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Special Issue Article

Recombinant yeast as a functional tool for understanding bitterness and cucurbitacin biosynthesis in watermelon (*Citrullus* spp.)

Rachel Davidovich-Rikanati[1], Lior Shalev[1], Nadine Baranes[1], Ayala Meir[1], Maxim Itkin[2], Shahar Cohen[2], Kobi Zimbler[1], Vitaly Portnoy[1], Yutaka Ebizuka[3], Masaaki Shibuya[3], Yosef Burger[1], Nurit Katzir[1], Arthur A. Schaffer[2], Efraim Lewinsohn[1] and Ya'akov Tadmor[1]*

[1]*Institute of Plant Sciences, Newe Ya'ar Research Centre, Agricultural Research Organization, Ramat Yishay, Israel*
[2]*Institute of Plant Sciences, Volcani Centre, Agricultural Research Organization, Bet Dagan, Israel*
[3]*Graduate School of Pharmaceutical Sciences, University of Tokyo, Japan*

**Correspondence to:*
Y. Tadmor, Institute of Plant Sciences, Newe Ya'ar Research Centre, Agricultural Research Organization, PO Box 1021, Ramat Yishay 30095, Israel.
E-mail: tadmory@volcani.agri.gov.il

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Abstract

Cucurbitacins are a group of bitter-tasting oxygenated tetracyclic triterpenes that are produced in the family Cucurbitaceae and other plant families. The natural roles of cucurbitacins in plants are probably related to defence against pathogens and pests. Cucurbitadienol, a triterpene synthesized from oxidosqualene, is the first committed precursor to cucurbitacins produced by a specialized oxidosqualene cyclase termed cucurbitadienol synthase. We explored cucurbitacin accumulation in watermelon in relation to bitterness. Our findings show that cucurbitacins are accumulated in bitter-tasting watermelon, *Citrullus lanatus* var. *citroides*, as well as in their wild ancestor, *C. colocynthis*, but not in non-bitter commercial cultivars of sweet watermelon (*C. lanatus* var. *lanatus*). Molecular analysis of genes expressed in the roots of several watermelon accessions led to the isolation of three sequences (*Cc*CDS1, *Cc*CDS2 and *Cl*CDS1), all displaying high similarity to the pumpkin *CpCPQ*, encoding a protein previously shown to possess cucurbitadienol synthase activity. We utilized the *Saccharomyces cerevisiae* strain BY4743, heterozygous for lanosterol synthase, to probe for possible encoded cucurbitadienol synthase activity of the expressed watermelon sequences. Functional expression of the two sequences isolated from *C. colocynthis* (*CcCDS1* and *CcCDS2*) in yeast revealed that only *Cc*CDS2 possessed cucurbitadienol synthase activity, while *CcCDS1* did not display cucurbitadienol synthase activity in recombinant yeast. *ClCDS1* isolated from *C. lanatus* var. *lanatus* is almost identical to *CcCDS1*. Our results imply that *CcCDS2* plays a role in imparting bitterness to watermelon. Yeast has been an excellent diagnostic tool to determine the first committed step of cucurbitacin biosynthesis in watermelon. Copyright © 2014 John Wiley & Sons, Ltd.

Keywords: **cucurbitacins; cucurbitadienol synthase; bitterness; watermelon**

Introduction

Watermelon, *Citrullus lanatus* var. *lanatus*, a member of the family Cucurbitaceae, is one of the most popular fruit crops worldwide. More than 500 diploid cultivars have been developed in the USA alone during the last two centuries, but there is an ongoing need to improve watermelon, particularly with respect to the development of disease- and pest-resistant cultivars (Levi *et al.*, 2001). The species *Citrullus lanatus* includes the sweet cultivated watermelons (*C. lanatus* var. *lanatus*) and C. *lanatus* var. *citroides*, known as citron or preserving melon. The genus *Citrullus* Schrad. ex Eckl. & Zeyh

includes additional three diploid ($n = 11$) species: the desert gourd, *Citrullus colocynthis* (L.) Schrad., the perennial wild species *Citrullus ecirrhosus* Cogn (Whitaker and Davis, 1962; Jeffrey, 1975; Whitaker and Bemis, 1976) and the annual wild species *Citrullus rehmii* De Winter (De Winter, 1990). Among these, *C. lanatus* (Thunb.) Matsum & Nakai is the ancestor of cultivated watermelon (*C. lanatus* var. *lanatus*), while *C. colocynthis* is considered to be a wild ancestor of watermelon (Levi *et al.*, 2001) and is now found in the temperate regions of Africa, Central Asia and the Mediterranean (Jeffrey, 1975; Whitaker and Davis, 1962). *C. colocynthis* fruits are small, with a maximum diameter of 75 mm, their flesh is bitter and the seeds are small and brown. Attempts to improve watermelon quality through breeding programmes involving primitive germplasm often resulted in the reappearance of bitterness, attributed to the accumulation of cucurbitacins (Navot *et al.*, 1990).

Cucurbitacins are a group of highly substituted triterpenoids characterized by a common tetracyclic cucurbitane backbone (Chen *et al.*, 2005). They are produced by many different plant species, but primarily in the family Cucurbitaceae, to which cucumber (*Cucumis sativus* L.), melon (*Cucumis melo* L.) and pumpkin (*Cucurbita pepo* L. and *C. maxima* L.) belong. Cucurbitacins are generally bitter and toxic to many organisms and are thus considered to act as plant defence constituents (Tallamy *et al.*, 1997; Torkey *et al.*, 2009; Koul, 2008). Some cucurbitacins also have considerable pharmaceutical value, since they possess anti-cancer, anti-inflammatory and cytotoxic properties (Recio *et al.*, 2012; Chen *et al.*, 2005). In contrast to these positive attributes of cucurbitacins, there are also a few undesirable attributes, such as bitter taste and cytotoxicity, that have motivated the study of their biosynthesis in different cucurbit crops (Zhang *et al.*, 2013; Shibuya *et al.*, 2004; Navot *et al.*, 1990). Very little information is available concerning cucurbitacin accumulation in watermelon species and their contribution to bitterness. Most of the studies describe cucurbitacins composition in *C. colocynthis* (Lavie *et al.*, 1964; Hatam *et al.*, 1989; Gurudeeban *et al.*, 2010), but the relation to bitterness has never been studied in detail. Cucurbitacins are generally bitter in taste, thus their accumulation in the cultivated varieties was selected out during breeding (Navot *et al.*, 1990). The genetic basis for bitterness in watermelon fruit was investigated in a backcross generation resulting from hybridization between an interspecific F1 hybrid of *C. lanatus* and *C. colocynthis*. Bitterness of the fruit, a trait that characterizes the wild *C. colocynthis*, was found to be governed by a single dominant gene (*Bi*). However, cucurbitacin accumulation in relation to bitterness was not evaluated in this study (Navot *et al.*, 1990).

Triterpenoids are synthesized from mevalonic acid via the isoprenoid pathway, the origin of >20 000 different plant metabolites built up by condensation of isopentenyl pyrophosphate (IPP, C_5) oligomers (Thimmappa *et al.*, 2014). Triterpenoids include the membranal sterols, steroids and triterpenoid saponins, synthesized from IPP via the 30-carbon intermediates squalene and 2,3-oxidosqualene (Figure 1). The diversity of the different triterpenoid backbones is primarily determined by the particular cyclization of 2,3-oxidosqualene by different oxidosqualene cyclases (OSCs) (Phillips *et al.*, 2006). For example, cycloartenol in plants and lanosterol in yeast and mammals, which serve as the main precursors to the membrane sterols and steroid hormones, are produced by two different OSC enzymes, cycloartenol synthase and lanosterol synthase, respectively, and are relatively well characterized in many organisms (Thimmappa *et al.*, 2014). In parallel, cucurbitacin synthesis starts with the cyclization of 2,3-oxidosqualene to cucurbitadienol (Figure 1). Indeed, a sequence encoding for such enzyme activity has been isolated and characterized from *C. pepo* roots and named *CpCPQ* (Shibuya *et al.*, 2004). Cucurbitadienol is further metabolized to a variety of different cucurbitacins by subsequent hydroxylation, acetylation and glucosylation steps (Chen *et al.*, 2005). Despite the considerable interest in this important group of natural products, the enzymes, genes and biochemical pathways involved in cucurbitacin biosynthesis are largely uncharacterized. A more detailed understanding of these secondary metabolite pathways, and of the genes that are involved, would facilitate the development of plants with altered or novel cucurbitacin content and enable the tracking and manipulation of negative and positive attributes of the cucurbitacins through classical plant breeding programmes, or by genetic modifications.

Saccharomyces cerevisiae is a well-studied organism that serves in many applications of metabolic engineering (Frommer and Ninnemann, 1995). Sterol biosynthesis in yeast is well established,

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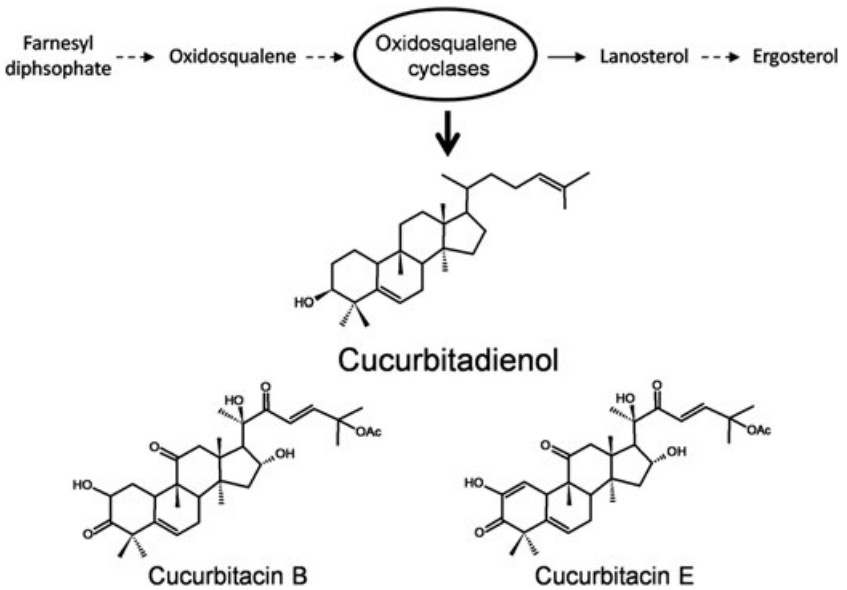

Figure 1. Cucurbitadienol biosynthesis in watermelon. Cucurbitadienol is a tetracyclic triterpene produced by a specific oxidosqualene cyclase from oxidosqualene. Cucurbitadienol is further metabolized to cucurbitacins with progressive hydroxylation, acylation and glucosylation reactions. Oxidosqualene is a common substrate to lanosterol *en route* to ergosterol biosynthesis in yeast

originating from the cytosolic terpenoid pathway via C15 farnesyl diphosphate, C30 squalene and oxidosqualene, converted to lanosterol and finally to the production of ergosterol (Corey *et al*., 1994; Shi *et al*., 1994; Karst and Lacroute, 1977). This pathway can be exploited for the production of other triterpenes by the expression of different members of the OSC family, and this concept has been used as a successful tool for the characterization of numerous OSC genes from plants (Corey *et al*., 1993; Hayashi *et al*., 2001; Husselstein-Muller *et al*., 2001; Kushiro *et al*., 1998).

Here we describe the utilization of a heterozygote yeast strain (BY4743) for lanosterol synthase for the characterization of two OSC members isolated from watermelon and their relation to bitterness in watermelon cultivars.

Materials and methods

Plant material

Citrullus accessions from three different species, as indicated in Table 1, were used in this study. They included cultivated sweet watermelon (*C. lanatus* var. *lanatus*; not bitter), cultivated preserving melon (*C. lanatus* var. *citroides*; bitter and non-bitter accessions) and wild colocynth (*C. colocynthis*; all bitter). Seeds were germinated in our local greenhouses in Newe-Ya'ar, Northern Israel. Young plantlets were then transferred to the open field, where they were grown under conventional agricultural conditions. Plants were open-pollinated by bees and were enabled to grow until full fruit maturation.

Bitterness evaluation

Mature fruits of the watermelon accessions shown in Table 1 were cut and tasted by two or three volunteers working in the Newe-Ya'ar Centre. Each sample was referred as bitter or non-bitter according to the sensorial evaluation of the panel.

Qualitative analysis of cucurbitacin content in watermelon cultivars using LC–TOF–MS

Mature fruits were collected, freeze-dried and ground with an Ultra-Turrax blender (Janke und Künkel, IKA Labortechnik). Tissue samples (0.3 g) were homogenized with 3 ml 80% MeOH, vortexed for 30 s and shaken for another 60 min

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Table 1. Summary of qualitative sensory analysis of bitterness and cucurbitacins* presence in watermelon leaf and fruit extracts

Cultivated and wild watermelon accessions	Bitterness	Cucurbitacins
C. lanatus var. *lanatus* 'Big Crimson'	Non-bitter	–
C. lanatus var. *lanatus* 'Au sweet Scarlet'	Non-bitter	–
C. lanatus var. *citroides* _PI 542114	Bitter	V
C. lanatus var. *citroides* _PI 482318	Non-bitter	–
C. lanatus var. *citroides*_PI 532666	Bitter	V
C. lanatus var. *citroides*_PI 221778_A	Non-bitter	–
C. lanatus var. *citroides*_PI 221778_B	Non-bitter	–
C. lanatus var. *citroides* _PI 220778_A	Bitter	V
C. lanatus var. *citroides*_PI 220778_B	Non-bitter	–
C. colocynthis 'Jourdan'	Bitter	V
C. colocynthis 'Hazeva'	Bitter	V

*Qualitative cucurbitacins distribution and bitterness evaluation in fruits of different watermelon cultivars originating from cultivated watermelon *C. lanatus* var. *lanatus*, C. *lanatus* var. *citroides* and the wild desert gourd *C. colocynthis*.
V, represents cucurbitacins presence.

on a vertical shaker, repeating the vortex every 10 min. Cell debris and particles were discarded by centrifugation (5000 × *g*) for 5 min; 1 ml of each sample was then filtered through Acrodisc® syringe filters with GHP membrane, 13 mm × 0.2 µm (PALL, USA), and transferred to vials for liquid chromatography time-of-flight- mass spectrometry (LC–TOF–MS) analysis, as described.

LC–TOF–MS analysis

Compounds were separated on a Zorbax Extend-C18 Rapid Resolution HT column with internal diameter of 2.1 X 50 mm length and particles size of 1.8 micron Agilent Technologies). The gradient elution mobile phase consisted of H_2O with 0.1% v/v formic acid (eluent A) and acetonitrile containing 0.1% v/v formic acid (eluent B). The column was equilibrated with 5% B at a flow rate of 0.3 ml/min for 1.5 min. Eluent B was then increased to 95% for 6 min, raised to 100% B for 12–15 min and restored to 5% by 16.5 min. The flow rate of the mobile phase was 0.3 ml/min and the column oven temperature was 40 °C.

Eluting compounds were subjected to a positive atmospheric pressure chemical ionization (APCI) source, with one sprayer for analytical flow and one for the reference compound (Agilent Technologies, Santa Clara, CA, USA). The APCI source was operated in positive mode, with the following settings: gas and vaporizer temperature, 350 °C; drying gas flow rate, 5 l/min; nebulizer set to 40 psig; VCap set to 3500 V; corona needle, 7 µA; and fragmentor, 140 V. Scan mode of the mass detector was applied (110–1000 *m/z*).

Cucurbitacin identification was done by comparison of retention time and exact mass spectrum of purchased cucurbitacin E and B standards (Sigma-Aldrich), and by the exact mass comparisons of cucurbitacin K, I and D and their potential glycosides.

Cloning of *OCS* genes from watermelon

The roots of 10 day-old seedlings were used for the extraction of RNA. The roots were gently washed with tap water, immediately frozen in liquid nitrogen and ground with a mortar and pestle. Total RNA was extracted from the powder using an Invisorb spin plant RNA mini kit (Invitek GmbH, Berlin, Germany) with RP lysis buffer, following the manufacturer's protocols. The RNA was then used for cDNA synthesis with a Verso cDNA Synthesis Kit (Thermo Scientific, USA), following the manufacturer's protocols. The resulting cDNA mixture served directly as a template for the following PCRs. Degenerate primers, designated C and E (see Table 2), were designed from the consensus sequences of the known pumpkin cucurbitadienol synthase (*CpCPQ*) and other plant OSCs with proven functionality. In addition, a set of full-length primers (A and B) were designed, based on the *CpCPQ* sequence, which yielded satisfactory results. The PCR was performed using Extensor Hi-Fidelity PCR Enzyme Mix (Thermo Scientific) on a Mastercycler gradient apparatus (Eppendorf, Germany), using the following basic programme: initial denaturation of 3 min at 94 °C, following

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Table 2. Primer sequences used for the isolation of CDS from *Citrullus* varieties

A	CPQFOR	ATGTGGAGGCTGAAGGTGG
B	CPQREV	TCATTCAGTAAGAACCCGATGG
C	OSCDEG1F	GGGAGTTTATGAATGGTCTGGAAAYAA YCC NYT
E	OSCDEG3R	CCAAAAGTTTCAGCTGGATTCATArdyTCnArCCA
Race S1	106	TGC TTT TGT CAT TGC CCC G
Race S2	180	CCA ATT CCC ATC GGG TTC T
Race S3	128	ATG GGC CCT CCA AGA TCC GA

25 cycles of 30 s at 94 °C, 1 min at 55 °C and 2 min at 68 °C; the reaction was ended with an additional 20 min at 68 °C; annealing temperature was optimized between 50–62 °C for each primer set. Products of the expected sizes were subcloned into pTZ57R/T vector (Thermo Scientific), using the T/A cloning protocol, and transformed in *Escherichia coli* strain DH5*α*. Positive colonies were picked for plasmid isolation and nucleotide sequencing.

About 40 clones harbouring 800 bp sequences obtained by using the degenerate primers set C and E on *C. colocynthis* and *C. lanatus* cDNA were analysed. All sequences were found to be closely related to *CpCPQ* from pumpkin, dispaying 93% identity. Two separate sequences were isolated from *C. colocynthis*, named *CcCDS1* and *CcCDS2*, while only one sequence was found in *C. lanatus* and was named *ClCDS1*. To obtain the full-length sequences, we first tried to use a set of primers based on the pumpkin *CpCPQ* full-length sequence (A and B). This strategy resulted in obtaining the full-length of *CcCDS1* and a 2000 bp sequence of *CcCDS2* and *ClCDS1*, both missing the 5′ end. As *CcCDS1* and *ClCDS1* were almost identical (99% identity, with only two amino acids difference), we elongated *CcCDS2* by RACE (FirstChoice® RLM-RACE Kit, Ambion, USA) using specific primers sets s1–s3 (Table 2). The full-length *CcCDS2* clone was then synthesized (Gen9Bio, Cambridge, MA, USA), according to the full-length sequence obtained by the RACE method.

Finally, each of the 2.3 kb clones was digested with appropriate restriction enzymes and ligated into the corresponding sites of yeast expression vector pYES2 (Invitrogen) under the control of the GAL1 promoter. The constructs were transformed into DH5*α*-competent cells and plasmid DNAs were prepared and used to transform the yeast strain BY4743_YHR072 (MAT**a**/*α* his3*Δ*1/his3*Δ*1 leu2*Δ*0/leu2*Δ*0 LYS2/lys2*Δ*0 met15*Δ*0/MET15 ura3*Δ*0/ura3*Δ*0 kanMax::erg7/ERG7), obtained from Thermo by the TRAFO method (Gietz and Woods, 2002).

Heterologous expression of cloned genes in recombinant yeast

Single transformed yeast colonies were incubated overnight in 3 ml synthetic complete medium without uracil at 30 °C and 220 rpm, and were then used for the inoculation of 20 ml medium. After 2% w/v galactose induction for 2 days, cells were collected and resuspended in 20 ml 0.1 m potassium phosphate buffer. The cells were collected by centrifugation and the pellet was disrupted with 2.5 ml hot 20% w/v KOH and 50% v/v EtOH and extracted twice with a similar volume of *n*-hexane. The hexane extract was evaporated and resuspended in 1 ml MeOH for analysis of the cucurbitadienol product, using a LC–TOF–APCIMS (Agilent) instrument (as described above).

Results

Cucurbitacins content in bitter and sweet watermelon fruits

Limited information is available concerning cucurbitacin composition and content in watermelon cultivars and their correlation to bitterness. According to previous reports (Gurudeeban *et al*., 2010), the main cucurbitacins found in watermelon are cucurbitacins B and E and their related glycosides (Figure 1). To investigate a possible relationship between fruit bitterness and cucurbitacins accumulation in the accessions under study, we analysed the MeOH-soluble extracts of watermelon fruit and leaf samples in parallel to organoleptic evaluation for bitterness of the corresponding fruits. The extracts were subjected to LC–TOF–MS analysis; representative chromatograms are shown in Figure 2 and summarized in Table 1. All accessions of the cultivated *C. lanatus* var. *lanatus* were non-bitter, as well as most of the accessions of *C. lanatus* var. *citroides*; in

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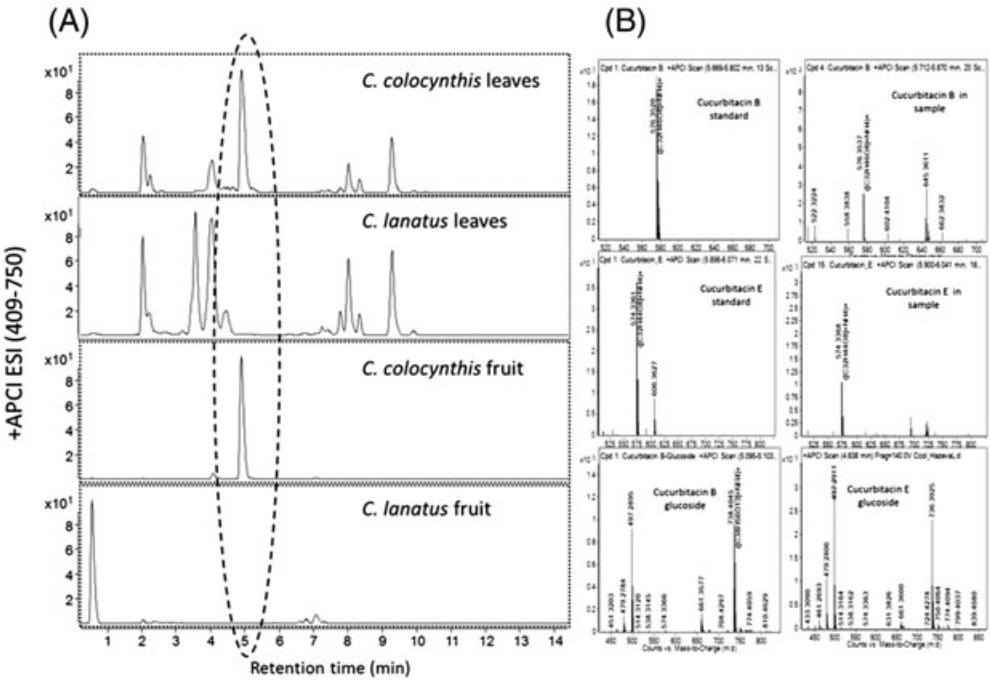

(C)

Compound name	Chemical formula	RT	Masses found
Cucurbitacin D-Glu	C36H54O12	3.8	499.305 M+H-Glu-H_2O 696.39 M+NH_4
Cucurbitacin I-Glu	C36H52O12	4.15, 4.3	497.290 M+H-Glu-H_2O 515.3004 M+H-Glu 694.3799 M+NH_4
Cucurbitacin E-Glu	C38H54O13	4.9	736.3907 M+NH_4+ 719.3697 M+H 557.31 M+H-Glu 574.337 M+H-Glu
Cucurbitacin B-Glu	C38H56O13	5.1	738.4045 M+NH_4+ 721.377 M+H 576.3551 M+H-Glu
*Cucurbitacin B	C32H46O8	5.7	576.3520 M+NH_4+ 541.3166 M+H-H_2O
*Cucurbitacin E	C32H44O8	5.95	574.3365 M+NH_4+ 539.3020 M+H-H_2O

Figure 2. LC–MS analysis of wild and cultivated watermelon extracts. (A) Extracted ion chromatograms of ions 409–750 Da, the representative range of ions for cucurbitacins and their glycosides. (B) Mass spectra of the main cucurbitacins in watermelon. (C) Exact masses and retention times of cucurbitacins found in bitter watermelon accessions

contrast, all of the tested accessions of *C. colocynthis* were bitter. LC–MS analysis of the fruits revealed that all the bitter accessions accumulated cucurbitacins E and B. The compounds were identified by comparison to their calculated exact mass and by identity of elution patterns and exact masses of commercially available authentic standards (see Figure 2, Table 1). In addition to these two cucurbitacins, we could detect their glycosides and additional cucurbitacins, such as cucurbitacin I, K and D, that were identified according to their exact mass (identification data summarized in Figure 2B, C). None of these cucurbitacins were detected in the non-bitter accessions of *C. citroides* and *C. lanatus*. Moreover,

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none of the non-bitter accessions accumulated cucurbitacins at detectable levels. These findings strengthen our hypothesis that the bitterness perceived in fruits of the watermelon accessions is related to the presence of cucurbitacins, both free and as glycosylated derivatives.

Following these findings, we turned to investigating the molecular and biochemical mechanisms involved in cucurbitacins accumulation in bitter watermelon, using recombinant yeast as a tool. We first aimed to identify the gene encoding the first step in the biosynthesis of cucurbitacins, an oxidosqualene cyclase that is able to convert 2,3-oxidosqualene to cucurbitadienol, the triterpenoid backbone of cucurbitacins (Figure 1).

Cloning of putative cucurbitadienol synthase from watermelon

To isolate the oxidosqualene cyclase involved in cucurbitadienol synthesis from watermelon, and to investigate the molecular differences between the bitter and non-bitter accessions, degenerate primers were designed based on the consensus sequences of known OSCs, including the characterized cucurbitadienol synthase from pumpkin roots. The clones originating from a bitter *C. colocynthis* accession included two closely related sequences (99% identity), which we designated *CcCDS1* and *CcCDS2*. Both sequences have 93% sequence identity to the pumpkin cucurbitadienol synthase (*CpCPQ*). The clones that originated from the non-bitter *C. lanatus* var. *lanatus* roots consisted only of a single sequence, designated *ClCDS1*, almost identical (99% nucleotide identity, with only two different amino acids) to *CcCDS1* and similarly having 93% sequence identity to the pumpkin cucurbitadienol synthase *CpCPQ*. The relationship between these three sequences with the pumpkin sequence is illustrated in the phylogenetic tree shown in Figure 3A. Based on the sequences of these fragments, and on subsequent PCR amplifications using a set of specific primers based on the full-length pumpkin cucurbitadienol synthase, we obtained the full-length clones *CcCDS1* and *CcCDS2*. Figure 3B depicts the encoded protein sequences of all the clones, together with pumpkin cucurbitadienol synthase (*CpCPQ*). Comparison of the protein sequences of the three clones to the functionally characterized pumpkin cucurbitadienol synthase reveals 10 amino acids that differentiate the three watermelon sequences. Some of these amino acids (at positions 211, 308, 595 and 599) are common to *Cc*CDS2 and *Cp*CPQ but are different in *Cc*CDS1 and *Cl*CDS1 (as shown in Figure 3C).

Heterologous expression of *CcCDS1* and *CcCDS2* in yeast

To further test the possible involvement of *CcCDS1* and *CcCDS2* in cucurbitadienol biosynthesis and to test their functionality, both clones were heterologously expressed in recombinant yeast and the products obtained were analysed by LC–TOF–MS. Both sequences were cloned in the pYES2 expression system downstream of the galactose-inducible GAL1 promoter, using appropriate restriction endonuclease sites. The resulting plasmids were transferred to *S. cerevisiae* strain BY4743_YHR072 (MAT**a**/α his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 LYS2/lys2Δ0 met15Δ0/MET15 ura3Δ0/ura3Δ0 kanMax::erg7/ERG7) originating from the yeast deletion project collection (Brachmann *et al*., 1998) that is heterozygous for lanosterol synthase, Erg7 (Corey *et al*., 1994). In parallel, we also transformed a construct harbouring the previously characterized *CpCPQ* to use as a positive control and as a source of authentic cucurbitadienol to serve as a standard. Transformed yeast were cultured and the GAL1 promoter was induced by replacing the glucose carbon source by galactose and extracting as described in Materials and methods. The resulting cell extracts were subjected to LC–TOF–MS analysis, using APCI interphase. The extracted ion chromatograms of the transformed yeast extracts are shown in Figure 4. In all cases, a compound identified as lanosterol eluted at 13.4 min. This is likely produced by the endogenous yeast lanosterol synthase and indicates that yeast produce 2,3-oxidosqualene. Notably, extracts derived from yeast expressing *CcCDS*2 displayed an additional peak, eluting at 12.8 min and possessing an extracted mass spectrum of *m/z* 427.393 as $(M+H)^+$ and *m/z* 409.382 as $(M+H-H_2O)^+$ in APCIMS. Both the elution volume and the exact mass spectrum are identical to those of the cucurbitadienol standard. These findings indicate that *CcCDS2* encodes an active oxidosqualene cyclase that is able to produce cucurbitadienol. As expected,

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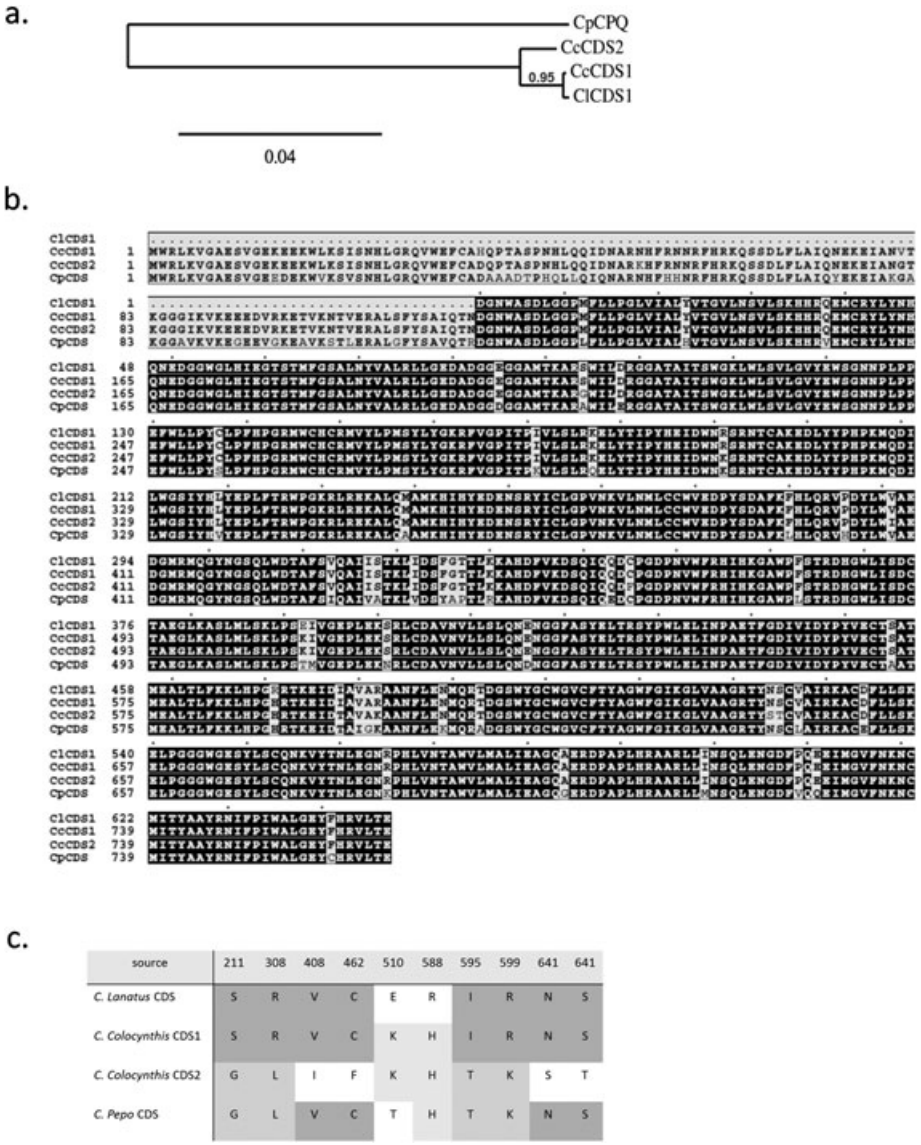

Figure 3. Nucleotide and encoded protein comparison of the three isolated CDS genes from cultivated and wild watermelon. (A) Phylogenetic tree deduced from the nucleotide sequences isolated from *Citrullus lanatus ClCDS1* and *Citrullus colocynthis CcCDS1* and *CcCDS2*, compared to the previously characterized cucurbitadienol synthase from *Cucurbita pepo*, *CpCPQ*. (B) Multiple alignment of deduced amino acid sequences of cucurbitadienol synthase homologues with (C) the representation of differentiating amino acids. GenBank Accession Nos ClCDS1_KM821403, CcCDS1_KM821404, CcCDS2_KM821405 and CpCPQ_ Q6BE24

yeasts expressing the *CpCPQ* gene, used as positive controls, also accumulated cucurbitadienol at high levels, as noted previously (Shibuya *et al*., 2004). Yeast harbouring empty vector controls did not accumulate cucurbitadienol at detectable levels. Similarly, yeast extracts harbouring *CcCDS1* did not show any additional compounds (besides lanosterol), indicating that this clone was not active as a cucurbitadienol synthase. Further analyses of single and total ion chromatograms indicated that no novel triterpenes were formed in the recombinant yeast (supplemental figure 1).

Discussion

To better understand the correlation between cucurbitacins accumulation and bitterness in different

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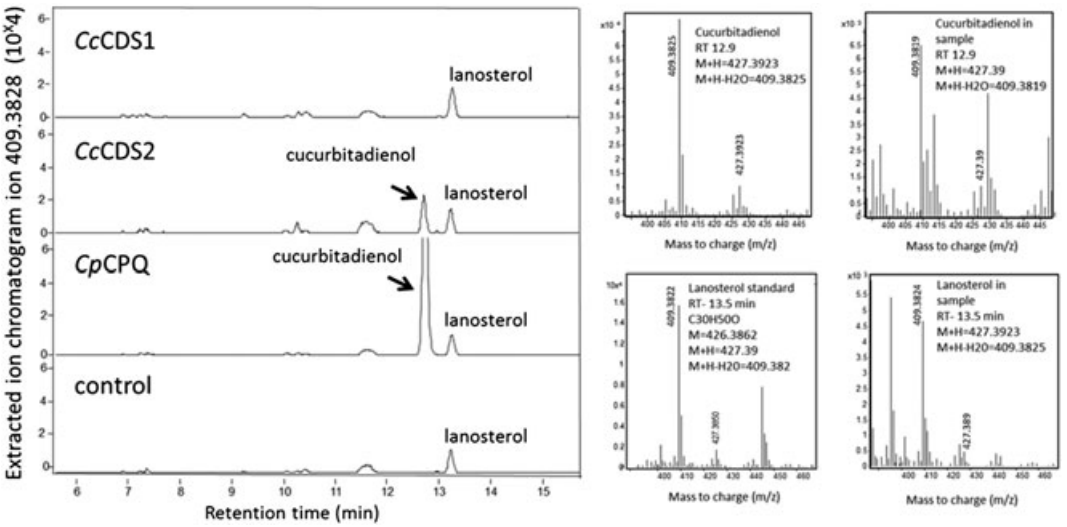

Figure 4. LC–MS analysis of products of watermelon CDS clones that were heterologously expressed in yeast. The extracted ion chromatograms of ion *m/z* = 409.38, the representative ion for cucurbitadienol (and other triterpenes with molecular formula of $C_{30}H_{50}O$, such as lanosterol) is shown for each extract of yeast harbouring *CcCDS1*, *CcCDS2* from *C. colocynthis*, in comparison to yeast extracts harbouring the previously characterized pumpkin *CpCPQ* producing cucurbitadienol (Shibuya *et al*., 2004). A chromatogram from yeast harbouring an empty vector is shown as a negative control (bottom panel). Mass spectra of cucurbitadienol and lanosterol are shown in the left panels

watermelon cultivars, we first conducted a qualitative analysis of cucurbitacins in the different cultivars. In parallel, samples were tasted and evaluated for bitterness. Our findings clearly show that bitter watermelon types accumulated cucurbitacins in fruits, whilst no cucurbitacins were detected in the non-bitter fruits. These findings are in agreement with previous reports (Gurudeeban *et al*., 2010; Torkey *et al*., 2009; Hatam *et al*., 1989; Lavie *et al*., 1964; Gamlath *et al*., 1988) indicating the presence of cucurbitacins E, B, I, J, L and their glycosides in wild bitter desert gourd (*C. colocynthis*). Following our hypothesis that bitterness is correlated to cucurbitacins accumulation in watermelon fruits, we elucidated a possible molecular and biochemical mechanism that likely controls cucurbitacin biosynthesis in watermelon. The first dedicated gene in cucurbitacin biosynthesis is cucurbitadienol synthase (CDS), which catalyses the first committed step in cucurbitacin biosynthesis, i.e. the formation of the cucurbitadienol skeleton (Shibuya *et al*., 2004). The first cucurbitadienol synthase gene was isolated from pumpkin and functionally characterized by Shibuya *et al*. (2004). The pumpkin gene was isolated from roots and its role in fruit bitterness has not been assessed. Degenerate primers based on this gene and other members of the OSC family led us to the isolation of three closely related sequences, designated *ClCDS1*, *CcCDS1* and *CcCDS2*, all having the highest homology to the already characterized pumpkin cucurbitadienol synthase. *CcCDS1* is almost identical to *ClCDS1*, with only two differing amino acids at positions 510 and 588. *CcCDS2* had eight different amino acids compared to *ClCDS1* and *CcCDS1*, while four of these amino acids were common to *CpCPQ*. We therefore turned to the heterologous expression of *CcCDS1* and *CcCDS2* in yeast, and explored their functionality in cucurbitadienol synthesis. Our analysis showed that only *CcCDS2* was active and produced cucurbitadienol, while *CcCDS1* did not produce any novel triterpenes in yeast. We cannot exclude the possibility that the lack of activity of *CcCDS1* is due to unforeseen conditions during the functional expression experiments. Still, our results clearly show that *CcCDS2* encodes a protein with the ability to mediate the production of cucurbitadienol. Our findings suggest that cucurbitadienol is produced in *C. colocynthis* as it expresses an active CDS, while the *lanatus* cultivars have lost the ability to produce cucurbitacins as a result of losing the active copy of CDS and the ability to convert oxidosqualene to cucurbitadienol. Deletion of gene sequences is a rare evolutionary event accompanying domestication, but it was recently reported that

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several disease-resistance genes that exist in *C. colocynthis* were similarly lost during domestication and that they are presently absent from *C. lanatus* var. *lanatus* (Levi, 2014). To further establish our hypothesis that non-bitter watermelon results from the absence of active CDS, a further exploration of the genes expressed in bitter vs non-bitter cultivars is needed.

The effect of numerous changes in amino acids sequences on several OSC catalytical functions has been demonstrated previously (Hart *et al*., 1999; Herrera *et al*., 2000; Lodeiro *et al*., 2004). Mutational analyses have shown that Tyr410, His477 and Ile481 cooperate to control product specificity in *Arabidopsis* cycloartenol synthase (CAS1), and that CAS1 can be converted to an accurate lanosterol synthase with only two amino acid substitutions: His477 to Asn and Ile481 to Val (Lodeiro *et al*., 2005). A similar attempt to change the *CpCPQ* product specificity by the substitution of the 488th leucine and/or the 489th isoleucine did not result in any change (Shibuya *et al*., 2004). There are four amino acids that discriminate between the active *Cc*CDS2 and the non-active *Cc*CDS1 and are also common to the amino acids of the active *Cp*CPQ; however, all of them fall out of the known functional sites of the protein. To understand their true influence on functionality, a deeper investigation is needed, including intended amino acid changes along with a comprehensive three-dimensional modelling of the encoded protein compared to functional tests as described above.

The bitterness of cucumber fruit and foliage is also believed to occur due to the presence of cucurbitacins. The inheritance of cucumber bitterness was studied and located using a recombinant inbred lines (RILs) population (Miao *et al*., 2011; Zhang *et al*., 2013). These inheritance analyses concluded that there were two loci controlling fruit bitterness in the population, one locus, *bi* (*bi-1*), making fruit and foliage bitter-free and *Bt* (*Bt-1*) making the fruit highly bitter. An additional locus was also identified, *bi-3*. The *bi-1* locus was positioned on chromosome 6 in a region spanning 160 predicted genes. The locus of *bi-3* was placed on chromosome 5 and limited to a region with nearly 200 predicted genes. Among the160 predicted genes for *bi-1*, an oxidosqualene cyclase (named Csa008595) was found with highest similarity to cucurbitadienol cyclase (Zhang *et al.*, 2013). Interestingly, all of the amino acid polymorphism that differentiated the active *Cc*CDS2 and the non-active *Cc*CDS1 were identical to the putative cucumber sequence (*Csa*008595). The genetic basis of bitterness of watermelon was also investigated in a backcross population resulting from hybridization between an interspecific F1, hybrid of *C. lanatus* and *C. colocynthis*, with the cultivated parent *C. lanatus*. In this case, the characteristic bitterness of wild *C. colocynthis*, was found to be governed by a single dominant gene (*Bi*) linked to the isozyme marker *Pgm-1* (Navot *et al*., 1990). Further investigation needs to be carried out in order to determine the relationship of watermelon *Bi* to *CcCDS*.

Yeast (*Saccharomyces cerevisiae*) offers a low-cost and efficient way to express heterologous genes involved in triterpenoid biosynthesis of plants (Frommer and Ninnemann, 1995; Moses *et al*., 2014; Shibuya *et al*., 2006; Haralampidis *et al*., 2001; Kim *et al*., 2009; Hayashi *et al*., 2001). The yeast sterol biosynthesis pathway, leading mainly to the production of ergosterol, has been successfully used to provide substrate for many heterologously expressed genes from the OSC family. Oxidosqualene availability can be further enhanced by the complete shut-down of its endogenous consumption by using lanosterol synthase-deficient mutant strains, such as GIL77 and SMY8, that accumulate 2,3-oxidosqualene, thus favouring the synthesis of novel 2,3-oxidosqualene cyclization products by heterologous expression of oxidosqualene cyclases (Gollub *et al*., 1977; Kushiro *et al*., 1998; Shibuya *et al*., 2004, 2006; Hayashi *et al*., 2001). However, the complete lanosterol synthase knockout strains are difficult to handle and grow. They usually possess an additional mutation in haemoglobin biosynthesis genes to allow ergosterol uptake from medium. Therefore, in addition to their marker amino acids, hemin, Tween80 and ergosterol are added to the growing media. Thus, they are relatively slow growing and have fewer options for selection markers (Gollub *et al.*, 1977; Kushiro *et al*., 1998; Shibuya *et al*., 2004, 2006; Hayashi *et al*., 2001). In this study we chose to use the yeast knockout BY4743 strain, heterozygous for the lanosterol synthase gene knockout, maintaining the ability to actively grow without any external supplementations. We found that oxidosqualene production is adequate for the production of heterologously produced triterpenes, as demonstrated with the already characterized *Cp*CPQ, and we further exploited this to the functional analysis of *CcCDS2* genes isolated from desert gourd (*C. colocinthis*). In addition, this

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strain can provide additional selection markers for the discovery of genes involved in cucurbitadienol hydroxylation, glucosylation and acylation by the co-expression of CDS with the putative genes. This approach has already been successfully used for the discovery of several cytochrome p450s and glucosyltransferases involved in plant triterpene modifications, such as sapogenins and glycirrhizin (Fukushima *et al*., 2013; Moses *et al*., 2014; Seki *et al*., 2008). Once the full biosynthetic pathways to cucurbitacins are solved, yeast systems such as the one described here can also serve as platforms for the large-scale production of specified compounds.

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Supporting information

Additional supporting information may be found in the online version of this article at the publisher's web site:

Figure S1. LC–MS analysis of products of watermelon CDS clones that were heterologously expressed in yeast

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