Noemi Controls Production of Flavonoid Pigments and Fruit Acidity and Illustrates the Domestication Routes of Modern Citrus Varieties

Graphical Abstract

Highlights

- Noemi is essential for the production of flavonoid pigments in citrus
- Noemi is essential for the regulation of fruit acidity in citrus
- Retrotransposons are associated with the acidless phenotype in commercial varieties
- A specific ancient mutation retraces the steps of citrus history and cultivation

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In Brief

In some varieties of citrus, exceptionally low fruit acidity is associated with absence of anthocyanin pigments in leaves and flowers and proanthocyanidins in seeds. Taking advantage of natural variation, Butelli et al. show that this pleiotropic phenotype is the result of mutations in a single gene, Noemi, encoding a bHLH transcription factor.

Butelli et al., 2019, Current Biology 29, 158–164
January 7, 2019 © 2018 The Author(s). Published by Elsevier Ltd.
https://doi.org/10.1016/j.cub.2018.11.040
Noemi Controls Production of Flavonoid Pigments and Fruit Acidity and Illustrates the Domestication Routes of Modern Citrus Varieties

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SUMMARY

In citrus, the production of anthocyanin pigments requires the activity of the transcriptional activator Ruby. Consequently, loss-of-function mutations in Ruby result in an anthocyaninless phenotype [1]. Several citrus accessions, however, have lost the ability to produce these pigments despite the presence of wild-type Ruby alleles. These specific mutants have captivated the interest of botanists and breeders for centuries because the lack of anthocyanins in young leaves and flowers is also associated with a lack of proanthocyanidins in seeds and, most notably, with an extreme reduction in fruit acidity (involving about a three-unit change in pH). These mutants have been defined collectively as “acidless” [2–4]. We have identified Noemi, which encodes a basic helix-loop-helix (bHLH) transcription factor and which controls these apparently unrelated processes. In accessions of Citron, limetta, sweet lime, and sweet orange, acidless phenotypes are associated with large deletions or insertions of retrotransposons in the Noemi gene. In two accessions of limetta, a change in the core promoter region of Noemi is associated with reduced expression and increased pH of juice, indicating that Noemi is a major determinant of fruit acidity. The characterization of the Noemi locus in a number of varieties of Citron indicates that one specific mutation is ancient. The presence of this allele in Chinese fingered Citrons and in those used in the Sukkot Jewish ritual [5] illuminates the path of domestication of Citron, the first citrus species to be cultivated in the Mediterranean. This allele has been inherited in Citron-derived hybrids with long histories of cultivation.

RESULTS AND DISCUSSION

Citrus is a complex group of flowering plants with notable nutritional, medicinal, and aromatic value. Citrus became one of the world’s most economically important fruit crops through a largely obscure history of evolution and domestication. The genus originated in a wide area spanning North-Eastern India to South China and South-East Asia and is thought to have been cultivated for at least 3,300 years [6–8].

Within citrus, the existence of a “syngameon”—a group of genetically related organisms linked by interspecific hybridization [9]—is more appropriate than distinguishing individual species, since marked sexual compatibility between species has generated hundreds of citrus varieties, often with broad morphological diversity [10]. However, genetic studies have identified Pummelo (C. maxima), Citron (C. medica), and Mandarin (C. reticulata) as the fundamental or primary species [11, 12] (Figure 1), with a fourth species from Papeda (C. micrantha) involved in the origin of some limes [13]. Other commercially important citrus are the result of hybridization among these four primary species [14]. We have used anthocyanin production to facilitate our understanding of the taxonomy and domestication of this important group of fruit crops. Among primary species, Citron produces anthocyanins naturally in its leaves and young flower buds, whereas most Pummelo and all Mandarin accessions do not, due to mutations affecting the expression or activity of the Ruby gene, which encodes an R2R3MYB transcription factor [1, 15]. The ability of hybrids to make anthocyanins depends on the functionality of the Ruby alleles inherited from their parental species [1]. The hybrids are able to maintain their unique genetic composition because they are propagated clonally, either by apomictic seeds or by grafting.

While almost all anthocyanin production in citrus can be explained by variations in the activity of the Ruby gene, one specific subset of mutants has lost anthocyanin pigmentation despite the presence of wild-type Ruby alleles. This group of anthocyaninless variants is characterized by two accompanying traits: the
absence of proanthocyanidins in the seeds (apparent phenotypically as the absence of the “chalazal spot,” a characteristically colored round area on the inner seed coat) and fruit with juice almost completely devoid of acidity [2–4]. The invariant combination of these three traits (low fruit acidity, white flowers and green leaves, seeds of light cream color) defines the so-called “acidless” phenotype (Figure 2).

Acidless varieties are often defined as “sweet,” reflecting their insipid flavor and the sharp increase in the sugar-acid ratio in their fruit, which is the most important determinant of fruit quality and taste in citrus. The multifaceted anthocyaninless, proanthocyanindin-less, acidless phenotype is likely to be the result of mutations in a single gene. From 33 citrus varieties completely unable to produce anthocyanins, acidless varieties are the only accessions containing functional alleles of *Ruby*, a key regulatory MYB gene essential for anthocyanin production [1], suggesting strongly for pleiotropic effects of mutations in a single gene.

In angiosperms, anthocyanin biosynthesis is regulated by conserved MYB/bHLH/WD40 (MBW) complexes, formed by the interaction between MYB transcription factors, basic helix-loop-helix (bHLH) transcription factors, and WD40-repeat proteins [16, 17]. The three elements of the complex act in pyramidal fashion. While the MYB factor provides the DNA-binding specificity for the activation of the target genes, the other two components are often involved in regulating additional processes including proanthocyanidin biosynthesis, which involves an MBW complex with the same bHLH transcription factor that regulates anthocyanin biosynthesis and a specific R2R3MYB protein that determines specificity for target genes. Pivotal studies in Petunia have linked regulation of anthocyanin biosynthesis and vacuolar acidification through a common bHLH protein (AN1) working in the complex [18–20]. Consequently, we tested whether members of the MBW complex might be responsible for the acidless phenotypes in citrus by searching the genome of *C. clementina* (https://phytozome.jgi.doe.gov) for potential citrus homologs of key regulatory genes in Petunia and *Arabidopsis*.

We first considered an acidless Citron mutant because Citron is a true species with a low level of heterozygosity that can facilitate genomic analysis. A wild-type Citron, “Poncire commun,” can synthesize both anthocyanins and proanthocyanidins and [21] (Figure 2). For both accessions, we sequenced *Ruby* (corresponding to Ciclev10013455 in the *C. clementina* genome), two candidate genes encoding proteins with homology to the WD-40 proteins of the MBW complex (Ciclev10005375 and Ciclev10015815), the bHLH gene MYC2 (Ciclev10019219) [22], and an uncharacterized gene (Ciclev10019118) encoding a bHLH protein closely related to TT8 in *Arabidopsis* and AN1 in Petunia, which we named *Noemi*. While the sequences of the WD-40, genes *Ruby* and MYC-2, were identical in both the Corsican and Poncire commun accessions of Citron, PCR analysis indicated the presence of a deletion of 1,313 nucleotides in the 3' region of *Noemi* (Figures 3A and S1A). The deletion was homozygous in Corsican Citron (*n<sup>DEL3</sup>; *n<sup>DEL3</sup>*) and heterozygous in Poncire commun (*N<sup>F</sup>; *n<sup>DEL3</sup>*). The wild-type allele (*N<sup>F</sup>*) contains seven introns and encodes a protein of 695 amino acids. The mutation in *n<sup>DEL3</sup>* results in deletion of the sequences encoding the last 275 amino acids and is predicted to generate a non-functional truncated protein lacking the entire bHLH domain required for dimerization and interaction with other proteins [23, 24] (Figure S1B).

Unlike most cultivated citrus, Citron produces zygotic monoembryonic seeds, offering the opportunity for segregation analysis using seeds from the heterozygous Poncire commun. PCR analysis indicated that five seedlings, showing unequivocal production of anthocyanins, contained at least one wild-type *Noemi* allele, while the green seedlings were homozygous for the deletion (*n<sup>DEL3</sup>*). Figure 2. For both accessions, we sequenced *Ruby* (corresponding to Ciclev10013455 in the *C. clementina* genome), two candidate genes encoding proteins with homology to the WD-40 proteins of the MBW complex (Ciclev10005375 and Ciclev10015815), the bHLH gene MYC2 (Ciclev10019219) [22], and an uncharacterized gene (Ciclev10019118) encoding a bHLH protein closely related to TT8 in *Arabidopsis* and AN1 in Petunia, which we named *Noemi*. While the sequences of the WD-40, genes *Ruby* and MYC-2, were identical in both the Corsican and Poncire commun accessions of Citron, PCR analysis indicated the presence of a deletion of 1,313 nucleotides in the 3’ region of *Noemi* (Figures 3A and S1A). The deletion was homozygous in Corsican Citron (*n<sup>DEL3</sup>; *n<sup>DEL3</sup>*) and heterozygous in Poncire commun (*N<sup>F</sup>; *n<sup>DEL3</sup>*). The wild-type allele (*N<sup>F</sup>*) contains seven introns and encodes a protein of 695 amino acids. The mutation in *n<sup>DEL3</sup>* results in deletion of the sequences encoding the last 275 amino acids and is predicted to generate a non-functional truncated protein lacking the entire bHLH domain required for dimerization and interaction with other proteins [23, 24] (Figure S1B).

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Flowers or fruit have not yet been obtained from these seedlings because of the long juvenile period. However, a few plants were grown from seeds of self-pollinated Poncire commun in 2013 in Corsica. Two plants were kept: one with purple leaves with pigmented flowers that produced fully acidic fruit and seeds with proanthocyanidins; the other produced no anthocyanins nor pigments, either anthocyanins in its leaves and flowers or proanthocyanidins in its seeds, and has very low juice acidity around pH 5.5 (Table 1). Poncire commun is the ideal control for Corsican Citron, since it is very closely genetically related to the Corsican Citron accession we tested. We then compared the MYC2 and MYC-2 sequences of Poncire commun to those of Corsican Citron, Citron and Citron×Noemi to confirm that the deletion allele, *n<sup>DEL3</sup>*, was homozygous in the plant with the acidless phenotype.
nDEL3 for the time of the destruction of the First Temple, is also heterozygous without juice sacs used in the Sukkot Jewish tradition since the remarkable, the ‘‘Yemen’’ Citron, a very ancient pulpless variety because alternative sweeter citrus varieties did not arrive in 160 Current Biology [25]. The underwent a wave of diversification in the Mediterranean region Chinese Citron varieties are genetically distinct from those that

**Figure 2. Wild-Type and Acidless Citrons (C. medica)**

(A) Young leaves and flowers of Poncire commun (left) and Corsican Citron (right), a variety unable to produce anthocyanins. (B) Germinating seeds in a fruit of Corsican Citron. The seeds are clear colored, the seedlings are anthocyaninless, and the juice is acidless. (C) Seeds of Poncire commun (left) and Corsican Citron (right) before (upper panel) and after (lower panel) staining with p-Dimethylaminocinnamaldehyde (DMACA) reagent, indicating presence or absence of proanthocyanidins. The red arrow indicates the chalazal spot (scale bars, 1 cm).

See also Figures S1 and S2 and Table S1.

Chinese Citron varieties are genetically distinct from those that underwent a wave of diversification in the Mediterranean region [25]. The \( n^{\text{DEL3}} \) allele might represent a recent mutation, consistent with the view of Hodgson that Corsican Citron originated in Corsica [4]. However, we found that another acidless variety, ‘‘Assads Moroccan’’ Citron [3], is also homozygous for the \( n^{\text{DEL3}} \) allele, suggesting a common origin with Corsican Citron despite phenotypic and genetic differences [25]. More remarkably, the ‘‘Yemen’’ Citron, a very ancient pulpless variety without juice sacs used in the Sukkot Jewish tradition since the time of the destruction of the First Temple, is also heterozygous for the \( n^{\text{DEL3}} \) allele. Another variety traditionally used for this religious ritual, ‘‘Greek’’ Citron, has the same heterozygous constitution at the Noemi locus (Figure S1C; Table S1). Historically, these findings support claims of the importance of religion and Jewish communities on the spread of Citron in the Mediterranean region [26, 27]. It also suggests that the ‘‘authentic’’ Jewish Citron, or Etrog (nongrafted and not hybridized with other varieties) could have been an acidless one, an idea supported by a reference to ‘‘sweet Citron’’ in the Talmud (200 AD) [28]. This probably refers to an acidless Citron, because alternative sweeter citrus varieties did not arrive in the Middle East until the 10th century and were introduced to Europe at the end of the 15th century [5].

We examined the Noemi locus in a number of fingered varieties of Citron (C. medica var. sarcodactylis), characterized by fruit segmented into finger-like sections (Figure S1D). While usually considered as a single variety under the name of ‘‘Buddha’s Hand,’’ many distinct varieties are known in Asia [5, 25]. Fingered Citrons have no juicy pulp or seeds and represent ‘‘genotypes frozen in time.’’ Both the common Buddha’s Hand and a Japanese fingered accession were heterozygous (\( N^{L} ; n^{\text{DEL3}} \)) at the Noemi locus. More interestingly, two related Chinese fingered Citron varieties, ‘‘Qingpi’’ and ‘‘Aihua,’’ used in traditional medicine, characterized by white flowers and green leaves [5], were homozygous for the \( n^{\text{DEL3}} \) allele (Figure S1C; Table S1). This confirmed that Noemi is essential for anthocyanin production and that the \( n^{\text{DEL3}} \) allele originated before the arrival of Citron in the Mediterranean, suggesting that it may have been inherited by some of the many hybrids derived from Citron [13].

One such hybrid is ‘‘Palestine sweet lime,’’ a true acidless variety still popular in India where it probably originated. The corresponding acidic form, which produces flavonoid pigments, is the rare ‘‘Soh Synteng’’ [4]. Palestine sweet lime and Soh Synteng (both also referred to as C. limettioides) are likely hybrids between Citron and a Pummelo × Mandarin hybrid [13] (Figure 1). We determined the allelic composition of Noemi in five acidless accessions and in Soh Synteng. All the accessions contained the same mutated \( n^{\text{DEL3}} \) allele, derived from Citron. Soh Synteng contained a second Noemi allele, which appears, from its sequence, to be functional while all the acidless accessions carried a second mutated allele of Noemi, \( n^{\text{DEL2}} \), involving a deletion of 741 bp spanning the 5‘‘ UTR, the first exon, and most of the second exon of the Noemi gene (Figures 3A and S3A).

Another interesting hybrid of Citron is C. limetta, a sour orange × Citron cross [13] (Figure 1). The acidless form, unable to produce flavonoid pigments, has a long history of cultivation as depicted in Italian books and paintings of the XVII and XVIII centuries (Figures 3C and S2D). The corresponding acidic variety, ‘‘Limonette de Marrakech,’’ is less well known [29]. Both varieties contain the non-functional allele of Noemi derived from Citron, \( n^{\text{TOS}} \) (Figure S3B), but the functional \( N^{L} \) allele found in Limonette de Marrakech has been disrupted by the insertion of a retrotransposon, Tc5, in the acidless ‘‘Limetta dolce’’ (\( n^{\text{TOS}} ; n^{\text{DEL3}} \); Figure 3A). Tc5 is a recently inserted and potentially active retroelement, as indicated by its identical long terminal repeats (LTRs) and intact coding region.

Lemon (C. limon) is the most widely distributed Citron-derived hybrid (arising from a sour orange × Citron cross). In regular acidic lemons, both Noemi alleles are wild-type and presumably functional (\( N^{L} ; N^{L} \)); Sweet varieties of lemon, unable to produce anthocyanins, have been described. All the varieties we have analyzed (often displaying variegated phenotypes, in terms of color and acidity, on the same plant; Figure S3C) contained new alleles not present in standard lemons (Figures S3B and S3D) and thought to be from periclinal graft chimeras [30, 31] where the L1 layer, which produces the epidermis and epidermal juice sacs of the fruit, came from an acidless limetta, while L2 and L3 came from lemon. Sweet lemons are not true lemons but chimeric accessions as observed in the XIX century [32] and reported in more recent studies [1, 32, 33].
Sweet orange (C. sinensis) is a complex Mandarin × Pummelo hybrid with no contribution from Citron. It contains two potentially functional Noemi alleles, both derived from the Mandarin genetic pool [34], which are expressed in wild-type fruit (Figure S4A). Sweet orange does not produce anthocyanins because of different mutations in both alleles of Ruby [1]. Despite the absence of anthocyanins, true acidless varieties can be identified based on seed color [4, 35]. Specific staining indicated that the light color of seeds is due to the absence of proanthocyanidins (Figure S4B). Two related acidless varieties, “Vaniglia biondo” and “Vaniglia sanguigno” [36], were identical at the Noemi locus, where both alleles were disrupted by the insertion of different retrotransposons within the sixth exon. One of them, Tcs4, is intact and putatively active; the other one, Tcs6x, is rearranged with two LTRs in tandem in the middle of the insertion (Figure 3A). Both elements are Copia-like LTR retrotransposons, similar to Tcl5 in Limetta dolce and to three elements inserted upstream of Ruby in blood orange varieties and in Citron [1, -15] (Figure S4D; Data S1). Consequently, this family of retroelements may be responsible for a large proportion of the

### Table 1. Species and Hybrids Used in This Study

| Common Name                | Tanaka’s Classification | Juice pH | Anthocyanins | Proanthocyanidins | Source | Accession |
|----------------------------|-------------------------|----------|--------------|-------------------|--------|-----------|
| Citron                     |                         |          |              |                   |        |           |
| Poncire commun             | C. medica               | 2.45     | yes          | yes               | D      | SRA701    |
| Corsican                   | C. medica               | 5.42     | no           | no                | D      | SRA613    |
| Diamante                   | C. medica               | 2.46     | yes          | yes               | B      | Palazzelli Certif. |
| Buddah’s hand              | C. medica               | NA       | yes          | NA                | C      | CRC3786   |
| Sweet lime                 |                         |          |              |                   |        |           |
| Soh Synteng                | C. limettioides         | 2.58     | yes          | yes               | C      | CRC3261   |
| Palestine sweet lime       | C. limettioides         | 5.41     | no           | no                | C      | CRC1482   |
| Mary Ellen                 | C. limettioides         | 5.51     | no           | no                | C      | CRC4053   |
| Unnamed                    | C. limettioides         | 5.39     | no           | no                | C      | CRC921    |
| Unnamed                    | C. limettioides         | 5.54     | no           | no                | C      | CRC919    |
| Unnamed                    | C. limettioides         | 5.32     | no           | no                | C      | CRC363    |
| Limetta                    |                         |          |              |                   |        |           |
| Limonette de Marrakech     | C. limetta              | 2.55     | yes          | yes               | C,D    | CRC3989; SRA829 |
| Limetta dolce              | C. limetta              | 5.88     | no           | no                | B      | C-F2P7    |
| Pomona                     | C. limetta              | 4.59     | yes          | yes               | C      | CRC4068   |
| Millsweet                  | C. limetta              | 4.5      | yes          | yes               | C      | CRC569    |
| Sweet Orange               |                         |          |              |                   |        |           |
| Navel                      | C. sinensis             | 3.47     | no           | yes               | B      | 2B-F1-P14 |
| Valencia                   | C. sinensis             | 3.34     | no           | yes               | B      | 6A2-F5P1  |
| Tarocco comune             | C. sinensis             | 3.61     | yes          | yes               | B      | 4-F20P9   |
| Moro                       | C. sinensis             | 3.50     | yes          | yes               | B      | 8-F1P2    |
| Tarocco Ferreri            | C. sinensis             | 6.32     | yes          | yes               | B      | 2B-F14P14 |
| Vaniglia biondo            | C. sinensis             | 6.02     | no           | no                | B      | 2A-F19P6  |
| Vaniglia sanguigno         | C. sinensis             | 6.74     | no           | no                | B      | 1A-F7P10  |
| Lemon                      |                         |          |              |                   |        |           |
| JIC lemon                  | C. limon                | 2.42     | yes          | yes               | A      | S72       |
| Politi apireno             | C. limon                | 2.39     | yes          | NA                | B      | C-F3P1    |
| Girotta (red leaf)         | C. limon                | 3.20     | yes          | yes               | B      | C-F2P9    |
| Girotta (green leaf)       | C. limon                | 5.70     | no           | no                | B      | C-F2P9    |
| Poros (red leaf)           | C. limon                | 2.78     | yes          | yes               | B      | C-F1P7    |
| Poros (green leaf)         | C. limon                | 5.69     | no           | no                | B      | C-F1P7    |
| Pispisa (red leaf)         | C. limon                | 2.41     | yes          | yes               | B      | C-F1P7    |
| Pispisa (green leaf)       | C. limon                | 5.68     | no           | no                | B      | C-F1P7    |
| ISA (red leaf)             | C. limon                | 2.30     | yes          | yes               | B      | C-F2P5    |
| ISA (green leaf)           | C. limon                | 5.36     | no           | no                | B      | C-F2P5    |

The pH of fruit juices and the presence or absence of flavonoid pigments are indicated. Citrus accessions were obtained from the following sources: A: John Innes Centre, Norwich, UK; B: CREA-OFA, Consiglio per la Ricerca in Agricoltura e l’Analisi dell’Economia agraria, Olivicoltura Frutticoltura Agrumicoltura, Acireale, Italy; C: USDA-ARS National Clonal Germplasm Repository for Citrus & Dates, Riverside, CA, USA; D: CRB CITRUS, INRA-CIRAD, Citrus Biological Resource Center, San Giuliano, Corsica, France. NA, not available because of absence of seeds or juice in the accession. See also Figures 3 and S1–S4 and Table S1.
Figure 3. Noemi Is Essential for the Biosynthesis of Flavonoid Pigments and Is a Major Determinant of Fruit Acidity
(A) Allelic constitution of Noemi in wild-type (boxed in purple) and acidless (boxed in green) citrus varieties. Molecular events that resulted in the acidless phenotypes are indicated by a green arrow.
(B) pH of juice, expression of Noemi in fruit and sequences of the promoters of four accessions of C. limetta. LM, Limonette de Marrakech; P, Pomona; M, Millsweet; LD, Limetta dolce. Error bars represent SE. The arrow indicates a 2-bp deletion polymorphism upstream of the TATA box.

(legend continued on next page)
phenotypic variation in citrus, particularly in the progeny of interspecific crosses which may carry more active Copia-like retroelements as a result of genome shock [37, 38].

The identification of five independent mutations associated with the acidless phenotype demonstrated that Noemi is essential for both the biosynthesis of flavonoid pigments and for fruit acidity and suggests that this regulatory gene may be, in part, responsible for the large spectrum of acidity in commercially grown citrus [14]. In sweet orange, a blood variety with very low acidity, “Tarocco Ferreri,” showed a 92% reduction in Noemi expression compared to the acidic variety from which it derived, “Tarocco comune” (Figure S4C). Tarocco Ferreri is not an acidless accession since it is able to produce flavonoid pigments and does not contain mutations in the coding sequence of Noemi, but the low expression suggests it carries a weak Noemi allele or a mutation in a gene controlling Noemi expression.

A more direct indication that Noemi is a major determinant of fruit acidity was provided by the analysis of C. limetta. As an apomictic hybrid, its complex genetic constitution (which combines all the three citrus primary species, Figure 1) is fixed within the population. Given that the origin of the hybrid is relatively recent, a single polymorphism in a candidate gene is significant. Besides the fully acidic form and the true acidless variety, we identified two additional accessions producing flavonoid pigments but intermediate levels of fruit acidity (Table 1; Figure S3E). The sequences of the intact Noemi gene in these accessions, named “Pomona” and “Millsweet” [4, 39], were identical to the sequence of the acidic Limonette de Marrakech; the sequences of the regions 1 kb upstream were also identical except for a 2-bp deletion, adjacent to a canonical TATA box in the core promoter, present in both accessions with intermediate acidity (Figures 3B and S3F). The deletion was associated with reduced Noemi expression, which correlated with fruit acidity (Figure 3B).

Our study has identified Noemi encoding the bHLH protein that interacts with Ruby to control anthocyanin production in citrus. In this context, it should be possible to use Noemi to increase the accumulation of anthocyanins induced by Ruby [15]. Noemi also controls proanthocyanidin biosynthesis in seeds and is essential for the regulation of fruit acidity. Identification of Noemi alleles in Citron allowed us to link modern Mediterranean Citron varieties to those of ancient China, quite possibly facilitated by the adoption of Citron in Jewish culture as an important religious symbol. Although detection of acidless varieties of citrus in the fossil record based on morphological characters of macro-remains is impossible, it might be possible to perform a DNA analysis to identify Noemi alleles in the fossil remains from the Mediterranean and to demonstrate their links to citrus of Asian origins.

Noemi is an important determinant of natural variation in fruit acidity in citrus, as evidenced by its alleles in interspecific hybrids like Palestine sweet lime and limetta as well as acidless oranges. This natural variation is associated with recent activity of a family of Copia-like retroelements, particularly evident in interspecific hybrids of citrus that may represent a classic example of genome shock as proposed by Barbara McClintock in her Nobel lecture of 1983 [37]. Citrus breeders appear to have used the outcomes of genome shock to select for new variants despite their limited capacity for improvement using conventional breeding.

STAR METHODS

Detailed methods are provided in the online version of this paper and include the following:

- **CONTACT FOR REAGENT AND RESOURCE SHARING**
- **EXPERIMENTAL MODEL AND SUBJECT DETAILS**
- **METHOD DETAILS**
  - Plant Material
  - Isolation of Noemi alleles
  - Segregation Analysis
  - Self-Pollination of ‘Poncire commun’ Citron
  - DNA Gel Blot
  - Expression Analysis of Noemi
- **QUANTIFICATION AND STATISTICAL ANALYSIS**

SUPPLEMENTAL INFORMATION

Supplemental Information includes four figures, one table, and two data files and can be found with this article online at https://doi.org/10.1016/j.cub.2018.11.040.

ACKNOWLEDGMENTS

E.B. and C.M. were supported by the Institute Strategic Programs: “Understanding and Exploiting Plant and Microbial Secondary Metabolism” (BB/J004398/1) and “Molecules from Nature” (BB/P01253/1) from the Biotechnological and Biological Scientific Research Council (BBSRC). We thank Robert Krueger and Manjunath Keremane (USDA-ARS National Clonal Germplasm Repository for Citrus and Dates, Riverside, California, USA) for providing DNA samples and for helpful discussion during the study. We also thank Michel Bachès (Pépinières Bachès, Eus, France) for providing leaves of the Citron accessions listed in Table S1 and information on “Assads Moroccan” Citron. We thank Eliezer Goldschmidt for his encouraging comments on the manuscript.

AUTHOR CONTRIBUTIONS

E.B. and C.M. planned and designed the research; E.B., C.M., C.L., Y.F., and M.D.-H. performed experiments; G.R.R. provided plant material and information; A.C. provided information and images; and E.B. and C.M. wrote the article with input and comments from the other authors.

DECLARATION OF INTERESTS

The authors declare no competing interests.

Received: October 2, 2018
Revised: November 7, 2018
Accepted: November 13, 2018
Published: December 20, 2018

(C) Engraving of citrus fruit from a rare copy of “Hesperides” by Ferrari (1646) kept at CREA, Acireale; C. limetta can be recognized by the distinctive prominent nipple. The ribbon is labeled in Latin with the name “Lima Dulcis et Lima Acris” to indicate acidless and acidic fruit, which are morphologically undistinguishable.

See also Figures S1, S3, and S4, Tables 1 and S1, and Data S1 and S2.
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STAR METHODS

KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Chemicals, Peptides, and Recombinant Proteins | | |
| 4-(Dimethylamino)cinnamaldehyde | Sigma-Aldrich Merck | D4506 |
| Critical Commercial Assays | | |
| DNasey Plant Mini Kit | QIAGEN | 69106 |
| RNeasy Plant Mini Kit | QIAGEN | 74904 |
| Phusion High-Fidelity DNA Polymerase | Thermo Fisher | F530L |
| pGEM®-T Easy Vector Systems | Promega | A1360 |
| SuperScript III Reverse Transcriptase | Thermo Fisher | 18080044 |
| High-Capacity cDNA Reverse Transcription Kit | Thermo Fisher | 4368814 |
| ABI PRISM 7000 Sequence Detection Systems | Thermo Fisher | 4328895 |
| SYBR Green PCR Master Mix | Thermo Fisher | 4309155 |
| Custom DNA sequencing | Eurofins | https://www.eurofinsgenomics.eu/ |
| Deposited Data | | |
| Nucleotide sequence of Noemi in citrus accessions | This study | GenBank: MK139964–MK139972 |
| Experimental Models: Organisms/Strains | | |
| List of citrus accessions presented in Tables 1 and S1 | This study | N/A |
| Oligonucleotides | | |
| List of oligonucleotides presented in Table S2 | This study | N/A |
| Software and Algorithms | | |
| Citrus Genome Assembly and Annotation | Citrus clementina v1.0 | https://phytozome.jgi.doe.gov/ |
| Citrus Genome Assembly and Annotation | Citrus sinensis v1.1 | https://phytozome.jgi.doe.gov/ |
| Citrus Genome Assembly and Annotation | Citrus sinensis Annotation project | http://citrus.hzau.edu.cn/ |
| Sequence alignment | MultAlin | http://multalin.toulouse.inra.fr/multalin/ |

CONTACT FOR REAGENT AND RESOURCE SHARING

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Eugenio Butelli (eugenio.butelli@jic.ac.uk).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Leaves and fruit were of citrus accessions were obtained from the sources listed in Table 1 and Table S1. Plant material was analyzed as described in Method Details.

METHOD DETAILS

Plant Material
Citrus leaves were ground in liquid nitrogen and DNA and RNA were extracted using the DNasey Plant Mini Kit and the RNeasy Plant Mini Kit (QIAGEN) according to the manufacturer’s instructions. Fruit juice was obtained using a conventional citrus squeezer and pH was measured in three replicates with a standard combination Ag/AgCl pH electrode. The presence of anthocyanins in young leaves and flowers was determined by visual inspection and confirmed measuring the absorbance at 530 nM of acidified methanol extracts. The presence of proanthocyanidins in seeds was determined by visual inspection and confirmed by staining for 30 min with 0.3% (w/v) DMACA (p-Dimethylaminocinnamaldehyde, Sigma) in methanol and 6 M HCl (1:1, v/v) followed by several washing steps with 70% ethanol.

Isolation of Noemi alleles
Full-length Noemi alleles were isolated by PCR using primers EB-072 and EB-076 designed within the 5’UTR and 3’UTR regions, respectively. PCR fragments were used directly for sequencing or cloned into the pGEM-T Easy vector (Promega) when two alleles
having the same size were present. The alleles containing retrotransposons where isolated using primers EB-088 and EB-076. Appropriate primers were used to obtain the complete sequences and confirm the presence of the retrotransposons. The alleles with a deletion in the 3’ region, nDEL3, where identified using primers EB-093 and EB-94 followed by sequencing. The alleles with a deletion in the 5’ region, nDEL5, and the core promoter regions where isolated using primers EB-111 and EB-076. The determination of the intron—exon structure was performed after isolation of full-length cDNA clones from leaves of wild-type Citron and sweet orange. Total RNA was retrotranscribed using Superscript III reverse transcriptase (Thermo Fisher) and amplified using primers EB-105 and EB-106 in Citron followed by direct sequencing or, for sweet orange, with primers EB-115 and EB-116, equipped with attB sites, followed by cloning into pDONR 207 (Invitrogen). Primer sequences are listed in Table S2. The sequences of the different alleles are provided in Data S2 and have been deposited in GenBank under the accession numbers MK139964 to MK139972.

Segregation Analysis
Seeds were extracted from three fruits of ‘Poncire commun’ Citron, washed with water and sterilized with 10% bleach for three hours with shaking. Germination of seeds was carried out in tissue culture conditions placing seeds in Phytatray II vessels (Sigma) containing MS medium with 0.8% agar; seeds were kept at 23°C with 16 h of light and 8 h of dark. One tray, accidentally contaminated by the common mold Cladosporium (as determined by sequencing), showed unequivocal production of anthocyanins in five out of eight seedlings. All the eight seedlings were used for PCR analysis using primers EB-093 and EB-094.

Self-pollination of ‘Poncire commun’ Citron
Self-pollination was realized between April 23rd and May 20th 2013 at the INRA-CIRAD Citrus germplam of San Giuliano, Corsica, France. One tree of ‘Poncire commun’ was covered with insect proof net. Fruits were harvested on December 9th and seeds were extracted and sown on the same day. Twenty plantlets were grafted on Citrus volkameriana rootstock on March 17th 2014. The first flowers were obtained during the spring 2015. Three trees (two with purple and one with white flowers) are conserved in a greenhouse in San Giuliano. Every year, the tree with white flowers produces acidless fruit and trees with purple flowers acidic fruit. Mature leaves from each phenotype were used for PCR analysis using primers EB-093 and EB-094.

DNA Gel Blot
Leaves of different varieties of lemon were ground in liquid nitrogen and DNA was extracted using caesium chloride density gradient purification. DNA (10 μg per sample) was digested with HindIII restriction enzyme for 5 h and then separated by electrophoresis. Denatured DNA was transferred to nitrocellulose membrane filters. Filters were hybridized with randomly primed 32P-labeled probe of the Ruby gene overnight at 60°C and washed in 0.1 x SSC, 0.5% SDS at 60°C for 2 h before exposure to X-ray film (Fuji RX-100).

Expression Analysis of Noemi
Quantification of Noemi expression in fruit of different varieties of limetta and sweet orange was performed by qRT-PCR. Total RNA was extracted from 3 mL of juice, previously filtered under sterile conditions. One volume of extraction buffer (0.2 M Tris–HCl pH 8, 50 mM EDTA, 0.2 M NaCl, 2% (w/v) SDS), one volume of phenol and 60 μl of β-mercaptoethanol were added to 3 mL of juice. After an incubation at 50°C for 5 minutes, samples were centrifuged at 4000 rpm for 15 minutes. The aqueous phase was extracted for two times with one volume of chloroform:isoamyl alcohol (24:1, v/v) after centrifugations at 4000 rpm for 15 minutes each. Half volume of 6 M LiCl was added to the new aqueous phase, and RNA was left to precipitate overnight at −20°C. After centrifugation and washing with 70% (v/v) ethanol, RNA was resuspended in RNase-free water. DNase-treated total RNA was further purified using the RNA Cleanup protocol (QIAGEN) and retrotranscribed into cDNA using a High-Capacity cDNA Archive kit (Thermo Fisher). Quantitative real-time PCR was performed in optical 96-well plates with an ABI Prism 7000 sequence detection system (Thermo Fisher). A PCR mixture (final volume 25 μl) containing 15 μL Power SYBR Green mix, 0.2 μM each of gene-specific primers and 100 ng of cDNA sample was prepared using the protocol for Power SYBR Green PCR Master Mix (Thermo Fisher). The following standard thermal profile was used for all PCR reactions: 50°C for 2 min, 95°C for 10 min, 40 cycles of 95°C for 15 s, and 60°C for 1 min. Three replicates were assayed, and a no-template negative control (water control) was performed. The analyses used the relative quantification standard curve method.

QUANTIFICATION AND STATISTICAL ANALYSIS
Quantification of Noemi expression was performed by qRT-PCR; three replicates were assayed. Error bars in Figures 3 and S4C represent standard error of the mean (SE) and were determined using Excel 2017 (Microsoft, Redmond, WA).