The genetics of vacuoles
*Biogenesis and function in plant cells*

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Hyperacidification of Citrus fruits by a vacuolar proton-pumping P-ATPase complex*

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CHAPTER 2

Abstract

The sour taste of Citrus fruits is due to the extreme acidification of vacuoles in juice vesicle cells via a mechanism that remained elusive. Genetic analysis in petunia identified two vacuolar P-ATPases, PH1 and PH5, which determine flower color by hyperacidifying petal cell vacuoles. Here we show that Citrus homologs, CitPH1 and CitPH5, are expressed in sour lemon, orange, pummelo and rangpur lime fruits, while their expression is strongly reduced in sweet-tasting “acidless” varieties. Down-regulation of CitPH1 and CitPH5 is associated with mutations that disrupt expression of MYB, HLH and/or WRKY transcription factors homologous to those activating PH1 and PH5 in petunia. These findings address a long-standing enigma in cell biology and provide targets to engineer or select for taste in Citrus and other fruits.
Introduction

*Citrus* are widely used for the consumption of fruit flesh and juices. Modern citrus varieties were generated over thousands of years by intra and interspecific crosses of a handful of species combined with clonal propagation\(^1\)\(^-\)\(^3\). Acidity is a major trait determining the taste and use of citrus fruits and selection by breeders and producers has generated a broad palette of sour and “sweet” (i.e. non-sour) varieties of lemons, oranges, pummelos and other citrus fruits\(^4\).

For a sour taste, food/liquid should have \((i)\) a high concentration of free H\(^+\) ions (low pH), which is sensed by acid-sensitive cells in taste buds, presumably via an H\(^+\)-selective channel\(^5\) and \((ii)\) a certain pH-buffering capacity to prevent that the liquid is neutralized by the saliva. The acidity (low pH) of *Citrus* fruits is determined by the pH of the vacuoles in juice vesicle cells, which can be as low as 2 in sour lemons and lime\(^6\)\(^-\)\(^8\). The steep proton gradient across the vacuolar membrane (tonoplast) drives massive transport of citrate into the vacuole via a mechanism that is only partially understood\(^9\),\(^10\). As citrate enters the vacuole in dissociated form (citrate\(^3^-\)) it increases its buffer capacity, which contributes to the sour taste\(^11\), but does not lower the pH. How juice vesicle cells can hyperacidify their vacuoles to such an extreme extent remained elusive, in spite of extensive biochemical work\(^8\),\(^9\),\(^12\)\(^-\)\(^15\).

In most plant cells the cytoplasm is about neutral and the vacuolar lumen mildly acidic. The (moderate) pH gradient across the tonoplast is generated by vacuolar-ATPases (V-ATPases)\(^16\),\(^17\), which are complex multi-subunit proton pumps found in both animals and plants\(^18\),\(^19\). V-ATPases translocate 2-4 protons per hydrolyzed ATP (H\(^+\)/ATP = 2-4) depending on the pH on both sides of the membrane\(^20\),\(^21\), and may in theory acidify vacuoles down to pH\(\approx\)3.5, when operating without kinetic inhibition, which it rarely if ever occurs *in vivo*, and in its “lowest gear” (H\(^+\)/ATP = 2). Further acidification to pH<3, as in lemon vacuoles, would require H\(^+\)/ATP ratios below 2 (ref\(^20\)). Acid lemons indeed contain a proton-pump activity with...
H\textsuperscript{+}/ATP=1 stoichiometry\textsuperscript{8,12,14,15}. However, the nature of this fruit-specific proton pump has remained elusive ever since, because it could not be completely purified\textsuperscript{13,15}.

In petunia, mutations in one of the seven PH loci (PH1 to PH7) reduce the acidity of petal vacuoles and petal homogenates, resulting in a blue flower color\textsuperscript{22,23}. PH3 and PH4 encode transcription factors of the WRKY and MYB family respectively, which together with the helix-loop-helix protein ANTHOCYANIN1 (AN1) and the WD-repeat protein AN11, form a complex (WMBW) that activates genes involved in vacuolar acidification\textsuperscript{24-27}. PH1 and PH5 are the major downstream genes involved in vacuolar hyperacidification\textsuperscript{28}. PH5 encodes a P\textsubscript{3A}\textsuperscript{+}-ATPase proton pump that resides in the tonoplast instead of the plasma membrane where other P\textsubscript{3A}\textsuperscript{+}-ATPases reside\textsuperscript{29,30}. PH1 is a P\textsubscript{3B}-ATPase similar to bacterial Mg transporters and also resides in the tonoplast. It has no known transport activity on its own, but can bind to PH5 and promote PH5 proton-pumping activity\textsuperscript{28} and has an additional role in membrane/protein trafficking to the vacuole\textsuperscript{31}.

PH1 and PH5 homologs are widely conserved among flowering plants, including species without colored petals, suggesting that their function is not confined to flower pigmentation\textsuperscript{30,32}. Since CitPH5 homologs are expressed in sour lemons and oranges\textsuperscript{33-35} and P-ATPase proton pumps can theoretically generate steeper proton gradients (because H\textsuperscript{+}/ATP =1), we investigated whether the PH5/PH1 complex might be the proton pump that acidifies Citrus vacuoles to such extreme extent.

Analyzing a collection of lemon, orange and pummelo varieties (Supplementary Table 1) we found that PH1 and PH5 homologs are highly expressed in all acidic (low pH) fruits, but are down regulated in non-acidic (high pH) fruits, due to inactivating mutations in CitAN1 (sweet lemon and sweet oranges) or regulatory mutations that inactivate CitAN1, CitPH3 and/or CitPH4 expression.
Results

CitPH1 and CitPH5 expression in ‘Faris’ lemons

Lemon trees of the variety ‘Faris’ produce branches bearing either sour or sweet (non-sour) fruits, enabling a comparison of sweet and sour fruits grown in the same conditions\textsuperscript{33}. However, sweet and sour fruits are not necessarily isogenic, because ‘Faris’ is a graft chimera in which the L1 tunica layer of the shoot meristem derives from an unknown variety related to ‘Millsweet’ limetta and the L2 layer from a standard sour lemon\textsuperscript{33}. Branches of ‘Faris’ trees carrying sour fruits (Fso) have purple immature leaves, a purple blush on the lower petal epidermis, and dark spots at the chalazal end of the seeds, whereas the branches bearing sweet fruits (Fsw) lack purple pigmentation on leaves, petals and seeds (Fig. 1a). The juice of Fsw fruits is less acidic (pH 5.1) than that of Fso or ‘Frost Lisbon’ (Fli) (pH 2.5), a standard lemon with strong sour taste, and Fsw fruits contain less titratable acid than Fso or Fli fruits (Fig. 1b). There was, however, no correlation between soluble solid content (Brix) and juice taste (Fig. 1b), indicating that juice pH and/or titratable acid rather than the sugars, which are major components of total soluble solids, determine the different taste of Fsw and Fso fruits.

In melon (Cucumis melo) and tomato (Solanum lycopersicum), which both lack PH1 and PH5 homologs\textsuperscript{30}, the (mild) acidification of the fruit flesh requires a membrane transporter of unknown function, known in melon as PH or SOUR\textsuperscript{36}. We identified the SOUR homolog of Citrus (CitSO) and found that CitSO mRNAs in Fsw and Fli fruits, are expressed at similar levels and lack mutations with an obvious effect on the expression and/or activity of the encoded protein, indicating that CitSO does not contribute to the differences in their acidity (Supplementary Fig. 1 and 2).

Analysis of the Citrus limon homologs CitPH1 and CitPH5 identified previously\textsuperscript{30} revealed that both are expressed in low-pH Fli and Fso lemons, but not in high-pH Fsw lemons (Fig. 1c, Supplementary Fig. 3a). Expression of the Citrus homolog of
MAC9F1, a petunia gene of unknown function that is activated by the same transcription factors as PH1 and PH5 (ref37), is also down regulated in Fsw juice vesicles similar to CitPH1 and CitPH5 (Fig. 1c).

These findings indicate that (i) the CitPH1-PH5 heterodimeric proton pump is involved in the hyperacidification of Fso and Fli fruits and (ii) that the reduced acidity of Fsw fruits results from a mutation in a regulatory gene controlling the expression of CitPH1, CitPH5, and other genes like CitMAC9F1.

**Figure 1.** Analysis of ‘Frost Lisbon’ (Fli), ‘Faris’ sour (Fso) and ‘Faris’ sweet (Fsw) lemons. (a) Phenotypes of Fli, Fso and Fsw fruits, flowers and leaves. Scale bars represent 1 cm. (b) pH, titratable acid and soluble solid content (°Brix) in juice from (nearly) mature Fli, Fso and Fsw fruits (mean ± SE). (c) Real time RT-PCR of CitPH1, CitPH5 and CitMAC9F1 mRNA in juice vesicles from (nearly) mature Fli, Fso and Fsw fruits. (d) Real time RT-PCR of CitAN1 mRNA in Fli, Fso and Fsw juice cells, using primers that amplify the 5’, middle and 3’ part of the mRNA. (e) Structure of the CitAN1 alleles and their transcripts in Fso and Fsw juice vesicles. (f) Real time RT-PCR of CitPH3, CitPH4 and CitAN11 mRNA in juice vesicles from (nearly) mature Fli, Fso and Fsw fruits. Values in (c), (d) and (f) are mean ± SE; n = number of samples from different fruits X number of technical replicates of each. Source data are provided as a Source Data file.
Inactivation of CitAN1 causes the reduced acidity in Fsw.

To identify the mutation(s) responsible for the reduced expression of CitPH1 and CitPH5 in Fsw fruits, we identified Citrus homologs of the transcription regulators AN1, AN11, PH4 and PH3 (Supplementary Fig. 4), which drive PH1 and PH5 expression in petunia petals and seeds.\textsuperscript{28,29} Public RNA-seq data\textsuperscript{38} indicate that in the Citrus sinensis (sweet orange) variety ‘Valencia’ CitPH4 is predominantly expressed in fruits, like CitPH5 and CitMAC9F1, whereas CitAN1 is also expressed in flowers, where it most likely drives anthocyanin synthesis in concert with other MYB proteins (Supplementary Fig. 5).

RT-PCR experiments using primers complementary to the 5’ and 3’ end of the CitAN1 coding sequence revealed that Fli and Fso juice vesicles express full size CitAN1 mRNAs (Fig. 1d; Supplementary Fig. 3b), which encode a functional protein (see below). Both CitAN1 alleles of Fso juice vesicles consist of 7 exons, of which one (exon 4) is skipped in a fraction of the mature CitAN1 mRNA (Fig. 1e; Supplementary Fig. 3), and are distinguishable by sequence polymorphisms in CitAN1 (single nucleotide polymorphism (SNPs) and a 1661-bp transposon insertion in the 5’ flanking region) and in the genes immediately upstream (CitFAR-like) and downstream (CitTFIIH-like) (Supplementary Figs. 6-8).

Fsw fruits, by contrast, lack full size CitAN1 mRNAs and instead express truncated CitAN1 transcripts, which span the 5’ and middle region of the mRNA, but lack the 3’ part (Fig. 1d-e and Supplementary Fig. 3). Analysis of genomic and 3’RACE cDNA fragments revealed that the citan1\textsuperscript{Fsw} allele(s) contain an identical 1.3-kb deletion with breakpoints in exon 7 and 143 bp downstream the normal polyadenylation site, resulting in a transcript that lacks exon 8 and most of exon 7 to terminate at a cryptic polyadenylation site 57 bp downstream from the deletion breakpoint (Fig. 1e and Supplementary Fig. 9). Sequencing of PCR products from CitAN1 and the flanking genes CitFAR-like and CitTFIIH-like revealed no sequence
polymorphism (Supplementary Fig. 8). This indicates that in Fsw fruits the genomic CitAN1 region is either homozygous or hemizygous over a larger deletion in the sister chromosome that spans CitAN1, CitFAR-like and CitTFIID-like. The latter possibility is the more likely, because varieties of C. limon (and other hybrid Citrus “species”) have been long propagated asexually, which prevents homozygosity, and are thought to be essentially clones that diverged by accumulating mutations.\textsuperscript{39}

Transcripts of the PH4 homolog CitPH4 accumulate at similar levels in Fso and Fsw fruits (Fig. 1f), excluding that the high-pH phenotype results from down-regulation of CitPH4. Fso and Fsw fruit both contain two distinct CitPH4 alleles distinguishable by few SNPs and triplet repeats of variable length, resulting in several amino acid replacements and polyglutamine (Q) track of variable length, which may affect protein function and/or stability (Supplementary Fig. 10-11). The sequences of CitAN1\textsuperscript{Fso} and CitAN1\textsuperscript{Fsw} did not reveal differences with an obvious negative effect on Fsw protein activity (Supplementary Fig. 12), and in fruits mRNA expression levels of the two alleles are comparable (Fig. 1e).

The finding that juice vesicles of Fso and Fsw fruits contain distinct alleles for multiple genes, including CitAN1, CitPH4, CitAN11, CitFAR-like and CitTFIID-like, suggests that the occurrence of sweet and sour fruits results from atypical periclinal cell division(s) by which a daughter of a cell in the L2 meristem layer, containing the genome of a standard sour lemon, invaded L1 and displaced the mutant ‘Millsweet’-like L1 cells, or vice versa, as in other chimeras.\textsuperscript{40,41} rather than the somatic reversion of an unstable (epi)allele.

To examine whether CitPH1 and CitPH5 are transcriptionally activated by CitAN1 and CitPH4 and whether the deletion in CitAN1 and/or the polymorphisms in CitPH4 coding sequence impair the transcription of CitPH1 and CitPH5 in Fsw lemons, we performed transient expression in protoplasts from ph4 petunia petals, where endogenous PH1 and PH5 promoters are inactive. Therefore, we expressed
various combinations of CitAN1\(^{Fso}\), CitAN1\(^{Fsw}\) and GFP fusions of CitPH4\(^{Fso}\) or CitPH4\(^{Fsw}\) from the constitutive 35S promoter and measured the expression of RFP reporter genes that were translationally fused to the CitPH1\(^{Fso}\) or CitPH5\(^{Fso}\) promoters (Fig. 2a). To identify transformed cells and normalize expression levels for transformation efficiency we co-transformed p35S:AHA10-GFP, which encodes a GFP fusion of the Arabidopsis PH5-homolog AHA10 that localizes in tonoplast of the central vacuole\(^{32}\), and the nuclear localized GFP-CitPH4.

**Figure 2.** Activation of CitPH1 and CitPH5 promoters by CitAN1 and CitPH4. (a) Structure of effector, reporter and control genes used for transient transformation of protoplast from the epidermis of petunia ph4 petals. (b) Relative expression (real-time RT-PCR) of effector (CitAN1 and CitPH4) and reporter (pCitPH1:RFP and pCitPH5:RFP) mRNA expression in petal protoplasts transformed with different effectors [mean ± SE from 2 technical PCR replicates of 4 independent transformation experiments (n=4x2)]. Expression of the co-transformed 35S:AHA10-GFP gene was used for normalization. (c) Confocal micrographs of petunia ph4 epidermal petal protoplasts, with anthocyanins in the central vacuole, from stage 6 flowers, 24 hrs after transformation 35S:AHA10-GFP, the reporters pCitPH1:RFP, pCitPH5:RFP and different combinations of the effectors 35S:GFP-CitPH4\(^{Fso}\),
Expression of either CitAN1 or GFP-CitPH4 alone was insufficient to induce the CitPH1 or CitPH5 reporters in protoplasts (Fig. 2b-c; Supplementary Fig. 13). However, co-expression of CitAN1 and GFP-CitPH4 strongly induced pCitPH1:RFP and pCitPH5:RFP expression (Fig. 2b) in most if not all cells expressing GFP-CitPH4 and AHA10-GFP (Fig. 2c), and was independent from other (petunia) regulators of the anthocyanin/pH pathway as it also occurred in white mesophyll cells (Supplementary Fig. 13) where endogenous AN and PH genes are not expressed\(^{26,27,29}\). The CitPH4\(^{Fso}\) and CitPH4\(^{Fsw}\) alleles activated the CitPH1 and CitPH5 reporters with similar efficiency, whereas the truncated AN1 protein encoded by an1\(^{Fsw}\) proved unable to induce pCitPH1:RFP or pCitPH5:RFP expression.

These findings indicate that the mutation in citan1\(^{Fsw}\) is responsible for the reduced CitPH1 and CitPH5 expression in the Fsw fruits and very likely also the loss of anthocyanins in leaves and flowers on the branches bearing Fsw fruits, whereas the polymorphisms in the CitPH4\(^{Fsw}\) and CitPH4\(^{Fso}\) have little or no effect. The reduced expression of CitPH3 in Fsw juice cells (Fig. 1f) is likely due to the citan1\(^{Fsw}\) mutation, since in petunia petals PH3 is (partially) regulated by AN1 and PH4 and essential for transcription of PH1 and PH5\((ref^{27})\).

**CitPH1 and CitPH5 expression in other sweet lemons.**

To support that the reduced acidity of Fsw fruit was caused by mutation in CitAN1 and consequent loss of CitPH1 and CitPH5 expression, rather than by independent mutations affecting unrelated pathway(s), we analyzed additional sweet and sour lemon varieties (Fig. 3a). ‘Schaub’ rough lemon is a non-edible citrus variety, unrelated to standard lemons and generally used as rootstock. ‘Millsweet’ limetta (Citrus limetta) and unnamed Sweet lemon (Citrus limettioides) bear sweet-tasting
fruits, which have a similar soluble solid content (Brix), but have reduced juice acidity and titratable acid content, as well as *CitPH1*, *CitPH5* and *CitMAC9F1* mRNAs expression compared to ‘Schaub’ and ‘Frost Lisbon’ fruits (Fig. 3b-c).

**Figure 3.** Analysis of ‘Schaub’, ‘Amber’, ‘Millsweet’ and Sweet lemon varieties. (a) Fruits, flower and leaf phenotypes of the analyzed varieties. Size bars represent 1 cm. (b) pH, titratable acid and soluble solid content (°Brix) in juice from different varieties (mean ± SE). (c) Real time RT-PCR of *CitPH1*, *CitPH5* and *CitMAC9F1* mRNA juice vesicles. (d) Real time RT-PCR of *CitAN1*, *CitPH3*, *CitPH4* and *CitAN11* mRNA in juice vesicles. Values in (c), (d) are mean ± SE; n = number samples from different fruits X number of technical replicates of each. For panels c-d full size nearly mature fruits were analyzed. Relative mRNA expression levels from ‘Frost Lisbon’ were taken from Fig. 1, and are repeated here for comparison. Source data are provided as a Source Data file.

*CitAN1*, *CitPH3* and *CitPH4* transcripts are low abundant in ‘Millsweet’ limetta and Sweet lemon compared to low-pH fruits, whereas *CitAN11* is expressed at similar levels as in low-pH fruits (Fig. 3d). ‘Schaub’, sweet ‘Amber’ and sour ‘Amber’ lemons have two *CitAN1* alleles with wild type structure (Fig. 4), differing only for a few SNPs in *CitAN1* and the flanking genes *CitFAR-like* and *CitTFI1H-like* (Supplementary Figs. 8-9). ‘Millsweet’ instead contains one allele with a wild type structure and a truncated allele (*citan1Msw*) with a 1.3 kb deletion. The deletion in *citan1Msw* has the same breakpoints as that in *citan1Fsw* (Fig. 4, Supplementary Fig. 9) suggesting that both alleles originate from the same deletion event, which
apparently occurred in the distant past as $citan_1^{FSw}$ and $citan_1^{Msw}$ and the flanking $CitFAR-LIKE$ and $CitTFIIH-like$ genes acquired since then several polymorphisms (Supplementary Figs. 8-9). As the first plant containing this 3’ deletion allele of $CitAN1$ was most likely heterozygous, homozygotes appeared later in progenies segregating for the deletion allele from various crosses, because of which today’s varieties can be homozygous ($FSw$) or heterozygous ($Msw$) for this allele.

**Figure 4. Structure and expression of $CitAN1$ alleles in distinct lemon, orange and pummelo varieties.** Exons are indicated by rectangles with 5’ and 3’ untranslated regions at reduced height. The hooked arrow marks the transcription start. Deleted sequences are indicated with dotted red lines and pink filling, and transposon insertions by triangles with different colors. The Faris sweet, Lima and Orange of Heaven alleles are most likely heterozygous over a complete deletion, and depicted as such, but that we cannot exclude homozygosity based on the analyses in this paper. The red dotted hooked
arrows in ‘Millsweet’ and Sweet lemon indicate (inferred) reduced transcription most likely caused by reduced activity of an upstream regulator(s). Gene structures confirmed by sequencing are depicted as dark grey lines (introns and flanking DNA), filled rectangles (exons), or filled triangles (transposon insertion). Gene structures inferred from PCR alone are marked by grey lines and white filling.

PCR analysis and partial sequencing showed that Sweet lemon contains a CitAN1 allele of normal size and a truncated allele with a deletion in 3’ end that may be similar to the deletion in citan1\textsuperscript{Fsw} and citan1\textsuperscript{Msw} (Supplementary Fig. 14). One of the Sweet lemon alleles, presumably the full size allele, contains in its upstream region the same 1.7-kb transposon insertion as found in the ‘Faris’ sour and ‘Frost Lisbon’ alleles (Fig. 4; Supplementary Fig. 6). Given that in ‘Millsweet’ limetta and Sweet lemon fruits mRNAs from both the full-size and a truncated CitAN1 allele are down-regulated, in spite of their very different origin, it is most likely that their reduced expression is caused by a mutation in an upstream trans-acting factor, rather than independent cis-acting mutations in each allele.

‘Amber’ is a chimera, like ‘Faris’, that originated as a variant tree in a grove of ‘Eureka’ lemons. Some branches on the ‘Amber’ tree bear pale green leaves, white flowers and sweet-tasting fruits with “amber” colored flesh, while other branches bear purplish young leaves, flowers with a purple blush and fruits with yellow flesh and sour taste (Fig. 3a). Sweet ‘Amber’ lemons have a low juice pH similar to sour ‘Amber’, ‘Schaub’ and ‘Frost Lisbon’ lemons and express similar CitPH1, CitPH5 mRNA levels as sour ‘Amber’ fruits and only slightly less than in ‘Frost Lisbon’.

Expression of the CitANI allele(s) in ‘Amber’ sour fruits, which contain the same transposon insertion as CitANI\textsuperscript{Fso}, and those in sweet ‘Amber’ fruit, which lack this insertion (Supplementary Figure 6) differs less than 2-fold, indicating that this transposon has little or no effect on the expression of CitANI and downstream genes. Thus ‘Amber’ sweet is essentially a low-pH variety that owes its sweet taste not from increased juice pH, from the reduced buffer capacity, as measured by the amount of titratable acid (Fig. 3b), which might result from genetic defects in distinct pathways affecting, for example, citrate transport into the vacuole.
Taken together we found that all varieties with low pH lemons (‘Frost Lisbon’, ‘Schaub’, sour ‘Amber’, sweet ‘Amber’) express relatively high levels of CitPH1 and CitPH5 mRNA, whereas in all varieties with strongly reduced acidity (‘Faris sweet’, ‘Millsweet’, ‘Sweet lemon’) CitPH1 and CitPH5 expression is reduced due to independent mutations that affect upstream transcription regulators. This strongly supports the view that CitPH1 and CitPH5 are essential for the hyperacidification of vacuoles in lemon juice vesicles and sour taste of the fruit.

**CitAN1 and CitPH4 are down-regulated in high-pH oranges.**

Next, we extended our investigation to orange varieties (Fig. 5a). Sweet (*Citrus sinensis*) and sour oranges (*Citrus aurantium*) are both hybrids originating from pummelo and mandarin\(^1\), with tastes ranging from sharply sour to insipidly sweet. We analyzed ‘Pineapple’ orange, a variety with moderate acid and sweet rich taste, three varieties with a bland (non-sour) taste (‘Lima’, ‘Vaniglia’ and ‘Orange of Heaven’), and sour oranges from two trees growing on different locations on Gran Canaria Island (small and large fruits) and one from Ostia, Italy. ‘Pineapple’ orange juice vesicles express CitPH1 and CitPH5 and CitMAC9F1 at similar levels as sour oranges, which correlates with the low pH of the fruit juice whereas the titratable acid content is much lower than in sour oranges (Fig. 5b-c).

The juice of ‘Lima’, ‘Vaniglia’ and ‘Orange of Heaven’ oranges has higher pH and lower titratable acidity than juice from the sour varieties from Ostia, Gran Canaria (GC1) and GC2, whereas the soluble solid content (Brix) is similar and therefore does not contribute to the taste differences (Fig. 5b). The high pH of ‘Lima’, ‘Vaniglia’ and ‘Orange of Heaven’ fruits correlates with strongly reduced amounts of CitPH1, CitPH5 and CitMAC9F1 mRNAs and ~4-fold less CitPH3 mRNA as compared to the low pH fruits from ‘Pineapple’ orange and the trees from Ostia, and Gran Canaria (GC1, GC2), possibly due to the strongly reduced levels of CitAN1 and CitPH4 mRNA (Fig. 5c-d). RNA seq data revealed that CitPH5, CitPH4 and
CitAN1 mRNAs are also down-regulated in the ‘Sucarri’ and ‘Bintang’ sweet oranges.35

Figure 5. Analysis of sweet and sour orange varieties. (a) Fruits, flower and leaf phenotypes of the analyzed varieties. Size bars represent 1 cm. (b) pH, titratable acid and soluble solid content (°Brix) in juice from full colored mature fruits (mean ± SE; n ≥ 3). Note that titratable acid was not determined for Ostia (nd). (c) Real time RT-PCR of CitPH1, CitPH5 and CitMAC9F1 mRNA in juice vesicles from full colored mature fruits. (d) Real time RT-PCR of CitAN1, CitPH3, CitPH4 and CitAN11 mRNA in juice vesicles from full colored mature fruits. Values in (c), (d) are mean ± SE; n = number samples from different fruits X number of technical replicates of each. Source data are provided as a Source Data file.

The low-pH oranges from Gran Canaria (GC1 and GC2) and Ostia all have two CitAN1 alleles, which differ for a few SNPs and the presence/absence of the same transposon insertion as in CitAN1Fso and CitAN1Aso (Supplementary Figs. 6-7; Fig. 4). The citan1 allele(s) of the high-pH oranges are disrupted by the insertion of a 6.9 kb transposable element containing 39bp inverted repeats (MudR-like element) in intron 2. We detected no sequence polymorphisms indicating the presence two distinct citan1 alleles, suggesting that ‘Lima’ and ‘Orange of Heaven’ in either homozygous or hemizygous condition. Given, that sweet orange varieties were propagated asexually for long time, the latter possibility seems the more likely. The
citan1 allele(s) of ‘Vaniglia’ are disrupted by the insertion of a 5.3-kb copia-like TCS1 retrotransposon in exon 6 (Fig. 4). The down-regulation of CitPH4 is most likely due to an independent mutation, as CitPH4 expression in lemons is independent from CitAN1 (Fig. 1).

No relevant differences in expression of CitAN11 were detected in fruits of low and high pH orange varieties, while CitPH3 is low expressed in high pH oranges as compared to the low pH ones, consistent with its (partial) regulation by CitAN1 and CitPH427. Because the CitPH4 alleles from these varieties contain no mutations in the coding sequence (Supplementary Fig. 10), it is conceivable that transcription of CitPH4 (and possibly CitAN1) transcription is reduced by additional mutations in higher rank regulator(s).

These data show that CitPH1 and CitPH5 are also in oranges responsible for hyperacidification in juice vesicles, and that independent mutations in CitAN1, and an (unknown) upstream regulator of CitPH4 have been selected to obtain fruit varieties with decreased acidity.

**Analyses of acidless pummelos and rangpur limes.**

We further broadened our survey to a group of pummelo accessions (Fig. 6a). Pummelos (C.maxima) are non-hybrid citrus fruits1, 3. They are similar to large grapefruit, variable in sweetness, and native of Southeast Asia, but now grown in tropical and subtropical areas all over Asia and the Pacific Islands as well as in California and Florida.

‘Pin Shan Kong Yau’ (PSKY) and an unnamed ‘Kao Panne’ pummelo (Punk) are acidic pummelos with blond fruit flesh (Fig. 6a), whereas the unrelated Siamese pummelo P2240 (also known as ‘Siamese sweet’) is an acidless pummelo with blond flesh that is commonly used in breeding to reduce acidity in the progeny. The “sweet” (non-sour) taste of P2240 fruits correlated with reduced acidity and titratable acid content of the fruit juice and reduced CitPH1, CitPH5 and
CitMAC9F1 mRNA levels in juice vesicles, as compared to the low pH PSKY and Punk (Fig. 6b-c). In the pink mildly sweet-tasting fruits of ‘Chandler’, a hybrid of P2240 and an acidic accession (P2241), the juice pH as well as CitPH1, CitPH5 and CitMAC9F1 mRNA levels are similar to those the low-pH PSKY and Punk fruits. This indicates the acidification of pummelo fruits also depends on CitPH1 and CitPH5, and that the causative mutation(s) that reduce the acidity and expression of CitPH1 and CitPH5 in P2240 fruits are recessive.

Figure 6. Analysis sweet and sour pummelo and Rangpur lime varieties. (a) Fruits of the analyzed pummelo varieties. Size bars represent 1 cm. (b) pH, titratable acid and soluble solid content (Brix) in juice from the analyzed varieties (mean ± SE). (c) Real time RT-PCR of CitPH1, CitPH5 and CitMAC9F1 mRNA in juice vesicles. (d) Real time RT-PCR of CitAN1, CitPH3, CitPH4 and CitAN11 mRNA in juice vesicles. (e) Fruits, flower and leaf phenotypes of the analyzed rangpur limes. Size bars represent 1 cm. Values in (c), (d) are mean ± SE; n = number samples from different fruits X number of technical replicates of each. Fruits analyzed in b-d were mature with a slight green blush (pummelos), or full colored mature (rangpurs and rangur x pummelo hybrids). Images of Punk and ‘Chandler’ fruits were provided by and reproduced with permission of the UC-Riverside Citrus Variety collection. Source data are provided as a Source Data file.
Since the reduced acidity of P2240 fruits is associated with down-regulation of at least three genes (Fig. 6c) the causative mutation(s) most likely affect one of more upstream transcription activators. Because the *CitAN1*, *CitPH3* and *CitPH4* alleles of *P2240* express in juice vesicles similar mRNA amounts as those from Punk and ‘Chandler’ (Fig. 6d), and because their coding sequences do not contain polymorphisms with obvious negative effects on protein activity (Supplementary Figs 11 and 15), the causative mutation likely affects an unknown transcription factor that operates in concert with *CitAN1*, *CitPH3*, and *CitPH4*, which may be encoded or controlled by an unknown locus (*acitric*) within 1.2 cM of the RFLP marker RFZ20 (ref. 42), which maps to genome sequences on chromosome 2.

The rangpur limes (*Citrus limonia*) ‘Philippine’ and ‘Weirick’ have flowers and leaves with anthocyanin pigments (Fig. 6e). While soluble solid content (Brix) is similar, the acidity and amount of titratable acid is reduced in juice from sweet tasting ‘Weirick’ fruit, compared to the sour fruits from ‘Philippine’ (Fig. 6b). This correlates with strongly reduced mRNA expression levels of *CitPH1*, *CitPH5* and *CitMAC9F1*, most likely due to reduced activity of an upstream transcription factor (Fig 6c). *CitAN1* and *CitPH4* transcripts accumulate at similar levels in sour ‘Philippine’ and sweet ‘Weirick’ juice vesicles, whereas *CitPH3* mRNA was essentially abolished in ‘Weirick’ fruits (Fig. 6d). PH3 is in petunia, like other WMBW complex components (AN1, PH4 and AN11), essential for *PH1*, *PH5* and *MAC9F1* expression27, suggesting that the strongly reduced *CitPH1* and *CitPH5* expression in Weirick fruits is most likely caused by abolished *CitPH3* expression.

We found no obvious defects in the coding part of the ‘Weirick’ *CitPH3* allele (Supplementary Fig. 16) or any large rearrangements in its promoter that may account for the reduced *CitPH3* transcription or mRNA processing. Hence, we infer that the reduced acidity and titratable acid is most likely due to a mutation in an (unknown) upstream regulator(s), which strongly reduces expression of *CitPH3* and its target genes *CitPH1*, *CitPH5* and *CitMAC9F1*. 
The hybrid Weirick x P2240 (7D-76-03) had sour fruits with high titratable acid, indicating that loss of acidity in Weirick and P2240 is due to recessive mutations in distinct genes. Since 7D-76-03 is no longer available we analyzed progeny with sour (6C-32-01) and sweet fruits (6C-32-02) from the cross ‘Chandler’ x 7D-76-03 in which segregation of acidity is observed. Whereas soluble solid content (Brix) is similar in both fruits, juice acidity, titratable acid content and expression of CitPH1, CitPH5 and CitMAC9F1 are all reduced in 6C-32-02 fruits compared to 6C-32-01. The latter is probably due to mutations originating from P2240 rather than ‘Weirick’, as CitPH3 expression in 6C-32-02 is strongly increased compared to ‘Weirick’ (Fig. 6d).

Discussion

How juice cell vacuoles in sour citrus fruits can be acidified to such an extreme extent is a long-standing question, as vacuolar proton pumps capable of generating the required steep pH gradient across the tonoplast were unknown. We have shown that juice vesicles of Citrus varieties with acidic (low pH) fruits express CitPH1 and CitPH5, encoding two interacting P-ATPases that constitute a vacuolar proton pump, while CitPH1 and CitPH5 expression levels are drastically decreased in fruit varieties with reduced acidity (high pH). The down regulation of CitPH1 and CitPH5 in distinct fruits results from independent mutations in multiple genes required for CitPH1 and CitPH5 expression, such as for example CitAN1, or upstream regulators thereof, suggesting that these are the causative mutations for the loss of acidity rather than being linked to them. A contemporaneous study also concluded that loss of fruit acidity is associated with mutations in CitAN1 (ref43). These results indicate that the long-sought vacuolar proton pump is a P-ATPase complex, encoded by CitPH1 and CitPH5, previously identified for its role in the pigmentation of flowers and seeds. The coupling ratio of P-ATPases (H+/ATP =1), the sensitivity of PH1/PH5 activity to vanadate and its insensitivity to bafilomycin28 fit perfectly with
the biochemical properties of the proton pump activity that is expressed in sour (low pH) fruits but absent in sweet fruit varieties\(^8,12-15\).

While our results show that the large pH differences between acidic (low pH) and acidless (high pH) varieties within a Citrus group (lemons, oranges or pummelos) are due to differences in CitPH1 and CitPH5 expression, the cause(s) of the much smaller pH variations between acidic (non-mutant) varieties of different Citrus groups or the even smaller pH differences within a group remain unclear. Given that the analyzed varieties are not isogenic and not grown under identical circumstances such small pH differences may originate from small differences in the expression of CitPH1/CitPH5 or other transporters, like CitSO or various vacuolar antiporters that import solutes in exchange for protons\(^17\).

It is noteworthy that fruits with strongly reduced CitPH1 and CitPH5 expression all contain reduced amounts of titratable acids, which is in Citrus mostly citric acid. This provides in vivo support of biochemical data, which indicated that most of the citrate transport into vacuoles is driven by the H\(^+\) gradient across the tonoplast (\(\Delta p\text{H}\)), while only a small part relies on ATP-driven transporters\(^9\). Hence, we infer that CitPH1 and CitPH5 promote sour taste by (i) hyperacidifying vacuoles resulting in low pH of the fruit (juice) and by (ii) generating the steep pH gradient required for the import and sequestration of citrate and conjugate bases of other acids into the vacuole, which increases pH-buffering capacity and prevents that juice pH is neutralized by saliva before the low pH can be sensed.

Citrus varieties have been subject to cultivation and selection for several thousands of years\(^3\). Our data show that citrus varieties with reduced acidity arose multiple times independently in different citrus lineages through mutations disrupting the expression of genes that encode transcription activators of CitPH1 and CitPH5. The finding that the citan\(_{Fsw}\) and citan\(_{Msw}\) alleles contain several SNPs, suggests that the inactivating 3’ deletion and, hence, varieties with reduced acidity arose in the
distant past, possibly hundreds if not thousands of years ago during early stages of *Citrus* domestication. It is, however, difficult to give a precise timing, without a better estimate of mutation rates in (inactivated) *Citrus* genes that are not under selection.

Inspection of public RNA-seq data\(^4^4\) indicates that *Malus domestica* homologs of *PH1* (MDP0000319016) and *PH5* (MDP0000303799) are expressed in developing apples and RT-PCR data\(^3^0\) indicate that the *Vitis vinifera* homologs *VvPH1* and *VvPH5* are expressed in developing grape berries. Hence, *PH1* and *PH5* and genes encoding upstream transcription activators are likely to be important determinants of the acidity and taste in many other fruits besides *Citrus*.

Taken together our genetic data show that a vacuolar proton-pump consisting of the P-ATPases PH1 and PH5 is required for the hyperacidification of vacuoles in juice vesicles and the very sour taste of Citrus fruits and juices, and that over thousands of years of *Citrus* breeding “sweet” (non-sour) tasting varieties were obtained many times via independent mutations in distinct transcription regulators driving *CitPH1* and *CitPH5* expression. This opens the way to develop molecular markers for fruit acidity and taste, to speed up the breeding in *Citrus* and other fruit crops, most of which are trees or shrubs with long generation times.

**Methods**

**Plant material**

Lemon, sweet orange, pummelo and rangpur lime fruits were collected from trees belonging to the Citrus Variety Collection or from other citrus orchards on the Agricultural Experiment Station at the University of California, Riverside, CA. Sour oranges were collected from a tree in Ostia, Italy (Ostia orange) and from trees grown in the botanical garden Jardin Botanico Canario Viera y Clavijo (GC1) and from the town Agüimes (GC2) in Gran Canaria, Spain. For transient expression
assays and isolation of protoplasts we used petals from the petunia line V74 (ph4 mutant) grown under normal greenhouse conditions.

**Fruit taste parameters**

Vesicles were excised from the fruits and frozen in liquid nitrogen. After grinding 800 mg of frozen pulp, and dissolving in 6 ml distilled water, pH was immediately measured with a pH meter (Consort P901). Brix was determined by directly reading the juice on a refractometer (Marius, Poland). The acid content (expressed as citric acid equivalents) was evaluated by titration using sodium hydroxide (0.1 M) and a phenolphthalein pH indicator. To deliver and measure the volumes we use a volumetric buret.

**Citrus homologs of petunia AN and PH genes and melon SO**

To identify *Citrus* homologs of petunia and melon genes, we searched *Citrus* genome sequences at Phytozome (https://phytozome.jgi.doe.gov/pz/portal.html) and *Citrus sinensis* Annotation Project (http://citrus.hzau.edu.cn/orange/index.php) with BLAST and confirmed homology by phylogenetic analysis\(^3\). Protein sequences were aligned using MUSCLE, and after curation by GBLOCKS phylogenetic trees were constructed with maximum likelihood (PHYML) using online tools\(^4\). For comparison of DNA and proteins, sequences were aligned with Clustal-Omega (https://www.ebi.ac.uk) and MUSCLE (http://phylogeny.lirmm.fr/phylo.cgi/index.cgi) respectively, optimized by hand using Aliview\(^4\), and colored using BOXSHADE (https://embnet.vital-it.ch/software/BOX_form.htm).

*CitANI*, *CitPH4*, *CitPH3*, *CitAN11* alleles from citrus varieties (Supplementary Table 2) were isolated by PCR with primers amplifying the entire genomic sequence (for *CitAN1* gDNA primer 7642 and 7590, for *CitAN1* cDNA primer 7642 and 6592, for *CitPH4* gDNA and cDNA primers 6572 and 6573, for *CitPH3* cDNA primer 6611 and 6612, for *CitAN11* cDNA primer 6613 and 6615). PCR fragments were
then directly sequenced or cloned into pDONR P1-P2 by Gateway cloning system. 

*CitSO* cDNAs were amplified by RT-PCR from juice vesicle RNA using primers 8932 and 8933.

To isolate *CitAN1* promoters, PCR fragments were generated using primers covering the 4-kb promoter (primer 7674 and 7473) and used directly for sequencing.

To isolate the flanking sequences of the *CitAN1* gene we designed primers to amplify fragments (1000 bp) of the *FAR like* gene located 29 kb upstream of the start of the *AN1* coding sequence (primer 8066 and 8068) and of the *TFIIH* gene (570 bp) located 617 bp downstream of the *AN1* stop codon (primer 8063 and 8065). PCR fragments were directly sequenced and nucleotide polymorphisms were depicted using the IUPAC code. Primer sequences are shown in Supplementary Table 3-7.

### DNA and RNA isolation from *Citrus* vesicles

For DNA and RNA extraction, 800 mg of frozen vesicles were ground in liquid nitrogen and 7.5 ml of preheated (65°C) extraction buffer (2% (w/v) CTAB, 2% (w/v) PVP (molecular weight 30,000-40,000), 25 mM EDTA pH 8.0, 2 M NaCl, 100 mM Tris-HCl (pH 8.0), 2% β-mercaptoethanol) was added to the frozen powder. To (re)adjust the solution to pH 7-8, we added for citrus vesicles from very acidic fruits (pH 2-2.5) approximately 800 µl 1M Tris/HCl pH 9.0 and for less acidic or acidless fruits 400 µl Tris/HCl pH 9.0 and verified the pH using pH paper. The samples were then incubated at 65°C for 15 min and extracted twice with chloroform-isooamyl alcohol (24:1), precipitated with 2-propanol, resuspended in sterile water, extracted with phenol/chloroform (1:1), precipitated with NaOAc and 2-propanol, washed in 70% ethanol and resuspended in RNAse free water. Total RNA was precipitated using 1 volume of 4M LiCl. The RNA pellet was washed with 70% ethanol, dissolved in sterile water and quantified by measuring OD$_{260/280}$.

To ensure absence of genomic DNA we performed a DNAse treatment.
Total DNA was obtained from the supernatant left after LiCl precipitation of RNA by precipitation with 2-propanol, washed with 70% ethanol, dissolved in water and checked by agarose electrophoresis and OD$_{260/280}$.

**Expression analysis**

To identify the truncated AN1 transcript in *Fsw* juice vesicles, we amplified the 3’ cDNA ends by 3’-RACE (5’/3’-RACe KIT 2nd generation; Roche). RT products from *Fso* and *Fsw* AN1 were amplified with a primer complementary to the 5’ UTR region (primer 6577) and an adaptor primer complementary to the poly(A) tail (primer 64a). Two nested PCR were then performed using gene-specific primers; nested1 PCR: primer 6577 designed on the 5’ UTR and adaptor primer 65a (tail adaptor); nested2 PCR: primer 6579 designed on the exon 5 sequence and adaptor primer 65a.

RT-PCR analysis of *CitPH1*, *CitPH5*, *CitAN1*, *CitPH3*, *CitPH4*, *CitAN11* and *CitACTIN* were performed as described previously$^{24, 26, 47}$, using primers shown in Supplementary Table 3. cDNA products were amplified using primers specific for *PH1* (primers 6524 and 4383), *PH5* (primer 6530 and 6529), *AN1* (primer 6623 and 6593, for the *Fsw* allele: primer 6623 and 6592), *PH3* (primer 6538 and 6540), *PH4* (primer 6544 and 6546), *AN11* (primer 6614 and 6615), *ACTIN* (primer 58a and 59a). All primer sequences are reported in Supplementary Table 3.

Quantitative RT-PCR was performed with an QuantStudio 3 Real-Time PCR System (Thermo Fisher Scientific) using the SensiMix (Bioline QT650-05) as described before$^{27}$, using primers shown in Supplementary Table 8. Relative expression was calculated by normalizing against *CitANKYRIN*, *CitANNEXIN2* and *CitRIBOSOMAL PROTEIN S10*, which are the most constantly expressed genes in broad range of tissues and *Citrus* species$^{33}$, and *CitACTIN11*. For ‘Schaub’, ‘Amber’ sour, ‘Amber’ sweet, Sweet lemon, GC1, GC2 and Ostia one biological replicate
was analyzed and for all other varieties two or three fruits. For each fruit two qPCR
reactions (technical replicates) were performed.

**Gene constructs and transient expression in protoplasts**

To generate 35S::CitAN1\textsuperscript{Fso} cDNA, the 2.2 Kb full size cDNA AN1 was amplified
from Fso cDNA with primers 6623 and 6593 and used to generate a Gateway Entry
cloning by BP reaction with pDONR P1-P2 (Gateway system; Life Technologies,
Invitrogen, Carlsbad, CA, USA) and then recombined into the pK2GW7.0
overexpression vector.

35S::CitAN1\textsuperscript{Fsw} cDNA construct was obtained by amplifying the PCR fragment of
Fsw cDNA, with primers 6623 and 6595 and cloning the 1.3 Kb cDNA fragment in
pDONR P1-P2. The insert was then recombined into pK2GW7.0 by Gateway
recombination. The 35S::GFP-CitPH4\textsuperscript{Fso} construct was generated by amplification of the
PH4\textsuperscript{Fso} cDNA with a 3' race PCR primer (6572) and a 65a (oligo dT), cloning in
pDONR P1-P2 and recombining in pK7WGF2.0 by Gateway system. The 35S::GFP-CitPH4\textsuperscript{Fsw} construct was obtained similarly from cDNA of Fsw juice
vesicles.

Promoter: RFP constructs of the target genes were generated as follows: a 3.3Kb
PH1 promoter fragment was amplified with primers 7326 and 7328, cloned in
pDONR P1-P2 and recombined into pWSK by Gateway system; a 3Kb PH5
promoter fragment was amplified with primers 7194 and 7195, and cloned with the
same procedure. In 35S::AtAHA10-GFP the 35S promoter drives expression of a
translational fusion of the coding sequence of the AHA10 gene (including all introns)
and GFP\textsuperscript{30, 32}. All primer sequences are reported in Supplementary Table 9. For
isolation and transformation of petunia protoplasts\textsuperscript{28, 31, 32} chopped corollas of 15
open flowers (stage 6-7) from line V74 (ph4) were incubated in the dark for 16
hours at room temperature in 0.2% (w/v) macerozyme R-10, 0.4% (w/v) cellulose
R-10 in TEX buffer (3.1 g/L Gamborg’s B5 salts (Sigma-Aldrich), 500 mg/l 4-
morpholineethanesulfonic acid (MES), 750 mg/l CaCl$_2$, 250 mg/l NH$_4$NO$_3$, 136.9 g/l sucrose, pH 5.7) and centrifuged for 10 minutes at 700 RPM. The floating protoplasts were recovered by removing the underlying layers, washed twice in TEX buffer, left for 2 hours at 4°C. After centrifugation (10 minutes, 700 RPM) floating protoplasts were recovered and resuspended in 5 ml MMM solution (0.1% (w/v) MES, 0.5 M Mannitol, 15 mM MgCl$_2$). For transformation 300 µl protoplast suspension, 20 µg plasmid DNA and 300 µl PEG solution (0.4 M Mannitol, 0.1 M Ca(NO$_3$)$_2$, 40% (w/v) Polyethyleneglycol 4000, pH 8) were mixed, and after 1 minute 2 ml of TEX buffer was added. After 2 hours at room temperature, 5 ml W5 buffer (154 mM NaCl, 125 mM CaCl$_2$, 5mM KCl, 5 mM glucose) was added and protoplasts were pelleted for 5 minutes at 700 RPM, resuspended in 2 ml TEX buffer and kept for 16 hours in the dark at room temperature and then analyzed by confocal microscopy and RNA isolation.

Total RNA was extracted from protoplasts using the NucleoSpin® RNA II kit (Macherey-Nagel, Düren, Germany). In the expression analysis after transient transformation, $AHA10$ mRNA was used as reference to normalize the data.

GFP, RFP and anthocyanin fluorescence were imaged with a LSM Pascal Zeiss confocal microscope.$^{28,31,32}$

**Data availability**

Identifiers for genes in distinct *Citrus* species and varieties can be found in Supplementary Table 2. Sequence data generated in this study have been deposited in NCBI Genbank and are accessible under accession numbers MH843936-MH843962 (cDNAs of *CitSO* and *CitPH3*), MH885854–MH885946 (genomic DNAs of *CitAN1*, *CitPH4*, *CitPH3*, *CitFAR*-like, *CitTFIIH*-like), and MH898434-MH898465 (cDNAs of *CitAN1*, *CitPH4*, *CitAN11*, and *CitPH3*). A reporting summary for this article is available as a Supplementary Information file. Source
data underlying Figs. 1-3, 5, and 6 and Supplementary Figs. 1, 2, 5 and 13 are provided as a Source Data file. Other data and biological materials are available from the authors on request. Requests for fruits from the Riverside Citrus Variety Collection should be addressed directly to Mikeal Roose (roose@ucr.edu). Availability is dependent on flowering and fruiting season and on the recipient providing an appropriate import permit if required.
Supplementary information

Supplementary Figure 1. Expression of CitSOUR in juice cells of various Citrus fruits. Relative gene CitSO expression levels in juice cells were determined by real-time RT-PCR. Four genes (CitANKYRIN, CitANNEXIN2 and CitRIBOSOMAL PROTEIN S10, and CitACTIN11) served as a constitutively expressed reference mRNAs for normalization. Gene identifiers of CitSO homologs are given in Supplementary Table 2. Values shown are mean ± SE; n = number of samples from different fruits X number of technical replicates of each. Source data are provided as a Source Data file.
Supplementary Figure 2. Alignment of predicted CitSO proteins expressed in fruits from distinct varieties. Cm_sour, AIC32551.1; Cm_sweet, XP_008463302.1.
Supplementary Figure 4. Identification of AN1, PH4, PH3, AN11 and MAC9F1 homologs from Citrus. (a) Phylogenetic analysis of AN1 homologs. The closely related HLH proteins JAF13 from petunia and GLABROUS3 (GL3) from Arabidopsis served as an outgroup. (b) Phylogenetic analysis of PH4 homologs. The closely related MYB protein ANTHOCYANIN2 (AN2) from petunia was used as an outgroup. (c) Phylogenetic analysis of PH3 homologs. The next most similar WRKY protein from Arabidopsis (WRKY4) was used as an outgroup. Additional evidence for orthology of PH3, TTG1 and Citrus homologs can be found elsewhere. (d) Phylogenetic analysis of AN11 homologs. The Arabidopsis homolog is TRANSPARENT TESTA GLABRA1 (TTG1). Two TTG1 paralogs, initially named AtAN11a and AtAN11b, and the human homolog HsAN11 served as an outgroup. (e) Phylogenetic analysis of MAC9F1 homologs. MAC9F1 is encoded by a single gene in all species analyzed and not part of a family. As an outgroup we used the next most similar protein from C. sinensis (orange 1.1g031889). For each protein a Genbank accession number and/or gene identifier is given in brackets. Branch support is calculated on the basis of 300 bootstraps and indicated as percentage. Prefixes denote the species of origin as follows: At, Arabidopsis thaliana; Cs, Citrus sinensis; Cc, Citrus clementina; Fv, Fragaria vesca; Gh, Gossypium hirsutum; Gm, Glycine max; Hs, Homo sapiens; Ph, Petunia hybrida; Pp, Prunus persica; St, Solanum tuberosum; Sl, Solanum lycopersicum; Vv, Vitis vinifera. Gene IDs of homologs in other Citrus species/varieties are given in Supplementary Table 2.
Supplementary Figure 5. Expression pattern of Citrus homologs of *PH5*, *MAC9F1*, *AN1*, *JAF13*, *PH3*, *PH4* and *AN11*. Expression data were extracted from publicly available genome-wide RNA-seq data."
Supplementary Figure 6. PCR analysis of CitAN1 promoter in different Citrus varieties. (a) Diagram showing the structure of a CitAN1 allele with an insertion of a 1661-bp hAT-type transposon (Tn; yellow/orange rectangle) in the promoter. (b) PCR analysis to detect the presence of transposon copies in the promoter of CitAN1 alleles from different Citrus fruits. Yellow circles indicate lemon varieties, orange circles indicate sweet and sour orange varieties and red circles indicate pummelo varieties. Note that PCR with primers 7706 and 7880 only yields a PCR product when the transposon is present in the fragment, while the size of the fragment indicates its position (top panels). PCR with primers 7706 and 7681 yields PCR products of two sizes when the transposon is heterozygous. Primer sequences used are shown in Supplementary Table 5-6.
**Supplementary Figure 7. Analysis of AN1 promoters.** Alignment of AN1 promoter sequences of ‘Frost Lisbon’, ‘Faris’ sour, ‘Schaub’, GC1, GC2, Ostia, Vaniglia, Heaven (primers 7706 and 7681). The left and right inverted repeats (LIR, RIR) are marked in blue. The 8-bp target site duplication (TSD) caused by the insertion is marked in red.
Supplementary Figure 8. Polymorphisms in CitFAR-like and CitTFIIH. (a) Diagram showing structure of the genomic region surrounding CitAN1 in Citrus sinensis cv Pineapple Ridge (top). The intron-exon structure CitFAR-like (orange1.1g014509) and CitTFIIH-like (orange 1.1g004358m) is shown below; the black line bar indicates the region (re)sequenced to establish homo- or heterozygosity of this genomic region. (b) Distribution of SNPs found in the (re)sequenced region (~1 kb) of CitFAR-like. (c) Distribution of SNPs found in (re)sequenced region (~0.6 kb) from the CitTFIIH-like. Nucleotide ambiguity codes indicating mixed sequences due to heterozygosity or low quality sequence reads are indicated in red letters, following IUPAC code (W = A/T; S=G/C; M=A/C; K=G/T; R=A/G; Y=C/T). Dashes on a white background indicate absence of nucleotides (indel). Grey shading indicates sequences not determined. Primer sequences used for PCR amplification and sequencing are described in Supplementary Table 4.
Supplementary Figure 9. Comparison of the CitAN1 alleles from ‘Faris’ sour (Fso), ‘Faris’ sweet (Fsw) and ‘Millsweet’ lemons (Msw) and C. medica. Blue bars overlying the sequences indicate exons. The deletion in \textit{citan1}^{Fsw} and \textit{citan1}^{Msw} is indicated by yellow shading, the downstream sequence until the polyadenylation site is indicated with an orange bar below the sequence. SNPs distinguishing \textit{citan1}^{Fsw} and \textit{citan1}^{Msw} are marked by magenta shading.
Supplementary Figure 10. Alignment of \textit{CitPH4} alleles from \textit{Citrus} species and varieties. Blue bars overlying the sequences indicate exons. Start and stop codons are indicated by red lettering.
Supplementary Figure 11. Comparison of CitPH3 and CitPH4 proteins. (a) Alignment of CitPH3 proteins from different Citrus varieties with PH3 from petunia and the functionally interchangeable homolog TTG2 from Arabidopsis. The two WRKY domains are marked in orange. (b) Alignment of CitPH4 proteins from different Citrus accessions with PH4 from Petunia and its apparent homolog, AtMYB5, from Arabidopsis. The R2 and R3 repeats, which make up the MYB DNA binding domain and G20 boxes, which are signatures for this subclass of MYB proteins, are marked in orange.
Supplementary Figure 12. Comparison of CitAN11 proteins.
Supplementary Figure 13. Confocal micrographs of petunia ph4 mesophyll petal protoplasts from stage 6 flowers 24 hours after transformation 35S:GFP-CitPH4<sup>Fso</sup>, 35S:GFP-CitPH4<sup>Fsw</sup>, 35S:CitAN1<sup>Fso</sup>, 35S:CitAN1<sup>Fsw</sup>, pCitPH1:RFP, pCitPH3:RFP in different combinations. (a) Petal mesophyll protoplast expressing 35S:GFP-CitPH4<sup>Fso</sup> showing accumulation of GFP-CitPH4<sup>Fso</sup> in nucleus and cytoplasm (b) Petal mesophyll protoplasts co-transformed with the reporter genes pCitPH1:RFP, pCitPH3:RFP, the control gene 35S:AtAHA10-GFP and different combinations of effector genes expressing <i>Fso</i> and <i>Fsw</i> alleles of CitAN1 and/or GFP-CitPH4, as indicated on the left. Note that the central vacuole lacks anthocyanins, indicating that these protoplasts originate from the petal mesophyll. Expression data on epidermal cells, which contain anthocyanins, are shown in Fig 2. Size bars equal 10 μm.
Supplementary Figure 14. Identification of truncated CitAN1 allele in Sweet lemon. (a) Diagram showing the structure of the functional CitAN1 allele (top) and the mutant citan1 Fsw in which the 3’end of the gene is deleted (blue dotted lines). (b) PCR analysis of genomic DNA obtained from the fruits of different Citrus varieties with primers 7193 and 6806 (PCR1: this gives 1048 bp product when CitAN1 is full size) and primers 7536 and 7522 (PCR2: this yield either 1619 bp in a full size allele or a smaller PCR product when the allele is truncated). Yellow circles indicate lemons, orange circles indicate sweet and sour oranges and red circles indicate pummelos. Primer sequences given in Supplementary Table 3. Source data are provided as a Source Data file.
Supplementary Figure 15. Comparison of CitAN1 proteins encoded by distinct Citrus alleles.
Supplementary Figure 16. Comparison of CitPH3 alleles. Blue bars overlying the sequences indicate exons. Red lettering indicates start and stop codon.
Supplementary Table 1. Description of Citrus varieties investigated in the study

<table>
<thead>
<tr>
<th>Variety</th>
<th>Species/Origin</th>
<th>General Description</th>
<th>Taste</th>
<th>CRC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Schaub rough lemon</td>
<td>C. jambhiri</td>
<td>Standard rough lemon</td>
<td>Sour</td>
<td>3879</td>
</tr>
<tr>
<td>Frost Lisbon lemon</td>
<td>C. limon</td>
<td>Standard commercial lemon</td>
<td>Sour</td>
<td>3176</td>
</tr>
<tr>
<td>Amber lemon Eureka</td>
<td>C. limon</td>
<td>Chimera. Trees have branches with purple flowers and yellow flesh and branches with white flowers and orange flesh. Yellow flesh-fruits are sour like a standard lemon, orange flesh-fruits are sweet.</td>
<td>Sour</td>
<td>2429</td>
</tr>
<tr>
<td>Faris lemon</td>
<td>C. limon</td>
<td>Chimera. Trees have branches with purple flowers and branches with white flowers. Both fruits have yellow flesh. Branches with purple flowers have acid tasting fruits, Branches with white flowers have sweet, almost acidless fruits.</td>
<td>Sour</td>
<td>2695</td>
</tr>
<tr>
<td>Millsweet limetta</td>
<td>C. limetta</td>
<td>It is the closest genotype to the sweet portion of Faris</td>
<td>Sweet, but with a relatively low pH</td>
<td>569</td>
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<tr>
<td>Unnamed sweet lemon</td>
<td>C. limettoides?</td>
<td>Sweet lime-type</td>
<td>Slightly acidic like limetta</td>
<td>3093</td>
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<tr>
<td>Pin Shan Kong Yau (PSKY)</td>
<td>C. maxima</td>
<td>Standard pummelo</td>
<td>Acid</td>
<td>2348</td>
</tr>
<tr>
<td>Siamese acidless pummelo</td>
<td>C. maxima</td>
<td>Commonly used in breeding to reduce acid in progeny</td>
<td>Sweet, acidless</td>
<td>2240</td>
</tr>
<tr>
<td>Pummelo unknown (Punk)</td>
<td>C. maxima</td>
<td>White flesh, yellow rind</td>
<td>Sour (mildly acidic)</td>
<td>none</td>
</tr>
<tr>
<td>Chandler</td>
<td>C. maxima (hybrid of P2240 × P2241)</td>
<td>The flesh varies in color from light pink to very dark pink</td>
<td>Sweet, but with a relatively low pH</td>
<td>3224</td>
</tr>
<tr>
<td>6C-32-01</td>
<td>[Chandler × (Weirick sweet rangpur lime × P2240)]</td>
<td>One progeny tree from a cross that segregates for acidity</td>
<td>Sour</td>
<td>none</td>
</tr>
<tr>
<td>6C-32-02</td>
<td>[Chandler × (Weirick sweet rangpur lime × P2240)]</td>
<td>One progeny tree from a cross that segregates for acidity</td>
<td>Sweet</td>
<td>none</td>
</tr>
<tr>
<td>Weirick sweet rangpur lime</td>
<td>C. limonia</td>
<td>It is the male parent of record for the male hybrid parent of 6C-32-01 and 6C-32-02</td>
<td>Sweet</td>
<td>1684</td>
</tr>
<tr>
<td>Philippine rangpur lime</td>
<td>C. limonia</td>
<td>Standard rangpur type. Results on same Affymetrix SNP chip as Weirick were nearly identical</td>
<td>Sour</td>
<td>2318</td>
</tr>
<tr>
<td>Pineapple orange</td>
<td>C. sinensis</td>
<td>Standard seedy orange</td>
<td>Sweet, but with a relatively low pH</td>
<td>3858</td>
</tr>
<tr>
<td>Lima orange</td>
<td>C. sinensis</td>
<td>Low acid type of orange</td>
<td>Sweet, taste almost acidless</td>
<td>950</td>
</tr>
<tr>
<td>Orange of Heaven</td>
<td>C. sinensis</td>
<td>Low acid type of orange</td>
<td>Sweet</td>
<td>371</td>
</tr>
<tr>
<td>Vaniglia Sanguigno</td>
<td>C. sinensis</td>
<td>Low acid type of orange. Pink flesh pigmented by lycopene.</td>
<td>Sweet</td>
<td>3801</td>
</tr>
<tr>
<td>unnamed sour orange (GC1)</td>
<td>C. aurantium</td>
<td>Fruit from tree in the botanical garden, Jardín Botánico Canario Viera y Clavijo, in Gran Canaria (Spain). It is a normal orange type.</td>
<td>Sour</td>
<td>none</td>
</tr>
<tr>
<td>unnamed sour orange (GC2)</td>
<td>C. aurantium</td>
<td>Fruit from tree in the town Agüimes, Gran Canaria (Spain). It is a more yellowish orange, smaller than an ordinary orange.</td>
<td>Sour</td>
<td>none</td>
</tr>
</tbody>
</table>

* CRC= catalog number used by the Citrus Variety Collection
Supplementary Table 3. Primers used for the amplification of CitPH1, CitPH5, CitAN1, CitPH3, CitPH4, CitAN11 genes and/or cDNA.

<table>
<thead>
<tr>
<th>primer gene</th>
<th>sequence 5'-3'</th>
<th>orientation</th>
</tr>
</thead>
<tbody>
<tr>
<td>CitISO</td>
<td>GAT TGT TGT ATC TCT TCT CAG AG</td>
<td>F</td>
</tr>
<tr>
<td>CitSO</td>
<td>CTG CGA AGA AAA AAT TCC TCC GAG</td>
<td>R</td>
</tr>
<tr>
<td>CitPH1</td>
<td>ATG GGA GGA CTC AAA AAT ATC TTC TCT</td>
<td>F</td>
</tr>
<tr>
<td>CitPH1</td>
<td>CCA GGA AAA AGG TCT CCA GGT TCT</td>
<td>R</td>
</tr>
<tr>
<td>CitPH5</td>
<td>GG AGC TCT GTT GAT GTG TGC A</td>
<td>F</td>
</tr>
<tr>
<td>CitPH5</td>
<td>TCA GAC GTG TGT AGC TGC TTT</td>
<td>R</td>
</tr>
<tr>
<td>CitPH3</td>
<td>TCA GAC GTG TGT AGC TGC TGT</td>
<td>F</td>
</tr>
<tr>
<td>CitPH3</td>
<td>AGG ACG ACT ATA TCA GCA GGT TTT</td>
<td>R</td>
</tr>
<tr>
<td>CitPH3</td>
<td>TGT CAG GTA ATA TGT TGG CTC GA</td>
<td>R</td>
</tr>
<tr>
<td>CitPH4</td>
<td>ATG ACG AAC TCA TCA ACA TCA C</td>
<td>F</td>
</tr>
<tr>
<td>CitPH4</td>
<td>CTT GGC TTA TCA GCT TCT TAC TCT</td>
<td>R</td>
</tr>
<tr>
<td>CitPH4</td>
<td>GAG GTG GAA CAA GTG CAT GAA</td>
<td>R</td>
</tr>
<tr>
<td>CitPH3</td>
<td>TGT CAG GTA ATA TGT TGG CTC GA</td>
<td>R</td>
</tr>
<tr>
<td>CitAN11</td>
<td>ATG GAA AAC TCA AGC CAA GAA TAC</td>
<td>F</td>
</tr>
<tr>
<td>CitAN11</td>
<td>AGT TAG CTA TCCCAA ACG AGC A</td>
<td>R</td>
</tr>
<tr>
<td>CitAN1</td>
<td>AGA AGA GAA CAA TCT CTG GCG GTG A</td>
<td>F</td>
</tr>
<tr>
<td>CitAN1</td>
<td>CGGT GAA TAC AGT CGG TAA AGG A</td>
<td>F</td>
</tr>
<tr>
<td>CitAN1</td>
<td>ATG GAT GCT CCG CCG CCG A</td>
<td>F</td>
</tr>
<tr>
<td>CitAN1</td>
<td>G AGT TAA TTG ACA TAC TGG GG</td>
<td>R</td>
</tr>
<tr>
<td>CitAN1</td>
<td>G AAA ATT TAA ATG CCA AAT GC</td>
<td>R</td>
</tr>
</tbody>
</table>
### Supplementary Table 3. Continue

<table>
<thead>
<tr>
<th>primer</th>
<th>gene</th>
<th>sequence 5'-3'</th>
<th>orientation</th>
</tr>
</thead>
<tbody>
<tr>
<td>7642</td>
<td>CitAN1</td>
<td>GAA TAC AGT CGG TAA AGG AAT GGA TG</td>
<td>F</td>
</tr>
<tr>
<td>7590</td>
<td>CitAN1</td>
<td>GTC AGA TTA GGA GGC AGT AG</td>
<td>R</td>
</tr>
<tr>
<td>6806</td>
<td>CitAN1</td>
<td>TCG TAC GTA AGG CTG ACT ACA TCT</td>
<td>R</td>
</tr>
<tr>
<td>7193</td>
<td>CitAN1</td>
<td>GTC ACA GTG GGT ATT GAA GTA CGT</td>
<td>F</td>
</tr>
<tr>
<td>7522</td>
<td>CitAN1</td>
<td>GAG ATA AAG ACG TGT CAG ATT CGG A</td>
<td>R</td>
</tr>
<tr>
<td>7536</td>
<td>CitAN1</td>
<td>GGA AGA AGA ATT GAC GCC AGA G</td>
<td>F</td>
</tr>
<tr>
<td>64a</td>
<td>attB2-oligo dT adaptor</td>
<td>GAGACTGGACCACTTTGTACA</td>
<td>R</td>
</tr>
<tr>
<td>65a</td>
<td>attB2-oligo dT adaptor</td>
<td>GGGGACCACCTTTGTACAAGAAG</td>
<td>R</td>
</tr>
</tbody>
</table>

### Supplementary Table 4. Primers used for the amplification of AN1 flanking sequences

<table>
<thead>
<tr>
<th>gene</th>
<th>sequence 5'-3'</th>
<th>orientation</th>
</tr>
</thead>
<tbody>
<tr>
<td>8063 CitTFIIH-like</td>
<td>GGA AAC ATT CGC AGA GCT GAG</td>
<td>F</td>
</tr>
<tr>
<td>8065 CitTFIIH-like</td>
<td>GGT ACT CAC TGG AAG TTG GTC</td>
<td>R</td>
</tr>
<tr>
<td>8066 CitFAR-like</td>
<td>GAG GAT GTG GCT CCT AAT GAG A</td>
<td>F</td>
</tr>
<tr>
<td>8068 CitFAR-like</td>
<td>GAA TGG AGT ACT GAC TTA CAA GAG</td>
<td>R</td>
</tr>
</tbody>
</table>

### Supplementary Table 5. Primers used for the amplification of AN1 promoters

<table>
<thead>
<tr>
<th>primer</th>
<th>sequence 5'-3'</th>
<th>orientation</th>
</tr>
</thead>
<tbody>
<tr>
<td>7706 CitAN1</td>
<td>TGT AGT AGT GAG AGT TGA GTC TTC</td>
<td>F</td>
</tr>
<tr>
<td>7681 CitAN1</td>
<td>GAA GGA AGA AAT GAG AGG CTA G</td>
<td>R</td>
</tr>
<tr>
<td>7880 CitAN1 (Tn-insertion)</td>
<td>CGT ACA TGA GTT ATG TCG ACG A</td>
<td>R</td>
</tr>
</tbody>
</table>

### Supplementary Table 6. Primers used for the sequencing of AN1 promoters

<table>
<thead>
<tr>
<th>primer</th>
<th>sequence 5'-3'</th>
<th>orientation</th>
</tr>
</thead>
<tbody>
<tr>
<td>7674  CitAN1</td>
<td>ACA GCA AAC AGC AGT AGG ACA G</td>
<td>F</td>
</tr>
<tr>
<td>7473  CitAN1</td>
<td>GGA AGG AAA CGA GAA GGA GAA AAC A</td>
<td>R</td>
</tr>
</tbody>
</table>

### Supplementary Table 7. Primers used for the cloning of CsPH4 and CsAN1 genes

<table>
<thead>
<tr>
<th>primer</th>
<th>gene</th>
<th>sequence 5'-3'</th>
<th>orientation</th>
</tr>
</thead>
<tbody>
<tr>
<td>6544</td>
<td>CitPH4</td>
<td>ATG AGG AAC CCA TCA ACA TCA C</td>
<td>F</td>
</tr>
<tr>
<td>6547</td>
<td>CitPH4</td>
<td>CTA CTC AAC GTG TTC ATC AAC C</td>
<td>R</td>
</tr>
<tr>
<td>6623</td>
<td>CitAN1</td>
<td>ATG GAT GCT CCG CCG CCG A</td>
<td>F</td>
</tr>
<tr>
<td>6596</td>
<td>CitAN1</td>
<td>TGA GTT AAT TGA CAT ACT GGG GT</td>
<td>R</td>
</tr>
</tbody>
</table>

### Supplementary Table 8. Primers used for the amplification and qRT-PCR analysis of distinct mRNAs

<table>
<thead>
<tr>
<th>primer</th>
<th>gene</th>
<th>sequence 5'-3'</th>
<th>orientation</th>
</tr>
</thead>
<tbody>
<tr>
<td>7233</td>
<td>CitACTIN11</td>
<td>GGC ATT GCC GAT AGA ATG AGC A</td>
<td>F</td>
</tr>
<tr>
<td>7234</td>
<td>CitACTIN11</td>
<td>TCA TAC TCA GCC TTT GCA ATC CA</td>
<td>R</td>
</tr>
<tr>
<td>8934</td>
<td>ANNEXIN2</td>
<td>TGC TGA GCA ACT CCA CAA AGC</td>
<td>F</td>
</tr>
<tr>
<td>8935</td>
<td>ANNEXIN2</td>
<td>TCT CGA ATC AAC TTG CGC TGA G</td>
<td>R</td>
</tr>
<tr>
<td>8936</td>
<td>ANKYRIN</td>
<td>ATG AGG AGT AGT CAA TTG TTC ATC</td>
<td>F</td>
</tr>
</tbody>
</table>
### Supplementary Table 8. Continue

<table>
<thead>
<tr>
<th>Primer</th>
<th>Gene</th>
<th>Sequence 5'-3' Orientation</th>
</tr>
</thead>
<tbody>
<tr>
<td>8937</td>
<td>ANKYRIN</td>
<td>GCA ATG CTG TCC TTC CTT CTG</td>
</tr>
<tr>
<td>8938</td>
<td>Ribosomal protein S10</td>
<td>GAA ATC TGC AAG TAC TCC TTC C</td>
</tr>
<tr>
<td>8939</td>
<td>Ribosomal protein S10</td>
<td>CAT CAG CTT AAT CAC CTG CAG G</td>
</tr>
<tr>
<td>7666</td>
<td>CitPH1</td>
<td>CCA GAG ACA GAT GTG TCG TCA</td>
</tr>
<tr>
<td>7667</td>
<td>CitPH1</td>
<td>CAT GAT TGC ACG GTG CAT GGT</td>
</tr>
<tr>
<td>6530</td>
<td>CitPH5</td>
<td>GG AGC AGC TTA AGT TCG TCA G</td>
</tr>
<tr>
<td>6531</td>
<td>CitPH5</td>
<td>ATG AAG TTA ATG ACG TTC AAT GGT</td>
</tr>
<tr>
<td>7633</td>
<td>CitMAC9F1</td>
<td>GCA GAA ACG GAT TGA TGA AGA G</td>
</tr>
<tr>
<td>7634</td>
<td>CitMAC9F1</td>
<td>CAT CAG GGA CAA CAC AGC CAG</td>
</tr>
<tr>
<td>7664</td>
<td>CitPH3</td>
<td>CTG AGA TTC TTA GTG AGT GCT TC</td>
</tr>
<tr>
<td>7665</td>
<td>CitPH3</td>
<td>CAG ATG TCA CTG GAG TTG TGC</td>
</tr>
<tr>
<td>6545</td>
<td>CitPH4</td>
<td>GAT CTC ATT CTT CCG CTA CAT C</td>
</tr>
<tr>
<td>6546</td>
<td>CitPH4</td>
<td>CTT GGC TTA TCA GCT TCT TAC TC</td>
</tr>
<tr>
<td>7670</td>
<td>CitAN11</td>
<td>GCT TCT CTA GAC CTG A</td>
</tr>
<tr>
<td>6615</td>
<td>CitAN11</td>
<td>AGT TAT CTA TCC CAA ACG AGC A</td>
</tr>
<tr>
<td>6577</td>
<td>CitAN1 5'</td>
<td>CGGT GAA TAC AGT CCG TAA AGG A</td>
</tr>
<tr>
<td>7575</td>
<td>CitAN1 5'</td>
<td>CTG CAC TGT TTT CCT AGT CCT GA</td>
</tr>
<tr>
<td>6579</td>
<td>CitAN1 mid</td>
<td>ACC GGT TAT GAT AGG TAG CAG T</td>
</tr>
<tr>
<td>6580</td>
<td>CitAN1 mid</td>
<td>GCT GCT TCT GAA GAA TGG TAG A</td>
</tr>
<tr>
<td>7598</td>
<td>CitAN1 3'</td>
<td>GAA CAA AGA AGG GTG GCT GCT</td>
</tr>
<tr>
<td>7599</td>
<td>CitAN1 3'</td>
<td>CTT TCT TCC CGT TTA CAT TGC CC</td>
</tr>
<tr>
<td>7807</td>
<td>CitSO 5'</td>
<td>CTT CTG GCA GAA AGC TTT TGA ATG</td>
</tr>
<tr>
<td>7808</td>
<td>CitSO 5'</td>
<td>CCA GAA CAA CAT TGA TAG GAA TGA</td>
</tr>
</tbody>
</table>

### Supplementary Table 9. Primers used for the preparation of construct for transient transformation

<table>
<thead>
<tr>
<th>Primer</th>
<th>Clone</th>
<th>Sequence 5'-3' Orientation</th>
</tr>
</thead>
<tbody>
<tr>
<td>6623</td>
<td>Fso AN1 2.2kb full size cDNA</td>
<td>ATG GAT GCT CCG CCG CCG A</td>
</tr>
<tr>
<td>6593</td>
<td>Faris Sour AN1 2.2kb full size cDNA</td>
<td>G AAA ATT TAA ATG CCA AAT GC</td>
</tr>
<tr>
<td>6623</td>
<td>Faris sweet AN1 1.3kb truncated cDNA</td>
<td>ATG GAT GCT CCG CCG CCG A</td>
</tr>
<tr>
<td>6595</td>
<td>Faris sweet AN1 1.3kb truncated cDNA</td>
<td>AGA AGA GAA TCT CTG CCG GTG A</td>
</tr>
<tr>
<td>6572</td>
<td>Faris Sour PH4 cDNA</td>
<td>ATG AGG AAC CCA TCA ACA T</td>
</tr>
<tr>
<td>65a</td>
<td>Faris Sour PH4 cDNA</td>
<td>GGGGACGACTTTTGACAAAGAAG</td>
</tr>
<tr>
<td>6572</td>
<td>Faris Sweet PH4 cDNA</td>
<td>ATG AGG AAC CCA TCA ACA T</td>
</tr>
<tr>
<td>65a</td>
<td>Faris Sweet PH4 cDNA</td>
<td>GGGGACGACTTTTGACAAAGAAG</td>
</tr>
<tr>
<td>7326</td>
<td>Faris Sour PH7 5kb promoter</td>
<td>CT CGT GAT TCT GAA TCT T</td>
</tr>
<tr>
<td>7328</td>
<td>Faris Sour PH1 3.3kb promoter</td>
<td>TCT CTT CCT CTT CAG TCT TAG AC</td>
</tr>
<tr>
<td>7194</td>
<td>Faris Sour PH5 3kb promoter</td>
<td>GAG GTA CAT TGA CCT ACA</td>
</tr>
<tr>
<td>7195</td>
<td>Faris Sour PH5 3kb promoter</td>
<td>CAG AGC AAA GTC CTT GTC GAG</td>
</tr>
</tbody>
</table>
Reference


