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ETHQV6.3 is involved in melon climacteric fruit ripening and is encoded by a NAC domain transcription factor

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SUMMARY

Fruit ripening is divided into climacteric and non-climacteric types depending on the presence or absence of a transient rise in respiration rate and the production of autocatalytic ethylene. Melon is ideal for the study of fruit ripening, as both climacteric and non-climacteric varieties exist. Two introgressions of the non-climacteric accession PI 161375, encompassed in the QTLs *ETHQB3.5* and *ETHQV6.3*, into the non-climacteric 'Piel de Sapo' background are able to induce climacteric ripening independently. We report that the gene underlying *ETHQV6.3* is *MELO3C016540* (*CmNAC-NOR*), encoding a NAC (NAM, ATAF1,2, CUC2) transcription factor that is closely related to the tomato *NOR* (*non-ripening*) gene. *CmNAC-NOR* was functionally validated through the identification of two TILLING lines carrying non-synonymous mutations in the conserved NAC domain region. In an otherwise highly climacteric genetic background, both mutations provoked a significant delay in the onset of fruit ripening and in the biosynthesis of ethylene. The PI 161375 allele of *ETHQV6.3* is similar to that of climacteric lines of the *cantalupensis* type and, when introgressed into the non-climacteric 'Piel de Sapo', partially restores its climacteric ripening capacity. *CmNAC-NOR* is expressed in fruit flesh of both climacteric and non-climacteric lines, suggesting that the causal mutation may not be acting at the transcriptional level. The use of a comparative genetic approach in a species with both climacteric and non-climacteric ripening is a powerful strategy to dissect the complex mechanisms regulating the onset of fruit ripening.

Keywords: non-climacteric ripening, *ETHQV6.3*, NAC transcription factor, TILLING mutant, *Cucumis melo*, introgression line.

INTRODUCTION

Fruit ripening is the last stage of the fruit developmental program in which fruit undergoes a series of physiological and metabolic changes that protect the seeds from environmental conditions and promote their dispersion (Giovannoni, 2001). Two types of ripening have been defined with respect to the role of the plant hormone ethylene: climacteric ripening, which is characterized by the autocatalytic biosynthesis of ethylene and the increase in respiration at the onset of ripening; and non-climacteric ripening, in which both ethylene production

and respiration rate remain low throughout the process (McMurchie *et al.*, 1972; Lelièvre *et al.*, 1997). Ethylene is involved in many plant developmental processes, including flower development and sexual determination, abscission and plant organ senescence, and biotic and abiotic stress responses (Abeles *et al.*, 1992). It plays a primary role in the regulation of climacteric fruit ripening, by acting as a triggering signal initiating the biochemical and physiological processes that lead to the characteristics of a ripe fruit (McMurchie *et al.*, 1972). These usually include the formation of an abscission layer, changes in fruit colour, development of aroma, fruit softening and a short post-harvest life. Conversely, non-climacteric fruits do not typically display these characteristics. Despite the physiological differences between climacteric and non-climacteric ripening, several common features exist, suggesting that common molecular and regulatory processes may underlie both types of ripening (Giovannoni, 2004).

Ripening has been a major focus of plant breeding in fleshy fruits, with special effort on the improvement of organoleptic quality and post-harvest durability (Handa *et al.*, 2014). Tomato is the model species for studying climacteric ripening, and important advances in the elucidation of the ethylene biosynthetic pathway (Alexander and Grierson, 2002), as well as its signalling (Klee, 2004) and transduction components, (Adams-Phillips *et al.*, 2004) have been achieved. The availability of ripening-impaired mutants in tomato allowed the identification of three main transcription factors (TFs) involved in its regulation: *RIN* (*ripening-inhibitor*), *CNR* (*Colorless non-ripening*) and *NOR* (*non-ripening*; Vrebalov *et al.*, 2002; Manning *et al.*, 2006; Giovannoni, 2007). The *rin*, *Cnr* and *nor* mutants produce completely developed fruits with fertile seeds that are unable to initiate fruit ripening and remain in a mature green stage. This phenotype is due to the inhibition of autocatalytic ethylene biosynthesis and respiration, and absence of flesh softening, aroma volatiles biosynthesis, chlorophyll degradation and pigment biosynthesis (Robinson and Tomes, 1968; Tigchelaar *et al.*, 1973; Thompson *et al.*, 1999; Kovács *et al.*, 2009). Although fruits from mutants in any of these three genes fail to ripen in response to exogenous ethylene, the expression of ethylene-responsive genes is not impaired in fruits or in other plant tissues (Giovannoni, 2007). It has been suggested that *RIN*, *CNR* and *NOR* may belong to a highly conserved ripening regulation system that controls not only ethylene biosynthesis, but the overall ripening process, and that this system is common to climacteric and non-climacteric species alike (Klee and Giovannoni, 2011). Additional TFs involved in the regulation of fruit ripening include the positive regulators *TAGL1* (Itkin *et al.*, 2009), *LeHB-1* (Lin *et al.*, 2009) and *SlNAC4* (Zhu *et al.*, 2014), and the negative regulators *LeAP2a* (Chung *et al.*, 2010) and *LeERF6* (Lee *et al.*, 2012). Recent studies also suggest the involvement of miRNAs (Gao *et al.*, 2015) and epigenetic regulation (Zhong *et al.*, 2013; Liu *et al.*, 2015) in fruit ripening. Despite these recent advances, the full complexity of the ethylene-dependent and -independent regulation of fruit ripening remains to be resolved.

Melon (*Cucumis melo* L.) has emerged as an interesting model for fruit ripening studies due to the existence of both climacteric and non-climacteric genotypes within the species (Ezura and Owino, 2008). The *cantalupensis* (e.g. 'Védrantais') and *reticulatus* ('Dulce') varieties show climacteric ripening and short shelf-life, whereas *inodorus* varieties like 'Piel de Sapo' (PS) are non-climacteric and show long shelf-life (Saladié *et al.*, 2015). The role of ethylene in melon fruit ripening regulation was demonstrated by reducing its biosynthesis in antisense *CmACO1* 'Védrantais' plants (Ayub *et al.*, 1996; Pech *et al.*, 2008). These experiments showed that the development of an abscission layer, the rind colour change and the production of aroma volatiles were processes strictly ethylene-dependent, while flesh softening only partially so. Conversely, carotenoid biosynthesis, sugar and organic acid accumulation were ethylene-independent.

The genetic basis of melon fruit ripening was first studied in a recombinant inbred line (RIL) population generated from the cross between the climacteric variety 'Védrantais' (*cantalupensis*) and the non-climacteric exotic accession PI 1611375 (SC, *conomon*; Perin *et al.*, 2002). *Al-3* and *Al-4* in chromosomes 8 and 9, respectively, were found to be involved in the development of an abscission layer and the autocatalytic ethylene biosynthesis, and four QTLs in chromosomes 1, 2, 3 and 11 were involved in the amount of ethylene produced. More recently, the near-isogenic line SC3-5-1, originated from the cross between PS and SC, showed climacteric ripening despite both parents being non-climacteric (Eduardo *et al.*, 2005). SC3-5-1 contains two QTLs, *ETHQB3.5* and *ETHQV6.3* in chromosomes 3 and 6, respectively, involved in the regulation of climacteric ripening (Moreno *et al.*, 2008; Vegas *et al.*, 2013). Both QTLs are capable of inducing climacteric ripening in the non-climacteric background of PS individually, but they also interact to increase ethylene biosynthesis and intensity of the ripening-associated processes (Vegas *et al.*, 2013). Interestingly, there was no commonality between the QTLs from this study and Perin *et al.* (2002), suggesting that the genetic basis of fruit ripening in melon is complex and variety-specific.

In previous work, *ETHQV6.3* was mapped to a 4.5-Mb region of melon LG VI (Vegas *et al.*, 2013). In this study we identified, characterized and functionally validated *MELO3C016540* (*CmNAC-NOR*) as the causal gene for *ETHQV6.3*.

RESULTS

Positional cloning of *ETHQV6.3*

The 2008-F_2 mapping population, obtained after crossing the near isogenic line SC3-5-1 (carrying both *ETHQV6.3* and *ETHQB3.5*) to PS, was used to map *ETHQV6.3* in a 4.5-Mb centromeric region of melon chromosome 6 (Vegas *et al*., 2013). We obtained the 2012-F_4 segregating population from 7M80-11.4, an individual of the 2008-F_2 mapping population, heterozygous for *ETHQV6.3* and fixed for the PS alleles for *ETHQB3.5* (Figure S1). The genotyping of 1131 2012-F_4 individuals with flanking markers SNP-64658 and SNP-2826073 allowed for the identification of 27 recombinants in the interval (Figure S2a). Twenty-four SNPs polymorphic between PS and SC and evenly distributed in the SNP-64658/SNP-2826073 interval (Table S1) were used to delimit the recombination point in each recombinant. A progeny test was performed with 15 informative recombinants, where 20 individuals per family were phenotyped for climacteric ripening after recording fruit abscission (Figure S3), which allowed the mapping of *ETHQV6.3* between markers SNP-2691690 and SNP-2826073 in a 139-kb interval (Figure S2a; Table S2).

This interval contains five annotated genes in the reference genome v3.5 (Garcia-Mas *et al*., 2012; Table S3), two of which are TFs of the NAC-domain family, *MELO3C016536* and *MELO3C016540*. Recombinants R24, R25 and R26 were genotyped with six additional SNPs between SNP-2691690 and SNP-2826073 (SEQ-1–SEQ-6; Table S1), which allowed a reduction of the interval to 80.7 kb between markers SEQ-3 and SNP-2826073 containing *MELO3C016538*, *MELO3C016539* and *MELO3C016540* (Figure S2b and c). *MELO3C016538* and *MELO3C016539* encode short mRNAs of 247 and 396 bp, respectively, with no homologies or reported expression in sequence databases. Thus, *MELO3C016540* (*CmNAC-NOR*), identified as a member of the NAC-domain TF family, was considered a good candidate for *ETHQV6.3*.

A QTL for climacteric ripening from a different genetic background maps to an identical genomic interval as *CmNAC-NOR*

An F_3 population obtained from the cross between the 'Noy-Amid' (non-climacteric, *inodorous* type) and 'Dulce' (climacteric, *reticulatus* type) was phenotyped for ethylene emission at harvest. Parental lines, F1 and 131 F_3 plants representing the whole scale of ethylene emission were selected for genotyping from 700 F_3 plants previously evaluated for ethylene emission in 2013. The same individuals were genotyped with 76 988 SNPs identified by RAD-seq and used to map QTLs for ethylene emission at harvest. One of the QTLs [logarithm of odds (LOD) 5.3, $r^2 = 0.16$] collocated with *ETHQV6.3* in chromosome 6. The QTL interval included 160 annotated genes and a bin of 17 genes at the LOD peak (*MELO3C016523* to *MELO3C016539*; Figure S4; Table S4). *CmNAC-NOR* was located adjacent to the peak, with only one SNP in the intergenic region separating them, strengthening the findings presented above and suggesting that the allele of *CmNAC-NOR* for *ETHQV6.3* may be common in climacteric/non-climacteric melon germplasm.

CmNAC-NOR belongs to the melon NAC domain TF family and is phylogenetically related to the tomato SlNAC-NOR

Transcription factors of the NAC family are plant specific. They contain a conserved domain NAC (NAM, ATAF1,2, CUC2) distributed with subdomains A–E in the N-terminal region, which is involved in DNA binding (Puranik *et al*., 2012). We identified 81 genes of the NAC-domain family in the melon genome, putatively encoding 92 proteins and evenly distributed in the 12 chromosomes. *CmNAC-NOR* is 1771 bp in length, contains three exons (184, 314 and 564 bp) and two introns (89 and 183 bp), and encodes a predicted 352 aa protein (Figures 1 and 2a).

CmNAC-NOR was aligned with 37 NAC proteins of known function from different plant species (Table S5) and the melon NAC-domain family (Figure 3). The alignment showed a highly conserved N-terminal region of approximately 200 aa containing the NAC domain. The proteins in the cladogram clustered according to their biological function: group 1 mainly includes NAC proteins involved in growth and development, but also cell wall metabolism and senescence; group 2a contains NAC proteins involved in stress response; and group 2b contains NAC proteins involved in senescence, but is more heterogeneous. Group 2b contains the tomato SlNAC-NOR (non-ripening) involved in fruit ripening, which clusters close to *CmNAC-NOR*. Another tomato NAC protein also involved in fruit ripening, SlNAC4, is clustered in group 2a. The phylogenetic analysis indicates that *CmNAC-NOR* is a closely related homologue of the tomato *SlNAC-NOR*, a regulator of climacteric fruit ripening (Giovannoni, 2004), reinforcing its potential as the candidate gene for *ETHQV6.3*.

Association of *CmNAC-NOR* with climacteric ripening

In a previous work, Leida *et al.* (2015) studied the association of candidate genes with climacteric behaviour in a panel of 175 melon accessions that included wild relatives, feral types, landraces and breeding lines, representing the diversity of the species. The accessions were phenotyped for fruit ripening behaviour and genotyped with a set of 251 SNPs, of which 60 were located in 34 candidate genes involved in ethylene and cell wall pathways. Two SNPs on chromosomes 11 and 12 were associated with ripening traits, but no association was detected with SNPs on chromosome 6, as none located near *CmNAC-NOR* was assayed.

A non-synonymous SNP G411T in *CmNAC-NOR* (Table S6) was previously identified after re-sequencing

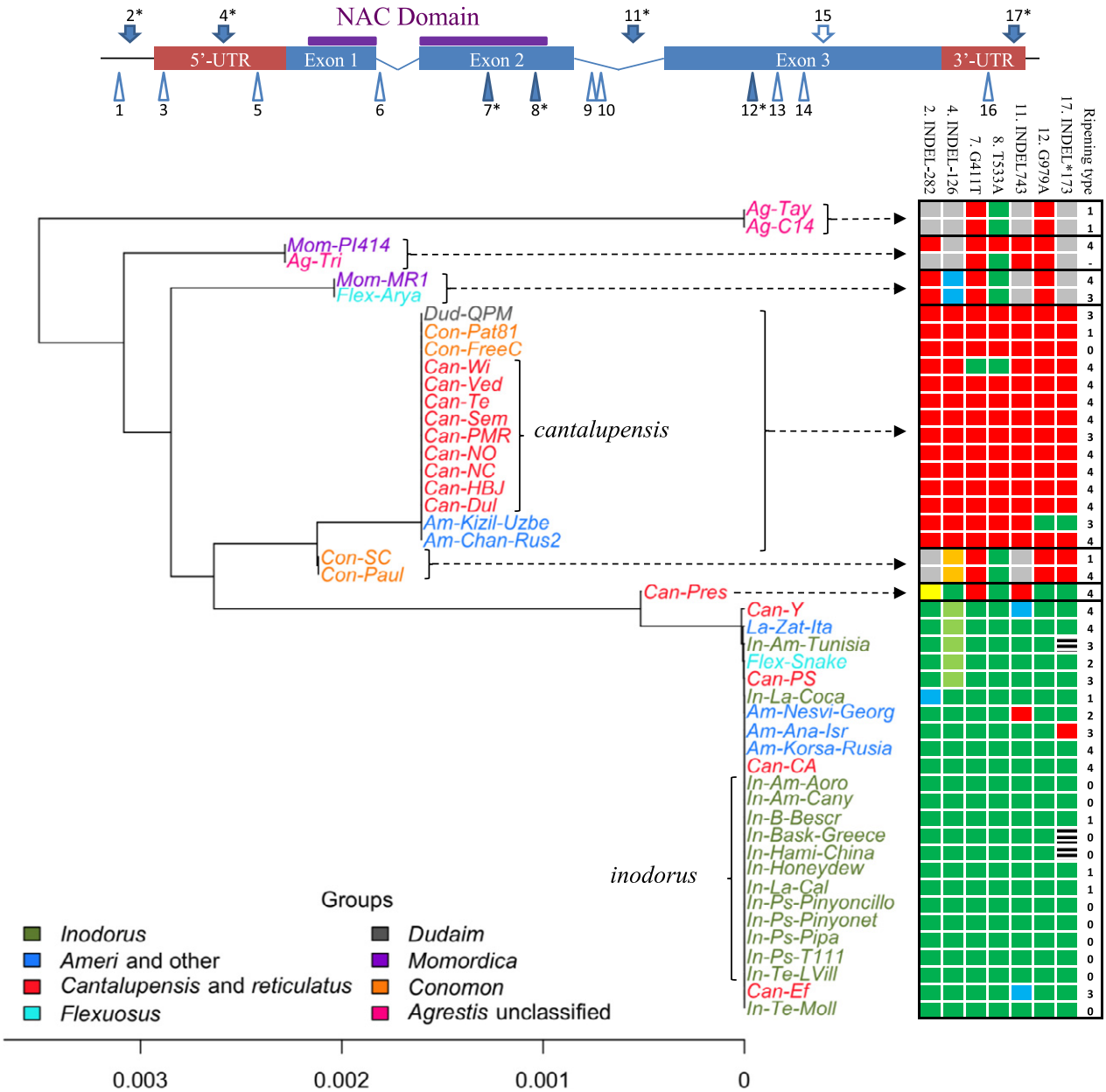

Figure 1. Sequence diversity of *CmNAC-NOR*.
Left panel: cladogram representing the sequence of *CmNAC-NOR* in 47 melon accessions. The scale indicates genetic distance. Colours for each accession represent the melon botanical classification: green, *inodorus*; dark blue, *ameri* and other European traditional varieties; red, *cantalupensis* and *reticulatus*; light blue, *flexuosus*; grey, *dudaim*; purple, *momordica*; orange, *conomon*; pink, *agrestis*.
Top panel: the structure of *CmNAC-NOR* with the position of SNPs (triangles) and indels (arrows), numbered from 1 to 17. Solid arrows and triangles marked with an asterisk indicate a significant association of each variation with the type of fruit ripening.
Right panel: genotyping of the collection for the seven variations significantly associated with the type of fruit ripening. Colours indicate the observed alleles for each SNP/indel. In green the PS (*inodorus*) allele and in red the *cantalupensis* allele. For indels, additional alleles are represented with different colours as in Table S8. The ripening type score for each accession (0 = non-climacteric as PS; 4 = highly climacteric as 'Védrantais') is included in the last column.

eight pools of accessions that represented the main melon botanical groups, and was polymorphic between climacteric and non-climacteric groups of melons (snv26555 available at www.melogene.net; Blanca *et al.*, 2012). Climacteric *cantalupensis* and *momordica* melons had the T allele, the non-climacteric *inodorus* melons had the G allele, and both alleles were present in the group of African *agrestis* showing variable climacteric behaviour (Leida *et al.*, 2015). We genotyped SNP G411T in the panel of 175 melon accessions used in Leida *et al.* (2015), and its

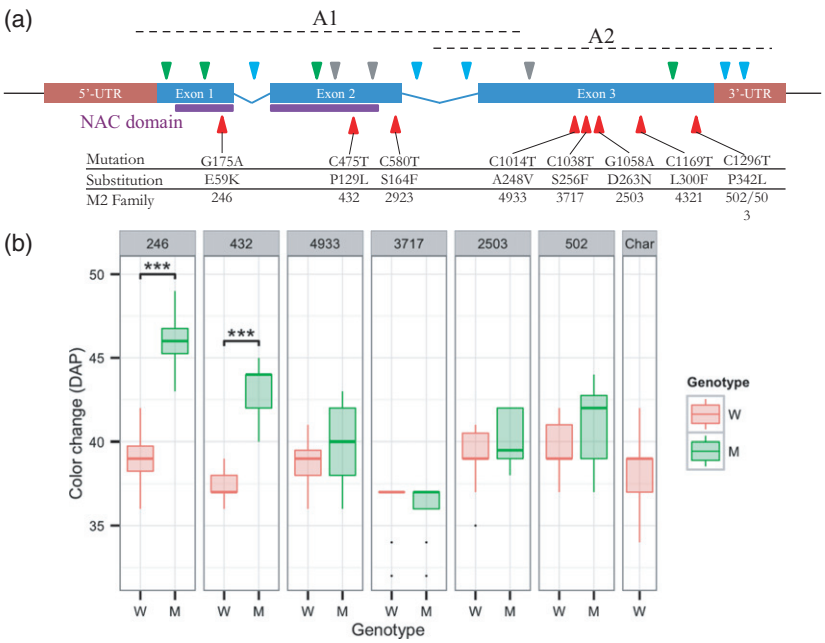

Figure 2. Mutants identified for *CmNAC-NOR*.
(a) Structure of *CmNAC-NOR*. A1 and A2 represent the regions amplified for TILLING. Red boxes represent UTRs, blue boxes represent exons and blue lines represent introns. The NAC domain is represented with a purple line under exons 1 and 2. Red triangles represent non-synonymous mutations; green triangles represent synonymous mutations; blue triangles represent mutations in non-coding regions; grey triangles represent discarded mutations corresponding to family 5388.
(b) Phenotypic differences according to external colour change in M2 families of mutant families 246, 432, 4933, 3717, 2503 and 502. On the *y*-axis, days between pollination and external colour change are represented. W (red) is homozygous for the wild-type allele; M (green) is homozygous for the mutated allele. Asterisks indicate statistically significant differences between each group after a Student's *t*-test. Significance level ***$P < 0.001$.

association with ripening related traits was assessed (Table S7). SNP G411T was found to be highly associated with ripening type ($P = 4.35 \times 10^{-5}$) and abscission layer formation ($P = 6.48 \times 10^{-4}$), further supporting the implication of *CmNAC-NOR* in climacteric fruit ripening.

Sequence diversity of *CmNAC-NOR* in melon germplasm

We selected a group of 54 melon accessions representing 11 of the 16 botanical groups (Pitrat, 2008) of the two subspecies *melo* and *agrestis* (Table S8) from the above-mentioned panel to characterize the genetic variability of *CmNAC-NOR*. We identified 12 SNP and five indel in 54 accessions, distributed in the promoter region (2), 5′UTR (3), exons (6), introns (4), 3′UTR (1) and the terminator region (1) (Figure 1; Table S8). A phylogenetic analysis of 47 of these sequences showed a clear separation of the *cantalupensis* and *inodorus* groups, although five *cantalupensis* accessions were clustered in the *inodorus* group (Figure 1). Interestingly, the allele of SC clustered close to the climacteric *cantalupensis* group. As there was a strong correlation between the three variables that were phenotyped in the accessions panel, ripening type was used in modelling the effects of the sequence differences. Seven polymorphisms in *CmNAC-NOR* were significantly related to ripening type (Figure 1; Tables S6 and S8). G411T and T533A produced non-synonymous amino acid changes A108S and S236N, respectively, although located outside the NAC domain region and predicted as neutral (Figure S5). The polymorphisms showing the strongest significance were INDEL-282 and INDEL-126, located in the promoter and the 5′UTR, respectively. INDEL-126 is particularly interesting as it shows a 26-bp indel in the 5′UTR (Figure S6). The INDEL-126 analysis in the accessions resulted in the identification of nine alleles, structured in four blocks with a polyA track (A), and three repeats GAGAAAA (B), GAAAAAA (C) and GAAATAA (D). The SC allele (CON) is similar to the *cantalupensis* one (CAN), containing only block A, whereas PS (INO) contains the block structure ABCD.

Functional validation of *CmNAC-NOR* through the characterization of TILLING mutants

In order to validate *CmNAC-NOR* as the candidate gene for *ETHQV6.3*, we screened for mutants using the climacteric 'Charentais Mono' TILLING platform (Dahmani-Mardas *et al.*, 2010). We screened 6200 M2 families with two overlapping amplicons, A1 and A2 of 920 and 807 bp, respectively, which covered the 1334-bp ORF of *CmNAC-NOR*. We identified 21 families containing 20 mutations (Table S9). Family 5388 was discarded as it contained three mutations (T411G, A533T and A978G) that are not the expected G:C to A:T change produced by EMS. This resulted in 20 mutant M2 families containing 17 mutations. We identified 12 mutations in exons, three in introns and two in the 3′UTR, with eight of them producing non-synonymous amino acid changes (Figure 2a; Table S9). Three of the non-synonymous mutations in the CmNAC-NOR protein were located between residues 15 and 178, corresponding to the NAC domain (Figure S7). E59K and P129L were located in subdomains B and D, respectively, and S164F was placed near subdomain E. We used PROVEAN (Choi *et al.*, 2012) to predict the effect of the mutations, which suggested E59K, P129L and S164F as deleterious mutations.

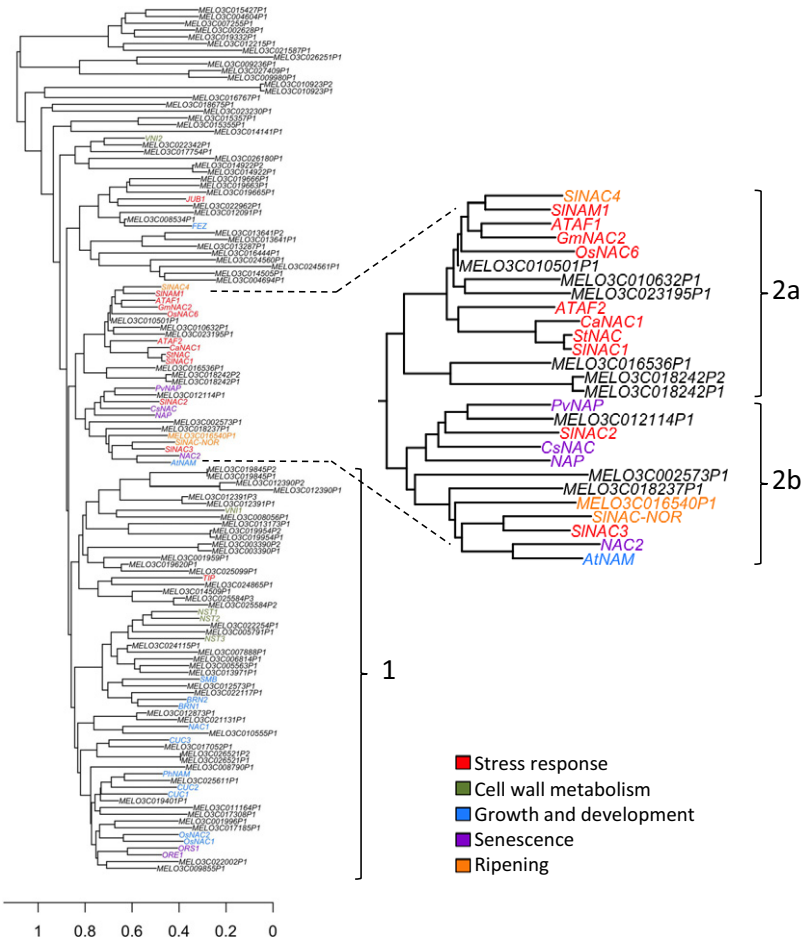

Figure 3. Cladogram containing the melon NAC family and NAC proteins of known function of other plant species. The zoom shows the clade that contains MELO3C016540 (CmNAC-NOR), and tomato SINAC-NOR and SINAC4. The prefix for each protein sequence indicates the plant species (Table S5). Colours indicate protein function: red, stress response; green, cell wall metabolism; blue, plant growth and development; purple, senescence; orange, fruit ripening. The lower scale represents the relative genetic distance. Group 1 contains proteins involved in growth, development and cell wall metabolism. Group 2a contains proteins involved in stress response. Group 2b contains proteins involved in senescence and fruit ripening.

The mutant families were phenotyped in two consecutive seasons, after discarding families 228, 4978 and 503 that shared the same mutation as 2923, 4321 and 502 (Table S9). The first season the number of seed for some of the eight mutant families was limited, which resulted in the availability of a low number (<5) of homozygous wild-type (W) and homozygous mutant (M) individuals per family. We used days from pollination to abscission to phenotype the mutant families, and families 246 (E59K) and 502 (P342L) significantly increased the time to abscission in M plants in 6.4 and 11.7 days, respectively. However, the low number of individuals phenotyped for each class in each family, the loss of several fruits that were severely affected by a fungal disease and the absence of fruit abscission in some control 'Charentais Mono' plants meant that the number of replicates was too low to apply a powerful statistics analysis. A new phenotyping assay was performed in a second season, where we chose to phenotype days from pollination to external colour change as a more robust measure of ripening. The external colour change is a good approximation of the peak of ethylene production in climacteric fruits, as we have observed in a RIL population from the cross of 'Védrantais' × PS. Six mutant families were phenotyped (246, 432, 4933, 3717, 2503 and 502), using a higher number of W and M allelic groups per family (between $n = 7$ and $n = 18$). In two families, both allelic groups showed statistically significant differences in ripening behaviour: 246 (E59K; $P = 4.4 \times e^{-8}$) and 432 (P129L; $P = 1.6 \times 10^{-6}$), with 7.2 and 5.6 additional days to external colour change, respectively, compared with the controls (Figure 2b; Table S10).

We applied a method for measuring ethylene fruit production, based on non-invasive ethylene quantification in attached fruit with chromatography-mass spectrometry (Pereira *et al.*, 2017), in the fruits of the mutant families 246 and 432. We observed a significant increase in the days from pollination to the production of the ethylene peak in both families (37.3 days W versus 45.7 days M in family 246; 38 days W versus 42 days M in family 432; Figure 4a; Table 1), coinciding with 6.1 and 5.7 additional days to external colour change. The values for days from pollination to abscission, although not significant, were also increased in both families (Table 1). However, we could not see a significant difference in the amount of

ethylene produced in both mutant families (Figure 4b), probably due to other genes in the 'Charentais Mono' genetic background that also control the ripening process.

The ripening delay observed in the mutant families 246 (E59K) and 432 (P342L) confirmed that *CmNAC-NOR* is involved in the control of climacteric fruit ripening. Both mutations are located in subdomains B and D of the NAC domain, in residues that are conserved in NAC proteins of known function (Figure S7).

CmNAC-NOR is expressed primarily in fruit

In order to know if *CmNAC-NOR* plays a role only in fruit, or if it is also expressed in other organs, we performed quantitative polymerase chain reaction (qPCR) in the non-climacteric PS, and in the near-isogenic lines (NILs) containing *ETHQB3.5* (GF35), *ETHQV6.3* (GF40) or both of them (GF31) (Figure 5a). *CmNAC-NOR* is highly expressed in fruit tissue of all four genotypes, both climacteric and non-climacteric, during fruit development at 20 days after pollination (DAP), 30 DAP and harvest, whereas the expression in leaves and roots is very low. We also tested the expression of another NAC-domain containing gene, *MELO3C016536*, which is also located in the original *ETHQV6.3* interval (Figure 5e). *MELO3C016536* is expressed in fruit tissue in GF31, GF35 and GF40, but it is not expressed in fruit of the non-climacteric PS. We also tested the expression of three genes known to be involved in ethylene biosynthesis in melon fruit: *CmACO1*, *CmACS1* and *CmACS5* (Saladié *et al.*, 2015; Figure 5b–d; Table S11). All three showed the highest expression in GF31 and GF35 at harvest, and much lower expression in GF40. *CmACO1* and *CmACS1* expression was also detected in root tissue.

DISCUSSION

The map-based cloning of the ripening QTL *ETHQV6.3*, identified in the PI 161375 (SC) × 'Piel de Sapo' (PS) genetic background, revealed that the underlying gene is *CmNAC-NOR*, which encodes a TF of the NAC (NAM, ATAF1,2 and CUC2) family. A QTL for climacteric ripening in a mapping population derived from distinct parental lines ('Noy Amid' × 'Dulce') also maps to the identical genome interval containing *CmNAC-NOR*. Furthermore, a genome-wide association analysis showed that SNP G411T, present in *CmNAC-NOR*, is strongly associated with ripening behaviour in a panel of 175 melon accessions. Taken together, these findings support the involvement of *CmNAC-NOR* in the climacteric ripening process.

The confirmation of *CmNAC-NOR* as the causal gene of *ETHQV6.3* was demonstrated after characterizing several TILLING mutants in the highly climacteric 'Charentais Mono' genetic background (Dahmani-Mardas *et al.*, 2010). Two mutant families containing the non-synonymous mutations E59K and P232L showed a significant delay in the onset of the climacteric ripening, both at the level of external colour change and presence of an ethylene peak, when compared with controls. The delay of the ripening process is therefore compatible with non-synonymous amino acid changes in subdomains B and D, respectively, of the highly conserved NAC domain region causing an alteration of gene function (Figure S7).

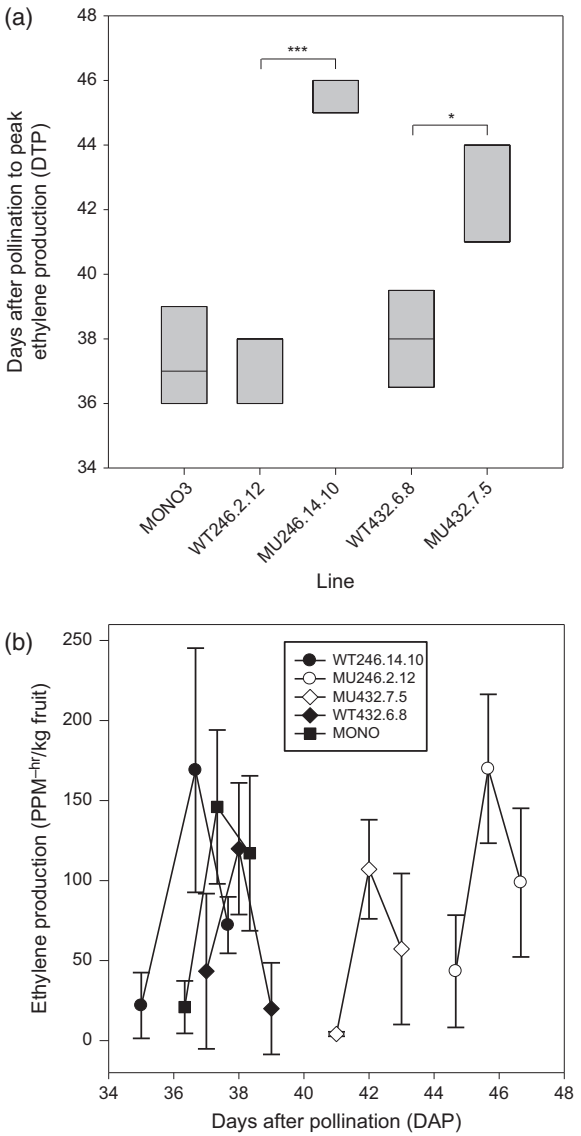

Figure 4. (a) Box plots for days after pollination (DAP) to peak ethylene production in (n = 4) fruits of 'Charentais Mono' (MONO), and (n = 3) fruits in each of two homozygous wild-type (WT) and two homozygous mutant (MU) families of *CmNAC-NOR.* Asterisks indicate significant differences between WT and MU families connected by horizontal bars at $^{***}P < 0.001$ and $^{*}P < 0.05$ with Tukey HSD.
(b) Three-day interval including the peaks of ethylene production in the MONO and WT (closed symbols) and MU families (open symbols) according to DAP. Means are plotted ± SD (n = 4) for MONO and (n = 3) for WT and MU families.

Table 1 Phenotyping of ethylene production in the mutant families 246 and 432

		Mean ± SD (DAP)		Mean differences in days		
	M2 Family	W	M	M–W	W–Char Mono	M–Char Mono
DAP to peak ethylene production	246	37.3 ± 1.1	45.7 ± 0.6	8.4***	0	8.4***
	432	38 ± 1.6	42 ± 1.7	4.0*	0.3	4.7*
	CharMono	37.3 ± 1.5	–	–	–	–
External colour change	246	38.6 ± 2.6	44.8 ± 1.9	6.1***	1.5	7.7***
	432	36.3 ± 1.6	42 ± 2.2	5.7***	−0.7	4.9***
	CharMono	37.1 ± 1.9	–	–	–	–
Abscission	246	42 ± 2.8	47.3 ± 2.9	5.3	−2.3	3
	432	38.7 ± 2.1	43.8 ± 3	5.1	−5.6*	−0.5
	CharMono	44.3 ± 2.7	–	–	–	–

Ethylene production during fruit ripening was measured in mutant families 246 and 432 and the CharMono line. The external colour change and the abscission dates were also recorded.

DAP, days after pollination; M, homozygote for the mutant allele; SD, standard deviation; W, homozygote for the wild-type allele.

Asterisks indicate the level of significance after a Tukey HSD test. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

The NAC domain TFs constitute one of the largest families of plant TFs (Puranik *et al.*, 2012). A phylogenetic analysis of the melon NAC gene family including NAC proteins of known function from different plant species suggests that *CmNAC-NOR* is a closely related homologue of the tomato *Nor* gene, which is involved in fruit ripening (*SlNAC-NOR*; Figure 3). Both proteins are included in a clade that contains other NAC proteins involved in stress response and senescence processes (Zhu *et al.*, 2014). The tomato *nor* (*non-ripening*) mutant (Tigchelaar *et al.*, 1973) produces fruit with mature seed. However, the characteristic respiration and ethylene peaks, the degradation of chlorophylls, and the biosynthesis of carotenes observed during ripening in wild type tomato are absent (Klee and Giovannoni, 2011). A network analysis combining transcriptome, proteome and metabolome data using the tomato mutants *nor*, *rin* (*ripening-inhibitor*) and *Nr* (*Never-ripe*) reported that *nor* exerts a global effect on ethylene-related gene expression and may be acting upstream of *rin* in the regulation of ethylene biosynthesis (Osorio *et al.*, 2011). Interestingly, the Spanish 'de Penjar' traditional tomato type is well known for its extraordinarily long shelf life. At least part of this characteristic is attributed to the alcobaça (*alc*) mutant, which is allelic to *nor* (Casals *et al.*, 2012). Similarly to the E59K and P232L melon mutants, the *alc* mutant is due to a non-synonymous V106D amino acid change in the NAC subdomain C region, producing a fruit with delayed ripening and long shelf life. On the other hand, the *nor* mutant is due to a 2-bp deletion in the third exon, producing a non-functioning protein and an extreme non-ripening phenotype (Casals *et al.*, 2012). The NAC gene *ppa008301m* has also been proposed as the candidate gene for a major locus controlling maturity date in peach (Pirona *et al.*, 2013), and although no functional validation has yet been reported, *ppa008301m* is also phylogenetically close to *Nor*. Other members of the NAC family have been involved in the ripening process, as *MaNAC1* and *MaNAC2* in banana (Shan *et al.*, 2012). These data suggest an important role of NAC genes in the control of fruit ripening among different plant clades.

Similar to the *nor* tomato mutant, the *inodorus* melon type PS does not show a peak of ethylene during ripening, fruit abscission is absent, the exocarp colour remains green through maturation, and fruit softening is reduced (Table S12). More interestingly, an exogenous ethylene treatment does not induce the onset of ripening in PS or in the tomato *nor* mutant (Vegas *et al.*, 2013; Saladié *et al.*, 2015). The SC allele of *ETHQV6.3* introgressed into the PS non-climacteric type in line GF40 shows a moderate climacteric type (Table S12). The SC allele of *ETHQB3.5*, another QTL in chromosome 3, is capable of independently rescuing the climacteric ripening capacity of PS in line GF35 (Table S12), suggesting that at least two genes may be impaired in the non-climacteric phenotype of PS.

The sequence of *CmNAC-NOR* in a panel 54 melon accession belonging to different botanical groups revealed 17 polymorphisms (SNP and indel), of which seven were strongly associated with the climacteric phenotype (Figure 1). Two features of the sequence diversity analysis of *CmNAC-NOR* deserve further attention. First, the *CmNAC-NOR* haplotypes of the non-climacteric SC (Con-SC) and three other *conomon* types (Con-Paul, Con-Pat81 and Con-FreeC) were included in the climacteric *cantalupensis* cluster. Second, all 15 *inodorus* haplotypes and other non-climacteric accessions were clustered together. Nine out of 14 *cantalupensis* and *reticulatus* climacteric accessions were in the climacteric *cantalupensis* cluster, but five (Can-Pres, Can-Y, Can-PS, Can-CA and Can-Ef) were included in

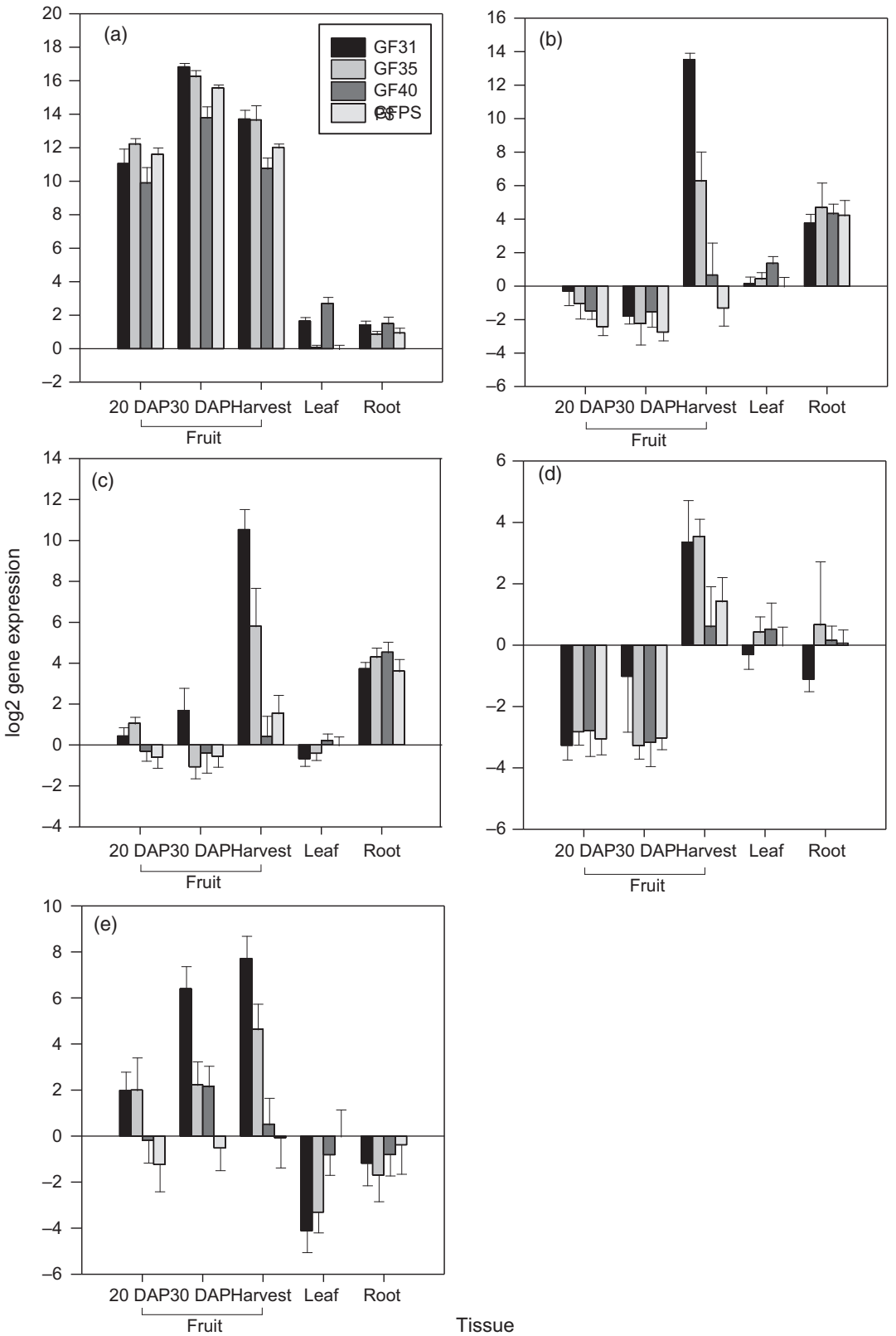

Figure 5. *CmNAC-NOR* quantitative polymerase chain reaction (qPCR) expression. *CmNAC-NOR* expression was measured in GF31, GF35, GF40 and PS (a), *CmACO1* (b), *CmACS1* (c), *CmACS5* (d) and *MELO3C016536* (e). Gene expression was plotted relative to PS expression in leaves and measured in developing fruit at 20 and 30 days after pollination (DAP), and at harvest, and in leaf and root tissue. Means are plotted ± SE (n = 3).

the *inodorus* group. A genetic analysis in a RIL population obtained from the cross between the climacteric variety 'Védrantais' (*cantalupensis*) and the non-climacteric SC revealed that the development of an abscission layer and the autocatalytic ethylene biosynthesis were controlled by *Al-3* and *Al-4*, and four additional QTLs were involved in regulating the amount of ethylene (Perin *et al.*, 2002). Interestingly, none of these QTL map in the same genomic intervals as *ETHQB3.5* and *ETHQV6.3*, suggesting that the non-climacteric phenotype of SC should be attributed to mutations in different QTLs alleles than *ETHQB3.5* and *ETHQV6.3*. This would also explain why the *ETHQV6.3* allele of SC, which is almost identical to that of the climacteric *cantalupensis* accessions, is able to partially rescue the climacteric phenotype when introgressed into the non-climacteric PS. The non-climacteric phenotypes of SC and PS are different (Table S12), as accumulation of carotenoids in the flesh and the induction of a set of ethylene biosynthetic genes are observed in SC (Vegas *et al.*, 2013; Saladié *et al.*, 2015). Recently, QTLs that delay fruit ripening of the climacteric 'Védrantais' containing introgressions of the exotic 'Ginsen Makuwa' line (*makuwa* type) have been reported in chromosomes 7 and 10 (Perpiñá *et al.*, 2016). The complexity of the climacteric phenotype, with at least 10 QTLs reported in melon, suggests that the division of ripening behaviour into just two classes may be revised into a more complex scenario that envisions ripening in a continuous spectrum with non-climacteric and highly climacteric types at the extremes. Thus, a group of climacteric *cantalupensis* accessions, which contain the PS allele of *CmNAC-NOR*, still show climacteric behaviour, probably due to the presence of the climacteric alleles for other QTLs involved in ripening. Similarly, the delayed ripening phenotype observed in the 'Charentais Mono' mutants E59K and P232L would also support this hypothesis (Table S12). The recent availability of a non-invasive method for the ethylene quantification in attached fruits will help in classifying melon accessions according to their ripening behaviour in a more precise manner (Pereira *et al.*, 2017).

Our current data do not allow the identification of the causal polymorphism of the climacteric phenotype among the seven polymorphisms highly associated with ripening behaviour identified in *CmNAC-NOR*. *CmNAC-NOR* is expressed in flesh at different stages of fruit development in both climacteric and non-climacteric types, peaking at about 30 DAP (Figure 5a) when the ripening process starts, and it shows very low expression in leaves and roots. The same pattern of expression in fruit tissue has also been reported in the climacteric 'Védrantais' and 'Dulce', and the non-climacteric SC (Saladié *et al.*, 2015). The lack of differential expression of *CmNAC-NOR* in fruit flesh among distinct ripening phenotypes suggests that its regulation may occur through other mechanisms. Two of the natural polymorphisms found in *CmNAC-NOR* produce non-synonymous changes A108S and S236N, which are located outside the NAC subdomains, but still may affect interaction with other proteins or binding to DNA. INDEL-126 is particularly interesting as it is located in the 5′UTR of the gene, the *conomon* and *cantalupensis* alleles being different from the non-climacteric *inodorus* types. The possible effect of INDEL-126 in the translation of *CmNAC-NOR* in both melon types deserves further attention.

Finally, the tomato NAC gene *SlNAC4* has a role in abiotic stress response and is a positive regulator of fruit ripening, affecting ethylene synthesis and carotenoid accumulation (Zhu *et al.*, 2014). SlNAC4 probably interacts with NOR and RIN, and it emerges as a new player in the complex regulatory network of fruit ripening in tomato (Zhu *et al.*, 2014). Among the clusters of NAC proteins 2a and 2b, which include CmNAC-NOR and SlNAC-NOR, other melon NAC proteins are also found (Figure 3). MELO3C016536, which is in the same genomic interval contained in *ETHQV6.3*, is phylogenetically related to SlNAC4 and other NAC proteins involved in stress responses, and shows a clear differential expression in fruit flesh of climacteric and non-climacteric lines (Figure 5e). It would not be surprising that, as in tomato, other NAC genes are also involved in regulating melon fruit ripening.

The use of comparative physiology and genetics in melon, a species that contains both climacteric and non-climacteric genotypes, has begun to help to elucidate the differences between these two types of ripening behaviours. It has also provided a link to a common mechanism of ripening shared with tomato, the classical climacteric model species for studying fruit ripening. Our results and other current genetic data suggest that several factors are involved in the regulation of fruit ripening in melon, and *ETHQV6.3*, the first one characterized, shows similarities with the well-studied tomato *Nor*. Further investigation of other melon QTLs involved in fruit ripening is required to complete the complex picture of this important process. It should, however, be noted that the ripening-associated changes observed in PS could lead to considering it a ripening mutant instead of a 'true' non-climacteric fruit, and that the mechanisms regulating fruit ripening in non-climacteric species may be different from those operating in melon non-climacteric types.

EXPERIMENTAL PROCEDURES

Plant material

A mapping population originated from the cross SC3-5-1 × PS (Figure S1) was used for the positional cloning of *ETHQV6.3*. SC3-5-1 (GF31) is a NIL that harbours two homozygous introgressions (carrying both *ETHQB3.5* and *ETHQV6.3*) in chromosomes 3 and 6 from the accession PI 161375 (*C. melo* var. *conomon*, SC) in the 'Piel de Sapo' (*C. melo* var. *inodorous*, PS) genetic background

(Vegas *et al.*, 2013). NILs GF35 and GF40 contain *ETHQB3.5* and *ETHQV6.3*, respectively. All plants were grown in a greenhouse in coco-fibre bags, and all flowers were self-pollinated manually allowing only one fruit per plant.

A melon germplasm collection from the COMAV-UPV, which includes 175 melon varieties (Leida *et al.*, 2015; Table S7), was used to study the association of the candidate gene with ripening behaviour. A subset of 54 accessions from this collection was selected for a detailed analysis (Table S8) (Esteras *et al.*, 2013).

Four-hundred and eighty F_2 plants from a cross between 'Noy-Amid' (*C. melo* var. *inodorous*, Yellow Canary type) and 'Dulce' (*C. melo* var. *reticulatus*, cantaloupe type; Harel-Beja *et al.*, 2010; NA × Dul) were phenotyped for ethylene emission at harvest in 2011. Twenty F_3 plants of each of 32 F_2 plants with extreme ethylene levels (16 plants > 7.5 and 16 plants <0.5 μg/kg fresh fruit/h) were grown in two repetitions in a greenhouse at Beit Elazari, Israel in 2013. Flowers were manually pollinated and tagged at anthesis, and one–two fruits were allowed to develop per plant.

DNA extraction and genotyping

Genomic DNA was extracted from young leaves according to CTAB method with some modifications to improve quality (Garcia-Mas *et al.*, 2000).

Eight SSRs and one Cleaved Amplified Polymorphic Sequence (CAPS; Table S1) were used to genotype the 2008-F_2 population (Vegas *et al.*, 2013) to identify 7M80-11.4. The 2012-F_4 population was screened with TaqMan probes (Thermo Scientific, Waltham, USA) SNP-64658 and SNP-2826073, designed by the Custom TaqMan Assay Design Tool (www.lifetechnologies.com/snpcadt) using two flanking SNPs between PS and SC (Table S1; Sanseverino *et al.*, 2015). PCR reactions were prepared in a final volume of 5 μl: 2.5 μl 2 × TaqMan Universal PCR Master Mix (Thermo Scientific, Waltham, USA), 2.375 μl genomic DNA (40 ng/μl) and 0.125 μl TaqMan probes mix. Amplification was performed in a Light Cycler 480 (Roche, Basel, Switzerland) with an initial cycle at 95°C for 1 min, 10 cycles of temperature gradient consisting of 90°C for 20 s and 61°C for 1 min diminishing the temperature from 61°C to 57°C at 0.4°C per cycle, and 26 cycles at 95°C for 20 s and 57°C for 1 min. Fluorescence was measured at 37°C. Twenty-four SNPs were genotyped using KASP chemistry (LGC, Teddington, UK) in a Biomark™ system (San Francisco, CA, USA). SNP primers were designed with Kraken (Table S1). SNPs SEQ-1 to SEQ-6 were genotyped by Sanger sequencing (Table S1). Sequences were analysed using Sequencher 5.0 (Gene Codes Corporation, Ann Arbor, MI, USA). Amplicons PRO40.1, CDS40.1, CDS40.2 and CDS40.3 (Table S1) were designed to sequence *CmNAC-NOR*, and to genotype the mutant families and the melon germplasm collection.

Restriction-site-associated DNA sequencing (RAD-seq; Miller *et al.*, 2007) and QTL analyses were performed by NRgene LTD (Nes Ziyyona, Israel) using 131 F_3 plants of the NA × Dul population, representing the whole scale of ethylene emission.

Fruit phenotyping

Fruits were collected at abscission or harvested when fully ripe (between 65 and 70 DAP). Fruit ripening behaviour was assessed with traits closely associated to melon climacteric ripening (Vegas *et al.*, 2013). The development of an abscission layer was measured using a scale from 0 to 4 (0: no abscission layer; 1: no-slip; 2: half-slip; 3: full-slip; 4: abscission), and days from pollination to abscission were recorded. External colour change was evaluated visually in fruit after abscission and harvested fruits. Days from pollination to external colour change were also measured for the phenotyping of the mutants. The production of characteristic climacteric aroma volatiles was detected by olfactory evaluation of fruit after abscission and harvested fruits.

The production of ethylene in the fruits of the mutant families was measured using a method based on non-invasive ethylene quantification in attached fruit headspace by gas chromatography-mass spectrometry (Pereira *et al.*, 2017).

Ripe fruits from the melon germplasm collection were phenotyped for fruit firmness and abscission layer development by COMAV (Leida *et al.*, 2015). The variable 'ripening type' represents the overall intensity of the climacteric ripening according to the germplasm collection curators. Scores range from 0 (non-climacteric as PS) to 4 (very climacteric as 'Védrantais').

NA × Dul fruits were sampled at ripening, determined by abscission layer development and/or change of rind colour. Ripening was verified by BRIX values. Evaluation of ethylene emission was performed on the day after harvest. Ripe detached fruits were enclosed for 3 h in containers, under controlled atmosphere conditions. Headspace gases were sampled by syringe through a septum in the lid. Ethylene was measured with a gas chromatograph equipped with flame ionization detector (Varian 3300: Varian, Palo Alto, CA, USA) and alumina column (HayeSep T Mesh- 100/120, Sciences, Deerfield, IL, USA).

Data analysis

DNA and protein multiple sequence alignments were obtained with Clustal Omega (ClustalO; Sievers *et al.*, 2011). The alignments were represented with Jalview 2.8 (Waterhouse *et al.*, 2009). Phylogenetic analyses were performed using the Neighbor-joining method in MEGA 6.06 (Tamura *et al.*, 2013) with 1000 Bootstrap iterations. Cladograms were represented with the ape package for R (Paradis *et al.*, 2004).

Association analysis of climacteric behaviour with SNP G411T in the germplasm collection was performed as by Leida *et al.* (2015). Mixed linear models implemented in TASSEL v.5.0 (Bradbury *et al.*, 2007; www.maizegenetics.org) were used with a kinship matrix to adjust for genetic structure using the full SNP data set as cofactors. Association analysis of the polymorphisms in *CmNAC-NOR* with the ripening type score for each accession were performed with ANOVA-GLM (*aov* and *glm* functions in R 3.2.1; R Development Core Team, 2016).

For phenotyping each mutant family, plants homozygous for each of the two alleles (M = mutant; W = wild type) were selected. The mean values obtained for each class were compared with a Student's *t*-test (*t.test* function in R 3.2.1; R Development Core Team, 2016) or a Tukey HSD test in JMP 8.0.1 (SAS Institute, NC, USA).

Identification of TILLING mutants in *CmNAC-NOR*

Mutant identification in *CmNAC-NOR* consisted of the screening of 6200 M_2 families using a nested PCR technique in the TILLING platform 'Charentais Mono' (Dahmani-Mardas *et al.*, 2010). PCR amplification and mutation detection were carried out as previously described (Dahmani-Mardas *et al.*, 2010) using specific primers for the amplification of regions A1 and A2 (Figure 2a Table S1). Additional primers were designed to validate the mutations by Sanger sequencing (Table S1). PROVEAN (Protein Variation Effect Analyzer; Choi *et al.*, 2012) was used to predict the impact of the mutation on the protein function. Seed from M_2 mutant families was obtained from URGV.

qPCR expression

RNA from three biological replicates for GF31, GF35, GF40 and PS was isolated from mesocarp, root and leaf tissue. RNA was isolated from 100 mg frozen sample and ground using TriZOL® reagent (Ambion®, Life Technologies, Carslbad, CA, USA). RNA samples were purified with RNeasy® spin columns (Qiagen, Hilden, Germany) and treated with RNAse free TURBO-DNase I (Turbo DNA-free™ Kit; Ambion®, Life Technologies) for 60 min at 37°C. RNA quality was as in Saladié *et al.* (2015).

Gene expression analysis by qPCR was performed on a LightCycler® 480 Real-Time PCR System using SYBR® Green I Mix (Roche Applied Science, Mannheim, Germany). The relative amounts of specific transcripts were determined using cyclophilin (*CmCYP7*) as a reference gene (Saladié *et al.*, 2015) and then normalized to PS expression in leaves. Primers were designed with Primer3 (http://primer3.wi.mit.edu/) and checked for the presence of secondary structures with NetPrimer (http://www.premierbiosoft.com/netprimer/; Table S11). Calculation of intra-assay variation, primer efficiencies and amplification specificity of the PCR by melting curve analysis were as described previously (Saladié *et al.*, 2015).

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CONFLICT OF INTEREST

The authors declare no conflicts of interest

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.

Figure S1. Scheme with the plant material used to identify *ETHQV6.3*.

Figure S2. High-resolution physical map of the *ETHQV6.3* interval.

Figure S3. Distribution of the fruit abscission dates of the progenies of 15 recombinants, expressed in days after pollination (DAP).

Figure S4. Integrative Genomics Viewer (IGV) snapshots of the genomic location of the QTL for ethylene levels in chromosome 6 in the 'Noy-Amid' × 'Dulce' population.

Figure S5. Mutations in the *CmNAC-NOR* sequence.

Figure S6. Sequence of INDEL-126 in the collection of melon accessions.

Figure S7. Mutations E59K (family 246) and P129L (family 432) in the NAC domain region of CmNAC-NOR and other NAC domain-containing proteins.

Table S1. Sequences of the markers and primers used during the high-resolution mapping of *ETHQV6.3* and for the TILLING screening.

Table S2. Phenotyping and genotyping of 15 informative recombinants and fine mapping of *ETHQV6.3*.

Table S3. Candidate genes annotated in the 139-kb interval between SNP-2691690 and SNP-2826073.

Table S4. QTL peak for ethylene measured at harvest in the RIL population of 'Noy-Amid' × 'Dulce'.

Table S5. NAC-domain-containing proteins of different plant species with known function.

Table S6. Polymorphisms in *CmNAC-NOR* associated with the climacteric behaviour.

Table S7. Genotyping of SNP G411T in a panel of 175 melon accessions.

Table S8. Melon germplasm used for assessing the variation analysis of *CmNAC-NOR*.

Table S9. Mutations identified in *CmNAC-NOR*.

Table S10. Phenotyping of external colour change in the mutants.

Table S11. Primer sequences for qPCR.

Table S12. Phenotypic information for the main genotypes discussed in the manuscript: PS, SC, Védrantais, Charentais Mono, both TILLING mutants, and the introgression lines containing *ETHQV6.3* and *ETHQB3.5*.

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