previous studies have shown that genes of the anthocyanin biosynthetic pathway in *A. thaliana* are upregulated in response to sucrose-induced signals resulting in a red pigmented phenotype and accumulation of anthocyanins, whereas plants without sucrose treatment do not show any pigmentation (Gollop et al., 2001; Abrahams et al., 2003; Solfanelli et al., 2006; Kovinich et al., 2014). In order to functionally characterize the *Rubus Ans* genes, transgenic lines were obtained through complementation of *ldox* mutant of *Arabidopsis* 'ldox:KO' harboring *RiAns* coding sequences from "Anne" and "Tulameen" expressed under the control of the constitutive *CaMV 35S* promoter. The plants transformed with '35S::Ans_Tulameen' showed a restored phenotype under sucrose stress while lines transformed with '35S::Ans_Anne' did not show any changes when compared to 'ldox:KO' line irrespective of the sucrose-induced signals (as shown in Figure 18; page 38). Chemical analysis confirmed the complementation of the anthocyanin pathway due to presence of anthocyanins in transgenic plants transformed with '35S::Ans_Tulameen' (Figure 19; page 39). However, no anthocyanins were detected from the plants transformed with 'Anne-Ans' similar to the 'KO' line. These complementation results confirm that 'Tulameen-Ans' encodes for a functional ANS protein while 'Anne-Ans' results in a non-functional protein. Thus, our results correspond to the findings of Ben-Simhon et al. (2015) who reported that an insertion in the coding region of *Ans* mediates a block in anthocyanin pathway and produces white fruits of pomegranates.

4.1.7 Polyphenolic metabolites in Rubus

The anthocyanin pathway mutation found at *Ans* level (Section 4.1.4) and elucidation of the role of ANS (Section 4.1.5) in "Anne" may predict the reason of other non-red varieties. The basis of the origin of other yellow or also orange varieties to their red counterparts might be that the same or maybe other mutations in *Ans* gene or in other steps of the anthocyanin pathway are involved, which could result in different profiles of metabolites. Thus, the metabolic profile of 22 available varieties including "Tulameen" and "Anne" was analysed (as described earlier

in Vrhovsek et al., 2012) all together to get an overview of other possible blocks in the pathway of yellow or orange fruits. The polyphenolic compounds in yellow varieties were not considerably different from red varieties except for the amounts of various single metabolites, as described earlier (Figure 20; page 41, Carvalho et al., 2013a). Taking into account not only yellow and red fruiting varieties, but also other fruit color groups, such as black, purple and orange varieties, there was no particular class of compounds absent (or present) in these fruits except anthocyanins. However, among the varieties, irrespective of fruiting color, concentrations of various phenolic compounds are different from each other. Many studies have suggested that environmental, developmental and genetic factors significantly influence the composition and constituents of raspberry bioactive compounds (Anttonen and Karjalainen, 2005; Beekwilder et al., 2005). Overall these findings do not support in a characteristic/typical pattern of polyphenolic compounds among yellow, orange and other different colored varieties excluding anthocyanins, hence it suggests the block quite late after flavonoid pathway of these varieties too. Based on metabolites identified, a pathway map has been created for various polyphenols including flavonoids and anthocyanins detected in different raspberry varieties (Figure 35).

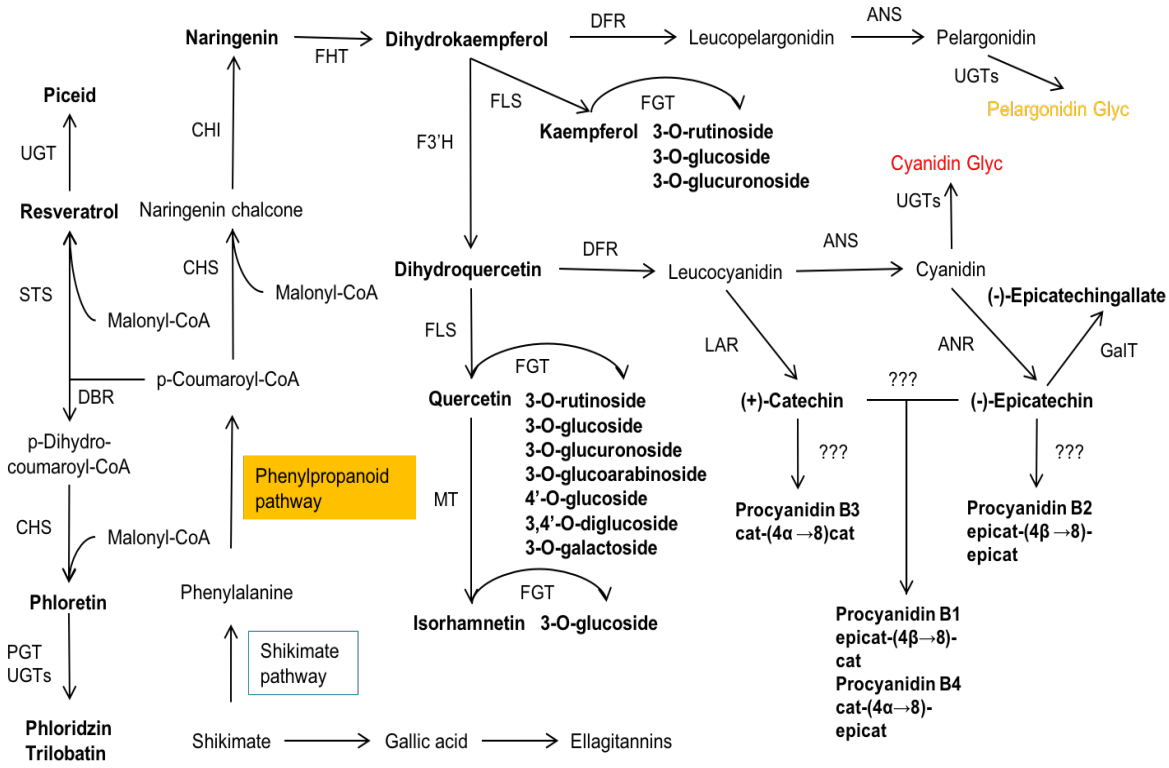


Figure 35: Preliminary pathway map of biosynthesis of polyphenols in raspberry; CHS, chalcone synthase, CHI, chalcone isomerase; STS, stilbene synthase, DBR, double-bond reductase, FHT, flavanone 3*β*-hydroxylase; F3'H, flavonoid 3'-hydroxylase; DFR, dihydroflavonol 4-reductase; ANS, anthocyanidin synthase; UGT, UDP-glycosyltransferase; ANR, anthocyanidin reductase; LAR, leucoanthocyanidin reductase and FLS, flavonol synthase; MT, methyltransferase; PGT, phloretin glycosyltransferase, FGT, flavonoid glycosyltransferase.

In order to get an idea of whether the overall variation of compounds (concentrations) relate to the entire berry color in a group or not, the obtained data of polyphenols were examined in the PCA biplot. Varieties containing similar concentrations of metabolites can be assigned in the same group and if these concentrations are different they lie in separate groups and plot planes (Figure 21; page 42). Yellow and red colored varieties are clustered together on the plot plane. "Black Jewel", "Tayberry" and "Buckingham Tayberry" have unique concentrations of polyphenolic metabolites and form a separate group (as indicated in Figure 21). The reason behind this may be that these varieties are genetically different from most of the diploid *R. idaeus* in the present study. "Black Jewel", belonging to *R. occidentalis* L., is an octaploid and resulted from a complex, unknown cross, while "Tayberry" and "Buckingham Tayberry" with purple fruits, emerged from a hybrid cross between octaploid black and tetraploid red raspberries (Jennings, 1979; Jennings and McNicol, 1989). Interestingly, varieties with orange berries were found isolated on the plot plane and lie close to the yellow group, indicating that they have some variation in the phenolic pattern that altered the metabolic profile compared to the other color groups. Even though, the different composition of polyphenols is not suitable to correlate with a pathway block, the absence of anthocyanins in yellow and orange varieties indicate that it would be of interest to screen all these varieties for possible mutations in the *Ans* gene of the anthocyanin pathway.

4.1.8 Molecular markers for Rubus screening

Since no clear evidence was obtained from polyphenolics data regarding putative pathway blocks, molecular markers were designed to screen *Rubus* for possible Anne-like mutation (ans^{+5}) in the *Ans* gene. DNA/RNA based genetic markers are common in use for identification of SNPs or small target sequences within particular amplified fragments (Wittwer et al., 1997). Taking advantage of this technique, a fluorogenic-labeled probe was designed that helped in detecting the "Anne" ans^{+5} mutations from genomic DNA and RNA/cDNA (Figure 22; page 44). In addition to screening with the probe, HRM analysis also validated the allelic discrimination for "Anne" and "Tulameen" (Figure 23; page 44). Being more efficient and

economical than the techniques described here, another molecular marker, the CAPS marker, was developed which also identified the 5 bp (ans^{+5}) mutation in "Anne" (Figure 24; page 46). Such molecular approaches (fluorogenic-labeled probe, HRM analysis, CAPS marker, etc.) based on a known DNA/RNA sequence have been an effective tool for molecular-genetic study, for example marker assisted selection which geared the germplasm selection (Collard et al., 2005; Shavrukov, 2016; Chatzidimopoulos et al., 2019). Thus, the molecular markers developed in the current study can further be utilized for characterization of ans^{+5} insertion/mutation inside *Ans* gene and to screen yellow but also red/orange varieties of raspberry and to characterize germplasm collections and off-springs in breeding programs at an early stage.

4.1.9 The origin of ans^{+5}

It is important to address the question from where this 5 bp (ans^{+5}) mutation originates in yellow variety "Anne". Either it occurred as a spontaneous mutation, and the variety was selected due to the color change during the breeding approach or it is directly derived from crossing of both its heterozygous parents implicating an earlier mutation event. The CAPS-marker applied on red fruiting parents of "Anne" ("Amity" and "Glen Garry") indicates that "Glen Garry" contains the same 5 bp insertion/mutation in one of the alleles, hence, apparently "Anne" originated as a crossing event. But unexpectedly, "Amity" does not have the ans^{+5} mutation and it contains all wild type *Ans* alleles (Figure 24B). In general, *Rubus* is a diploid with two *Ans* alleles, however, "Glen Garry" was apparently found to contain at least three *Ans* alleles. It is not clear how the "Anne" *ans* alleles can be derived only from a single parent. There are shreds of evidence explaining that in addition to interspecific hybridization, the existence of automictic and apomictic events exist in *Rubus* (Antonius and Nybom, 1995; Clark and Jasieniuk, 2012; Nybom, 1988). The origin of several other yellow varieties have been reported as a sport (a faulty chromosomal replication due to a genetic mutation) of red varieties, e.g. "Sugana Giallo" from "Sugana Red" (Anonymous), "Kiwigold" and "Graton Gold" (Goldie, Fall Gold) from "Heritage" (Thomas, 2000; Dixon, 1991), "Lisa" from "Meeker" (Nikolić and Milivojević, 2008), "Golden Queen" from "Cuthbert" (Szalatnay et al., 2011) and "Golden Bliss" ("All Gold") from "Autumn Bliss" (Bundessortenamt, 2006). Similarly, a cross between two red fruiting varieties "Autumn Bliss" and "Tulameen" resulted in a yellow fruit variety "Lumina", but sequence analysis of *Ans* of "Tulameen" did not indicate the presence of any mutation which leads to the suggestion that there might be some other phenomenon involved. A study based on SSR marker reveals that the fingerprint of "Lumina" is identical to that of "Autumn Bliss"

(Girichev et al., 2015), minimizing the involvement of a direct crossing event and indicating a spontaneous mutation. Further screening of yellow varieties indicated a similar type of three alleles in yellow fruiting “Citria”, “Alpen Gold” and “Giallo Mutant” as observed in “Glen Garry” (“Anne” parent). Interestingly, another variety, “Valentina” was found with orange fruit phenotype having the same ans^{+5} mutation in the homozygous state. Thus, it is expected that the mutation is revolving in *Rubus* genome with yellow/orange phenotypes but none of the red fruit-bearing variety under study except “Glen Garry” contains the ans^{+5} mutation. It seems that these yellow varieties have a parental ans^{+5} connection at some point for its transmission further into the genome. However, other independent mutations resulting in the loss of anthocyanins cannot be excluded at this stage and are subjected for further analysis of *Ans* gene not only in yellow but also in orange fruiting *Rubus* in comparison to red fruiting varieties.

4.1.10 Other Ans mutations in yellow fruiting Rubus

Molecular analysis of the *Ans* gene in selected yellow, orange and red fruiting varieties indicate the existence of further mutations or variations in the *Ans* gene in most of the *Rubus* varieties under study. All these identified mutations were named as RAMT-1 to RAMT-7. RAMT-1 observed in yellow “Herbert Gold”, orange “Valentina” and red “Glen Garry” is the same ans^{+5} found in "Anne”, while, RAMT-2, a 4 bp insertion (ans^{+4}) was found in many yellow varieties (“Golden Everest”, “All Gold”, “Gelbe Antwerpener”, “Lumina”, “Him13K39-8” and “Herbert Gold”) and an orange variety “Orange Marie”. Similarly, RAMT-3 (ans$^{+2, +5}$) and RAMT-4 (ans^{+2}) indicate 2 bp and 5 bp insertions in the same allele of red variety “Glen Garry”, and a 2 bp insertion in yellow variety “Herbert Gold”, respectively. Here, almost all the yellow and orange varieties contain such kind of mutations those lead to truncated proteins lacking the conserved crucial elements necessary for their functional activity as described previously (Saito et al., 1999; Koehntop et al., 2005; Clifton et al., 2006; Gebhardt et al., 2007; Cheng et al., 2014). In view of previous reports, it is suggested that *Ans* gene is most probably the *T* locus which plays key role in determining the phenotype of raspberry fruits. It is evident that to synthesize anthocyanins at least a wild type allele is necessary as retained in red fruiting “Glen Garry” and “Amity”, parents of yellow fruiting “Anne” together with all other red fruiting varieties under molecular analysis. However, there is an exception as yellow fruiting “Golden Queen” and “Sugana Gold” contain wild type *Ans* alleles, otherwise, all yellow and orange phenotypes contain any of the RAMT-1 to RAMT-6. A report, indicating the absence of anthocyanins in “Golden Queen” and “Sugana Gold” (Carvalho et al. 2013a), proposes the existence of other potential mutations in the anthocyanin pathway. Moreover, sequence analysis

indicated that apparently "Glen Garry" has 3 alleles for *Ans* gene, which is in line with the findings of CAPS analysis. Allele 1 is wild type (like "Tulameen") and allele 2 contains RAMT-1 (ans^{+5}), while allele 3 contains RAMT-3 ($ans^{+2, +5}$). "Glen Garry", being diploid, having more than two *Ans* alleles, may indicate a local gene duplication. The reason of "Anne" *Ans* gene retaining both alleles which contain ans^{+5} (RAMT-1) is not clear yet because only one parent ("Glen Garry") has a single allele with ans^{+5} and the second parent ("Amity") does not seem to contain RAMT-1. One explanation can be that "Amity" is not the true "Amity" or the crossing parents could be different than reported. Another possible reason could be that "Glen Garry", being a complex cross, may contain more than one *ans* (ans^{+5}) like alleles, and transferred to "Anne" by some unknown mechanism during hybridization rather involving the second parent "Amity". The process of meiotic gene conversion is also possible by which a gene in a heterozygous diploid replaces the homologous sequence such that both the alleles become identical (Stahl, 2001).

4.1.11 Variation in RiANS and PA routes in Rubus

Despite the interest in functional RiANS polypeptide predicted from the *Ans* gene, a high identity was found among ANS protein sequences derived from different *Rubus* varieties under study. Comparison of the deduced ANS amino acid sequences revealed natural variation among different yellow, orange and red fruiting varieties (Annex IX). For example, "Heritage", "Autumn Bliss" and "Sugana Red" showed 3, 2 and 2 SAAPs, respectively when compared to "Tulameen" sequence. However, these SAAPs apparently do not have a significant impact on ANS function as the fruits were red pigmented in all these genotypes and all known conserved and catalytically important residues are present. The RiANS protein sequence from "Tulameen" is 99% identical to the other sequences of red varieties ("Autumn Bliss", "Heritage", "Sugana Red") explored during this study (Annex X). Likewise, few SAAPs were also observed among RiANS of yellow/orange varieties. These ANSs also presented high similarities to the "Tulameen" ANS but the shorter ANS proteins lack the essential conserved residues, known to be responsible for substrate and co-substrate binding (Annex IX), giving strong support for inactive protein in these varieties and therefore the genetic block in the pathway.

In the current study, in spite of the fact that the ANS enzyme is obviously inactive in yellow/orange raspberries, the chemical analysis indicated still the synthesis of epicatechin based procyanidins. This finding together with inactive ANS and the actual knowledge on flavan-3-ol biosynthesis led to the assumption, that a yet unknown step is needed to enable the

biosynthesis of epicatechin. Within the reductase-epimerase-dehydrogenase (RED) family, *Lar* and *Anr* are considered to be remotely linked but their epimeric activities have been discussed previously (Stafford, 1990; Gargouri et al., 2009; Qian et al., 2015). Some reports are available accounting the formation of epicatechin where ANS is potentially not active (similar to the finding here in *Rubus*), and few hypothetical alternatives have been explained (Szankowski et al., 2009; Liu et al., 2013). According to these reports, one possible biosynthetic route for the formation of epicatechin in such mutants is the epimerization of catechin to epicatechin as described in apple (Szankowski et al., 2009). Yet another explanation for the formation of epicatechin-based-procyanidins can be the non-stereospecific de-polymerization of polymeric catechins when ANS is not functional, e.g. in *Rubus* ("Anne"). Thus, catechin-epicatechin derived PA-oligomers were observed with a decrease in epicatechin-based oligomers in yellow raspberry fruits in comparison to red ones; however, oligomeric-catechins were not significantly different but being higher in concentration in yellow than in red varieties (Figure 20; page 41; Carvalho et al. 2013a). This phenomenon is in agreement to the previous findings,

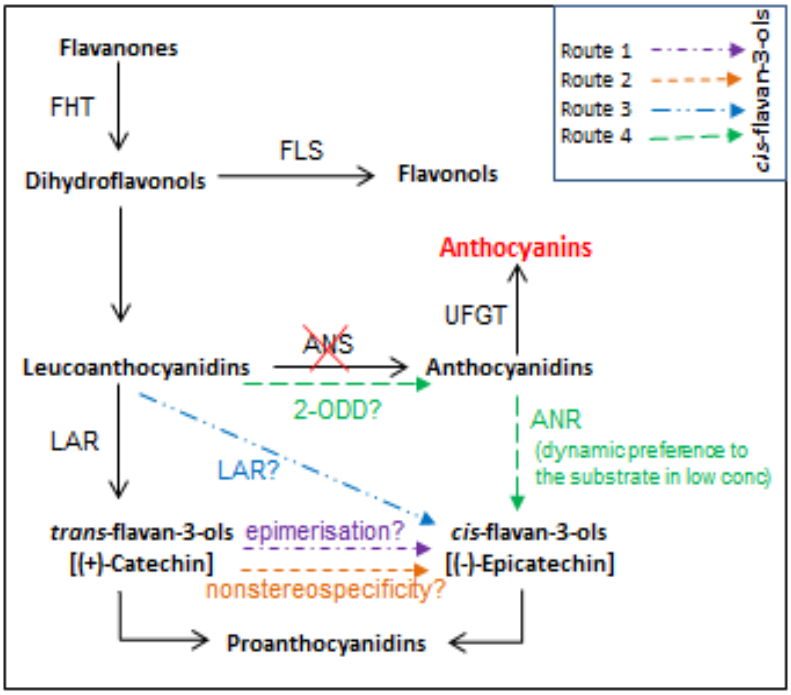


Figure 36: Proposed flavonoid biogenesis pathway in yellow raspberry; FHT, flavanone 3*β*-hydroxylase; DFR, dihydroflavonol 4-reductase; ANS, anthocyanidin synthase; UFGT, UDPG-flavonoid-glycosyltransferase; ANR, anthocyanidin reductase; LAR, leucoanthocyanidin reductase and FLS, flavonol synthase. The red cross indicates the blocked step of the anthocyanin biosynthetic pathway at ANS level leading to the absence of anthocyanin pigments. Dotted arrows present various possible routes for the formation of *cis*-flavan-3-ols.

where silenced *Ans* gene of apple, resulted in elevated concentrations of both, polymeric catechin and catechin-epicatechin derivatives with a decrease in polymeric epicatechins (Szankowski et al. (2009). Moreover, it has been described by Liu et al. (2013) that overexpression of *Theobroma Lar* in *ldox* mutant of *A. thaliana* led to the enzymatic conversion of leucocyanidin resulting into a mixture of catechins and epicatechins (Route 3). There is also another way to explain that some other 2-ODD could by-pass the ANS for leucocyanidin substrate and dynamic preference of *Anr* for the substrate could produce epicatechin-based-procyanidins (Route 4). Thus, any of the above-mentioned possibilities or combinations with other routes can explain the deviation of metabolic flow into other directions leading to the biosynthesis of epicatechin-based proanthocyanidins (Figure 36).

In the present study, a genetic block at ANS level is evident in the anthocyanin pathway of "Anne". However, the involvement of other mechanisms (as discussed in Section 4.1.9) cannot be excluded as some varieties with wild type *Ans* gene also produce yellow fruits apparently due to accumulation of carotenoid pigments. More studies of carotenoid biosynthetic genes will be useful to predict the type of carotenoid pigments and co-pigments that provide distinct colors to raspberry fruits.

4.2. Analysis of carotenoid pathway genes

The yellow/orange fruit characters of the varieties under study originate apparently from mutation events in the anthocyanin pathway (e.g. *Ans* gene). Hence, carotenoids seem to be responsible for the yellow/orange fruit color and they might be just masked by anthocyanins in case of the red varieties. Due to a range of raspberry fruit colors, the current study of pathway genes leading to carotenoid accumulation was carried out for understanding of their biosynthesis and role in imparting color to the raspberry fruits as described here.

4.2.1 Biosynthesis of carotenoids

In general, there are various ways that control the biosynthesis and accumulation of carotenoids in plants, such as feedback regulation (Rodríguez-Villalón et al., 2009), other interacting/competing pathways (Sauret-Güeto et al., 2006), metabolic sink capacity of plastids (Lu et al., 2006) and degradation of carotenoids (Brandi et al., 2011). Mechanisms controlling the accumulation of carotenoids have not been studied yet in *Rubus*. The red, orange and yellow pigments are the most prominent carotenoid pigments and these are also the prominent color patterns existing in *Rubus* fruits. Herein, a yellow fruiting variety "Anne" was used for

functional characterization of carotenoid pathway genes and red fruiting variety “Tulameen” for comparative analysis to “Anne” to postulate a pathway map in raspberry fruits.

4.2.2 Transcriptomic analysis

Expression analysis of carotenoid pathway genes

The level of expression of the biosynthetic genes determines the production and accumulation of carotenoid pigments. In view of entering the carotenoid pathway, GGPS is an important branch point enzyme, which catalyzes the formation of primary substrate geranylgeranyl diphosphate (GGPP) in the pathway (Figure 37; page 84). In *Rubus*, expression of *RiGgps* is higher in later fruit ripening stages as compared to early stages, especially in “Tulameen”, indicating the distribution of carbon flow in the form of GGPP, a substrate to yield first carotene (phytoene) of the pathway. *RiPsy* encoding phytoene synthase is highly expressed at early stages and downregulated at fruit turning stage and then again start increasing with fruit ripening (Figure 31; page 54). Comparatively higher *RiGgps* expression and lower *RiPsy* is correlated to the production of higher tocopherols but lower phytoene in “Tulameen” as compared to “Anne” where metabolic flux tends to produce higher phytoene and lower tocopherols (Carvalho et al., 2013b). Similarly, it is evident from expression analysis of most of the pathway genes that enzymes may also compete for a common substrate causing fluctuation in the downstream carotenoid metabolic flux and resulting in different pigmentation pattern in fruits (Wang et al., 2011). Upregulation of *Psy* is in line with the accumulation of phytoene in ripe raspberry fruits as reported in a previous study (Carvalho et al., 2013b) and also indicates the *de novo* biosynthesis of carotenoids in later ripening stages. The present work reveals that due to the transcript abundance of *RiPds* in very early stages, e.g. in “Tulameen” (Figure 31), the enzyme RiPDS transforms phytoene to ζ-carotene and regulates the production of high contents of downstream compounds. However, low expression of *RiPds* affects the accumulation of ζ-carotene and downstream carotenoids, similarly as lower transcription due to silenced *Pds* in tomato resulted in reduced ζ-carotene and lycopene affecting the fruit phenotype from red to yellow (Fantini et al., 2013). Thus, downregulation of the *RiPds* gene at this important step can be responsible for the origin of other yellow, especially orange varieties under study due to the production of reduced lycopene contents.

Lycopene is an important precursor for two branches leading to the formation of *β*-carotene and various xanthophylls. In both varieties, *RiLcy-b* was highly expressed at early fruit development

stages then decreased in later stages, which is concordant with the presence of high to low *β*-carotene profile from unripe to ripe raspberries (Carvalho et al., 2013b). A trend of increase in transcripts of *RiLcy-e* was observed in both varieties from unripe to ripe raspberries being maximum at stage 4 and 5, comparatively almost double in "Anne" than "Tulameen" (Figure 31). These expression patterns of *RiLcy-b* and *RiLcy-e* correspond to the production of *β*-carotene that is higher in unripe fruits, whereas accumulation of lutein occurs mainly in ripe raspberry fruits (Carvalho et al., 2013b). Similarly, reduced expression levels of lycopene cyclases have been reported to determine the accumulation of lycopene which impart pink and red color to tomato fruits and cassava roots, respectively (Ronen et al., 1999; Carvalho et al., 2016). At this point, downregulation or overexpression of cyclases can lead to the accumulation of lycopene or formation of *β*-carotene, that can cause the orange or yellow raspberry fruits patterns, respectively.

Downstream in the pathway, hydroxylases regulate the biosynthesis of xanthophylls through hydroxylation of *α*-carotene and *β*-carotene (Figure 37; page 84). A downregulation of hydroxylases can decrease xanthophylls and increase accumulation of upstream *β*-carotene, similarly as it has been reported in pale-yellow cassava roots which contain high amounts of *β*-carotene isomers due to reduced *Chy-b* transcripts (Carvalho et al., 2016). There is no distinct expression pattern for *RiChy-b* in "Anne" and "Tulameen" as relatively higher expression levels were observed at early stages of "Anne" and "Tulameen" and also ripe fruits of "Tulameen". Thus, small fruits (T0) and ripe fruits (T4, T5) of "Tulameen" may show the formation of increased xanthophylls but "Anne" may accumulate higher *β*-carotene than "Tulameen". Thus, accumulation of *β*-carotene in unripe "Anne" corresponds to the accumulation of higher *β*-carotene content in yellow pigmented cassava varieties due to reduced transcripts abundance of direct downstream *Chy-b* gene (Carvalho et al., 2016). Similarly, downregulation of *Chy-b* also led to an increase of *β*-carotene in maize (Yan et al., 2010). On the other hand, expression of *Chy-e* is higher in unripe fruits of "Anne" and "Tulameen" than ripe ones. In accordance to that, a major component of unripe raspberries was found as lutein in free form along with *β*-carotene, and further metabolic flux of esterified forms of lutein increased from unripe to ripe raspberries (Carvalho et al., 2013b). Accumulation of lutein and especially its esterified forms in ripe raspberry fruits indicate their stability and *de novo* synthesis in contrast to degradation of lutein in strawberry ripe fruits due to lutein specific CCD (Cazzonelli and Pogson, 2010) that apparently do not exist in *Rubus*. Downstream of the hydroxylases, the expression of the important *Zep* gene controls the level of zeaxanthin and downstream branch of xanthophylls.

Natural mutations or induced silencing of the *Zep* gene has been reported to cause enhanced accumulation of zeaxanthin in potato tubers (Römer et al., 2002; Wolters et al., 2010). In early stages of fruit development, i.e. unripe fruits, relatively higher amounts of xanthophylls (e.g. antheraxanthin, violaxanthin, neoxanthin) are expected to be produced due to relatively higher expression of *RiZep* and *RiNsy* genes, in contrast to ripe fruits of "Anne" and "Tulameen". These findings are in agreement with the detection of xanthophylls (e.g. zeaxanthin) relatively twice as high in "Tulameen" as compared to unripe "Anne" fruits (Carvalho et al., 2013b).

Not any obvious downregulation in the expression pattern of any carotenoid pathway gene at all development stages was observed in "Anne" and "Tulameen". Overall expression pattern of most of the carotenoid genes (*Psy*, *Lcy-b*, *Lcy-e*, *Zep*, *Nsy*) was not found significantly different, but with some variations in both varieties. So, variation in the carotenoid contents may be due to environmental conditions and genetic variability in addition to variation in expression patterns as it has been proposed for various red and yellow pigmented fruit varieties (Carvalho et al., 2013b). Expression analysis indicates that accumulation of carotenoids, especially *β*-carotene and xanthophylls (mainly lutein), are important for yellow pigmentation of *Rubus* fruits, especially in "Anne".

Expression analysis of carotenoid cleavage dioxygenases

The existence of carotenoid cleavage dioxygenases (*RiCcd1.1*, *RiCcd1.2* and *RiCcd1.3*) in *Rubus*, that might degrade carotenoids, i.e. *β*-carotene to produce apo-carotenoids, is not only an important quality determinant of fruits, but also play a role in their pigmentation (Figure 37). Downregulation of all the three *RiCcd1s* in later ripening stages indicates that *de novo* synthesis of *β*-carotene may not be substantial. In spite of expression of *Ccd1*, availability of the suitable substrate is also crucial for the formation of apo-carotenoids and determination of fruit pigmentation. For example, white-fleshed melons have been described lacking both, the carotenoids and apo-carotenoids, whereas orange and pale-green melons contain both constituents (Ibdah et al., 2006) as observed also in "Anne" (Carvalho et al. 2013b). Lutein content remains unchanged (rather transformed to lutein esters) during ripening in raspberry, but *δ*-carotene and *β*-carotene compounds were reduced to *α*-ionone and *β*-ionone as a degradation product contributing to the characteristic flavor. Thus, high expression of the three *Ccd1s* in the early fruit development stages shows that apparently none of the *Ccd1s* is specific to lutein degradation in raspberry as found in strawberry and wheat (Roca and Mínguez-Mosquera, 2001, Cazzonelli and Pogson, 2010, Qin et al., 2016). Degradation process of lutein

is also considered slower than other carotenoid components by the activity of carotenoid cleavages (Roca and Mínguez-Mosquera, 2001; Giovanelli and Brenna, 2006; Cazzonelli and Pogson, 2010), hence due to stability they are most abundant in ripe raspberries and most probably they are the most important constituents along with other carotenoids responsible for pigmentation of raspberry fruits. A clear link between the activity of *Ccd1* and pigmentation has been described in melon where expression of *CmCcd1* is very high in ripe fruits (Ibdah et al., 2006) in contrast to the findings of *RiCcd1s* expression in the current study. On the other hand, higher expression of *RiCcd*, *FaCcd1*, *VmCcd1*, *VvCcd1* within ripe fruits led to increase in the level of VOCs ultimately causing a decrease in the carotenoid profile (Beekwilder et al., 2008, García-Limones et al., 2008, Lashbrooke et al., 2013, Karppinen et al., 2016).

Other *Ccds*, such as *Ccd4*, are also considered to be important for degradation of carotenoids and are known to change the fruit phenotype. For example, reduced expression of *Ccd4* gene has been reported to result in yellow to white phenotypic character of peach fruits (Brandi et al., 2011, Adami et al., 2013). However, *in-silico* search indicated that *Ccd4* apparently do not exist in *Rubus*. Considering the expression of *Ccds*, the current study supports the idea proposed of Carvalho et al. (2013b), that *Ccd1* in *Rubus* is specific to catalyze the oxidative cleavage of *β*-chain carotenoids, especially *β*-carotene, to produce aroma compounds particularly *β*-ionone in early fruit development stages. In later fruit development stages, *Ccd1s* activity is reduced due to its relatively lower expression and accumulation of *β*-carotene in ripe fruits may occur due to the *de novo* synthesis of *β*-carotene as described previously (Beekwilder et al., 2008). In the current study, most of the carotenoid pathway genes and *Ccds* are fairly expressed and there is no complete inhibition of transcripts of any gene. As transcription levels do not always indicate the functional role of genes, it is of interest to address the important pathway genes at the genetic and biochemical level.

4.2.3 Sequencing analysis and complementation

The carotenoid pathway genes have not been studied nor functionally characterized so far in *Rubus*. Therefore, it is not well understood, how the carotenoid biosynthesis is regulated in this important soft fruit species. Thus, the most important carotenoid pathway genes and the cleavage dioxygenases from “Anne” were subjected to sequence analysis and functional characterization (as described in Sections 3.2.3 and 3.2.4). Co-transformation and complementation in *E. coli* (as presented in Table 4&5) enabled the successful characterization of these pathway genes (Figure 33; page 61) as it has been reported previously (Cunningham

and Gantt, 1998; Cunningham and Gantt, 2007). The first committed step of the carotenoid pathway, the phytoene synthase, plays a key role in the biosynthesis of carotenoid pigments in different plant parts. The gene encoding phytoene synthase enzyme has been reported for some members of the Rosaceae and other plant species (Ampomah-Dwamena et al., 2015). Biosynthesis of downstream carotenoid pigments correlates to the production of primary pathway compounds, e.g. phytoene (Rodríguez-Villalón et al., 2009; Welsch et al., 2010). In this study, a full-length *RiPsy* sequence from "Anne" gave a polypeptide of 249 aa. It contained the active site DXXXD (DELVD and DVGED) including four conserved aspartate residues necessary for its activity (as previously described in López-Emparán et al., 2014). Comparison with other published PSY sequences indicated that RiPSY share high percentage of homologies, such as 81% to *Citrus* (ABB72444; Inoue et al., 2006), 74% to *Adonis* (AAV74394; Cunningham and Gantt, 2007) and *Actinidia* (ACO53104; Ampomah-Dwamena et al., 2009), 72% to *Capsicum* (CAA48155; Guzman et al., 2010), 71% to *Arabidopsis* (AAA32836; Scolnik and Bartley, 1994) and 60% to *Solanum* (AAA34153; Bartley et al., 1992) (Annex XII). Further, the RiPSY enzyme was functionally characterized by complementation in a GGPP accumulating strain of *E. coli* (Figure 33a). On the other hand, a *psy1* mutant in yellow tomato has been reported to cause down-regulation and downstream block of all kinds of carotenoid pigments and overexpression of a functional PSY enzyme restored the red pigmentation in tomato fruits (Fray and Grierson, 1993). However, in the current study, sequence analysis and functional activity confirmed the active role of PSY enzyme in "Anne". It also corresponds to the detection of phytoene and downstream carotenoids in this variety (Carvalho et al., 2013b).

In metabolic engineering approaches of plant carotenoids, synthesis of carotenes, being the precursors of Vitamin-A, is of key interest (Nisar et al., 2015). They are synthesized from lycopene as precursor by the catalytic function of LYC-e and LCY-b, respectively. At the two-branch step of lycopene cyclization, LCY-e catalyzes the formation of carotenes, i.e. δ-carotene and γ-carotene, eventually leading to the accumulation of β-carotene and a number of xanthophylls (zeaxanthin, antheraxanthin, violaxanthin, neoxanthin) in one branch and lutein (xanthophyll) as end product of the second branch. Herein, the sequencing approach indicated that the conserved motifs for RiLCY-e are to a high degree identical to the respective gene from *Arabidopsis* (U50738) (Cunningham et al., 1996). RiLCY-e protein sequence of "Anne" was found 100% identical to *Rubus* draft ("Heritage"). It also showed high similarities to other species, such as 83% to *Coffea* (ABC87738; Simkin et al., 2008), 76% to *Adonis* (AAK07432; Cunningham and Gantt, 2001), 75% to *Arabidopsis* (AAB53336; Cunningham et al., 1996) and

71% to *Solanum* (CAA74745; Ronen et al., 1999) as presented in Annex XIV. The functional activity of *RiLcy-e* gene was noticed through the complementation experiment when the color of cultures turned from red to light yellow. It indicates the successful complementation through the conversion of the red lycopene precursor to light yellow *δ*-carotene by the activity of RiLCY-e. On the other hand, silencing and mutations of *Lcy-e* genes change the carotenoid metabolic flux to the *β*-branch giving rise to the enhanced production of *β*-carotene, e.g. in potato and maize (Diretto et al., 2006, Harjes et al., 2008). However, in *Rubus* ("Anne") accumulation of higher amounts of lutein xanthophyll during ripening as compared to *β*-carotene and other xanthophylls indicate functional activity of RiLCY-e (Carvalho et al., 2013b). Further, its activity was found to be completely diminished at 13.5 µM CPTA. Similar inhibition of LCY-e from tomato has been described at 5.0 µM CPTA (Ronen et al., 1999). Downstream in the pathway, mutations or SNPs in the LCY-b sequences may alter the fate of compounds and fruit pigments as previously reported in red to yellow water melons (Bang et al., 2007). In the present work, the RiLCY-b sequences from "Anne" were found exactly same as of *Rubus* "Heritage". It contained all the conserved and essential motifs for LCY-b, such as plant *β*-cyclase conserved region, dinucleotide binding domain on N-terminus, cyclase motifs I and II in middle and charged region and *β*-LCY essential motif on C-terminus (Hugueney et al., 1995; Cunningham et al., 1996; Bouvier et al., 1997). All these conserved and important motifs, which are essential for the activity of LCY-b, have also been described in *Capsicum* (Alquézar et al., 2009). Moreover, phylogenetic analysis indicated the high similarity to other plant species, such as 87% to *Vitis* (AFP28799; Young et al., 2012), 85% to *Solanum* and (CAA60170; Pecker et al., 1996) and *Daucus* (ABB52071; Just et al., 2007) and 84% to *Nicotiana* (CAA57386; Pecker et al., 1996) and *Capsicum* (ADH04271; Guzman et al., 2010) and 82% to *Arabidopsis* (AAA81880; Scolnik and Bartley, 1995). However, *Zea* (AAO18661; Singh et al., 2003) and *Citrus* (BAM66329; Zhang et al., 2012) show comparatively low, i.e. 75% and 60%, similarity to the RiLCY-b (Annex XIII). The cyclization of lycopene directs the formation of cyclic compounds playing key roles in photosynthesis as well as precursors for regulatory molecules in plants. The *Lcy-b* gene catalyzes the cyclization reaction producing provitamin A carotenoids, such as *β*-carotene. Visible color change and production of *β*-carotene through complementation clearly indicate the functional activity of RiLCY-b in "Anne". Whereas a non-functional enzyme will not consume lycopene and by this not change the red color to the cultures like the precursor line. Accumulation of *β*-carotene in "Anne" further supports the active role of this gene (Carvalho et al., 2013b). *In planta*, accumulation of lycopene in cassava pink roots has been reported due to proposed mutations in the *Lcy-b* gene

(Carvalho et al., 2016). In contrast to it, conversion of lycopene to *β*-carotene in "Anne" supports the active and functional role of this gene (Carvalho et al., 2013b). Moreover, CPTA (10.5 µM) completely inhibited the RiLCY-b activity and inhibition of LCY-b from tomato has been reported at 13.5 µM in a similar type of cultures (Ronen et al., 1999).

Down at the two-branch step, two hydroxylases (CHY-e, CHY-b) catalyze the introduction of hydroxyl groups onto the ionone rings resulting in the formation of lutein and other xanthophylls as end products through cyclic *α*-carotene and *β*-carotene, respectively (Figure 37). In the present study, the protein sequence predicted from *RiChy-e* amplicon contains all three conserved elements, i.e. threonine (T, Thr), cytochrome P450 (CYP) pocket, cysteine (C; Cys) motif, essential for its activity, molecular oxygen binding and nitrogen remobilization, respectively. It also contains a predicted transmembrane domain and a cleavage site of the putative chloroplast-targeting sequence as described in *Arabidopsis* (Tian et al., 2004). It is presented in Annex XVI, that the CHY-e sequence is similar to the sequence derived from the *Rubus* draft genome and showed a high level of identity to the protein sequences of other Rosaceae family members but also other plant species. Phylogenetic tree constructed using other known sequences show that RiCHY-e is highly identical to *Fragaria* (90%; XP_004306170), *Prunus* (87%; XP_007204192), *Malus* (85%; XP_008392557) and *Pyrus* (85%; XP_009375781). It also presents high identity to other plant species, such as 81% to *Arachis* (XP_015951517), 79% to *Vitis* (XP_002265015) and 78% to *Cucumis* (XP_008462512) as shown in the phylogenetic tree (Annex XVI). Translation of *RiChy-b* gene predicted a 300 aa polypeptide containing a highly conserved domain (HDGLVHKRFP) which is required for its activity as described by Linden (1999). The protein sequence presented 99% identity to the *Rubus* draft. It also shows high identity to the other published sequences, such as 75% to *Capsicum* (CAA70888; Bouvier et al., 1998), 74% to *Coffea* (ABA43903; Simkin et al., 2008), 68% to the *Arabidopsis* (AAC49443; Sun et al., 1996), *Adonis* (ABI93208; Cunningham and Gantt, 2007) and *Diospyros* (ACN86365; Zhao et al., 2010) as shown in the phylogenetic analysis (Annex XV). The accumulation of lutein in "Anne" fruits is in agreement to the previous findings regarding the activity of RiCHY-e as it provides the precursor (*δ*-carotene) for the synthesis of lutein as an end product in this branch (Carvalho et al., 2013b). As following associated CHY-b enzyme catalyzes the formation of lutein xanthophyll in one branch and other xanthophylls in *β*-branch. However, previous findings propose that inactive or downregulated *Chy-b* gene enhance the accumulation of upstream yellow *β*-carotene owing the yellow pigmentation in cassava varieties (Carvalho et al., 2016). Similarly, *Chy-b* silencing

also led to increase in the synthesis of *β*-carotene in potato tubers (Diretto et al., 2007). In contrast, together with sequence and transcriptional analysis, the presence of *β*-carotene and two-branch xanthophylls during fruit development in both, yellow and red fruiting raspberries (Carvalho et al. 2013b), indicates the functional role of *RiChy-b* gene in the pathway. On the basis of these findings within the current study regarding transcriptional, molecular and functional regulation of carotenoid pathway genes and together with previous published carotenoid profiles, a general pathway map has been created for the synthesis of various carotenoids in raspberry fruits (as shown in Figure 37).

The *Ccds* play key roles in determining the accumulation of carotenoids, composition of volatiles and final fruit pigmentation. Carotenoids can be cleaved via the activity of *Ccds* into smaller units to give rise to various carotenoid derived volatile molecules known as ap-carotenoids. Apo-carotenoids, especially *β*-ionones, contribute to the aroma in fruits and flowers, for example, apo-carotenoids significantly increase in nectarine and melon fruits during ripening and contribute to the aroma by *Ccd* activities (Aubert et al., 2003; Ibdah et al., 2006). However, *ccd* mutants of tomato fruits and petunia flowers have been reported with reduced contents of apo-carotenoids (Simkin et al., 2004a,b). Similarly, *ccd* mutants or downregulation of *Ccds* determine the accumulation of carotenoids and pigmentation in peach fruits, potato tubers and chrysanthemum flowers (Ohmiya et al., 2006; Campbell et al., 2010; Brandi et al., 2011). Among CCD1 and CCD4 dioxygenases, the conserved residues are four histidines (H), along with two glutamates (E) and an aspartate (D) giving stability to the complex. However, RiCCD1.1 sequence contained two of the four conserved iron-ligating H residues and one of the two known conserved glutamates (E). The two conserved histidines and one glutamate residues are missing towards the N-terminus which may affect the protein activity and stability. Comparative sequence analysis of RiCCD1.1 between “Anne” and “Heritage” showed 100% identity. Phylogenetic tree of deduced amino acids of RiCCD1.1 clustered with CCD1 of Rosaceae family and other plant members in comparison to CCD4s, CCD7s, CCD8s and NCEDs (Annex XVII). It shows high identity to the other CCD1s, such as 94% to *Rosa* CCD1 (ABY47994; Huang et al., 2009), 90% to *Cucumis* CCD1 (ABB82946; Ibdah et al., 2006), 87% to *Coffea* CCD1 (ABA43904; Simkin et al., 2008) and 83% to *Vitis* CCD1 (AGT63320; Lashbrooke et al., 2013). However, the similarity of RiCCD1.1 sequence to the CCD4s, CCD7s, CCD8s and NCEDs sequences are less than 50% as shown in Annex XVII.

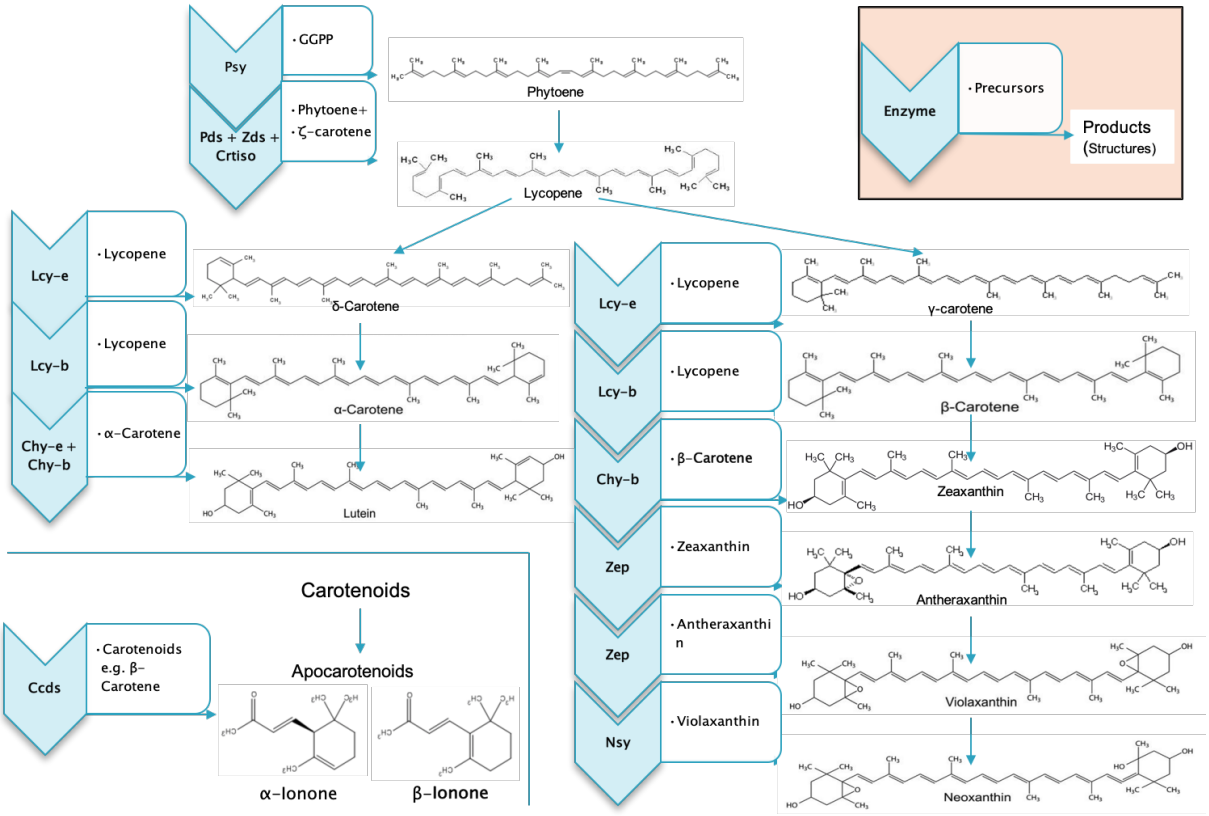


Figure 37: Pathway map of biosynthesis of carotenoids in raspberry; *Ggps*, geranylgeranyl diphosphate synthase; *Psy*, phytoene synthase; *Pds*, phytoene desaturase; *Lcy-b*, lycopene *β*-ring cyclase; *Lcy-e*, lycopene *ε*-ring cyclase; *Chy-b*, carotenoid *β*-ring 3-hydroxylase; *Chy-e*, carotenoid *ε*-ring 3-hydroxylase; *Zep*, zeaxanthin epoxidase; and *Nsy*, neoxanthin synthase; *Ccd*, carotenoid cleavage dioxygenases.

In the present study, overexpression of *RiCcd1.1* in *E. coli* containing pACCRT-EIB plasmid (producing lycopene) degraded the lycopene in to smaller derivatives as evident from clearly reduced color (Figure 34; page 62). The amount of lycopene after complementation is 5-6 times reduced compared to that produced by pACCRT-EIB (without *RiCcd1.1*) most probably due to the formation of apo-carotenoids. Similar reports are available indicating CmCCD1 activity to degrade the lycopene by 50%, produced by pBCAR-EIB causing color loss of red cultures forming colorless apo-carotenoids (Ibdah et al., 2006). CCD4 enzymes targeting plastid based carotenoids have been described to play an active role in determining the phenotypic character in some fruits rather than cytosolic CCD1 and NCED (Rubio et al., 2008, Gómez-Gómez and Moraga-Rubio, 2010). Not only *Ccd4* gain of function mutation and increased transcript abundance but also loss of function mutation and reduced expression by the NMD mechanism has been reported to affect the phenotype of peach fruits (Brandi et al., 2011; Adami et al., 2013; Falchi et al., 2013). However, other *Ccds* does not seem to be present in raspberry, thus

it is suggested that *Ccd1* degrade carotenoids, i.e. *β*-carotene, more specifically to produce apo-carotenoids. However, accumulation of *β*-carotene resulted most probably from the *de novo* synthesis in ripe raspberry fruits as described by Beekwilder et al., (2008). Thus, most important *β*-carotene and xanthophylls, especially, lutein are considered to accumulate and responsible for yellow fruit pigmentation in ripe raspberry fruits.

5. SUMMARY AND PROSPECTS

Pigmentation is an important and complex fruit quality trait that may be controlled not just by one gene but rather, by multiple genes. Among various colored raspberries, anthocyanins and carotenoids are responsible for giving color to the fruits. It was found that various colored raspberries contain different concentrations of phenolic metabolites, however, a block of a certain class of compounds was not observed except for anthocyanins. Thus, the study of anthocyanin and carotenoid pathways can elucidate the role of certain pigments involved in raspberry fruit colors. Within the anthocyanin pathway, a 5 bp insertion (ans^{+5}) was identified in the *Ans* gene of the yellow variety "Anne", as compared to different red fruiting varieties ("Tulameen", "Heritage", "Autumn Bliss" and "Sugana Red") of raspberry. Deletion or insertion of a few bases that is not a multiple of 3, can introduce pre-mature STOP codons in addition to a significant change in amino acid composition ultimately causing loss of function of a protein sequence. It is therefore concluded, that the ans^{+5} (*TGGCC*) in "Anne" generated a truncated polypeptide lacking the consensus motifs for co-factor and substrate binding, posing a complete loss-of-function in this *ans* mutant. The function of the ANS protein was shown from "Tulameen" that imparts red fruiting color but non-functional ANS protein in yellow fruiting raspberry "Anne". A block in a step of the anthocyanin pathway at ANS level is reported herein resulting in yellow fruit phenotype as indicated in the proposed pathway (Figure 35; page 69). It also supports the phenomenon that such nonsense mutations can abolish splicing resulting by the NMD mechanism. Furthermore, in the current study, various molecular markers, such as a probe, HRM and CAPS were developed for screening other red, yellow and orange varieties of raspberry for identification of "Anne" like mutations inside *Ans* genes. For example, a CAPS marker was efficiently utilized in successfully screening of not only yellow fruiting "Citria", "Giallo Mutant", and "Alpen Gold" varieties but also orange fruiting "Valentina" variety for ans^{+5} mutation in heterozygous and homozygous states, respectively. Heterozygosity for the *Ans* gene in red fruiting "Glen Garry" was also found. This homozygous/heterozygous mutation nature suggested that the presence of other mutations in the pathway cannot be excluded. Hence, upon molecular analysis of many yellow and red fruiting varieties indicated various *Rubus Ans* mutation types (RAMT-1 to RAMT-6) which produce short/truncated proteins in yellow and orange fruiting varieties. Therefore, it is suggested that these raspberry genotypes might also have originated from a mutation event in the *Ans* gene from different ancestors.

The anthocyanin pathway mutants have led to the loss of anthocyanins and generated yellow and possibly also orange pigmented raspberry fruits. In case of reduced anthocyanins, carotenoids are considered to be the main pigments accounting for the fruit color in raspberries. Differential expression patterns or mutation events in the carotenoid pathways may determine the accumulation of different carotenoids, hence, pigmentation in the raspberry fruits. The expression analysis of carotenoid pathway genes in "Anne" suggested an adequate biosynthesis of carotenoids. Moreover, molecular analysis of important genes, such as *Psy*, *Lcy-e*, *Lcy-b*, *Chy-e*, *Chy-b*, *Ccd1* did not indicate the presence of mutation events at least in "Anne". Moreover, functional characterization of important carotenoid pathway genes indicates the accumulation of *β*-branch carotenoids like *β*-carotene and xanthophylls as principal components at different fruit stages of raspberry. Here, it is suggested that the functional lycopene *β*-cyclase efficiently converts cyclic lycopene to *β*-carotene that in part give yellow color to yellow fruit raspberries along with lutein and other carotenoids. However, other colored fruits like orange raspberries might also suggest the differential expression or existence of mutations in the carotenoid pathway genes in addition to anthocyanins in these varieties. Therefore, carotenoid pathway blocks or quantitative differences of carotenoids might indicate the involvement of genetic components of the pathway in originating the orange pigmentation of raspberry fruits with reduced anthocyanins in addition to yellow ones. Based on the results of the current study, a preliminary scheme has been made which can further be exploited for the biotechnological production of specific carotenoids and aroma compounds. Furthermore, carotenoids play a key role in fruit pigmentation and act as precursors for flavoring compounds during fruit ripening in raspberries. Thus, it will be interesting to assess the carotenoid composition and molecular analysis of the pathway genes not only to improve the understanding of the fruit pigmentation but also their possible link to the other fruit quality traits in raspberry color mutants. Taken together, the data can further be utilized in breeding programs for indirect selection of genetic determinants of a trait of interest.

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7. ANNEXES

Annex I: Background of *Rubus* varieties under study

Sr. No.	Variety	Code	Location	Color	Lineage
1	Anne	AN	TN	Yellow	Amity x Glen Garry
2	Golden Queen	GQ	TN	Yellow	Sport of Cuthbert (occidentalis)
3	Citria	CT	TN	Yellow	Cayuga x cross seedling
4	All Gold	AG	TN	Yellow	Yellow sport of Autumn Bliss
5	Gelbe Antwerpener	GA	DN	Yellow	Unknown
6	Gelbe Siebenkugel	GSi	DN	Yellow	Unknown
7	Gelbe Sugana	GSu	DN	Yellow	Unknown
8	Alpen Gold	Alp	VN	Yellow	Polka × Tulameen
9	Giallo Mutant	GM	---	Yellow	Unknown
10	Fall Gold	FG	TN	Yellow	NH-R7 x (Taylor x R. Pungens var. Oldhamii)
11	Juan de Metz	Jdm	---	Yellow	Unknown
12	Sugana Gold	SG	TN	Yellow	Unknown
13	Golden Everest	GE	DN	Yellow	Unknown
14	Lumina	Lum	DN	Yellow	Unknown
15	Him13K39-8	Him	DN	Yellow	Seedling of Zheltyi Gigant open pollinated
16	Herbert Gold	HG	MT	Yellow	Unknown, may be yellow sport/mutant of Herbert cv.
17	Valentina	Val	DN	Orange	Unknown
17	Orange Marie	OM	DN	Orange	Unknown
18	Amity	AT	TN	Red	ORUS 1835 x ORUS 1837
19	Glen Garry	GG	TN	Red	Malling Delight x SCRI 7331/1
20	Tulameen	TL	TN	Red	Nootka x Glen Prosen
21	Nootka	NK	TN	Red	Carnival x Willamette
22	Glen Prosen	GP	TN	Red	SCRI 6531/84 x SCRI 6549/1
23	Heritage	Her	TN	Red	(Milton × Cuthbert) × Durham
24	Autumn Bliss	AB	TN	Red	Complex cross of Lloyd George, R. idaeus strigosus, R. arcticus and R. occidentalis
25	Sugana Red	SR	TN	Red	Autumn Bliss × Tulameen
26	Meeker	MK	TN	Red	Willamette x Cuthbert
27	Pocahontas	PH	TN	Red	Hilton x (Taylor x St. Regis)
28	Himbo Top	HT	TN	Red	Autumn Bliss x Himbo Queen
29	Tayberry	TB	DN	Purple	R. fruticosus x R. idaeus (Aurora x SCRI)
30	Buckingham Tayberry	BT	DN	Purple	Red raspberry x Blackberry (some refer as sport of Tayberry)
31	Black Jewel	BJ	DN	Black	(Bristol x Dundee) x Dundee

*DN: Julius Kuehn-Institute, Federal Research Centre for Cultivated Plants, Institute for Breeding Research on Horticultural and Fruit Crops Dresden.
*MT: ENEA - Trisaia Research Centre S.S. 106, Km 419+500 I-75026 Rotondella (MT) Italy.
*TN: Fields of Vigalzano Trentino (TN), Italy
*VN: Fields of company Berry Plant Verona (VN), Italy

Annex II: Primers designed for expression analysis and cloning of flavonoid and carotenoid pathway genes, copy number analysis, CAPS analysis and Arabidopsis complementation in *Rubus*

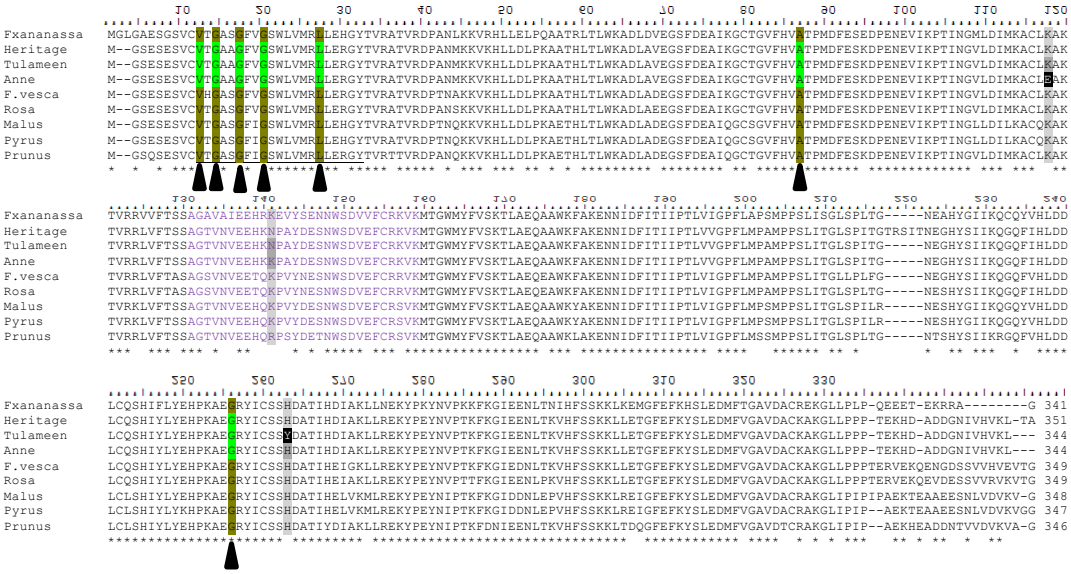
Forward primer	Primer sequence	Reverse primer	Primer sequence
Primers for expression analysis of flavonoid pathway genes			
MYB10-F	GTATTCATTCACACCTGTAG	MYB10-R	ATGCCAAAGATCCATTTCAAA
Chs-F	CCGACTACTACTTTCGTATCACCA	Chs-R	ACTACCACCATGTCTTGTCTTGC
Fht-F	GTGCGCCACCGTGACTACTC	Fht-R	ATGCCTTTGTCAATGCCTCC
Dfr-F	GGGTGGTGTTTACATCTTCGG	Dfr-R	CTGCTTGCTCGGCTAGAGTTT
Ans-F	ATCGTCATGCACATAGGCGACACC	Ans-R	CCTTGGGCGGCTCAGAGAAAA
Ufgt-F	ATCGTGGCTTGACAAACAGAA	Ufgt-R	TGACCACAAGAATGGAACCCTA
Fls-F	TTATCTTTTGGGTTAGGGCTTGAA	Fls-R	GAGAATGGTGAGGGCGGACA
Lar-F	GGTGATGGCACGGTTAAAGC	Lar-R	CTCCCACAGTGAAGCAAGTCC
Anr-F	CATCCAAGGCGAAGACCAT	Anr-R	TCATACTTAAACAACTGAGACCACC
Adh-F	TTGTGGAGAATACATGAACAAGG	Adh-R	GAAACTGATCTAATGCTCCATGC

Primers for expression analysis of carotenoid pathway genes			
RiGgps-F	CCGACCCATTTCCTTCGTTTC	RiGgps-R	TTCTTCTTGCTGTTGCTCATCAA
RiPsy-F	GCTGGAACTGTTGGCCTAATGA	RiPsy-R	CCAATACCCAAGTAGAGTGCAGCAT
RiPds-F	TGTGTCTCTGCCACCAACTTGAA	RiPds-R	CCTTGTGAGCCCAGAAATCTCA
RiLcy-b-F	AAGCAGCTCAAGTCCAAAATG	RiLcy-b-R	GCGATTTTTCGTCCTCATGA
RiLcy-e-F	CGCGGATGAGGAAGATTACGT	RiLcy-e-R	GTCAGCGAGCTTGGACTGCTT
RiChy-b-F	AGCCAAGTACCCACGTCGATAAG	RiChy-b-R	TCGGATCTCTTCTTGGCCAATC
RiChy-e-F	GTGCTCTGTTCCTGCCCCTAT	RiChy-e-R	TGCGAGCCGGTAAATTGG
RiZep-F	AGGTGGTAACAGCTCAAAACTTGAAG	RiZep-R	CCAGTGCATCATCGTCCTCAAA
RiNsy-F	GACGCACACAGTTAGTGCTCTCCTA	RiNsy-R	AAGAGTAGTGGTTGCTTCTTCATTCG
RiCcd1.1-F	GGTGAAGGAAGGCAAGCTGATATT	RiCcd1.1-R	CTCAAACCATCTGATTAGCAACTCATC
RiCcd1.2-F	TGGAACCACTCTGGATAGCA	RiCcd1.2-R	TCCTCCAACCTCAATTTTCG
RiCcd1.3-F	TTGGTGGCATTGACACAGTT	RiCcd1.3-R	CCGTATTTCCACTCGTGTTG
Actin-F	TTCGTGTTGCCCCAGAAGAGCAC	Actin-R	ACCAGTTGTACGGCCACTTGCAT
Pap2-F	GCGTGAGGGACAAAGCTGTGGAG	Pap2-R	ACACGCAGAGACTCGGGCTGTAA
Sand-F	GTGGGATGCGTGTCGAGGAGTTG	Sand-R	AAATGCCAAAGCCCAGCAGGACC
Primers for copy number analysis, CAPS and Arabidopsis complementation			
Basta-F	CCATCGTCAACCACTACATCGAGAC	Basta-R	AAACCCACGTCATGCCAGTTC
RubUni-F	TGGAGAAGGAGGTCGGTGG	RubUni-R	GGGAACCATGTTGTGGAGGAT
FAM-probe	CTGCACTCACCTGGC		
HRM-F	TGGAGAAGGAGGTCGGTGG	HRM-R	GGGAACCATGTTGTGGAGGAT
CAPS-F	CCTGGGATTAGAAGAAGGGAGGC	CAPS-R	TCCCTACCTGCAGCTCTTCTACGAAG
Primers for cloning of flavonoid pathway genes in *Rubus*			
Ans-utr-F	ATGCTCATTAAAGCATAACAAAGGCCC	Ans-utr-R	TTAAACGGCTCCATTAATTAAGCAGCA
Ans-orf-F	ATGGTGACTGCTGCATCC	Ans-orf-R1	GCAGCATCTTATGTAGAGATGAGAGC
Ans-orf-F	ATGGTGACTGCTGCATCC	Ans-orf-R2	CGTCACCCATTTGTCTTCGTAGAA
Dfr-orf-F	ATGGGATCGGAGTCCGAATCC	Dfr-orf-R	TTAGGCGGTGAGTTTGACATGGACG
Primers for cloning of carotenoid pathway genes in *Rubus*			
RiPsy-orf-F	ATGAGTTCTACATTTTCACTTGCACC	RiPsy-orf-R	TCAAAGAAGGGTATATACCG
RiLcy-b-orf-F	ATGGATACATTACTCAAAACACATAACAAGC	RiLcy-b-orf-R	CTAATCTCTATCCTGTATCAAGTTGTTGATC
RiLcy-e-orf-F	ATGGACTGCGTTGGACTCG	RiLcy-e-orf-R	TTAAGGGAAGGAATTCAAGTTAACAGTG
RiChy-b-orf-F	ATGGCGGTCGCCTCCA	RiChy-b-orf-R	CTATGAGTTCTTGGATGCTTTGATTCTCC
RiChy-e-orf-F	ATGCCTTGCTCTCTCCACTCCT	RiChy-e-orf-R	TTAGATAGACGAAGCAGCAAACG
RiCcd1.1-orf-F	ATGGTGAAGGAAGGCAAGCTG	RiCcd1.1-orf-R	TTAGAGCCTTGCTTGTTCTTGC

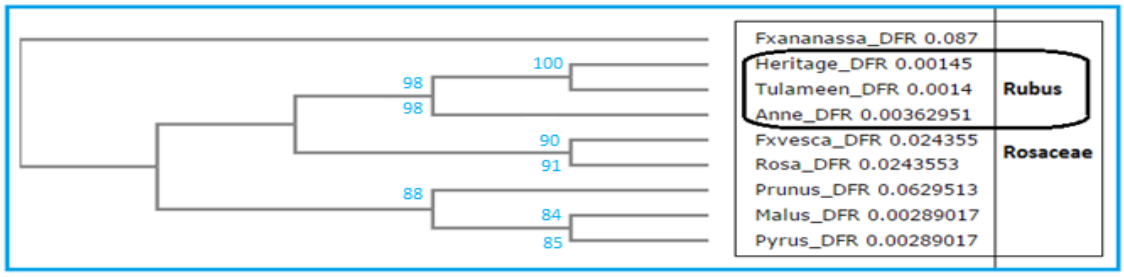
Annex III: Identification of flavonoid and proanthocyanidin pathway genes in *Rubus* and GenBank Accessions (GB Acc.) of the cloned genes

Query	Template GB Acc. No.	Enzyme encoded	*Rubus* gene	*Rubus* GB Acc. No.
Flavonoid pathway genes in *Rubus* and GenBank Accessions				
Fragaria	EU155162	Transcription factor	RiMYB10	---
Fragaria	AY997297	Chalcone synthase	RiChs	---
Fragaria	AY691919	Flavanone 3*β*-hydroxylase	RiFht	---
Fragaria	AY695812	Dihydroflavonol 4-reductase	RiDfr (AN)	MF850337
			RiDfr (TL)	MF850338
Fragaria	AY695817	Anthocyanidin synthase	RiAns (AN	KX950788
			RiAns (TL)	KX950789
Fragaria	AY575056	Flavonoid-3-*O*-glucosyltransferase	RiUfgt	---
Fragaria	DQ087252	Flavonol synthase	RiFls	---
Proanthocyanidin pathway genes in *Rubus*				
Fragaria	DQ087253	Leucoanthocyanidin reductase	RiLar	---
Fragaria	DQ664193	Anthocyanidin reductase	RiAnr	---
Vitis	AJ865335	Leucoanthocyanidin reductase	VtLar	---

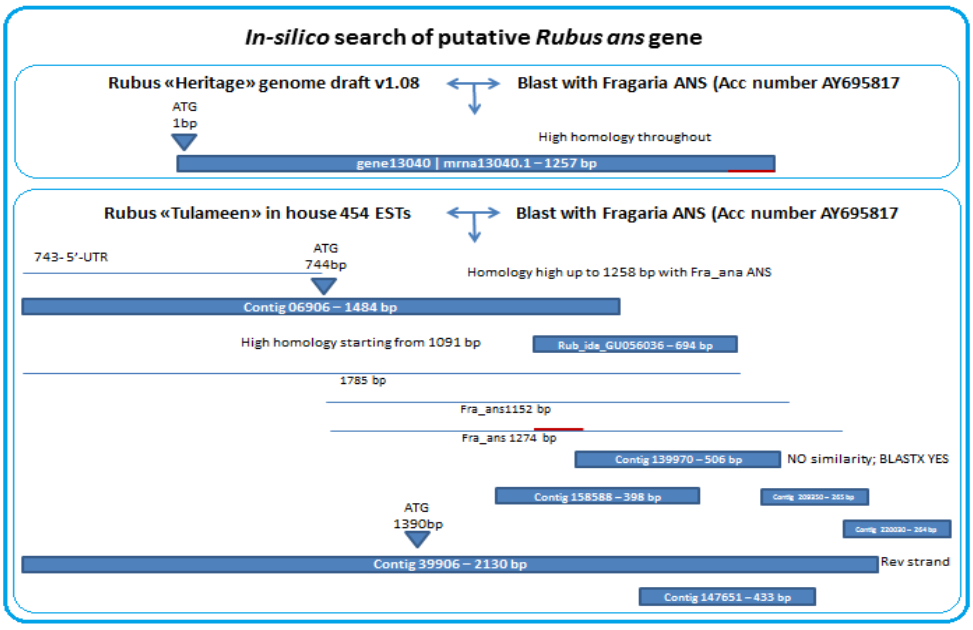
Annex IV: Multiple alignment of deduced amino acid sequences of dihydroflavonol 4-reductase gene involved in reduction of dihydroflavonols to leucoanthocyanidins in anthocyanin biosynthesis from *Rubus* ("Anne", "Tulameen" and "Heritage") with the known related nucleotide sequences from Rosaceae species, such as *F.* × *ananassa* (AY695812); *F. vesca* (NM_001305268); *Rosa* (KM203111); *Malus* (NM_001293939); *Pyrus* (KC460395) and *Prunus* (JF740093). The amino acid residues strictly conserved in the mammalian 3*β*-hydroxysteroid dehydrogenase/DFR superfamily (Baker and Blasco 1992, Lacombe et al. 1997) are marked with green color sequences shown by black triangles. The underlined region is a putative NAD(P) binding domain (Lacombe et al. 1997) in the N-terminal of sequence. The region predicted to be related to substrate specificity (Johnson et al. 1999, Johnson et al. 2001) is marked with violet color. Important SAAPs are shaded in black with white letters in "Anne" and "Tulameen", while similar SAAPs in other Rosaceae member are presented as grey shades.



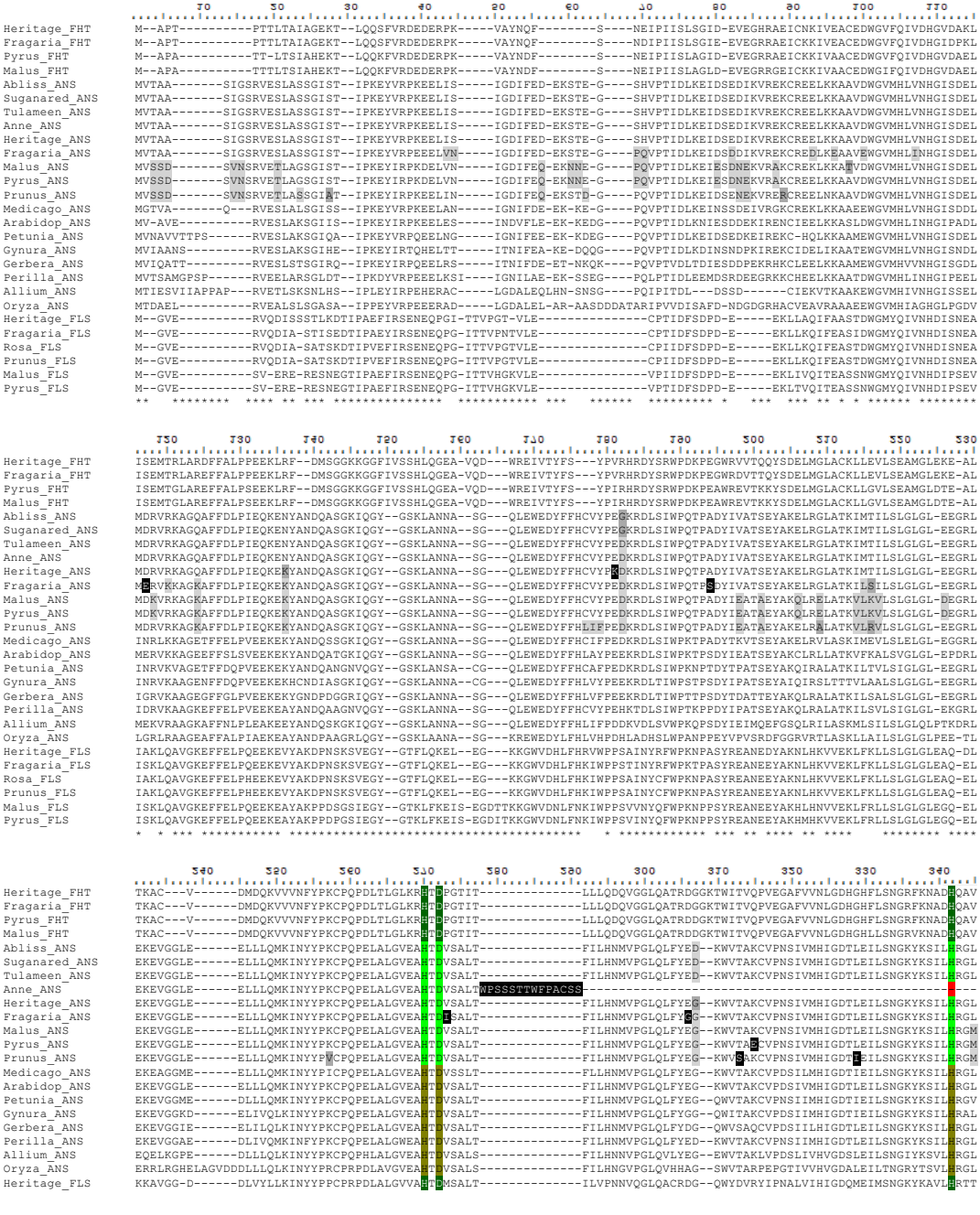
Annex V: Phylogenetic tree of the deduced amino acid sequences of RiDFR from *Rubus* and other members of Rosaceae family clustered according to UPGMA. All DFR sequences are presented in percentage value from *Rubus* genome draft "Heritage". DFR sequences from *Rubus* are boxed.



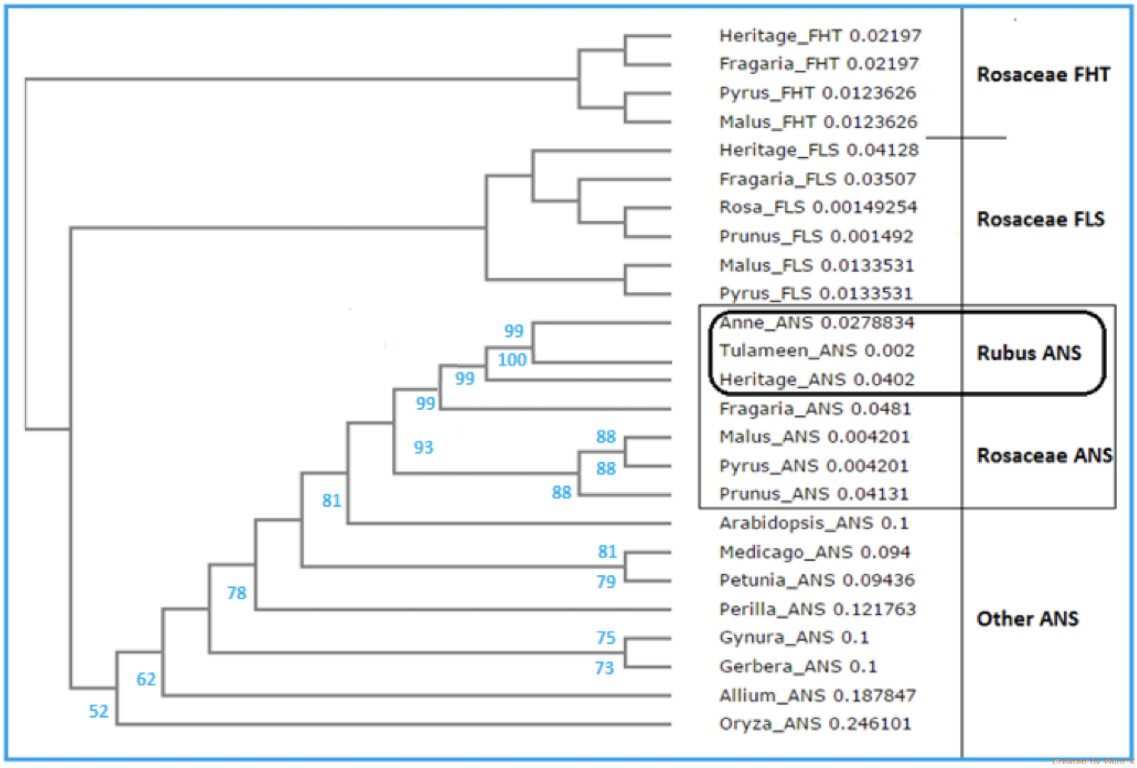
Annex VI: *In-silico* search of putative *Rubus Ans* gene by exploiting the *Rubus* ("Heritage") genome draft and in house EST library of fruit stages of "Tulameen" made by 454 sequencing using *Fragaria Ans* (GenBank Accession Number AY695817) as query.



Annex VII: Multiple alignments of deduced amino acid sequences of 2-ODDs from *Rubus* (“Anne”, “Tulameen”, “Heritage”, “Autumn Bliss”, “Sugana Red”) with the known related nucleotide sequences from other Rosaceae members and several other plant species. Shown are the sequences of anthocyanidin synthase (ANS) from Rosaceae members *Fragaria* (AY695817); *Malus* (AB074487); *Pyrus* (DQ230994); *Prunus* (KF974776), other plant species *Medicago* (ABU40983); *Arabidopsis* (U70478); *Petunia* (X70786); *Gynura* (AB550241); *Gerbera* (HB755946); *Perilla* (AB003779); *Allium* (EF192475); *Oryza* (Y07955), flavonol synthase (FLS) from “Heritage” (*Rubus* draft v1.08: gene 04708); *Fragaria* (DQ087252); *Rosa* (KM099095); *Prunus* (KP050782); *Malus* (AY965343); *Pyrus* (DQ230993) and flavanone 3*β*-hydroxylase (FHT) from “Heritage” (*Rubus* draft v1.08: gene 03814); *Fragaria* (AY691919); *Pyrus* (AY965342) and *Malus* (AY965339). Different green color shades shown by black triangles indicate the conserved His (H) and Asp (D) residues required for ferrous-iron coordination, Arg (R) and Ser (S) residues in black circles for binding site of 2-oxoglutarate, Phe (F) residue in black arrow-heads for substrate binding and dashes indicate gaps in the sequences. Group of SAAPs between *Rubus* and other Rosaceae members are indicated by black letters in grey shades; however, SAAPs among all Rosaceae species are indicated by white letters in black shades. Asterisks show the identical amino acids among all Rosaceae family members.

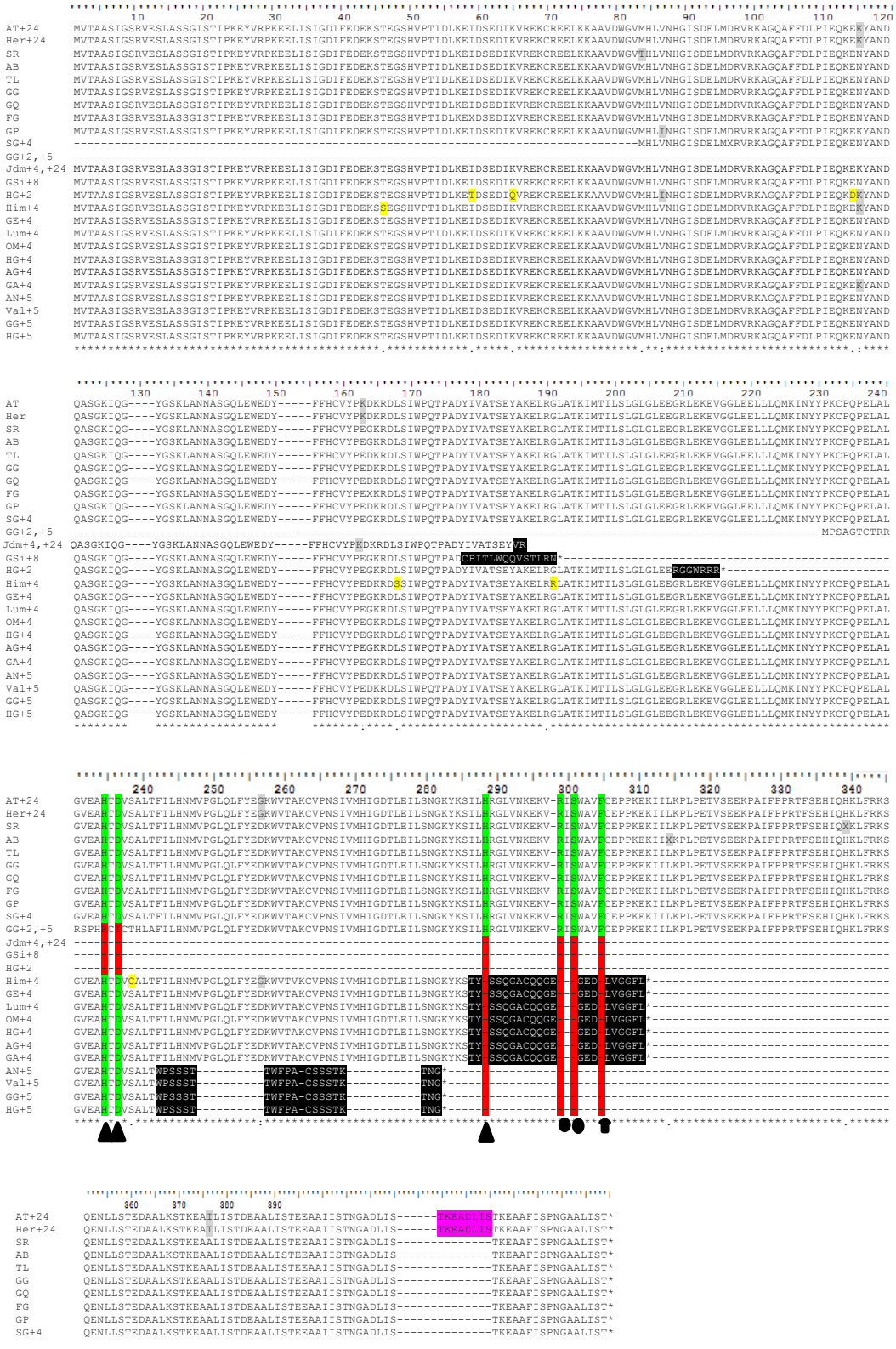


Annex VIII: Phylogenetic tree of the deduced amino acid sequences of ANS from *Rubus idaeus* and other related 2-ODDs from several plant species clustered according to UPGMA. *Rubus* ANS clustered together with ANS from Rosaceae and other plant families and separated from other flavonoid pathway 2-ODDs (FLS & FHT). ANS sequences of *Rubus* and other Rosaceae species are boxed and presented in percentage value from "Tulameen".



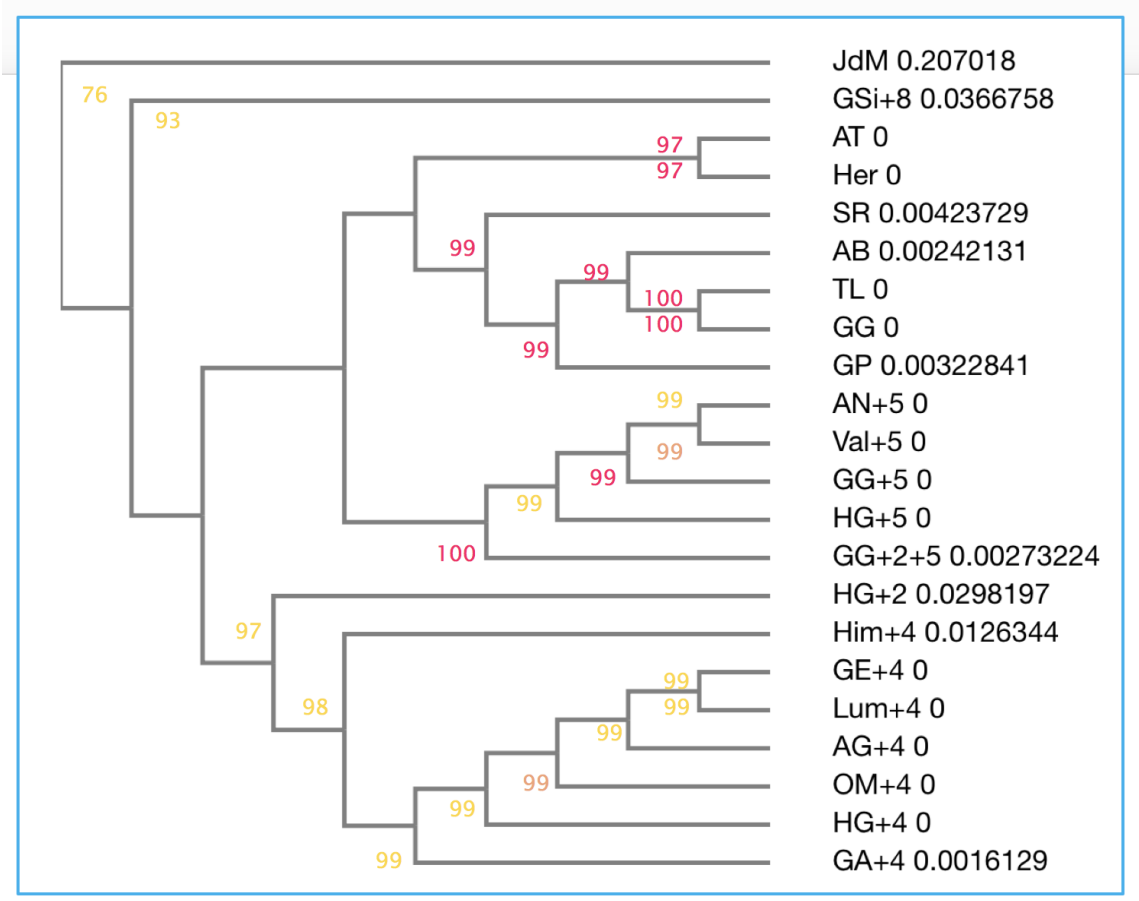
Annex IX: Multiple alignments of deduced amino acid sequences of ANS from different *Rubus* varieties. Shown are the sequences of ANS from yellow "Golden Everest" (GE), "All Gold" (AG), "Gelbe Antwerpener" (GA),"Lumina" (Lum), "Him13K39-8" (Him), "Herbert Gold" (HG), "Gelbe Siebenkugel" (GSi), "Golden Queen" (GQ),, orange "Valentina" (Val), "Orange Marie" (OM) and red

“Glen Garry” (GG), “Amity” (AT), “Glen Prosen” (GP), “Heritage” (Her), “Autumn Bliss” (AB), “Sugana Red” (SR) varieties. Different green color shades shown by black triangles indicate the conserved His (H) and Asp (D) residues required for ferrous-iron coordination, Arg (R) and Ser (S) residues in black circles for binding site of 2-oxoglutarate, Phe (F) residue in black arrow-heads for substrate binding and broken lines indicate gaps in the sequences. Group of SAAPs in yellow/orange varieties different from red ones are indicated yellow letters, while grey letters represent common SAAPs among all varieties. White letters in black shades indicate frameshift in protein sequence caused by mutations. Asterisks show the consensus for identical amino acids among all *Rubus* varieties.



```
GG+2,+5    QENLLSTEDAALKSTKEAALISTDEAALISTEEAAIISTNGADLIS---------------TKEAAFISPNGAALIST*
Jdm+4,+24  QENLLSTEDAALKSTKEATLISTDEAALISTEEAAIISTNGADLIS------TKEADLISTKEAAFISPNGAALIST*
GSi+8      ------------------------------------------------------------------------------
HG+2       ------------------------------------------------------------------------------
Him+4      ------------------------------------------------------------------------------
GE+4       ------------------------------------------------------------------------------
Lum+4      ------------------------------------------------------------------------------
OM+4       ------------------------------------------------------------------------------
HG+4       ------------------------------------------------------------------------------
AG+4       ------------------------------------------------------------------------------
GA+4       ------------------------------------------------------------------------------
AN+5       ------------------------------------------------------------------------------
Val+5      ------------------------------------------------------------------------------
GG+5       ------------------------------------------------------------------------------
HG+5       ------------------------------------------------------------------------------
                                                                                          
```

Annex X: Molecular phylogenetic tree of the deduced amino acid sequences of ANS from *Rubus idaeus* from yellow, orange and red varieties clustered according to UPGMA. ANS sequences of yellow, orange and red varieties presented in percentage value from "Tulameen" according to their fruiting color.

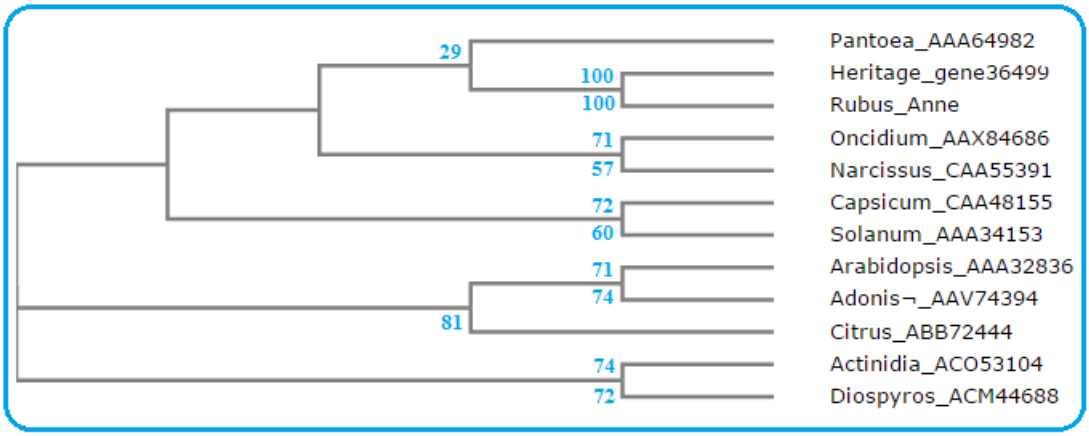


Annex XI: Identification of carotenoid pathway genes in *Rubus* and GenBank Accessions of cloned genes

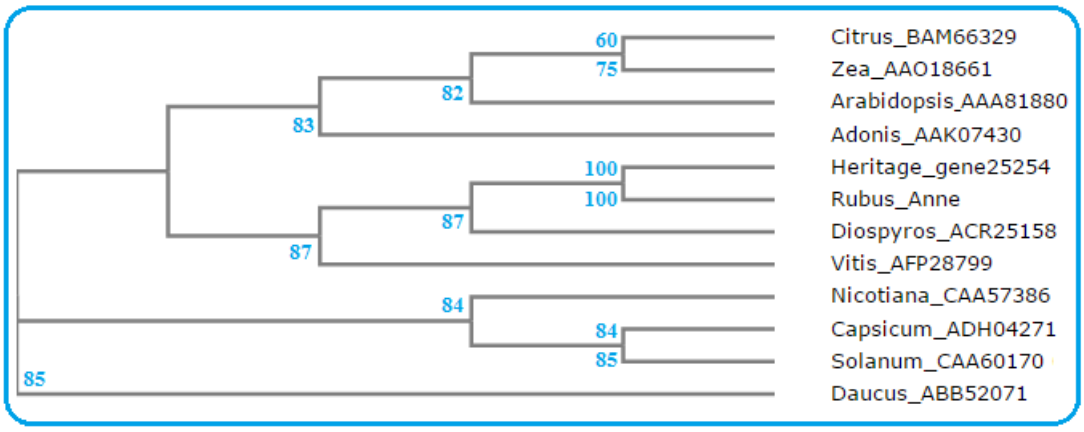
Template	**Template GB Acc No**	***Rubus* draft**	**Enzyme encoded**	***Rubus* (Anne)**	***Rubus* GB Acc No**
Fragaria	XM_004300550	gene27828	Geranylgeranyl diphosphate synthase	RiGgps1	---
Fragaria	XM_004296142	gene36499	Phytoene synthase	RiPsy	MF850339
Fragaria	XM_004296916	gene16633	Phytoene desaturase	RiPds	---
Fragaria	XM_004303559	gene25254	Lycopene *β*-ring cyclase	RiLcy-b	MF850340
Fragaria	XM_004287534	gene26936	Lycopene *ε*-ring cyclase	RiLcy-e	MF850341
Fragaria	XM_004308006	gene00755	Carotenoid *β*-ring 3-hydroxylase	RiChy-b	MF850342
Pyrus	XM_009377507	gene20154	Carotenoid *ε*-ring 3-hydroxylase	RiChy-e	MF850343
Malus	XM_008340094	gene30561	Zeaxanthin epoxidase	RiZep	---
Malus	JN941557	gene05983	Neoxanthin synthase	RiNsy	---

Malus	EU871633	gene25674	Carotenoid cleavage dioxygenases	RiCcd1.1	MF850344
Malus	EU871633	gene22899	Carotenoid cleavage dioxygenases	RiCcd1.2	---
Malus	EU871633	gene08372	Carotenoid cleavage dioxygenases	RiCcd1.3	---

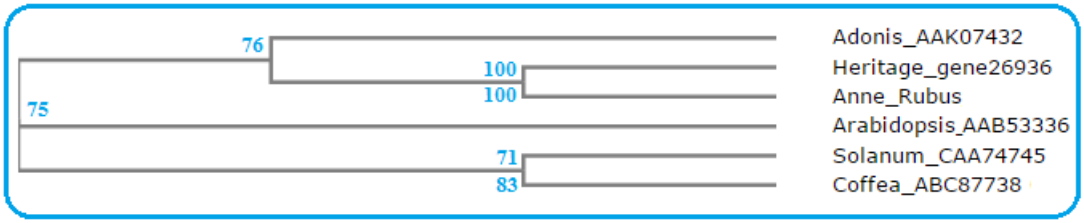
Annex XII: Molecular phylogenetic tree of the deduced amino acid sequences of PSY from *Rubus* and some other members clustered according to neighbor-joining method. All sequences are presented as percentage value from *Rubus* genome draft "Heritage".



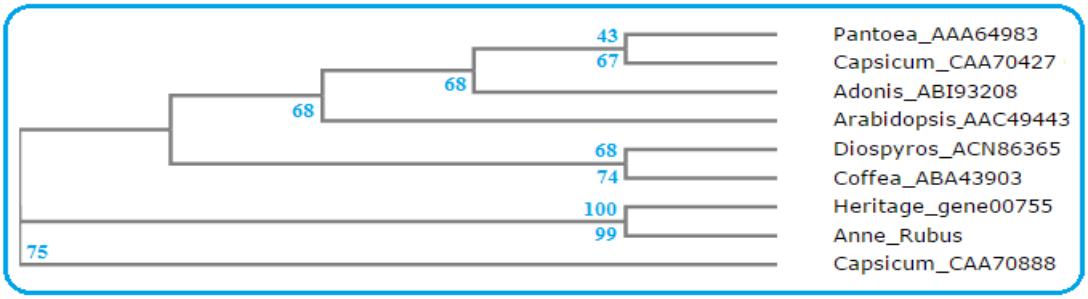
Annex XIII: Molecular phylogenetic tree of the deduced amino acid sequences of LCY-b from *Rubus* and some other members clustered according to neighbor-joining method. All sequences are presented as percentage value from *Rubus* genome draft "Heritage".



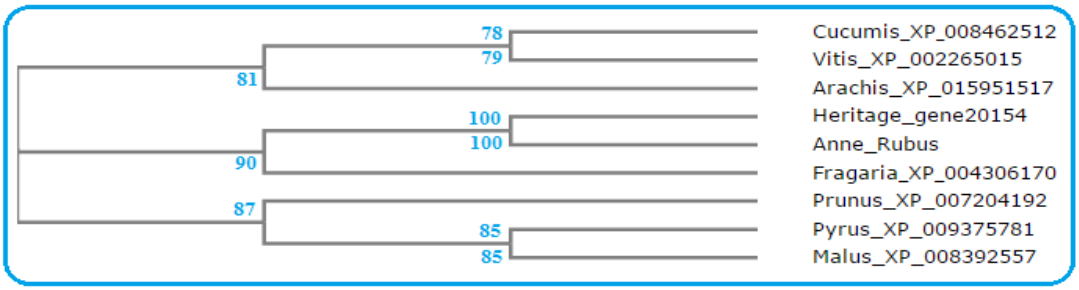
Annex XIV: Molecular phylogenetic tree of the deduced amino acid sequences of LCY-e from *Rubus* and some other clustered according to neighbor-joining method. All sequences are presented as percentage value from *Rubus* genome draft "Heritage".



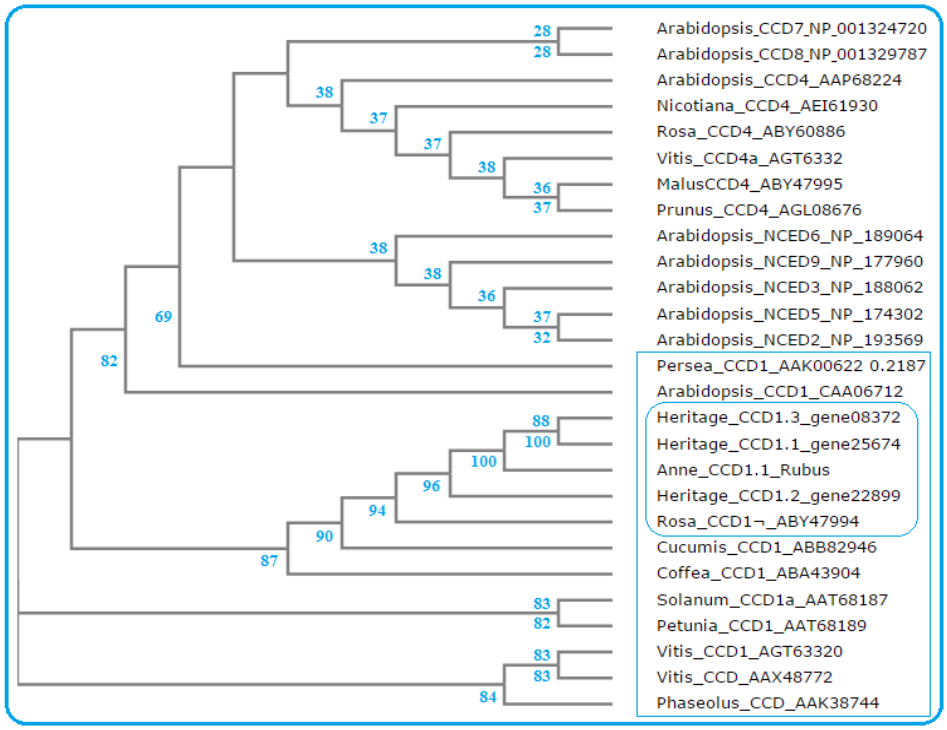
Annex XV: Molecular phylogenetic tree of the deduced amino acid sequences of CHY-b from *Rubus* and some other members clustered according to neighbor-joining method. All sequences are presented as percentage value from *Rubus* genome draft "Heritage".



Annex XVI: Molecular phylogenetic tree of the deduced amino acid sequences of CHY-e from *Rubus* and some members of Rosaceae family and other plants clustered according to neighbor-joining method. All sequences are presented as percentage value from *Rubus* genome draft "Heritage".



Annex XVII: Molecular phylogenetic tree of the deduced amino acid sequences of CCD1 from *Rubus* "Anne" and some other members of Rosaceae family clustered according to neighbor-joining method. All sequences are presented as percentage value from *Rubus* genome draft "Heritage".



8. ABBREVIATIONS

2-ODD:	2-oxoglutarate-dependant dioxygenases
ANR:	Anthocyanidin reductase;
ANS/LDOX:	Anthocyanidin synthase/Leucoanthicyanidin dioxygenase
ANS-KO:	Arabidopsis *ans* (*ldox*) T-DNA mutant complemented with *Rubus* Anthocyanidin synthase
CAPS	Cleaved amplified polymorphic sequences
CHS	Chalcone synthase
CHY-b	Carotenoid β-ring 3-hydroxylase
CHY-e	Carotenoid ε-ring 3-hydroxylase
DFR	Dihydroflavonol 4-reductase;
DTT	Dithiothreitol
EMR	East Malling Research, Kent, UK
F3H/FHT	Flavanone 3β-hydroxylase
FLS	Flavonol synthase
GGPS	Geranylgeranyl diphosphate synthase
HRM	High-Resolution Meltinng
IPTG	Isopropyl β-D-1-thiogalactopyranoside
KO	Arabidopsis *ans* (*ldox*) T-DNA mutant
LAR	Leucoanthocyanidin reductase
LCY-b	Lycopene β-ring cyclase
LCY-e	Lycopene ε-ring cyclase
NSY	Neoxanthin synthase
NTC	Non-template control
NMD	Nonsense-mediated messenger RNA decay
PA	Proanthocyaninidin
PDS	Phytoene desaturase
PMSF	Phenylmethylsulfonyl fluoride
PSY	Phytoene synthase
RT	Room temperature
SAAP	Single amino acid polymorphisms
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SNP	Single nucleotide polymorphism
UFGT	UDPG-flavonoid-glycosyltransferase
ZEP	Zeaxanthin epoxidase

9. ACKNOWLEDGEMENTS

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“I do appreciate all those who remembered me in their prayers and encouraged me throughout my life and education career”.

Muhammad Zubair Rafique

ERKLÄRUNG

Ich versichere, dass ich meine Dissertation

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selbständig ohne unerlaubte Hilfe angefertigt und mich dabei keiner anderen als der von mir ausdrücklich bezeichneten Quellen bedient habe. Alle vollständig oder sinngemäß übernommenen Zitate sind als solche gekennzeichnet.

Die Dissertation wurde in der jetzigen oder einer ähnlichen Form noch bei keiner anderen Hochschule eingereicht und hat noch keinen sonstigen Prüfungszwecken gedient.

Marburg (Lahn), den 23.09.2019

Muhammad Zubair Rafique