# Breeding for pepper fruit quality: A genetical metabolomics approach

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# Breeding for pepper fruit quality: A genetical metabolomics approach

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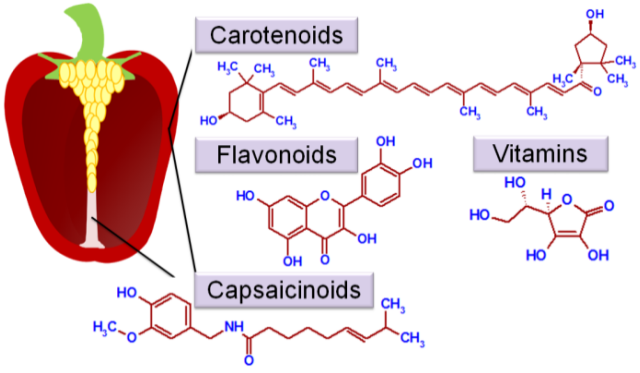
## General Introduction: Secondary Metabolites of *Capsicum* species and Their Importance in the Human Diet

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## ABSTRACT

The genus *Capsicum* (pepper) comprises a large number of wild and cultivated species. The plants are grown all over the world, primarily in the tropical and subtropical countries. The fruits are an excellent source of health-related compounds, such as ascorbic acid (vitamin C), carotenoids (provitamin A), tocopherols (vitamin E), flavonoids, and capsaicinoids. Pepper fruits have been used for fresh and cooked consumption, as well as for medicinal purposes, such as treatment of asthma, coughs, sore throats, and toothache. Depending on its uses, there are several main characters important for product quality; pungency, bright attractive colors, highly concentrated extracts, and a small number of seeds are the main characters on which quality is based and priced. Herein, a general overview of biochemical composition, medical properties of these compounds, and characteristics of quality attributes of pepper fruits is presented.

# 1. INTRODUCTION

The genus *Capsicum*, pepper, belongs to the family Solanaceae and consists of up to 30 species, among which the five major cultivated species are *Capsicum annuum* L., *Capsicum chinense* Jacq., *Capsicum frutescens* L., *Capsicum baccatum* L., and *Capsicum pubescens* Ruiz and Pav. (Barboza et al. 2005; Basu and De 2003). Biosystematically, the genus *Capsicum* is classified by its flower and fruit structure, and the number of chromosomes (2n = 24, 26) (Hunziker 2001). Although the wild ancestor of *Capsicum* species remains unclear, species with 2n=26 chromosomes represent the more primitive clade, while the cultivated, semi-domesticated and some wild species have a predominant chromosome number of 2n=24 (Djian-Caporilano et al. 2007; Moscone et al. 2007; Pozzobon et al. 2006; Walsh and Hoot 2001). Wild species have small, red, erect fruits and seeds, which are dispersed by birds attracted by the bright fruit color (Paran and van der Knaap 2007; Pickersgill 1971). During domestication, breeders selected for increased fruit size and weight and the fruit morphology changed from erect to pendant (Paran and van der Knaap 2007).

On the basis of the geographic origin, morphological traits, reproductive behavior, karyotype analysis and biochemical and molecular marker information, *Capsicum* species are grouped into three main complexes: *C. annuum*, *C. baccatum*, and *C. pubescens* (Djian-Caporilano et al. 2007; Ibiza et al. 2011). These complex groups are organized in two main phylogenetic branches: the white-flowered group (including *C. annuum* and *C. baccatum*) and the purple-flowered group (*C. pubescens*; Fig. 1). The *C. annuum* complex consists of *C. annuum*, *C. frutescens*, and *C. chinense*, which can be intercrossed with each other and are probably derived from a recent common ancestor (Djian-Caporilano et al. 2007; Ibiza et al. 2011; Pickersgill 1971; Walsh and Hoot 2001). The wild *C. annuum* var. *glabriusculum* (Dunal) Heiser and Pickersgill is the most probable progenitor of the domesticated *C. annuum*. In addition, *C. chinense* is suggested to be developed from *C. frutescens* (Djian-Caporilano et al. 2007). The wild species *C. galapagoense* (endemic to the Galapagos Islands) and *C. chacoense* (native to Paraguay, Argentina, and Bolivia) are morphologically and genetically most similar to the white-flowered group complex (Ibiza et al. 2011; Ince et al. 2010). The *C. baccatum* complex includes the domesticated species *C. baccatum* var. *pendulum* and the wild species *C. baccatum* var. *baccatum* and *C. praetermissum* (Djian-Caporilano et al. 2007; Ibiza et al. 2011). The purple-flowered group consists of *C. pubescens* and the wild species *C. eximium* and *C. cardenasii* (Mcleod et al. 1982). *C. pubescens* is

unique for its black seeds, and only the domesticated form is known (Fig. 1; Djian-Caporilano et al. 2007).

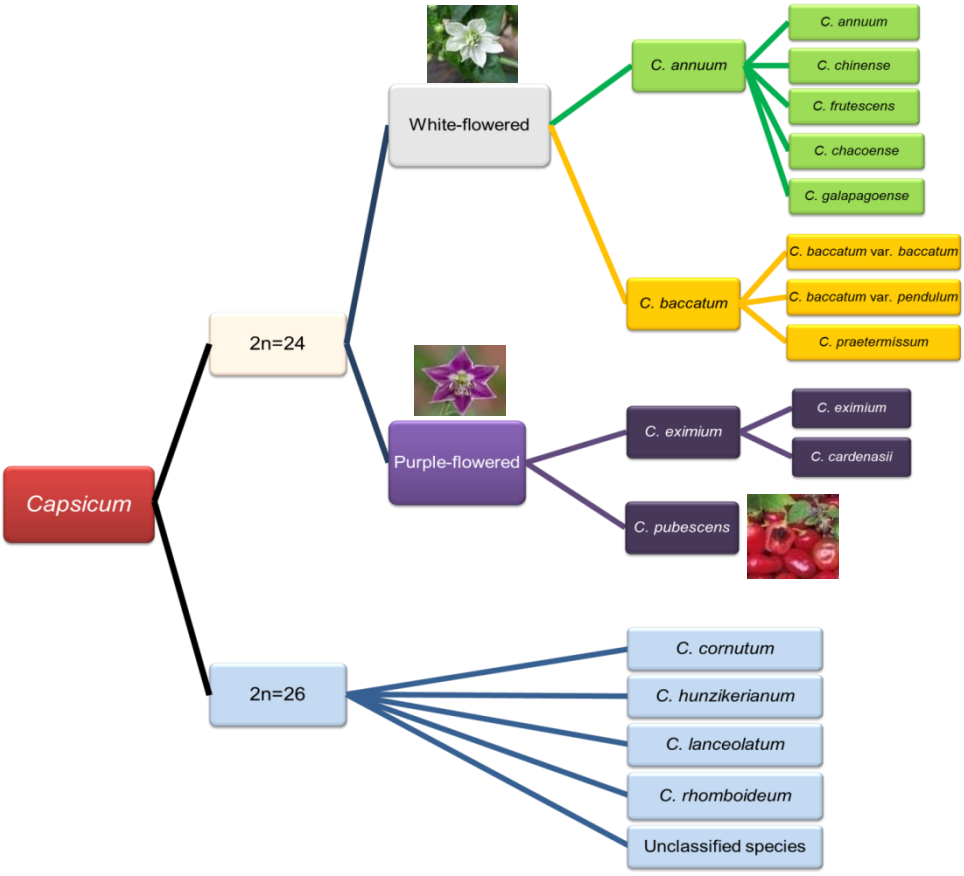



**Fig. 1** Phylogenetic relationships of *Capsicum* species. The photograph of *C. pubescens* is based on http://www.wildfirechilli.com.au/red-rocoto-seeds-c-pubescens-rrcs.html.

Five domesticated species have been widely cultivated in different parts of America. Thus, *C. annuum* is widespread in Mexico and the northern part of Central America; *C. frutescens* is mainly grown in the Caribbean and South America; *C. chinense* is cultivated in the West Indies, northern South America and the Amazon basin; *C. baccatum* is grown in Peru and Bolivia, while *C. pubescens* is

more tolerant to low temperatures than the other cultivated peppers and is found at higher altitudes in the Andes (Knapp 2007; Pickersgill 1971).

In the world spice trade, the market for *Capsicum* is expanding (UNCTAD/WTO 2006). The production of peppers has consistently increased during the last 30 years. World pepper production in 2010 reached 1.8 million Ha with more than 29 million metric tonnes harvested (FAO 2012). Indonesia is the world's fourth largest producer, with more than 1.3 million tonnes and pepper production challenges the production of other common fruits such as tomato and potato. Peppers are used as a fresh or cooked vegetable, a condiment or a spice. The industry uses peppers intensively as a spice or coloring agent in many foodstuffs worldwide.

## 2. BIOCHEMICAL COMPOSITION

*Capsicum* fruits are a rich source of capsaicinoids, carotenoids (some of them with provitamin A activity), flavonoids, ascorbic acid (vitamin C), and tocopherols (vitamin E) (Howard and Wildman 2007; Wahyuni et al. 2011). The amount and composition of these metabolites varies among genotypes, and are affected by many conditions such as fruit maturity, cultivation systems, and processing methods (Howard et al. 2000; Markus et al. 1999). The presence of some of these metabolites may function as general defense mechanism against various biotic and abiotic stresses (Aloni et al. 2008; Park et al. 2012; Schulze and Spiteller 2009). For example, pepper contains polyphenols such as feruloyl *O*-glucosides, kaempferol *O*-pentosyldihexosides, and dihydroxyflavone *O*-hexoses that can act as phytoanticipins (Park et al. 2012). Other polyphenols are known phytoalexins, such as *N*-caffeoyl putrescine and caffeoyl *O*-hexoside, which are induced in *C. annuum* fruits upon infection with the fungus *Colletotrichum gloeosporioides*, the cause of anthracnose (Park et al. 2012). In addition, capsaicin is suggested as one of the defense mechanisms of pepper plants against frugivore (fruit-eating) animals and *Fusarium* fungi (Schulze and Spiteller 2009). Ascorbic acid in the apoplast of bell pepper fruit may protect the plant against physiological disorders caused by environmental stresses, such as the calcium deficiency known as blossom-end rot (Aloni et al. 2008).

The biochemical content of *Capsicum* species not only is valuable for the plant itself, but may also be advantageous for human use. The antioxidant activity of polyphenol compounds, such as cinnamic acid derivatives and flavonoids towards free radicals and reactive oxygen, and their potential anticancer activity, such as

antiproliferation, draws attention to their possible health-protecting role in the human diet (Jeong et al. 2011; Tundis et al. 2011; Zimmer et al. 2012).

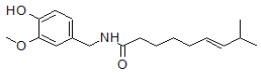
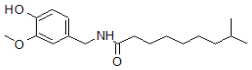
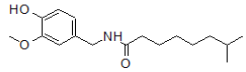
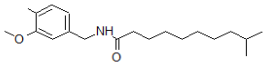
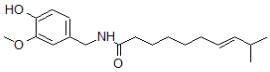
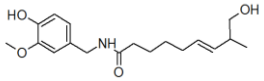
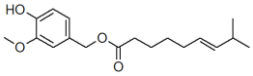
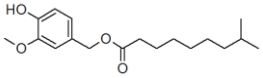
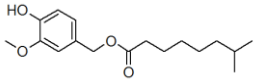
## 2.1. Capsaicinoids

Capsaicinoids are amides produced by *Capsicum* species. These secondary metabolites are responsible for the strong and hot taste of the fruits, also known as pungency. Capsaicinoids consist of compounds that differ in the structure of branched fatty acid (acyl) moieties attached to the benzene ring of vanillylamine (Table 1). These compounds are capsaicin, dihydrocapsaicin, nordihydrocapsaicin, and homocapsaicin. Any variation in the chemical structures of the capsaicinoids, including the structure of the acyl moiety, affects the degree and the level of the pungency (Wang et al. 2011a). Capsaicin and dihydrocapsaicin are the two major capsaicinoids found in hot peppers (more than 90% of the total capsaicinoids), whereas nordihydrocapsaicin, homodihydrocapsaicin, and homocapsaicin are present in lower amounts (Howard and Wildman 2007). Levels of total capsaicinoids can be converted to Scoville Heat Units (SHU), a measurement for pungency developed by Wilbur Scoville (Zachariah and Gobinath 2008). The number of Scoville units equals the approximate number of times a pepper extract would need to be diluted for the spiciness to be imperceptible. By definition, one part per million (ppm) of capsaicin has a pungency of 15 SHU. Scoville categorized four pungency groups: mild (0-5,000 SHU), medium (5,000-20,000 SHU), hot (20,000-70,000 SHU), and extremely hot (70,000-300,000; Eich 2008). The most pungent cultivar is “Naga Viper”, which has more than 1.3 million SHU (Narain 2010).

Capsaicinoids are synthesized in the placenta of the fruits by an enzymatic condensation of vanillylamine and medium-length branched-chain fatty acids (Curry et al. 1999; Mazourek et al. 2009). Vanillylamine originates from the phenylpropanoid pathway and the branched-chain fatty acid derives from branched-chain amino acid metabolism (Mazourek et al. 2009). The key enzyme leading to the production of capsaicinoids is capsaicin synthase (CS), an acyltransferase enzyme involved in the condensation of vanillylamine with the short-chain fatty acid moiety (Curry et al. 1999; Díaz et al. 2004; Mazourek et al. 2009; Stewart et al. 2005, 2007). The *Pun1* locus, formerly known as the *C* locus, encodes a putative acyltransferase AT3, of which the allelic state functions as an on-off switch for pungency in pepper. Hot (pungent) peppers have a functional CS (*Pun1*) allele, but in sweet (non-pungent) peppers the predominant *pun1-1* allele has a deletion in the promoter and in the first intron of the *AT3* gene and, as a consequence, capsaicinoids cannot be formed (Lee et al. 2005; Stewart et al. 2005,

2007). The branched-chain fatty acid pathway was suggested to contribute to the production of the aroma of pungent pepper fruits (Wahyuni et al. 2013). This was particularly true for branched fatty acid esters present in pungent accessions, which may reflect the activity through the acyl branch of the metabolic pathway leading to capsaicinoids.

**Table 1** Chemical Structures and Pungency Levels of Capsaicinoid and Capsinoid Derivatives[a]

| compound | typical relative amount | scoville heat units (SHU) | chemical structure |
|---|---|---|---|
| capsaicin | 69% | 15,000,000 |  |
| dihydrocapsaicin | 22% | 15,000,000 |  |
| nordihydrocapsaicin | 7% | 9,100,000 |  |
| homodihydrocapsaicin | 1% | 8,600,000 |  |
| homocapsaicin | 1% | 8,600,000 |  |
| ω-hydroxycapsaicin | | --- |  |
| capsiate | | --- |  |
| dihydrocapsiate | | --- |  |
| nordihydrocapsiate | | --- |  |

[a]Chemical structures and pungency levels of capsaicinoid analogues are summarized from previous reports (Zachariah and Gobinath 2008; Eich 2008; Ravishankar et al. 2003).

The levels of capsaicinoid production and the relative abundance of capsaicinoids in various pepper cultivars are at least in part determined by genetic and/or environmentally driven differences in the expression of capsaicinoid pathway

genes such as phenylalanine ammonia lyase (*Pal*), cinnamic acid 4-hydroxylase (*C4h*), caffeic acid *O*-methyltransferase (*Comt*), and 3-keto-acyl ACP synthase genes (Aza-González et al. 2011; Curry et al. 1999; Kirschbaum-Titze et al. 2002).

Most capsaicinoids are pungent, but there are also non-pungent capsaicinoids, such as ω-hydroxycapsaicin (Ochi et al. 2003). Moreover, a group of non-pungent capsaicinoid-like substances, named capsinoids, has been reported in the sweet pepper, *C. annuum* CH-19 Sweet, which was derived from a hot Thai pepper variety, CH-19 (Kobata et al. 1998; Yazawa et al. 1989). Capsinoids do have the same two building blocks as capsaicinoids – a vanillyl core bound to a fatty acid moiety – but they are connected through an ester rather than an amide bond (Fig. 2). On the basis of the structure of their fatty acid moiety, these analogues have been named capsiate, dihydrocapsiate, and nordihydrocapsiate (Eich 2008; Kobata et al. 1998, 1999). Capsiate, similar to capsaicin, is the major compound found in CH19-Sweet fruits and is a thousand times less pungent than capsaicin. The production of capsinoids rather than capsaicinoids in CH-19 Sweet is due to a mutation in the putative aminotransferase gene (*pAMT*), which leads to a loss of function of the pAMT enzyme that catalyzes the formation of vanillylamine from vanillin in the capsaicinoid biosynthetic pathway (Lang et al. 2009). As a result, the latter compound is used as a substrate to produce capsinoids (Sutoh et al. 2006). The *pAMT* gene has been validated functionally in a heterologous system using *Agrobacterium*-mediated genetic transformation studies in *Nicotiana tabacum* and *Capsicum frutescens* callus cultures (Gururaj et al. 2012). In addition to CH19-Sweet cultivar, capsinoids have also been detected in some other pepper cultivars (Singh et al. 2009; Wahyuni et al. 2011).

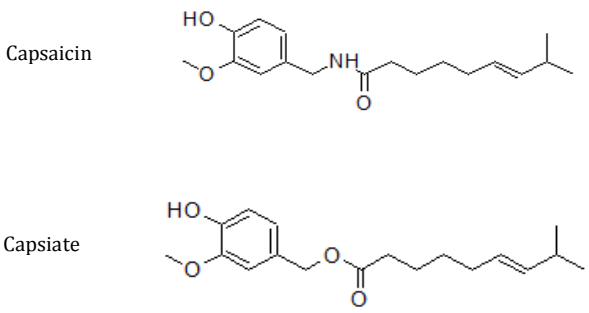



**Fig. 2** Structures of capsaicin and capsiate.

Capsaicinoids are synthesized and accumulated in the epidermis cells of the placenta and dissepiment tissues of the *Capsicum* fruit (Díaz et al. 2004; Iwai et al. 1979). These compounds are transported out of the epidermal cells and stored in the vesicles of the placenta, also called "blisters". Seeds do not produce capsaicin, but can draw capsaicin from the surrounding tissue (Aza-González et al. 2011; Broderick and Cooke 2009; Díaz et al. 2004; Stewart et al. 2007; Zamski et al. 1987). Capsaicinoids start to accumulate when fruits begin to ripen (Suzuki et al. 1980) and reach their highest content when fruits reach their maximum size, e.g., in *C. annuum* (Eich 2008) or their maximum length, e.g., in *C. frutescens* (Sukrasno and Yeoman 1993). Capsaicinoids not only are present in the fruits but can also be found in vegetative organs such as the leaves and stems, although at a much lower concentration than found in the fruits (Estrada et al. 2002; Kim et al. 2011). The presence of capsaicinoids in those organs suggests that capsaicinoids are translocated from placenta to pericarp tissue via the cell walls of the epidermal layer of the placenta, as observed recently by Broderick and Cooke (2009).

All cultivated non-pungent *Capsicum* accessions have a mutation in the *pun-1* gene encoding acyltransferase AT3. To date, three different pun-1 mutations have been described (Stellari et al. 2010; Stewart et al. 2005, 2007). Phylogenetic analysis of these *pun-1* alleles revealed that they do not occur in a single *Capsicum* clade, suggesting multiple origins of the loss-of-pungency alleles (Stellari et al. 2010). Variation in pungency has also been observed in populations of wild *Capsicum* accessions. Characterization of such populations revealed the occurrence, in addition to *pun-1*, of at least one other *pun* gene, *pun-2*. This gene is not allelic to *pun-1* and has been found in wild *C. chacoense* populations (Stellari et al. 2010). The gene has not been cloned yet and its function remains to be elucidated. The non-pungent (*pun-2*) *C. chacoense* plants dominate populations in low-elevation, dry regions and as the elevation increases, pungent plants predominate in the populations (Tewksbury et al. 2006). Recently, these patterns showed a correlation with protection against fungal infection (Haak et al. 2011). It has been shown that the proportion of pungent plants in *C. chacoense* populations increases as the moisture in the regions increases, whereas pungency is less advantageous in low-moisture environments as the risk of fungal infection is lower.

It is not clear why all domesticated non-pungent pepper species only have *pun-1* alleles. This may be due to strong selection for other fruit and seed characteristics linked to the *pun-1* gene on chromosome 2. Alternatively it is possible that the capsaicinoid biosynthetic pathway may be metabolically linked to a trait that is under strong selective pressure for an agronomically desirable trait

(Stellari et al. 2010). Additional studies using various wild populations with varying degrees of pungency are needed to obtain a clear understanding about the origin of pungency and the contribution of natural and domestic selection to the evolution of pungency in *Capsicum*.

## 2.2. Carotenoids

Carotenoids are an important group of pigments responsible for the attractive colors of many flowers and fruits, and likewise play an important role in attracting pollinators and seed dispersers. In addition, they are essential constituents of the photosynthetic machinery and are involved in many aspects related to photosynthesis, such as photosystem assembly, light harvesting, photoprotection, photomorphogenesis, and nonphotochemical quenching (DellaPenna and Pogson 2006). *Capsicum* is one of the richest sources of carotenoids among vegetable crops (Palevitch and Craker 1995). The colors of pepper fruits, which are green, red, yellow, brown, orange, and salmon, all derive from carotenoids, except for the purple-fruited pepper. In the case of purple-fruited peppers, anthocyanins (flavonoid derivatives) contribute to the purple colour (Lightbourn et al. 2008). Unripe pepper fruits contain chlorophylls and carotenoids, such as lutein, violaxanthin, and neoxanthin. During ripening, chloroplasts degenerate into chromoplasts and give rise to the colored carotenoids (Camara et al. 1995; Egea et al. 2010; Paran and van der Knaap 2007). At this stage, chlorophylls are degraded by a tandem activity of chlorophyllase, magnesium dechelatase, and pheophorbide *a* oxygenase (Minguez-Mosquera and Hornero-Mendez 1994b; Moser and Matile 1997; Roca and Minguez-Mosquera 2006). The major carotenoids in red-fruited peppers are capsanthin and capsorubin, whereas violaxanthin, β-carotene, lutein, antheraxanthin, and zeaxanthin are the most important pigments in yellow-fruited peppers (Hornero-Mendez et al. 2000; Matus et al. 1991; Topuz and Ozdemir 2007; Wahyuni et al. 2011). Brown-colored peppers contain both chlorophyll b and carotenoids in the mature fruit (Hornero-Mendez et al. 2000; Wahyuni et al. 2011). The variation in the orange color in pepper fruits is due to different carotenoid profiles. Some orange-fruited cultivars, e.g., Numex Sunset and Habanero, contain the same carotenoid profiles as in red peppers but the levels of these secondary metabolites are lower in the orange peppers (Guzman et al. 2010; Huh et al. 2001). Other orange-fruited cultivars, e.g., Valencia, Orange Granada, Oriole, as well as orange fruits of the segregating population from a cross between *C. annum* cultivar msGTY-1 (orange fruit) and 277long (red fruit), accumulate β-carotene and zeaxanthin (Guzman et al. 2010; Lang et al. 2009; Paran and van der Knaap 2007).

Different fruit colors are caused by the variation in the expression and functionality of genes encoding for enzymes in the carotenoid biosynthetic pathway. To explain the variation in carotenoid composition, a model has been proposed in which three independent pairs of genes, encoded by the *y*, *c1*, and *c2* loci, control the ripe fruit color (Hurtado-Hernandez and Smith 1985). The *Y* locus encodes capsanthin-capsorubin synthase (CCS), the enzyme that converts antheraxanthin and violaxanthin into the red pigments capsanthin and capsorubin, respectively (Bouvier et al. 1994; Lefebvre et al. 1998; Paran and van der Knaap 2007). The *C2* gene encodes phytoene synthase, the first step in the carotenoid biosynthesis pathway. Mutation of this gene leads to a strongly decreased carotenoid content, resulting in pale-colored fruits. The *C1* gene has not been isolated to date, but it has been proposed to affect the levels rather than the composition of carotenoids (Thorup et al. 2000). Red peppers require functional alleles for all three genes and therefore have the genotype *YC1C2*. In yellow peppers, fruit color is determined by the presence of a homozygous recessive *y* allele, which contains a deletion of the *Ccs* gene (Lefebvre et al. 1998; Paran and van der Knaap 2007). Segregation analysis of the offspring from a cross between an orange and a yellow pepper has suggested that yellow peppers also have a recessive *c1* allele (Hurtado-Hernandez and Smith 1985) and thus may have the genotype *yc1C2*. Orange peppers can result from different mutations, at least including the above-mentioned three loci (Guzman et al. 2010). The orange color in cultivars with a reduced level of red carotenoids is determined by a mutation in the *Psy* gene and these cultivars have the genotype *YC1c2*. Orange fruits accumulating β-carotene and zeaxanthin are determined by a deletion in the *Ccs* gene. These cultivars may have genotype *yC1C2* (Lang et al. 2004). Recently, a new *Ccs* variant (*Ccs-3* allele) was detected in the orange-colored pepper cultivar Fogo, which accumulates β-carotene and zeaxanthin, but neither capsanthin nor capsorubin (Guzman et al. 2010). This *Ccs-3* allele contains a frameshift mutation in its coding region, leading to a premature translation stop. Another molecular polymorphism underlying orange color was identified in the orange-colored cultivar Canary, which has a wildtype *Ccs* gene, but which surprisingly did not accumulate any red carotenoids (Rodriguez-Uribe et al. 2012). In this cultivar, the *Ccs* transcript could be detected, but the protein was not found, suggesting that translational control is preventing expression of the CCS protein. Recently, an EMS-induced orange-fruited mutant was found using the blocky red-fruited cultivar 'Maor' as progenitor (Borovsky et al. 2013). This orange mutant mainly accumulates β-carotene in its fruit and the orange phenotype showed a complete co-segregation with a single nucleotide polymorphism (SNP) in the *β-carotene hydroxylase2* (*CHY2)* gene, which is required for the conversion of β-ring

carotenes into zeaxanthin. The SNP leads to an amino acid substitution in the encoded CHY2 protein and the authors hypothesize that this gene is responsible for the observed orange phenotype (Borovsky et al. 2013). Brown-colored pepper fruits retain chlorophylls due to a mutation in the chlorophyll retainer gene (*cl*), in combination with a dominant *Y* locus that is responsible for the production of capsanthin and capsorubin (Efrati et al. 2005).

In ripe pepper fruits, carotenoids may be present in an esterified form when conjugated to one or two fatty acids (Schweiggert et al. 2005). Esterification is initiated during chromoplast development to ease the integration of these compounds in the matrix of chromoplast membranes (Hornero-Mendez and Minguez-Mosquera 2000; Schweiggert et al. 2005). During this process, chloroplast degeneration leads to disorganization of the thylakoid membranes, where the chlorophylls and other early pigments are found associated to membrane proteins and lipids. The carotenoids are consequently liberated to the stroma, where they are not well soluble. Esterification facilitates their integration into the membranes and plastoglobules (Hornero-Mendez and Minguez-Mosquera 2000). Pepper carotenoid esters consist of saturated fatty acids, mainly myristic, lauric, palmitic, and linoleic acid (Minguez-Mosquera and Hornero-Mendez 1994a; Schweiggert et al. 2005).

### 2.3. Flavonoids

Flavonoids are a large group of secondary plant metabolites, which all share the same basic skeleton, the flavan nucleus, consisting of two aromatic rings with six carbon atoms (rings A and B) interconnected by a heterocycle including three carbon atoms (ring C; Fig. 3A). According to modifications of the central C-ring, they can be divided in different structural classes like flavanones, isoflavones, flavones, flavonols, flavan-3-ols, and anthocyanins (Fig. 3B; Rhodes and Price 1996). Flavones are characterized by a 2-phenylchromen-4-one backbone and the natural flavones include apigenin and luteolin. Flavonols differ from flavones in that they have a hydroxy group at the 3-position (3-hydroxyflavones). This group includes quercetin, kaempferol, myricetin, and isorhamnetin (Corradini et al. 2011). Flavanones are characterized by the presence of a chiral center at the 2-position and by the absence of the double bond between the 2- and 3-positions. Naringenin is a flavanone abundant in plants. Anthocyanins form a class of flavonoids which give color to fruits, vegetables, and plants. Anthocyanin pigments are red, purple or blue according to pH. The most common anthocyanidins are pelargonidin, cyanidin, peonidin, delphinidin, petunidin, and malvidin (Corradini et al. 2011). In plants, flavonoids occur as glycosides and glucose is the most

commonly encountered sugar, followed by galactose, rhamnose, xylose, and arabinose, whereas glucuronic and galacturonic acids are rare (Corradini et al. 2011).

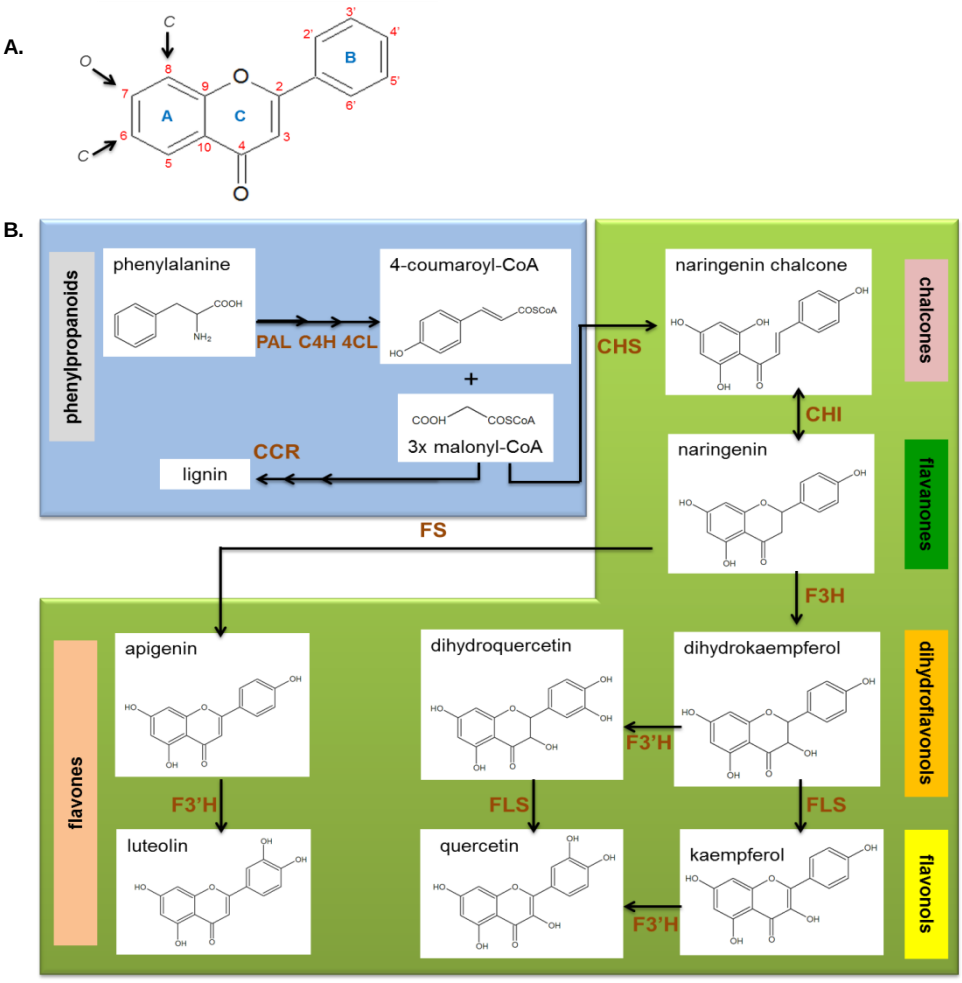



**Fig. 3** (A) Basic structures of flavonoids consisting of malonyl residues (ring A) and hydroxycinnamic acid (ring B and carbon atoms of ring C). Common *O*- and *C*-glycosylation positions are indicated by arrows. (B) The biosynthetic pathway of flavonoids involves the enzymes phenylalanine ammonia lyase (PAL), cinnamate 4-hydroxylase (C4H), 4-coumarate:CoA ligase (4CL), chalcone synthase (CHS), chalcone isomerase (CHI), flavanone 3-hydroxylase (F3H), flavonol synthase (FLS), flavonoid 3`-hydroxylase (F3`H), and flavone synthase (FS). Cinnamoyl Co-A reductase (CCR) catalyzes lignin formation using the precursor of flavonoid biosynthesis.

Most of the research studying flavonoid and phenolic acids in pepper fruits has concentrated (only) on the detection of flavonoid aglycones, particularly quercetin, luteolin, and apigenin, obtained after hydrolysis (Lee et al. 1995). More recently, several studies have focused on the characterization of flavonoid glycosides in the pepper fruit (Iorizzi et al. 2001; Marín et al. 2004; Materska et al. 2003; Sukrasno and Yeoman 1993). In addition to *O*-glycosylated flavonols and flavones, *C*-glycosylated flavones have been identified in the fruits (Marín et al. 2004; Wahyuni et al. 2011). Flavonoid levels vary greatly among pepper cultivars and the composition depends on the fruit maturation stage (Howard et al. 2000; Lee et al. 1995; Marín et al. 2004; Materska et al. 2003; Sun et al. 2007; Wahyuni et al. 2011).

The flavonoid biosynthetic pathway has been well-described in many plants, including *Arabidopsis* (Graham 1998) and tomato (Bovy et al. 2007). Flavonoids are derived from the phenylpropanoid pathway by the activity of a multi-enzyme complex (Fig. 3B; Pfeiffer and Hegedűs 2011; Wang et al. 2011b). The first three general steps of the phenylpropanoid pathway are catalyzed by phenylalanine ammonia lyase (PAL), cinnamate 4-hydroxylase (C4H), and 4-coumarate:CoA ligase (4CL). The product of 4CL, 4-coumaroyl-CoA, serves as a precursor for both the capsaicinoid as well as the flavonoid pathway: this compound can be converted to feruloyl-CoA, an intermediate of the capsaicinoid pathway, but it can also react with three molecules of malonyl-CoA to form the flavonoid naringenin chalcone, a reaction catalyzed by chalcone synthase (CHS). Chalcone isomerase (CHI) isomerizes naringenin chalcone into the flavanone naringenin and from this point onwards the pathway diverges into several side branches, each resulting in a different class of flavonoids. Dihydrokaempferol and dihydroquercetin are synthesized from naringenin by flavanone 3-hydroxylase (F3H) and these intermediates, finally, are converted to kaempferol and quercetin, respectively, by flavonol synthase (FLS). In addition, flavonols and flavones (apigenin and luteolin) are abundantly present in *Capsicum* fruits. In plants, two types of flavone synthase (FS) have been described: FS-1, found in the plant family Apiaceae, and FS-2, found in a wide range of higher and lower plant species (Martens and Mithöfer 2005). It is expected that *Capsicum* has an *FS-2* gene, which leads to the conversion of naringenin into apigenin. The flavone apigenin can be transformed into luteolin by flavonoid 3`-hydroxylase (F3`H). The biosynthesis of anthocyanidins involves the reduction of dihydroflavonols to flavan-3,4-diols by dihydroxyflavonol 4-reductase (DFR) and the conversion of flavan-3,4-diols by anthocyanidin synthase (ANS; Pfeiffer and Hegedűs 2011).

Molecular regulation of the pathway involves two classes of genes: structural genes encoding enzymes that directly participate in the formation of flavonoids and regulatory genes that control the expression of structural genes (Bovy et al. 2007). Some of these genes may be part of gene families. In *Arabidopsis*, the enzymes of CHS, CHI, F3H, F3`H, DFR, and ANS are encoded by single gene, but in contrast, there are six gene homologues of FLS (Owens et al. 2008). In the tomato, some genes, such as chalcone synthase and chalcone isomerase, are encoded by multiple genes, whereas F3H and FLS seem to be encoded by single gene. In addition to the structural genes, flavonoid biosynthesis regulation also involves the combinatorial actions of transcription factors. In general, three classes of transcription factors are involved in regulating the flavonoid pathway: R2R3-MYB, basic helix-loop-helix (bHLH), also called MYC, and WD40 transcription factors (Baudry et al. 2004). MYB transcription factors are required to activate transcription of downstream target genes and they function either alone or as a heterodimer with a bHLH transcription factor. The WD40 transcription factors function in the stabilization of the transcription factor complex (Schaart et al. 2012). Recently, the *Arabidopsis thaliana* R2R3-MYB factors MYB12, MYB11, and MYB111 were identified as flavonol-specific regulators of flavonoid biosynthesis. These genes were able to activate CHS, CHI, F3H, and FLS1 (Stracke et al. 2007). In addition, tomato MYB12, denoted as SIMYB12, plays a role in regulating the production of naringenin chalcone (Adato et al. 2009; Ballester et al. 2010). In *Capsicum*, the activity of two transcription factors called $MYB_A$ and MYC has been demonstrated to be required for anthocyanin accumulation in flower and in fruit tissues (Stommel et al. 2009). Recently, two R2R3-MYB genes, designated as CaMYB1 and CaMYB2, were isolated from hot pepper fruit (*C. annuum* L.) and the expression level of these MYBs correlated with DFR gene expression (Li et al. 2011).

## 2.3. Vitamins

### 2.3.1. Vitamin C (Ascorbic Acid)

Ascorbic acid is a water-soluble vitamin that is an essential antioxidant and a cofactor for enzymes involved in human metabolism (Food and Nutrition Board 2011). *Capsicum* fruits are a rich source of ascorbic acid in the human diet along with other vegetables, such as broccoli, carrots, lettuce, cucumber and tomatoes (Howard and Wildman 2007; Palevitch and Craker 1995). Vitamin C levels in pepper genotypes may range from 43 mg/100 grams to 247 mg/100 grams of fresh fruits (Eggink et al. 2012; Wahyuni et al. 2011) and may contribute 50% to over 100% of the Recommended Daily Intake (RDI), as shown in Fig. 4. The

current RDI for vitamin C is 90 mg/day for men and 75 mg/day for women (Food and Nutrition Board 2011; Howard and Wildman 2007).

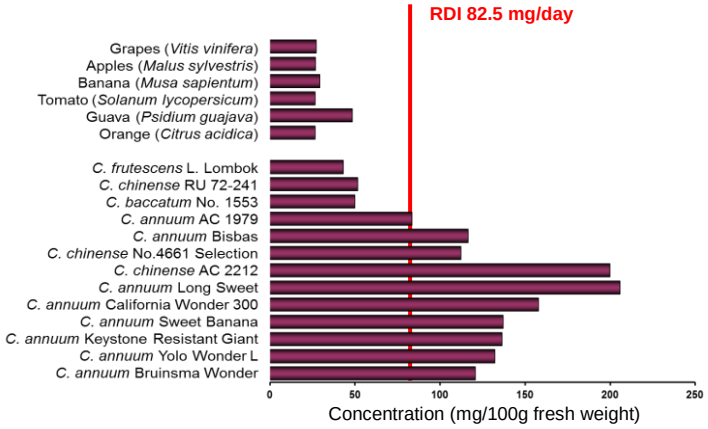



**Fig. 4** Vitamin C variation in ripe fruits of *Capsicum* accessions and several other fruits. *Capsicum* data were derived from the previous report of Wahyuni et al. (2011) and other fruits data were derived from Hussain et al. (2008). The red line indicates the recommended daily intake (RDI) for adults, which is 82.5 mg/day, as described in Food and Nutrition Board (2011).

In pepper, ascorbic acid content increases during fruit ripening (Gnayfeed et al. 2001; Lee et al. 1995; Marín et al. 2004). Higher levels of ascorbic acid observed during ripening may be related to light intensity and greater levels of glucose, the precursor of ascorbic acid (Howard and Wildman 2007; Osuna-Garcia et al. 1998). In addition to maturation, differences in genetic background and environmental growing conditions also affect the ascorbic acid content (Aloni et al. 2008; Howard and Wildman 2007).

### 2.3.2. Vitamin E (Tocopherols)

Tocopherols are a group of (poly)isoprenoid derivatives, which have a polar head group derived from aromatic amino acid metabolism and a saturated tail derived from geranylgeranyl diphosphate. Tocopherols occur as α-, β-, γ- and δ-forms, determined by the number and position of methyl groups on the aromatic ring (DellaPenna and Pogson 2006). Each individual tocopherol compound has a different relative contribution to the total vitamin E activity. α-Tocopherol has the highest vitamin E activity, followed by β-tocopherol, γ-tocopherol, and δ-tocopherol (DellaPenna and Pogson 2006). For the human diet, peppers are one of

the richest sources of natural vitamin E besides some other vegetables such as asparagus, cabbage, broccoli, and eggplant (Isabelle et al. 2010).

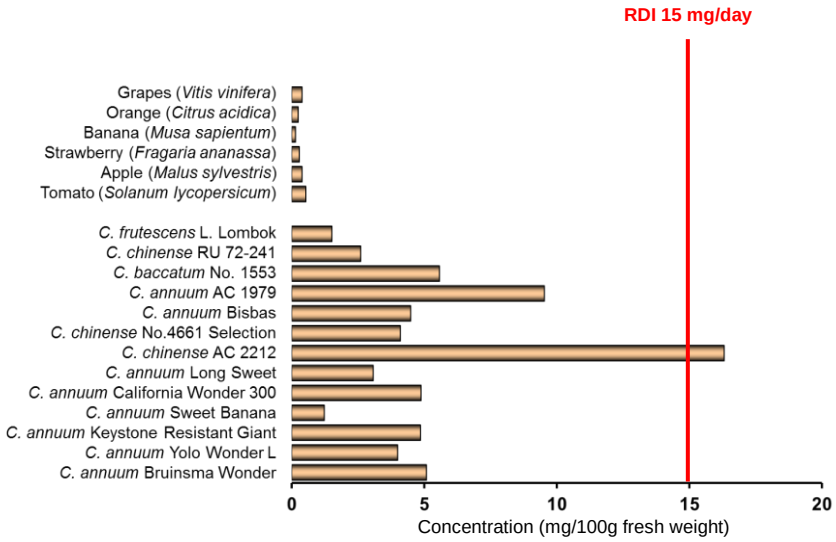



**Fig. 5** Vitamin E variation in ripe fruits of *Capsicum* accessions and several other fruits. *Capsicum* data were derived from the previous report of Wahyuni et al. (2011) and other fruits data was derived from Chun et al. (2006). The red line indicates the recommended daily intake (RDI) for adults, which is 15 mg/day, as described in Food and Nutrition Board (2011).

In pepper fruits, α-tocopherol accumulates in the pericarp tissue, whereas γ-tocopherol is found in the seeds (Osuna-Garcia et al. 1998). Tocopherol levels are influenced by the fruit maturity stage (Markus et al. 1999; Matsufuji et al. 1998; Osuna-Garcia et al. 1998) and vary among pepper cultivars (Wahyuni et al. 2011). For example the amount of α-tocopherol in the *C. annuum* cultivar Mild California was more than twice that of the bell type cultivar Vinedale, at 650 μg/g and 275 μg/g respectively (Palevitch and Craker 1995). The RDI of vitamin E for both men and women is 15 mg/day of α-tocopherol (Food and Nutrition Board 2011). Depending on the pepper cultivar, this fruit can thus provide over 100% of the α-tocopherol RDI per 100 g serving (Fig. 5).

### 2.3.3. Provitamin A (Carotenoids)

Three carotenoids, including α-carotene, β-carotene, and β-cryptoxanthin, contribute to provitamin A activity (IOM 2001). After consumption, carotenoids

are converted by oxygenase enzymes to retinol (Simpson and Chichester 1981; Topuz and Ozdemir 2007). Provitamin A values are expressed as retinol activity equivalent (RAE), in which 1 RAE is equivalent to 12 μg β-carotene, 24 μg α-carotene, and 24 μg β-cryptoxanthin. The RDI values for men and women are 900 and 700 μg RAE/day, respectively (Food and Nutrition Board 2011). Peppers are a rich source of provitamin A and the levels of this vitamin are linked to the specific color of the fruit. Red-fruited, orange-fruited, and brown-fruited cultivars have 3-5 fold higher levels of provitamin A per 100 g fresh weight fruits than those in green-fruited, yellow-fruited and purple-fruited cultivars and contribute to 5-10% RDI/100 g fresh weight of the edible portion, as shown in Fig. 6 (Rodríguez-Burruezo et al. 2010; Simonne et al. 1997; Topuz and Ozdemir 2007).

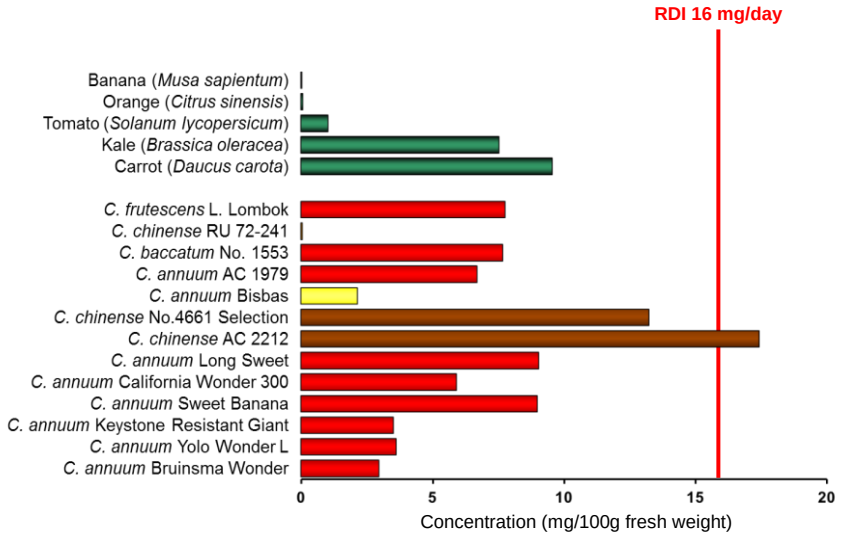



**Fig. 6** Provitamin A variation in ripe fruits of *Capsicum* accessions and several other sources. Provitamin A content is equal to the total β-carotene, α-carotene, and β-cryptoxanthin detected. *Capsicum* data was derived from the previous report of Wahyuni et al. (2011) and other sources data was derived from Müller (1997). Colored-bars represent the ripe fruit colour of *Capsicum*. The red line indicates the recommended daily intake (RDI) for adults, which is 800 μg retinol activity equivalents (RAE)/day, as described by the Food and Nutrition Board (2011). 1 RAE = 1 μg retinol, 12 μg β-carotene, 24 μg α-carotene, or 24 μg β-cryptoxanthin. On the basis of the conversion formula in the previous report (Topuz and Ozdemir 2007), 800 μg RAE/day is equal to 16 mg edible portion/day.

# 3. USES AND MEDICAL PROPERTIES

Although pepper fruits are consumed mainly for their unique taste, aroma and color, some native American tribes have used these fruits in traditional medicine for treating asthma, coughs, sore throats, and toothache (Singletary

2011; Zachariah and Gobinath 2008). The pharmaceutical industry markets capsaicin as a balm for external application to counter the pain in sore muscles. Capsaicin is also used for neuropharmacological studies concerning the handling of pain and for thermoregulation. Creams containing capsaicin may help to reduce postoperative pain for mastectomy patients and for amputees suffering from phantom limb pain (Zachariah and Gobinath 2008). In addition, capsaicin provides relief in osteoarthritis (De Silva et al. 2011). Carotenoids, capsaicinoids, flavonoids and vitamins are also well known for their antioxidant activity. Capsanthin, capsorubin, β-carotene, lutein and zeaxanthin may suppress hydroperoxide formation and may help to quench singlet oxygen (Matsufuji et al. 1998; Palevitch and Craker 1995; Stahl and Sies 2005). Capsaicin has been studied for its effect in decreasing membrane lipid peroxidation in human erythrocytes subjected to oxidative stress (Luqman and Rizvi 2006). At a concentration of 2 mM, capsaicin was found to decrease the production of lipid hydroperoxide by 60% (Henderson et al. 1999; Qureshi et al. 2008). Flavonoids have been known for their biochemical and pharmacological effects, including antioxidants, anti-inflammation, and anti-allergenic activity (Grassi et al. 2010; Lee et al. 1995).

Vitamins present in the pepper fruit have also been claimed for other health benefits. For instance, vitamin A (after being converted from provitamin A) is important for the differentiation of the cornea, the conjunctival membranes, and the retina, as well as for the integrity of epithelial cells (IOM 2001). Combination of vitamins E, C, and A may exhibit cooperative antioxidant synergistic effects to scavenge reactive oxygen and nitrogen species generated during metabolism (Stahl and Sies 2005). The cooperative interaction between lipophilic antioxidants, such as α-tocopherol and β-carotene, showed an inhibition of lipid peroxidation greater than the individual effect of each compound alone (Stahl and Sies 2005).

Some compounds of pepper are claimed to have anticarcinogenic activity, for example, vitamin E has been tested for its inhibitory effect on tumorigenesis in animal models (Ju et al. 2010). The application of β-carotene or a carotenoid mixture, consisting of β-carotene and lutein from pepper fruits, showed a photoprotective effect against solar light irradiation and may help to suppress skin carcinogenesis (Nishino et al. 1999; Stahl and Sies 2005). A polyphenol mixture of cinnamic acid and quercetin derivatives has been shown to decrease the proliferation of cancer cells (Jeong et al. 2011) and capsaicin has been demonstrated to induce growth arrest and apoptosis in human breast and leukemia cancer cells in vitro (Dou et al. 2011; Ito et al. 2004; Min et al. 2004). In contrast, capsaicin has also been reported to be mutagenic and to induce proliferation of prostate cancer cells (Bode and Dong 2011). However, other

studies showed ambiguous results, for example, at low concentrations (0.13-160 μM) in rat, capsaicin is reported to protect the gastric mucous against ulceration by ethanol, whereas at high concentrations (1 or 2 mg/mL) it is reported to deteriorate the tissue (Reyes-Escogido et al. 2011). These controversial results indicate the need for additional studies into the causal effect of such applications. Also, capsinoids have been claimed to inhibit the processes involved in tumor formation and inflammation (Luo et al. 2011; Macho et al. 2003).

## 4. DEFINING THE QUALITY OF PEPPER

*Capsicum* fruits are among the most important vegetables and spices currently used in the world market. Depending on their uses, there are several main characters related to product quality. The required level of pungency, bright attractive colors, highly concentrated extracts, and a paucity of seeds are the main characters on which quality is based and priced (Pruthi 2003). For consumption as a vegetable, the Western consumer prefers to have a fruit with a sweet taste, large size, thick flesh, and bright color (Buckenhüskes 2003). Bell pepper or so called paprika-typed cultivars, such as California Wonder, Bruinsma Wonder, and Yolo Wonder, have been especially developed for this purpose. As a spice, the most important characters are hotness, fruit size, and skin thickness (Buckenhüskes 2003). Some commercial cultivars that are especially bred for their spice are Cayenne, Jatilaba, and Laris.

For industrial food purposes, pepper is used in a dehydrated and powdered form. The most prized powder has a bright rich red color and a sweet taste and is used as a colorant for food and cosmetics. The more pungent powder is used to give flavor to processed food products. The quality of fruit colors is measured by the official analytical method of the American Spice Trade Association (ASTA). In trade, generally, the lower red color limit allowable for pungent pepper powder is 120 and for non-pungent paprika 160-180 ASTA units (Zachariah and Gobinath 2008). Breeding programs select for cultivars that combine a good fruit appearance, including color and size, spiciness, high yield, disease resistance, and adaptation to less favorable growing conditions (Joshi and Berke 2005; Paran and van der Knaap 2007; Prakash and Eipeson 2003). During the last few decades, there has been an increase in the interest of consumers in a diverse diet of fruits and vegetables of a high nutritional quality. For this reason, the development of high-quality foods and food products is becoming increasingly important. Many studies have been performed to unravel the biochemical composition of pepper fruits (Wahyuni et al. 2011; 2013) and to address the beneficial effects of pepper

extracts and their constituents (Jeong et al. 2011; Tundis et al. 2011; Zimmer et al. 2012). These studies have revealed that *Capsicum* species are a good source of health-related metabolites and that the levels of these compounds in these fruits may be affected by maturity, genotype, environmental conditions, and processing methods. To assist breeding programs, more insight is needed, as to how the interplay between genotype, environment and cultural practices affects the biochemistry of the rich metabolic profile in pepper fruits.

## 5. CONCLUDING REMARKS

Fruits of *Capsicum* species are well-known worldwide as a tasty spice with a characteristic smell and taste. In addition, secondary metabolites found in these fruits, including flavonoids, carotenoids, capsaicinoids, and vitamins A, C, and E, have meant increasing interest from the food and pharmaceutical industries, due to their antioxidant activity and their capacity to induce protective mechanisms against free radicals. The composition and levels of these compounds vary among genotypes and may be affected also by maturity and processing. Molecular regulation is one of the key mechanisms underlying the variation in the composition and levels of the secondary metabolites. To date, our understanding of the molecular regulation of metabolic pathways in pepper fruits is still scarce and certainly insufficient to explain the variation in metabolite levels and composition among pepper genotypes. Therefore, further studies, aimed at characterizing large germplasm collections using various X-omics approaches are needed in order to obtain detailed insight into the genetic and molecular regulation of metabolite production in pepper fruit. This knowledge can eventually be applied for the genetic improvement of pepper.

## 6. AIM AND OUTLINE OF THE THESIS

This thesis aims to explore the biochemical diversity present within the wild and cultivated pepper germplasm, using both targeted and untargeted metabolomic approaches. In addition, we aim to gain insight into the molecular mechanisms underlying the production of health-related metabolites in pepper, by linking expression profiles of selected candidate genes with metabolic data. **Chapter 2** explores the variation in levels and composition of well-known health-related compounds in 32 *Capsicum* accessions, including the four pepper species, *C. annuum*, *C. frutescens*, *C. chinense* and *C. baccatum*. Accessions were selected based on their variation in morphological characteristics such as fruit colour,

pungency and origin. We analysed and quantified targeted metabolites, including carotenoids, capsaicinoids, capsinoids, flavonoid glycosides, and vitamins C and E, using high performance liquid chromatography equipped with a photodiode array and a UV detector. The analysis showed that levels of these metabolites vary greatly among fruits of different accessions. Two of 32 accessions, *C. annuum* Long Sweet (no. 12) and *C. chinense* AC2212 (no. 24) contained fruits with outstanding levels of health-related metabolites, such as vitamins, flavonoids and carotenoids. **Chapter 3** focuses on the characterisation of the semi polar and volatile metabolites in 32 accessions. Semi-polar metabolites were analysed by untargeted LC-QTOF-MS(n) and volatile metabolites by headspace SPME-GC-MS. AFLP markers were used to evaluate the genetic diversity among pepper accessions and to correlate metabolite profiling with genetic diversity. The untargeted metabolomic analysis revealed a large variation in semi-polar and volatile metabolites. Variations in semi-polar metabolites demonstrate species-driven metabolite differences, whereas pungency is the main driver for variations in volatiles. Based on the data of both chapters, five accessions were selected for further analyses. **Chapter 4** describes the effect of fruit ripening on the metabolic composition of five selected accessions: *C. annuum* Long Sweet (no. 12), *C. annuum* AC1979 (no. 19), *C. chinense* No. 4661 Selection (no. 18), *C. chinense* AC2212 (no. 24) and *C. frutescens* Lombok (no. 28). The composition and levels of carotenoids, capsaicinoids and flavonoids were analysed in three ripening stages: unripe, turning and ripe. In addition, gene expression analysis of candidate genes putatively involved in the flavonoid biosynthesis pathway was carried out by correlating candidate gene expression patterns with metabolite accumulation profile, aiming to find the key genes responsible for the high flavonoid phenotype of pepper accessions. **Chapter 5** covers the untargeted metabolite analysis of segregating F2 population from the cross of *C. chinense* No. 4661 Selection (no. 18) with *C. annuum* AC1979 (no. 19). These accessions differed in both their metabolite composition as well as in resistance to thrips. The metabolite data were integrated with molecular marker data to find metabolite QTLs. Metabolic analysis showed the segregation of semi-polar metabolites among the F2 individuals. Two mQTL hotspots were found on two different regions on chromosome 9, which regulated the levels of 35 and 103 metabolites, respectively. In addition, we found a co-localization of flavonoid mQTLs and flavonoid gene expression QTLs (eQTLs) on chromosome 1, 6 and 9. Confirmation of flavonoid mQTLs using a candidate gene approach, in which flavonoid mQTL, gene expression (eQTL) and candidate gene marker data were combined, indicated several flavonoid genes as the causative gene underlying important flavonoid QTLs and provided valuable insight in the molecular regulation of the flavonoid pathway in pepper fruit. **Chapter 6**

summarizes the overall findings, starting from the genotypic and metabolic biodiversity of *Capsicum* germplasm, to the metabolic regulation in different fruit ripening stages followed by the expression profile of candidate genes and the metabolite segregation in the F2 population. In the discussion, we emphasize the value of our exploration to analyze the metabolic variation as can be found in pepper fruits from various origins with different analytical platforms and to couple metabolomics with genetic analysis to target future pepper breeding programs to phenotypic diversity for important quality traits.

## ACKNOWLEDGEMENTS

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# CHAPTER 2

## Metabolite Biodiversity in Pepper (*Capsicum*) Fruits of Thirty-Two Diverse Accessions: Variation in Health-Related Compounds and Implications for Breeding

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## ABSTRACT

A comprehensive study on morphology and biochemical compounds of 32 *Capsicum* spp. accessions has been performed. Accessions represented four pepper species, *Capsicum annuum*, *Capsicum frutescens*, *Capsicum chinense* and *Capsicum baccatum* which were selected by their variation in morphological characters such as fruit colour, pungency and origin. Major metabolites in fruits of pepper, carotenoids, capsaicinoids (pungency), flavonoid glycosides, and vitamins C and E were analyzed and quantified by high performance liquid chromatography. The results showed that composition and level of metabolites in fruits varied greatly between accessions and was independent of species and geographical location. Fruit colour was determined by the accumulation of specific carotenoids leading to salmon, yellow, orange, red and brown coloured fruits. Levels of both *O*- and *C*-glycosides of quercetin, luteolin and apigenin varied strongly between accessions. All non-pungent accessions were devoid of capsaicins, whereas capsaicinoid levels ranged from 0.07 up to 80 mg/100 g fresh weight in fruit pericarp. In general, pungent accessions accumulated the highest capsaicinoid levels in placenta plus seed tissue compared to pericarp. The non-pungent capsaicinoid analogues, capsiates, could be detected at low levels in some pungent accessions. All accessions accumulated high levels of vitamin C, up to 200 mg/100 g fresh weight. The highest vitamin E concentration found was 16 mg/100 g fresh weight. Based on these metabolic data, five accessions were selected for further metabolic and molecular analysis, in order to isolate key genes involved in the production of these compounds and to assist future breeding programs aimed at optimizing the levels of health-related compounds in pepper fruit.



**Keywords**:
*Capsicum* spp.; metabolites; ascorbic acid; capsaicinoids; capsinoids; carotenoids; flavonoid glycosides; provitamin A; tocopherols

# 1. INTRODUCTION

Pepper is a member of the *Solanaceae* family, together with other crops such as tomatoes, eggplants and potatoes. Based on floral characteristics, such as the shape of the calyx and the number and orientation of flowers per node, the pepper (*Capsicum*) genus is categorized into 25 species (Barboza et al. 2005; Basu and De 2003). Five of these species, *Capsicum annuum*, *Capsicum frutescens*, *Capsicum baccatum*, *Capsicum chinense* and *Capsicum pubescens,* are inter-crossable and can produce fertile and heterogeneous hybrids (Djian-Caporilano et al. 2007). Wild pepper species form a genetic resource of economically valuable traits that potentially could be introduced into the cultivated varieties. Some of these traits (e.g. taste, colour and nutritional value) are determined by the metabolic composition of the fruit and it is therefore important to gain a further insight in the metabolic variation present in fruits of both cultivated and wild pepper genotypes.

Pepper fruits are a rich source of metabolites with potential health-promoting properties, for example carotenoids (provitamin A), ascorbic acid (vitamin C), tocopherols (vitamin E), flavonoids and capsaicinoids (Howard and Wildman 2007; Topuz and Ozdemir 2007). Many of these compounds are antioxidants that exert their biological effects through free-radical scavenging, protein binding and interaction with human signal transduction pathways (Edge et al. 1997; Padayatty et al. 2003). Fruits of different pepper varieties show variation in the composition and level of health-related metabolites (Hornero-Mendez et al. 2002; Minguez-Mosquera and Hornero-Mendez 1993; Osuna-Garcia et al. 1998; Topuz and Ozdemir 2007). The metabolic composition is influenced by genotypic, developmental and environmental factors (Conforti et al. 2007; Daood et al. 1996; Howard et al. 1994; Lee et al. 2005b). Comparison of these data is limited as most studies generally included a small number of pepper varieties and/or a few selected phytochemicals. In order to obtain a more comprehensive picture of the biochemical diversity we determined the composition of capsaicinoids, flavonoids, carotenoids and vitamins (tocopherols and ascorbic acid) in the fruit of a set of 32 pepper accessions, consisting of breeding varieties, land races and wild accessions from four different inter-crossable pepper species. These accessions were selected based on variation in geographical origin, fruit morphology (size, shape and colour) and pungency level. Our results showed a large variation in both the level and the composition of all metabolites analysed. Results may help to further unravel the biochemical and molecular mechanisms underlying this metabolic variation and can serve to steer pepper breeding programs to develop new commercial hybrids with fruits enriched for health-related compounds.

# 2. MATERIALS AND METHODS

## 2.1. Plant material, growth conditions and sample preparation

A total of 32 *Capsicum* accessions were selected from the Plant Research International (PRI) and the Centre for Genetic Resources, the Netherlands (CGN) seed collections. Out of all accessions, 17 were from *C. annuum*, nine from *C. chinense*, four from *C. baccatum* and two from *C. frutescens*. The selection was based on variations in fruit colours, shapes, sizes and geographical origins (Table 1 and Fig. 1).

Plants were cultivated in rock wool media under irrigated conditions during December 2007 until June 2008. Plants were grown in a greenhouse located in Wageningen (The Netherlands). The experimental design was completely randomized in two blocks, each one including all 32 accessions grown in plots of four plants. For each accession, 10-50 ripe fruits (depending on fruit size) were harvested from the two innermost plants of each plot. Pericarp tissue was separated from placenta plus seeds, frozen in liquid nitrogen, ground and stored at -80 °C until analysis.

Before harvesting fruits for metabolic analyses, we carefully followed the ripening stages of the first set of fruits produced by each accession. Fruit ripeness was indicated by a combination of (i) its mature colour based on the description in the CGN databases, (ii) fruit firmness and (iii) number of days after fruit setting (on average 70-80 days after fruit setting).

## 2.2. Extraction and analysis of carotenoids and tocopherols

Carotenoids and tocopherols were extracted as described previously by López-Raéz et al. (2008). Briefly, 500 mg of pericarp tissue were extracted with 4.5 ml of methanol:chloroform (2.5:2.0, v/v) containing 0.1% butylated hydroxytoluene (BHT). After shaking and incubation on ice in the dark for 10 min, 2.5 ml of 50 Mm Tris-chloride (pH 7.5) containing 1 M sodium chloride were added. Samples were incubated on ice for another 10 min, centrifuged at 1350*g* for 10 min at room temperature, and the chloroform layer was transferred to a clean tube. Samples were re-extracted twice with 1.0 ml of chloroform containing 0.1% BHT. Pooled chloroform phases were dried under a nitrogen flow, tubes were closed and kept at -20 °C until high-pressure liquid chromatography (HPLC) analysis. Samples were prepared for HPLC by dissolving the dried residues in 1.0 ml of ethyl acetate containing 0.1% BHT. To confirm the presence of carotenoid esters in the extracts, the dried residues were saponified according to Ittah et al.

(1993). HPLC analysis was performed according to Bino et al. (2005) using a YMC-Pack reverse-phase $C_{30}$ column (250 x 4.6 mm; 5 µm) coupled to a 20 x 4.6 mm $C_{30}$ guard (YMC Inc. Wilmington, NC, USA), maintained at 40 °C. The mobile phase used was methanol, tert-methyl butyl ether and water:methanol (20:80, v/v) containing 0.2% ammonium acetate. Flow rate of 1 ml/min was used. Chromatography was carried out on a Waters system consisting of a No. 600 quaternary pump, No. 996 photo diode array detector (PDA) and No. 2475 fluorescence detector (FD). Data were collected and analyzed using the Waters Empower software supplied. Carotenoids were detected by setting the PDA to scan from 220 to 700 nm. All carotenoids were quantified at 478 nm, except for violaxanthin, violaxanthin-esters compounds and neoxanthin, which were quantified at 440 nm. Tocopherols were detected by FD at excitation and emission wavelengths of 296 and 405 nm. Quantitative determination of compounds was conducted by comparison with dose-response curves constructed from authentic standards. Carotenoids esters, denoted as capsanthin-esters and violaxanthin-esters compounds, were quantified using dose-response curves of their free forms, since the ester and free forms showed the same UV absorption spectrum (Schweiggert et al. 2007). Each value was the mean of two biological replicates with two independent technical replicates.

### 2.3. Extraction, separation and detection of ascorbic acid (vitamin C)

The extraction method and HPLC-PDA analysis for ascorbic acid (vitamin C) were carried out according to Moco et al. (2007). Vitamin C was extracted using 500 mg fresh frozen pericarp powder in 2 ml of 5% meta-phosphoric acid containing 1 mM aqueous diethylene triamine pentaacetic acid (DTPA). The extracts were sonicated for 15 min, filtered through 0.2 µm polytetrafluoroethylene (PTFE) filter and analyzed by the same HPLC-PDA system used as for the detection of carotenoids. Extracts were kept in amber vials to keep the stability of the vitamin. A YMC-Pro $C_{18}$ (YMC Europe GmbH; 150 x 3.9 mm) column was used and ascorbic acid was separated at 30 °C using 50 mM of phosphate buffer (pH 4.5) as eluent. For ascorbic acid detection, after 6 min separation using a flow of 0.5 ml/min, the column was washed with 50% of pure acetonitrile and reconstructed before the next injection. Quantification of ascorbic acid was made at 262 nm using a calibration curve of the authentic chemical standard of ascorbic acid from Merck (Darmstad, germany).

### 2.4. Determination of capsaicinoid and capsinoid analogues

For the analysis of capsaicinoids and capsinoids, 500 mg of frozen ground samples of pericarp or placenta plus seeds were extracted with 1.5 ml of 100% methanol (final methanol concentration in the extract approximately 75%). The extracts were sonicated for 15 min and filtered through a 0.2 µm PTFE membrane filter into an amber vial to prevent the degradation of the compounds. Ten microlitres of sample were injected into the same Waters system used to detect carotenoids but equipped with a Luna C18(2) pre-column (2.0 x 4 mm) and analytical column (2.0 x 150 mm, 100 Å, particle size 3 µm) from Phenomenex (Torrance, CA, USA) for chromatographic separation. Degassed solutions of formic acid (FA):ultrapure water (1:$10^3$, v/v, eluent A) and FA:acetonitrile (1:$10^3$, v/v, eluent B) were used as a mobile phase. The gradient applied started at 50% B and increased linearly to 75% B in 30 min. Then, the column was washed with 80% B during 10 min and re-equilibrated before the next injection. The flow rate of the mobile phase was 1 ml/min. The column temperature was kept at 40 °C. Capsaicinoid and capsinoid analogues were detected by PDA at wavelength of 280 nm and quantified by comparison with commercial capsaicinoids (capsaicin and dihydrocapsaicin) from Sigma (St Louis, USA) and capsinoids (capsiate and dihydrocapsiate) from Konectia (Cádiz, Spain).

### 2.5. Extraction, separation and detection of flavonoid glycosides

Flavonoid glycosides were extracted using a protocol described previously by de Vos et al. (2007). Briefly, freeze-ground material of pepper pericarp (500 mg) was extracted with 1.5 ml of 99.875% methanol acidified with 0.125% formic acid and mixed immediately for 10 s. The extracts were sonicated for 15 min and filtered through 0.2 µm PTFE filter. The LC-PDA-QTOF-MS-MS system was set up in electrospray negative mode, as previously described by Moco et al. (2007). The separation of flavonoid glycosides from 5 µl extract/sample was performed in a gradient flow containing ultrapure water/0.1% formic acid (A) and acetonitrile/0.1% formic acid (B) with a flow rate of 1 ml/min. The gradient flow started with 95% of A and 5% of B for 45 min, continued with 35% A and 65% B for 2 min, 25% A and 75% B for 5 min and returned to 95% A and 5% B for 6 min. The MS-MS measurements were set up with increasing collision energies according to the following program: 10, 15, 25, 35 and 50 eV. Leucineenkaphalin, $[M-H]^-$ = 554.2620, was used as a lock mass and injected through a separate inlet.

Quercetin, luteolin and apigenin glycoside identification was carried out by means of the UV spectra, molecular weight and MS-MS fragmentation profile in

negative ion mode at 25 eV collision energy. Putative names of flavone glycosides were obtained based on Marin et al. (2004). Peak areas of quercetin, luteolin and apigenin glucosides were obtained by integration at 340 nm. The concentration of quercetin *O*-glycosides and apigenin *C*-glycosides were calculated and expressed as mmol of quercetin 3-rutinoside equivalents/100 g fresh weight (FW) and as mmol of luteolin 6-*C*-glucoside equivalents/100 g FW, respectively. Levels of both luteolin *O*- and *C*-glycosides were expressed as mmol of luteolin 6-*C*-glucoside equivalent/100 g FW.

# 3. RESULTS

## 3.1. Morphological and agronomical variations among 32 pepper accessions

The morphological and agronomical information of the 32 pepper accessions studied are shown in Table 1 and Fig. 1. The agronomical information, such as accession name, country of origin, population type and pungency was based on the information obtained from the CGN website (http://www.cgn.wur.nl/UK/CGN+Plant+Genetic+Resources). The fruit morphological features such as colour, type, shape and size, were determined on 10 ripe fruits of the first harvest. Fruit colour of unripe fruit was determined from immature fruit before harvest. The accessions used originate from America, Europe, Asia and Africa (Table 1). Most accessions are breeders' varieties or land races. Two accessions from South America, Bolivia and Jamaica, *C. baccatum* var. *baccatum* (no. 15) and *C. annuum* AC1979 (no. 19) respectively, were wild species. Pungency was based on taste trials performed on whole ripe fruit conducted by CGN. The pungency level varied greatly among all accessions, ranging from low to very high (Table 1). The pungency level was very low in most breeders' varieties, such as *C. annuum* Bruinsma Wonder (no. 6), California Wonder 300 (no. 10), Keystone Resistant Giant (no. 11), Sweet Banana (no. 13), Yolo Wonder L (no. 14) and Sweet Chocolate (no. 21). Two breeders' varieties had very high pungency level, e.g. *C. frutescens* Tabasco (no. 29) and *C. baccatum* Aji Blanco Christal (no. 30).

In all accessions, the fruit colour at the unripe stage was green, with a different level of colour ranging from light green to dark green. One accession, *C. frutescens* Lombok (no. 28), had a very light green unripe fruit colour, which was categorized as white by CGN. These unripe colours turned into different colours during ripening. Most accessions had red-coloured ripe fruit (Fig. 1), but the level of red varied from dark red, e.g. in *C. annuum* I2 Tit Super (no. 1), to light red, such as in *C. baccatum* var*. pendulum* (no. 30-32). Several accessions had fruit with special colours (Fig. 1), such as yellow (no. 9), salmon (no. 26), orange (no. 27)

and brown (no. 3, 17, 18, 21, and 24). *Capsicum chinense* AC2212 (no. 24) had a special brown colour which is a mix between dark green and dark brown.

The fruit shapes, types and sizes varied in the accessions studied. Based on the CGN criteria, they can be grouped in different types, such as blocky, pointed, round or conical. There were four accessions with a blocky fruit type (also called bell peppers). The blocky type is equal in shape between the fruit height and width. Most accessions had a pointed fruit type. Several *C. chinense* accessions had a round shape and the conical shape was found in both *C. chinense* and *C. annuum*.

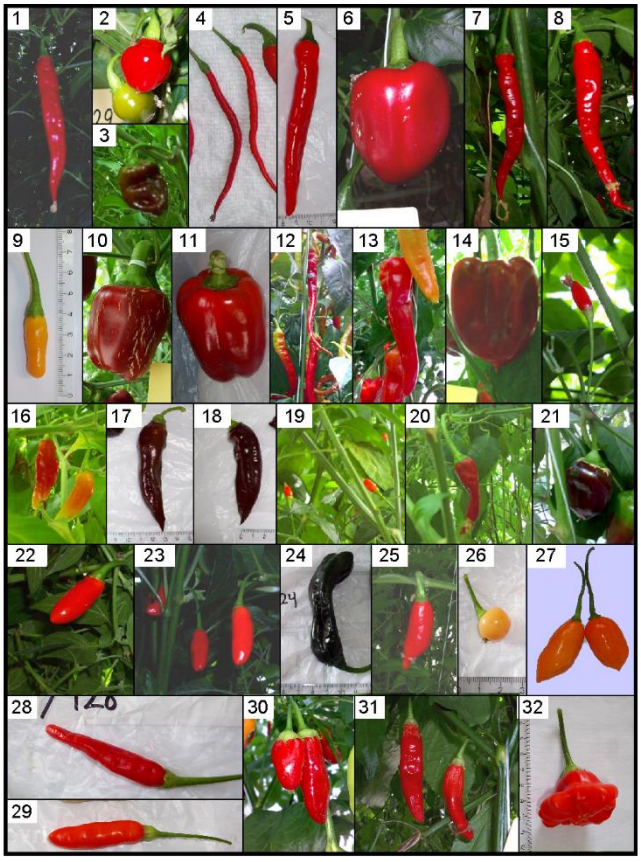


**Fig. 1** Variation in fruit colour and shape among 32 pepper accessions. Each number in this figure corresponds with the number in the first column of Table 1.

**Table 1** Morphological characters of 32 pepper accessions

| No. | Accession no. | Species | Accession name* | Reference | Country of origin* | Population type* | Pungency* | Fruit colour | | Fruit type | Fruit shape | Fruit size |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | Unripe | Ripe | | | |
| 1 | PRI1996048 | *C. annuum* | I2 Tit super | | Indonesia | n.d. | n.d. | Dark green | Red | Pointed | Very oblong | Medium |
| 2 | PRI1996108 | *C. chinense* | I1 PI 281428 | | Surinam | n.d. | n.d. | Green | Red | Roundish | Almost round | Medium |
| 3 | PRI1996112 | *C. chinense* | I1 PI 315023 (Mishme Black) | | Peru | n.d. | n.d. | Green | Brown | Conical | Campanulate | Small |
| 4 | PRI1996236 | *C. annuum* | Laris HS | | Indonesia | Breeders variety | n.d. | Dark green | Red | Pointed | Very oblong | Medium |
| 5 | PRI1999049 | *C. annuum* | Jatilaba | | Indonesia | Breeders variety | n.d. | Dark green | Red | Pointed | Very oblong | Medium |
| 6 | PRI2004001 | *C. annuum* | Bruinsma Wonder | | Netherlands | Breeders variety | low | Dark green | Red | Blocky/bell | Height equals width | Large |
| 7 | PRI2007007 | *C. annuum* | PBC 473 - none - cayenne | | SriLanka | n.d. | High | Green | Red | Pointed | Very oblong | Medium |
| 8 | PRI2007008 | *C. annuum* | PBC 535 - IR - 12x1cm - cayenne | | SriLanka | n.d. | High | Green | Red | Pointed | Very oblong | Medium |
| 9 | CGN20503 | *C. annuum* | Bisbas | | Yemen | Land variety | n.d. | Green | Yellow | Other | Other | Small |
| 10 | CGN19189 | *C. annuum* | California Wonder 300 | Wall et al. 2001; Saga and Sato 2003 | USA | Breeders variety | low | Dark green | Red | Blocky/bell | Height equals width | Medium |
| 11 | CGN23222 | *C. annuum* | Keystone Resistant Giant | | USA | Breeders variety | Low | Dark green | Dark red | Blocky | Very flattened | Medium |
| 12 | CGN23289 | *C. annuum* | Long Sweet | | Zambia | Land variety | Low | Light green | Red | Pointed | Very oblong | Large |
| 13 | CGN22173 | *C. annuum* | Sweet Banana | Howard and Wildman 2007; | USA | Breeders variety | Low | Light green | Red | Pointed | Very oblong | Medium |
| 14 | CGN23098 | *C. annuum* | Yolo Wonder L | Bouvier et al. 1998 | USA | Breeders variety | Low | Dark green | Red | Blocky/bell | Oblong | Large |
| 15 | CGN17042 | *C. baccatum* var. *baccatum* | No. 1553; PI 238061 | | Bolivia | Wild | Very high | Green | Red | Pointed | Oblong | Very small |
| 16 | CGN22829 | *C. chinense* | Miscucho colorado; PI 152225; 1SCA no.6 | | Peru | n.d. | Very high | Light green | Red | Conical | Oblong | Small |
| 17 | CGN21557 | *C. chinense* | No.4661; PI 159236 | | USA | n.d. | Very high | Green | Brown | Pointed | Oblong | Small |
| 18 | CGN17219 | *C. chinense* | No.4661 Selection; PI 159236 Selection | | Netherlands | Research material | Very high | Green | Brown | Pointed | Very oblong | Large |
| 19 | CGN16975 | *C. annuum* | AC1979 | | Jamaica | Wild | Very high | Green | Red | Conical | Oblong | Very small |
| 20 | CGN23765 | *C. annuum* | CM 331; Criollos de Morelos | | Mexico | Land variety | n.d. | Green | Red | Conical | n.d. | Medium |
| 21 | CGN16922 | *C. annuum* | Sweet Chocolate | | USA | Breeders variety | Low | Dark green | Brown | Roundish | Flattened | Small |
| 22 | CGN22830 | *C. annuum* | Chili Serrano; PI 281367; No. 999 | | Mexico | Land variety | Very high | Dark green | Red | Pointed | Oblong | Small |

**Table 1** *Continued*

| No. | Accession no. | Species | Accession name* | Reference | Country of origin* | Population type* | Pungency* | Fruit colour | | Fruit type | Fruit shape | Fruit size |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | Unripe | Ripe | | | |
| 23 | CGN21534 | *C. annuum* | Chili de Arbol; PI 281370; No. 1184 | Thomas et al. 1998 | Mexico | Land variety | Very high | Dark green | Red | Other | Oblong | Very small |
| 24 | CGN21469 | *C. chinense* | AC2212 | | n.d. | n.d. | Very high | Dark green | Dark brown | Pointed | Very oblong | Large |
| 25 | CGN22862 | *C. chinense* | No.1720; PI 281426; 1GAA | | Surinam | Land variety | Very high | Green | Red | Pointed | Oblong | Small |
| 26 | CGN16994 | *C. chinense* | RU 72-194 | | Brazil | Land variety | Very high | Light green | Salmon | Roundish | Oblong | Small |
| 27 | CGN16995 | *C. chinense* | RU 72-241 | | Brazil | Land variety | Very high | Green | Orange | Other | Oblong | Small |
| 28 | CGN22817 | *C. frutescens* | Lombok | | Indonesia | Land variety | Very high | White | Red | Pointed | Very oblong | Small |
| 29 | CGN21546 | *C. frutescens* | Tabasco | Collins et al. 1995; Howard et al. 2000 | USA | Breeders variety | Very high | Light green | Red | Pointed | Oblong | Small |
| 30 | CGN21470 | *C. baccatum* var. *pendulum* | Aji Blanco Christal; CAP 333 | | Chile | Breeders variety | Very high | Light green | Red | Pointed | Oblong | Medium |
| 31 | CGN17028 | *C. baccatum* var. *pendulum* | | | n.d. | n.d. | Very high | Green | Red | Pointed | Very oblong | Medium |
| 32 | CGN23206 | *C. baccatum* var. *pendulum* | RU 72-51 | | Brazil | Land variety | n.d. | Green | Red | Other | Other | Medium |

n.d.: data not available;
* Denotes that information is derived from the Centre for Genetic Resources in The Netherlands (CGN)

## 3.2. Biochemical analysis of health-related compounds

### 3.2.1. Carotenoids

In *Capsicum*, fruit colour is mainly determined by the composition and concentration of carotenoids (Lightbourn et al. 2008). A quantitative analysis of carotenoids in ripe fruit pericarp extracts of the 32 different accessions was carried out using HPLC with photodiode array (PDA) detection. The chromatograms revealed that, in addition to the free carotenoids, many other carotenoid peaks were present in the extracts. These peaks disappeared upon saponification while the levels of the free carotenoids increased, suggesting that they represent carotenoids conjugated as fatty acid esters (not shown). This is in line with several other studies showing the presence of mono- and di-esterified carotenoids in pepper fruit (Hornero-Mendez and Minguez-Mosquera 2000; Schweiggert et al. 2005). As shown in Fig. 2, red-fruited pepper accessions accumulated six major carotenoid pigments, capsanthin, β-cryptoxanthin, β-carotene, capsorubin, zeaxanthin and antheraxanthin, at variable relative amounts. In red-fruited peppers, a large proportion of capsanthin was present as fatty acid esters (denoted as capsanthin-ester). In general, the levels of capsanthin and capsanthin-ester, β-cryptoxanthin and β-carotene were higher than those of capsorubin, zeaxanthin and antheraxanthin. Among the red-fruited accessions, *C. annuum* I2 Tit Super (no. 1) and *C. annuum* PBC 473-none-cayenne (no. 7) contained the highest levels of carotenoid pigments.

The typical colour of brown-fruited peppers is due to the accumulation of carotenoid pigments found in red-fruited genotypes, in combination with the green chlorophyll b. Chlorophyll b levels varied greatly among the brown peppers, ranging from 2.15 mg/100 g FW in *C. annuum* Sweet Chocolate (no. 21), to 27.22 mg/100 g FW in *C. chinense* AC2212 (no.24). Lutein, a carotenoid present in unripe green fruit, was also detected in all brown pepper accessions. Of all accessions analyzed, *C. chinense* AC2212 (no. 24) contained the highest levels of chlorophyll b, the green-fruit carotenoid lutein, the red-fruit carotenoids β-carotene, β-cryptoxanthin, zeaxanthin, anteraxanthin and capsanthin and the yellow carotenoids violaxanthin and neoxanthin. This accession accumulated low levels of capsorubin (Fig. 2).

In contrast to the red accessions, the yellow-fruited pepper accession *C. annuum* Bisbas (no. 9) did not contain any capsanthin or capsorubin at detectable levels (Fig. 2). Instead, other carotenoids such as violaxanthin and its fatty acid esters as well as lutein accumulated at higher levels in this accession compared to red accessions (up to a concentration of 8.64 mg/100 g FW and 0.84 mg/100 g FW,

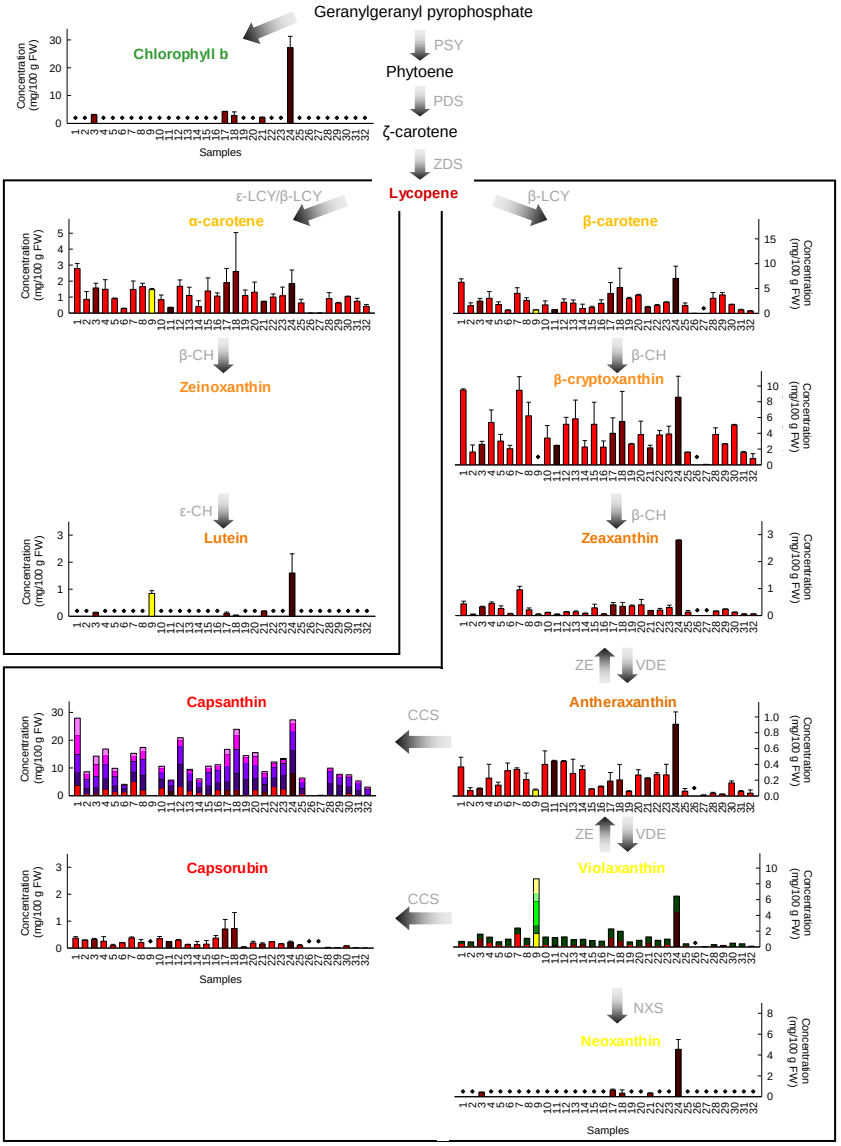


**Fig. 2** Carotenoid levels in ripe pepper fruit. The colour of each bar corresponds to the colour of the ripe pepper fruit. The capsanthin and violaxanthin graphics also include levels of violaxanthin isomer (denoted as dark green bar) and different capsanthin and violaxanthin esters. The latter are coloured in a different way. Each bar represents the average of two biological replicates plus standard deviation. All values (in case of both free and esterified carotenoids) represent the amount of the free form per 100 g FW. The diamond symbol indicates "not detected". Abbreviations: PSY, phytoene synthase; PDS, phytoene desaturase; ZDS, ζ-carotene desaturase; β-LCY, lycopene-β-cyclase; ε-LCY, lycopene-ε-cyclase; β-CH, β-carotene hydroxylase; ε-CH, ε-carotene hydroxylase; ZE, zeaxanthinepoxidase; VDE, violaxanthin de-epoxidase; NXS, neoxanthin synthase; CCS, capsanthin-capsorubin synthase.

respectively). In addition yellow fruit contained α-carotene, β-carotene, zeaxanthin andantheraxanthin.

The orange fruit of accession *C. chinense* RU 72-241 (no. 27) contained low, but significant levels of the red carotenoids capsanthin, β-cryptoxanthin and antheraxanthin. Besides these, the orange accession accumulated detectable levels of the yellow-coloured violaxanthin and traces of α-carotene, which together with the red pigments may lead to an orange colour. Orange fruit did not accumulate any detectable levels of carotenoids more upstream in the pathway, such as zeaxanthin and β-carotene (Fig. 2).

The salmon fruit of *C. chinense* RU 72-194 (no. 26) was virtually devoid of carotenoids, except for trace levels of capsanthin and capsanthin-ester (0.04 mg/100 g FW), β-carotene (0.01 mg/100 g FW) and α-carotene (0.003 mg/100 g FW) (Fig. 2).

### 3.2.2. Capsaicinoids and Capsinoids

Nordihydrocapsaicin, capsaicin and dihydrocapsaicin were the major capsaicinoids in the fruit pericarp and placenta plus seed tissues of all accessions. The capsaicinoid concentrations varied greatly, as presented in Fig. 3. In general, capsaicin and dihydrocapsaicin were relatively more abundant than nordihydrocapsaicin. Among all accessions, *C. frutescens* Lombok (no. 28) contained the highest total amount of capsaicinoids in the pericarp, with capsaicin and dihydrocapsaicin levels up to 60 and 15 mg/100 g FW, respectively (Fig. 3B, C). As presented in Fig. 3A-C, seven accessions had no detectable levels of capsaicinoids in the pericarp. These were all *C. annuum* accessions, including California Wonder 300 (no. 10), Keystone Resistant Giant (no. 11), Long Sweet (no. 12), Sweet Banana (no. 13), Yolo Wonder L (no. 14), Criollos de Morelos (no. 20) and Sweet Chocolate (no. 21). Out of all *C. chinense* accessions, AC2212 (no. 24) was the one with the lowest capsaicinoid concentration (0.07 mg/100 g FW) in the pericarp.

Placenta plus seed tissue contained levels of capsaicinoids reaching up to 400 mg/100 g FW (Fig. 3E-H). Several accessions that did not show capsaicinoids in the pericarp did contain low levels of capsaicinoids in placenta plus seed. This was the case for the blocky paprika type peppers *C. annuum* California Wonder 300 (no. 10) and Yolo Wonder L (no. 14) and the pointed peppers *C. annuum* Long Sweet (no. 12) and *C. chinense* AC2212 (no. 24) (Fig. 3D, H).

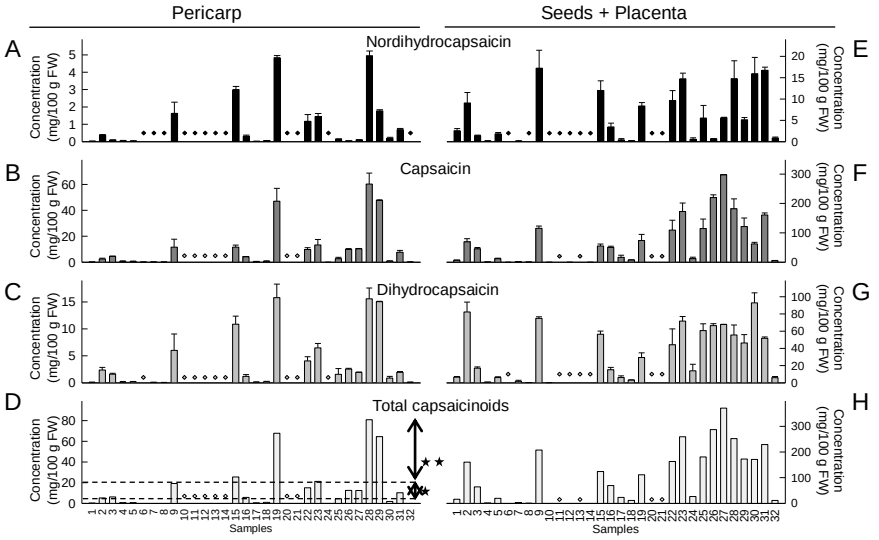


**Fig. 3** Levels of the capsaicinoids nordihydrocapsaicin (A, E) capsaicin (B, F) and dihydrocapsaicin (C, G) in pericarp (A-D) and placenta plus seeds (E-H) of 32 pepper accessions. Figures D and H represent the total amount of capsaicinoids in pericarp (D) and placenta plus seeds (H), respectively. Each bar represents the average of two biological replicates plus standard deviation. Dotted bars indicate the pungency level according to (Eich 2008): one star - mildly pungent; two stars - highly pungent. Diamond symbol: not detectable.

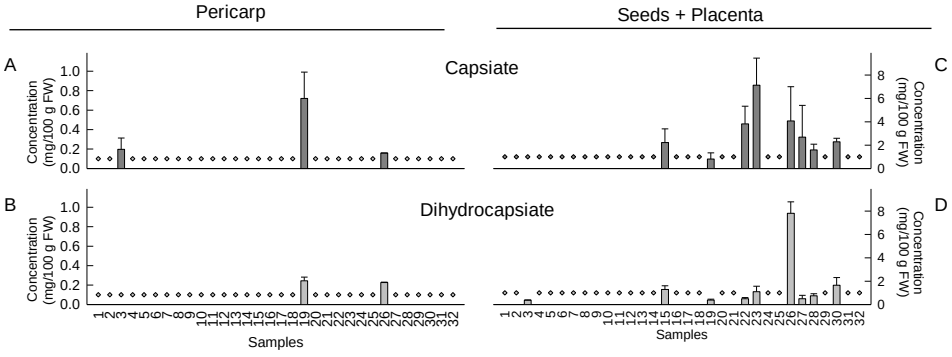


**Fig. 4** Levels of the capsinoids capsiate (A and C) and dihydrocapsiate (B and D) detected in pericarp (A and B) and placenta plus seeds (C and D) of 32 pepper accessions. Each bar represents the average of two biological replicates plus standard deviation. Diamond symbol: not detectable.

Capsinoids, the sweet analogues of capsaicinoids, were found at low (less than 1 mg/100 g FW) levels in the pericarp as well as in placenta plus seed tissue of some accessions. In the pericarp, three accessions had detectable levels of capsiate and/or dihydrocapsiate (Fig. 4A, B). Also capsinoids were more abundant in placenta plus seed tissues (Fig. 4C, D) than in the pericarp. Nine accessions accumulated capsinoids in placenta plus seed, up to 12 mg/100 g FW in *C. chinense* RU 72-194 (no. 26).

### 3.2.3. Flavonoids

Flavonoid *C*- and *O*-glycosides, previously reported by Marin et al. (2004), were detected and identified based on MS-MS fragmentation and UV spectra (Marin et al. 2004). Quercetin was only found in the form of *O*-glycosides. Of the four quercetin glycosides detected, quercetin 3-*O*-rhamnoside and quercetin 3-*O*-rhamnoside-7-*O*-glucoside have been previously reported in hot and sweet peppers (Marin et al. 2004; Materska et al. 2003). In addition, two new quercetin glycosides, quercetin 3-*O*-glucoside-7-*O*-rhamnoside and quercetin glycosylated with rhamnoside-glucoside attached either at the *C*-3 or *C*-7 position were found for the first time in this study (Table 2). Two luteolin *O*-glycosides, luteolin (apiosyl-acetyl)-glucoside and luteolin 7-*O*-(2-apiosyl)-glucoside (Table 2), and five luteolin *C*-glycosides, luteolin 6-*C*-hexoside, luteolin 8-*C*-hexoside, luteolin 6-*C*-pentoside-8-*C*-hexoside, luteolin 6-*C*-hexoside-8-*C*-pentoside and luteolin 6,8-di-*C*-hexoside (Table 3), were detected in the pericarp of pepper fruits. In addition, two apigenin *C*-glycosides were found: apigenin 6-*C*-pentoside-8-*C*-hexoside and apigenin 6,8-di-*C*-hexoside (Table 3).

The above-mentioned flavonoids were quantified by comparing their absorption at 340 nm with standard curves of commercially available standards representative for quercetin, luteolin, and apigenin*O*- and *C*-glycosides (as described in the Materials and Methods section). Both the composition and levels of the flavonoid glycosides varied among the set of 32 pepper accessions (Fig. 5A-D). Quercetin 3-*O*-rhamnoside and quercetin 3-*O*-rhamnoside-7-*O*-glucoside were the predominant quercetin conjugates present. Accession *C. annuum* Long Sweet (no. 12) had high levels of quercetin *O*-glycosides, in total up to 0.0403 mmol of quercetin 3-*O*-rutinoside equivalents/100 g FW (Fig. 5A). This accession was also extreme in the levels of luteolin *C*-glycosides, reaching up to 0.0068 mmol of luteolin 6-*C*-glucoside equivalents/100 g FW (Fig. 5B). Levels of luteolin *O*-glycosides and apigenin *C*-glycosides varied from undetectable levels, e.g. in the orange-fruited *C. chinense* RU 72-241 (no. 27), up to 0.0034 and 0.014 mmol of luteolin 6-*C*-glucoside equivalents/100 g FW, respectively (Fig. 5C-D).

**Table 2** LC-PDA-QTOF-MS-MS analysis of quercetin *O*-glycosides and luteolin *O*-glycosides in 32 pepper accessions.

| RT (min) | Formula | Observed mass | Theoretical mass | ppm error | Putative Name | MS-MS, m/z (%) | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | -42 | -(42+18) | -132 | -146 | -162 | $[Agl-H]^-$ |
| 12.920 | C27H30O16 | 609.1431 | 609.1461 | 4.9374 | Quercetin 3-*O*-rhamnoside-7-*O*-glucoside | | | | 463 (100) | 447 (59) | 301 (84) |
| 16.537 | C27H30O16 | 609.1437 | 609.1461 | 3.9524 | Quercetin 3-*O*-glucoside-7-*O*-rhamnoside | | | | 463 (1) | 447 (100) | 301 (39) |
| 17.190 | C26H28O15 | 579.1348 | 579.1355 | 1.2840 | Luteolin 7-*O*-(2-apiosyl)-glucoside | | | 447 (34) | | | 285 (100) |
| 18.556 | C27H30O16 | 609.1407 | 609.1461 | 8.8773 | Quercetin rhamnoside-glucoside | | | | | 447 (1) | 301 (100) |
| 19.238 | C21H20O11 | 447.0907 | 447.0933 | 5.7809 | Quercetin 3-*O*-rhamnoside | | | | | | 301 (100) |
| 19.530 | C28H30O16 | 621.1429 | 621.1461 | 0.0032 | Luteolin (apiosyl-acetyl)-glucoside | 579 (14) | 561 (5) | 489 (74) | | 459 (2) | 285 (100) |

**Table 3** LC-PDA-QTOF-MS-MS analysis of luteolin *C*-glycosides and apigenin *C*-glycosides 32 pepper accessions.

| RT (min) | Formula | Observed mass | Theoretical mass | ppm error | Putative Name | MS-MS, m/z (%) | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | -18 | -60 | -90 | -120 | Agl +113 | Agl +83 |
| 12.220 | C27H30O16 | 609.145 | 609.146 | 1.982 | Luteolin 6,8-di-*C*-hexoside | 591 (5) | | 519 (26) | 489 (100) | 399 (34) | 369 (30) |
| 13.230 | C26H28O15 | 579.133 | 579.136 | 3.701 | Luteolin 6-*C*-hexoside-8-*C*-pentoside | 561 (4) | 519 (5) | 489 (38) | 459 (100) | 399 (68) | 369 (48) |
| 13.390 | C27H30O15 | 593.149 | 593.151 | 4.373 | Apigenin 6,8-di-*C*-hexoside | 575 (6) | 533 (1) | 503 (29) | 473 (100) | 383 (34) | 353 (51) |
| 14.050 | C26H28O15 | 579.133 | 579.136 | 4.392 | Luteolin 6-*C*-pentoside-8-*C*-hexoside | 561 (4) | 519 (21) | 489 (100) | 459 (19) | 399 (78) | 369 (71) |
| 14.350 | C26H28O14 | 563.141 | 563.141 | 0.660 | Apigenin 6-*C*-pentoside-8-*C*-hexoside | 545 (15) | 503 (44) | 473 (80) | 443 (62) | 383 (73) | 353 (100) |
| 15.037 | C21H20O11 | 447.094 | 447.093 | 1.153 | Luteolin 6-*C*-hexoside | 429 (5) | | 357 (100) | 327 (83) | | |
| 15.499 | C21H20O11 | 447.092 | 447.093 | 2.426 | Luteolin 8-*C*-hexoside | | | 357 (27) | 327 (100) | | |

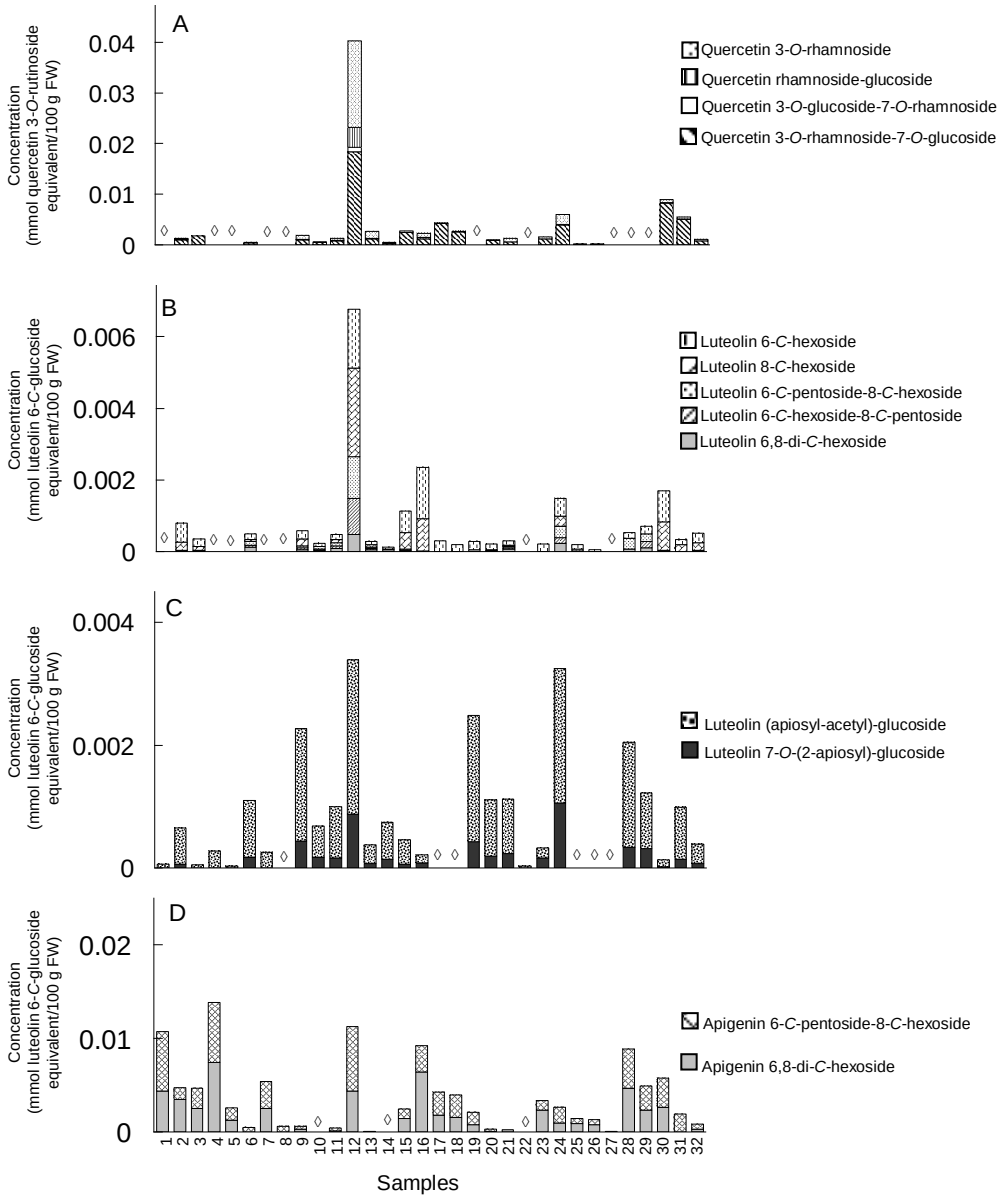


**Fig. 5** Content of quercetin *O*-glycosides (A), luteolin *C*-glycosides (B), luteolin *O*-glycosides (C) and apigenin *C*-glycosides (D) in the pericarp of 32 pepper accessions. Each bar represents the average of two biological replicates. Diamond symbol: not detectable.

3.2.4. Vitamins

Vitamin E (α- and β- tocopherols) was present at detectable levels in the pericarp tissue of all pepper accessions studied (Fig. 6A and B). Both the level and the relative abundance of α- and β- tocopherols varied between the accessions, but α-tocopherol was always the predominant form. The highest level of α-tocopherol was found in *C. chinense* AC2212 (no. 24), reaching up to 16.32 mg/100 g FW. The level of α-tocopherol in the other accessions varied from 0.84 mg/100 g FW in *C. chinense* RU 72-194 (no. 26) to 9.53 mg/100 g FW in *C. annuum* AC1979 (no. 19). The latter accession had the highest amount of β-tocopherol, containing up to 4.24 mg/100 g FW.

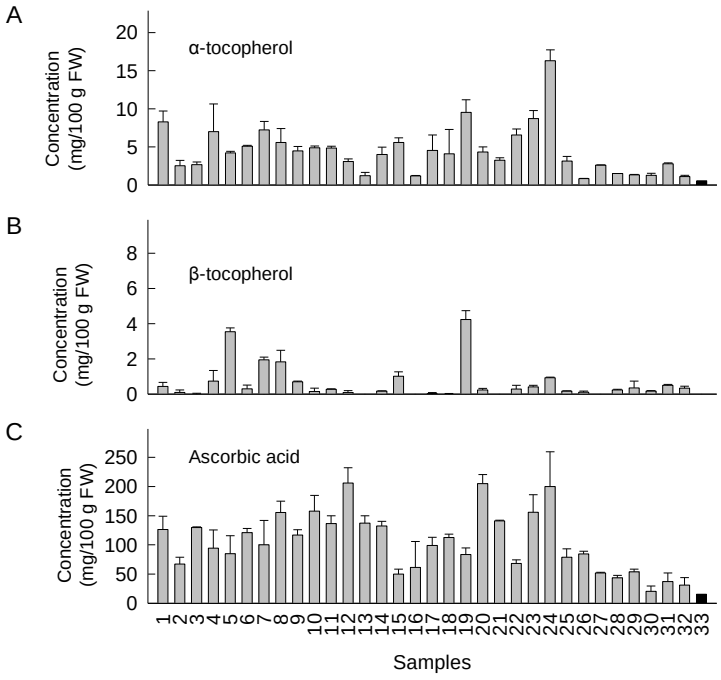


**Fig. 6** Concentration of α- and β-tocopherols (vitamin E) and ascorbic acid (vitamin C) in the pericarp of 32 pepper accessions (grey bars) compared to tomato (black bar). Each bar represents the average of two biological replicates plus standard deviation.

Vitamin C or ascorbic acid content varied from 20.45 mg/100 g FW in *C. baccatum* Aji Blanco Christal (no. 30) to 205.94 mg/100 g FW in *C. annuum* Long Sweet (no. 12) (Fig. 6C). Sixteen of 32 accessions, including the yellow, four brown

and some red accessions, had ascorbic acid levels equal to or above 100 mg/100 g FW. Of all accessions analysed, the four *C. baccatum* and two *C. frutescens* accessions contained the lowest levels of ascorbic acid (<50 mg/100 g FW).

Three carotenoids contribute to provitamin A activity, α-carotene, β-carotene and β-cryptoxanthin (IOM 2001). Based on the total of these carotenoids, provitamin A activity of red and brown accessions was higher than of yellow, salmon, and orange accessions. The provitamin A levels ranged from 18.52 mg/100 g FW in *C. annuum* I2 Tit Super (no. 1) up to trace amounts in *C. chinense* RU 72-194 (no. 26) and *C. chinense* RU 72-241 (no.27).

# 4. DISCUSSION

The composition and level of metabolites in ripe fruits varied greatly between the 32 *Capsicum* accessions analyzed and was independent of species, geographical location and environmental conditions. Apparently, the genetic diversity among the accessions contributes to the large variation in the biochemical composition of the pepper fruits. Accessions differed in fruit colour, a character which can be attributed to several pigments, including chlorophylls, carotenoids and flavonoids (Lightbourn et al. 2008). Although the genetics of fruit colour in pepper is not fully understood, a three loci model (*y*, *c1* and *c2*), has been proposed to explain the variation in carotenoid composition leading to the different fruit colours found in pepper (Hurtadohernandez and Smith 1985).

Red-fruited genotypes contain two red carotenoid pigments, capsanthin and capsorubin, which are characteristic for pepper (Minguez-Mosquera and Hornero-Mendez 1993; Paran and van der Knaap 2007). Capsanthin is the most abundant red pigment and was mainly found in the form of fatty acid esters (denoted capsanthin-esters compounds; Fig. 2). Capsanthin and capsorubin are end-products of the carotenoid pathway and are formed from antheraxanthin and violaxanthin, respectively. This conversion is catalysed by the bifunctional enzyme capsanthin-capsorubin synthase (CCS; Bouvier et al. 1994), encoded by the dominant *Y* locus (Lefebvre et al. 1998; Paran and van der Knaap 2007). In addition to capsanthin and capsorubin, red pepper fruits contain a number of minor carotenoids such as the orange α- and β-carotene, β-cryptoxanthin, antheraxanthin and zeaxanthin as well as the yellow violaxanthin and neoxanthin. These represent intermediates in the pathway leading to capsanthin and capsorubin and may contribute to the tone of the red fruit colour.

According to the three loci model, there are several gene combinations which lead to orange pepper fruit. Analysis of carotenoid profiles in seven different

orange pepper accessions indeed revealed that an orange colour can be derived from at least two different biochemical phenotypes: a genotype which produces high amounts of β-carotene and a genotype which produces a mixture of yellow- and red-coloured carotenoids, but at much lower levels than in red-fruited genotypes (Guzman et al. 2010). In our collection, the salmon (no. 26) and orange (no. 27) coloured-pepper fruits belonged to the latter group. This phenotype is, at least in part, due to a mutation in the *C2* locus, which in its recessive form leads to a 6-10-fold reduction in carotenoid pigments (Huh et al. 2001). There is strong genetic evidence that the *C2* locus encodes phytoene synthase (PSY), the first step in the carotenoid biosynthesis pathway (Ha et al. 2007; Huh et al. 2001).

The yellow fruited pepper accession (no. 9) did not contain any capsanthin and capsorubin. The yellow fruit trait has been correlated with a strongly reduced expression of *Ccs*. Several studies reported that the lack of *Ccs* expression may be due to either a partial deletion or the complete absence of the *Ccs* gene (Ha et al. 2007; Lang et al. 2004; Lefebvre et al. 1998; Popovsky and Paran 2000). Recently, a new *Ccs* variant (*Ccs-3* allele) was detected in the orange pepper cultivar Fogo, which accumulates β-carotene and zeaxanthin, but neither capsanthin nor capsorubin (Guzman et al. 2010). This *Ccs-3* allele contained a frameshift mutation in its coding region leading to a premature translation stop. The yellow accession no. 9 did not show any decrease in *Ccs* expression compared to red fruited genotypes, but contained the same *Ccs-3* allele as found in cv Fogo (data not shown), suggesting that a non-functional Ccs protein rather than a reduced expression causes the lack of red pigments in this accession. The yellow colour in accession no. 9, is due to accumulation of the Ccs precursor violaxanthin (free or as ester) and the α-carotenoid lutein, as reported by Azevedo-Meleiro and Rodrigueza-Amaya (2009).

Brown fruited pepper accessions accumulate typical red fruit carotenoids, such as capsanthin and capsorubin, in addition to chlorophyll b. Brown fruited pepper accessions have a single recessive *chlorophyll retainer* (*cl*) locus (Efrati et al. 2005; Roca et al. 2006) combined with a dominant allele of *Y (Ccs)*. This combination of genes allows red carotenoids and green chlorophylls to accumulate simultaneously in the ripe fruit and cause the brown colour (Borovsky and Paran 2008). One of the brown fruited accessions in our study, *C. chinense* AC2212 (no. 24) coincidently accumulated high levels of chlorophyll b and carotenoids. This phenotype resembles the so-called high pigment (*hp*) mutants in tomato, which contain increased levels of chlorophyll, carotenoids and flavonoids in their fruit (Bino et al. 2005). We intend to use a candidate gene approach to investigate if *C. chinense* AC2212 (no. 24) is an ortholog of a known tomato *hp* mutant.

Spiciness is caused by capsaicinoids, a group of pungent alkaloids which are synthesized and accumulate in hot pepper fruit (Nelson and Dawson 1923). For consumer's preference, breeders reduce or exclude this trait in commercial sweet pepper cultivars and in our studies all *C. annuum* breeders varieties indeed did not contain any significant levels of capsaicinoids in either pericarp or placenta plus seed tissues. In contrast, land races and wild accessions contained high levels of capsaicinoids. Pungency is controlled by *Pun1*, formerly known as the *C* locus, which is mapped on chromosome 2 (Blum et al. 2002; Mazourek et al. 2009; Stewart et al. 2005). *Pun1* encodes a putative acyltransferase (AT3), involved in the last step of capsaicin production, the condensation of vanillylamine with a short-chain fatty acid moiety (Curry et al. 1999; Mazourek et al. 2009; Stewart et al. 2005, 2007). Therefore, AT3 is also called capsaicin synthase (CS). The recessive *pun1* allele is the main cause for the absence of capsaicinoids in most sweet pepper accessions due to a large 2.5 kb deletion spanning the promoter region and the first exon of the *At3* gene, which leads to strongly reduced *At3* transcript accumulation (Lee et al. 2005a; Stewart et al. 2005, 2007). Recently, a second *pun1* allele was found in sweet pepper germplasm. This *pun1*$^{2}$ allele had a 4 bp deletion in the first exon resulting in a frameshift mutation leading to the production of a truncated protein (Stewart et al. 2007). In pungent accessions, quantitative variation in capsaicinoid levels may be due to genotypic variation at other loci (Abraham-Juarez et al. 2008; Ben-Chaim et al. 2006), differences in fruit maturation (Contreras-Padilla and Yahia 1998) or environmental effects (Harvell and Bosland 1997; Zewdi and Bosland 2000).

We observed that capsaicinoid levels in placenta plus seed tissues were higher than in pericarp. This is consistent with observations by Stewart et al. (2007), showing that capsaicin production is restricted to the epidermal cells of the interlocular septum (=placenta), due to the specific expression of capsaicinoid biosynthetic genes in this tissue, in particular *AT3* (Kim et al. 2009), but also *Pal* (phenylalanine ammonia lyase; Stewart et al. 2007), *Kas* (3-keto-acyl-acyl carrier protein synthase; Stewart et al. 2007) and *pAMT* (putative aminotransferase; Lang et al. 2009). The presence of capsaicinoids in the pericarp of our pungent accessions suggests that capsaicinoids are translocated from placenta to pericarp tissue via the cell walls of the epidermal layer of the placenta, as observed recently by Broderick and Cooke (2009). We can not exclude, however, that capsaicinoid levels in pericarp tissue are over-estimated due to contamination with placenta tissue. This may particularly be the case in very small-fruited accessions such as *C. baccatum* var. *baccatum* (no. 15), *C. annuum* AC1979 (no. 19), *C. frutescens* Lombok (no. 28), and *C. frutescens* Tabasco (no. 29).

Capsinoids are the non-pungent analogues of capsaicinoids and were first detected in the CH-19 Sweet cultivar, a non-pungent mutant derived from the pungent pepper strain, *C. annuum* CH-19 (Yazawa et al. 1989). In capsinoids vanillylalcohol, instead of vanillylamine, is used as substrate for condensation with the branched-chain fatty acid moiety, leading to an ester rather than an amide bond. Recent studies revealed that CH-19 Sweet carries a nonsense mutation in the *pAMT* gene, leading to a premature stop codon and loss of function of the encoded putative aminotransferase (pAMT) which catalyzes the amination of vanillin to produce vanillylamine, the immediate precursor of capsaicinoids in pungent peppers (Lang et al. 2009; Tanaka et al. 2010). As a consequence of the *pAMT* mutation, there is increased flux towards vanillylalcohol, which can be used as substrate for AT3 to form capsinoids. We found trace levels of capsiates in pungent accessions, but no detectable levels of capsiates in sweet peppers. The latter is likely due to the absence of a functional AT3 in sweet varieties. Three pungent accessions were detected to have capsinoids, i.e. capsiate and dihydrocapsiate, in the pericarp whereas in nine accessions, including the aforementioned three accessions, these compounds were detected in the placenta plus seed tissues. The capsinoids levels observed in those accessions were in line with those reported in Singh et al. (2009). The presence of capsaicinoids and capsinoids in pungent accessions was also found in other pungent pepper varieties (Lang et al. 2009; Yazawa et al. 1989) and suggests that the two branches of the pathway leading to vanillylalcohol and vanillinamide, respectively, exist naturally in pungent accessions (Lang et al. 2009). The fact that in the majority of accessions capsiate and dihydrocapsiate were restricted to placenta plus seed tissues indicates that these compounds are synthesized and accumulated in a similar way as capsaicinoids.

Studies concerning the quantification of flavonoids in pepper have been mainly based on acid hydrolysis (Howard et al. 2000; Lee et al. 1995). However, peppers also contain high levels of flavonoid *C*-glycosides which are not hydrolyzed under acidic conditions (Lee et al. 1995; Marin et al. 2004). We used LC-PDA-QTOF-MS-MS to determine the levels and composition of both *O*-glycosyl and *C*-glycosyl derivatives of the three major flavonoids in pepper fruit: quercetin, luteolin and apigenin. Quercetin glycosides were only present in the form of *O*-glycosides, of which quercetin 3-*O*-rhamnoside and quercetin 3-*O*-rhamnoside-7-*O*-glucoside were the most abundant in the set of 32 pepper accessions studied. In addition, we detected two apigenin *C*-glycosides as well as two *O*-glycosylated and five *C*-glycosylated forms of luteolin. In accordance with previous results (Howard and Wildman 2007; Lee et al. 2005b), we observed a large genotypic variation (up

to 2000-fold) in total flavonoid levels (the sum of all quercetin- , luteolin- and apigenin glycosides) and there was no correlation between levels of quercetin and luteolin glycosides, neither between levels of luteolin *O*- and luteolin *C* glycosides (results not shown). In addition, we did not observe any correlation of flavonoid levels with *Capsicum* type, as suggested by Howard et al. (2000), neither with fruit colour, as suggested by Sun et al. (2007). The red-fruited accession *C. annuum* Long Sweet (no.12) had outstanding total flavonoid levels, up to 0.12 mmol/100 g FW, consisting for two-third of quercetin-glycosides. The amount of quercetin (aglycone) in this accession was equal to 24.58 mg/100 g FW, a level comparable to those in yellow onions, which are regarded as a very good source of flavonols in the human diet (Hubbard et al. 2006; Manach et al. 2004). This accession would therefore be an interesting candidate for breeding programs aimed at the development of high flavonoid pepper varieties. In addition, we selected this accession to elucidate the molecular basis underlying the high-flavonol phenotype as part of a follow-up study.

Vitamin C (ascorbic acid), vitamin E (tocopherols), and provitamin A analyses revealed that *Capsicum* is a very good source of these three vitamins. Vitamin C levels were up to 10-fold higher than the levels found in tomato (Fig. 6), which is regarded as a good source of vitamin C (Padayatty et al. 2003). Vitamin C levels in our germplasm collection were fully in line with those observed in other studies (Howard et al. 1994; Khadi et al. 1987; Simonne et al. 1997). The level of α-tocopherol (vitamin E) measured in our accessions, especially in *C. chinense* AC2212 (no. 24), was moderately high compared to other edible plants, as reviewed by Ching and Mohamed (2001).

Three pepper carotenoids, α-carotene, β-carotene, and β-cryptoxanthin, contribute to provitamin A activity. Red and brown-fruited accessions, such as *C. annuum* Long Sweet (no. 12) and *C. chinense* AC2212 (no. 24), respectively, contained relatively high levels of these carotenoids and likewise have a high level of provitamin A equivalents. Yellow, orange and salmon-fruited accessions contained significantly lower levels of one or more of the above-mentioned carotenoids and therefore are a poorer source of provitamin A.

## 5. CONCLUDING REMARKS

Our results showed that levels of the metabolites analyzed vary greatly among fruits of different accessions, demonstrating the potential of the current germplasm collection for genetic improvement of metabolic traits. Two accessions, *C. annuum* Long Sweet (no.12) and *C. chinense* AC2212 (no.24) contained fruits

with outstanding levels of several health-related compounds and therefore are potential candidates for breeding programs aimed at developing new pepper cultivars with improved consumer quality characteristics. In a follow-up study, we will focus on the molecular and genetic regulation of the biosynthetic pathways underlying these health-related compounds.

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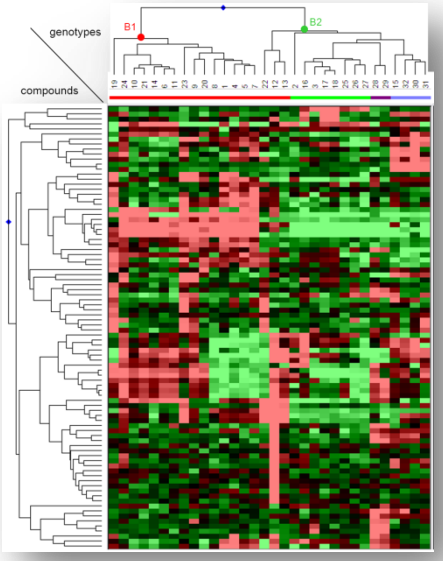
# CHAPTER 3

## Metabolomics and Molecular Marker Analysis to Explore Pepper (*Capsicum* sp.) Biodiversity

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## ABSTRACT

An overview of the metabolic diversity in ripe fruits of a collection of 32 diverse pepper (*Capsicum* sp.) accessions was obtained by measuring the composition of both semi-polar and volatile metabolites in fruit pericarp, using untargeted LC-MS and headspace GC-MS platforms, respectively. Accessions represented *C. annuum*, *C. chinense*, *C. frutescens* and *C. baccatum* species, which were selected based on variation in morphological characters, pungency and geographic origin. Genotypic analysis using AFLP markers confirmed the phylogenetic clustering of accessions according to *Capsicum* species and separated *C. baccatum* from the *C. annuum*–*C. chinense*–*C. frutescens* complex. Species-specific clustering was also observed when accessions were grouped based on their semi-polar metabolite profiles. In total 88 semi-polar metabolites could be putatively identified. A large proportion of these metabolites represented conjugates of the main pepper flavonoids (quercetin, apigenin and luteolin) decorated with different sugar groups at different positions along the aglycone. In addition, a large group of acyclic diterpenoid glycosides, called capsianosides, was found to be highly abundant in all *C. annuum* genotypes. In contrast to the variation in semi-polar metabolites, the variation in volatiles corresponded well to the differences in pungency between the accessions. This was particularly true for branched fatty acid esters present in pungent accessions, which may reflect the activity through the acyl branch of the metabolic pathway leading to capsaicinoids. In addition, large genetic variation was observed for many well-established pepper aroma compounds. These profiling data can be used in breeding programs aimed at improving metabolite-based quality traits such as flavour and health-related metabolites in pepper fruits.


**Keywords**:
*Capsicum*; metabolite profiling; semi-polar compounds; volatiles; pungency; AFLP

# 1. INTRODUCTION



Pepper (*Capsicum* spp.) is one of the most important fruit crops worldwide. It is cultivated all over the world, primarily in the tropical and subtropical countries. Pepper fruit production reached up to 1.8 million HA with more than 28 million metric tonnes harvested from the crop (FAO 2011). The fruits are mainly consumed as a fresh or cooked vegetable, a condiment or a spice and used as a colouring agent in the food industry. Pepper is increasingly recognized as an excellent source of health-related metabolites, such as ascorbic acid (vitamin C), carotenoids (provitamin A), tocopherols (vitamin E), flavonoids and capsaicinoids (Howard and Wildman 2007). We recently showed that different *Capsicum* accessions display a large variation in morphological characters as well as in the levels and composition of the above-mentioned health-related metabolites (Wahyuni et al. 2011). The accessions belong to four widely cultivated species: *Capsicum annuum*, *Capsicum chinense*, *Capsicum baccatum*, and *Capsicum frutescens*. These four species can be intercrossed and produce fertile hybrids (Pickersgill 1997). Variation in morphological characters included fruit features, such as colour, type, shape and size. Fruit colour in the accessions varied from red, orange and yellow to brown and salmon and this was determined by the accumulation of specific carotenoids, i.e. capsanthin and capsanthin-esters for red accessions, violaxanthin and violaxanthin-esters for yellow accession. Fruit spiciness or pungency is determined by total levels of capsaicinoids, which varied strongly among the accessions analyzed (Wahyuni et al. 2011). Based on the range of capsaicinoid levels the accessions can be categorized into non-pungent, low, mild and highly pungent (Howard and Wildman 2007).

While the targeted metabolite analyses described above focused on the analyses of specific groups of known pepper compounds, untargeted metabolomics approaches based on mass spectrometry (MS) allow the simultaneous detection of metabolites in a biological sample, without *a priori* knowledge of the identity of the metabolites detected. Such untargeted profiling approaches have been used to obtain an overview of the metabolic diversity in germplasm collections, such as *Arabidopsis* (Keurentjes et al. 2006) and tomato (Tikunov et al. 2005). These analyses may help to understand metabolic pathways underlying the main metabolic contrasts between genotypes and, in combination with genetic analyses, to identify mQTLs and genes underlying key steps in metabolic pathways. In addition, metabolic profiling allows the detection and subsequent identification of unknown compounds correlating with a trait of interest, such as pungency, colour or flavour. There is no doubt that the concept of metabolomics-assisted breeding is

a novel and powerful approach leading to new targets for breeding programs aimed at the improvement of metabolite-based quality traits (Fernie and Schauer 2009).

In addition to phenotypic information, knowledge about genetic diversity among accessions is also important to maximize the rate of crop improvement (Geleta et al. 2005). Genetic diversity can be measured using molecular markers and one of the methods is amplified fragment length polymorphism (AFLP) developed by Vos et al. (1995), which has been used extensively for genetic mapping and fingerprinting in plants. Combining genetic and phenotypic information, such as metabolite profile, may help breeders to develop new varieties with desired specific metabolite compositions and related traits.

Here we present a broad overview of the metabolic variation in the fruits of a collection of 32 diverse pepper accessions, by measuring the composition of semi-polar and volatile metabolites using LC-MS and headspace GC-MS, respectively. Our results showed that the diversity of volatile and semi-polar metabolites detected in the accessions separated them according to species and pungency, which correlated well with the phylogenetic relationships measured using AFLP molecular markers. Metabolite identification revealed the metabolic pathways underlying the metabolic differences between genotypes, including several known flavour-related pathways. This result offers both new sources and novel traits for the genetic improvement of the quality of cultivated pepper.

# 2. MATERIALS AND METHODS

## 2.1. Plant materials and sampling protocol

A total of 32 accessions from *Capsicum annuum*, *Capsicum chinense*, *Capsicum baccatum* and *Capsicum frutescens* were selected based on the variations in morphological characters and geographical origins as presented in Table 1 (Wahyuni et al. 2011). Plant growth condition, experimental design and sampling protocol were performed as described by Wahyuni et al. (2011). Briefly, the experimental design consisted of two randomized blocks, each comprising all 32 accessions grown in plots of four plants. Fruits of two biological replicates, consisting of 10-50 ripe fruits (depending on fruit size) from the two innermost plants of a plot, were harvested for each accession. Before harvesting fruits for metabolic analyses, we carefully followed the ripening stages of the first set of fruits produced by each accession.

**Table 1** Description of 32 *Capsicum* accessions

| Accession | Species | Accession Name | Population Type | Fruit Type | Pungency[a] |
|---|---|---|---|---|---|
| 1 | *C. annuum* | I2 Tit super | n.d. | Pointed | Low pungent |
| 2 | *C. chinense* | I1 PI 281428 | n.d. | Roundish | Mildly pungent |
| 3 | *C. chinense* | I1 PI 315023 (Mishme Black) | n.d. | Conical | Mildly pungent |
| 4 | *C. annuum* | Laris HS | Breeders variety | Pointed | Low pungent |
| 5 | *C. annuum* | Jatilaba | Breeders variety | Pointed | Low pungent |
| 6 | *C. annuum* | Bruinsma Wonder | Breeders variety | Blocky/bell | Low pungent |
| 7 | *C. annuum* | PBC 473 - none – cayenne | n.d. | Pointed | Low pungent |
| 8 | *C. annuum* | PBC 535 - IR - 12x1cm - cayenne | n.d. | Pointed | Low pungent |
| 9 | *C. annuum* | Bisbas | Land variety | Other | Mildly pungent |
| 10 | *C. annuum* | California Wonder 300 | Breeders variety | Blocky/bell | Non pungent |
| 11 | *C. annuum* | Keystone Resistant Giant | Breeders variety | Blocky/bell | Non pungent |
| 12 | *C. annuum* | Long Sweet | Land variety | Pointed | Non pungent |
| 13 | *C. annuum* | Sweet Banana | Breeders variety | Pointed | Non pungent |
| 14 | *C. annuum* | Yolo Wonder L | Breeders variety | Blocky/bell | Non pungent |
| 15 | *C. baccatum* var. *baccatum* | No. 1553 | Wild variety | Pointed | Highly pungent |
| 16 | *C. chinense* | Miscucho colorado; PI 152225; 1SCA no.6 | n.d. | Conical | Mildly pungent |
| 17 | *C. chinense* | No.4661; PI 159236 | n.d. | Pointed | Low pungent |
| 18 | *C. chinense* | No.4661 Selection; PI 159236 Selection | Research material | Pointed | Low pungent |
| 19 | *C. annuum* | AC1979 | Wild variety | Conical | Highly pungent |
| 20 | *C. annuum* | CM 331; Criollos de Morelos | Land variety | Conical | Non pungent |
| 21 | *C. annuum* | Sweet Chocolate | Breeders variety | Roundish | Non pungent |
| 22 | *C. annuum* | Chili Serrano; PI 281367; No. 999 | Land variety | Pointed | Mildly pungent |
| 23 | *C. annuum* | Chili de Arbol; PI 281370; No. 1184 | Land variety | Other | Mildly pungent |
| 24 | *C. chinense* | AC2212 | n.d. | Pointed | Low pungent |
| 25 | *C. chinense* | No.1720; PI 281426; 1GAA | Land variety | Pointed | Mildly pungent |
| 26 | *C. chinense* | RU 72-194 | Land variety | Roundish | Mildly pungent |
| 27 | *C. chinense* | RU 72-241 | Land variety | Other | Mildly pungent |
| 28 | *C. frutescens* | Lombok | Land variety | Pointed | Highly pungent |
| 29 | *C. frutescens* | Tabasco | Breeders variety | Pointed | Highly pungent |
| 30 | *C. baccatum* var. *pendulum* | Aji Blanco Christal; CAP 333 | Breeders variety | Pointed | Low pungent |
| 31 | *C. baccatum* var. *pendulum* | | n.d. | Pointed | Mildly pungent |
| 32 | *C. baccatum* var. *pendulum* | RU 72-51 | Land variety | Other | Low pungent |

[a] Denotes that the information is derived from the previous report of Wahyuni et al. (2011)
n.d.: data not available

Fruit ripeness was indicated by a combination of (i) its mature colour based on the description in the CGN database, (ii) fruit firmness and (iii) number of days after fruit setting (on average 70-80 days after fruit setting). Young leaves and fruit pericarp tissue (separated from placenta plus seeds) were collected, frozen in liquid nitrogen, ground and stored at -80 $^{0}$C prior to analysis.

### 2.2. Genomic DNA extraction and AFLP analysis

Genomic DNA from young pepper leaves was extracted by using the automated protocol of AGOWA® mag Maxi DNA isolation kit and KingFisher 96 instrument (LGC Genomics). AFLP analysis was performed according to the protocol previously described by Vos et al. (1995). In brief, 250 ng of genomic DNA was used for restriction/ligation reaction with two restriction enzymes *Eco*RI and *Mse*I. In order to selectively amplify a smaller number of genomic DNA fragments, a pre-amplification was performed using two primers: E01 (5'-GAC TGC GTA CGA ATT CA-3') and M02 (5'-GAT GAG TCC TGA GTA AC-3'). Amplification primers were selected as followed: E32 (E01+AAC), E35 (E01+ACA), E36 (E01+ACC), E40 (E01+AGC), M47 (M02+CAA), M49 (M02+CAG), M59 (M02+CTA) and M61 (M02+GCG). Primers E32, E36 and E40 were labeled with fluorescent IRD700 and primer E35 was labeled with fluorescent IRD 800 dye (LI-COR®, Lincoln, USA). DNA fragments were amplified using five primer combinations of E40–M49, E36–M47, E35–M61, E35–M59 and E32–M49. AFLP products were separated and visualized on 6.5% denaturing polyacrylamide gel using LI-COR 4200 Global System® sequencer. AFLP bands were scored based on the presence or absence of amplified DNA fragments on gel by Quantar software (Keygene®). Genetic distance was calculated and constructed using unweighted pair group mean average (UPGMA) and Jaccard similarity coefficient in Genemath XT version 1.6.1 software (www.applied-math.com). The reliability of the hierarchical cluster analysis (HCA) was tested using bootstrap analysis with 100 replications.

### 2.3. Extraction and analysis of semi-polar metabolites

Semi polar metabolites were extracted using the protocol described previously by de Vos et al. (2007). Briefly, 500 mg of freeze-ground material of pepper pericarp were extracted with 1.5 ml of 99.875% methanol acidified with 0.125% formic acid. The extracts were sonicated for 15 min and filtered through a 0.2 µm polytetrafluoroethylene (PTFE) filter. For each accession two biological replicates were prepared, resulting in a total of 64 extracts. To check the technical variation, including extraction, sample analysis and data-processing, quality

control samples were prepared by pooling fruit material of several randomly chosen accessions, extracted using the same procedure and injected after every 16 accession sample extracts.

All the extracts were analysed using reversed phase liquid chromatography coupled to a photodiode array detector and a quadrupole time of flight high-resolution mass spectrometry (LC-PDA-QTOF-MS) system, using C18-reversed phase chromatography and negative electrospray ionization, as described previously (de Vos et al. 2007). For LC-PDA-QTOF-MS, 5 µl of the extract were injected and separated using a binary gradient of ultrapure water (A) and acetonitrile (B), both acidified with 0.1% formic acid, with a flow rate of 0.19 ml/min. The initial solvent composition consisted of 95% of A and 5% of B; increased linearly to 35% A and 65% B in 45 min and maintained for 2 min. The column was washed with 25% A and 75% B for 5 min and equilibrated to 95% A and 5% B for 2 min before the next injection.



The putative identification of differential semi-polar metabolites was performed by re-injection of the extract from one of the biological replicates of each accession on a LC-LTQ-Orbitrap FTMS hybrid mass spectrometer (Thermo Fisher Scientific) and performing $MS^n$ fragmentation The LTQ-Orbitrap hybrid mass spectrometer system was set up in negative ionization mode, as previously described by van der Hooft et al. (2011). Xcalibur software (Thermo Fisher Scientific) was used to control all instruments and for data acquisition and data analysis.

### 2.4. Extraction and analysis of volatile metabolites

Volatile metabolites were extracted using the protocol previously described by Tikunov et al. (2005). Briefly, 750 mg of freeze-ground pepper pericarp of each sample was weighed in a 5-ml screw-cap vial, closed, and incubated at 30 °C for 10 min in a water bath. An aqueous 0.75 ml of EDTA-NaOH solution (100 mM EDTA solution pH adjusted to 7.5 with NaOH) was added to the incubated sample and followed by the addition of solid $CaCl_2$ (5 M final concentration). The closed vials were sonicated for 5 min and 1 ml of the extract was transferred into a 10-ml crimp vial (Waters), capped, and used directly for headspace SPME-GC-MS analysis as described in Tikunov et al. (2005). As for LC-MS, quality control samples were prepared after eight accession samples to analyse the analytical variation. Each accession was analysed with two biological replicates.

## 2.5. Metabolite data processing

Both volatile and semi-polar data were processed separately through some steps as described in several points below:

### 2.5.1. Mass spectral alignment, filtering and clustering

Volatile and semi-polar metabolite profiles derived using the SPME-GC-MS and the LC-PDA-QTOF-MS platforms, respectively, were processed as described by Tikunov et al. (2005, 2010). Both datasets were processed independently by the MetAlign software package (www.metalign.nl) for baseline correction, noise estimation, and ion-wise mass spectral alignment. The MetAlign outputs for both the GC-MS and LC-MS data were processed separately with MSClust software for data reduction and compounds mass spectra extraction (Tikunov et al. 2011).

### 2.5.2. Putative identification of semi-polar and volatile metabolites

The identification of semi-polar metabolites was carried out by means of their UV spectra, exact molecular weight and $MS^n$ fragmentation pattern. Putative identification of semi-polar metabolites was obtained using different metabolite databases such as Dictionary of Natural Products (http://dnp.chemnetbase.com), KNApSAcK (http://kanaya.naist.jp/KNApSAcK) and in-house metabolite databases, and using previous results on pepper described by Marin et al. (2004) and Wahyuni et al. (2011).

Putative identification of volatile metabolites was performed by automatic matching of their mass spectra extracted by MSClust with the National Institute of Standards and Technology (NIST) mass spectral library entries, using the NIST MS Search v2.0 software (http://chemdata.nist.gov/mass-spc/ms-search/). The compound hit that showed the highest matching factor (MF) value ($\geq$600) and the lowest deviation from the retention index (RI) value was used for the putative metabolite identity. Additional manual spectral matching was performed for selected metabolites with low MF and high RI deviations by deconvoluting the chromatographic peak at the corresponding retention time using AMDIS (version 2.64) (http://www.amdis.net/) followed by matching the resulting spectra with those in the NIST library database.

### 2.5.3. Multivariate analysis

Semi-polar and volatile metabolite data sets containing the intensity levels of all centrotypes for all pepper samples were analysed separately using multivariate

statistical analyses included at the Genemath XT version 1.6.1 software. Pre-treatment of the data was performed by $log_2$ transformation and mean centering. The pre-treatment data were subjected to analysis of variance (ANOVA) and multiple testing error rates (Bonferroni procedure) to determine the least significance variances among pepper accessions. Metabolites that showed significant differences between accessions, determined by *p*-values lower than 0.001, were subjected to principal component analysis (PCA) and hierarchical cluster analysis (HCA). HCA was performed by using the UPGMA method and Pearson's coefficient matrix in Genemath XT software. To test the reliability of the dendrogram produced by HCA, bootstrap analysis was performed with 100 replications.

# 3. RESULTS

## 3.1. Genetic diversity of *Capsicum* accessions based on AFLP analysis

The collection of 32 pepper accessions derived from diverse origins (Table 1). To get insight into the genetic relationships, all plants were genotyped using AFLP analysis. Screening of the accessions with five *Eco*RI x *Mse*I primer combinations resulted in the identification of 255 polymorphic bands. A dendrogram resulting from HCA was constructed by counting the presence or absence of these polymorphic bands. The dendrogram showed that the 32 accessions were separated based on *Capsicum* species (Fig. 1). Two main clusters were observed, differentiating between *C. baccatum*, denoted as A-1, and the *C. annuum–C. frutescens–C. chinense* cluster, denoted as A-2. Cluster A-1 grouped all *C. baccatum* accessions analysed, including *C. baccatum* var. *baccatum* (no. 15) and three accessions of *C. baccatum* var. *pendulum*: Aji Blanco Christal (no. 30), no. 31, and RU 72-51 (no. 32). Cluster A-2 contained two sub-clusters that distinguished all *C. annuum* accessions from *C. frutescens–C. chinense*.

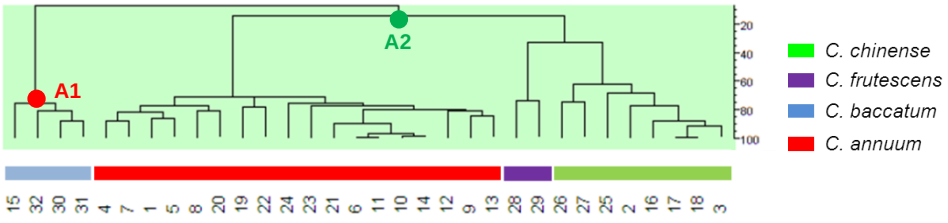


**Fig. 1** Dendrogram resulting from a hierarchal cluster analysis (HCA) based on the distribution patterns of the AFLP markers. The numbers at the bottom of the figure correspond to the accession numbers shown in the first column of Table 1.

In the *C. frutescens*–*C. chinense* sub-cluster, the two *C. frutescens* accessions, Lombok (no. 28) and Tabasco (no. 29) clustered together and were separated from the *C. chinense* cluster. Within the *C. annuum* sub-cluster, several subgroups could be observed, e.g. accessions with pointed fruits (accession no. 4, 7, 1, 5, 8, and 20) and the blocky-type breeding varieties (accession no. 21, 6, 11, 10 and 14). In addition, we observed that accession *C. chinense* AC2212 (no. 24) was positioned in the *C. annuum* cluster, suggesting that this accession, genetically, more likely belongs to *C. annuum* than to *C. chinense*. Based on this dendrogram, *C. chinense* AC2212 (no. 24) is denoted as *C. annuum* for further discussions.

## 3.2. Principal component and hierarchical cluster analyses of *Capsicum* accessions based on their metabolites profiles

In order to explore the metabolic variation in pepper fruits, two different analytical platforms were used: LC-PDA-QTOF-MS for the detection of semi-polar metabolites, such as alkaloids, flavonoids and other phenolic compounds, and headspace GC-MS to detect flavour-related volatiles.

### 3.2.1. Semi-polar metabolites

Pericarp extracts from ripe fruits of the 32 pepper accessions were analysed by LC-PDA-QTOF-MS and the corresponding chromatograms were subjected to full mass spectral alignment using MetAlign software followed by filtering out of low intensity ions. Using the MSClust algorithm, a total of 11,372 ions were grouped into 881 ion clusters, representing reconstructed metabolites. From each mass cluster, the most abundant ion was selected as the representative of each putative compound and subsequently used for analysis of variance. In total 297 compounds showed significant intensity differences (ANOVA; $p < 0.001$) between *Capsicum* accessions. These were used for further analyses, supplemented with 34 additional metabolites, which did not meet the above criteria, but for which a putative identification was obtained (Supplemental Table S1).

PCA revealed a separation of the 32 pepper accessions into species groups, based on their semi-polar metabolite patterns (Fig. 2a). The first principal component (PC1) explained 43.4% of the variation and separated a group of 15 *C. annuum* accessions, denoted as *C. annuum*-spI, from the other 17 *Capsicum* accessions. The second principal component (PC2) explained 9.6% of the variation and separated the *C. chinense* group from *C. baccatum*, *C. frutescens* and the sub-group of *C. annuum*, denoted as *C. annuum*-spII. The latter group contained *C.*

*annuum* Long Sweet (no. 12), *C. annuum* Sweet Banana (no. 13) and *C. annuum* Chili Serrano (no. 22).

The dendrogram produced by HCA of the pepper accessions, based on the variation in 331 semi-polar metabolites (Fig. 3), confirmed the results observed with PCA and provided a more detailed view on the relationships between accessions. HCA distinguished two major clusters, denoted as B-1 and B-2. Cluster B-1 consisted of the *C. annuum*-spI accessions and showed a clear separation between the wild variety *C. annuum* AC1979 (no. 19) and the land races and breeders' varieties in this group. Cluster B-2 contained two sub-groups, one consisting of the *C. annuum*-spII and the second one separating the *C. chinense*, *C. frutescens*, and *C. baccatum* accessions, conform the PCA results. Accession no. 2, *C. chinense* I1 PI 281428, which in the PCA plot was located in close proximity to the *C. annuum*-spII group (Fig. 2a), was biochemically different from the other *C. chinense* accessions and forms an outlier in cluster B2.



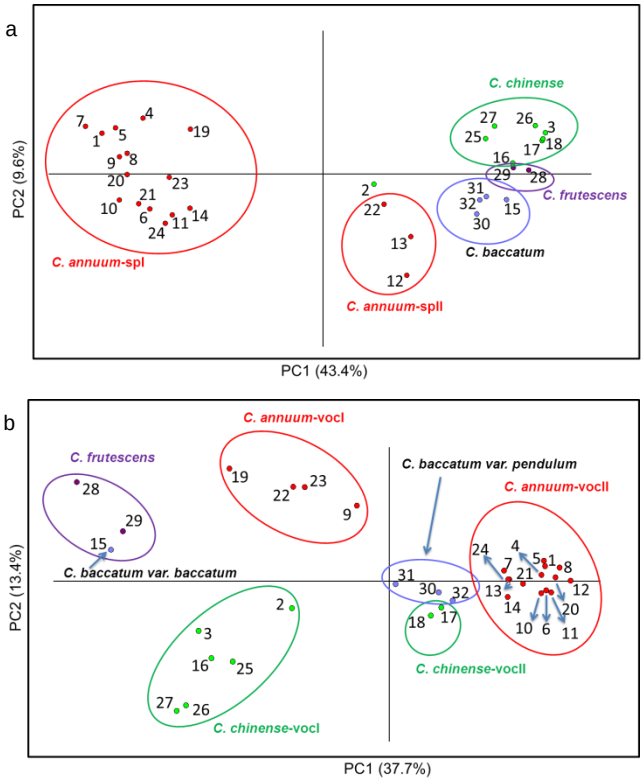


**Fig. 2** Principal Component Analysis (PCA) of pepper accessions based on semi-polar (a) and volatile (b) metabolite profiles. The numbers in PCA correspond to the accession numbers as shown in the first column of Table 1.

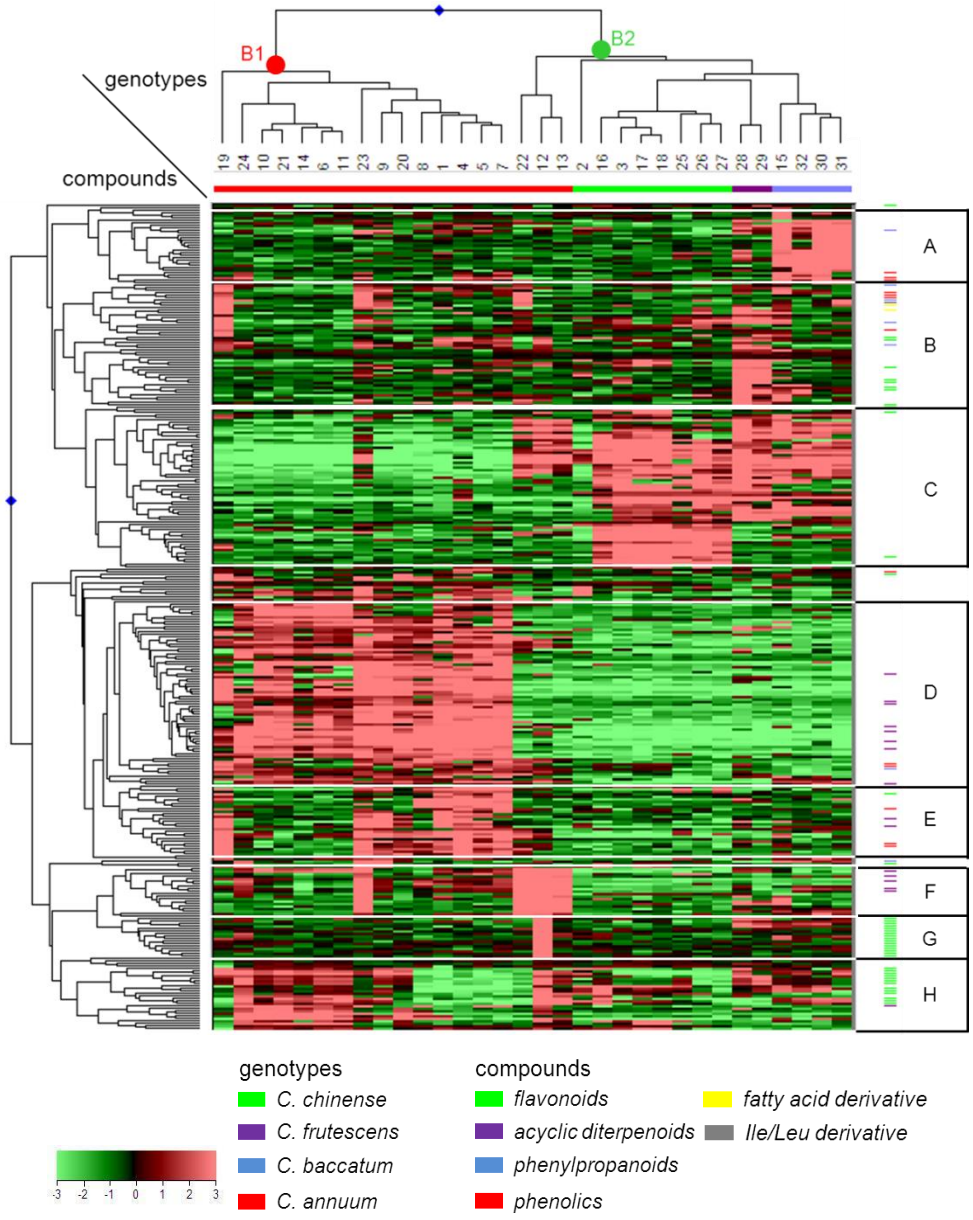


**Fig. 3** Heat map of 331 semi-polar metabolites in 32 pepper accessions. A color-coded matrix represents the mean values of the metabolite intensity in two biological replicates of pepper accessions, which has been $\log_2$ transformed and mean-centered. The alphabetes (A-H) represent metabolite clusters. Characteristics of the underlying metabolites are presented in the Supplemental Table S1. The numbers below the dendrogram correspond to the accession numbers in the first column of Table 1.

HCA of the set of 331 semi-polar metabolites revealed the presence of several metabolite groups, arbitrarily denoted A to H, characterised by their specific expression pattern across the 32 pepper accessions (Fig. 3). By using different metabolite databases, in combination with accurate mass $MS^n$ fragmentation experiments, we could putatively identify in total 88 out of the 331 semi-polar metabolites (Supplemental Table S1). These metabolites belonged to a number of compound classes, such as flavonoids, phenylpropanoids, phenolics, acyclic diterpenoids, branched chain amino acid derivatives and fatty acid derivatives. Structurally related metabolites derived from the same metabolic pathway clustered together, as shown previously for volatiles in tomato (Tikunov et al. 2005) and pepper (Eggink et al. 2011). Compared to the three other species, all *C. annuum* accessions accumulated relatively high levels of the acyclic diterpenoid phytatetraene, which was present in the form of different conjugated derivatives (groups D, E and F). These diterpenoid glycosides are also called capsianosides (De Marino et al. 2006; Izumitani et al. 1990; Lee et al. 2009) and currently receive increasing interest for their presumed antioxidant (Shin et al. 2012) and pharmaceutical activities (Hashimoto et al. 1997). The difference between *C. annuum*-spI (cluster B-1) and *C. annuum*-spII (part of cluster B-2) was mainly due to variation in the decoration of capsianosides, as shown in metabolite groups D and E versus F, respectively. The *C. baccatum* group could be distinguished from all other genotypes due relatively high levels of several phenolic and phenylpropanoid glycosides, i.e. several coumaroyl, feruloyl and benzyl glycosides. Interestingly, three of these compounds had a sulfate conjugation as well. In addition, the *C. baccatum* accessions contained several unique double charged compounds which we have not been able to annotate so far.

A large proportion of the identified metabolites consisted of flavonoid glycosides. These represented different intermediates in the biosynthetic pathway leading from chalcones (naringenin chalcone), through flavanones to the major pepper flavonoids belonging to both flavones (luteolin and apigenin) and flavonols (quercetin and kaempferol). The abundance of specific flavonoid glycosides appeared to be, at least partly, responsible for the separation of *C. chinense* and *C. frutescens* from the other species (group B and C). Other flavonoid glycosides showed accumulation patterns which were subspecies or genotype-specific rather than species-specific (Group G and H). This was most obvious for *C. annuum* Long Sweet (no. 12), which contained strongly elevated levels of various flavanone, flavone and flavonol glycosides. This accession was previously shown to be a high-flavonoid pepper accession (Wahyuni et al. 2011). Unfortunately, it was not possible to see a species- or genotype-specific pattern in the flavonoid conjugation

suggestive of the presence of specific modifying enzymes in certain genotypes or species groups.

Capsaicinoids were generally not detected in the LC-MS set-up used. However, we could detect one capsaicin analogue (Fig. 3; group B). The levels of this compound showed a very high correlation with the total capsaicinoid content in pericarp ($R^2$=0.98; Table 2), which was measured previously (Wahyuni et al. 2011). Other metabolites present in the same cluster showed a good correlation with capsaicinoid content as well (Table 2) and, interestingly, those metabolites for which we had a putative annotation all derived from the phenylpropanoid and the branched chain fatty acid pathway (consisting of methyl-branched amino acid degradation and fatty acid synthesis modules), both of which also serve to provide the immediate precursors for capsaicin biosynthesis (Mazourek et al. 2009).

**Table 2** Pearson correlation of volatile and semi-polar metabolites with total capsaicinoid levels in pericarp, placenta & seeds and with the capsaicin analogue detected by LCMS

| Type | Ret (min) | Mass | RI | Putative identity | HCA cluster | Correlation to capsaicin pericarp | Correlation to capsaicin seed and placenta | Correlation to capsaicin analog LCMS |
|---|---|---|---|---|---|---|---|---|
| Volatile | 38.7 | 55 | 1657.50 | 9-Tetradecenal, (Z)- | A | 0.90 | 0.53 | 0.88 |
| | 27.3 | 70 | 1253.70 | Heptyl isobutanoate | A | 0.86 | 0.41 | 0.82 |
| | 29.9 | 82 | 1332.70 | Hexanoic acid, 3-hexenyl ester, (Z)- | A | 0.85 | 0.57 | 0.79 |
| | 25.0 | 71 | 1187.30 | Butanoic acid, hexyl ester | A | 0.85 | 0.59 | 0.83 |
| | 23.8 | 56 | 1153.40 | Hexyl 2-methylpropanoate | A | 0.82 | 0.66 | 0.82 |
| | 38.8 | 41 | 1664.00 | Oxacyclotetradecan-2-one | A | 0.81 | 0.71 | 0.81 |
| | 21.7 | 71 | 1093.60 | Butanoic acid, pentyl ester | A | 0.81 | 0.36 | 0.79 |
| | 38.8 | 55 | 1662.00 | Oxacyclotetradecan-2-one | A | 0.80 | 0.72 | 0.81 |
| | 24.9 | 64 | 1183.00 | cis-3-Hexenyl Butyrate | A | 0.79 | 0.36 | 0.75 |
| | 23.3 | 103 | 1138.00 | Pentyl 2-methylbutanoate | A | 0.79 | 0.73 | 0.81 |
| | 27.0 | 56 | 1244.70 | Hexyl *n*-valerate | A | 0.78 | 0.70 | 0.78 |
| | 20.2 | 43 | 1051.40 | Pentylisobutyrate | A | 0.78 | 0.59 | 0.81 |
| | 28.1 | 56 | 1278.60 | Hexyl *n*-valerate | A | 0.78 | 0.58 | 0.78 |
| | 22.0 | 70 | 1101.70 | Isopentyl 2-methylbutanoate | A | 0.78 | 0.71 | 0.79 |
| | 16.7 | 43 | 956.08 | 3-Heptanone, 5-methyl- | A | 0.77 | 0.29 | 0.67 |
| | 23.7 | 68 | 1148.30 | 3-Methyl-3-butenyl 3-methylbutanoate | A | 0.76 | 0.71 | 0.79 |
| | 39.4 | 43 | 1683.60 | 13-Tetradecanolide | A | 0.76 | 0.63 | 0.77 |
| | 23.7 | 70 | 1150.60 | iso-Amyl isovalerate | A | 0.75 | 0.58 | 0.75 |
| | 33.8 | 55 | 1466.50 | 1-Pentadecene | A | 0.74 | 0.69 | 0.78 |
| | 35.6 | 56 | 1530.80 | 1-Tridecanol | A | 0.74 | 0.66 | 0.74 |
| | 33.8 | 43 | 1468.20 | *n*-Heptyl hexanoate | A | 0.73 | 0.65 | 0.76 |

**Table 2** *Continued*

| Type | Ret (min) | Mass | RI | Putative identity | HCA cluster | Correlation to capsaicin pericarp | Correlation to capsaicin seed and placenta | Correlation to capsaicin analog LCMS |
|---|---|---|---|---|---|---|---|---|
| Volatile | 18.8 | 43 | 1014.90 | 3-Methyl-1-butanol, 2-methylpropanoate | A | 0.71 | 0.60 | 0.72 |
| | 38.5 | 43 | 1647.50 | Hexadecane, 2-methyl- | A | 0.70 | 0.47 | 0.68 |
| | 30.0 | 117 | 1337.70 | 4-methylpentyl 4-methylpentanoate | A | 0.69 | 0.72 | 0.71 |
| | 28.0 | 82 | 1273.40 | 3-Hexen-1-ol valerate, (Z)- | A | 0.69 | 0.47 | 0.61 |
| | 25.8 | 70 | 1209.70 | Pentanoic acid, 4-methyl-, pentyl ester | A | 0.68 | 0.66 | 0.66 |
| | 23.6 | 43 | 1145.60 | Hexyl 2-methylpropanoate | A | 0.67 | 0.71 | 0.70 |
| | 23.4 | 43 | 1141.70 | *n*-Amyl isovalerate | A | 0.66 | 0.76 | 0.70 |
| | 22.4 | 43 | 1111.80 | Hexyl 2-methylpropanoate | A | 0.65 | 0.73 | 0.68 |
| | 32.8 | 69 | 1430.70 | alpha-Ionone | A | 0.64 | 0.71 | 0.68 |
| | 22.1 | 70 | 1106.30 | Butanoic acid, 3-methyl-, 3-methylbutyl ester | A | 0.63 | 0.72 | 0.63 |
| | 20.4 | 43 | 1057.50 | Pentyl 2-methylpropanoate | A | 0.62 | 0.73 | 0.68 |
| | 25.4 | 56 | 1196.70 | 4-methylpentyl 3-methylbutanoate | A | 0.61 | 0.76 | 0.64 |
| | 35.6 | 71 | 1531.70 | gamma-Macrocarpene, (E)- | A | 0.60 | 0.78 | 0.64 |
| | 12.2 | 56 | 829.52 | 1-Pentanol, 4-methyl- | A | 0.60 | 0.86 | 0.64 |
| | 26.9 | 70 | 1242.40 | Isopentyl hexanoate | A | 0.59 | 0.38 | 0.60 |
| | 28.9 | 70 | 1301.00 | 4-Methyl-1-hexanol 2-methylbutanoate | A | 0.58 | 0.76 | 0.60 |
| | 34.8 | 91 | 1499.60 | gamma-Humulene | A | 0.58 | 0.69 | 0.65 |
| | 25.9 | 69 | 1212.10 | a`-Citronellol | A | 0.57 | 0.75 | 0.62 |
| | 34.8 | 93 | 1502.90 | gamma-Humulene | A | 0.56 | 0.67 | 0.63 |
| | 31.0 | 43 | 1368.90 | alpha-Longipinene | A | 0.53 | 0.65 | 0.59 |
| | 31.7 | 91 | 1392.00 | Benzyl 3-methylbutanoate | A | 0.51 | 0.75 | 0.51 |
| | 26.5 | 67 | 1230.60 | Hexyl 2-methylbutanoate | A | 0.48 | 0.68 | 0.52 |
| | 26.8 | 43 | 1238.10 | Heptyl isobutanoate | A | 0.48 | 0.50 | 0.52 |
| | 34.5 | 177 | 1490.70 | beta-Ionone | A | 0.47 | 0.64 | 0.51 |
| | 26.7 | 56 | 1234.20 | Hexyl 3-methylbutanoate | A | 0.47 | 0.68 | 0.51 |
| | 29.1 | 73 | 1308.80 | Nonanoic acid | A | 0.46 | 0.72 | 0.50 |
| | 31.3 | 82 | 1380.00 | Hexenyl (3Z)-hexenoate, (3Z)- | A | 0.45 | 0.56 | 0.48 |
| | 13.4 | 56 | 863.91 | 1-Hexanol | A | 0.44 | 0.75 | 0.47 |
| Semi-polar metabo-lites | 39.5 | 318.17 | | capsaicin/capsaicin analogue | B | 0.98 | 0.55 | 1.00 |
| | 17.9 | 395.19 | | Hexanol–pentose–hexose | B | 0.87 | 0.33 | 0.87 |
| | 21.6 | 665.17 | | Chrysoeriol diglucose | B | 0.76 | 0.28 | 0.66 |
| | 19.2 | 651.16 | | Luteolin-*O*-acetyl-diglucose | B | 0.73 | 0.27 | 0.63 |
| | 13.9 | 903.24 | | Quercetin-dihexose-deoxyhexosepentose | B | 0.65 | 0.29 | 0.62 |
| | 12.6 | 447.15 | | Benzyl alcohol-hexose-pentose + FA | B | 0.63 | 0.21 | 0.67 |

**Table 2** *Continued*

| Type | Ret (min) | Mass | RI | Putative identity | HCA cluster | Correlation to capsaicin pericarp | Correlation to capsaicin seed and placenta | Correlation to capsaicin analog LCMS |
|---|---|---|---|---|---|---|---|---|
| Semi-polar metabo-lites | 18.4 | 725.19 | | 3,4',5,7-Tetrahydroxyflavone; 3-*O*-[Rhamnosyl-(1-[?)-galactoside], 7-*O*-arabinoside | B | 0.58 | 0.22 | 0.56 |
| | 13.3 | 337.09 | | Coumaroylquinic acid | B | 0.57 | 0.12 | 0.60 |
| | 16.0 | 425.20 | | 1-Hexanol; *O*-[?-D-Glucopyranosyl-(1?2)-?-D-glucopyranoside] | B | 0.53 | 0.14 | 0.59 |

*Ret* retention time (minutes); *Mass* nominal mass (in case of volatiles) or exact mass (in case of semi-polar metabolites); *RI* retention index; *HCA cluster* refers to the cluster in the HCA for volatiles (Fig. 3) or semi-polar metabolites (Fig. 4). Only metabolites with a putative identity are shown. Total capsaicinoids used in the correlation analysis was measured previously (Wahyuni et al. 2011)

Metabolite cluster C consists of compounds with relatively high abundance in accession cluster B2, consisting of *C. annuum*-spII, *C. chinense*, *C. frutescens* and *C. baccatum*. Apart from three flavonoids, most metabolites within this cluster could not be identified so far.

### 3.2.2. Volatile metabolites

Headspace SPME-GC-MS chromatograms of ripe fruit pericarp samples from the 32 pepper accessions were subjected to full mass spectral alignment using MetAlign software, followed by grouping of the resulting 13,833 ion fragments into 408 putative volatile metabolites, using MSClust software. Of those 408 metabolites, 347 showed differential expression patterns based on ANOVA ($p < 0.001$) and were selected for further analysis. These 347 volatiles were putatively identified on the basis of both their mass spectrum and retention index, and are listed in Supplemental Table S2. In total 194 volatiles had a NIST-match factor above 600 and a retention index deviation of less than 50 units, and their putative identification was therefore considered as reliable. PCA analysis based on 347 volatiles (Fig. 2b) revealed that the separation into species groups was not as clear as observed for both the genetic data (Fig. 1) and the semi-polar metabolites (Fig. 2a). In fact, PC1, which explained 37.7% of the total variation, differentiated the species groups according to pungency, as previously determined by measuring total capsaicinoid levels (Wahyuni et al. 2011). Accessions of *C. annuum* were clustered into two groups, denoted as *C. annuum*-vocI and *C. annuum*-vocII. *C.*

*annuum*-vocI consisted of three mild pungent accessions: *C. anuum* Bisbas (no. 9), Chili Serrano (no. 22) and Chili de Arbol (no. 23), and one high pungent accession: *C. annuum* AC1979 (no. 19). The second group, *C. annuum*-vocII, contained the remaining *C. annuum* accessions, consisting of seven non-pungent accessions and seven low pungent accessions. In addition, PC1 also differentiated the *C. chinense* accessions into two groups based on pungency. Group one, denoted as *C. chinense*-vocI, consisted of six *C. chinense* accessions with a mild level of pungency, whereas *C. chinense*-vocII contained two low pungent accessions: *C. chinense* No. 4661 (no. 17) and No. 4661 Selection (no. 18). Also the four *C. baccatum* accessions were differentiated based on pungency: the high pungent *C. baccatum* var. *baccatum* (no. 15) was clearly separated from the three low/mild pungent accessions of *C. baccatum* var. *pendulum* and plotted close to the two high pungent accessions of *C. frutescens*.

HCA revealed two main accession clusters, denoted as D-1 and D-2, (Fig. 4). This separation was driven by a large set of volatiles which were much more abundant in fruits of accession cluster D-1 compared to D-2. We arbitrary divided the volatiles characteristic for cluster D-1 into two groups A and B based on their accumulation pattern (Fig. 4). On average the similarity distances between volatiles of group B were the shortest over the whole dendrogram, indicating a very high pattern similarity across the accessions. A major portion of this volatile cluster consisted of various methyl-branched esters, which are products of the branched chain amino acid degradation pathway. Some of these compounds could also be observed in the neighbouring cluster A together with sesquiterpenes, which were clustered in this group. In summary, methyl-branched esters and sesquiterpenes were much more abundant in fruits of accession cluster D-1 compared to D-2. Interestingly, these two accession clusters correspond to the division that could be made based on fruit pungency, which was also apparent in the PCA results. Cluster D-1 was comprised of accessions with mild and high levels of pungency, corresponding to *C. annuum*-vocI, *C. chinense*-vocI, *C. frutescens* and *C. baccatum* var. *baccatum*. Cluster D-2 contained the low and non pungent accessions and consisted of three sub-clusters containing *C. annuum*-vocII, *C. chinense*-vocII and *C. baccatum* var. *pendulum*. The total amount of methyl-branched esters, in general, fits well with the quantitative patterns of capsaicinoids in both pericarp ($R^2$=0.9) and placenta ($R^2$=0.7) across all 32 accessions, as previously determined by Wahyuni et al. (2011), but detailed analysis of the pungent group shows that accumulation of these volatiles fit the capsaicinoid accumulation in pericarp better than capsaicinoid amounts found in placenta (Fig. 5; Table 2).

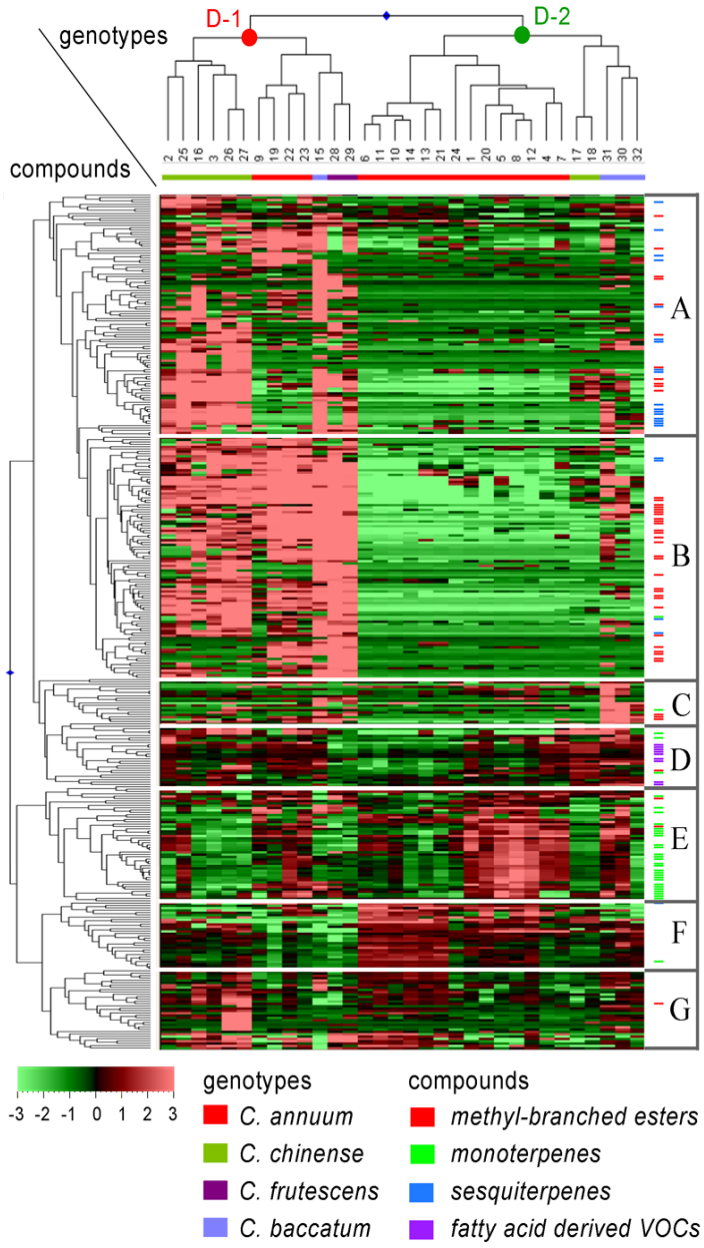


**Fig. 4** Heat map of 347 volatile metabolites in 32 pepper accessions. A color-coded matrix represents the mean values of the metabolite intensity in two biological replicates of pepper accessions, which has been log2 transformed and mean-centered. The alphabetes (A-G) represent metabolite clusters. Characteristics of the underlying metabolites are presented in the Supplemental Table S2. The numbers below the dendrogram correspond to the accession numbers in the first column of Table 1.

The apparent division of genotypes based on pungency likely reflects the activity of the branched-chain amino-acid degradation pathway, which not only leads to the production of methyl-branched esters, but also provides precursors for the production of the acyl moiety of capsaicinoids.

Monoterpene volatiles, clustered in compound group E, drove further differentiation of *C. annuum*-vocII into six sweet breeders' varieties (accession no. 6, 11, 10, 14, 13 and 21) and the low pungent, pointed *C. annuum* accessions (accession no. 24, 1, 20, 5, 8, 12 , 4 and 7), which showed a significantly higher emission of monoterpenes. In addition, *C. chinense* accessions generally showed a low emission of monoterpenes. Another group of volatiles showing biochemically driven compound clustering is group D, in which several volatiles originating from the lipoxygenase pathway showed very similar intensity patterns across the accessions and were on average low in the six sweet breeder's *C. annuum* accessions mentioned above. Group G did not reveal a clear biochemically driven compound clustering as it consisted of volatiles of various biochemical origin.

In general, methyl-branched esters and sesquiterpenes appeared to be the most differentiating compounds between the accessions analysed. Their quantitative patterns show a high similarity to the pungency pattern of the accessions suggesting that these volatiles and capsaicinoids may have common factors which control their accumulation in pepper fruit.

# 4. DISCUSSION

The dendrogram produced using AFLP markers revealed the phylogenetic relationship between the four *Capsicum* species used in this study. The phylogenetic relationships fit well with the geographic dispersion of the species and the common ancestor of the *C. annuum*–*C. chinense*–*C. frutescens* species complex, which diverged from *C. baccatum* at an early stage during evolution (Basu and De 2003; Djian-Caporilano et al. 2007; Eshbaugh 1970). This is also in line with results from previous studies in which different *Capsicum* germplasm was compared (Ince et al. 2010; Kochieva et al. 2004; Toquica et al. 2003). Further differentiation can be seen within the *C. annuum* group, in which bell pepper breeding lines and pointed peppers formed separate groups. This molecular differentiation most likely reflects breeders' efforts to develop and genetically select for specific pepper fruit types.

The overall composition of semi-polar metabolites was strongly determined by the species group, indicating that genetic differences between species are

reflected in metabolic differences. This may be due to differences in the regulation of metabolic pathways, differences in the activity of key enzymes determining the flux through the pathway, or the activity or substrate specificity of specific modifying enzymes. The importance of the latter enzyme group was illustrated by the large, species-specific, variation in the "decoration" of flavonoids and capsianosides. In contrast to the AFLP data, semi-polar metabolites differentiated the *C. annuum* group from *C. chinense*–*C. frutescens*–*C. baccatum*. This differentiation may be due to the domestication and selection history of the accessions studied. Indeed, *C. annuum* accessions have been most commonly used for breeding purposes and therefore may have been exposed to the strongest selection history, including selection for desirable traits during breeding. Selection on specific mutations will have a marginal impact on the total genetic make-up of the species group, but may strongly affect the expression of genes thereby altering specific metabolic pathways, as shown for tomato ripening (Kovács et al. 2009) , or high pigment (Bino et al. 2005; Levin et al. 2006) mutants. In pepper, this is clearly demonstrated by comparing the position of the wild variety *C. annuum* AC1979 (no. 19) relative to those of the cultivated *C. annuum* accessions in the phylogenetic tree based on AFLP markers (Fig. 1) with its position in the HCA based on semi-polar metabolites (Fig. 3).

Unlike the overall metabolite profiles, targeted analyses of a specific set of metabolites, such as carotenoids, capsaicinoids and vitamins C and E, revealed a large variation between individual accessions rather than between species groups (Wahyuni et al. 2011). Variation in these health-related metabolites may have resulted from breeding efforts aimed at selecting for consumer-driven attributes such as carotenoids for fruit color and capsaicinoid levels to enhance or avoid pungency, rather than from differences between species.

In case of volatiles, the accessions were primarily clustered based on pungency rather than on species (Fig. 2b). Identification of the volatiles driving this contrast revealed a relatively high abundance of sesquiterpenes and methyl-branched fatty acid esters in the pericarp of pungent accessions. The total amount of methyl-branched fatty acid esters showed a strong correlation with the total amount of capsaicinoids in the pericarp and, to a lesser extent, to capsaicinoids in the placenta (Fig. 5) and the same holds for the individual volatiles in metabolite cluster A of the HCA, including four sesquiterpenes (Table 2). In this respect we also observed a close clustering of methyl-branched esters with the capsaicin analog in a HCA based on a combined dataset of both semi-polar and volatile metabolites (Supplemental Fig. 1; Table S3). However, there is no obvious relation between the metabolic pathways leading to sesquiterpenes and the pungent

capsaicinoids, suggesting that this correlation is most likely due to population structure in this germplasm collection rather than to a causal relationship.

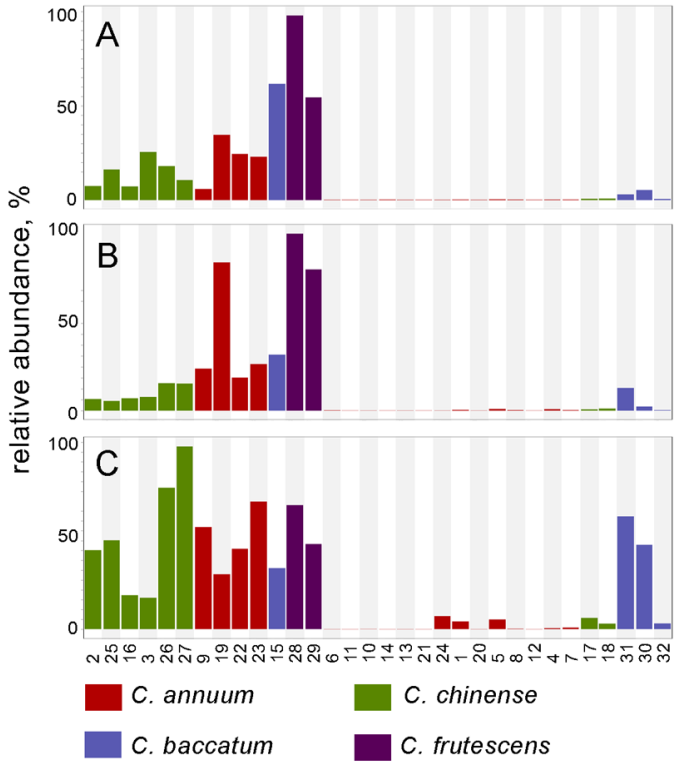


**Fig. 5** Bar-plots representing relative abundance of metabolites in 32 pepper accessions: (a) total methyl-branched esters in pericarp, (b) total capsaicinoids in pericarp and (c) total capsaicinoids in combined placenta and seeds. Total capsaicinoids in pericarp and placenta and seeds were based on Wahyuni et al. (2011). The numbers below the bar plots correspond to the accession numbers in the first column of Table 1.

This can, however, only be addressed by the use of segregating populations. We can not exclude that population structure may also account for the correlation of methyl-branched esters with pungency. However, methyl-branched fatty acid esters are derived from the catabolism of branched-chain amino acids, such as valine, leucine and isoleucine, which also serve as direct precursors for the acyl branch of capsaicinoid biosynthesis (Mazourek et al. 2009). Hence, methyl-branched fatty acid esters may reflect the activity of the capsaicinoid pathway. The primary site of capsaicinoid production is the placenta, from where capsaicinoids are transported into the apoplast and stored in so-called 'blisters', or towards

other tissues or organs, such as the fruit pericarp, stems and leaves (Broderick and Cooke 2009; Estrada et al. 2000; Kim et al. 2011; Stewart et al. 2007). The strong correlation of methyl-branched fatty acid ester levels with capsaicinoid levels in the pericarp suggests that these methyl-branched esters are also synthesized in the placenta from where they are subsequently transported to the pericarp. Indeed, Moreno et al. (2012) recently showed a 12-48-fold higher accumulation of methyl-branched esters in the placenta compared to the pericarp.

In pepper, pungency is primarily determined by the *Pun1* gene, which encodes capsaicin synthase, the final step in capsaicinoid biosynthesis. This putative acyltransferase is responsible for the formation of capsaicinoids through transfer of the acyl moiety of the capsaicin pathway to vanillin-amide, the product of the aromatic branch of the capsaicin pathway (Mazourek et al. 2009; Stewart et al. 2005). Loss of function mutations in this gene lead to the absence of capsaicinoids and a lack of pungency. Quantitative differences in pungency likely reflect the activity through the aromatic and acyl branches of the capsaicin pathway and several QTLs affecting pungency have been described (Ben-Chaim et al. 2006; Blum et al. 2003; Mazourek et al. 2009). Most of the current sweet pepper breeding germplasm has a loss of function mutation in the *pun1* gene (Mazourek et al. 2009). It is evident that these accessions not only lack capsaicinoids, but are also low in methyl-branched fatty acid esters, suggesting that the entire pathway leading to capsaicinoids is down-regulated in these accessions. There are several possible explanations for this observation. Firstly, it is possible that the *Pun1* locus or capsaicinoids, directly or indirectly, regulate the flux through the entire upstream biochemical pathway, as suggested by increased and co-regulated expression of several capsaicin pathway genes in *Pun1* compared to *pun1* genotypes (Stewart et al. 2005, 2007). Secondly, we cannot exclude that the capsaicin synthase enzyme has a broad substrate preference and also functions as an ester-forming enzyme required for the esterification of methyl-branched fatty acid esters (Stewart et al. 2005). Thirdly, it is possible that breeding efforts selecting for sweet bell peppers not only targeted the *pun1* mutation, but also selected for genotypes with a low activity of the upstream branches of the capsaicin pathway, which may be due to mutations in one or more genes encoding enzymes or regulatory factors of this pathway. The latter option would make it possible to introgress the pungent aroma into a non-pungent pepper background.

**Table 3** Relative abundance of 16 odour-contributing volatiles in 32 pepper accessions

| Accession cluster | ID | Compound name | 2,6-Nonadienal, (E,Z)- | 2-nonenal | 2-heptanethiol | Hexyl 2-methylbutanoate | Hexyl 3-methylbutanoate | 4-methylpentyl 3-methylbutanoate | 4-methylpentyl 4-methylpentanoate | Methyl salicylate | alpha-Ionone | bet.-Ionone | Hexanal | alpha-Pinene | 1,8-Cineole | Linalool | ectocarpane | 2-Isobutyl-3-methoxypyrazine |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Odour description | cucumber | cucumber | paprika, green, kerosine-like | fruity | fruity | fruity, peach | soapy, weak fruity | green, sweet, phenolic | floral | fruity, floral | green | pine wood | eucalyptus | citrus, fruity, floral | green, sweet | paprika, green, earthy |
| **Volatile cluster** | | | A | A | A | A | A | B | B | B | B | B | D | E | E | E | E | E |
| Chinense | **27** | RU 72-241 | 13.4 | 15.8 | 18.1 | 21.8 | 21.7 | 20.6 | 16.4 | 20.1 | 19.7 | 16.5 | 21.1 | 13.9 | 9.9 | 17.7 | 14.5 | 16.7 |
| | **26** | RU 72-194 | 15.9 | 16.8 | 17.7 | 21.7 | 21.7 | 21.3 | 17.3 | 20.4 | 18.6 | 15.5 | 20.7 | 14.1 | 9.8 | 17.5 | 14.6 | 17.6 |
| | **16** | Miscucho colorado; PI 152225; 1SCA no.6 | 14.9 | 13.9 | 13.8 | 20.8 | 21.1 | 19.3 | 19.9 | 20.5 | 14.9 | 17.9 | 19.7 | 10.9 | 9.7 | 18.0 | 14.5 | 18.5 |
| | **25** | No.1720; PI 281426; 1GAA | 16.7 | 14.4 | 16.0 | 21.9 | 21.8 | 20.7 | 17.5 | 20.0 | 19.1 | 16.3 | 19.2 | 13.8 | 12.4 | 20.0 | 17.1 | 18.8 |
| | **3** | I1 PI 315023 (Mishme Black) | 14.3 | 14.5 | 11.9 | 21.3 | 20.9 | 21.9 | 16.5 | 20.1 | 21.4 | 17.5 | 19.9 | 9.8 | 9.8 | 17.6 | 14.2 | 18.4 |
| | **2** | I1 PI 281428 | 14.8 | 14.3 | 12.6 | 21.4 | 20.6 | 19.7 | 17.6 | 20.9 | 16.0 | 18.1 | 21.1 | 10.7 | 9.8 | 17.1 | 13.7 | 17.8 |
| Frutescens | **29** | Tabasco | 13.2 | 14.0 | 15.5 | 21.5 | 21.3 | 21.9 | 21.1 | 20.2 | 21.0 | 14.9 | 18.9 | 11.9 | 9.8 | 18.5 | 15.9 | 18.3 |
| | **28** | Lombok | 15.0 | 15.8 | 14.4 | 20.5 | 21.8 | 22.0 | 22.0 | 20.0 | 21.4 | 17.1 | 20.2 | 15.3 | 13.7 | 9.8 | 18.4 | 18.5 |
| Baccatum baccatum | **15** | No. 1553 | 11.1 | 16.9 | 17.2 | 21.5 | 21.8 | 20.3 | 22.0 | 19.5 | 19.3 | 13.6 | 21.7 | 16.2 | 15.9 | 18.2 | 14.1 | 17.2 |
| Pungent Annuum | **23** | Chili de Arbol; PI 281370; No. 1184 | 20.2 | 14.9 | 13.9 | 16.2 | 14.7 | 21.6 | 17.4 | 21.6 | 18.1 | 17.7 | 20.7 | 13.6 | 9.9 | 20.3 | 15.1 | 18.9 |
| | **22** | Chili Serrano; PI 281367; No. 999 | 9.9 | 15.3 | 13.0 | 16.1 | 15.3 | 21.7 | 18.2 | 22.0 | 20.3 | 18.0 | 21.3 | 12.5 | 9.9 | 20.8 | 17.0 | 19.5 |
| | **19** | AC 1979 | 18.3 | 14.4 | 11.6 | 17.9 | 15.6 | 21.9 | 17.8 | 20.2 | 20.6 | 17.9 | 21.4 | 16.1 | 12.3 | 17.8 | 15.5 | 18.3 |
| | **9** | Bisbas | 16.2 | 14.9 | 13.6 | 9.9 | 10.5 | 19.9 | 13.1 | 20.5 | 19.0 | 16.3 | 20.4 | 16.4 | 15.2 | 18.6 | 13.5 | 19.3 |
| Annuum pointed | **20** | CM 331; Criollos de Morelos | 12.9 | 14.7 | 14.6 | 11.3 | 9.8 | 9.8 | 9.9 | 16.8 | 13.7 | 13.5 | 20.0 | 15.2 | 14.6 | 21.5 | 16.6 | 17.9 |
| | **12** | Long Sweet | 11.2 | 14.8 | 15.2 | 12.1 | 9.9 | 9.8 | 10.3 | 16.8 | 13.3 | 12.9 | 20.1 | 14.4 | 15.0 | 21.5 | 17.8 | 18.0 |
| | **8** | PBC 535 - IR - 12x1cm - cayenne | 13.3 | 14.8 | 14.1 | 12.5 | 11.8 | 12.9 | 11.4 | 19.1 | 12.8 | 11.5 | 19.7 | 14.3 | 15.0 | 21.9 | 18.2 | 18.8 |
| | **5** | Jatilaba | 13.8 | 14.5 | 12.0 | 12.0 | 9.7 | 13.8 | 11.2 | 19.4 | 11.1 | 12.1 | 20.0 | 14.6 | 14.6 | 21.8 | 17.4 | 19.9 |
| | **4** | Laris HS | 15.7 | 14.1 | 13.8 | 9.8 | 12.6 | 9.8 | 10.7 | 17.0 | 9.8 | 11.6 | 20.6 | 12.3 | 9.8 | 20.8 | 16.4 | 19.3 |
| | **1** | I2 Tit super | 10.6 | 14.2 | 13.3 | 11.8 | 9.9 | 9.7 | 10.7 | 18.7 | 13.0 | 12.5 | 20.9 | 13.7 | 13.5 | 19.9 | 16.1 | 18.5 |
| | **24** | AC 2212 | 16.0 | 14.5 | 11.9 | 10.3 | 9.9 | 9.9 | 9.8 | 16.5 | 15.9 | 10.8 | 19.8 | 14.5 | 12.5 | 18.9 | 14.3 | 16.6 |
| | **7** | PBC 473 - none - cayenne | 16.6 | 13.9 | 12.5 | 11.1 | 12.5 | 13.9 | 10.8 | 19.5 | 16.9 | 13.6 | 20.5 | 14.2 | 9.8 | 20.8 | 16.1 | 20.1 |
| **Annuum breeding lines** | **14** | **Yolo Wonder L** | 9.9 | 14.8 | 15.5 | 10.3 | 11.7 | 13.3 | 12.0 | 13.8 | 11.7 | 12.6 | 19.5 | 11.7 | 9.8 | 17.9 | 13.9 | 16.9 |
| | **10** | **California Wonder 300** | 10.2 | 15.0 | 15.8 | 10.5 | 9.7 | 12.2 | 9.7 | 19.2 | 10.9 | 13.1 | 20.1 | 11.7 | 11.7 | 19.5 | 15.4 | 18.8 |
| | **6** | **Bruinsma Wonder** | 9.8 | 14.6 | 14.8 | 10.3 | 11.6 | 9.9 | 12.9 | 16.3 | 9.9 | 12.0 | 18.9 | 13.4 | 12.8 | 18.8 | 15.1 | 16.6 |
| | **11** | **Keystone Resistant Giant** | 9.9 | 14.7 | 15.6 | 9.8 | 9.8 | 9.8 | 9.8 | 14.8 | 11.1 | 11.5 | 17.8 | 12.5 | 11.7 | 18.8 | 14.7 | 15.7 |
| | **21** | **Sweet Chocolate** | 10.8 | 15.0 | 13.0 | 9.9 | 9.9 | 9.7 | 9.7 | 17.3 | 15.1 | 12.2 | 20.6 | 10.6 | 12.8 | 18.3 | 13.4 | 17.9 |
| | **13** | **Sweet Banana** | 9.8 | 14.9 | 13.6 | 11.7 | 9.9 | 9.8 | 9.7 | 15.1 | 15.7 | 14.3 | 18.7 | 13.6 | 13.5 | 18.9 | 14.6 | 16.1 |
| Chinense wild | **18** | No.4661 Selection; PI 159236 Selection | 12.5 | 14.0 | 12.2 | 16.3 | 16.8 | 17.8 | 11.3 | 20.5 | 15.3 | 15.3 | 20.3 | 9.8 | 9.8 | 19.0 | 14.4 | 18.3 |
| | **17** | No.4661; PI 159236 | 13.1 | 14.2 | 12.3 | 15.5 | 15.5 | 16.7 | 10.0 | 20.5 | 15.6 | 15.0 | 21.2 | 9.8 | 9.9 | 18.9 | 14.6 | 18.6 |
| Baccatum pendulum | **32** | RU 72-51 | 13.1 | 14.5 | 15.5 | 12.3 | 11.5 | 12.5 | 12.2 | 12.6 | 13.6 | 12.1 | 21.8 | 11.2 | 9.9 | 17.0 | 13.4 | 19.0 |
| | **30** | Aji Blanco Christal; CAP 333 | 13.1 | 14.5 | 16.2 | 17.1 | 17.1 | 17.6 | 15.2 | 19.7 | 14.3 | 15.1 | 21.9 | 15.4 | 16.3 | 20.0 | 15.8 | 18.8 |
| | **31** | | 9.8 | 14.6 | 15.1 | 15.2 | 16.0 | 15.1 | 16.3 | 21.7 | 19.4 | 13.5 | 21.9 | 13.1 | 9.9 | 19.5 | 15.5 | 18.7 |

Odour descriptions are derived from Rodríguez-Burruezo et al. (2010). Genotypes are represented in the same order as in the HCA (Fig. 4). Values represent $\log_2$ values of mass peak intensities determined for each volatile in the set of 32 pepper accessions. Colours represent relative intensities for each volatile from dark green (low intensity) to dark red (high intensity)

(Sub)species-specific clustering of volatiles was mainly due to qualitative and quantitative differences in the accumulation of metabolites derived from the major pathways leading to volatile production: aromatic and branched chain amino acid catabolism, fatty acid degradation, mono- and sesquiterpene biosynthesis and, to a lesser extent, carotenoid degradation, reflecting variation in the activities and substrate specificities of the corresponding enzymes. Although we did not conduct any sensorial analyses on these accessions, it may be expected that the observed metabolic differences form the basis for species-specific differences in aroma and opens up possibilities to breed for novel aromas in cultivated pepper backgrounds. Rodríguez-Burruezo et al. (2010) determined the volatile composition in ripe fruits of 16 *Capsicum* accessions from the *annuum-chinense-frutescens* complex and combined their metabolite analysis with taste panel data and sniffing port analyses. They concluded that the diversity in aromas found in their accessions was due to variation in the levels of at least 23 odour-contributing volatiles. Of those, 16 could also be detected in our data set (Table 3). In agreement with Rodríguez-Burruezo et al. (2010), we found that pungent accessions contain the highest levels of aroma-contributing volatiles, such as the sweet, fruity methyl-branched esters hexyl-2/3-methylbutanoate, 4-methylpentyl-3-methylbutanoate and 4-methylpentyl-4-methylpentanoate, the fatty acid derived aldehydes nonenal and nonedienal, which give rise to a green cucumber-like odour impression, as well as the fruity, floral carotenoid-derived volatiles alpha- and beta-ionone (Fig. 4, clusters A and B). The characteristic green bell pepper volatiles pyrazine and 2-heptanethiol were detected at varying levels in all genotypes analysed, irrespective of pungency level or species. Pointed *C. annuum* accessions were relatively rich in the monoterpenes alpha-pinene (pine, wood-like), linalool (citrus, fruity, floral) and 1,8-cineole (eucalyptus-like), as well as the hydrocarbon ectocarpene which has a green, sweet odor description (Fig. 4, cluster E). Finally, we observed that the important fatty-acid derived volatile hexanal was most abundant in all *C. baccatum* accessions analysed (Fig. 4, cluster D).

# 5. CONCLUDING REMARKS

The results of this study clearly demonstrate that there is a large metabolic variation present in the pepper germplasm collection analysed. Species-driven metabolic differences are the major determinants of the variation in semi-polar metabolites, whereas pungency was the main driver responsible for the variation in aroma volatiles. The levels of the metabolites analysed varied greatly among fruits of different accessions, demonstrating the potential of the current

germplasm collection for genetic improvement of metabolic traits. In addition to promising sources of health-related flavonoids and capsianosides, we identified accessions with high levels of several established flavour-related volatiles, such as methyl-branched fatty acid esters, fatty acid derived volatiles, such as hexanal, nonenal and nonedienal, and monoterpenes. These accessions are potential candidates for breeding programs aimed at developing new pepper cultivars with improved flavour and other consumer quality characteristics. Our results also indicate the value to explore the metabolic variation with different analytical platforms and to couple metabolomics with genetic analysis as a strategy to target crop breeding programs to phenotypic diversity for important quality traits.



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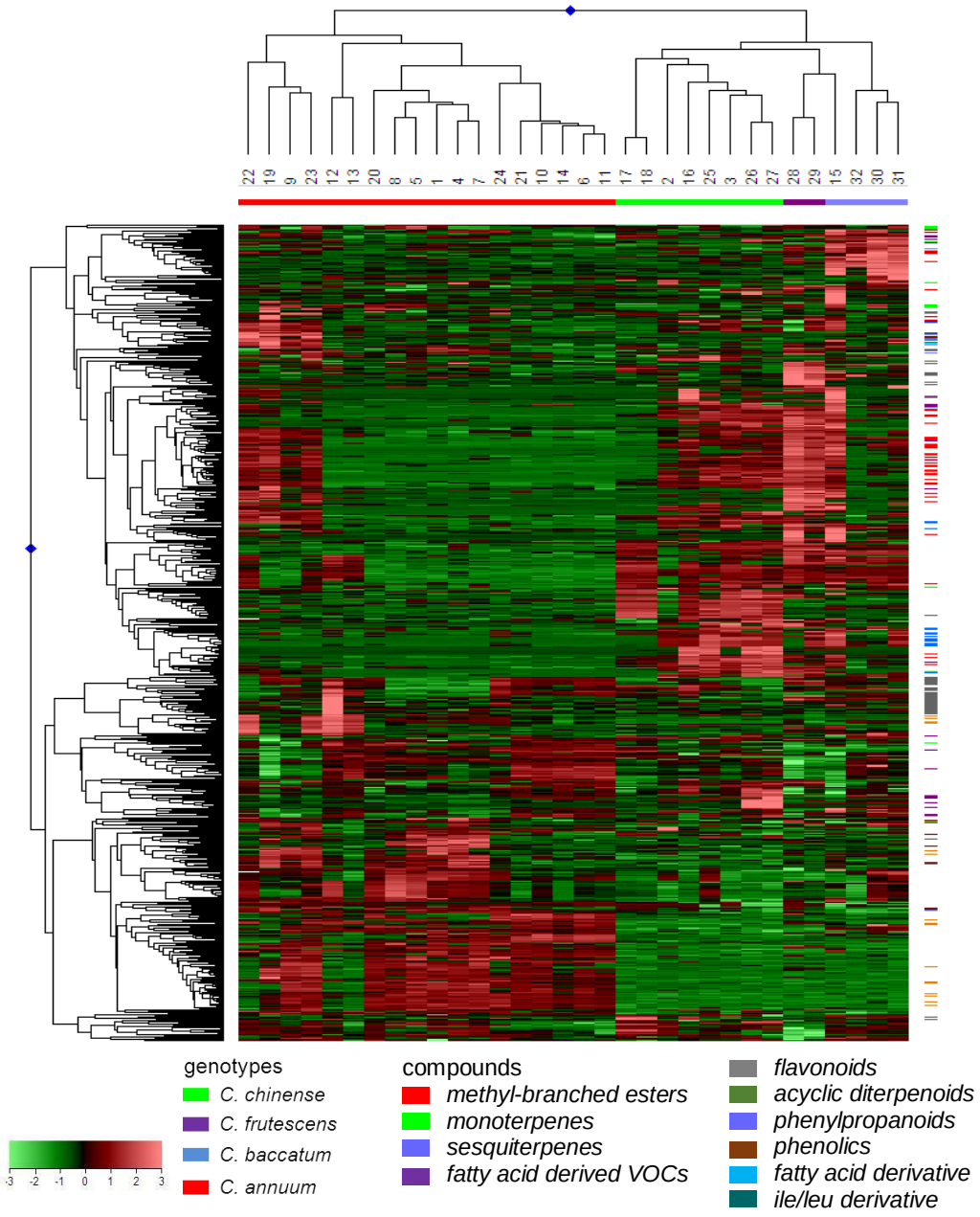
# Supplemental Materials

**Supplemental Table Sl** Semi-polar metabolite composition of 32 *Capsicum* accessions (available at http://edepot.wur.nl/287153)

**Supplemental Table S2** Volatile metabolite composition in 32 *Capsicum* accessions (available at http://edepot.wur.nl/287154)

**Supplemental Table S3** Metabolite data based on hierarchal cluster analysis using a combined data set of semi-polar and volatile metabolites (available at http://edepot.wur.nl/287155)

**Supplemental Fig. 1** Hierarchal Cluster Analysis of a combined data set based on semi-polar and volatile metabolites (available on page 89)

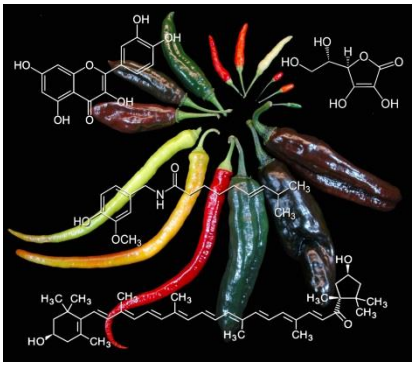



**Supplemental Fig. 1** Hierarchal Cluster Analysis based on a combined data set of semi-polar and volatile metabolites. The order of each metabolite is represented in **Supplemental Table S3**

# CHAPTER 4

## The Influence of Fruit Ripening on the Accumulation of Health-Related Metabolites in Five Different *Capsicum* Accessions

Wahyuni Y, Ballester AR, Sudarmonowati E, Bino RJ, Bovy AG



## ABSTRACT

To elucidate the influence of fruit ripening on the accumulation of health-related metabolites, we analyzed the composition and levels of carotenoids, capsaicinoids and flavonoids in unripe, turning and ripe fruits of five *Capsicum* accessions. These metabolites were analyzed and quantified by HPLC and LC-QTOF-MS. Results showed that the composition and levels of metabolites changed during fruit ripening in all accessions. Chlorophyll and the red carotenoids capsanthin and capsorubin were the major pigments characterizing the fruit colour at the three ripening stages tested. In red-ripened fruits, chlorophyll disappeared upon ripening, concomitant with an increase of capsanthin and capsorubin. In brown-ripened fruits red carotenoids increased upon ripening, and chlorophyll levels remained high throughout ripening. In all pungent fruits, three major capsaicinoids were detected, which were already present at unripe stage and increased slightly along the ripening process. Quercetin and naringenin chalcone were the main flavonoids accumulating in *C. annuum* Long Sweet (no. 12) and *C. chinense* No. 4661 Selection (no. 18) fruits, respectively. Gene expression analyses suggest that the high quercetin levels observed in *C. annuum* Long Sweet (no. 12) may be caused by a strong flux through the pathway to quercetin in unripe fruits, due to strong expression of all flavonoid genes leading to quercetin, in combination with a relatively low expression of *CCR*, a key gene in lignin biosynthesis, a pathway competing with quercetin for their common phenylpropanoid precursors. The high naringenin chalcone levels observed in *C. chinense* No. 4661 Selection (no. 18) are likely due to the relatively high and sustained expression of transcription factor *Ca-MYB12* and its target flavonoid gene *CHS-2* in ripe fruits of this accession, resulting in the accumulation of the CHS product naringenin chalcone. These results offer new insights into the regulation of flavonoid production in pepper fruit.


**Keywords**:
fruit ripening; *Capsicum*; metabolites; flavonoid gene expression

# 1. INTRODUCTION

*Capsicum*, pepper, is a member of the Solanaceae family and around 25 to 30 *Capsicum* species have been discovered in America (Moscone et al. 2007). Five *Capsicum* species have been widely domesticated and the fruits are economically important worldwide. Depending on the application, pepper fruits can be consumed and used at mature green or at ripe stage as vegetables, food colourant or as a spice. Pepper fruits are an excellent source of ascorbic acid (vitamin C), carotenoids (provitamin A), tocopherols (vitamin E), flavonoids and capsaicinoids (Howard et al. 1994; Howard and Wildman 2007; Topuz and Ozdemir 2007; Wahyuni et al. 2011). These compounds have been recognized for their biological effects, for example carotenoids have been suggested to prevent certain cancers, cardiovascular diseases, age related macular degeneration, cataracts, and other degenerative diseases (Nishino et al. 2009); capsaicinoids to treat pain in anti-inflammatory drugs (Bode and Dong 2011; Dou et al. 2011) and flavonoids to prevent cardiovascular disease (Grassi et al. 2010; Jeong et al. 2011).



The composition and levels of the above-mentioned metabolites may vary among pepper genotypes, environmental conditions and cultivation techniques (Butcher et al. 2012; Howard and Wildman 2007; Wahyuni et al. 2011). In addition, fruit maturity is another important factor that influences the composition and levels of fruit metabolites due to their functional roles during ripening, for example in providing fruit colour, flavour, aroma and firmness. Metabolite accumulation is partly mediated through the expression of genes encoding biosynthetic enzymes of a metabolic pathway, and of regulatory proteins, such as the transcription factor MYB12 which is responsible for the production of naringenin chalcone in tomato (Ballester et al. 2010) and pepper (Wahyuni et al. 2013) and MYB11, MYB12 and MYB111 which regulate flavonol accumulation in Arabidopsis seedlings (Stracke et al. 2010).

In peppers, the molecular regulation of carotenoids (Guzman et al. 2010; Ha et al. 2007; Huh et al. 2001; Rodriguez-Uribe et al. 2012) and capsaicinoids (Abraham-Juarez et al. 2008; Ben-Chaim et al. 2006; Blum et al. 2002, 2003; Kim et al. 2009; Mazourek et al. 2009; Stewart et al. 2005, 2007) has been studied extensively, since these metabolites are important determinants of the main qualities of pepper fruit: colour and pungency. Pepper fruit colours are controlled by three independent genes encoded by the *y*, *c1* and *c2* loci. The dominant *Y* locus, which encodes Capsanthin-Capsorubin Synthase (CCS), is responsible for the production of red carotenoids (Lefebvre et al. 1998). In yellow pepper, the fruit colour is determined by the presence of a homozygous recessive *y* allele, for which

different *CCS* mutations, including a partial or complete deletion, a premature stopcodon or a frameshift mutation, have been described (Lefebvre et al. 1998; Li et al. 2013; Paran and van der Knaap 2007). The *c2* locus corresponds to phytoene synthase (PSY), an enzyme that is responsible for phytoene production and mutation of this locus is associated with an orange fruit colour in crosses between red-coloured *Capsicum annuum* and orange-coloured *Capsicum chinense* (Huh et al. 2001). The production of capsaicinoids is regulated by the presence of a functional *Pun1* gene, which encodes the acyltransferase AT3 required for the formation of capsaicin from its aromatic precursor vanillylamine and various acyl moieties derived from the catabolism of branched-chain amino acids (Mazourek et al. 2009). A loss-of function allele of this gene, *pun1-1*, is present in all commercial non-pungent pepper varieties. Up to six QTLs affecting the capsaicinoid levels in pungent accessions have been identified on chromosomes 3, 4 and 7 (Ben-Chaim et al. 2006; Blum et al. 2003).

In this paper we studied the influence of fruit ripening on the composition and levels of carotenoids, capsaicinoids and flavonoids in five pepper accessions. These accessions were selected from a diverse set of 32 pepper accessions based on their contrasts in metabolite content (Wahyuni et al. 2011). In addition, gene expression analysis of candidate flavonoid genes was performed to get insight in the molecular regulation of flavonoid accumulation during ripening.

# 2. MATERIALS AND METHODS

## 2.1. Plant materials and sampling protocol

Five accessions, *Capsicum annuum* Long Sweet (no. 12), *Capsicum chinense* No. 4661 Selection (no. 18), *Capsicum annuum* AC2212 (no. 24), *Capsicum annuum* AC1979 (no. 19) and *Capsicum frutescens* Lombok (no. 28), were selected from the set of 32 accessions due to the higher accumulation of health-related metabolites, as observed in the previous experiment (Wahyuni et al. 2011). Seeds of these accessions, collected from the Centre for Genetic Resources, the Netherlands (CGN), were sowed and grew in a greenhouse located in Wageningen, The Netherlands during January 2009 until September 2009. The experimental design was completely randomized in three blocks, each containing all five accessions grown in two plots of three plants. Fresh fruits were collected from all the plants in both plots at three ripening stages: unripe (mature green), turning and ripe. For each accession, 10-50 fruits (depending on fruit size) from each ripening stages were harvested from all plants in two plots. Pericarp tissue was separated from placenta plus seeds, frozen in liquid nitrogen, ground and stored at

-80 °C until analysis. Fruit ripeness behaviours were studied before the harvest by following the ripening stages of the first set of fruits produced by each accession and using information based on the description in the CGN databases, such as the fruit colours, fruit firmness and the number of days after fruit setting.

### 2.2. Extraction and analysis of carotenoids

Carotenoids were extracted as described previously by López-Raéz et al. (2008). Briefly, 500 mg of freeze-ground pericarp tissue from three different ripening stages were extracted with 4.5 ml of methanol:chloroform (2.5:2.0, v/v) containing 0.1% butylated hydroxytoluene (BHT). Samples were homogenized by vortex and incubated on ice for 10 min in the dark. After the incubation, 2.5 ml of 50 mM Tris-chloride (pH 7.5) containing 1 M NaCl were added and incubated again on ice for another 10 min. In order to separate the chloroform layer containing carotenoids, samples were centrifuged at 1350*g* for 10 min at room temperature and the chloroform layer was transferred to a clean tube. Samples were re-extracted twice with 1.0 ml of chloroform containing 0.1% BHT. Pooled chloroform phases were dried under a nitrogen flow, tubes were closed and kept at -20 °C until high-pressure liquid chromatography (HPLC) analysis. For HPLC analysis, dried extracts were dissolved in 1.0 ml of ethyl acetate containing 0.1% BHT and transferred to amber vials. HPLC analysis was performed according to Bino et al. (2005) using a YMC-Pack reverse-phase $C_{30}$ column (250 x 4.6 mm; 5 µm) coupled to a 20 x 4.6 mm $C_{30}$ guard (YMC Inc. Wilmington, NC, USA), maintained at 40 °C. The mobile phase used was methanol, tert-methyl butyl ether and water:methanol (20:80, v/v) containing 0.2% ammonium acetate. Flow rate of 1 ml/min was used. Chromatography was carried out on a Waters system consisting of a No. 600 quaternary pump, No. 996 photo diode array detector (PDA). Data were collected and analyzed using the Waters Empower software supplied. Carotenoids were detected by setting the PDA to scan from 220 to 700 nm. All carotenoids were quantified at 478 nm, except for violaxanthin, violaxanthin-ester and neoxanthin, which were quantified at 440 nm. Quantitative determination of each carotenoid was conducted by comparison with dose-response curves constructed from authentic standards. Carotenoids esters, denoted as capsanthin-esters and violaxanthin-ester, were quantified using dose-response curves of their free forms, since the ester and free forms showed the same UV absorption spectrum (Schweiggert et al. 2007). Each value represented the average of three biological replicates.

### 2.3. Extraction and analysis of capsaicinoids

Capsaicinoids were quantified in pericarp and placenta plus seeds tissues from three different ripening stages. Capsaicinoids were extracted from 500 mg of frozen ground samples of pericarp or placenta plus seeds with 1.5 ml of 100% methanol (final methanol concentration in the extract approximately 75%). The extracts were sonicated for 15 min and filtered through a 0.2 µm polytetrafluoroethylene (PTFE) membrane filter into an amber vial to prevent the degradation of the compounds. Ten microliters of sample were injected into the same HPLC Waters system used to detect carotenoids but equipped with a Luna C18(2) pre-column (2.0 x 4 mm) and analytical column (2.0 x 150 mm, 100 Å, particle size 3 µm) from Phenomenex (Torrance, CA, USA) for chromatographic separation. Degassed solutions of formic acid (FA):ultrapure water (1:$10^3$, v/v, eluent A) and FA:acetonitrile (1:$10^3$, v/v, eluent B) were used as a mobile phase. The gradient applied started at 50% B and increased linearly to 75% B in 30 min. Then, the column was washed with 80% B during 10 min and re-equilibrated before the next injection. The flow rate of the mobile phase was 1 ml/min. The column temperature was kept at 40 °C. Capsaicinoid analogues were detected by PDA at wavelength of 280 nm and quantified by comparison with commercial capsaicinoids (capsaicin and dihydrocapsaicin) from Sigma (St Louis, USA).

### 2.4. Extraction and analysis of flavonoid glycosides

Flavonoid glycosides were extracted using a protocol described previously by de Vos et al. (2007). In a brief, 500 mg of freeze-ground pepper pericarp from three different ripening stages was extracted with 1.5 ml of 99.875% methanol acidified with 0.125% formic acid (FA) and mixed immediately for 10 s. The extracts were sonicated for 15 min and filtered through 0.2 µm PTFE membrane filter. In order to avoid saturation of some compounds shown in the preliminary analysis, the extracts were diluted 50 times from the original extracts. For each accession, three biological replicates containing three ripening stages were prepared, resulting in a total of 45 extracts. Quality control samples were prepared by pooling fruit material from several randomly chosen accessions, extracted at the same time as accession samples and injected after every 11 accession sample extracts.

All the extracts were analysed using C18-reversed phase liquid chromatography coupled to a photodiode array detector and a quadrupole time of flight high-resolution mass spectrometry (LC-PDA-QTOF-MS) system and negative electrospray ionization as described previously (de Vos et al. 2007). For LC-PDA-

QTOF-MS, 5 µl of the extract were injected and separated using a binary gradient of ultrapure water (A) and acetonitrile (B), both acidified with 0.1% formic acid, with a flow rate of 0.19 ml/min. The initial solvent composition consisted of 95% of A and 5% of B; increased linearly to 35% A and 65% B in 45 min and maintained for 2 min. The column was washed with 25% A and 75% B for 5 min and equilibrated to 95% A and 5% B for 2 min before the next injection. Leucineenkaphalin, $[M-H]^-$ = 554.2620, was used as a lock mass and injected through a separate inlet.

Flavonoid glycosides identification was carried out by means of their UV spectra, exact molecular weight and different metabolite databases such as Dictionary of natural Products (http://dnp.chemnetbase.com), KNApSAcK (http://kanaya.naist.jp/KNApSAcK) and in-house metabolite databases, and based on previous results on pepper described by Marin et al. (2004) and Wahyuni et al. (2011).



### 2.5. Total RNA extraction and cDNA synthesis

Total RNA was extracted using the same plant materials as those for metabolite analyses. The extraction was performed from 50 mg freeze-ground pericarp of three different ripening stages using QuickGene RNA cultured cell kit S (FujiFilm Life Science) according to manufacturer's instructions. The quality and quantity of total RNA were measured by Nanodrop spectrophotometer model ND-1000 (Thermo Scientific) and evaluated by electrophoresis using 200 ng of the total RNA from each sample on 1.5% w/v agarose gel. cDNA was synthesized from 500 ng total RNA using Taqman® Reverse Transcription (Applied Biosystem) according to the manufacturer's instructions. This reaction resulted 10 ng of cDNA for gene expression analysis.

### 2.6. Candidate genes selection, primers design and gene expression analysis using Fluidigm platform

Structural genes and regulatory genes of flavonoid biosynthetic pathway were targets for gene expression analysis. Candidate genes were selected from pepper gene index database (http://compbio.dfci.harvard.edu/cgi-bin/tgi/gimain.pl?gudb=pepper), which showed the best homology with structural genes and regulatory genes underlying flavonoids biosynthesis in tomato. Primers were designed for each candidate gene using Primer3Plus (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi) with the criteria suggested by Udvardi et al. (2008). Candidate genes and primer sequences were described in Supplemental Table 1. In order to validate the performance of primers and cDNA samples,

quantitative real-time PCR analysis was done using iCycler iQ machine (Bio-Rad Laboratories) in the presence of fluorescent dye (iQ SYBR Green Supermix) before Fluidigm analysis.

Fluidigm was performed with 45 samples, composing of three biological replicates of unripe, turning and ripe fruits of five *Capsicum* accessions. A total of 92 candidate genes underlying phenylpropanoids, phenolics and flavonoids biosynthesis, including structural genes, regulatory genes and glycosyltransferase genes were included in Fluidigm. Four reference genes: *actin*, *ubiquitin*, *α-tubulin* and *glyceraldehyde-3-phosphate dehydrogenase* (subunit GapA1) were included in the analysis. Of four reference genes, *α-tubulin* gene was chosen to normalized the expression levels of candidate genes due to the stability of its Ct (threshold cycle) value over all samples. Expression levels were determined by delta Ct (ΔCt) values, calculated by subtracting Ct value of each candidate gene with Ct value of α-tubulin gene, converted by $2^{-\Delta Ct}$ calculation and multiplied by 100. To validate Ct values resulted from Fluidigm, qRT-PCR analysis for several genes was performed using the same cDNA and primer pairs as those for Fluidigm using iCycler iQ machine (Bio-Rad Laboratories) in the presence of fluorescent dye (iQ SYBR Green Supermix).

# 3. RESULTS

## 3.1. Metabolic profiles in unripe, turning and ripe fruits

We monitored changes in the composition and level of carotenoids, capsaicinoids and flavonoids at three fruit ripening stages in five *Capsicum* accessions. Upon ripening, changes in fruit colours from green to red in red-fruited accessions or to brown in brown-fruited accessions (Fig. 1) were in-line with the modification of carotenoid composition and levels detected in fruits. Chlorophyll b was abundantly present in unripe fruits of all accessions. In addition, several carotenoids were also detected at that stage, including several ε-cyclic carotenoids (α-carotene and lutein) and β-cyclic carotenoids (β-carotene, β-cryptoxanthin, antheraxanthin, violaxanthin and neoxanthin; Fig. 1). The level of chlorophyll b, however, varied among accessions and resulted in different intensity of the green colour of unripe fruits. Accessions *C. chinense* No. 4461 Selection (no.18), *C. annuum* AC1979 (no. 19) and *C. annuum* AC2212 (no. 24) with dark green unripe fruits showed higher levels of chlorophyll b and early carotenoids, compared to *C. annuum* Long Sweet (no. 12) and *C. frutescens* Lombok (no. 28) with light green unripe fruits (Fig. 1). At turning stage, all carotenoids accumulated to higher levels than those at unripe stage.

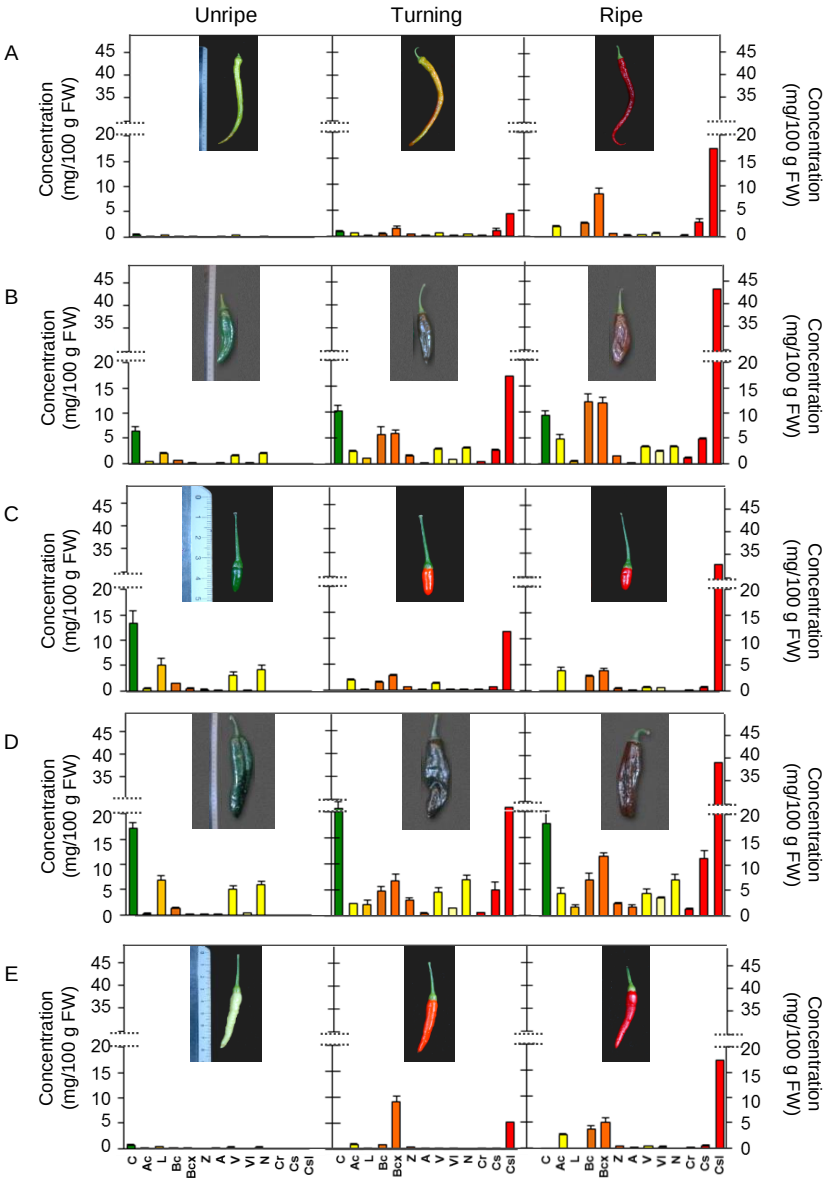



**Fig. 1** Ripening-dependent changes in fruit phenotypes and carotenoid content in fruit pericarp of pepper accessions: A, *C. annuum* Long Sweet (no. 12); B, *C. chinense* No. 4661 Selection (no. 18); C, *C. annuum* AC1979 (no. 19); D, C. *annuum* AC2212 (no. 24) and E, *C. frutescens* Lombok (no. 28). Values represent the average value from three biological replicates. The ripening stages include unripe (U), turning (T) and ripe (R). Carotenoids detected in fruits were C (chlorophyll b), Ac (alpha-carotene), L (lutein), Bc (beta carotene), Bcx (beta cryptoxanthin), Z (zeaxanthin), A (astaxathin), V (violaxanthin), Vl (violaxanthin-like), N (neoxanthin), Cr (capsorubin), Cs (capsanthin), Csl (capsanthin-like). FW: fresh weight.

The levels and relative abundance of various carotenoids, however, varied among accessions, resulting in different phenotypes (Fig. 1). Fruits of accessions no. 19 and no. 28 turned orange and this was due to the accumulation of orange-coloured carotenoids, i.e. β-cryptoxanthin and β-carotene and a concomitant decrease of chlorophyll b levels at this stage. In contrast, chlorophyll b did not show any degradation in accessions no. 18 and no. 24, but rather increased during ripening in parallel with the accumulation of the main red carotenoids capsanthin and capsorubin. Fruits of accession no. 12 showed a variegated green/orange pattern at turning stage reflecting remaining traces of the green chlorophyll b and the accumulation of the red carotenoids capsanthin and capsorubin. At the final stage of the ripening process, capsorubin and capsanthin were the major carotenoids accumulating in ripe fruits of both red and brown fruited accessions. They were mainly present in an esterified form, as shown previously in Wahyuni et al. (2011). Chlorophyll b completely disappeared in ripe fruits of red accessions, while it remained present in brown fruits. This combination of green and red pigments is responsible for the colour of brown–fruited accessions. The levels of the 'green' carotenoids, i.e. lutein, violaxanthin and neoxanthin decreased up to 4-fold during ripening in red fruits, while in fully ripe brown fruits their levels increased upon ripening.

Three major capsaicinoids, i.e. nordihydrocapsaicin, capsaicin and dihydrocapsaicin, have been detected in both pericarp and placenta plus seeds tissues. Levels of capsaicinoids in both pericarp and placenta plus seeds tissues increased during fruit ripening in mild (no. 18 and no. 24) and highly pungent accessions (no. 19 and no. 28; Fig. 2). Accession *C. annuum* Long Sweet (no. 12), which has been characterized previously as a low pungent pepper based on the very low levels of capsaicinoids in placenta and seed tissues of its ripe fruits (Wahyuni et al. 2011), showed higher levels of these compounds (less than 0.7 mg/100 g fresh weight; FW) in placenta plus seeds tissues of unripe fruits.

In fruit pericarp of the five accessions, different flavonoid *O*- and *C*-glycosides could be detected (Fig. 3 and 4) and their levels varied at different ripening stages. Glycosides of quercetin and luteolin were the major flavonoids detected in all samples, in addition to apigenin glycosides and naringenin chalcone (Fig. 4). The flavonoid glycosides were already present at the unripe stage in all accessions. Of four quercetin glycosides, quercetin 3-*O*-rhamnoside and quercetin 3-*O*-rhamnoside-7-*O*-glucoside, were the predominant flavonol glycosides in all accessions. Levels of quercetin 3-*O*-rhamnoside decreased upon ripening, whereas quercetin 3-*O*-rhamnoside-7-*O*-glucoside levels increased during ripening (Fig. 4A).

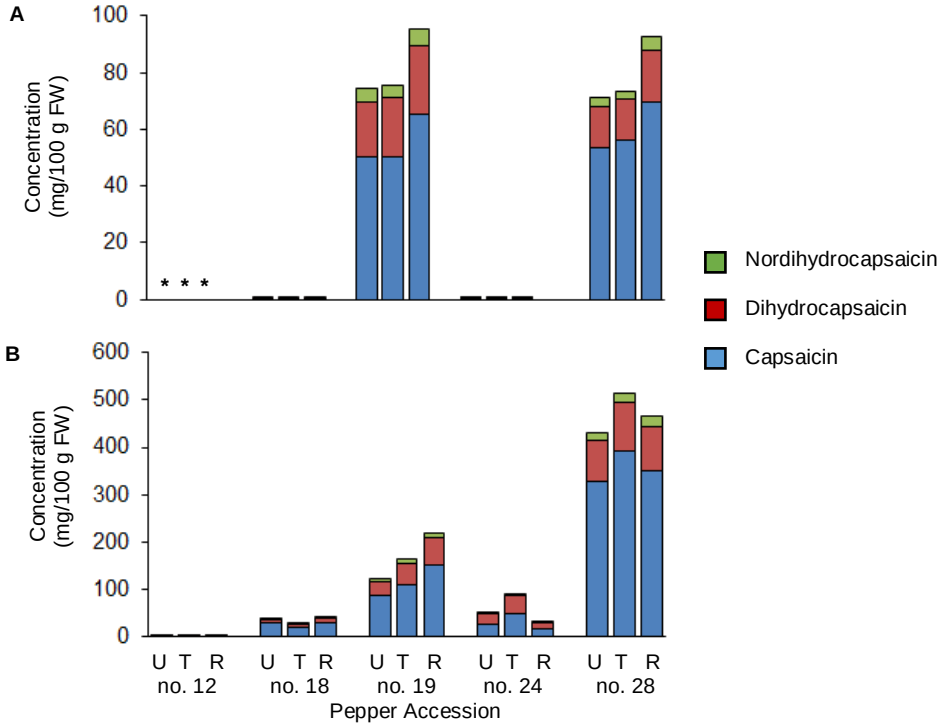



**Fig. 2** Capsaicinoid levels upon ripening stages detected in pericarp (A) and placenta and seeds tissues (B) of pepper accessions: *C. annuum* Long Sweet (no. 12), *C. chinense* No. 4661 Selection (no. 18), *C. annuum* AC1979 (no. 19), *C. annuum* AC2212 (no. 24) and *C. frutescens* Lombok (no. 28). Values represent the average of the value from three biological replicates. The ripening stages include unripe (U), turning (T) and ripe (R). * = not detectable.

Two apigenin *C*-glycosides, apigenin 6,8-di-*C*-hexose and apigenin 6-*C*-pentoside-8-*C*-hexose, accumulated upon ripening in four accessions, no. 12, 18, 19 and 24 (Fig. 4B), while these apigenin glycoside levels decreased upon ripening in *C. frutescens* Lombok (no. 28). Five luteolin *C*-glycosides have been detected and their levels varied among accessions (Fig. 4C). Two *O*-glycosides of luteolin, luteolin (apiosyl-acetyl)-glucoside and luteolin 7-*O*-(2-apiosyl)-glucoside, accumulated highly in ripe fruits of *C. annuum* AC1979 (no. 19) reaching up to 0.014 and 0.005 mmol luteolin 6-*C*-glucoside equivalent/100 g FW, respectively (Fig. 4D). The yellow flavonoid naringenin chalcone accumulated at high levels in ripe fruits of *C. chinense* No. 4661 Selection (no. 18), reaching up to 0.06 mmol naringenin equivalent/100 g FW (Fig. 4E). This chalcone was the main contributor to the total levels of flavonoid glycosides in accession no. 18, up to 0.09 mmol/100 g FW. This level was similar to the total levels of flavonoid glycosides detected in

ripe fruits of *C. annuum* Long Sweet (no. 12). As shown previously (Wahyuni et al. 2011), this accession predominantly accumulates quercetin *O*-glycosides in its ripe fruits, at much higher levels of quercetin glycosides in its ripe fruits than the other four accessions (Fig. 4), and was therefore regarded as a 'high flavonoid' accession. Even higher levels of total quercetin aglycone (as detected after acid hydrolysis) were present in unripe fruits (up to 34 mg/100 g FW) of accession no. 12 and the levels decreased upon ripening to a final level of 14 mg/100 g FW.

In addition to flavonoids, we also analyzed total lignin content in pericarp tissue of all samples (Fig. 4F). Total lignin levels varied among pepper accessions. Accession no. 19 accumulated the highest levels of lignin at all fruit stages, reaching the highest concentration, up to 30 mg/g dry weight, at turning stage. In contrast, the concentration of total lignin accumulated in accession no. 12 was significantly lower (only 2.4 mg/g dry weight at turning stage) than those in the other four accessions.

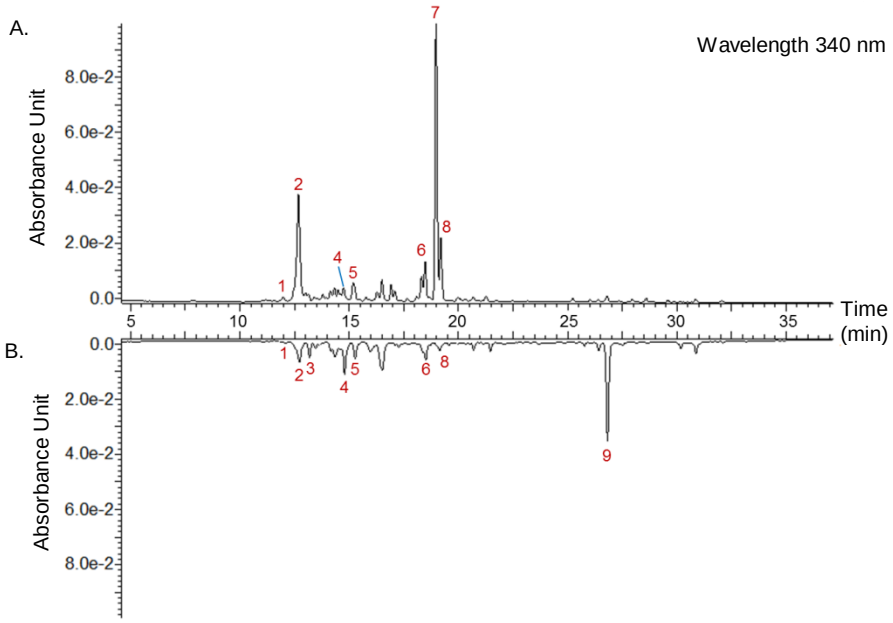



**Fig. 3** LC-PDA chromatogram of pericarp extract of (A), *C. annuum* Long Sweet (no. 12) and (B), *C. chinense* No. 4661 Selection (no. 18) at ripe stage showing several flavonoid glycosides, which are putatively identified as (1) luteolin 6,8-di-*C*-hexoside; (2) quercetin 3-*O*-rhamnoside-7-*O*-glucoside; (3) apigenin 6,8-di-*C*-hexoside; (4) luteolin 6-*C*-hexoside; (5) luteolin 8-*C*-hexoside; (6) quercetin rhamnoside-glucoside; (7) quercetin 3-*O*-rhamnoside; (8) luteolin (apiosyl-acetyl)-glucoside; and (9) naringenin chalcone.

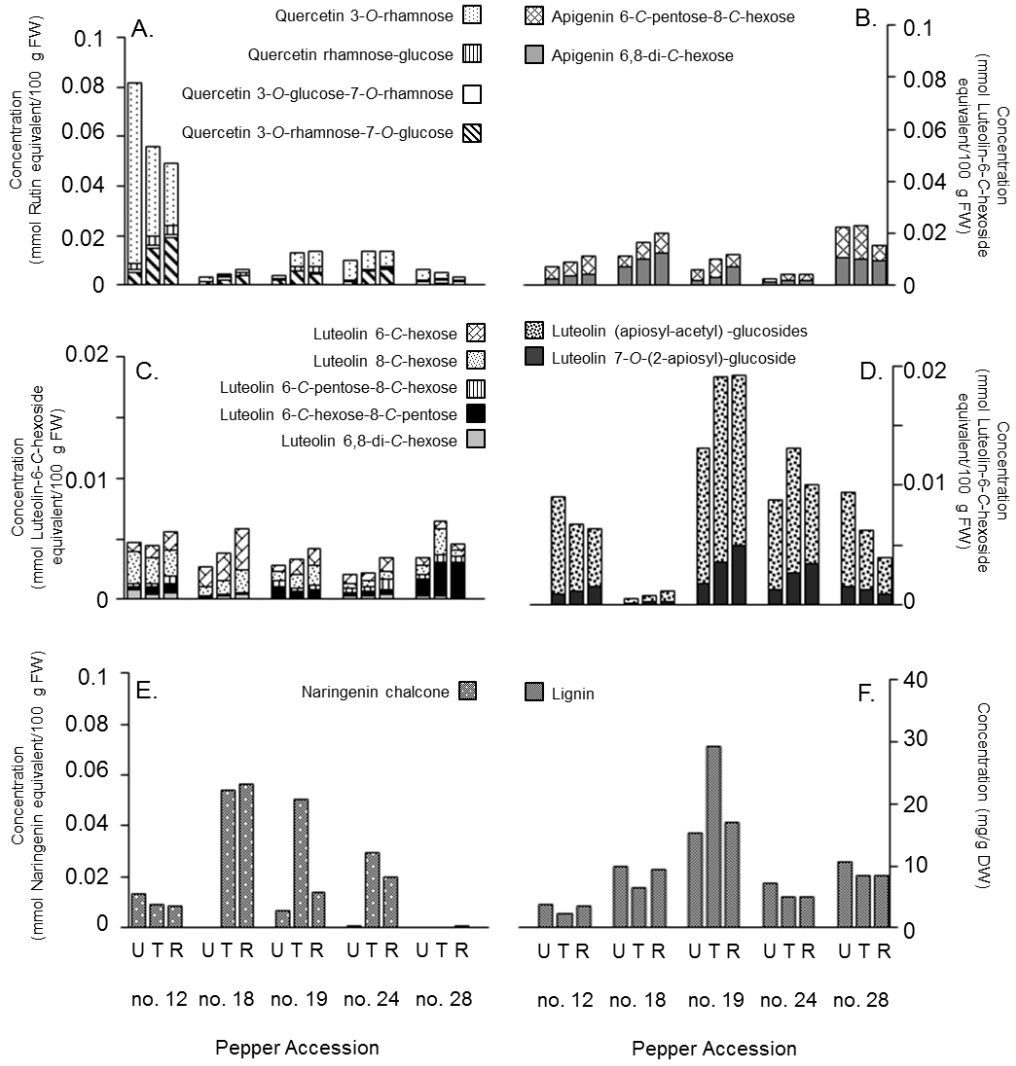



**Fig. 4** Concentrations of flavonoids and lignin at unripe (U), turning (T) and ripe (R) stages in fruit pericarp of *C. annuum* Long Sweet (no. 12), *C. chinense* No. 4661 Selection (no. 18), *C. annuum* AC1979 (no. 19), *C. annuum* AC2212 (no. 24) and *C. frutescens* Lombok (no. 28). A, quercetin glycosides; B, apigenin glycosides; C, luteolin *C*-glycosides; D, luteolin *O*-glycosides; E, naringenin chalcone; and F, lignin. Each bar represents the average value of three biological replicates. DW: dry weight.

### 3.2. Relative expression of genes underlying differential flavonoids biosynthesis by using quantitative gene expression analysis

The above-mentioned metabolic analyses revealed large differences between these five accessions in flavonoid accumulation during ripening. To gain insight in the molecular regulation of this pathway, the expression of several candidate structural genes and regulator genes involved in the flavonoid and the upstream phenylpropanoid pathway was analysed using real-time quantitative RT-PCR. As possible targets, we recently identified the *Ca-MYB12* transcription factor gene and 13 candidate structural flavonoid pathway genes based on homology with known flavonoid genes from tomato and *Lobelia erinus* (Supplemental Table 1; Wahyuni et al. 2013). This set was extended with several candidate genes from the upstream phenylpropanoid pathway, i.e. three *phenylalanine ammonia-lyase* (*PAL*), a *cinnamate 4-hydroxylase* (*C4H*), a *4-coumarate:CoA ligase* (*4CL*), a *hydroxycinnamoyl transferase* (*HCT*), a *p-coumaroyl shikimate 3-hydroxylase* (*C3H*), two *caffeic acid O-methyltransferase* (*CAO*) and a *cinnamoyl-CoA reductase* (*CCR*) genes (Supplemental Table 1). Of all genes tested, five showed very low expression levels in fruits compared to others: *CHS-A*, *CHS-1*, *CHI-3*, *F3'H-2*, *F3'H-3* (Supplemental Table 2 and Supplemental Fig. 1), suggesting that these genes do not play an important role in the regulation of the flavonoid pathway in pepper fruit. We decided to focus our analyses on a set of 18 candidate structural genes with a high expression level in fruit. The phenylpropanoid genes *PAL*, *C4H* and *4CL* showed their highest expression at unripe stage and their levels decreased upon ripening. The three phenylpropanoid genes were detected at similar levels in all five accessions (Fig. 5). In addition, a candidate gene involved in the production of lignin, *CCR*, decreased upon ripening, but had 20-fold lower levels in fruits of *C. annuum* Long Sweet (no. 12) compared to the other four accessions (Supplemental Table 2 and Supplemental Fig. 1).

In general, all flavonoid genes tested showed their highest mRNA expression at green stage and their levels decreased upon ripening (Fig. 5). However, the expression of the *CHS-2* gene decreased upon ripening in accessions no. 12 and 19, but increased strongly in accession no. 18 and showed a similar, but less pronounced trend in accessions no. 24 and 28.

To determine if there is a clear correlation between flavonoid gene expression and flavonoid accumulation, Hierarchical cluster analysis (HCA) was performed using both gene expression and flavonoid accumulation data. HCA showed that several flavonoids clustered with specific flavonoid candidate genes (Fig. 6).

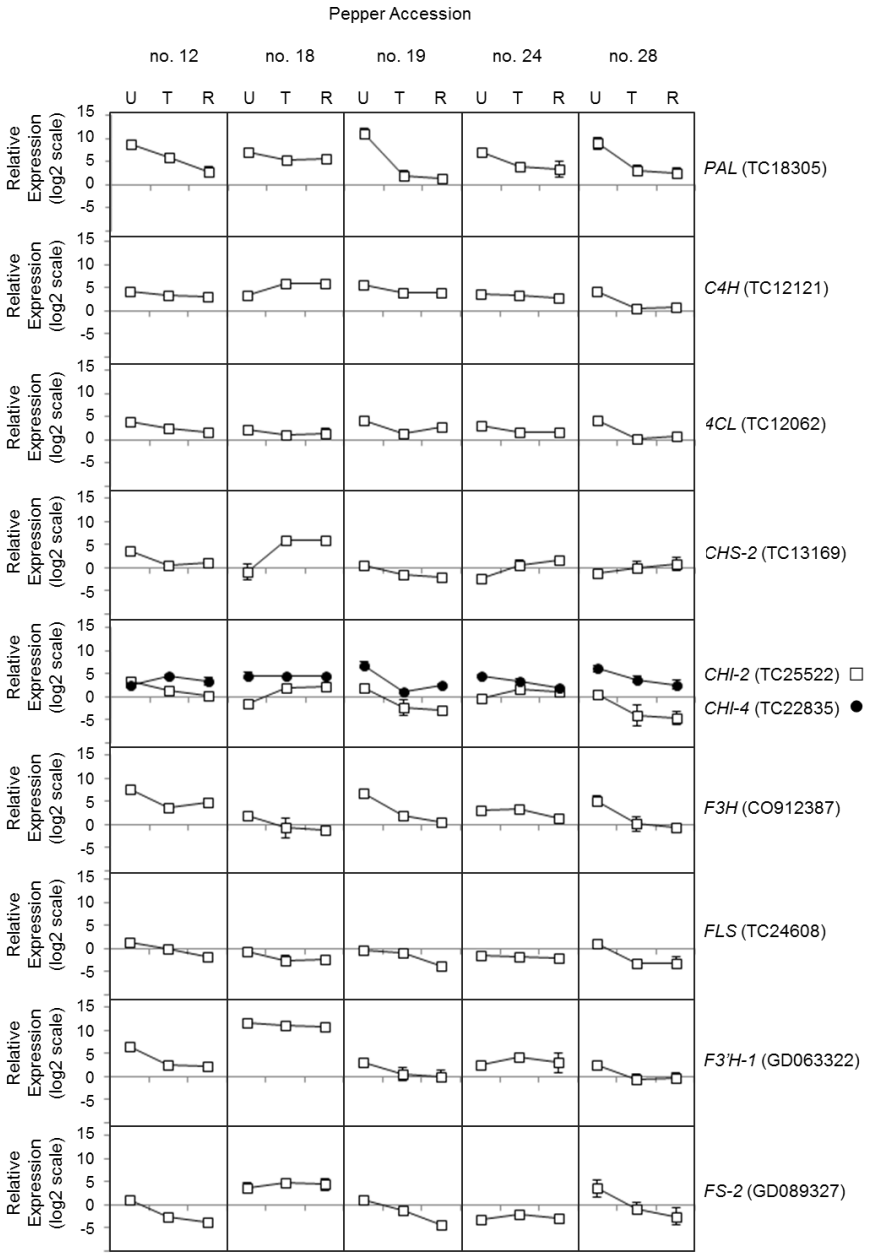



**Fig. 5** Relative expression of candidate genes encoding structural enzymes involved in flavonoid production in unripe (U), turning (T) and ripe (R) fruits of five accessions. Values represent averages of three biological replicates. Abbreviations: PAL, phenylalanine ammonia-lyase; 4CL, 4-coumarate:CoA ligase; CHS, chalcone synthase; CHI, chalcone isomerase; F3H, flavanone-3-hydroxylase; FLS, flavonol synthase; F3'H, flavonoid-3'-hydroxylase; FS, flavone synthase

The main branch of the dendrogram differentiated a cluster with naringenin chalcone from a cluster with quercetin and luteolin aglycone subclusters. Naringenin chalcone clustered with *Ca-MYB12* and *CHS-2*, which catalyses the production of this compound. Quercetin aglycone formed a cluster with *F3H* and *FLS*, the two last genes in the pathway towards quercetin.

*C. annuum* Long Sweet (no. 12) is characterized by very high flavonol levels, which are already present at unripe stage. It is therefore possible that the flavonol levels observed at later stages of ripening do not correlate very well with gene expression levels due to differences in degradation rate of metabolites vs. gene transcripts. To get a better view on the differences between the five accessions at unripe stage, we carried out a second HCA using unripe staged fruit samples only (Fig. 7). In contrast to the other four accessions, all the genes leading to the production of flavonols (from *PAL* to *FLS*) were well expressed in accession no. 12 at unripe stage and all flavonoid genes were present in one single cluster of the dendrogram. In addition, this accession has the lowest lignin levels and the lowest expression of *CCR*, a key gene in the pathway towards lignin. This suggests that the accumulation of high flavonol levels in accession no. 12 is due to a strong flux through the flavonoid pathway, which is partly resulting from a good expression of flavonoid genes at unripe stage, in combination with a suppression of the phenylpropanoid pathway branch towards lignin.

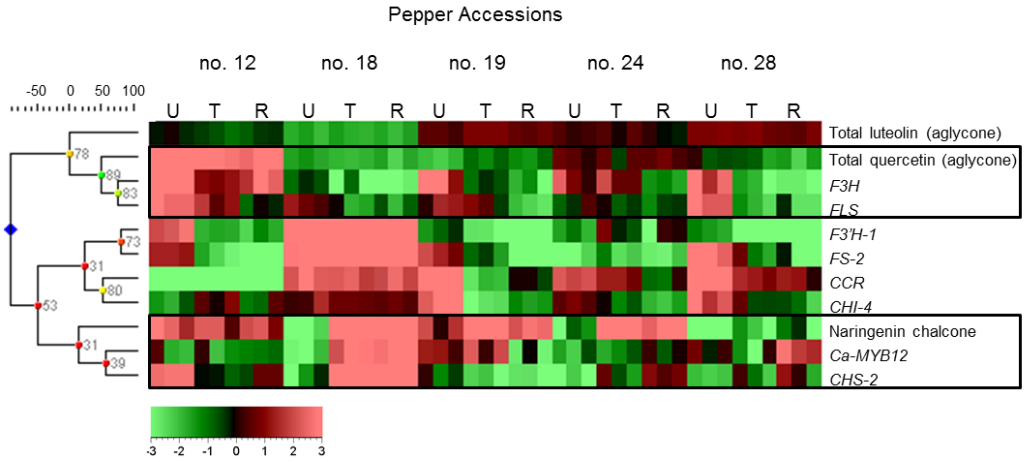



**Fig. 6** Hierarchal Cluster Analysis based on a combined data set of flavonoid metabolite levels and flavonoid candidate gene expression levels in unripe (U), turning (T) and ripe (R) fruits of five pepper accessions. Each genotype/stage combination is represented by three biological replicates. Abbreviations: CHS-2, chalcone synthase-2; CHI-4, chalcone isomerase-4; F3H, flavanone-3-hydroxylase; FLS, flavonol synthase; F3'H-1, flavonoid-3'-hydroxylase-1; FS-2, flavone synthase-2; Ca-MYB12, transcription factor MYB12 of *Capsicum.*

# 4. DISCUSSION

We observed changes in the composition and levels of health-related metabolites during fruit ripening of five contrasting pepper accessions. The change in carotenoid composition was associated with the conversion of chloroplasts into chromoplasts, which led to the degradation of chlorophyll and the formation of coloured-carotenoids (Camara et al. 1995; Egea et al. 2010; Paran and van der Knaap 2007). In red-ripened fruits, chlorophyll was no longer detectable at ripe stage due to the activity of chlorophyllase–magnesium dechelatase–pheophorbide a oxygenase which degrades this green pigment (Minguez-Mosquera and Hornero-Mendez 1994b; Moser and Matile 1997; Roca and Minguez-Mosquera 2006). In parallel, the red carotenoids capsanthin and capsorubin were produced from turning stage onwards. The accumulation of both red carotenoids is controlled genetically by the dominant *Y* locus encoding capsanthin-capsorubin synthase (CCS). In contrast to red peppers, brown-ripened pepper varieties retained their chlorophyll at ripe stage. The degradation of chlorophyll is not occurring in this typical pepper due to a mutation in the chlorophyll retainer gene (*cl*). The presence of green chlorophyll in combination with red carotenoids (in a *Y* background) leads to a brown fruit colour (Efrati et al. 2005).



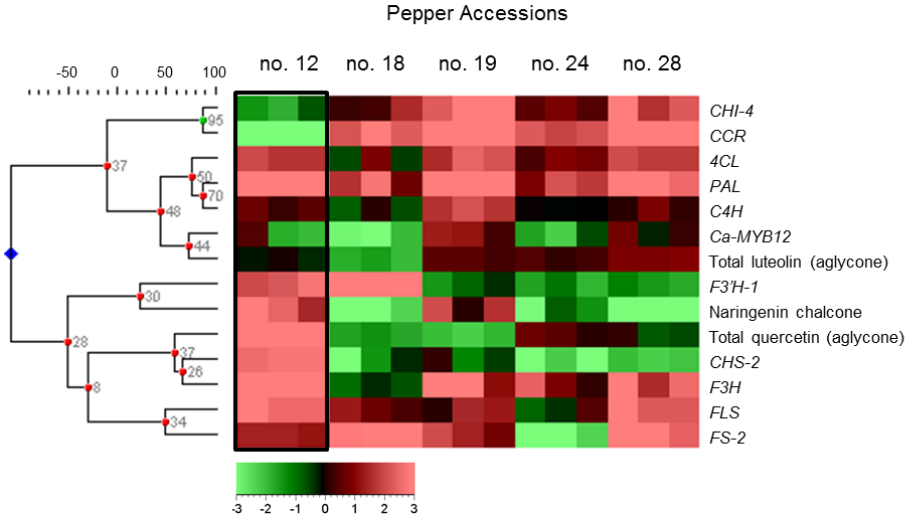



**Fig. 7** Hierarchal Cluster Analysis based on a combined data set of flavonoid metabolite levels and flavonoid candidate gene expression in unripe fruits of five pepper accessions. Each genotype is represented by three biological replicates. Abbreviations: PAL, phenylalanine ammonia-lyase; C4H, cinnamate 4-hydroxylase; 4CL, 4-coumarate:CoA ligase; CHS-2, chalcone synthase-2; CHI-4, chalcone isomerase-4; F3H, flavanone-3-hydroxylase; FLS, flavonol synthase; F3'H-1, flavonoid-3'-hydroxylase-1; FS-2, flavone synthase-2; CCR, cinnamoyl Co-A reductase; Ca-MYB12, transcription factor MYB12 of *Capsicum*.

*C. annuum* Long Sweet (no.12) accumulates high levels of flavonols in its ripe fruits (Wahyuni et al. 2011). This study revealed that even higher levels of flavonol glycosides were already present at unripe stage, suggesting that these compounds are produced at this stage or even earlier. Indeed we observed that all genes in the pathway leading to flavonols were highly expressed in early staged fruits of this accession, in line with the metabolite profiling results. In the four other accessions, expression of at least one gene in the pathway leading to flavonols, for example *CHS-2*, was limiting and this may be the reason for the large difference in flavonols observed between accession no. 12 and all other accessions. Another factor that may contribute to the high flavonol levels in this accession may be caused by the low expression of a key gene in the lignin pathway (*CCR*) and the concomitant low lignin levels in accession no. 12, which suggests that a larger proportion of phenylalanine entering the phenylpropanoid pathway may be targeted to flavonoids rather than to lignin.

Although total quercetin levels remained high in ripe fruits of accession no. 12, there was a clear ripening-related change in the type of quercetin glycosides: levels of quercetin 3-*O*-rhamnose decreased upon ripening, while levels of quercetin 3-*O*-rhamnose-7-*O*-glucose increased. The chemical structures of these quercetin derivatives differ only in one sugar group, attached to the C-7 position of the quercetin molecule. This suggests that, upon ripening, quercetin 3-*O*-rhamnose is converted into quercetin 3-*O*-rhamnose-7-*O*-glucose through the action of a ripening-dependent 7-*O*-glucosyltransferase, as observed in other fruits, such as strawberry, as well (Cheng et al. 1994; Griesser et al. 2008; Halbwirth et al. 2006).

Accumulation of the yellow-coloured flavonoid naringenin chalcone in ripe fruits of *C. chinense* No. 4661 Selection (no. 18) was in accordance with the up-regulation of the *CHS-2* gene, whose encoded enzyme is responsible for the production of naringenin chalcone from 4-coumaroyl CoA and malonyl CoA. The HCA cluster containing naringenin chalcone and *CHS-2* also contained the *Ca-MYB12* transcription factor. In line with these results, we recently showed in a QTL study based on an F2 population derived from a cross between accession no. 18 and 19 that the *Ca-MYB12* gene co-localised with expression QTLs for *Ca-MYB12* and *CHS-2* and a metabolite QTL for naringenin chalcone (Wahyuni et al. 2013). This strongly suggested that MYB12 is a key regulator of the pathway towards naringenin chalcone in pepper fruit.

Also in tomato it was demonstrated that accumulation of naringenin chalcone was dependent on *MYB12* gene expression and that in pink mutants, which no longer accumulate naringenin chalcone upon fruit ripening, the ripening-

dependent induction of *MYB12*, as well its downstream target flavonoid genes, such as *CHS*, was severely reduced (Adato et al. 2009; Ballester et al. 2010). In addition, fruit-specific over-expression of the Arabidopsis *MYB12* gene in transgenic tomato plants led to a strong induction of the flavonoid and phenylpropanoid pathway (Luo et al. 2008), once more supporting the pivotal role of *MYB12* as a key regulator of the flavonoid pathway.

## 5. CONCLUDING REMARKS

In conclusion, we observed ripening-dependent changes in the composition and levels of carotenoids, flavonoids and capsaicinoids in five different pepper accessions. The accumulation of flavonoids was accompanied with changes in flavonoid gene expression. Our results revealed two possible mechanisms underlying the accumulation of the flavonol quercetin and the naringenin chalcone in accession no. 12 and 18, respectively. Up-regulation of all genes in the pathway leading to the production of quercetin at early stages of fruit development may be responsible for the higher accumulation of quercetin in accession no. 12. Accumulation of naringenin chalcone in accession no. 18 may be due to the combined activity of three genes: high expression levels of *Ca-MYB12* and its target *CHS-2*, leading to production of naringenin chalcone, in combination with relatively low levels of *CHI-4* (compared to *CHS-2*) whose product catalyses the conversion of naringenin chalcone into naringenin. These results provide novel insight into the molecular regulation of flavonoid accumulation in pepper fruit.



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We thank Dewi Wulansari and Tammy Soputro for their excellent support in the gene expression analysis. We also thank Harry Jonker and Bert Schipper for their technical help and discussions, Maarten Peters for excellent greenhouse management. This work was supported by the Priority Programme of the Indonesia–Netherlands Koninklijke Nederlandse Akademie van Wetenschappen (SPIN KNAW) INDOSOL (05-PP-21).

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# Supplemental Materials

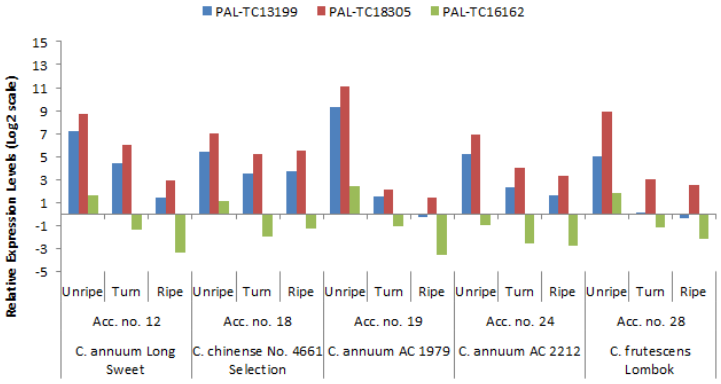
**Supplemental Table 1** Candidate flavonoid genes of *Capsicum* and primers for gene expression analysis (available online via http://edepot.wur.nl/287157)

**Supplemental Table 2** Relative gene expression of candidate flavonoid genes (available online via http://edepot.wur.nl/287156)

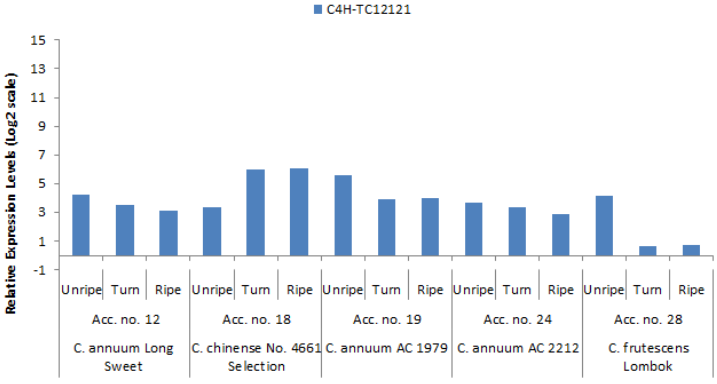
**Supplemental Fig. 1** Relative expression levels of phenylpropanoid and flavonoid candidate genes during fruit ripening (available on page 116 – 120)

**Supplemental Fig. 1** Relative expressions of phenylpropanoid and flavonoid candidate genes during fruit ripening

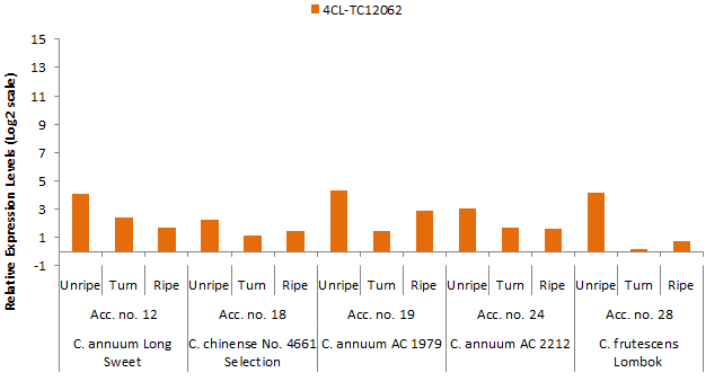
A. *Phenylalanine ammonia lyase* (*PAL*) genes



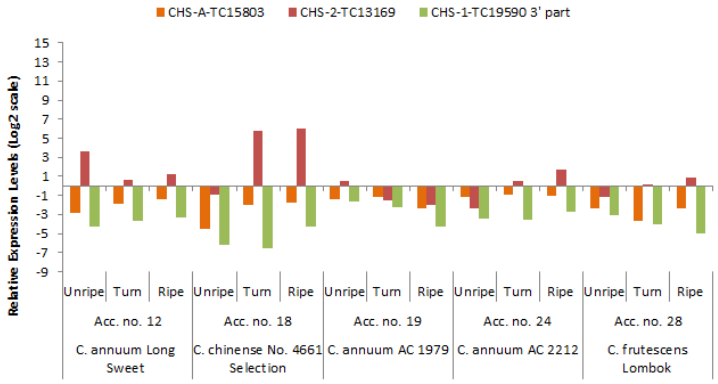

B. *Cinnamate 4-hydroxylase* (*C4H*) gene



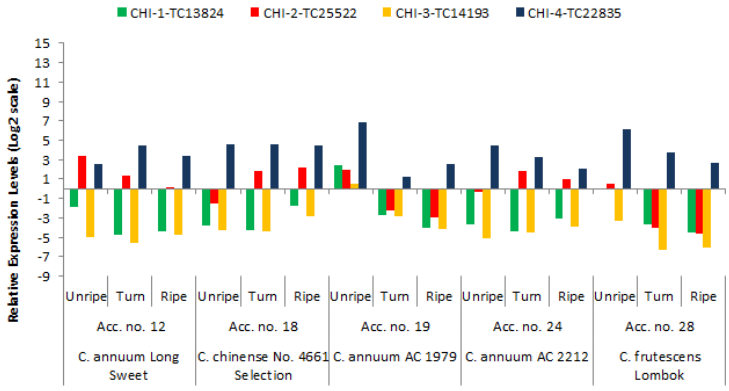

C. *4-coumarate:CoA ligase* (*4Cl*) gene



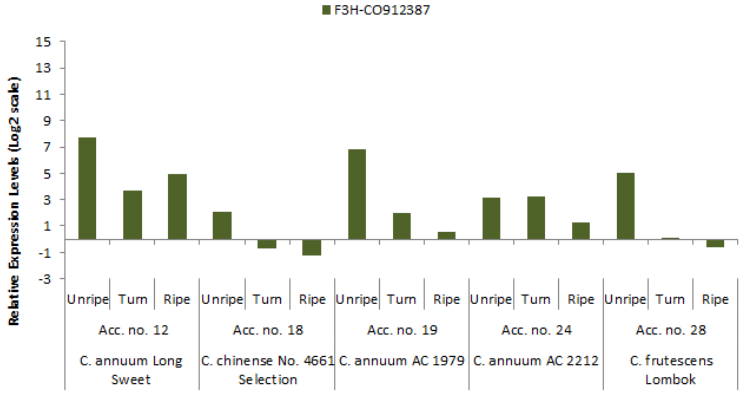
D. *Chalcone synthase* (*CHS*) genes



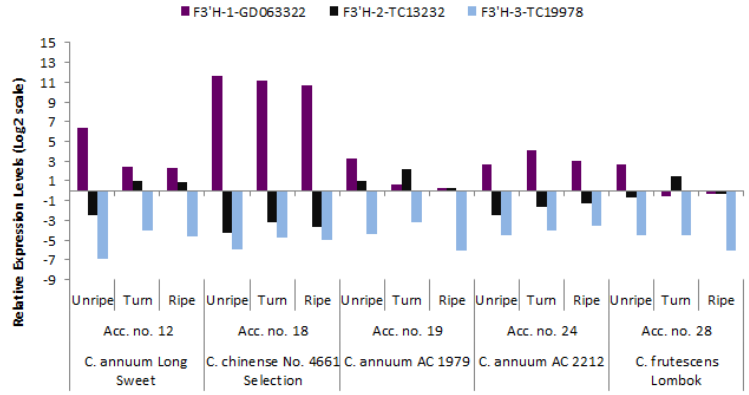

E. *Chalcone isomerase* (*CHI*) genes



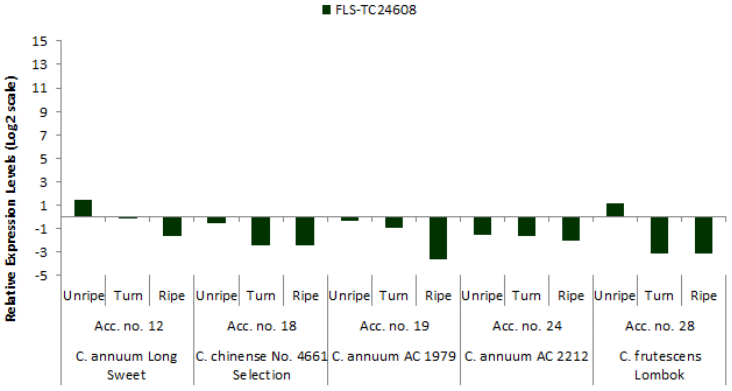

F. *Flavanone 3-hydroxylase* (*F3H*) gene



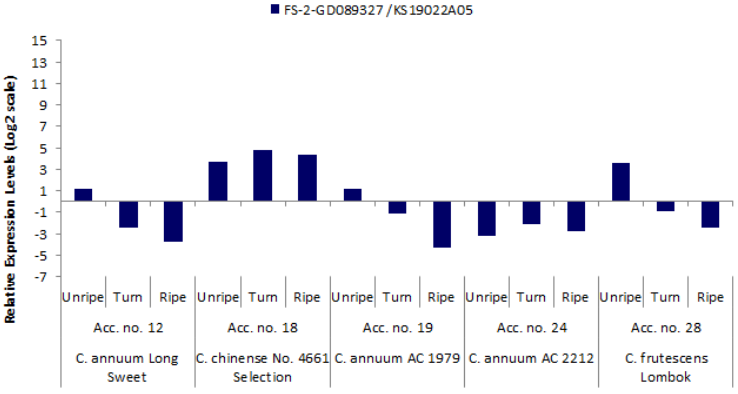
G. *Flavonoid 3`-hydroxylase* (*F3'H*) genes



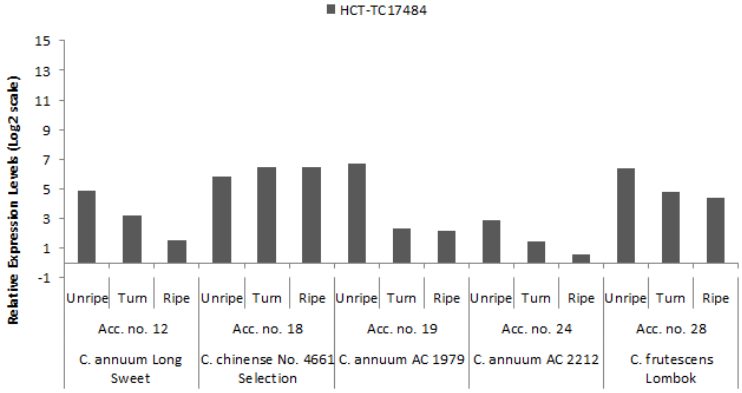

H. *Flavonol synthase* (*FLS*) gene



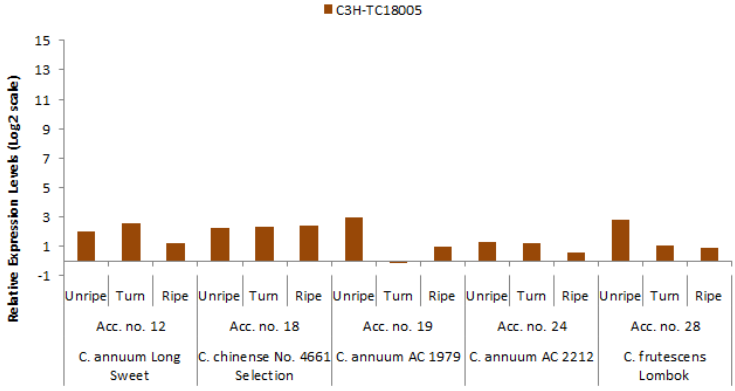

I. *Flavone synthase-2* (*FS-2*) gene



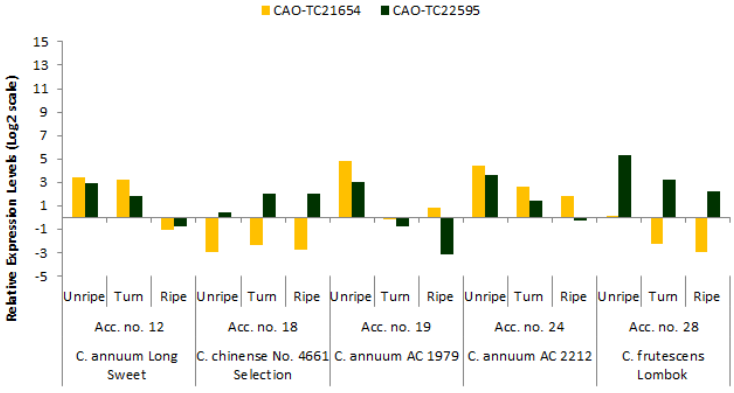
J. *Hydroxycinnamoyltransferase* (*HCT*) gene



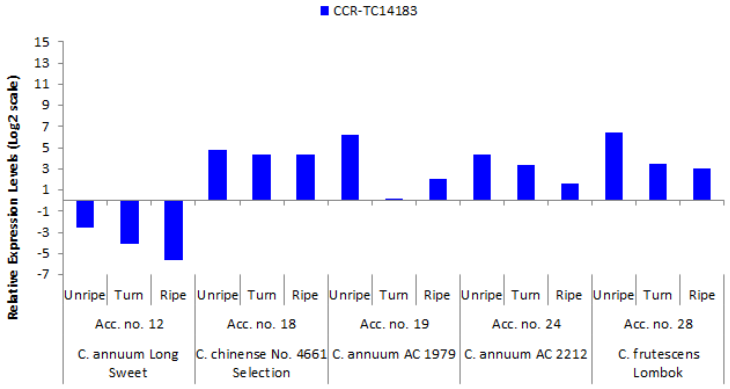

K. *p-coumarate 3-hydroxylase* (*C3H*) gene



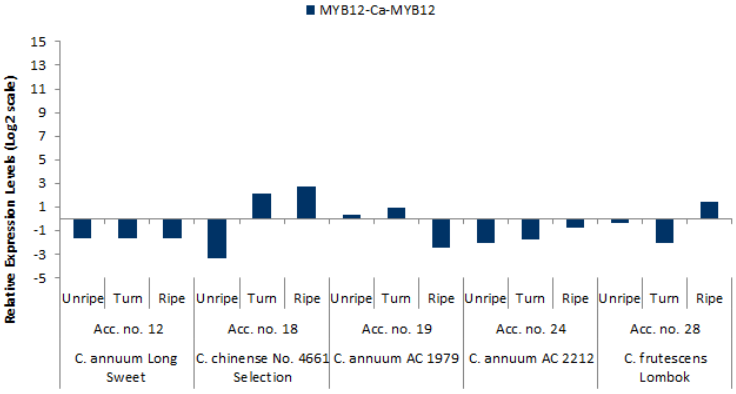

L. *Caffeoyl-CoA O-methyltransferase* (*CAO*) genes



M. *Cinnamoyl-CoA reductase* (*CCR*) gene




N. *MYB12 Transcription factor* (*CaMYB12*) gene



# CHAPTER 5

## Genetic Mapping of Semi-Polar Metabolites in Pepper Fruits (*Capsicum* sp.): towards Unravelling the Molecular Regulation of Flavonoid Quantitative Trait Loci

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# Both authors contributed equally to this manuscript

## ABSTRACT

Untargeted LCMS profiling of semi-polar metabolites followed by metabolite QTL (mQTL) analysis was performed in ripe pepper fruits of 113 F2 plants derived from a cross between *Capsicum annuum* AC1979 (no. 19) and *Capsicum chinense* No. 4661 Selection (no. 18). The parental accessions were selected based on their variation in fruit morphological characteristics and fruit content of some target phytonutrients. Clear segregation of fruit colour and fruit metabolite profiles was observed in the F2 population. The F2 plants formed three clusters based on their metabolite profiles. Of the total of 542 metabolites, 52 could be annotated, including a range of flavonoids, such as flavone *C*-glycosides, flavonol *O*-glycosides and naringenin chalcone, as well as several phenylpropanoids, a capsaicin analogue, fatty acid derivatives and amino acid derivatives. Interval mapping revealed 279 mQTLs in total. Two mQTL hotspots were found on chromosome 9. These two chromosomal regions regulated the relative levels of 35 and 103 metabolites, respectively. Analysis also revealed an mQTL for a capsaicin analogue, located on chromosome 7. Confirmation of flavonoid mQTLs using a set of six flavonoid candidate gene markers and their corresponding expression data (expression QTLs) indicated the *Ca-MYB12* transcription factor gene on chromosome 1 and the gene encoding flavone synthase (*FS-2*) on chromosome 6 as likely causative genes determining the variation in naringenin chalcone and flavone *C*-glycosides, respectively, in this population. The combination of large-scale metabolite profiling and QTL analysis provided valuable insight into the genomic regions and genes important for the production of (secondary) metabolites in pepper fruit. This will impact breeding strategies aimed at optimising the content of specific metabolites in pepper fruit.


**Keywords**:
*Capsicum*, F2 population, semi-polar metabolites, mQTL, flavonoids, *MYB12*

# 1. INTRODUCTION

Pepper (*Capsicum* spp.) is a member of the Solanaceae family, together with other important crops such as eggplant (*Solanum melongena*), tomato (*Solanum lycopersicum*) and potato (*Solanum tuberosum*). The genus *Capsicum* is categorized into 25 species and displays a wide range of genetic diversity. To introduce new traits into commercial hybrids, breeding programs based on interspecific crosses between cultivated and wild species, including *Capsicum annuum* L., *Capsicum chinense* Jacq., *Capsicum frutescens* L., *Capsicum baccatum* L. and *Capsicum pubescens* Ruiz and Pav. are underway. Such crosses produce fertile and heterogeneous progenies (Djian-Caporilano et al. 2007) and give opportunities to introduce economically valuable traits from wild species into the cultivated varieties. One such opportunity is the enrichment of pepper fruits with phytonutrients that encompass potential health-promoting properties.



Metabolite studies in pepper fruits have been mainly focused on the targeted analyses of specific groups of well-known pepper phytonutrients, including ascorbic acid (vitamin C), tocopherols (vitamin E), carotenoids (provitamin A), flavonoids and capsaicinoids (Howard and Wildman 2007; Wahyuni et al. 2011). Untargeted metabolite profiling techniques, such as those based on mass spectrometry (MS), allow the detection of metabolites in a biological sample, without *a priori* knowledge of the identity of the metabolites detected. Such a profiling approach gives the opportunity to analyse hundreds of metabolites simultaneously and provides the necessary information to elucidate metabolic relationships at the biochemical level. For instance, untargeted profiling approaches have been used to obtain an overview of the metabolic diversity in germplasm collections of *Arabidopsis* (Keurentjes et al. 2006), tomato (Tikunov et al. 2005) and pepper (Wahyuni et al. 2013), and have helped scientists to understand the genetic control of the metabolic pathways underlying the main biochemical contrasts between the genotypes under study. Metabolic pathways are generally controlled by multiple genes encoding specific biosynthetic enzymes as well as regulatory factors. The dramatic effect that both structural and transcription factor genes can have on the flux through a metabolic pathway has been demonstrated in, for example, transgenic tomatoes that produce high amounts of flavonols due to overexpression of the petunia *CHI* gene, which encodes the rate-limiting enzyme in the flavonoid pathway in tomato (Muir et al. 2001), or as the result of the introduction of transcription factor genes that up-regulate the endogenous flavonoid pathway genes in the fruit (Bovy et al. 2002; Butelli et al. 2008). The combination of metabolic and genetic analyses helps to

identify genetic markers and key genes that underlie so-called metabolite quantitative trait loci (mQTLs), genomic regions which are associated with increased (or decreased) metabolite levels. Using targeted metabolite analyses approaches, the genetic regulation of specific metabolites, such as chlorophyll (Brand et al. 2012) and the pungent capsaicinoids (Ben-Chaim et al. 2006; Blum et al. 2002, 2003), has been analysed in pepper fruit. Two major QTLs, *pc8.1* and *pc10.1*, that control chlorophyll content were identified in a population based on a cross between a dark green *C. annuum* and a light green *C. chinense* accession (Brand et al. 2012). In addition, the QTL *pc8.1* also affected carotenoid content in ripe fruit in that population. In pungent peppers, the ability to produce capsaicinoids is determined by the presence of a functional *Pun1* gene on chromosome 2, which encodes the acyltransferase AT3 required for the formation of capsaicin from its aromatic precursor vanillylamine and various acyl moieties derived from the catabolism of branched-chain amino acids (Mazourek et al. 2009). A loss-of-function allele of this gene, *pun1-1*, is present in all commercial non-pungent pepper varieties. Up to six QTLs affecting the capsaicinoid levels in pungent accessions have been identified on chromosomes 3, 4 and 7 (Ben-Chaim et al. 2006; Blum et al. 2003).

In order to find novel mQTLs in pepper fruit, we analysed a segregating F2 population derived from a cross between the wild pepper accession *C. annuum* AC1979 (no. 19) and the cultivated accession *C. chinense* No. 4661 Selection (no. 18) for variation in semi-polar metabolites, using accurate mass LC-QTOF-MS. Subsequently we carried out an mQTL analysis using Amplified Fragment Length Polymorphism (AFLP) and microsatellite marker data available for this population (Maharijaya et al. 2012b). The parental accessions were selected based on the results of a previous study with 32 pepper accessions (Wahyuni et al. 2011, 2013) and differed in several metabolic traits: *C. annuum* AC1979 (no. 19) is highly pungent and has a high level of capsianosides (diterpene glycosides) and luteolin-*O*-glycosides (Wahyuni et al. 2011), whereas *C. chinense* No. 4661 Selection (no. 18) showed high levels of the flavonoid naringenin chalcone (Wahyuni et al. 2013). The two accessions also differed for their susceptibility to two thrips species, *Frankliniella occidentalis* and *Thrips parvispinus*: leaves of *C. annuum* AC1979 (no. 19) showed a high thrips resistance, while *C. chinense* No. 4661 Selection (no. 18) was very susceptible (Maharijaya et al. 2011, 2012a). This approach revealed valuable insight into the genomic regions important for the production of (secondary) metabolites in pepper fruit. Confirmation of the results using a candidate gene approach, in which flavonoid mQTL, gene expression (eQTL) and candidate gene marker data were combined, indicated several flavonoid genes as

the causative gene underlying important flavonoid QTLs and provided valuable insight in the molecular regulation of the flavonoid pathway in pepper fruit. The impact of this approach on future breeding strategies aimed at developing new pepper varieties with a desired composition of specific metabolites will be discussed.

# 2. MATERIALS AND METHODS

## 2.1. Plant materials

An interspecific F2 population was derived from a cross between *C. annuum* AC1979 (no. 19) as the female and *C. chinense* No. 4661 Selection (no. 18) as the male (Supplemental Fig. 1), previously described by Maharijaya et al. (2012b). In short, seeds of the two parental accessions were obtained from the Centre for Genetic Resources, the Netherlands (CGN). After crossing the two accessions, the F1 plants were grown in a controlled environmental greenhouse located in Wageningen (The Netherlands) from December 2007 until September 2008. Two F1 plants showing homogeneous phenotypes were chosen and were self-pollinated to obtain the F2 generation. The F2 population was subsequently cultivated from December 2008 until September 2009 and consisted of 201 plants: 81 plants from F1-1 and 120 plants from F1-2. These plants were randomly arranged in eight rows of 32 plants: including F2 plants, cuttings of F1 plants and the parental plants. Of the 201 F2 plants, only 113 plants produced enough fruits for further analysis and, depending on fruit size, 10-50 ripe fruits were harvested from each of these plants. Whole fruits were frozen in liquid nitrogen, ground and stored at -80 °C for further metabolite analysis.



## 2.2. Extraction and analysis of semi-polar metabolites

Semi-polar metabolites were analysed from whole ripe fruits of 113 F2 plants, two F1 plants, and the parents *C. annuum* AC1979 (no. 19) and *C. chinense* No. 4661 Selection (no. 18). Metabolite extraction was performed according to de Vos et al. (2007). Briefly, ripe pepper fruits were frozen in liquid nitrogen and ground to a powder. Aliquots of 500 mg frozen powder were extracted with 1.5 ml of 99.875% methanol acidified with 0.125% formic acid. The extracts were sonicated, centrifuged and filtered through a 0.2 µm polytetrafluoroethylene (PTFE) filter. To check for total technical variations (i.e. extraction, sample analysis and data-processing), quality control samples were prepared by pooling fruit material that

was extracted 10 times using the same procedure and injected after every 15 extracts.

All extracts were analysed using reversed phase liquid chromatography (Waters Alliance HPLC 2695) coupled to a photodiode array detector and a quadrupole time of flight high-resolution mass spectrometry (LC-PDA-QTOF-MS) system (Waters QTOF-Ultima), using C18-reversed phase chromatography (Phenomenex C-18 Luna column) and negative electrospray ionization, mainly as described previously (de Vos et al. 2007). Extracts were diluted 10 times to prevent ionisation saturation and 5 µl of the diluted extract was injected and separated using a binary gradient of ultrapure water (A) and acetonitrile (B), both acidified with 0.1% formic acid, with a flow rate of 0.19 ml/min. The initial solvent composition consisted of 95% of A and 5% of B, increased linearly to 35% A and 65% B in 45 min and maintained for 2 min. The column was washed with 25% A and 75% B for 5 min and equilibrated to 95% A and 5% B for 2 min before next injection.

### 2.3. Metabolite data processing

Semi-polar metabolite data were processed as described below:

#### 2.3.1. Mass spectral alignment, filtering and clustering

The dataset was processed using the MetAlign software package (www.metalign.nl) for baseline correction, noise estimation, and ion-wise mass spectral alignment (Lommen and Kools 2012). Absent values were replaced by the local noise. The MetAlign output was processed with MSClust for data reduction and extraction of compound mass spectra through clustering of individual ions originating from the same metabolite (Tikunov et al. 2012). A total of 542 so-called reconstructed metabolites were thus obtained and used for mQTL analyses.

#### 2.3.2. Putative identification of semi-polar metabolites

The identification of semi-polar metabolites was based on their UV spectra (if present), observed accurate mass (LC-QTOF-MS) and $MS^n$ fragmentation patterns (LTQ-Orbitrap FTMS), as described by Wahyuni et al. (2012). Putative identification of semi-polar metabolites was obtained using different metabolite databases such as Dictionary of Natural Products (http://dnp.chemnetbase.com) and KNApSAcK (http://kanaya.naist.jp/KNApSAcK) and based on comparison with an accurate mass in-house metabolite database developed from literature data

(Marín et al. 2004) and previous metabolomics experiments in combination with accurate $MS^n$ fragmentation experiments (Wahyuni et al. 2011, 2013).

2.3.3. Multivariate analysis

Semi-polar metabolite datasets containing the intensity levels of all centrotypes for all pepper samples were analysed separately using multivariate statistical analyses included in the Genemath XT version 1.6.1 software. Pre-treatment of the data was performed by $\log_2$ transformation and mean centering. 542 metabolites were subjected to principal component analysis (PCA) and hierarchical cluster analysis (HCA). HCA was performed by using the UPGMA method and Pearson's coefficient matrix in Genemath XT software. To test the reliability of the dendrogram produced by HCA, bootstrap analysis was performed with 100 replications.



## 2.4. Genetic linkage map construction and metabolic QTL mapping analysis

A genetic linkage map was constructed with AFLP and microsatellite markers as described by Maharijaya et al. (2012b). In brief, 15 primer combinations were used to develop the following AFLP markers: P17-M39, P17-M32, P14-M50, P14-M49, P14-M48, P14-M41, P11-M61, P11-M48, E38-M49, E36-M48, E35-M58, E35-M49, E35-M48, E34-M48 and E32-M49. The sequence of these primers followed the standard list for AFLP primer nomenclature (http://wheat.pw.usda.gov/ggpages/keygeneAFLPs.html). For microsatellite marker amplification, 56 simple sequence repeat (SSR) primers were used and the primer sequences were described in Maharijaya et al. (2012b). The microsatellite markers were used to assign linkage groups to known pepper markers (Lee et al. 2009; Wu et al. 2009; Yi et al. 2006). Based on all markers a linkage map was constructed using JoinMap 4.1 (van Ooijen 2006).

Metabolite QTL mapping analysis was performed using MapQTL 6.0 (van Ooijen 2009). Briefly, the dataset containing the relative intensities of 542 metabolites were subjected to QTL analysis. To define the significance threshold of logarithm of odds (LOD), 1,000 permutation tests ($P \leq 0.05$) were performed on several metabolites having low LOD score values for QTL detection. This led to a LOD threshold value of 3.7.

### 2.5. RNA extraction

Total RNA was extracted from 50 mg freeze-ground pericarp of ripe fruits of 113 F2 segregating plants and the two parents *C. annuum* AC1979 (no.19) and *C. chinense* No. 4661 Selection (no.18) using RNeasy Plant Minikit (Qiagen) according to the manufacturer's instructions. The quality and quantity of total RNA were measured by Nanodrop spectrophotometer ND1000 (ISOGEN Life Science) and evaluated by electrophoresis using 200 ng of the total RNA from each sample on 1.2% w/v agarose gel. cDNA was synthesized from 1 μg of total RNA using Taqman® Reverse Transcription (Applied Biosystems) according to the manufacturer's instructions.

### 2.6. Selection and isolation of pepper flavonoid candidate genes

Putative flavonoid candidate gene sequences were selected from the pepper gene index database (http://compbio.dfci.harvard.edu/cgi-bin/tgi/gimain.pl?gudb=pepper), based on homology with known flavonoid genes from tomato, i.e. *chalcone synthase* (*CHS*), *chalcone isomerase* (*CHI*), *flavanone 3-hydroxylase (F3H), flavonol synthase (FLS), flavonoid 3'-hydroxylase (F3'H)*. In short, the tomato genes were compared with the pepper gene index using BLAST-N and the five best hits for each gene were re-BLASTed against the tomato genome. Only those expressed sequence tags showing their best hit with a known tomato flavonoid gene were considered as a candidate for gene expression and/or marker analysis. In addition, a candidate *flavone synthase-2* (*FS-2*) was selected based on homology with the *Lobelia erinus FS-2* gene. Furthermore, a *MYB12* transcription factor gene (coded as *Ca-MYB12* in this report) was isolated from pepper cDNA using the rapid amplification of cDNA ends (RACE)-PCR technique, using primer sets derived from the *SlMYB12* gene. For each candidate gene, a set of primers was designed using Primer3Plus (http://probes.pw.usda.gov/cgi-bin/batchprimer3/batchprimer3.cgi). Candidate genes and primer sequences are described in Supplemental Table 1.

### 2.7. Flavonoid gene expression analysis

Gene expression analysis was carried out by quantitative real-time PCR (qRT-PCR) using iCycler iQ machine (Bio-Rad Laboratories) with iQ SYBR Green Supermix (Bio-Rad Laboratories) as fluorescent dye. The housekeeping gene, *α-tubulin* was chosen to normalize the expression levels of candidate genes, due to the stability of its Ct (threshold cycle) value over all samples (results not shown). Expression levels of candidate genes were determined by the delta Ct (ΔCt) values,

calculated by subtracting the Ct value of each candidate gene with the Ct value of *α-tubulin*. For easy representation, $2^{-\Delta Ct}$ was calculated, multiplied by 100 and converted to $\log_2$, as described by Livak and Schmittgen (2001).

### 2.8. Detection of SNPs in flavonoid candidate genes and mapping of the genetic position of flavonoid SNP markers

Single nucleotide polymorphism (SNP) detection was carried out by sequence analysis of PCR fragments of 14 flavonoid genes and one *R2R3-MYB12* transcription factor gene obtained from cDNA and/or genomic DNA of the two parents of the F2 population. Based on SNPs detected on sequences from both parents, a pair of specific primers was designed using Primer-Picker (KBioscience, UK) for each SNP (Supplemental Table 1) and genotyping of the F2 population was carried out using KASPar SNP Genotyping (KBioscience, UK). A subset of SNP profiles was integrated with AFLP and microsatellite markers to reconstruct the genetic map using JoinMap 4.1 (van Ooijen 2006).



### 2.9. Flavonoid QTL and flavonoid eQTL analyses

Flavonoid QTLs obtained in previous analysis of this research were re-analysed with the genetic map improved with flavonoid SNP markers using MapQTL 6.0 (van Ooijen 2009). Subsequently, flavonoid gene transcript QTL (eQTL) analysis was performed with the same genetic map and software as those used to re-analysed flavonoid QTLs. The significance LOD thresholds was defined with a permutation test (1,000; $P \leq 0.05$) on genes and several metabolites having low LOD score values for QTL detection, leading to a LOD threshold value for eQTL of 3.6 and mQTL of 3.7.

## 3. RESULTS

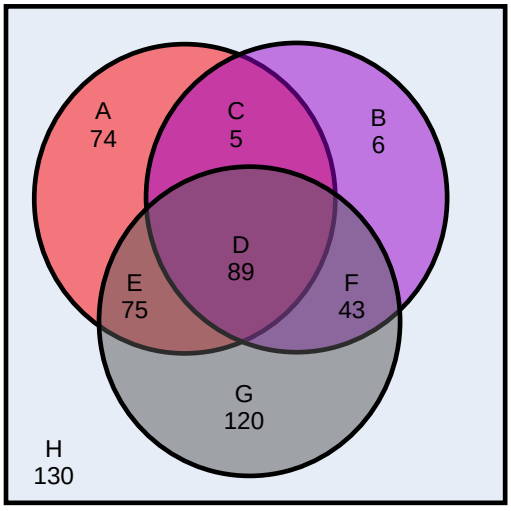
### 3.1. Phenotype of parental, F1 and F2 plants

The two parental accessions that were used for generating the F2 population (Supplemental Fig. 1), *C. annuum* AC1979 (no. 19) and *C. chinense* No. 4661 Selection (no. 18), varied in fruit colour, shape and size. Fruits of *C. annuum* AC1979 (no. 19) were small (1.5 cm) and the colour of ripe stage fruits was red (Supplemental Fig. 2a), while *C. chinense* No. 4661 Selection (no. 18) produced large (8.5 cm) brown ripe fruits (Supplemental Fig. 2b). Ripe fruits of F1 plants were red [similar to the female parent *C. annuum* AC1979 (no. 19)], with a length

of about 2.5 cm (Supplemental Fig. 2c). The ripe fruit colour of the F2 plants segregated among individuals and ranged over dark red, red, brown and dark brown with a frequency of 20, 56, 13 and 11%, respectively (Supplemental Fig. 2d). In addition, the fruit size segregated among F2 plants: the smallest fruit was 1 cm and the biggest reached up to 5 cm (Supplemental Fig. 2d).

### 3.2. Semi-polar metabolites in ripe fruits of parental, F1 and F2 plants

From previous work we observed that *C. annuum* AC1979 (no. 19) accumulated relatively high levels of capsianosides, capsaicinoids, chlorogenic acid and luteolin *O*-glycosides in its fruits, while *C. chinense* No. 4661 Selection (no. 18) fruits showed relatively high levels of naringenin chalcone and several specific flavonoid glycosides, such as a flavanone-tri-methyl-hexose-pentose and quercetin 3-*O*-rhamnoside-7-*O*-glucoside (Wahyuni et al. 2011, 2013). Using untargeted LC-PDA-QTOF-MS we determined the metabolite profiles of ripe fruits from parental, F1 and F2 plants. In total, 542 semi-polar metabolites were detected over all samples, of which 74 metabolites (14%) were detected only in parent *C. annuum* AC1979 (no. 19) and six metabolites (1%) were detected only in *C. chinense* No. 4661 Selection (no. 18) (Fig. 1; circles A and B).




| Codes: | No. of Metabolites |
|---|---|
| A: P1 | 74 |
| B: P2 | 6 |
| C: P1+P2 | 5 |
| D: P1+P2+F1 | 89 |
| E: P1+F1 | 75 |
| F: P2+F1 | 43 |
| G: F1 | 120 |
| H: Only present in F2 | 130 |
| **Total:** | **542** |

**Fig. 1** Venn diagram of 542 semi-polar metabolites detected in ripe fruits of two parental accessions: P1=*C. annuum* AC1979 (no. 19) and P2=*C. chinense* No. 4661 Selection (no. 18), and F1 and F2 plants. Values in *circles* and *intersections* illustrate the number of metabolites accumulating in each of the sample groups. A metabolite was denoted as "detected" in a given sample when the mass intensity level of its centrotype mass peak was above the local noise. According to the Metalign output the local noise was set at a mass intensity of 37.

Despite those differences, the two parents also shared five metabolites (0.9%) which were not detected in the two F1 plants (Fig. 1; intersection C). The fruit metabolite profiles of the two F1 plants showed that these plants were identical and contained 120 metabolites (22%) that were below detection limits in either parent (Fig. 1; circle G). In addition, the contribution of each parent to the metabolite profiles of the F1 was shown by the presence of 75 metabolites (14%) derived from female parent no. 19 (Fig. 1; intersection E) and 43 (8%) derived from male parent no. 18 (Fig. 1; intersection F). Another 89 metabolites (16%) were shared between all F1 plants and both parents (Fig. 1; intersection D). The other 130 metabolites (24%) were detected neither in parents nor in F1 plants, thus they were uniquely detected in fruits from F2 plants (Fig. 1; H).

Out of the 542 metabolites, 52 could be putatively identified based on comparison with an accurate mass in-house metabolite database developed from literature data (Marín et al. 2004) and from previous metabolomics experiments using the same LCMS system, in combination with accurate $MS^n$ fragmentation experiments (Wahyuni et al. 2013; Supplemental Table 2). These metabolites belonged to a number of compound classes, such as flavonoids, phenylpropanoids, acyclic diterpenoids and fatty acid derivatives. Of all putatively identified metabolites, 43 accumulated at detectable levels in fruits of one or both parents and/or the F1 plants (Fig. 2), whereas 9 metabolites were uniquely found in F2 plants only. Acyclic diterpenoids with different sugar decorations, a capsaicin analogue, a lignan, fatty acid derivatives and amino acid derivatives, as well as the flavone luteolin 7-*O*-(2-apiosyl)-glucoside were found in the female parent and F1 plants. In addition, we detected naringenin chalcone, quercetin 3-*O*-rhamnoside-7-*O*-glucoside, luteolin *C*-glycosides and some yet unknown metabolites in the male parent and F1 plants. Flavonoids, including apigenin, luteolin and quercetin glycosides were relatively abundant in F1 plants compared to the parents.

The semi-polar metabolite profiles in ripe fruits of all F2 plants were subjected to PCA and HCA. Based on the variation in semi-polar metabolite profiles, PCA revealed a separation of the samples into three main clusters: Cluster I contained eight F2 plants, Cluster II consisted of 11 F2 plants plus the accession *C. annuum* AC1979 (no. 19) and Cluster III contained 92 F2 plants together with the F1 plants (Fig. 3a). Three F2 plants and the male parent *C. chinense* No. 4661 Selection (no. 18) were not assigned to a specific cluster and they showed an intermediate behaviour. The first principal component (PC1) explained 32.5% of the variation and separated Cluster I and Cluster II from Cluster III. The second principal component (PC2) explained 11.2% of the variation and separated Cluster I from Cluster II.

HCA of the set of 542 semi-polar metabolites revealed the presence of specific metabolite groups, arbitrarily denoted A to F, as characterised by their specific accumulation pattern across the genotypes (Fig. 3b). Structurally related metabolites derived from the same metabolic pathway may cluster together, as shown previously for volatiles in tomato (Tikunov et al. 2005) and pepper (Eggink et al. 2012; Wahyuni et al. 2013). The heat map of the 542 metabolites shows a group of 98 metabolites in group D, which contained all flavone *C*-glycosides next to flavonol *O*-glycosides, naringenin chalcone and its glycoside derivative, phenylpropanoids, a capsaicin analogue, fatty acid derivatives and amino acid derivatives (Fig. 3b; Supplemental Table 2). The metabolites in this group and all metabolites in group C were present at different relative abundances in all plants tested and their segregation pattern in the F2 plants was not specific for any of the three genotype clusters, since genotypes with high and low abundance of these metabolites were present in each cluster. Metabolites in group B accumulated at relatively high abundance in the ripe fruits from the F2 plants of Cluster I only. Three out of the 90 metabolites of group B were putatively identified as capsicosides, a group of steroidal glycosides detected previously in *C. annuum* var. *acuminatum* seeds (Iorizzi et al. 2002; Yahara et al. 1994). These compounds were reported to have antimicrobial activity against yeast and fungi (Iorizzi et al. 2002). All 94 metabolites in group A accumulated at relatively high levels in Cluster II which contained several F2 plants and the female parent (no. 19). Group A contained five capsianosides and six flavonoid *O*-glycosides, including several luteolin *O*-glycosides as well as quercetin and kaempferol *O*-glycosides. These compounds were previously shown to accumulate at relatively high levels in female parent no. 19 (Wahyuni et al. 2013) and in the F1 plants (Fig. 3b; Supplemental Table 2), in comparison to parent no. 18. The 187 metabolites in groups E and F were detected at low levels in genotype Clusters I and II and contributed to the characteristic metabolite pattern of genotype Cluster III. Metabolites of group E accumulated at relatively high levels in all plants of genotype Cluster III and contained several unique but as yet unknown metabolites, detected as doubly charged negative ions, as well as one capsianoside and one capsoside (Fig. 3b; Supplemental Table 2). Capsoside is a cell wall galactolipid that has been previously detected in very small amounts in ripe fruits of *C. annuum* var. *acuminatum* (Iorizzi et al. 2001). Other capsianosides with different glycoside decorations were present in group D, E and F. In addition, one specific flavonol glycoside, present in group F, was detected at relatively high abundance in several F2 plants of Cluster III.

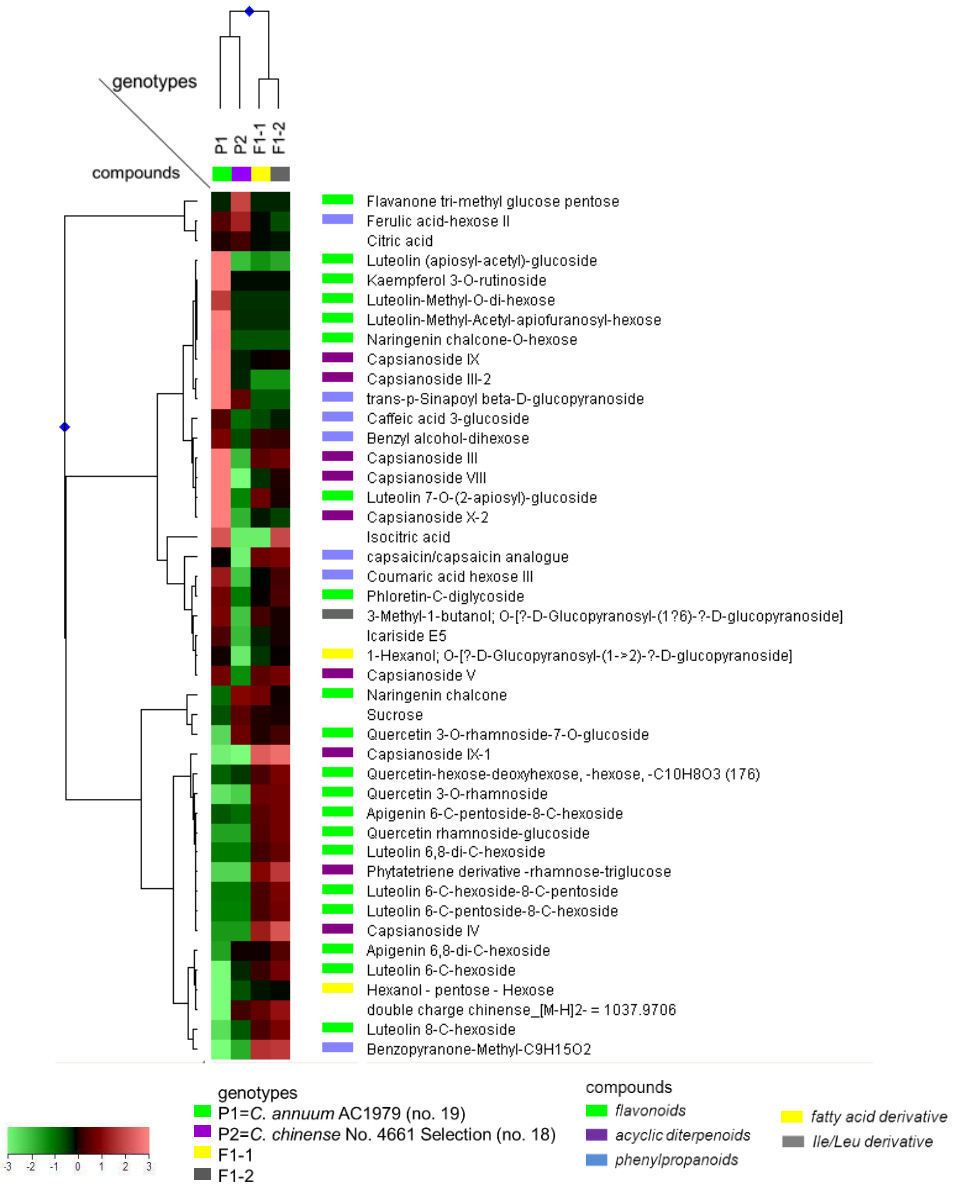



**Fig. 2** Heat map of 43 putative semi-polar metabolites which accumulated in ripe fruits of the parental accessions: *C. annuum* AC1979 (no. 19) and *C. chinense* No. 4661 Selection (no. 18), and/or the two F1 plants. A *colour-coded matrix* represents values of the metabolite intensity in each genotype, which has been $\log_2$-transformed and mean-centered.

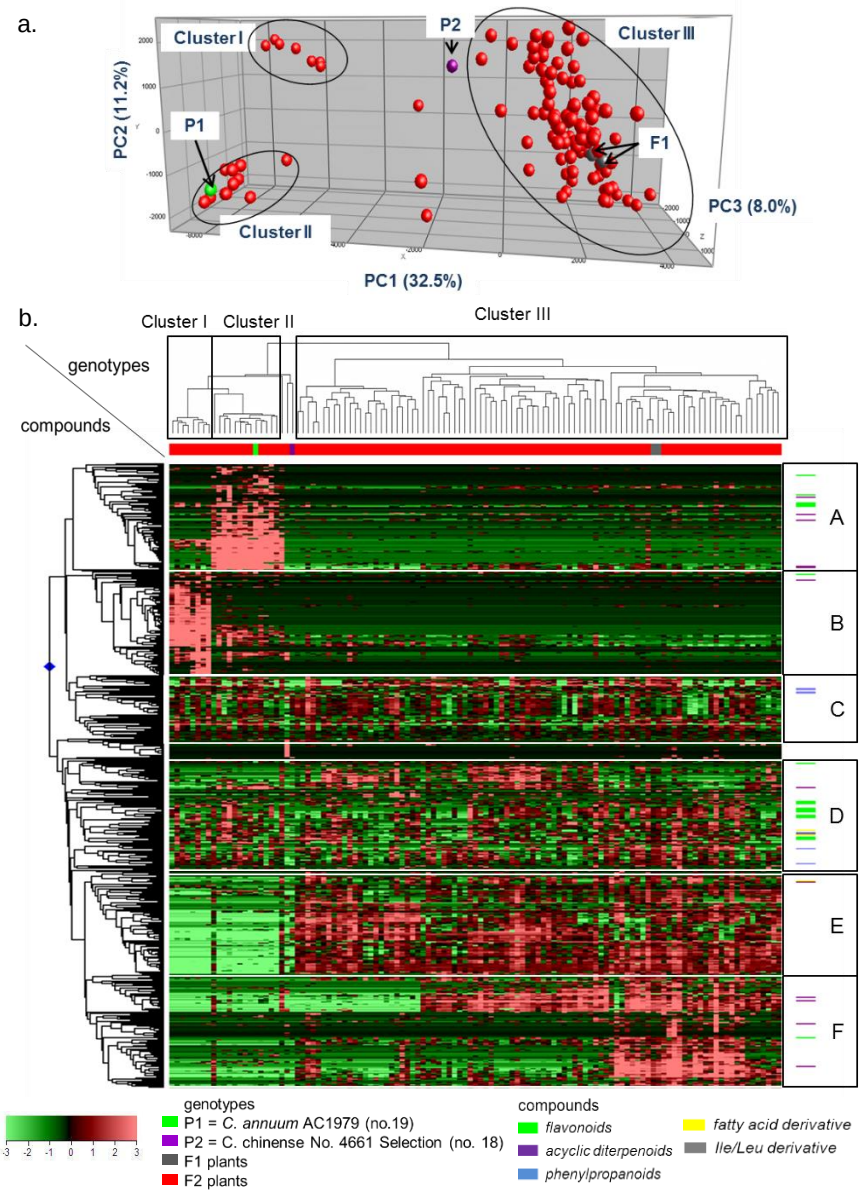



**Fig. 3** Principal component and hierarchical cluster analysis of F2 plants, two F1 plants and two parental accessions. **a** Principal component analysis. P1=*C. annuum* AC1979 (no. 19) and P2=*C. chinense* No. 4661 Selection (no. 18), based on 542 semi-polar metabolite profiles in ripe fruits. **b** Hierarchical cluster analysis. Heat map of 542 metabolites in ripe fruits of F2, two F1 and two parental accession plants, *C. annuum* AC1979 (no. 19) and *C. chinense* No. 4661 Selection (no. 18). A *colour-coded matrix* represents the value of the metabolite intensity in pepper samples, which has been $\log_2$-transformed and mean-centered. The alphabets (A-F) represent metabolite clusters. Characteristics of the underlying metabolites are presented in Supplemental Table 2.

### 3.3. QTL analyses of the F2 population

To uncover genetic loci controlling the observed variation in metabolic profiles, we subsequently analysed the genetic distribution of the 542 metabolite patterns using QTL analysis. We could significantly link 231 metabolites to one or more genetic markers (based on interval mapping with a LOD threshold of 3.7), accounting for a total of 279 mQTLs. In total 24 out of these 231 metabolites were putatively annotated as belonging to several metabolite classes, including acyclic diterpenoids/capsianosides, phenylpropanoids, fatty acid derivatives, amino acid derivatives and flavonoids (Supplemental Table 4). In addition, we found several mQTLs for as yet unknown, doubly charged compounds (Supplemental Table 4). The 279 mQTLs were not evenly distributed over the genome and in particular two major QTL hotspots were detected on chromosome 9 (Fig. 4a): one mQTL hotspot harbouring 35 mQTLs co-localised with the marker HpmsE143-9_Q1 at 76 cM and a second hotspot harbouring 103 mQTLs co-localised with the marker P11M48-517 at 100 cM (Fig. 4b). The first group included several doubly charged compounds, which were detected at significant levels in both the male parent (no. 18) and the F1 plants (Supplemental Table 2). The mQTL hotspot adjacent to marker P11M48-517 included seven putatively annotated metabolites, of which five were putatively identified as capsianosides. The QTL for these capsianosides was derived from the female parent (no. 19), as demonstrated by the positive effect of the homozygous A allele on their accumulation (Supplemental Table 4) and their relative abundance in the female parent (Fig. 2). In addition, we revealed 11 mQTLs for flavonoid glycosides distributed over several genomic locations (Supplemental Table 4). QTLs for quercetin, luteolin and apigenin glycosides were located at different positions on different chromosomes, i.e. we found a QTL for quercetin rhamnoside-glucoside on chromosome 2 at 144 cM and a QTL for quercetin 3-*O*-rhamnoside on chromosome 10 at 7 cM (linkage group 10b). On chromosome 6, several QTLs for flavone *C*-glycosides, including two apigenin *C*-glycosides and two luteolin *C*-glycosides, were found in the region from 160 to 167 cM. In addition, QTLs for two methylated luteolin glycosides were found on chromosome 9 at 68 and 97 cM, in the QTL hotspot region. On the top of chromosome 9 we also found a QTL for naringenin chalcone (Fig. 4b). Furthermore, two QTLs for flavanone-3-methylglucose pentose were found on chromosome 6 and 7, respectively. QTLs for other phenylpropanoids and acyclic diterpenoids were observed at different linkage groups (Supplemental Table 4). The capsaicin analogue, which has been shown previously to be highly correlated with total capsaicinoid levels (Wahyuni et al. 2011) was linked to a QTL on chromosome 7 (98 cM).

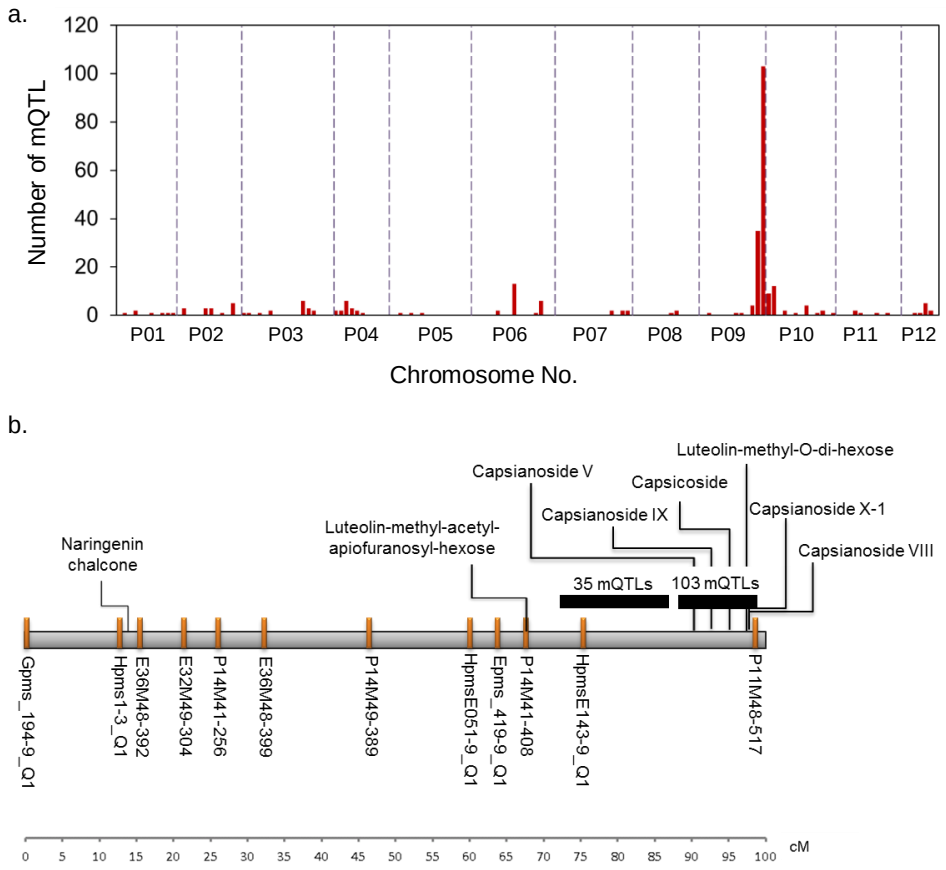



**Fig. 4** Overview of mQTLs in pepper fruit. **a** Frequency distribution of total mQTLs detected at each marker position on pepper chromosomes, which is represented by *red bars*. The *dotted vertical lines* represent the chromosomal borders. **b** mQTLs likelihood map on chromosome 9

To validate the flavonoid QTLs detected using the untargeted metabolomics approach and to gain insight in the underlying molecular regulation, three experiments were conducted: (1) mapping the genetic position of flavonoid candidate genes, (2) re-analysis of the flavonoid mQTLs using the flavonoid candidate genes as markers and (3) expression QTL (eQTL) analysis using flavonoid gene expression data. Firstly, a *Ca-MYB12* transcription factor gene, three *chalcone synthase* (*CHS-A*, *CHS-1* and *CHS-2*), four *chalcone isomerase* (*CHI-1*, *CHI-2*, *CHI-3* and *CHI-4*), a *flavanone-3-hydroxylase* (*F3H)*, a *flavonol synthase* (*FLS*) and three *flavanone-3'-hydroxylase* (*F3'H-1, F3'H-2 and F3'H-3*) candidate genes were identified based on homology with known tomato flavonoid genes. In addition, a *flavone synthase* candidate gene (*FS-2*) was identified based on

homology with the *Lobelia erinus flavone synthase* (*FS-2)* gene. For ten genes (*Ca-MYB12*, *CHS-2*, *CHI-1*, *CHI-2*, *CHI-4*, *F3H*, *F3'H-1*, *F3'H-3*, *FLS* and *FS-2*), SNPs between the two population parents were obtained and used to genotype the segregating F2 population. These data were used to locate the position of the candidate genes on the pepper genetic map. Out of these ten candidate genes, five genes could be located on chromosome regions, i.e. *Ca-MYB12* at 89.6 cM on chromosome 1, *CHS-2* on the top (0.0 cM) of chromosome 5 (linkage group 5a), *CHI-2* at 15.4 cM on chromosome 5 (linkage group 5a), *FS-2* at 162.1 cM on chromosome 6 and *CHI-4* at 176.8 cM on chromosome 6 (Fig. 5; Supplemental Table 3). *FLS* could not be linked to this genetic map. Secondly, all detected flavonoids were re-analysed for mQTLs using the updated genetic map including the flavonoid candidate genes markers. Several flavonoids showed their strongest linkage (based on LOD score) to one or more of the flavonoid candidate gene markers (Fig. 5; Supplemental Table 5). For example, in addition to the one on chromosome 9, we identified a second naringenin chalcone mQTL linked to the *Ca-MYB12* marker on chromosome 1 (LOD score = 4.1). Together, these two mQTLs explain 37% of the variation in naringenin chalcone. A strong mQTL for flavone *C*-glycosides was found on chromosome 6, having its strongest association with the *FS-2* gene (162 cM; LOD = 15.3), which encodes the last step in the biosynthesis of flavones. This QTL explains up to 40% of the variation in flavone *C*-glycosides. A significant association of flavone *C*-glycosides was also found with the nearby *CHI-4* (176.8 cM; LOD = 7.7) marker. We are not sure, however, whether *CHI-4* represents a separate mQTL, since this locus overlaps with the *FS-2* QTL interval. Thirdly, we analysed the expression of the candidate genes in ripe fruits of the F2 individuals and subjected them to an eQTL analysis. For eleven genes (*Ca-MYB12*, *CHS-1*, *CHS-2, CHI-1, CHI-2*, *CHI-4*, *F3H*, *F3'H-1*, *F3'H-3*, *FLS* and *FS-2*) significant expression levels could be observed and these could be used for eQTL analysis, while three candidate genes (*CHS-A*, *CHI-3*, and *F3'H-2*) were not expressed in pepper fruit. Strong eQTLs were found for *CHS-1*, *CHS-2*, *CHI-2, FLS* and *Ca-MYB12*, overlapping with the position of the naringenin chalcone mQTL at the *Ca-MYB12* marker on chromosome 1 (Fig. 5). eQTLs for *CHS-1*, *CHS-2, CHI-1, CHI-2* and *FS*-2 also co-localised with the naringenin chalcone mQTL on the top of chromosome 9. In addition to the flavone *C*-glycosides, the *FS-2* marker on chromosome 6 was associated with an eQTL of the corresponding *FS-2* gene. Similarly, a *CHI-4* eQTL showed the strongest association with the SNP marker in the *CHI-4* gene. Additional eQTLs were found for *F3'H-1* on chromosome 3, *F3'H-4* on chromosome 4 and *F3H* on chromosome 8. None of these eQTLs co-localised with a flavonoid mQTL, neither did they have an association with any of the flavonoid genes

mapped in this study. It should, however, be noted that these three genes were not among the set of mapped flavonoid genes.

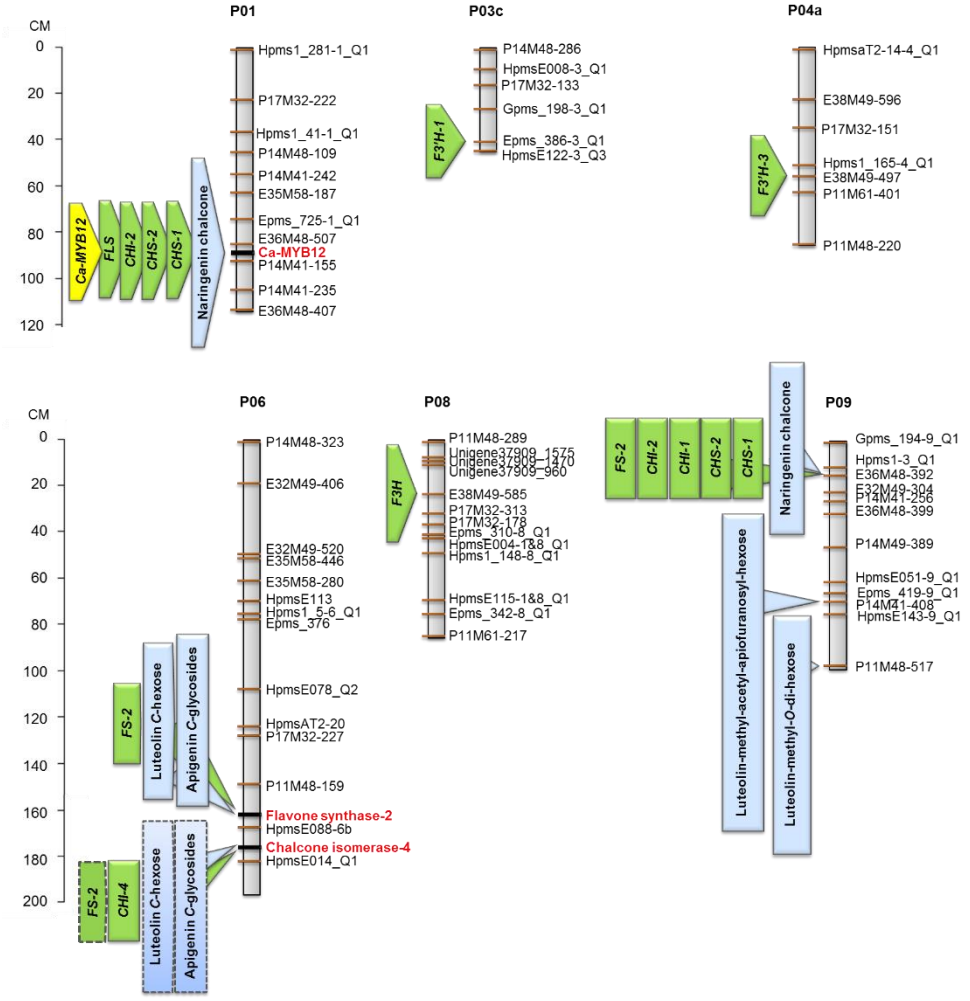



**Fig. 5** Flavonoid mQTLs and flavonoid candidate gene eQTLs on pepper chromosomes. *Light blue boxes* indicate flavonoid mQTLs, the *yellow box* indicates the *Ca-MYB12* transcription factor eQTL and *green boxes* indicate eQTLs of flavonoid structural candidate genes. *Boxes with grey dotted outlines* indicate flavone *C*-glycoside mQTLs and an *FS-2* eQTL with significant LOD scores (mQTL ≥3.7; eQTL ≥3.6) at the CHI-4 locus, but these are not considered as a separate locus for those metabolites, since they overlap with the *FS-2* QTL interval. *CHS* chalcone synthase, *CHI* chalcone isomerase, *F3H* flavanone-3-hydroxylase, *FLS* flavonol synthase, *F3'H* flavonoid-3'-hydroxylase, *FS* flavone synthase.

# 4. DISCUSSION

The cross between *C. annuum* AC1979 (no. 19) and *C. chinense* No. 4661 Selection (no. 18) generated a varied F2 population that genetically segregated for fruit colour as well as for the composition and level of metabolites in ripe fruits. The red fruit colour of the female parent *C. annuum* AC1979 (no. 19) is regulated by the dominant *Y* locus, encoding capsanthin-capsorubin synthase (CCS) which catalyses the production of the red carotenoids capsanthin and capsorubin from antheraxanthin and violaxanthin, respectively (Lefebvre et al. 1998; Paran and van der Knaap 2007). The male parent, *C. chinense* No. 4661 Selection (no. 18), is brown-fruited, caused by the simultaneous accumulation of both red carotenoids and green chlorophyll B. Brown-fruited pepper contain a single recessive *cl* (*chlorophyll retainer*) locus combined with the dominant *Y* locus (Efrati et al. 2005; Roca et al. 2006) and the crossing of both parents ($y^+cl^+$ x $y^+cl$) resulted in a red-fruited F1 ($y^+y^+cl^+cl$). The F2 fruit colour segregated into the expected 3:1 (red:brown) ratio, due to segregation of the *cl* gene.



The crossing of the parents with different metabolite profiles revealed the segregation of metabolites detected in either or both parents over the F2 plants, as we observed for the metabolites in groups A, C, and D (Fig. 3b). Based on metabolite profile, the F1 hybrid was most similar to the male parent *C. chinense* No. 4661 Selection (no. 18), since they clustered together into genotype cluster III in both the PCA (Fig. 3a) as well as the HCA (Fig. 3b). This cluster also contained the majority of F2 plants, indicating that the biochemical profile of the male parent (no. 18) was dominant over the female parent (no. 19) in both the F1 and the F2. This may suggest that the male parent (no. 18) contains many dominant alleles for metabolite production. However, we cannot exclude that several mQTLs from the female parent may be linked to lethal traits coming from the wild female parent, which may lead to lethality, or reduced flower and fruit set and thus to a skewed segregation. This is supported by the fact that from 201 F2 plants, only 113 set fruit and hence could be used in this study. In addition, a skewed segregation for several markers has indeed been observed by genetic analysis of the F2 population (Maharijaya et al. 2012b). Ripe fruits from both the F1 hybrid as well as several F2 plants contained metabolites that were not detected in any of the parents (metabolite clusters B, E and F). These metabolites are likely the result of epistatic effects of alleles from both parents. In addition to such epistatic effects, we found several metabolites that were significantly (up to 12-fold) more abundant in some F2 plants compared to both parents and the F1 hybrid (particularly flavonoid glycosides in metabolite group D, such as flavone *C*-glycosides, flavonol *O*-

glycosides and naringenin chalcone). This may be caused by a combination of positive alleles derived from both parents, a phenomenon called transgressive segregation. Alternatively, such extreme phenotypes may also reflect environmental variation or an interaction between genotype and environment. Replicated trials would be needed to distinguish between these different possibilities. Using the segregation in metabolite profiles we could define various mQTLs from the correlation between the genetic and metabolic data sets. At least 231 (43%) of the 542 metabolites could be linked to one or more genetic markers. The mQTLs were unevenly distributed over the pepper genome, as we could identify mQTL hotspots on chromosome 9 showing an association with up to 103 metabolites. This suggests that large groups of metabolites share a common genetic region, encoding one or more genes that regulate the accumulation of those metabolites. For example, on the QTL hotspot at 75 cM on chromosome 9 we found co-localisation of 16 as-yet-unknown metabolites which all showed a typical doubly charged ionisation behaviour in our LCMS set-up, suggesting the presence of a regulatory gene driving the synthesis of these compounds or a structural gene at an early step in the biosynthetic pathway leading to these double charged compounds.

The mQTL hotspot on the bottom of chromosome 9 (99 cM) was linked to four acyclic diterpenoids, also called capsianosides (Supplemental Table 4). Accurate mass MS and $MS^n$ fragmentation information revealed that these capsianosides contained different decorations of the basic diterpene skeleton (Supplemental Table 2). For example, capsianoside VIII has a diglucoside conjugated to the C-3 position of the capsianoside aglycone, whereas capsianoside IX has a triglucoside at this position (Lee et al. 2006; Supplemental Fig. 3). These five capsianosides accumulated in genotypes homozygous for the maternal allele (parent no. 19 and F2 plants) of the corresponding marker, suggesting that this allele may encode a regulatory gene which up-regulates the capsianoside biosynthetic pathway. Alternatively, it is possible that this QTL region encodes one or more modifying enzymes, such as glycosyltransferases, which lead to the production of these structurally different capsianosides.

Both parents used in this study were pungent, but showed quantitative differences in capsaicin content (Wahyuni et al. 2011). In this study we mapped a QTL for the capsaicin analogue, which was shown to be highly correlated with total capsaicin content (Wahyuni et al. 2013), on chromosome 7. In pepper, QTLs that control the capsaicinoid content in ripe fruits have been reported in a F3 population derived from an interspecific cross between the mildly pungent *C. annuum* cv. 'NuMex RNaky' and the highly pungent *C. frutescens* accession

BG2814-6 (Ben-Chaim et al. 2006). Two capsaicinoid QTLs in this F3 population were located on chromosome 2 and 7. The QTL on chromosome 2 encodes *AT3* (acyltransferase), the structural gene encoding the enzyme responsible for capsaicinoid condensation (Blum et al. 2002; Mazourek et al. 2009). Candidate genes have also been proposed for the QTL on chromosome 7, for example the putative aminotransferase gene *pAMT*, which may encode the enzyme catalysing the formation of vanillylamine from vanillin (Mazourek et al. 2009). The co-localisation of a QTL for capsaicin content on chromosome 7 in both populations may point to the same underlying candidate gene. This will be examined in a follow-up study.

Several flavonoid mQTLs were detected in this F2 population (Supplemental Table 4). To get more insight into the genes and molecular mechanisms underlying these QTLs, we (1) mapped the position of five flavonoid candidate genes and integrated these data into an updated genetic map, (2) performed an eQTL analysis based on gene expression data of 14 flavonoid genes and (3) re-analysed the flavonoid mQTL data using the updated genetic map (Fig. 5; Supplemental Table 5). This allowed the detection of a new QTL for naringenin chalcone on chromosome 1, with its QTL maximum at the *Ca-MYB12* gene. Expression QTLs for *Ca-MYB12*, *CHS-1*, *CHS-2*, *CHI-2* and *FLS* mapped at the same position. This is fully in line with results observed in tomato, where the *MYB12* gene has been shown to regulate the level of naringenin chalcone in the peel of ripening tomato fruits, through induction of the same set of flavonoid structural genes (Adato et al. 2009; Ballester et al. 2010). Although the mQTL for naringenin chalcone on chromosome 9 was confirmed, none of the candidate genes tested mapped within this QTL interval. However, significant eQTLs for *CHS-1*, *CHS-2*, *CHI-1*, *CHI-2* and *FS-2* mapped at the same position, suggesting that this naringenin chalcone mQTL is also driven by the action of an as-yet-unknown transcription factor gene, in analogy to the *MYB12* QTL on chromosome 1. On chromosome 6, the mQTLs for four flavone *C*-glycosides and the eQTL for flavone synthase (*FS-2*) showed their strongest association with the corresponding *FS-2* marker at 162.1 cM, explaining up to 40% of the variation in these compounds. This suggests that *FS-2*, which catalyses the conversion of flavanones into flavones, is the gene regulating flavone *C*-glycoside levels in this material, through differences in its expression. Our current efforts are geared towards the elucidation of the causative mutations in the *FS-2* gene or its promoter leading to the observed gene expression differences.

In addition to these four flavone *C*-glycosides, three additional flavone *C*-glycosides were detected in metabolite group D of the HCA (Fig. 3b). These seven flavone *C*-glycosides were highly correlated with each other ($R^2 > 0.6$;

Supplemental Table 6). Although only four of these seven flavone glycosides were significant in interval mapping, both the HCA and correlation analysis suggest that the *FS-2* locus regulates the formation of all flavone *C*-glycosides, as would be expected based on the function of the FS-2 enzyme. The lack of significance of the additional three flavone *C*-glycosides in interval mapping might be due to the limited population size, the presence of additional, undiscovered QTLs and/or environmental variation influencing the efficiency of QTL detection.

The CHI-4 locus at 176.8 cM on chromosome 6 showed an eQTL for the corresponding *CHI-4* gene. In addition this locus revealed significant associations with the flavone *C*-glycosides and *FS-2* gene expression. We cannot distinguish whether *CHI-4* represents a separate locus for flavone *C*-glycosides, since it overlaps with the *FS-2* QTL interval. However, the fact that the direction of the *CHI-4* eQTL is opposite to that of the *FS-2* and flavone *C*-glycoside QTLs argues against a second QTL for flavone-*C*-glycosides (Supplemental Table 5). For several other flavonoid metabolite and expression QTLs, we did not observe a link to any of the flavonoid genes tested. This is mainly due to the limited number of flavonoid genes mapped in this study. Our results, however, demonstrate the power of a candidate gene approach to find the genes underlying important mQTLs, in the case of a well-studied metabolic pathway. We are convinced that genetic mapping of additional flavonoid candidate genes would uncover the genes underlying several additional flavonoid QTLs in this population.

All QTL results have been obtained from the analysis of a segregating F2 population. In such a population, all F2 individuals are unique and mortal. Despite the lack of biological replicates in such a population, reliable QTLs can be found, due to the fact that every genomic region is represented, and therefore replicated, in 75% of the F2 individuals. Although reliable, the size of the QTL intervals in an F2 population is rather large, since only few meioses, and hence recombinations, have taken place during its development. On the other hand, the relatively high level of heterozygosity present in an F2 population makes it possible to estimate the degree of dominance for detected QTLs (Semagn et al. 2010). Currently we are developing a recombinant inbred line (RIL; > F6 generation) population based on the same cross between *C. annuum* AC1979 (no. 19) and *C. chinense* No. 4661 Selection (no. 18). Individuals of such a population are homozygous for more than 97%, can be propagated indefinitely through seeds and form a stable resource for future research. This material will be used to further confirm and test the robustness of selected QTLs, using biological replicates in different environmental conditions.

In summary, our results suggest that the extensive biochemical variation in pepper fruit is under the genetic control of a limited number of chromosomal regions (QTL hotspots), encoding genes that regulate the accumulation of large sets of metabolites. In the coming years we expect the release of the complete sequencing of the pepper genome (Park et al. 2012) and this will aid strongly in the identification of the key genes underlying important agronomic traits. Combining genetic and biochemical datasets will help breeders to develop new pepper varieties that unite a desirable taste and nutritional profile with genetic resistance to important diseases.

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# Supplemental Materials

**Supplemental Table 1** Flavonoid candidate genes and primers for SNPs (available on page 149)

**Supplemental Table 2** Semi-polar metabolite composition and mQTLs of F2 population (available online via http://edepot.wur.nl/287158)

**Supplemental Table 3** Position of flavonoid SNP markers on genetic map in pepper (available on page 150 – 151)

**Supplemental Table 4** Putative metabolites associated with a quantitative trait locus (available on page 152)

**Supplemental Table 5** Flavonoid QTLs and flavonoid candidate gene expression QTLs in pepper (available on page 153)

**Supplemental Table 6** Pearson correlation analysis of flavone *C*-glycosides (available on page 154)

**Supplemental Fig. 1** A crossing scheme to develop F2 plants from *C. annuum* AC1979 (no. 19) and *C. chinense* No. 4661 Selection (no. 18). Two homogenous F1 plants were obtained by crossing two pepper accessions. Each of F1-plants was self-pollinated to produce F2 progeny. The representation of plants shown in the scheme is not in true scaled (available on page 155)

**Supplemental Fig. 2** Variation in fruit morphological characters of: a, *C. annuum* AC1979 (no. 19); b, *C. chinense* No. 4661 Selection (no. 18); c, F1 plants; and d, F2 plants (available on page 156)

**Supplemental Fig. 3** $MS^n$ fragmentation pattern of Capsianoside VIII and IX using LTQ-Orbitrap FTMS in negative ionization mode (available on page 157)

**Supplemental Table 1** Flavonoid candidate genes and primers for SNPs

A. Flavonoid Candidate Genes

| Gene Name | Gene Code | Candidate Gene from DFCI Pepper Gene Index | Primer Sequence |
|---|---|---|---|
| α-Tubulin | tub | 145388967 | F TGAAGCACGTGAAGATCT |
| | | | R GTCCAATTCAGCACCAACCT |
| CaMYB12 | Ca-MYB12 | | F CAGATGATGGTAGACTTGAATG |
| | | | R CTAGGAATTGATGATGACCCTA |
| Chalcone synthase | CHS-1 | TC19590 | F AGTGGTGAAGGGCTTGAATG |
| | | | R CAGCCCAGCCCACTTAAATA |
| | CHS-2 | TC13169 | F AAGGCCTTTGTTTGAGCTTG |
| | | | R GACCATCTATAGCGCCTTCG |
| Chalcone isomerase | CHI-1 | TC13824 | F CCCTTGACGGGTAAGCAATA |
| | | | R TTTCGATGGCCTCACTCTCT |
| | CHI-2 | TC25522 | F GATGGGCCATGGAATAACTG |
| | | | R TCCATTGCTGCAAATGAGTC |
| | CHI-3 | TC14193 | F TGCCATCAAGAAGCTCACTG |
| | | | R CATATCCTGGGAGCCTTGAA |
| | CHI-4 | TC22835 | F GGTGTTTATGCTGACGATGC |
| | | | R ACAGAGTGCTGTGCATGCTT |
| Flavanone 3-hydroxylase | F3H | CO912387 | F ATCAGAAGCAATGGGTTTGG |
| | | | R ATCAGGCTGTGGACACTTTG |
| Flavone synthase | FS-2 | KS19022A05 | F AGAACCCAATGGAGTTTAGGC |
| | | | R AAATATTGGGCCTCCCTCTC |
| Flavonol synthase | FLS | TC24608 | F CCCATCAGAGCATGAAGTTG |
| | | | R TGGGTTGATTGGCCTCAT |
| Flavonoid 3'-hydroxylase | F3'H-1 | GD063322 | F AAGCCCAGTTAAACGAATGG |
| | | | R GGGTATACGCATGGTCCAGT |
| | F3'H-2 | TC13232 | F GCAGGAGATATGAAGCCAGAA |
| | | | R ATCGGCTTATTTGGGTGTGT |
| | F3'H-3 | TC19978 | F TTCTCGTCCTGCATTAGCTG |
| | | | R TAAGGTGCCCACGTCATGT |

B. SNPs profile on flavonoid candidate genes

| Gene Name | Gene Code | Sequence |
|---|---|---|
| Chalcone synthase | CHS-2 | GGGGCAGCCGCACTCATTGTAGGTTCAGATCCATTACCAGAGGTTGAAAG**[G/A]**CCTTTGTTTGAGCTTGTT TCTGCGGCCCAAACTCTTCTCCCTGATAGCGA |
| Chalcone isomerase | CHI-1 | CAACTGTCAGAAGCGGTGCTGGAATCCATAATTGGCAAGCACGGAGTTTC**[A/C]**CCTGCCGCAAAGAATAG TCTTGCCAAAAGAGTATCGGAATTGTTAAAGAG |
| | CHI-2 | TCTGCTCCCGTTTTGCCCTTCCATTGCTGCAAATGAGTCACAATTTCTGG**[T/A]**TCCAAGTAAACTCCAATT GCAGTGAATTTAATTTGGAGAAAGTGTATCTC |
| | CHI-4 | AGTCAATATCACCACAACTCGATTCCTTGTGTTGATATATTCTCTTTATG**[C/A]**CAAAGAAGTAGCTACTTG TGAAGTAGAGAAGCCAAGTTCGACTCGACATC |
| Flavonoid 3'-hydroxylase | F3'H-1 | ACCAATCGACTGCAGCCATGGCCCAAACTTACGGGCCACTCATGCATCTT**[G/C]**ACTTGGGGTTCGTGGATG TGGTGGTTGCGGCTTCGGCTTCAGTGGCGGCT |
| | F3'H-3 | GAGGAAAGACGAATTTTGACCTCTCGCCTTTTTCCTCTCTCAGGAAAACC**[A/G]**TTTTTCTTAAAGACCATT TACCTCTATTTGCTCTTCGCATGATAAGTAGG |
| Flavanone 3-hydroxylase | F3H | CATTTGATTTATCACTTTATAATCATTTCTCCATAAATAATAAAAAGAAA**[C/G]**TACCTGGAAAATGCCCC AATCTTCGCATGCCT**[C/T]**TACAATTTTTTCACATATTTCACCTCTTGTTCCAATCTCATCAATATCCT |
| Flavone synthase | FS-2 | GAGAACAAGCTATTAAGATTCTCCACACATTTTATTTAACTAATATCTAT**[T/A]**ACACATAATATATCCTC ACGCAACGAGGTTAAAATTTTTTTTTATCT |
| Flavonol synthase | FLS | CAAGAAGTACCATATCCTTCTATACTCTGCGATCCTGGATTCTTCGCAATC**[A/C]**ACTCTTTCTCCTCTTGT GGTACTTGCTCAAAGAACTCCTTCCCAACTTTCT |
| *Capsicum* MYB12 | Ca-MYB12 | TTTTCTCATTTGTTGATGACACTGCCTCCTGACTTGATACAGTTTTATT**[A/T]**CGTTCATCAATTCTGTGCG TGGTCCTGCAGTAATTATTTCCTGTTTCGTT |

**Supplemental Table 3** Position of flavonoid SNP markers on genetic map in pepper

| No | Locus | Position | Linkage Group |
|---|---|---|---|
| 1 | Hpms1-281-1_Q1 | 0.0 | P01 |
| 2 | P17M32-222 | 20.3 | |
| 3 | Hpms1_41-1_Q1 | 38.4 | |
| 4 | P14M48-109 | 46.8 | |
| 5 | P11M48-242 | 56.0 | |
| 6 | E35M58-187 | 61.1 | |
| 7 | Epms_725-1_Q1 | 74.2 | |
| 8 | E36M48-507 | 84.9 | |
| 9 | CaMYB12 | 89.6 | |
| 10 | P14M41-155 | 93.6 | |
| 11 | P14M41-235 | 106.6 | |
| 12 | E36M48-407 | 116.4 | |
| 13 | Gpms_169-2_Q1 | 0.0 | P02 |
| 14 | E38M49-457 | 24.1 | |
| 15 | Gpms_37-2_Q1 | 34.4 | |
| 16 | P11M48-448 | 42.1 | |
| 17 | P11M48-475 | 44.4 | |
| 18 | E32M49-267 | 49.0 | |
| 19 | E35M58-303 | 66.1 | |
| 20 | E35M48-250 | 82.9 | |
| 21 | P11M48-142 | 102.7 | |
| 22 | P11M61-253 | 112.7 | |
| 23 | P17M39-130 | 136.7 | |
| 24 | E34M48-371 | 168.6 | |
| 25 | P17M39-242 | 0.0 | P03a |
| 26 | P14M48-166 | 20.9 | |
| 27 | E38M49-668 | 34.2 | |
| 28 | Gpms_93-3_Q1 | 43.2 | |
| 29 | E38M49-672 | 52.1 | |
| 30 | P17M32-201 | 0.0 | P03b |
| 31 | P17M32-110 | 6.4 | |
| 32 | HpmsE073-3_Q2 | 10.2 | |
| 33 | P17M32-191 | 13.3 | |
| 34 | P17M32-172 | 27.8 | |
| 35 | E32M49-202 | 39.1 | |
| 36 | P14M48-286 | 0.0 | P03c |
| 37 | HpmsE008-3_Q1 | 7.5 | |
| 38 | P17M32-133 | 16.2 | |
| 39 | Gpms_198-3_Q1 | 25.2 | |
| 40 | Epms_386-3_Q1 | 40.4 | |
| 41 | HpmsE122-3_Q3 | 46.1 | |
| 42 | HpmsaT2-14-4_Q1 | 0.0 | P04a |
| 43 | E38M49-596 | 23.7 | |
| 44 | P17M32-151 | 34.0 | |
| 45 | Hpms1_165-4_Q1 | 50.6 | |
| 46 | E38M49-497 | 55.0 | |
| 47 | P11M61-401 | 62.9 | |
| 48 | P11M48-220 | 84.2 | |
| 49 | HmpsE099-4_Q2ac | 0.0 | P04b |
| 50 | HpmsE055-4_Q1 | 53.1 | |
| 51 | HpmsE085-4_Q1 | 64.2 | |
| 52 | HpmsE111-4_Q1 | 73.8 | |
| 53 | CHS2 | 0.0 | P05a |
| 54 | LM_2001 | 2.2 | |
| 55 | HpmsE116_5_Q1 | 8.8 | |
| 56 | CHI2 | 15.4 | |
| 57 | LM_2004 | 18.2 | P05a |
| 58 | LM_2002 | 21.2 | |
| 59 | E38M49-544 | 22.7 | |
| 60 | LM_2006 | 23.9 | |
| 61 | Hpms2_45-5_Q1 | 25.4 | |
| 62 | P14M49-195 | 28.8 | |
| 63 | E36M48-451 | 31.8 | |
| 64 | E36M48-310 | 37.6 | |
| 65 | Gpms_165-5_Q2 | 51.9 | |
| 66 | Hpms2_23-5_Q1 | 0.0 | P05b |
| 67 | HpmsE015-5nw | 22.0 | |
| 68 | E36M48-432 | 36.2 | |
| 69 | P14M50-333 | 51.8 | |
| 70 | P14M48-323 | 0.0 | P06 |
| 71 | E32M49-406 | 19.8 | |
| 72 | E32M49-520 | 48.9 | |
| 73 | E35M58-446 | 53.6 | |
| 74 | E35M58-280 | 61.1 | |
| 75 | HmpsE113 | 71.2 | |
| 76 | Hpms1_5-6_Q1 | 77.7 | |
| 77 | Epms_376 | 79.9 | |
| 78 | Isotig18917_234 | 106.6 | |
| 79 | Isotig18917_361 | 106.7 | |
| 80 | Isotig18917_or55 | 107.4 | |
| 81 | HpmsE078_Q2 | 108.1 | |
| 82 | Isotig18067_441 | 110.6 | |
| 83 | HpmsAT2-20 | 123.7 | |
| 84 | P17M32-227 | 129.1 | |
| 85 | P11M48-159 | 148.1 | |
| 86 | FS | 162.1 | |
| 87 | HpmsE088-6b | 168.6 | |
| 88 | CHI4 | 176.8 | |
| 89 | HpmsE014_Q1 | 182.0 | |
| 90 | E35M49-332 | 197.9 | |
| 91 | E36M48-274 | 0.0 | P07 |
| 92 | P14M48-282 | 23.7 | |
| 93 | E32M49-334 | 26.7 | |
| 94 | E32M49-389 | 29.2 | |
| 95 | E38M49-306 | 32.2 | |
| 96 | HpmsE068-7_Q1 | 33.7 | |
| 97 | E38M49-319 | 40.7 | |
| 98 | E36M48-497 | 42.0 | |
| 99 | E38M49-211 | 45.1 | |
| 100 | HpmsE057-7_Q1 | 56.6 | |
| 101 | P14M48-251 | 67.6 | |
| 102 | P14M41-259 | 93.9 | |
| 103 | Hpms1-227_Q1 | 98.5 | |
| 104 | P17M39-199 | 104.3 | |
| 105 | P11M48-289 | 0.0 | P08 |
| 106 | Unigene37909_1575 | 7.6 | |
| 107 | Unigene37909_1470 | 8.2 | |
| 108 | Unigene37909_960 | 9.0 | |
| 109 | E38M49-585 | 23.7 | |
| 110 | P17M32-313 | 32.9 | |
| 111 | P17M32-178 | 36.8 | |
| 112 | Epms_310-8_Q1 | 41.2 | |

| No | Locus | Position | |
|---|---|---|---|
| 113 | HpmsE004-1&8_Q1 | 42.2 | P08 |
| 114 | Hpms1_148-8_Q1 | 48.2 | |
| 115 | HpmsE115-1&8_Q1 | 68.2 | |
| 116 | Epms_342-8_Q1 | 76.2 | |
| 117 | P11M61-217 | 85.4 | |
| 118 | Gpms_194-9_Q1 | 0.0 | P09 |
| 119 | Hpms1-3_Q1 | 12.7 | |
| 120 | E36M48-392 | 15.1 | |
| 121 | E32M49-304 | 21.6 | |
| 122 | P14M41-256 | 25.9 | |
| 123 | E36M48-399 | 32.8 | |
| 124 | P14M49-389 | 46.6 | |
| 125 | HpmsE051-9_Q1 | 60.4 | |
| 126 | Epms_419-9_Q1 | 64.1 | |
| 127 | P14M41-408 | 67.9 | |
| 128 | HpmsE143-9_Q1 | 75.6 | |
| 129 | P11M48-517 | 99.5 | |
| 130 | Gpms159-10_Q1 | 0.0 | P10a |
| 131 | P17M32-244 | 16.5 | |
| 132 | E32M49-192 | 0.0 | P10b |
| 133 | E38M49-534 | 9.3 | |
| 134 | HpmsE013-10_Q1 | 14.9 | |
| 135 | HpmsE059-10_Q1 | 16.5 | |
| 136 | Hpms2_21-10_Q2 | 18.6 | |
| 137 | HpmsE-065_Q3 | 23.0 | |
| 138 | P14M41-176 | 36.1 | |
| 139 | HpmsE031_10_Q1 | 40.5 | |
| 140 | P14M41-162 | 43.1 | |
| 141 | P17M39-172 | 47.5 | |
| 142 | HpmsE096-10_Q1 | 83.0 | |
| 143 | E35M48-209 | 0.0 | P11 |
| 144 | E32M49-401 | 13.6 | |
| 145 | E32M49-484 | 17.5 | |
| 146 | Epms_561-11_Q1 | 23.7 | |
| 147 | Epms_410-11_Q1 | 25.3 | |
| 148 | E38M49-460 | 27.6 | |
| 149 | P17M39-228 | 33.9 | |
| 150 | Epms_391-11_Q1 | 39.1 | |

| No | Locus | Position | |
|---|---|---|---|
| 151 | Hpms2_2-11_Q1 | 45.9 | P11 |
| 152 | P17M39-318 | 56.3 | |
| 153 | P17M32-130 | 74.8 | |
| 154 | E32M49-501 | 91.6 | |
| 155 | HpmsE094-12_Q3 | 0.0 | P12a |
| 156 | HpmsE128_12_Q2 | 21.7 | |
| 157 | E35M49-121 | 0.0 | P12b |
| 158 | HpmsE064-12_Q1 | 6.4 | |
| 159 | E32M49-597 | 33.1 | |
| 160 | E32M49-590 | 44.0 | |
| 161 | P14M50-332 | 0.0 | X01 |
| 162 | E38M49-287 | 11.5 | |
| 163 | E36M48-178 | 15.7 | |
| 164 | P14M48-97 | 21.8 | |
| 165 | E34M48-360 | 35.7 | |
| 166 | E35M58-427 | 0.0 | X02 |
| 167 | P11M61-323 | 25.8 | |
| 168 | E32M49-412 | 45.6 | |
| 169 | E35M48-232 | 62.9 | |
| 170 | E32M49-414 | 73.9 | |
| 171 | E35M58-239 | 88.6 | |
| 172 | P14M50-309 | 0.0 | X03 |
| 173 | E36M48-369 | 17.0 | |
| 174 | E35M48-227 | 31.8 | |
| 175 | E35M48-377 | 50.2 | |
| 176 | P14M48-87 | 0.0 | X04 |
| 177 | E36M48-473 | 6.4 | |
| 178 | E35M58-308 | 17.8 | |
| 179 | E35M58-236 | 23.5 | |
| 180 | E35M58-554 | 30.7 | |
| 181 | P11M48-333 | 0.0 | X05 |
| 182 | P17M39-137 | 13.3 | |
| 183 | E32M49-584 | 0.0 | X06 |
| 184 | E35M48-254 | 18.5 | |
| 185 | E35M49-329 | 61.9 | |

**Supplemental Table 4** Putative metabolites associated with a quantitative trait locus

| Putative metabolites | Metabolite Group[a] | Linkage Group[b] | Position | Max. LOD[c] | mu_A[d] | mu_H[e] | mu_B[f] | Closest marker |
|---|---|---|---|---|---|---|---|---|
| Trihydroxy-phytatetraen-one-acetyl-O-glucose | Acy. Diterp. | P02 | 21.00 | 4.4 | 5.29 | 6.85 | 5.11 | E38M49-457 |
| Quercetin rhamnoside-glucoside | Flav. | P02 | 143.72 | 4.2 | 5.51 | 7.22 | 7.00 | P17M39-130 |
| 3-Methyl-1-butanol; O-[?-D-Glucopyranosyl-(1?6)-?-D-glucopyranoside] | Ile/leu deriv. | P03c | 12.51 | 3.9 | 8.20 | 7.17 | 6.27 | P17M32-133 |
| Flavanone tri-methyl glucose pentose | Flav. | P06 | 79.86 | 7.8 | 5.21 | 5.20 | 5.84 | Epms_376 |
| Icariside E5 | Lignan | P06 | 141.09 | 4.2 | 8.59 | 9.39 | 8.18 | P11M48-159 |
| Apigenin 6-C-pentoside-8-C-hexoside | Flav. | P06 | 161.35 | 5.6 | 6.68 | 8.74 | 8.77 | HpmsE088-6b |
| Apigenin 6,8-di-C-hexoside | Flav. | P06 | 162.35 | 8.7 | 6.97 | 9.19 | 9.20 | HpmsE088-6b |
| Luteolin 8-C-hexoside | Flav. | P06 | 165.35 | 3.8 | 6.28 | 8.13 | 7.95 | HpmsE088-6b |
| Luteolin 6-C-hexoside | Flav. | P06 | 167.35 | 6.9 | 7.29 | 9.42 | 9.30 | HpmsE088-6b |
| capsaicin/capsaicin analogue | Phenylprop. | P07 | 97.03 | 3.7 | 9.88 | 9.68 | 9.12 | Hpms1-227_Q1 |
| Flavanone tri-methyl glucose pentose | Flav. | P07 | 101.03 | 6.1 | 7.02 | 5.12 | 5.41 | P17M39-199 |
| Naringenin chalcone | Flav. | P09 | 13.71 | 6.7 | 7.96 | 8.72 | 9.86 | Hpms1-3_Q1 |
| Luteolin-methyl-acetyl-apiofuranosyl-hexose | Flav. | P09 | 67.86 | 4.1 | 5.93 | 5.28 | 5.22 | P14M41-408 |
| Capsianoside V | Acy. Diterp. | P09 | 90.57 | 6.4 | 5.84 | 10.04 | 10.47 | P11M48-517 |
| Capsianoside IX | Acy. Diterp. | P09 | 93.57 | 4.2 | 9.22 | 7.38 | 6.15 | P11M48-517 |
| Capsicoside | Saponin | P09 | 94.57 | 3.7 | 7.04 | 5.26 | 5.21 | P11M48-517 |
| Luteolin-methyl-O-di-hexose | Flav. | P09 | 96.57 | 6.5 | 9.95 | 6.53 | 5.95 | P11M48-517 |
| Capsianoside X-1 | Acy. Diterp. | P09 | 97.57 | 4.3 | 10.27 | 6.45 | 6.89 | P11M48-517 |
| Capsianoside VIII | Acy. Diterp. | P09 | 98.57 | 4.6 | 11.40 | 7.53 | 6.97 | P11M48-517 |
| Quercetin 3-O-rhamnoside | Flav. | P10b | 7.00 | 4.0 | 6.28 | 8.31 | 8.24 | E38M49-534 |
| Ferulic acid-hexose II | Phenylprop. | P11 | 23.68 | 4.3 | 5.90 | 6.77 | 7.01 | Epms_561-11_Q1 |
| Icariside E5 | Lignan | P11 | 42.12 | 6.0 | 8.08 | 8.91 | 9.51 | Epms_391-11_Q1 |
| Capsianoside IV | Acy. Diterp. | P12b | 38.15 | 3.9 | 8.72 | 6.67 | 5.40 | E32M49-597 |

[a] Metabolites were grouped into flavonoids (Flav.), acyclic diterpenoids (Acy. Diterp.), phenylpropanoids (Phenylprop.), ile/leu derivatives (Ile/leu deriv.), fatty acid derivative (FA deriv.), lignan and saponin; [b] Linkage group was annotated by the chromosome number after the alphabet P and followed by the small alphabet. Linkage map was constructed with AFLP and microsatellite markers as described by Maharijaya et al. (2012b); [c] LOD = logarithm of odds corresponds to a genome wide confidence level. LOD corresponds to the maximum LOD score; [d] mu_A = trait means of AA homozygous P1, *C. annuum* AC1979 (no. 19); [e] mu_H = trait means of AB heterozygous progeny; [f] mu_B = trait means of BB homozygous P2, *C. chinense* No. 4661 Selection (no. 18)

**Supplemental Table 5** Flavonoid QTLs and flavonoid candidate gene expression QTLs in pepper

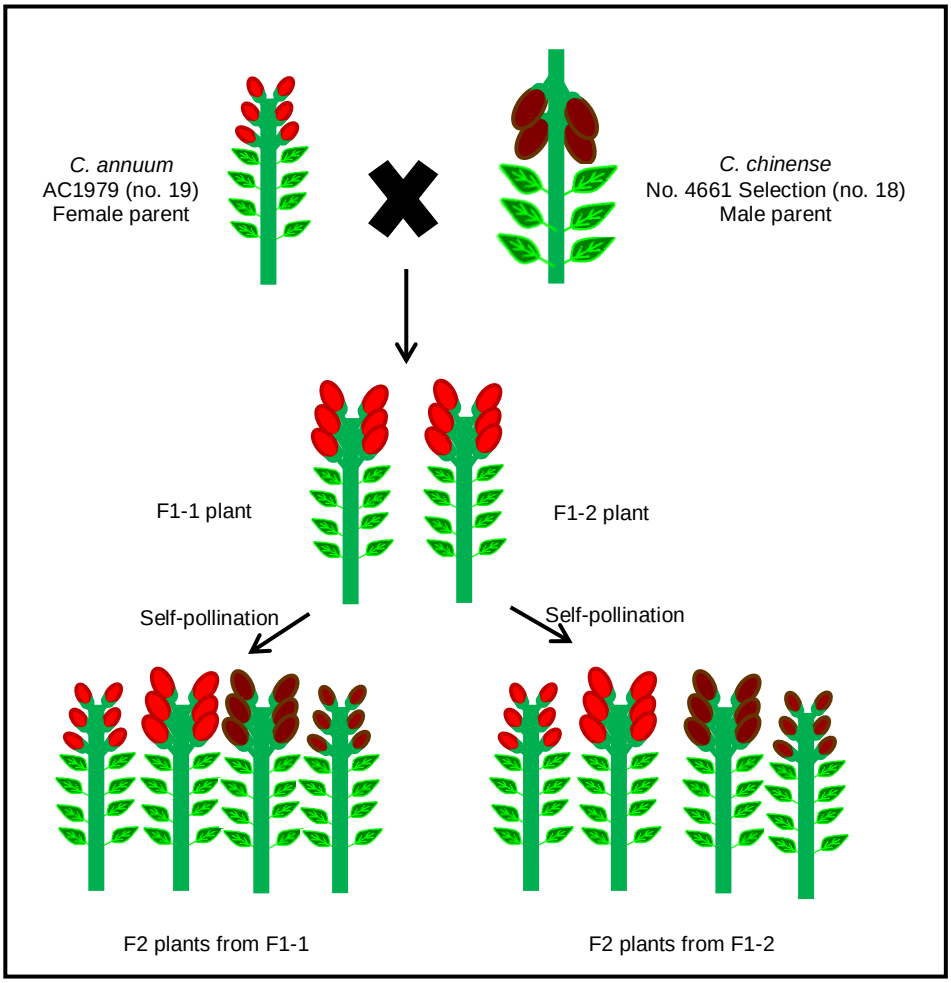
| Linkage Group[a] | Gene transcript/ Metabolite | Position | LOD[b] | mu_A[c] | mu_H[d] | mu_B[e] | Locus |
|---|---|---|---|---|---|---|---|
| P01 | *Ca-MYB12* | 89.63 | 21.9 | 7.56 | 10.68 | 12.47 | Ca-MYB12 |
| | *CHS-1* | 89.63 | 9.6 | 13.05 | 15.24 | 16.53 | Ca-MYB12 |
| | *CHS-2* | 89.63 | 9.9 | 13.15 | 15.39 | 16.57 | Ca-MYB12 |
| | *CHI-2* | 89.63 | 4.6 | 9.99 | 10.65 | 12.35 | Ca-MYB12 |
| | *FLS* | 89.63 | 3.6 | 6.22 | 7.18 | 7.95 | Ca-MYB12 |
| | Naringenin Chalcone | 89.63 | 4.1 | 7.87 | 8.99 | 9.38 | Ca-MYB12 |
| P03c | *F3'H-1* | 40.38 | 9.4 | 12.60 | 15.40 | 16.21 | Epms_386-3_Q1 |
| P04a | *F3'H-3* | 84.17 | 7.61 | 3.19 | 5.70 | 5.97 | P11M48-220 |
| P06 | *FS-2* | 162.13 | 7.8 | 7.79 | 9.08 | 11.08 | FS-2 |
| | Apigenin 6-*C*-pentoside-8-*C*-hexoside | 162.13 | 9.0 | 6.63 | 8.84 | 8.83 | FS-2 |
| | Luteolin 6-*C*-hexose | 162.13 | 13.9 | 6.78 | 9.64 | 9.54 | FS-2 |
| | Luteolin 8-*C*-hexose | 162.13 | 8.4 | 5.82 | 8.28 | 8.26 | FS-2 |
| | Apigenin 6,8-di-*C*-hexoside | 162.13 | 15.3 | 6.86 | 9.28 | 9.33 | FS-2 |
| | *CHI-4* | 176.82 | 33.0 | 13.13 | 11.67 | 6.15 | CHI-4 |
| | *FS-2* | 176.82 | 4.5 | 8.20 | 9.03 | 10.48 | CHI-4 |
| | Apigenin 6-*C*-pentoside-8-*C*-hexoside | 176.82 | 5.0 | 7.21 | 8.61 | 8.81 | CHI-4 |
| | Apigenin 6,8-di-*C*-hexoside | 176.82 | 7.7 | 7.53 | 8.98 | 9.31 | CHI-4 |
| | Luteolin 6-*C*-hexose | 176.82 | 6.1 | 7.62 | 9.32 | 9.47 | CHI-4 |
| | Luteolin 8-*C*-hexose | 176.82 | 3.6 | 6.59 | 8.12 | 8.04 | CHI-4 |
| P08 | *F3H* | 23.72 | 3.9 | 11.90 | 12.45 | 13.29 | E38M49-585 |
| P09 | *CHS-1* | 15.08 | 5.8 | 13.99 | 14.56 | 16.34 | E36M48-392 |
| | *CHS-2* | 15.08 | 5.7 | 14.11 | 14.71 | 16.42 | E36M48-392 |
| | *CHI-1* | 15.08 | 3.6 | 6.28 | 7.10 | 8.41 | E36M48-392 |
| | *CHI-2* | 15.08 | 4.2 | 10.31 | 10.35 | 11.75 | E36M48-392 |
| | *FS-2* | 15.08 | 3.6 | 8.60 | 9.12 | 10.65 | E36M48-392 |
| | Naringenin chalcone | 15.08 | 6.6 | 8.00 | 8.68 | 9.77 | E36M48-392 |
| | Luteolin methyl acetyl apiofuranosyl hexose | 67.86 | 4.1 | 5.93 | 5.28 | 5.22 | P14M41-408 |
| | Luteolin methyl-*O*-di-hexose | 96.57 | 6.5 | 9.95 | 6.53 | 5.95 | P11M48-517 |

[a] Linkage group was annotated by the chromosome number after the alphabet P and followed by the small alphabet. Linkage map was constructed with AFLP and microsatellite markers as described by Maharijaya et al. (2012b); [b] LOD = logarithm of odds corresponds to a genome wide confidence level. LOD corresponds to the maximum score at the position of marker; [c] mu_A = trait means of AA homozygous P1, *C. annuum* AC1979 (no. 19); [d] mu_H = trait means of AB heterozygous progeny
[e] mu_B = trait means of BB homozygous P2, *C. chinense* No. 4661 Selection (no. 18); The grey boxes indicate the parent of which the trait was derived from

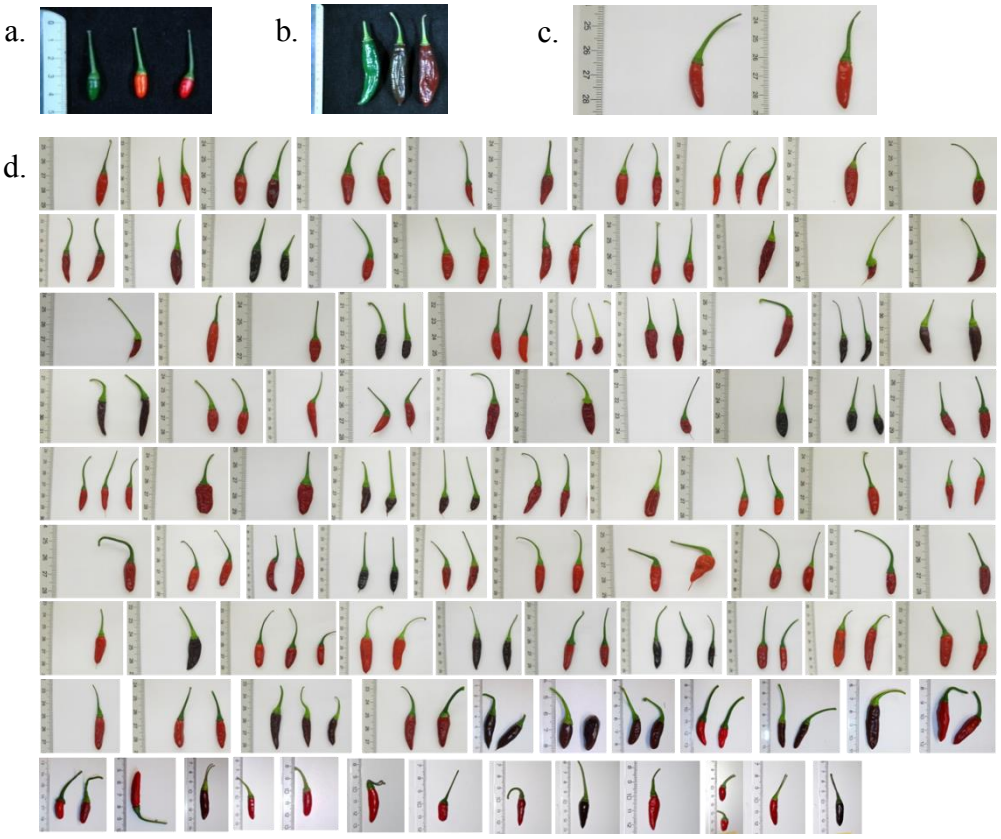
**Supplemental Table 6** Pearson correlation analysis of flavone *C*-glycosides

| HCA Order | HCA Group | Putative ID | Correlation value to Apigenin 6,8-di-*C*-hexoside ($R^2$) |
|---|---|---|---|
| 249 | D | Apigenin 6,8-di-*C*-hexoside | 1 |
| 242 | D | Apigenin 6-*C*-pentoside-8-*C*-hexoside | 0.9 |
| 244 | D | Luteolin 6,8-di-*C*-hexoside | 0.7 |
| 245 | D | Luteolin 6-*C*-hexoside-8-*C*-pentoside | 0.6 |
| 246 | D | Luteolin 6-*C*-pentoside-8-*C*-hexoside | 0.6 |
| 248 | D | Luteolin 8-*C*-hexoside | 0.8 |
| 250 | D | Luteolin 6-*C*-hexoside | 0.9 |

**Supplemental Fig. 1** A crossing scheme to develop F2 plants from *C. annuum* AC1979 (no. 19) and *C. chinense* No. 4661 Selection (no. 18). Two homogenous F1 plants were obtained by crossing two pepper accessions. Each of F1-plants was self-pollinated to produce F2 progeny. The representation of plants shown in the scheme is not in true scaled

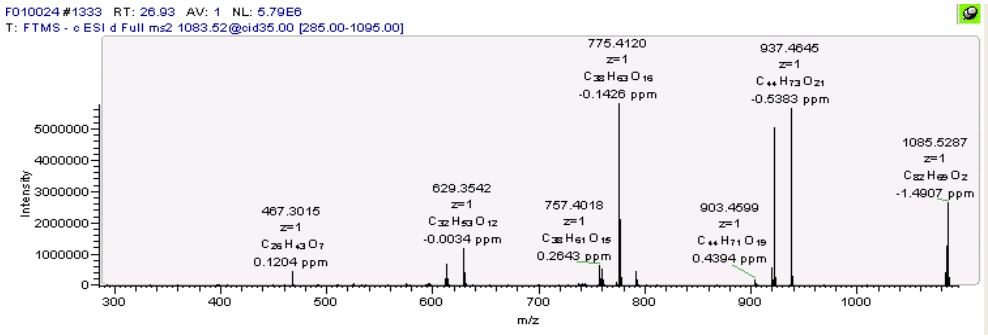


**Supplemental Fig. 2** Variation in fruit morphological characters of: a, *C. annuum* AC1979 (no. 19); b, *C. chinense* No. 4661 Selection (no. 18); c, F1 plants; and d, F2 plants

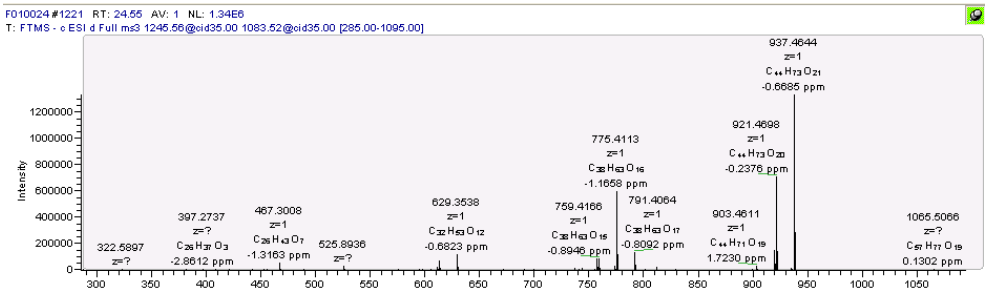


**Supplemental Fig. 3** $MS^n$ fragmentation pattern of Capsianoside VIII and IX using LTQ-Orbitrap FTMS in negative ionization mode

**Capsaicinoid VIII**




**Capsaicinoid IX**



# CHAPTER 6

## General Discussion: Improving Quality Attributes of Pepper (*Capsicum*) Fruits by Using Metabolomics and Molecular Breeding Approaches

# 1. INTRODUCTION

Pepper (*Capsicum* spp.) is one of the most important fruit crops worldwide. The *Capsicum* genus has been domesticated and cultivated for more than 6000 years, as indicated by archeological microfossils found in South and Central America (Perry 2007). Pepper fruits are mainly consumed as a fresh or cooked vegetable, a condiment or a spice and are used as a colouring agent in the food industry. Main goals of professional pepper breeding are to develop cultivars with high yield (i.e. larger fruit size and more fruits per plant), to improve quality attributes (such as bright fruit colour and balanced spiciness) and to enhance field performance (improved pathogen resistance and stress tolerance). These characteristics have been successfully combined in some commercial cultivars such as 'California Wonder', 'Keystone Resistant Giant' and 'Sweet Banana'. In the last years, consumers are becoming more concerned about the nutritional value of their diet and are increasingly interested in the potential value of the many health-related metabolites of fruits and vegetables. This also includes pepper fruits, which are a rich source of metabolites such as ascorbic acid (vitamin C), carotenoids (provitamin A), tocopherols (vitamin E), flavonoids and capsaicinoids. The occurrence of these metabolites in various wild and cultivated pepper accessions gives pepper breeders the opportunity to develop cultivars that combine a balanced metabolite level with the other breeding targets. However, breeding for metabolite composition is difficult since (i) metabolite composition is influenced by many external factors (i.e. environment, stress, etc.); (ii) only a few metabolites are directly visible by the eye as a phenotypic character; (iii) metabolite accumulation may involve other metabolites as precursors in a series of multiple enzymatic steps, and (iv) the metabolic phenotype may be regulated by many genes encoding for diverse biosynthetic steps. One strategy to accomplish the combination of the various breeding targets and to accelerate the selection process is to combine genetic and metabolomic approaches.

Targeted and untargeted metabolomic approaches may help to understand the biochemical pathways underlying the metabolic diversity between genotypes. In addition to studying genetic effects on the metabolic content, metabolomic approaches can be used to study the influence of developmental cues, environmental effects and the interactions between genotype and environment on the composition of metabolites. In addition, metabolomics may help to unravel complex multifactorial traits, such as taste and nutritional value, which may be determined by a combination of metabolites. Untargeted metabolite analyses allow the simultaneous detection of hundreds of metabolites in a biological sample,

without *a priori* knowledge of the identity of the metabolites detected, while targeted metabolite analyses cover specific metabolites, such as carotenoids, vitamins and flavonoids. These analyses, in combination with genetic approaches, may help to identify markers and genes that underlie key steps in metabolite biosynthesis. The concept of metabolomics-assisted breeding, combining metabolite profiling and molecular markers, will be invaluable for breeding programs aimed at the introduction or improvement of metabolite-driven quality traits, such as colour, taste and nutritional value, compared to classical breeding approaches (Table 1; Dixon et al. 2006; Fernie and Schauer 2009). However, in pepper, the currently available information on genetic and molecular markers for fruit metabolites is limited. It is known that pepper fruits contain many metabolites, but most studies are based on a small number of varieties and a few selected phytochemicals.



**Table 1** Different aspects in classical breeding and metabolomic-assisted molecular breeding

| Aspects | Classical Breeding | Metabolomic-Assisted Molecular Breeding |
|---|---|---|
| Quality traits | Mostly related to phenotypic characters, including fruit phenotypes, yields, abiotic tolerance and biotic resistance. | All traits, including metabolites related to plant phenotypes, nutrition and defense materials. |
| Data | Phenotypic characters. | Use comprehensive data platforms: phenotypic characters, reliable molecular markers, metabolomic profiling data and transcriptomic profiling data. |
| Cultivar selection | Phenotypic characters. | Molecular markers-tightly-linked to metabolite traits continued with metabolomic analyses. |
| Time needed until the improved cultivars are developed | 10 – 15 years. | Less than 10 years. |
| Cost | – Laborious due to selection and re-selection until the candidate cultivar with improved traits is detected.<br>– Need space to grow all progenies gained after crossing. | – Less cost for selection and space, since the selection is performed in all progenies at the very young age and therefore only limited number of candidate progenies that must be grown |

## 2. METABOLITE BIODIVERSITY IN PEPPER GERMPLASM

Breeding for metabolite composition requires the availability of candidate cultivars that manifest stable and high metabolite levels as an initial source for a breeding program. Therefore, a comprehensive picture of the metabolite biodiversity of a wide pepper germplasm collection may help to select for the best candidate genotypes. For this, the composition and levels of targeted metabolites, i.e. carotenoids, capsaicinoids, flavonoids and vitamins C and E, and untargeted metabolites, i.e. semi-polar and volatile metabolites, were analyzed in a set of 32 *Capsicum* accessions (Chapter 2 and 3 of this thesis). The accessions consisted of commercial breeding varieties, land races and wild accessions, which originated from different geographical regions and belong to four widely cultivated species: *C. annuum, C. chinense, C. baccatum,* and *C. frutescens* that can be intercrossed and produce fertile hybrids (Pickersgill 1997). Variation in morphological characters included fruit features, such as colour, type, shape and size. The accessions showed a high variation in the composition of carotenoids, capsaicinoids, flavonoids and vitamins C and E. All *Capsicum* accessions accumulated high levels of vitamin C (up to 200 mg/100 g fresh weight (FW)). The brown-fruited *C. chinense* AC2212 (no. 24) had the highest vitamin E concentration (16 mg/100 g FW). Especially, levels of flavonoid *O*- and *C*-glycosides, including quercetin, luteolin and apigenin, showed a high variation between the various accessions. Accession *C. annuum* Long Sweet (no. 12) had a quercetin level (24.58 mg/100 g FW) that is comparable to levels found in yellow onions, which are regarded as a very good source of flavonols in the human diet (Hubbard et al. 2006; Manach et al. 2004; Chapter 4 of this thesis). *C. chinense* No. 4661 Selection (no. 18) accumulated higher naringenin chalcone levels in its ripe fruits (60 μmol naringenin equivalent/100 g FW) compared to the other accessions of the panel. The wild type pepper, *C. annuum* AC1979 (no. 19), is highly pungent and has a high level of capsianosides (diterpene glycosides) and luteolin-*O*-glycosides (Chapter 3 of this thesis; Wahyuni et al. 2013). In addition to metabolic variation, accessions also differed for their susceptibility to biotic stresses, such as thrips (*Frankliniella occidentalis* and *Thrips parvispinus*). For example, accession no. 18 was very susceptible to thrips, while accession no. 19 was highly resistant to thrips (Maharijaya et al. 2011; 2012). The observed biodiversity demonstrated the potential of the used *Capsicum* germplasm for genetic improvement of the above-mentioned metabolic traits and five accessions, *C. annuum* Long Sweet (no. 12), *C. chinense* No. 4661 Selection (no. 18), *C. annuum* AC1979 (no. 19), *C. annuum* AC2212 (no. 24) and *C. frutescens* Lombok (no. 28), were selected as candidates for further breeding programs

aimed at developing pepper cultivars with improved consumer quality characteristics.

Untargeted metabolite analyses were included to generate a more detailed view of the metabolic biodiversity of the 32 *Capsicum* accessions (Chapter 3 of the thesis). Untargeted profiling approaches have been already used to obtain an overview of the metabolic diversity in germplasm collections, such as *Arabidopsis* (Keurentjes et al. 2006) and tomato (Tikunov et al. 2005). Fruits of the 32 pepper accessions were analyzed for semi-polar and volatile metabolites using liquid chromatography (LC) mass spectrometry (MS) and headspace gas chromatography (GC)-MS, respectively. Untargeted LC-MS and GC-MS metabolomic profiles demonstrated the large metabolic variation that was present in the *Capsicum* germplasm. The overall composition of semi-polar metabolites was strongly determined by the species group, indicating that genetic differences between pepper species lead to metabolic variations. This causality might be due to genetic differences leading to 1) effects on the regulation of metabolic pathways, 2) differences in the activity of key enzymes determining the flux through a metabolic pathway, or 3) differences in the activity or substrate specificity of specific modifying enzymes. The clustering of accessions based on their volatile composition primarily reflected differences in pungency rather than species differences. Identification of the volatiles driving this contrast revealed a relatively high abundance of sesquiterpenes and methyl-branched fatty acid esters in the pericarp of pungent accessions. However, there was no obvious relation between the metabolic pathways leading to sesquiterpenes and the pungent capsaicinoids, suggesting that the correlation was due to the structure of the germplasm collection rather than to a causal relationship. The strong correlation of methyl-branched fatty acid ester levels with capsaicinoid levels in the pericarp might suggest that these esters are synthesized in the placenta, from where they are transported to the pericarp. Indeed we demonstrated that methyl-branched fatty acid ester levels were even more abundant in seed and placenta tissue than in pericarp. GC-MS analysis furthermore showed that the 32 pepper accessions manifested a large variation in many other flavour-related volatiles, such as hexanal, nonenal, nonedienal and monoterpenes.

The metabolic composition of pepper fruits is controlled by genetic factors and is influenced by the stage of development. Chapter 4 of this thesis demonstrates the fruit-ripening dependent changes in metabolite levels and composition in mature unripe, turning and ripe pepper fruits. Chlorophylls are the main pigment from the beginning of fruit development up to the mature unripe fruit stage and levels of chlorophylls contained in the pericarp tissue give rise to

the young fruit greenness. The genetic effect on fruit colour at the young stage is exemplified by the 5 to 20 times lower chlorophyll b level in the very pale green to almost white unripe fruits of *C. annuum* Long Sweet (no. 12) and *C. frutescens* Lombok (no. 28), compared to the green unripe fruits of *C. chinense* No. 4661 Selection (no. 18), *C. annuum* AC1979 (no. 19) and *C. annuum* AC2212 (no. 24). Chlorophyll levels decreased at the turning stage, followed by an increase in levels of red carotenoids, capsanthin and capsorubin at the ripe stage of red-ripened fruits. Brown-ripened fruits (accessions no. 18 and 24) retained high chlorophyll b levels, at the ripe stage, in combination with increased levels of capsanthin and capsorubin. The molecular regulation of capsanthin and capsorubin involves the dominant *Y* locus, which encodes capsanthin-capsorubin synthase, an enzyme catalyzing the conversion from asteraxanthin to capsanthin and violaxanthin to capsorubin (Paran and van der Knaap 2007). In brown-ripened fruits, however, the presence of a recessive *cl* locus prevents the degradation of chlorophyll due to an inactive chlorophyllase enzyme, while the activity of the dominant *Y* locus results in the accumulation of the two different coloured-pigments (Efrati et al. 2005). Apart from the changes in fruit colour, also flavonoid levels altered during fruit ripening in the five accessions. In the high-quercetin accession, *C. annuum* Long Sweet (no. 12), the total quercetin level was the highest at the unripe fruit stage and decreased during further fruit ripening. In contrast, naringenin chalcone levels increased upon ripening and reached the highest concentration in ripe fruits of *C. chinense* No. 4661 Selection (no. 18). Such differences in the timing of metabolite accumulation and breakdown can be used in breeding programs that aim for the presence of specific metabolites at a particular stage of fruit development.

## 3. METABOLOMICS-ASSISTED MOLECULAR BREEDING OF PEPPER

As discussed in Chapter 5, an F2 population based on a cross of *C. chinense* No. 4661 Selection (no. 18) and *C. annuum* AC1979 (no. 19) was developed and analyzed as part of this thesis. The F2 population showed a segregation of fruit colour and semi-polar metabolites from the two parents. The colour of F2 fruits segregated 3:1 (red:brown) and is likely due to the segregation of the single recessive *cl* (*chlorophyll retainer*) locus (Efrati et al. 2005). In addition, the composition and levels of metabolites of the two parents segregated over all 113 F2 plants, including flavonoids, phenylpropanoids, a capsaicin analogue, fatty acid and amino acid derivatives. Most F2 plants contained metabolites that were similar to those detected in the male parent accession no. 18. This indicates that the

biochemical profile of the male parent (no. 18) was dominant over the female parent (no. 19) in the F2. Additionally, some F2 plants contained metabolites that have not been found in either of the two parents. Higher accumulation of these metabolites might be due to epistatic effects of alleles from both parents, since these metabolites also accumulated in the F1 hybrid. Furthermore we observed the (up to 12-fold) accumulation of several metabolites (particularly flavonoid glycosides, such as flavone *C*-glycosides, flavonol *O*-glycosides and naringenin chalcone) in some F2 plants compared to both parents and the F1 hybrid. Such extreme phenotypes may be the result of a transgressive segregation pattern caused by several factors, including i) the combination of positive alleles and elimination of negative alleles derived from both parents, ii) environmental variation or (iii) a specific interaction between genotype and environment. Additional replicated trials would be needed to address these different options and this would preferably be done in stable, homozygous genetic backgrounds, such as recombinant inbred lines (RILs).



In addition to phenotypic information on metabolite variation, genetic data of pepper accessions will help to increase breeding efficiency (Geleta et al. 2005). Genetic diversity can be analyzed by using molecular markers (such as amplified fragment length polymorphism = AFLP), microsatellites, simple sequence repeats (SSR) and single nucleotide polymorphism (SNP) markers. AFLP analysis of four *Capsicum* species, i.e. *C. annuum, C. chinense, C. baccatum,* and *C. frutescens* (Chapter 3 of this thesis) showed that the phylogenetic relationships reflected the geographic dispersion (see Fig. 1 of Chapter 1 of this thesis) of the common ancestor of the *C. annuum–C. chinense–C. frutescens* species complex from *C. baccatum* (Basu and De 2003; Djian-Caporilano et al. 2007; Eshbaugh 1970). Comparing these AFLP marker data with semi-polar metabolite profiles of ripe pepper fruits might help to reconstruct the effects of breeding on the pepper phylogeny. Semi-polar metabolites differentiated the *C. annuum* group from the *C. chinense–C. frutescens–C. baccatum* species complex (Chapter 3 of this thesis). This differentiation might be connected to the domestication and selection history of the accessions studied. Indeed, *C. annuum* accessions have been most commonly used for breeding purposes and therefore may have been exposed to the strongest selection pressure. Selection on specific mutations may only marginally impact the total genetic composition of the species group, but might strongly affect the expression of single or multiple downstream genes thereby altering specific metabolic pathways, as has been shown for tomato ripening mutants (Kovács et al. 2009) and high pigment mutants (Bino et al. 2005; Levin et al. 2006). In pepper, such a situation was observed for the wild accession *C. annuum* AC1979 (no. 19),

which genetically was closely related to the cultivated *C. annuum* accessions in the diversity panel, but which showed a markedly different metabolic phenotype, in particular for phenolic acids and flavonoids (luteolin *O*-glycosides). Based on these LC-MS metabolomic data, accession no. 19 was positioned as an outgroup in the phylogenetic tree (Chapter 3 of this thesis). This indicates that genetic differences can not always be assumed to reflect differences in the metabolic phenotype. This is likely due to the fact that small genetic differences (e.g a mutation), even in a homogeneous genetic background, may lead to large metabolic differences, for example the induction of an entire metabolic pathway leading to production of various metabolites. This may strongly affect a principle component analysis (PCA) or hierarchal cluster analysis (HCA), resulting in a different metabolite-based genotype clustering. Similar effects can be expected due to the interaction between genotype and environment.

A better insight in the relation between genetic information and final metabolite content can be obtained by analyzing the entire process from gene to metabolite at every possible level, including gene expression and proteomics data. This type of studies have been carried out in Arabidopsis, tomato and potato, in which the comparison of metabolic and gene transcript profiles resulted in a significant correlation between gene activity and metabolite composition (*Arabidopsis*: Keurentjes et al. 2006; tomato: Carrari et al. 2006; potato: Urbanczyk-Wochniak et al. 2003). In pepper, the combined analysis of gene expression and metabolite production has been reported in particular for carotenoids (Guzman et al. 2010; Ha et al. 2007; Huh et al. 2001; Rodriguez-Uribe et al. 2012;) and capsaicinoids (Abraham-Juarez et al. 2008; Ben-Chaim et al. 2006; Blum et al. 2002, 2003; Kim et al. 2009; Mazourek et al. 2009; Stewart et al. 2005, 2007), but such information is still lacking for flavonoid genes. Using a candidate gene approach, putative flavonoid genes were selected from a pepper gene index database based on the homology to flavonoid genes of tomato (Chapter 4 and 5 of this thesis). As discussed in Chapter 4, high transcript levels of all flavonoid genes could be detected in unripe fruits of the high-quercetin accession, *C. annuum* Long Sweet (no. 12). Interestingly, in this accession, high quercetin levels corresponded with low transcript amounts for *cinnamoyl-CoA reductase* (*CCR*), a gene encoding a key enzyme involved in lignin production. This finding might suggest that the decreased lignin pathway generated an additional flux to quercetin. In *C. chinense* No. 4661 Selection (no. 18), high naringenin chalcone levels in ripe fruits corresponded with an up-regulation of transcription factor *Ca-MYB12* and its molecular target *chalcone synthase* (*CHS-2*) gene expression.

Further information on phenotype-genotype interactions might be gained by using molecular markers that are linked to the metabolic attributes. In pepper, there are only a limited number of trait-associated markers that have been linked to fruit characteristics and disease resistance (Lefebvre 2005) but several so called, metabolite QTLs (mQTLs) have been identified for chlorophyll (Brand et al. 2012), carotenoids (Chaim et al. 2003; Huh et al. 2001; Lefebvre et al. 1998; Thorup et al. 2000) and capsaicinoids (Ben-Chaim et al. 2006; Blum et al. 2002, 2003). These mQTLs are important as they make it possible to select for a particular metabolic fruit phenotype even at the young plant stage. In addition, QTL and candidate gene marker data can help to identify genomic regions that are important for metabolite accumulation (Chapter 5 of this thesis). Metabolites and flavonoid gene expression profiles of F2 plants were used in a QTL analysis using random genetic markers, as well as flavonoid gene-based SNP markers. In total, 279 mQTLs were found in the segregating F2 population, consisting of yet unknown and putatively identified semi-polar metabolites. The detected mQTLs were not evenly distributed over the genome, as two major QTL hotspots for 35 and 103 metabolites, respectively, were detected on chromosome 9. This indicated that a major group of metabolites do share a common genetic region that encodes for one or more genes that regulate the accumulation of those metabolites. Analysis also revealed an mQTL for a capsaicin analogue, located on chromosome 7. Flavonoid mQTLs have also been observed on several genome regions, including naringenin chalcone mQTLs on chromosome 1 and 9 and flavone *C*-glycosides on chromosome 6. These flavonoid QTLs co-localized with flavonoid gene expression QTLs. For example, the mQTL for naringenin chalcone and the expression QTLs of *Ca-MYB12*, *chalcone synthase* (*CHS-1* and *CHS-2*), *chalcone isomerase* (*CHI-2*) and *flavonol synthase* (*FLS*) all mapped at the position of the *Ca-MYB12* marker on chromosome 1. Possibly, naringenin chalcone levels are controlled by the Ca-MYB12 transcription factor, which has also been found to regulate naringenin chalcone accumulation in tomato (Adato et al. 2009; Ballester et al. 2010).



## 4. CHALLENGES IN BREEDING FOR METABOLITE-DRIVEN TRAITS

The combination of molecular breeding and metabolomics might build into a strong approach for the generation of pepper cultivars that combine high yield and enhanced field performance with improved quality attributes. The complete pepper genome has been sequenced and published (Kim et al. 2014). Although the annotation of the pepper genome is limited to gene family analysis and a more detailed characterization of selected genes involved in regulation of fruit ripening,

ethylene synthesis and capsaicinoid biosynthesis in hot pepper (Kim et al. 2014), it is clear that this genome sequence will strongly aid to the identification of key genes that underlie important agronomic traits. Metabolomics-assisted molecular breeding depends on such advancements in DNA sequencing, since it allows the identification of the genes and alleles underlying metabolite-driven traits. The responsible genes are the best possible genetic markers and are invaluable tools in a precision breeding strategy, aimed at efficient trait selection, while minimizing unwanted linkage drag. The present thesis revealed valuable insight into the genomic regions important for the production of (secondary) metabolites in pepper fruit. Confirmation of the results using a candidate gene approach, in which flavonoid mQTL, gene expression (eQTL) and candidate gene marker data were combined, provided valuable insight in the molecular regulation of the flavonoid pathway in pepper fruit. Having these sets of information will help breeders to monitor and select pepper cultivars with improved flavonoid levels.

Apart from quality attributes, metabolites are important for the induction of resistances against abiotic stresses and biotic pathogens (Aloni et al. 2008; Park et al. 2012; Schulze and Spiteller 2009). Various pathogens influence plant metabolism and do affect the composition of metabolites in fruits. For example, the polyphenol content in yellow bell pepper fruits changes after an infection with the fungus *Colletotrichum gloeosporioides*, which causes anthracnose (Park et al. 2012). In countries such as Indonesia, many abiotic and biotic stresses are a major reason for a low yield of pepper fruits. The typical climatic conditions with high temperatures and high humidity easily lead to many different stress responses which negatively affect production. These particular conditions challenge breeders to aim for developing cultivars that combine the various quality attributes and that are active also under the very stressful conditions in the tropic regions of the world. It requires more experiments to fully understand the phenotypic and genotypic interactions under the various environmental conditions. A deeper and more mechanistic understanding of the regulatory networks that control metabolite composition in fruits is important to dissect the various metabolic pathways and is essential for the further advancement of metabolic-assisted breeding programs.

This thesis shows that metabolomics-assisted breeding in combination with genetic analysis helps to identify markers and genes that underlie key steps in metabolite biosynthesis. Such information may steer breeding programs to introduce metabolite quality traits in commercial pepper cultivars. Deciding which metabolites are important for both farmers and consumers will be the breeders' consideration for the future. This will require a close collaboration among all

stakeholders, including farmers, consumers, biochemists, geneticists and breeding specialists from research institutes and universities as well as seed companies.

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# SUMMARY
# SAMENVATTING
# RANGKUMAN

# SUMMARY

Pepper is one of the most important vegetable fruit crops worldwide. Pepper fruits are widely appreciated for their unique taste, colour, flavour and nutritional value and both fresh and dried fruit components spice up many dishes in the world and are used for pharmaceutical purposes. Pepper belongs to the *Capsicum* genus which consists of almost 30 domesticated and wild species. The genetic diversity of the *Capsicum* genus is reflected by the many colours, sizes and shapes as well as the diversity in metabolites (i.e. carotenoids, capsaicinoids, flavonoids and vitamins C and E) that can be found between fruits of the different species. To improve the agronomic performance of pepper, breeders select for cultivars that are more resistant to biotic and abiotic stresses. Lately, the nutritional value of fruits and vegetables has become more important and breeders have started to select for cultivars that combine good agronomic performances with traits that are valued by consumers. The rich diversity of the *Capsicum* germplasm for the many fruit attributes may be used to improve both the agronomic and economic value of cultivated pepper varieties. To aid this breeding scope, it is helpful to have a more comprehensive insight in the metabolic variation between fruits of different *Capsicum* genotypes. The study presented in this thesis aimed to better understand and explore the phenotypic diversity in the composition and levels of metabolites in fruits of various *Capsicum* species by using a combination of metabolomic and genetic approaches.

In this study a total of 32 pepper accessions from *Capsicum annuum*, *Capsicum frutescens*, *Capsicum chinense* and *Capsicum baccatum* were selected based on variations in morphological characteristics and geographical origin. The 32 accessions varied for traits such as fruit colour, pungency and fruit shape and consisted of breeders' varieties, land races and wild species that originated from America, Europe, Asia and Africa. AFLP markers revealed the phylogenetic relationship between the four *Capsicum* species and fitted well with the geographic dispersion of the species. A broad overview of the metabolic variation in the fruits of the different *Capsicum* accessions was generated by using both targeted and untargeted metabolomic approaches. A targeted and quantitative HPLC analysis showed that composition and level of health-related metabolites, such as carotenoids, capsaicinoids, flavonoids and the vitamins C and E in fruits varied greatly between accessions and was independent of species and geographical location (Chapter 2). Fruit colour was determined by the accumulation of specific carotenoids leading to salmon, yellow, orange, red and brown coloured fruits. All accessions accumulated high levels of vitamin C, up to

200 mg/100 g FW. Levels of many metabolites varied among accessions, showing the potential for using the genetic diversity for breeding programs aimed at developing new pepper cultivars with improved consumer quality characteristics. For instance, one accession (the red fruited *C. annuum* Long Sweet accession no. 12) showed a quercetin level of 24.58 mg/100 g FW, a level comparable to those in yellow onions, which are regarded as a very rich source of flavonols in the human diet. The composition of semi-polar and volatile metabolites in pepper fruits of the different accessions was determined using untargeted LC-MS and headspace GC-MS analyses, respectively (Chapter 3). The overall composition of semi-polar metabolites was strongly determined by the species group, indicating that species-driven metabolic differences determined the variation in semi-polar metabolites, whereas pungency was found to be a main driver for the variation in aroma volatiles.

Fruit ripening is known to have an important effect on the accumulation of metabolites. Therefore, the composition and levels of carotenoids, capsaicinoids and flavonoids in unripe, turning and ripe fruits was analyzed and quantified by HPLC and LC-MS in fruits of five *Capsicum* accessions that were selected from the set of 32 accessions based on their contrast in metabolite content (Chapter 4). Both metabolite composition and levels changed during fruit ripening in all the five accessions. Chlorophyll and the red carotenoids capsanthin and capsorubin were the major pigments characterizing fruit colours. In red-ripened fruits, chlorophyll disappeared upon ripening, concomitant with an increase of capsanthin and capsorubin. In brown-ripened fruits red-carotenoids increased upon ripening, and chlorophyll levels remained high throughout ripening. In all pungent fruits, three major capsaicinoids were detected, which were already present at the unripe stage and increased slightly upon ripening. The flavonoids quercetin, luteolin, apigenin and naringenin chalcone were present in all accessions. Quercetin levels accumulated in unripe fruits of *C. annuum* Long Sweet (no. 12) and decreased upon ripening. Naringenin chalcone accumulated in *C. chinense* No. 4661 Selection (no. 18) fruits and its levels reached the highest concentration at the ripe stage.

To gain a better insight in the molecular regulation of flavonoid accumulation during fruit ripening, the expression of some candidate phenylpropanoid and flavonoid genes, as well as a regulator gene were analyzed in correspondence with the quantitative metabolite analysis. Interestingly, flavonoid accumulation during pepper fruit development was accompanied with changes in the expression of three flavonoid genes: i.e. a MYB transcription factor (*Ca-MYB12*), *chalcone synthase-2* (*CHS-2*) and *chalcone isomerase-1* (*CHI-1*). It was postulated that quercetin accumulation in unripe fruits of accession no. 12 was the combined

result of a strong expression of all genes in the flavonoid pathway together with a suppression of the phenylpropanoid pathway branch towards lignin. The combined activity of the genes may result in an induced flux through the pathway towards flavonols.

The correspondence between flavonoid accumulation and candidate gene expression profiles was the base to further analyze the molecular regulation of flavonoid quantitative trait loci (Chapter 5). Untargeted LC-MS profiling of semi-polar metabolites followed by metabolite quantitative trait locus (mQTL) analysis was performed in ripe fruits of 113 F2 plants that derived from an interspecific cross between *C. annuum* AC1979 (no. 19) as the female parent and *C. chinense* No. 4661 Selection (no. 18) as the male parent. The parental accessions were selected based on their variation in fruit morphological and biochemical characteristics and for their difference in the susceptibility to thrips species. Two mQTL hotspots were found on chromosome 9. These two chromosomal regions regulated the relative levels of 35 and 103 metabolites, respectively. Analysis also revealed an mQTL for a capsaicin analogue, located on chromosome 7. Co-localizations of flavonoid mQTLs and flavonoid gene expression QTLs (eQTLs) were found on chromosome 1, 6 and 9. Confirmation of flavonoid mQTLs using a set of six flavonoid candidate gene markers and their corresponding eQTLs indicated the *Ca-MYB12* transcription factor gene on chromosome 1 and the gene encoding flavone synthase (*FS-2*) on chromosome 6 as likely causative genes determining the variation in naringenin chalcone and flavones *C*-glycosides, respectively, in this population.

Results suggest that the extensive biochemical variation in pepper fruit is under the genetic control of a limited number of chromosomal regions (QTL hotspots), encoding genes that regulate the accumulation of large sets of metabolites. In the coming years the complete sequence of the pepper genome is expected to be released and this will aid strongly in the identification of the key genes underlying important agronomic traits. Combining genetic and biochemical datasets will help breeders to develop new pepper varieties that unite a desirable taste and nutritional profile with genetic resistance to important diseases and stressful environmental conditions.

# SAMENVATTING

Peper is één van de meest belangrijke groentegewassen in de wereld. Pepers worden als vrucht enorm gewaardeerd voor hun unieke smaak, kleur, aroma en voedingswaarde. Verse en gedroogde componenten uit peper versterken de smaak van heel veel gerechten overal in de wereld en worden ook voor verschillende farmaceutische toepassingen gebruikt. Peper hoort tot het geslacht *Capsicum*, dat uit bijna 30 gedomesticeerde en wilde soorten bestaat en die genetisch heel divers zijn en bijvoorbeeld ook paprika (*Capsicum annuum*) is een soort binnen het geslacht van de pepers. Vruchten van de verschillende *Capsicum* soorten kunnen verschillen in kleur, grootte en vorm en kunnen ook heel verschillende inhoudstoffen bevatten (zoals carotenoïden, capsaicinoïden, flavonoïden en vitamine C en E). Om de agronomische kwaliteiten van peper te verbeteren selecteren veredelaars met name voor cultivars die meer resistent zijn tegen biotische en abiotische stress omstandigheden. De afgelopen jaren wordt de voedingswaarde van vruchten en groenten steeds belangrijker en veredelaars zoeken daarom naar cultivars die een goede groei combineren met eigenschappen die ook voor de consument belangrijk zijn. De rijke genetische diversiteit voor vruchteigenschappen zoals die binnen het geslacht *Capsicum* wordt gevonden, kan worden gebruikt om zowel de agronomische als economische waarde van gecultiveerde peper variëteiten te verhogen. Om deze veredelingsdoelstelling te ondersteunen is het belangrijk om een beter beeld te hebben van de variatie in de samenstelling van metabolieten in vruchten van de verschillende *Capsicum* genotypes. Het onderzoek gepresenteerd in dit proefschrift had tot doel om de fenotypische variatie in de samenstelling en het niveau van metabolieten in vruchten van verschillende *Capsicum* soorten beter te begrijpen en te kunnen gebruiken. Daarvoor is een multidisciplinaire aanpak gevolgd waarbij biochemische en genetische technieken zijn gecombineerd.

In dit onderzoek zijn in totaal 32 verschillende herkomsten van peper gebruikt uit de soorten *Capsicum annuum* (paprika), *Capsicum frutescens*, *Capsicum chinense* en *Capsicum baccatum*. De herkomsten zijn gekozen op basis van het verschil in morfologische kenmerken en geografische oorsprong. De 32 herkomsten verschilden in de kleur en vorm van de vrucht, maar ook in bijvoorbeeld smaakscherpte, en bestond uit variëteiten, landrassen en wilde soorten uit Amerika, Europa, Azië en Afrika. Met AFLP merkers zijn de fylogenetische relaties tussen de vier verschillende *Capsicum* soorten bevestigd en de resultaten sloten goed aan op de geografische herkomst van de soorten. Door een combinatie van een gerichte en ongerichte metabolomics aanpak werd een

breed overzicht gemaakt van de biochemische variatie tussen de vruchten van de verschillende *Capsicum* herkomsten. De gerichte en kwantitative HPLC analyse gaf aan dat de samenstelling en het gehalte van gezondheid gerelateerde metabolieten zoals carotenoïden, capsaicinoïden, flavonoïden en vitamine C en E tussen vruchten van de verschillende herkomsten enorm verschilden (Hoofdstuk 2). De kleur van de vrucht (zalm, geel, oranje, rood of bruin) werd bepaald door de ophoging van verschillende carotenoïden. Alle herkomsten bezaten hoge vitamine C gehaltes, tot wel 200 mg/100 g vers gewicht. Het onderlinge niveau van de metabolieten in de rijpe peper vrucht bleek afhankelijk te zijn van de specifieke herkomst. Dit resultaat gaf het potentieel aan om de gevonden genetische diversiteit te kunnen gebruiken in een veredelingsprogramma dat is gericht op het ontwikkelen van nieuwe peper cultivars met een verbeterde kwaliteit voor de consument. Bijvoorbeeld, een bepaalde herkomst (de rode vrucht dragende *C. annuum* Long Sweet nummer 12) had een quercetine gehalte van 24.58 mg/100 g vers gewicht, een gehalte dat ook gevonden wordt in gele uien die bekend staan als een rijke bron van flavonoïden in ons dieet. De samenstelling van semi-polaïre en vluchtige metabolieten in vruchten van de verschillende peper herkomsten werd gemeten door gebruik te maken van ongerichte LC-MS en zogenaamde 'head-space' GC-MS analyse (Hoofdstuk 3). De gevonden verschillen in de algemene samenstelling van de semi-polaïre metabolieten was gecorreleerd met de *Capsicum* soort, terwijl de smaakscherpte de meest bepalende factor was voor het gevonden verschil in de samenstelling van de vluchtige stoffen tussen de 32 herkomsten.

Het is bekend dat de rijping van de vrucht een belangrijk effect heeft op de metabolietsamenstelling. Daarom werd de compositie en het gehalte van carotenoïden, capsaicinoïden en flavonoïden bepaald in onrijpe, kleurende en rijpe vruchten van vijf *Capsicum* herkomsten die werden geselecteerd uit de groep van 32 op basis van het contrast in de samenstelling van de metabolieten (Hoofdstuk 4). Zowel de samenstelling als het gehalte van de metabolieten veranderde gedurende de rijping van de vrucht in alle vijf herkomsten. Vooral het gehalte van chlorofyl en de rode carotenoïden capsanthin en capsorubin was bepalend voor de vruchtkleur. In rood afrijpende vruchten verdween chlorofyl en steeg het gehalte aan capsanthin en capsorubin tijdens rijping. In bruin afrijpende vruchten steeg het gehalte aan rode carotenoïden tijdens rijping, terwijl het chlorofyl gehalte gelijk bleef. Scherp smakende vruchten bevatten drie belangrijke capsaicinoïden, die al aanwezig waren in de onrijpe vrucht en waarvan het gehalte licht steeg tijdens afrijping. Alle vruchten van de vijf onderzochte herkomsten bevatten de flavonoïden quercetine, luteoline, apigenine en naringenine chalcon. Het

quercetine gehalte was het hoogst in onrijpe vruchten van *C. annuum* Long Sweet nummer 12 en nam af tijdens afrijping. In vergelijking met de andere 4 herkomsten was het naringenine chalcon gehalte hoog in rijpe vruchten van *C. chinense* No. 4661 Selection nummer 18.

Om een beter inzicht te krijgen in de moleculaire regulatie van de verhoging van het gehalte aan flavonoïden tijdens vrucht rijping is de expressie van een aantal kandidaat phenylpropanoïd- en flavonoïdgenen en een flavonoid regulatorgen onderzocht en vergeleken met een kwantitatieve analyse van de metabolieten. De verhoging van het flavonoïdegehalte gedurende de ontwikkeling van de pepervrucht ging gepaard met een verandering in de expressie van drie flavonoïdgenen, namelijk een MYB transcriptiefactor (*Ca-MYB12*), een chalcone synthase 2 (*CHS-2*) en een chalcone isomerase-1 (*CHI-1*) gen. De ophoging van quercetine in de onrijpe vruchten van de *C. annuum* herkomst nummer 12 was mogelijk een resultaat van de gecombineerde sterke expressie van alle genen die betrokken zijn bij de flavonoid synthese in combinatie met een expressie van de phenylpropanoïd route naar lignine. De gecombineerde activiteit van de genen zou een verklaring kunnen geven voor een verhoogde flux door de biosyntheseroute naar flavonolen zoals quercetine.

De vergelijking van de flavonoïde ophoging met de kandidaatgen profielen gaf de basis om de moleculaire regulatie van de overerving van de kwantitatieve eigenschappen verder te analyseren (Hoofdstuk 5). Daartoe werd de ongerichte LC-MS profilering van semi-polaïre metabolieten gecombineerd met een zogenaamde 'metabolic quantitative trait locus' (mQTL) analyse. Deze gecombineerde biochemische en genetische analyse werd uitgevoerd met rijpe vruchten van 113 F2 planten die waren voortgekomen uit een interspecifieke kruising tussen *C. annuum* AC1970 (nummer 19) als de vrouwelijke ouder en *C. chinense* No. 46661 Selection (nummer 18) als de pollen donor. De herkomst van de ouders was bepaald op basis van het verschil in morfologische en biochemische eigenschappen en op basis van het verschil in resistentie tegen het plaaginsect trips. Twee zogenaamde 'mQTL hotspots' werden gevonden op chromosoom 9, die konden worden gekoppeld aan een verschil in expressie van respectievelijk 35 en 103 metabolieten. Een verdere analyse gaf een mQTL voor een capsaicine analoog op chromosoom 7. Een gekoppelde locatie van flavonoïd mQTL's en flavonoïd genexpressie markers (eQTL's) werd gevonden op de chromosomen 1,6 en 9. Door gebruik te maken van zes flavonoïd kandidaat gen merkers en de gekoppelde eQTL's kon worden aangetoond dat de transcriptiefactor *Ca-MYB12* op chromosoom 1 en het gen coderend voor flavon synthase (*FS-2*) op chromosoom 6

de mogelijke kandidaatgenen zijn voor de variatie in naringenin chalcon en flavone-*C*-glycosides in de gebruikte populatie.

Resultaten uit dit proefschrift geven aan dat de biochemische variatie in vruchten van peper ook bepaald wordt door de genetische oorsprong en dat er bepaalde zogenaamde 'hotspots' op het genoom zijn die de expressie van een groot aantal metabolieten tegelijk bepalen. De komende jaren wordt verwacht dat de volledige DNA volgorde van het *Capsicum* genoom beschikbaar zal komen. Deze genetische kennis zal helpen om de sleutelgenen te identificeren voor belangrijke agronomische eigenschappen. De combinatie van genetische en biochemische datasets zal veredelaars helpen om nieuwe peper variëteiten te ontwikkelen die een goede smaak en een goede voedingswaarde combineren met een genetische resistentie tegen belangrijke ziektes en een sterke weerstand tegen de diverse externe omstandigheden.

# RANGKUMAN

Cabai adalah salah satu jenis sayuran yang paling penting di dunia. Buah cabai diminati secara luas karena keunikan rasa, warna, aroma dan kandungan nutrisi buah. Buah cabai segar maupun kering telah digunakan sebagai komponen bumbu dari berbagai masakan di dunia dan bahan baku dalam industri farmasi. Cabai termasuk ke dalam genus *Capsicum* dan genus ini terdiri dari hampir 30 spesies, baik yang telah didomestikasi maupun liar. Keragaman genetik genus *Capsicum* tercermin pada keragaman warna, ukuran dan bentuk buah serta keragaman metabolit yang terkandung dalam buah (yaitu karotenoid, kapsaisinoid, flavonoid dan vitamin C dan E) dari spesies yang berbeda. Untuk meningkatkan performa agronomi tanaman cabai, pemulia tanaman harus merakit kultivar yang lebih tahan terhadap cekaman biotik dan abiotik. Saat ini, kandungan nutrisi buah dan sayuran menjadi sangat penting dan oleh sebab itu pemulia tanaman harus merakit kultivar baru dengan menggabungkan antara sifat unggul untuk performa agronomi yang baik dengan sifat unggul lain yang diinginkan konsumen. Kekayaan biodiversitas karakter buah cabai pada plasma nutfah *Capsicum* dapat dimanfaatkan untuk meningkatkan nilai agronomi dan ekonomi varietas cabai yang telah ada saat ini. Untuk mempermudah usaha pemuliaan ini, pengetahuan yang komprehensif tentang variasi metabolit yang terkandung pada buah dari berbagai genotipe *Capsicum* perlu dimiliki. Oleh karena itu, penelitian yang dilakukan dalam tesis ini bertujuan untuk lebih memahami dan mengeksplorasi keragaman fenotipik komposisi dan kadar metabolit pada buah cabai berbagai jenis spesies *Capsicum* dengan menggunakan kombinasi antara pendekatan metabolomik dan genetik.

Penelitian ini menggunakan 32 aksesi cabai yang berasal dari spesies *Capsicum annuum*, *Capsicum frutescens*, *Capsicum chinense* dan *Capsicum baccatum* yang dipilih berdasarkan variasi karakter morfologi buah dan asal geografisnya. Seluruh aksesi ini memiliki variasi pada beberapa karakter buahnya, yaitu warna, tingkat kepedasan dan bentuk, serta terdiri dari varietas hasil pemuliaan, *landraces* dan *wild spesies* yang berasal dari Amerika, Eropa, Asia dan Afrika. Penanda AFLP mengungkapkan adanya hubungan kekerabatan antara empat spesies *Capsicum* yang sesuai dengan penyebaran geografis dari ke empat spesies tersebut. Dua pendekatan metabolomik, *targeted* dan *untargeted metabolomics*, memberikan gambaran yang menyeluruh mengenai variasi metabolit dari seluruh aksesi. Analisis kuantitatif HPLC menunjukkan bahwa komposisi dan kadar metabolit buah sangat bervariasi antar aksesi dan tidak tergantung pada jenis spesies serta asal geografis cabai (Bab 2). Warna buah

ditentukan oleh kandungan karotenoid tertentu yang akan mempengaruhi warna buah menjadi *salmon*, kuning, oranye, merah dan coklat. Kandungan vitamin C buah cabai dari seluruh aksesi sangat tinggi, yang mencapai 200 mg/100 g berat basah buah. Kandungan metabolit bervariasi pada buah dari seluruh aksesi cabai yang diteliti, yang menunjukkan bahwa keragaman genetik dari aksesi tersebut sangat berpotensi untuk digunakan dalam perakitan kultivar baru melalui program pemuliaan. Sebagai contoh, aksesi cabai merah *C. annuum* Long Sweet (no. 12) memiliki kadar quersetin 24,58 mg/100 g berat basah buah dan kadar ini setara dengan kadar quersetin yang terkandung dalam bawang bombay yang merupakan sumber flavonol terbaik untuk manusia. Komposisi metabolit semi polar dan volatil dianalisis dengan *untargeted metabolomic*, masing-masing menggunakan LC-MS dan *headspace* GC-MS (Bab 3). Komposisi dari keseluruhan metabolit semi- polar sangat ditentukan oleh jenis spesies cabai dan hal ini menunjukkan bahwa perbedaan genetik antar spesies juga mencerminkan perbedaan metabolik. Oleh karena itu, jenis spesies mempengaruhi variasi senyawa semi-polar sedangkan tingkat kepedasan adalah faktor utama yang mempengaruhi variasi dalam volatil aroma.

Tingkat kematangan buah telah diketahui memiliki dampak pada komposisi metabolit yang terakumulasi pada buah cabai. Oleh sebab itu, komposisi dan kadar karotenoid, kapsaisinoid dan flavonoid dianalisis serta dikuantifikasi menggunakan HPLC dan LC-MS pada buah mentah, buah setengah matang dan buah matang dari lima aksesi *Capsicum* yang dipilih dari 32 aksesi sebelumnya berdasarkan kandungan metabolitnya (Bab 4). Komposisi dan kadar metabolit mengalami perubahan selama tahapan kematangan buah pada ke lima aksesi yang diuji. Klorofil dan karotenoid merah: kapsanthin dan kapsorubin adalah pigmen yang dominan yang mempengaruhi karakteristik warna buah. Pada buah cabai merah yang matang, klorofil menghilang dan diiringi dengan peningkatan kadar kapsanthin dan kapsorubin. Pada buah cabai coklat yang matang, pigmen karotenoid merah meningkat serta diiringi dengan bertahannya kadar klorofil. Pada cabai yang pedas, tiga senyawa utama kapsaisinoid terdeteksi dan ketiga senyawa tersebut sudah dapat terdeteksi pada buah mentah dan konsentrasi ketiga senyawa tersebut semakin meningkat seiring dengan kematangan buah. Kuersetin, luteolin, apigenin dan naringenin chalcone adalah senyawa flavonoid utama yang terdeteksi pada buah dari seluruh aksesi. Konsentrasi kuersetin tertinggi terdapat pada buah mentah dari aksesi *C. annuum* Long Sweet (no. 12), yang kemudian mengalami sedikit penurunan pada buah matangnya. Naringenin chalcone terakumulasi sangat besar pada buah matang *C. chinense* No. 4661 Selection (no. 18). Untuk mengetahui regulasi akumulasi flavonoid pada tahap

molekuler selama proses pematangan buah cabai, ekspresi kandidat gen fenilpropanoid dan flavonoid, serta protein regulator dianalisis dan dihubungkan dengan analisis kuantitatif metabolit. Analisis tersebut menunjukkan bahwa akumulasi flavonoid selama tahapan kematangan buah cabai diikuti dengan perubahan ekspresi tiga gen terkait biosintesis flavonoid yang menyandi faktor transkripsi MYB (*Ca-MYB12*), *chalcone synthase-2* (*CHS-2*) dan *chalcone isomerase-1* (*CHI-1*). Akumulasi kuersetin pada buah mentah dari aksesi no. 12 diduga disebabkan oleh kombinasi antara tingginya ekspresi seluruh gen yang terlibat dalam jalur biosintesis flavonoid dan supresi jalur biosintesis fenilpropanoid yang mengarah ke lignin. Kombinasi kedua aktivitas tersebut menginduksi fluks pada jalur biosintesis flavonol.

Korelasi antara profil akumulasi flavonoid dan ekspresi kandidat gen flavonoid adalah dasar untuk melakukan analisis yang lebih dalam terhadap regulasi molekuler lokus sifat kuantitatif flavonoid (Bab 5). Penentuan profil metabolit semi-polar dengan *untargeted* LC-MS yang diikuti oleh analisis *metabolite quantitative trait locus* (mQTL) dilakukan pada buah matang dari 113 tanaman F2 yang berasal dari persilangan interspesifik antara *C. annuum* AC1979 (no. 19) sebagai induk betina dan *C. chinense* No. 4661 Selection (no. 18) sebagai induk jantan. Kedua aksesi tersebut dipilih sebagai induk dalam persilangan karena memiliki perbedaan pada morfologi buah, karakteristik biokimia yang terkandung dalam buah dan tingkat kerentanan terhadap spesies thrips. Dua mQTL *hotspot* telah ditemukan pada kromosom 9. Kedua daerah kromosom ini mengatur masing-masing 35 dan 103 metabolit. Hasil analisis juga mengungkapkan adanya mQTL untuk kapsaisin analog yang terletak pada kromosom 7. Ko-lokalisasi beberapa mQTL flavonoid dengan beberapa QTL ekspresi gen flavonoid (eQTL) ditemukan pada kromosom 1, 6 dan 9. Konfirmasi mQTL flavonoid dengan menggunakan penanda genetik dari enam kandidat gen flavonoid dan korelasi antara mQTL dan eQTL menunjukkan bahwa gen faktor transkripsi *Ca-MYB12* pada kromosom 1 dan gen yang mengkode flavon synthase (*FS-2*) pada kromosom 6 merupakan faktor utama yang menentukan variasi naringenin chalcone dan flavon *C*-glikosida pada populasi F2.

Hasil penelitian pada tesis ini menunjukkan bahwa variasi biokimia yang besar pada buah cabai dikontrol secara genetik oleh beberapa daerah pada kromosom cabai (*QTL hotspots*), di mana terdapat gen yang meregulasi akumulasi sejumlah besar metabolit. Pada beberapa tahun mendatang diharapkan sekuens seluruh genom cabai telah tersedia dan ini akan sangat membantu pengidentifikasian gen-gen kunci yang meregulasi sifat-sifat agronomi yang penting. Kombinasi antara data genetik dan biokimia dapat membantu pemulia

tanaman untuk merakit varietas baru cabai yang memiliki rasa dan kandungan nutrisi yang diinginkan serta memiliki ketahanan genetik terhadap penyakit dan cekaman lingkungan.

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care of Adhitya with gentle and love. The best gratitude from me is never enough for both of you…

Wahyuni
Jakarta, May 2014

# About the author

Wahyuni was born on 23 June 1978 in Jakarta, Indonesia. In 2001, she obtained her BSc degree majoring Biology at the University of Indonesia. She continued her study and obtained her Master degree in Biomedical at the University of Indonesia in 2004. After her graduation, she obtained a position as candidate researcher in the Research Centre for Biotechnology, Indonesian Institute of Sciences (LIPI), in Cibinong, Indonesia, where she worked from 2005 until now. During her work in LIPI, she has been awarded a fellowship to do a-one-year research from L'ORÉAL-UNESCO for Women in Science National 2005. In 2007, she has been accepted as a PhD candidate at Wageningen University, the Netherlands, in March 2008. All chapters in this thesis represent the results of her PhD project. She could be contacted through her email address wahyu004@gmail.com.

## Publications

In peer-reviewed journal:

Wahyuni Y, Ballester AR, Sudarmonowati E, Bino RJ, Bovy AG (2011) Metabolite biodiversity in pepper (*Capsicum*) fruits of thirty-two diverse accessions: Variation in health-related compounds and implications for breeding. Phytochemistry 72 (11-12):1358-1370

Wahyuni Y, Ballester A-R, Tikunov Y, de Vos R, Pelgrom K, Maharijaya A, Sudarmonowati E, Bino R, Bovy A (2013) Metabolomics and molecular marker analysis to explore pepper (*Capsicum* sp.) biodiversity. Metabolomics:130-144

Wahyuni Y, Ballester AR, Sudarmonowati E, Bino RJ, Bovy AG (2013) Secondary metabolites of *Capsicum* species and their importance in the human diet. J Nat Prod 76 (4):783-793

Wahyuni Y, Stahl-Hermes V, Ballester AR, de Vos RCH, Voorrips RE, Maharijaya A, Molthoff J, Viquez Zamora M, Sudarmonowati E, Arisi ACM, Bino RJ, Bovy AG (2014) Genetic mapping of semi-polar metabolites in pepper fruits (*Capsicum* sp.): towards unravelling the molecular regulation of flavonoid quantitative trait loci. Mol Breed 33:503-518

In seminar proceeding:

Sudarmonowati E, Cahyani Y, Hartati NS, Wahyuni (2010) Provision of superior genotypes of *Jatropha curcas* for biodiesel production: Integrating morphology and yield variation with DNA-based marker. Proceeding of ASEAN-Korea Symposium and Workshop on Biorefinery Technology for Sustainable Production of Biofuel and Industrial Biochemicals: Converging Biorefinery to Response Climate Change. pp131-137

Wahyuni Y, Ballester AR, Sudarmonowati E, Bino RJ, Bovy AG (2013) Evaluasi kandungan mikronutrien pyridoxine (vitamin B6) pada 32 aksesi buah cabai (*Capsicum* spp.) (Evaluation of pyridoxine (vitamin B6) content in 32 *Capsicum* accessions). Proceeding of National Seminar on Food, Medicines and Environmental Research for Health. pp31-37

In preparation:

Wahyuni Y, Ballester AR, Sudarmonowati E, Bino RJ, Bovy AG The influence of fruit ripening process on the accumulation of health-related metabolites in five different *Capsicum* accessions.

## Education Statement of the Graduate School
## Experimental Plant Sciences

The Graduate School EXPERIMENTAL PLANT SCIENCES

**Issued to:** Yuni Wahyuni
**Date:** 4 June 2014
**Group:** Plant Physiology, Wageningen University & Research Centre

| **1) Start-up phase** | *date* |
|---|---|
| ► **First presentation of your project** | |
| Quality Attributes to Pepper Metabolites : A Functional Genomic Approach | Mar 17, 2008 |
| ► **Writing or rewriting a project proposal** | |
| Quality Attributes to Pepper Metabolites : A Functional Genomic Approach | 2008 |
| ► **Writing a review or book chapter** | |
| Metabolite biodiversity of Capsicum germplasm: variation in health-related compounds among 32 diverse pepper accessions and implication for breeding, J. Nat. Prod. 2013, 76, pp 783 - 793 | 2010 |
| ► **MSc courses** | |
| ► **Laboratory use of isotopes** | |
| *Subtotal Start-up Phase* | *13.5 credits** |

| **2) Scientific Exposure** | *date* |
|---|---|
| ► **EPS PhD student days** | |
| EPS PhD student day, Leiden University | Feb 26, 2009 |
| EPS PhD student day, Wageningen University | May 20, 2011 |
| ► **EPS theme symposia** | |
| EPS theme 3 'Metabolism and Adaptation', University of Amsterdam | Feb 18, 2009 |
| EPS theme 3 'Metabolism and Adaptation', Leiden University | Feb 19, 2010 |
| EPS theme 3 'Metabolism and Adaptation', Wageningen University | Feb 10, 2011 |
| ► **NWO Lunteren days and other National Platforms** | |
| ALW meeting 'Experimental Plant Sciences', Lunteren | Apr 06-07, 2009 |
| ALW meeting 'Experimental Plant Sciences', Lunteren | Apr 19-20, 2010 |
| ► **Seminars (series), workshops and symposia** | |
| Annual INDOSOL Meeting 2008 | Nov 05, 2008 |
| Plant Breeding Research Day | Mar 2009 |
| KNAW-Open Science Meeting 2009 | Nov 16-17, 2009 |
| VLAG Mini Symposium: Towards high productivities of microalgae in photobioreactors | Mar 05, 2010 |
| Annual INDOSOL Meeting 2010 | May 31-Jun 01, 2010 |
| ► **Seminar plus** | |
| ► **International symposia and congresses** | |
| Metabolomic Conference 2010 | Jun 27-Jul 01, 2010 |
| EUCARPIA Meeting 2010 | Aug 30-Sep 01, 2010 |
| EUSOL Meeting 2010 | Nov 13-16, 2010 |
| Indonesia Biotechnology Conference: An International Forum | Jul 04-07, 2012 |
| ► **Presentations** | |
| Oral Presentation of research progress in the Annual INDOSOL Meeting | Nov 05, 2008 |
| Oral Presentation of in the Cluster meeting of Bioscience Department | Jan 28, 2009 |
| Oral Presentation of research progress in the Cluster meeting of Bioscience | Nov 04, 2009 |
| Laptop Poster Presentation of research in Open Science Meeting | Nov 16, 2009 |
| Poster Presentation of research in ALW meeting 'Experimental Plant Sciences' Lunteren | Apr 19-20, 2010 |
| Oral Presentation of research progress in Metabolomic Group meeting | May 17, 2010 |
| Oral Presentation of research progress in the Annual INDOSOL Meeting | May 31-Jun 01, 2010 |
| Poster Presentation of research in Metabolomic Conference 2010 | Jun 28-30, 2010 |
| Oral Presentation of research in EUCARPIA Meeting 2010 | Aug 30-Sep 01, 2010 |
| Oral Presentation of research progress in Metabolomic Group meeting | Nov 01, 2010 |
| Poster Presentation of research in EUSOL meeting | Nov 15, 2010 |
| Oral Presentation of research in Indonesia Biotechnology Conference: An International Forum | Jul 05, 2012 |
| ► **IAB interview** | |
| Meeting with a member of the International Advisory Board | Feb 17, 2011 |
| ► **Excursions** | |
| Scientific Excursion to a seed company namely East West Indonesia | Nov 06, 2008 |
| Excursion to RijkZwaan | Mar 06, 2009 |
| *Subtotal Scientific Exposure* | *22.8 credits** |

| **3) In-Depth Studies** | *date* |
|---|---|
| ► **EPS courses or other PhD courses** | |
| INDOSOL Course "Application of molecular marker to phylogenetic study | Nov 10-13, 2008 |
| Metabolomics Course: Basics and Applications to Plant Sciences | Apr 20-24, 2009 |
| Masterclass "Agriculture Beyond Food" | Nov 18-20, 2009 |
| Metabolomics Course: An Introduction to SIMCA-P | Mar 01, 2010 |
| System Biology: Statistical Analysis of ~Omics Data | Dec 13-17, 2010 |
| Introduction Bioinformatics - a user's approach Course | Aug 29-Sep 02, 2011 |
| ► **Journal club** | |
| ► **Individual research training** | |
| *Subtotal In-Depth Studies* | *6.9 credits** |

| **4) Personal development** | *date* |
|---|---|
| ► **Skill training courses** | |
| Course: Techniques for writing and presenting a scientific paper | Apr 13-16, 2010 |
| How to write a world-class paper | Oct 26, 2010 |
| Presentation skill | Oct 14-28, 2011 |
| Writing a Grant proposal | Oct 21–Nov 18, 2011 |
| ► **Organisation of PhD students day, course or conference** | |
| ► **Membership of Board, Committee or PhD council** | |
| *Subtotal Personal Development* | *4.4 credits** |

| **TOTAL NUMBER OF CREDIT POINTS*** | **47.6** |
|---|---|

Herewith the Graduate School declares that the PhD candidate has complied with the educational requirements set by the Educational Committee of EPS which comprises of a minimum total of 30 ECTS credits

** A credit represents a normative study load of 28 hours of study.*