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Characterization of Furanocoumarin Profile and Inheritance Toward Selection of Low Furanocoumarin Seedless Grapefruit Cultivars

Chunxian Chen
University of Florida, Citrus Research and Education Center, 700 Experiment Station Road, Lake Alfred, FL 33850

Paul Cancalon and Carl Haun
Florida Department of Citrus, Scientific Division, 700 Experiment Station Road, Lake Alfred, FL 33850

Fred Gmitter, Jr.[1]
University of Florida, Citrus Research and Education Center, 700 Experiment Station Road, Lake Alfred, FL 33850

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Abstract. **Furanocoumarins are organic chemical components in grapefruit (*Citrus paradisi*) juice that have been shown to induce potentially deleterious drug interactions. In this study we measured seven furanocoumarins (FCs) [bergamottin, 6′,7′-dihydroxybergamottin (6,7-DHB), paradisin C, bergaptol, isoimperatorin, 5′,8′-dimethylallyloxypsoralen (5,8-DMP), and epoxybergamottin (EBM)] in fruit of three grapefruit cultivars [Foster (Fos), Low Acid Foster (LAF), and Hudson (Hud)], one pummelo (*C. maxima*) cultivar [Hirado Buntan (HBP)], 17 randomly selected hybrids from HBP× Hud, and 31 other triploid hybrids. Bergamotton, 6,7-DHB, and paradisin C were not detected or extremely low in HBP (0.00, 0.11, and 0.00 mg·L^{-1}) and LAF (0.40, 3.83, and 0.00 mg·L^{-1}) compared with Hud (13.03. 9.58, and 6.11 mg·L^{-1}) and Fos (6.48, 14.38, and 6.11 mg·L^{-1}). In these hybrids, 6,7-DHB, bergamottin, and paradisin C obviously cosegregated in an approximate rate of 1:1. The three FCs in eight hybrids were not detected or extremely low, like HBP, the maternal parent; those in the other nine were as high as or higher than Hud, the paternal parent. The same segregation tendency was also observed in these triploid hybrids. Based on all the cultivars and hybrids, strong correlations existed among 6,7-DHB, bergamottin, and paradisin C (coefficient up to 0.909). Such strong correlations may reflect their metabolic links in the bergamottin pathway. The 1:1 cosegregation and strong correlation among the three FCs suggested that the trait of FCs is likely controlled by one single enzymatic or regulatory gene in the pathway. The FC profiles and inheritance may lead to a genomic and breeding solution to the grapefruit FC–drug interaction issue. Selection of FC-low or FC-free seedless grapefruit cultivars is underway.**

Furanocoumarins are secondary metabolites found in some plants (Diawara and Trumble, 1997; Murray et al., 1982), among which some vegetables and fruits such as celery (*Apium graveolens*), parsnip (*Pastinaca sativa*), carrot (*Daucus carota*), and grapefruit are among the human diet (Aronson, 2001; Genser, 2008). The feature chemical structure of FCs is a furan ring fused with coumarin that belongs to phenylpropanoids and has a function mainly against insect herbivores (Nitao et al., 2003). The fusion can generate different isomers, specifically, psoralen and angelicin, the precursors of linear and angular FCs (Bourgaud et al., 2006; Diawara and Trumble, 1997; Larbat et al., 2007, 2009; Murray et al., 1982). The predominant FC derivatives greatly vary among these plants and tissue types as well (De Castro et al., 2006; Manthey and Buslig, 2005). For example, there are no angular FCs in citrus but they are found in celery and parsnip (Diawara and Trumble, 1997).

It has been clinically reported that FCs in grapefruit juice increase the bioavailability of some drugs (Bailey et al., 2004; Dahan and Altman, 2004; Oda et al., 2007). Such interaction, also called the grapefruit juice effect (GJE), occurs through inhibition of the human intestinal enzyme cytochrome P450 (CYP) 3A4 and the subsequently increased blood levels of a.i. of these drugs (Bailey et al., 1998, 2004; Ohnishi et al., 2000). Among the grapefruit FCs, ranked in terms of inhibitory potency are: paradisin C > 6′,7′-dihydroxybergamottin > bergamottin > isoimperatorin > bergapten > bergaptol (Ohnishi et al., 2000; Row et al., 2006). The former three are also most abundant. According to the bergamottin biosynthesis pathway (Fig. 1), these compounds are either intermediate metabolites or end FC products (Bourgaud et al., 2006; Larbat et al., 2007, 2009; Murray et al., 1982); their profile concentration levels are different during the biosynthesis process and also variable with conditions; e.g., maturity, storage, cultivar, tissue, and production environment (Manthey and Buslig, 2005; Widmer, 2005; Widmer and Haun, 2005). Similar pharmacokinetic interactions were observed among white and colored grapefruit juice (Uesawa and Mohri, 2008; Uesawa et al., 2008). P450s are responsible for the first-pass elimination of various xenobiotics including drugs. FCs act on CYP3A4 by a mechanism-based inhibition. After grapefruit juice ingestion, the FCs induce the catabolism of CYP3A4 from the intestinal enterocytes and it takes up to 3 d for the enzymes to be restored (Bailey et al., 2004; Guo et al., 2000). In the absence of CYP, the blood level of some drugs can be greatly increased, which may produce some adverse effects (Bailey et al., 2004; Dahan and Altman,

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[1]Corresponding author. E-mail: fgmitter@ufl.edu.

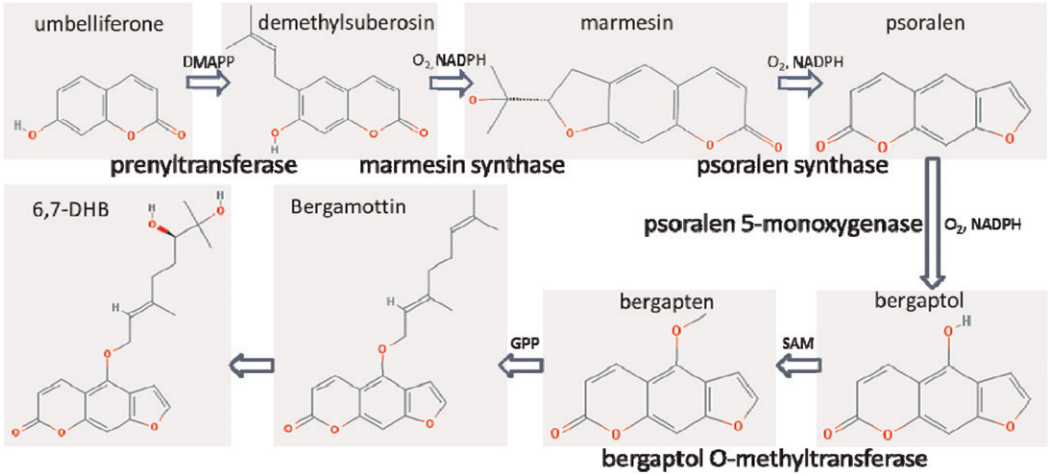

Fig. 1. Diagram of bergamottin biosynthesis pathway. The chemical steps were connected by the arrows where some essential substances and enzymes involved in the biosynthesis were marked. All the chemical structures were retrieved from the PubChem database (Wang et al., 2009) maintained at the National Center for Biotechnology Information (Bethesda, MD). DMAPP = dimethylallyl pyrophosphate; NADPH = nicotinamide adenine dinucleotide phosphate (the reduced form); SAM = S-adenosyl methionine; GPP = geranyl pyrophosphate; 6,7-DHB = 6′,7′-dihydroxybergamottin.

2004). As a result, the GJE has had a significant negative impact on marketing and consuming grapefruit and grapefruit juice for years, although grapefruit possesses numerous other phytochemicals and micronutrients that have been shown to have various health benefits (Gao et al., 2006; Genser, 2008). The consumption of grapefruit juice has greatly declined in the last few years. Therefore, it would be of interest to produce FC-free fruit and juice or at least with substantially lower FC levels, at which FCs are too low to induce the interaction (Widmer, 2005).

Recently some approaches have been attempted to remove FCs from grapefruit juice based on the chemical and physical properties of FCs (Myung et al., 2008a, 2008b; Paine et al., 2006; Uesawa and Mohri, 2006a, 2006b). FCs could be removed by a series of chemical extractions and reconstitutions (Paine et al., 2006), inactivated by ultraviolet radiation (Uesawa and Mohri, 2006b), degraded by heat (Uesawa and Mohri, 2006a), or absorbed by autoclaved fungi (Myung et al., 2008a, 2008b). These additional treatments on grapefruit juice need extra cost and may compromise juice quality. Development of grapefruit cultivars with low or free of FCs is a genetic improvement priority and an ultimate solution to the GJE issue. Identification of such cultivars and understanding the FC inheritance are essential for marketing and breeding. Recent reports revealed not all grapefruit and pummelo cultivars contain high level of FCs (Widmer, 2005; Widmer and Haun, 2005); some selections and low acid mutants have undetectable (read zero) or little amount of the components. Currently, little is known about the genetic control of FC inheritance among the cultivars. In this initiative study, the change and amount of different FCs in selected FC-free grapefruit and pummelo cultivars and their hybrids were monitored. These FC profiles greatly facilitate understanding of the possible inheritance mechanism and therefore isolation of controlling genes and breeding of low or FC-free grapefruit cultivars in the near future.

Materials and Methods

Cultivars and hybrids. Three grapefruit cultivars (Foster, Low Acid Foster, and Hudson), one pummelo cultivar (Hirado Buntan), and 17 HBP × Hud hybrids were used in this study. Three fruit from each tree were picked four times, on 9 Dec. 2008, 9 Jan. 2009, 10 Feb. 2009, and 19 Mar. 2009. It is well known that most current commercial grapefruit cultivars are multigenerational seedling selections or chance mutants from one ancestor (Gmitter, 1995). Fos is the first pink-fleshed grapefruit cultivar, a bud sport from white-fleshed 'Walters',

Table 1. Diploid and triploid grapefruit/pummelo hybrids from 'Hirado Buntan' pummelo (HBP) × 'Hudson' grapefruit (Hud), 'Low Acid Pummelo' (LAP) × tetraploid 'Walters' grapefruit (TWG), HBP × 'Succari + HBP' [SHB (a somatic hybrid)], and LAP × SHB.

Hybrids	Ploidy	Cross	Hybrids	Ploidy	Cross
RR1T19B	2x	HBP × Hud	RR17T11B	3x	LAP × TWG
RR1T25B	2x	HBP × Hud	RR17T2B	3x	LAP × TWG
RR1T28B	2x	HBP × Hud	RR17T3B	3x	LAP × TWG
RR1T27B	2x	HBP × Hud	RR17T7B	3x	LAP × TWG
RR1T26B	2x	HBP × Hud	RR17T8B	3x	LAP × TWG
RR1T29B	2x	HBP × Hud	RR17T9B	3x	LAP × TWG
RR1T30B	2x	HBP × Hud	RR17T13B	3x	LAP × TWG
RR28T44A	2x	HBP × Hud	RR17T14B	3x	LAP × TWG
RR7T10A	2x	HBP × Hud	RR17T15B	3x	LAP × TWG
RR7T16A	2x	HBP × Hud	RR17T6B	3x	LAP × TWG
RR7T17A	2x	HBP × Hud	RR17T10B	3x	LAP × TWG
RR7T19A	2x	HBP × Hud	RR13T35B	3x	HBP × SHB
RR7T23A	2x	HBP × Hud	RR13T36B	3x	HBP × SHB
RR7T25A	2x	HBP × Hud	RR13T38B	3x	HBP × SHB
RR7T30A	2x	HBP × Hud	RR13T39B	3x	HBP × SHB
RR7T31A	2x	HBP × Hud	RR13T40B	3x	HBP × SHB
RR7T9A	2x	HBP × Hud	RR13T42B	3x	HBP × SHB
RR14T29B	3x	LAP × SHB	RR13T43B	3x	HBP × SHB
RR14T31B	3x	LAP × SHB	RR13T44B	3x	HBP × SHB
RR14T43B	3x	LAP × SHB	RR13T46B	3x	HBP × SHB
RR14T46B	3x	LAP × SHB	RR13T47B	3x	HBP × SHB
RR14T47B	3x	LAP × SHB	RR13T48B	3x	HBP × SHB
RR16T10B	3x	LAP × SHB	RR14T27B	3x	HBP × SHB
RR16T9B	3x	LAP × SHB	RR14T28B	3x	HBP × SHB

a seedling selection from white-fleshed 'Duncan' that is the first named grapefruit cultivar in Florida. Both LAF and Hud are bud sports from Fos. LAF features very low acid. Hud is the first red-fleshed grapefruit cultivar. Fos and Hud are believed to contain high FCs of the most potent interaction (GJE), and LAF and HBP low or little. HBP was used as female parent in a cross with Hud also because it is monoembryonic, thus enabling the recovery of true sexually derived hybrid offspring.

Additionally, 31 triploid hybrids from three populations used for selection of low FC (or FC-free) seedless grapefruit cultivars were also chosen to measure FC profiles, including 13 hybrids from HBP × SHB, 11 LAP × TWG, and seven LAP × SHB (Table 1). Only their pollen parents are tetrapoids. SHB stands for 'Succari + HBP', an allotetraploid somatic hybrid of 'Succari' acidless sweet orange (*C. sinensis*) cultivar with a seedling derived from the original HBP clone. The tetraploid fruit of SHB is somewhat similar to that of 'Duncan' grapefruit (Grosser and Gmitter, 2005, 2010; Grosser et al., 2000). LAP and TWG are the abbreviations for 'Low Acid Pummelo' and tetraploid 'Walters' grapefruit, respectively.

Juice preparation, high-performance liquid chromatography, and mass spectrometry assay. Fresh fruit juice was prepared by manually squeezing. FCs were identified and concentrations estimated in high-performance liquid chromatography by comparing their elution times, ultraviolet absorbance at 320 nm, and mass spectrometry data to authentic FCs, as previously described (Yu et al., 2009). Examined are seven FCs, 6,7-DHB, bergamottin, bergaptol, isoimperatorin, epoxybergamottin, 5′,8′-dimethylallyloxypsoralen, and paradisin C.

Statistical analyses. Most analysis and all plotting were performed using Excel (Office 2007; Microsoft, Redmond, WA), which has extra tools in the Data Analysis add-in besides built-in statistical functions. The SAS (Version 9.0; SAS Institute, Cary, NC) general linear model procedure was used to perform Duncan's multiple range test to determine statistical significance among FC derivatives, sampling dates, and parental and hybrid cultivars, respectively.

Results

Overall profiles of seven furanocoumarin derivatives. The concentration of each FC varied greatly among the individual cultivars and hybrids (Fig. 2A–H). Across all the samples, 6,7-DHB was detected from 0 to 106.60 mg·L^{-1}, bergamottin 0 to 62.10

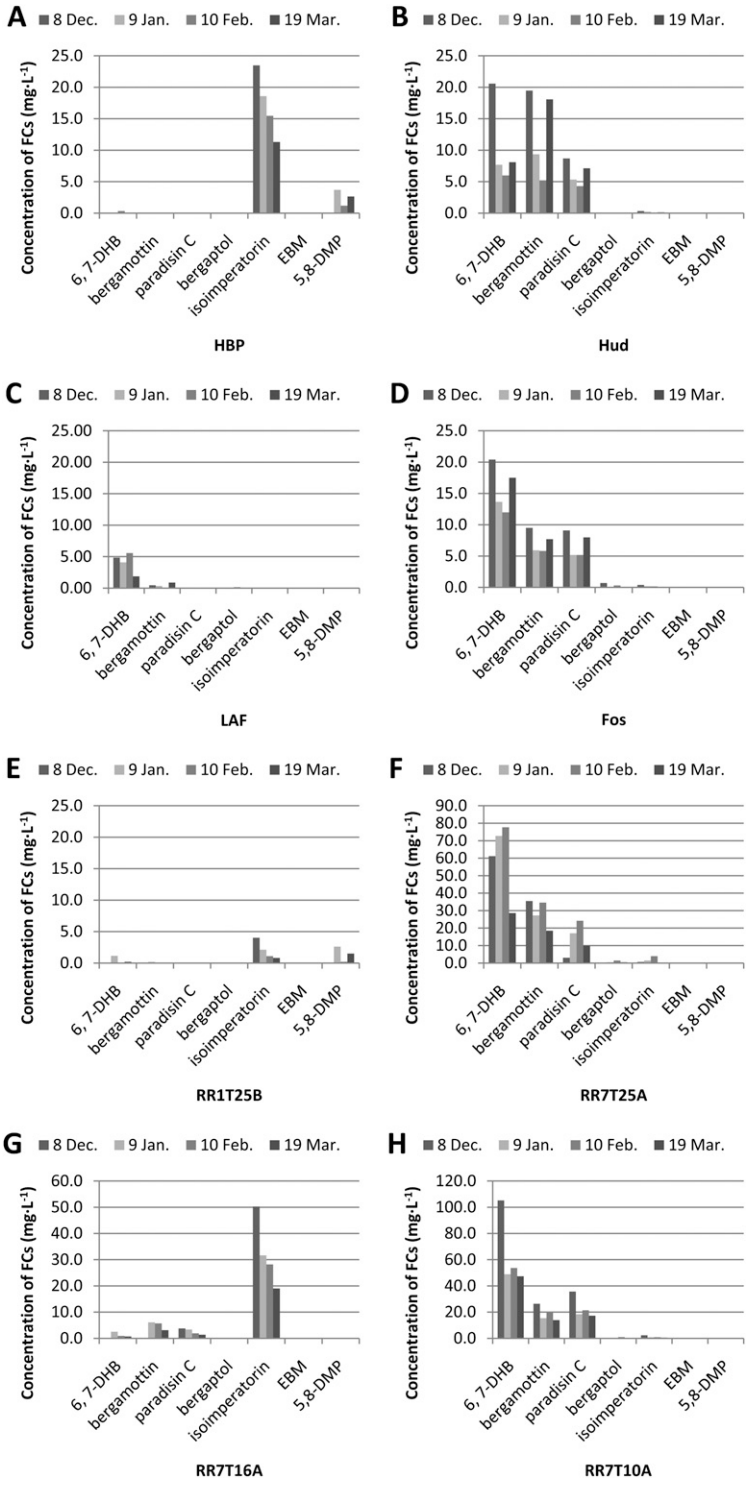

Fig. 2. The representative profiles of seven furanocoumarin (FC) derivatives in four grapefruit and pummelo cultivars (**A–D**): 'Hirado Buntan' pummelo (HBP), 'Hudson' grapefruit (Hud), 'Low Acid Foster' grapefruit (LAF), and 'Foster' grapefruit (Fos); and four HBP × Hud hybrids (**E–H**): RR1T25B, RR7T25A, RR7T16A, and RR7T10A. The fruit were picked on 4 d (8 Dec. 2008, 9 Jan. 2009, 10 Feb. 2009, and 19 Mar. 2009). HBP (**A**), LAF (**C**), RR1T25B (**E**), and RR7T16A (**G**) represented those cultivars and hybrids with low concentration of bergamottin, 6′,7′-dihydroxybergamottin (6,7-DHB), and paradisin C, whereas Hud (**B**), Fos (**D**), RR7T25A (**F**), and RR7T10A (**H**) with high concentration of these FCs. Isoimperatorin varied greatly among the samples. Bergaptol and 5′,8'-dimethylallyloxypsoralen (5,8-DMP) are extremely low or undetected in most samples and epoxybergamottin (EBM) not detected in all the samples. The concentration changes at the four harvest times were generally irregular although a similarly decreasing tendency of 6,7-DHB, bergamottin, and paradisin C was observed in some samples. The y-axis scales for the concentration of FCs in **F, G**, and **H** are different from each other, and A to E.

mg·L^{-1}, paradisin C 0 to 42.7 mg·L^{-1}, and isoimperatorin 0 to 50.3 mg·L^{-1}. Bergaptol was detected in many samples, but at extremely low concentrations (from 0 to 2.9 mg·L^{-1}). 5,8-DMP was detected very inconsistently, and no EBM was detected in any of the samples, so neither 5,8-DMP nor EBM was included in the statistical analysis or presentation. Among the seven FC derivatives measured, bergamottin, 6,7-DHB, and paradisin C were abundant in some cultivars (Figs. 2B and 2D) and hybrids (Figs. 2F and 2H) but low or undetectable in others (Figs. 2A, 2C, 2E, and 2G); the same was true of isoimperatorin. Seen from the individual samples, the concentrations of 6,7-DHB, bergamottin, and paradisin C changed slightly but were maintained at the detected level in most samples at the four harvest times. However, isoimperatorin appeared to constantly decrease in the period, up to almost none in some samples at the last sampling time. The average concentrations (Fig. 3) also reflected the same tendency as the individual concentration changes.

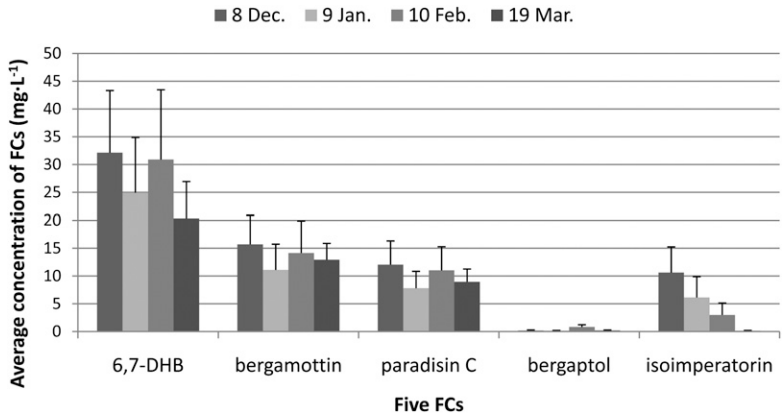

Fig. 3. The average concentrations (milligrams per liter of juice) of five different furanocoumarins (FCs) in all the 'Hirado Buntan' pummelo (HBP) × 'Hudson' grapefruit (Hud) hybrids. 6′,7′-dihydroxybergamottin (6,7-DHB), bergamottin, and paradisin C at high concentrations and bergaptol at low concentrations had similar slight changes at the four sample times, but isoimperatorin concentration was reduced consecutively. The bar above each column represents the SD.

As shown in Figure 2A–D, the concentrations of 6,7-DHB, bergamottin, and paradisin C were undetectable or extremely low in HBP (0.11, 0.00, and 0.00 mg·L^{-1}) and LAF (3.83, 0.40, and 0.00 mg·L^{-1}) compared with Hud (9.58, 13.03, and 6.11 mg·L^{-1}) and Fos (14.38, 6.48, and 6.11 mg·L^{-1}). The difference was not statistically significant between HBP and LAF or Hud and Fos but significant between either HBP or LAF and Hud or Fos ($P < 0.05$), implying that genetic segregation of the components may be observed in the hybrids from these low and high FC cultivars and indicating the possibility of selecting low FC or FC-free grapefruit cultivars.

To show overall patterns of five well-detected FC derivatives, the average concentrations and SDs of all the samples were calculated on the basis of the four sampling days (Fig. 3). The averages showed 6,7-DHB was the most abundant FC (27.07 mg·L^{-1}), bergamottin the second (13.47 mg·L^{-1}), and paradisin C the third (9.96 mg·L^{-1}), which is consistent with early reports that they were among most abundant FCs in grapefruit juice (Ohnishi et al., 2000; Row et al., 2006). Bergaptol and isoimperatorin were 0.34 and 4.98 mg·L^{-1}, respectively. Given relatively stable concentration levels during the four sampling times, 6,7-DHB, bergamottin, paradisin C, and bergaptol were likely accumulated up to their own peak concentrations before December and the levels exhibited very slow decrease rates in the coming months. However, isoimperatorin decreased consecutively and on average was almost undetectable at the last sampling time.

Strong correlation among 6′,7′-dihydroxybergamottin, bergamottin, and paradisin C. Correlation coefficients were calculated based on all the individual cultivars and hybrids, indicating certain positive correlations existed among 6,7-DHB, bergamottin, paradisin C, and bergaptol that each also had weak negative correlations against isoimperatorin (Table 2). The highest positive coefficient was between 6,7-DHB and bergamottin (0.909) and the lowest between paradisin C and bergaptol (0.433). It was worth noting that 6,7-DHB, bergamottin, and paradisin C were among the highest average concentrations and bergaptol the lowest (Fig. 3). Given bergaptol is one essential precursor and bergamottin (monomer), 6,7-DHB (modified monomer), and paradisin C (dimer) are the end FC products, such strong correlations among them might simply reflect their tight metabolic links in the bergamottin pathway.

Table 2. The correlation coefficients between any two of 6′,7′-dihydroxybergamottin (6,7-DHB), bergamottin, paradisin C, bergaptol, and isoimperatorin

	6,7-DHB	Bergamottin	Paradisin C	Bergaptol
Bergamottin	0.909			
Paradisin C	0.775	0.854		
Bergaptol	0.626	0.760	0.433	
Isoimperatorin	–0.433	–0.500	–0.453	–0.330

Cosegregation of 6′,7′-dihydroxybergamottin, bergamottin, and paradisin C in the hybrids. In Figure 4A–B, the concentrations of 6,7-DHB, bergamottin, and paradisin C are shown in all the diploid and triploid hybrids, where their strong statistical correlations can be more directly visualized. The three strongly correlated FCs appeared to cosegregate at an approximate 1:1 rate, according to the numbers of hybrids with low and high concentration FCs, particularly in the HBP × Hud diploid hybrids (Fig. 4A) and HBP × SHB triploid hybrids (Fig. 4B). The three FCs in eight HBP × Hud hybrids, RR7T17A, RR1T25B, RR7T19A, RR1T30B, RR1T28B, RR7T23A, RR7T16A, and RR1T29B, were undetectable or extremely low, like HBP, the maternal parent; in contrast, those in the other nine were as high or higher than Hud, the paternal parent. Similarly, the three FCs in seven HBP × SHB triploid hybrids were undetectable or extremely low, whereas those in the other six were high. A similar tendency was noted in the two other triploid hybrid families (LAP × TWG and LAP × SHB). From a genetic perspective, such cosegregation suggests that biosynthesis of all the three FCs arrayed in the same pathway is likely controlled by a single enzymatic or regulatory gene.

Discussion

Co-segregating 6′,7′-dihydroxybergamottin, bergamottin, and paradisin C may be a single gene-controlled trait. One primary goal for this study was to assess

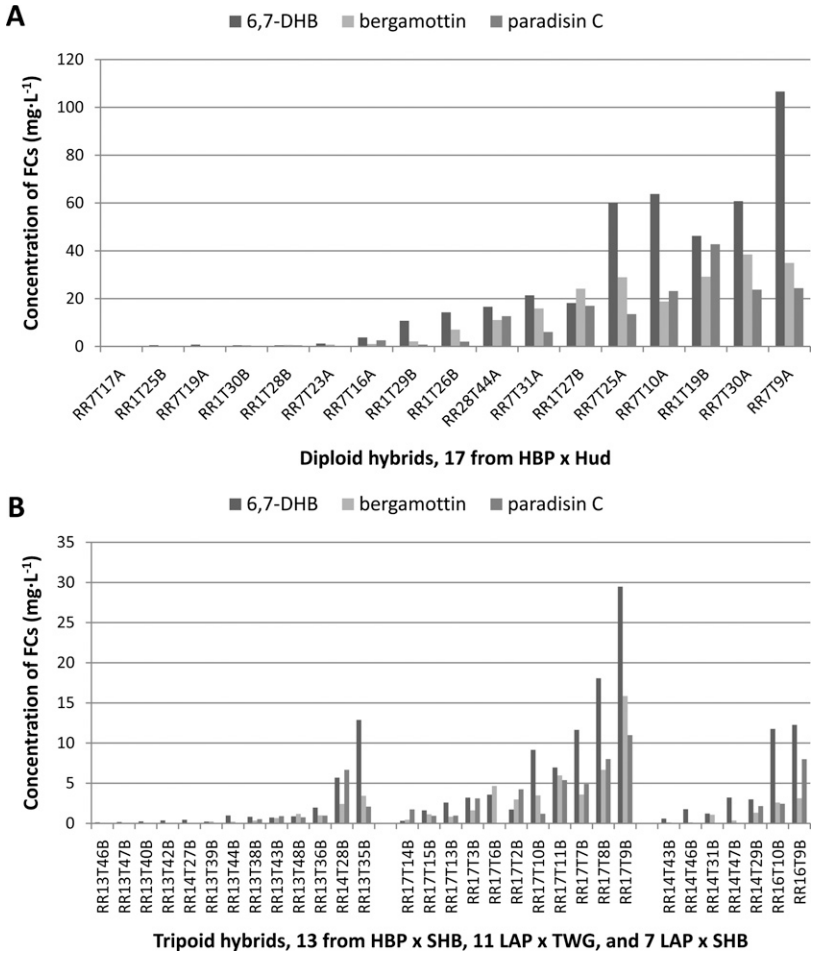

Fig. 4. The cosegregation of 6′,7′-dihydroxybergamottin (6,7-DHB), bergamottin, and paradisin C in the diploid (**A**) and triploid (**B**) hybrids. The three furanocoumarins (FCs) in half of them were undetectable or extremely low, like the maternal parents; and the other half were as high as or higher than the paternal parents, between which the ratio about was 1:1. HBP = 'Hirado Buntan' pummelo; Hud = 'Hudson' grapefruit; LAP = 'Low Acid Pummelo'; TWG = tetraploid 'Walters' grapefruit; SHB = 'Succari + HBP', a somatic hybrid.

the possible inheritance mode of these FCs (traits) in the randomly selected hybrids with low and high FCs from pummelo crossed with grapefruit. Interestingly, three FC components (traits), 6,7-DHB, bergamottin, and paradisin C, are strongly correlated (statistically) and obviously cosegregating (genetically). Although 48 hybrids may be too few to draw a solid conclusion supporting a single locus hypothesis, the approximate 1:1 segregation rate of the 48 randomly selected hybrids from four populations in the three FCs still encourages speculation that the traits are inherited in a simple Mendelian fashion. Support for this speculation is the result comparing Fos (featured high FC) with its low FC bud mutant, LAF. In many cases, such chance mutations usually result from on or off of a single functional or regulatory gene in a certain pathway (Sun et al., 2006). Given bergamottin, 6,7-DHB, and paradisin C are essential FC precursors or products, all of them are very likely controlled by one single gene involved in the biosynthetic pathway. Sampling more hybrids would help support the single gene hypothesis and potentially enable construction of a localized linkage map for the gene as well, if it is true.

Controlled points (genes) may be before bergaptol in the bergamottin biosynthesis pathway. Typically, the bergamottin biosynthesis pathway in plants undergoes multiple steps (Fig. 1), starting from dimethylallyl pyrophosphate, a product of the mevalonate pathway and the shikimate pathway. After the starting precursor, the chemicals synthesized in turn are umbelliferone, demethylsuberosin, marmesin, psoralen, bergaptol, bergapten, and finally bergamottin, and the involved enzymes prenyltransferase, marmesin synthase, psoralen synthase, psoralen 5-monoxygenase, and bergaptol O-methyltransferase to catalyze the reaction of each chemical (with or without other components) to produce the immediate next chemical in the pathway (Bourgaud et al., 2006; Larbat et al., 2007, 2009; Murray et al., 1982). In grapefruit, the most abundant FCs are bergamottin, 6,7-DHB, and paradisin C. Considering that the concentrations of all the three FCs are undetectable or extremely low in the low FC cultivars and hybrids, and that the mevalonate pathway plays a broad and complex role in plants, the gene controlling the FC synthesis flow might be in a point before the synthesis of bergaptol but after the mevalonate pathway and other pathways involved, which only provide some early precursors for the bergamottin biosynthesis pathway. Particular attention may be paid to these functional or regulatory genes in the pre-defined points during gene database mining or differential expression experiments to expedite the process of gene identification.

Toward selection of seedless furanocoumarin-free grapefruit cultivars. Strong correlation and cosegregation of 6,7-DHB, bergamottin, and paradisin C, the three FC components most abundant in many commercial grapefruit and most potent in juice–drug interaction, have led to a possibility to eliminate them through conventional hybridization. The diploid pummelo–grapefruit hybrids all produced seedy fruit; by contrast, as would be expected, the triploid hybrids yielded essentially seedless fruit. Among the randomly selected hybrids, a few were low in or free of the three FCs and also possessed excellent fruit and juice quality, from which some grapefruit alternative hybrids could be selected. It is significant to point out that the more commercially valuable seedless triploid hybrids also demonstrated the apparent segregation of high and low FC content. Further evaluation of these selected clones and extended screening of more triploid hybrids are underway toward selection of low FC or FC-free seedless grapefruit-like cultivars.

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