***eth6.3* is involved in melon climacteric fruit ripening and is encoded by a NAC domain transcription factor**

Pablo Ríos[1], Juan Vegas[1], Jason Argyris[1], Nurit Katzir[2], Abdel Bendahmane[3], Belén Picó[4], Antonio J. Monforte[5], Jordi Garcia-Mas[1]

[1]IRTA, Center for Research in Agricultural Genomics CSIC-IRTA-UAB-UB, Barcelona, Spain

[2]Institute of Plant Science, Agricultural research Organization (ARO), Newe Ya'ar Reseach Center, Ramat Yishay, Israel

[3]INRA-CNRS, UMR1165, Unité de Receché en Génomique Végétale, Evry, France.

[4]COMAV-UPV, Institute for the Conservation and Breeding of Agricultural Biodiversity, Valencia, Spain

[5]Instituto de Biología Molecular y Celular de Plantas (IBMCP), Universitat Politècnica de València (UPV)-Consejo Superior de Investigaciones Científicas (CSIC), Valencia, Spain

[*] Corresponding author: jordi.garcia@irta.cat

Abstract

Fruit ripening has been classically divided in climacteric and non-climacteric depending on the presence of a transient rise in respiration rate and the production of autocatalytic ethylene. Melon is an interesting model system to study fruit ripening, as both climacteric and non-climacteric varieties exist. Two introgressions of the non-climacteric PI 161375 accession in the non-climacteric "Piel de Sapo" line background, containing QTLs *eth3.5* and *eth6.3*, are able to independently induce climacteric ripening, and when combined in a single line the ripening behaviour is more pronounced. We report that the gene underlying *eth6.3* is *MELO3C016540*, which encodes a NAC (<u>N</u>AM, <u>A</u>TAF1,2 and <u>C</u>UC2) transcription factor that is orthologue to the tomato *nor* (non-ripening). *MELO3C016540* has been functionally validated with the identification of two lines carrying non-synonymous mutations in the NAC domain region. Both mutant lines significantly delay both the onset of fruit ripening and the biosynthesis of ethylene in a highly climacteric genetic background. We provide evidence that the *eth6.3* allele of PI 161375 is similar to that of climacteric lines of the *cantalupensis* type and when

introgressed into the non-climacteric "Piel de Sapo", it is able to partially restore its climacteric ripening capacity. *MELO3C016540* is expressed in fruit flesh of both climacteric and non-climacteric lines, suggesting that the causal mutation may be acting at the post-transcriptional level. We show that the use of a species with both climacteric and non-climacteric ripening is a useful strategy to help in dissecting the complex mechanisms regulating the onset of fruit ripening. Future characterization of additional genes contributing to fruit ripening, as *eth3.5*, will shed light into the poorly understood ripening in non-climacteric species.

Introduction

Fruit ripening is a complex and highly regulated process in which fruit undergoes a series of physiological and metabolic changes that protect the seeds from environmental conditions and promote their dispersion (Giovannoni, 2001). Fruit ripening is the last stage of the fruit developmental program. Two types of ripening have been defined regarding the role of the plant hormone ethylene in the process: 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, where both ethylene production and respiration rate remain low throughout the process (McMurchie *et al.*, 1972, Lelièvre *et al.*, 1997). There is great variability in the ripening-associated processes between species, but they commonly involve the accumulation of pigments, biosynthesis of aroma volatiles, sugar accumulation and fruit softening (Hiwasa-Tanase and Ezura, 2014). Despite the differences between climacteric and non-climacteric ripening there are several common features, suggesting that common molecular processes and regulation mechanisms may be shared between both types of ripening (Giovannoni, 2004). The plant hormone ethylene is involved in the control of a great variety of plant developmental processes, such as flower development and sexual determination, fruit ripening and abscission and plant organ senescence, and also biotic and abiotic stress responses, among others (Abeles *et al.*, 1992). All plants synthesise ethylene at a basal level or as a response to stress, but the hormone also plays a key role in the regulation of climacteric fruit ripening, acting as a triggering signal for the initiation of the biochemical and physiological processes that lead to a ripe fruit (McMurchie *et al.*, 1972). Non-climacteric ripening has been much less studied and there is currently no candidate main regulatory hormone analogous to ethylene.

Due to the importance of fruits for human diet, ripening has been a major focus of plant breeding with special effort on the improvement of the organoleptic quality and post-harvest durability of fleshy fruits (Handa *et al.*, 2014). Tomato has been used as a model species for climacteric ripening studies, with important advances in the comprehension of the ethylene biosynthetic pathway (Alexander and Grierson, 2002), signalling components (Klee, 2004) and transduction (Adams-Phillips *et al.*, 2004) during ripening. The availability of ripening-impaired mutants in tomato allowed the identification of three main transcription factors involved in the regulation of ripening: *RIN* ("ripening-inhibitor"), *CNR* ("Colorless non-ripening") and *NOR* ("non-ripening") (Vrebalov *et al.*, 2002, Manning *et al.*, 2006, Giovannoni, 2007). *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 ripening-associated processes such as the transition to autocatalytic ethylene biosynthesis, an increase in respiration rate, 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). Furthermore, fruits from the three mutants do not ripe in response to exogenous ethylene but the expression of ethylene-responsive genes increases in fruits as well as 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 is common to climacteric and non-climacteric species (Klee and Giovannoni, 2011). Additional downstream transcription factors involved in the regulation of fruit ripening include *TAGL1* (Itkin *et al.*, 2009), *LeHB-1* (Lin *et al.*, 2009) and *SlNAC4* (Zhu *et al.*, 2014) as positive regulators, and *LeAP2a* (Chung *et al.*, 2010) and *LeERF6* (Lee *et al.*, 2012) as negative regulators. Recent studies also suggest links between fruit ripening and miRNAs (Gao *et al.*, 2015) and epigenetic regulation (Zhong *et al.*, 2013, Liu *et al.*, 2015). Despite these recent advances, the full complexity of the ethylene-dependent and independent regulation of fruit ripening is still to be uncovered.

Melon (*Cucumis melo* L.) has emerged as an interesting model species for fruit ripening studies due to the existence of both climacteric and non-climacteric genotypes within the species (Ezura and Owino, 2008). Typical *cantalupensis* and *reticulatus* varieties like "Védrantais" and "Dulce", respectively, show climacteric ripening and short shelf-life, whereas "Piel de Sapo", an *inodorus* variety, is non-climacteric and shows long shelf-life

(Saladié *et al.*, 2015). The role of ethylene in melon fruit ripening regulation was studied by drastically reducing this hormone biosynthesis in antisense *CmACO1* "Védrantais" plants (Ayub *et al.*, 1996, Pech *et al.*, 2008). Authors observed that the development of an abscission layer, the rind color change from green to yellow and the aroma volatiles biosynthesis were processes strictly ethylene-dependent, while flesh softening only partially. Conversely, carotenoid biosynthesis, sugar and organic acid accumulation were ethylene-independent processes in melon.

Due to the economic importance of melon, a series of genetic and genomic tools have been developed in an effort to facilitate comprehensive studies in this species regarding various topics including fruit ripening. Some of the tools available include genetic populations such as double haploid lines (DHL) (Monforte *et al.*, 2004), recombinant inbred lines (RIL) (Perin *et al.*, 2002b) and near-isogenic lines (NIL) (Eduardo *et al.*, 2005), saturated genetic maps (Diaz *et al.*, 2011, Argyris *et al.*, 2015), reverse genetics platforms (Dahmani-Mardas *et al.*, 2010, Gonzalez *et al.*, 2011) and the genome sequence (Garcia-Mas *et al.*, 2012).

The genetic control of melon fruit ripening was first studied using a RIL population generated from the cross between the climacteric variety "Védrantais" (*cantalupensis*) and the non-climacteric Korean exotic variety "Songwhan Charmi" PI 1611375 (*conomon*) (Perin *et al.*, 2002a). Two *loci*, *Al-3* and *Al-4* in chromosomes 8 and 9, respectively, were found to control the development of an abscission layer and the autocatalytic ethylene biosynthesis in that population. Four additional QTLs in chromosomes 1, 2, 3 and 11 controlled the amount of ethylene produced. More recently, the SC3-5-1 line from a NIL population originated from the cross between "Piel de Sapo" and "Songwhan Charmi" showed climacteric ripening despite both parents being non-climacteric varieties (Eduardo *et al.*, 2005). The characterization of SC3-5-1 allowed the identification of two QTLs, *eth3.5* and *eth6.3* in chromosomes 3 and 6, respectively, involved in the regulation of climacteric ripening (Moreno *et al.*, 2008, Vegas *et al.*, 2013). Both QTLs are able of inducing climacteric ripening in the non-climacteric background of "Piel de Sapo" individually, but when together they interact increasing ethylene biosynthesis and intensity of the ripening-associated processes like fruit abscission (Vegas *et al.*, 2013). Interestingly, there is not overlapping between the QTLs from this study and Perin et al. (2002), suggesting that the genetic control of fruit ripening in melon in complex and differs between varieties.

A comparative transcriptional profiling analysis of developing melon fruit using two climacteric ("Védrantais", "Dulce") and two non-climacteric ("Piel de Sapo", PI 161373) varieties has shown that the expression of genes related to ethylene metabolism, accumulation of carotenoids and cell-wall re-organization is correlated with both types of ripening (Saladié *et al.*, 2015). It has also been observed that the classification of ripening behaviour in two extreme classes is a simplification, as genotypes with intermediate ripening phenotypes exist (Saladié *et al.*, 2015).

Following a previous work that allowed the mapping of *eth6.3* in a 4.5 Mb region of melon LG VI (Vegas *et al.*, 2013), the objectives of this study were the identification of a candidate gene for *eth6.3* using a positional cloning strategy, the characterization of the candidate gene in a collection of melon germplasm and its functional validation using a TILLING mutant collection.

Material and Methods

Plant material

The plant material used for the positional cloning of *eth6.3* derives from the climacteric near-isogenic line (NIL) SC3-5-1, harbouring two homozygous introgressions in chromosomes 3 and 6 from the Korean accession "Songwhan Charmi" PI 161375 (*C. melo* var. *conomon*, SC) in the "Piel de Sapo" T111 (*C. melo* var. *inodorous*, PS) genetic background (Vegas *et al.*, 2013). The mapping population was originated from the cross SC3-5-1 x PS (Figure S1). SC3-5-1 (also named GF31), contains both *eth3.5* and *eth6.3*. NILs GF35 and GF40 contain *eth3.5* and *eth6.3*, respectively. All plants were grown in a greenhouse in coco-fibre bags and fruits were hand pollinated as described previously (Vegas *et al.*, 2013). All flowers were self-pollinated manually allowing only one fruit per plant.

The mutant families identified for *MELO3C016540* belong to the melon TILLING platform that was developed by the URGV-INRA in the "Charentais Mono" (*C. melo* var. *cantalupensis*, CharMono) genetic background, which is described in (Dahmani-Mardas *et al.*, 2010).

The melon germplasm collection used for the association of *MELO3C016540* with the climacteric behaviour belongs to the Institute for the Conservation and Improvement of Valencian Agrodiversity (COMAV) and includes fifty-four melon varieties previously described (Esteras *et al.*, 2013, Leida *et al.*, 2015) (Table S1).

An F3 population from a cross between Noy-Amid cultivar (*C. melo* var. *inodorous*, NA, Yellow Canary type) and 'Dulce' (*C. melo* var. *reticulatus*, Dul, cantaloupe type, (Harel-Beja *et al.*, 2010)) (NA × Dul) was also used. Four hundred and eighty F2 plants were phenotyped for ethylene emission in 2011. Thirty-two F3 families were grown from F2 plants with extreme ethylene levels (16 plants >7.5 and 16 plants <0.5 microgram/kg fresh fruit/hr). Twenty plants of each of the 32 families were grown in two repetitions in a greenhouse at Beit Elazari, Israel in 2013. Flowers were manually pollinated and tagged at anthesis and 1–2 fruits were allowed to develop per plant.

DNA extraction and genotyping

Genomic DNA was extracted from young leaves according to CTAB method (Doyle, 1990) with some modifications to improve quality (Garcia-Mas *et al.*, 2000).

Eight SSR markers and one Cleaved Amplified Polymorphic Sequence (CAPS) marker (Table S2) were used to genotype the 2008-F_2 population described in (Vegas *et al.*, 2013).

The 2012-F_4 population was screened using TaqMan probes (Thermo Scientific, Waltham, USA) SNP-64658 and SNP-2826073 that were designed by the Custom TaqMan Assay Design Tool (www.lifetechnologies.com/snpcadt) using two high-quality flanking SNPs between PS and SC (Table S2) detected by the SUPER software (Sanseverino *et al.*, 2015). PCR reactions were prepared in a final volume of 5μl: 2.5 μl 2xTaqMan 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) and consisted in an initial cycle at 95ºC for 1 min, followed by 10 cycles of temperature gradient consisting in 90ºC during 20 s and 61ºC during 1 min diminishing the temperature from 61ºC to 57ºC at 0.4ºC per cycle, and 26 cycles at 95ºC during 20 s and 57ºC during 1 min. Fluorescence was measured at 37ºC. Twenty-four additional SNPs were used to genotype the recombinants using KASP chemistry (LGC, Teddington, UK) using their proprietary software Kraken to design the

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primers (Table S2). Genotyping was carried out by the Genomics Service at CRAG using Fluidigm (San Francisco, CA, USA) and following manufacturer's instructions.

For the fine mapping of the R24, R25 and R26 recombinants, six SNPs (SEQ-1 to SEQ-6) were genotyped by PCR amplification and Sanger sequencing (Table S2). The PCR mix was prepared in a final volume of 25μl containing 40-100 ng of genomic DNA, 2mM $MgCl_2$, 1xBioline Reaction Buffer (London, UK), 1mM dNTPs, 0.13 mM of each primer and 2 U of Taq DNA polymerase. Amplification was performed in a 2720 thermal cycler (Applied Biosystems, Foster City, CA, USA) and consisted in an initial cycle at 94ºC during 1 min; 35 cycles at 94ºC, 30 s, Tm, 30 s, 72ºc, 1 min; and 72ºC during 5 min. PCR products were purified using Sephadex G50 (Buckinghamshire, UK) and quantified by NanoDrop ND-1000 (NanoDrop® Technologies, Wilmington, Delaware) before being submitted to the Capillary Sequencing Service at CRAG using the amplification primers as sequencing primers. Sequences were analysed using Sequencher 5.0 (Gene Codes Corporation, Ann Arbor, MI, USA).

Four amplicons (PRO40.1, CDS40.1, CDS40.1 and CDS40.3, Table S2) were designed to sequence the ORF, 5' and 3' UTR of *MELO3C016540*, and were used in the genotyping of the TILLING mutant families and the melon germplasm collection. PCR, sequencing and sequence analysis were made as previously described.

In the NA × Dul F3 population, Restriction-site-associated DNA sequencing (RAD-seq) and QTL analyses were performed by NRgene LTD using 131 individual F3 plants representing the whole scale of ethylene emission.

Fruit phenotyping

Fruits were collected at dehiscence or harvested when fully ripe (between 65 and 70 DAP). Fruit ripening was assessed using three traits closely associated to climacteric ripening in melon: the development of an abscission layer, the external color change from green to yellow and the production of characteristic aroma volatiles (Pech *et al.*, 2008, Obando-Ulloa *et al.*, 2010, 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: dehiscence) and the days from pollination to dehiscence were recorded. External color change was evaluated visually in dehiscent and harvested fruits. The days

from pollination to external color change, prior to dehiscence, were also measured for the phenotypic characterization of the TILLING families. The production of characteristic climacteric aroma volatiles, mainly esters, was detected by olfactory evaluation of dehiscent and harvested fruits. Additionally, the production of ethylene in the fruits of the TILLING mutant families was measured using a novel method based in non-invasive ethylene quantification in attached fruit headspace by gas chromatography-mass spectrometry GC-MS (Pereira et al, under review).

Ripe fruits from the melon germplasm collection were phenotyped for fruit firmness and abscission layer development by COMAV as described previously (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), and values in between represent intermediate phenotypes.

All statistical analysis were performed in R 3.2.1 (R Development Core Team, 2016), using built-in functions and the package multcomp 1.4-1 (Hothorn *et al.*, 2008).

NA $\times$ Dul fruits were sampled at ripening, determined by abscission layer development and/or change of rind color. Ripening was verified by BRIX values. Evaluation of ethylene emission was performed on the day after harvest. Ripe detached fruits were enclosed for three hours 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).

Identification of TILLING mutants in MELO3C016540

Mutant identification in *MELO3C016540* consisted on the screening of 6,200 M_2 families from the CharMono TILLING platform using a nested PCR technique. PCR amplification and mutation detection was carried out as previously described (Dahmani-Mardas *et al.*, 2010) using specific primers for the amplification of two regions (A1 and A2, Figure 2a) (Table S2). When needed, additional primers were designed to validate the mutations by Sanger sequencing (Table S2). PROVEAN (Protein Variation Effect Analyzer, (Choi *et*

al., 2012)) was used to predict the impact of the mutation on the protein function. Seeds from M_2 mutant families were obtained from URGV for phenotyping.

qPCR expression analyses

RNA isolation, quality control, cDNA synthesis, and qPCR analyses were performed as in (Saladié *et al.*, 2015) with the following modifications: total RNA from three different biological replicates for each of three NILs GF31, GF35, and GF40 and control PS, was isolated from mesocarp, root, and leaf tissue. Total RNA was isolated from fruit tissue as described above, while 100 mg ± 2 mg sample of previously frozen and ground leaf and root tissue was isolated using TriZOL® reagent (Ambion®, Life Technologies, Inc.) following the manufacturer's instructions. RNA samples were then purified with RNeasy® spin columns (Qiagen, Hilden, Germany) and treated with RNAse free TURBO-DNase I (Turbo-DNA-freeTM Kit; Applied Biosystems, Ambion®, USA) for 60 min at 37º C, before use as a template for cDNA synthesis. RNA quality was assessed by gel electrophoresis, quantified on a NanoDrop ND-1000 (NanoDrop® Technologies, Wilmington, Delaware), and reversed transcribed into cDNA from 400 ng of total RNA with an oligo(dT)20 primer and a SuperScript™ III Reverse Transcriptase kit (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions.

Gene expression analysis by qPCR was performed on a LightCycler® 480 Real-Time PCR System using SYBR® Green I Mix (Roche Applied Science, USA). The relative amounts of specific transcripts were determined using cyclophilin (*CmCYP7*) as a reference gene, as in previous experiments (Mascarell-Creus *et al.*, 2009, Saladié *et al.*, 2015) and then normalized to PS expression in leaves. Primers for amplification of target and reference genes were designed with Primer3 software (http://primer3.wi.mit.edu/). To maximize efficiency of qPCR reactions, primer pairs were checked for the presence of secondary structures with NetPrimer (http://www.premierbiosoft.com/netprimer/) and redesigned if necessary. Calculation of intra-assay variation, primer efficiencies, and amplification specificity of the PCR by melting curve analysis, were as described previously. For each assay, reverse transcriptase minus controls (RT-) and non-template controls (NTC) were included in each plate to assess the presence of genomic contamination (RT-), and primer dimers and/or primer contamination (NTC). The relative expression of target genes was calculated using Cq values by the LC480 software. Primer sequences used for qPCR are displayed in Table S3.

Results

Positional cloning of eth6.3

The 2008-F_2 mapping population, obtained after crossing the near isogenic line SC3-5-1 (carrying both *eth6.3* and *eth3.5*) and PS, was previously used to map *eth6.3* in a 4.5 Mb centromeric region of melon chromosome 6 (Vegas *et al.*, 2013). We obtained the 2012-F_4 segregating population from a single individual (7M80-11.4) of the 2008-F_2 mapping population that was heterozygous for *eth6.3* and fixed for the PS alleles for *eth3.5* (Figure S1). The genotyping of 1,131 individuals of the 2014-F4 population with the flanking markers SNP-64658 and SNP-2826073 allowed identifying 27 recombinants in the interval (Figure 1a). A set of 24 SNPs markers polymorphic between PS and SC and evenly distributed in the SNP-64658/SNP-2826073 interval (Table S2) was used to delimit the recombination point in each recombinant. A progeny test was performed with 20 individuals per family from 16 informative recombinants, which were phenotyped for climacteric ripening (Figure S2), allowing to map *eth6.3* between markers SNP-2691690 and SNP-2826073 in a 139 kb interval (Figure 1a; Table S4).

The 139 kb interval contains 5 annotated genes in the melon genome sequence v3.5 (Garcia-Mas *et al.*, 2012) (Table 1), two of them being transcription factors of the NAC-domain family, *MELO3C016536* and *MELO3C016540*. In order to discard some of the candidate genes in the interval, recombinants R24, R25 and R26 were genotyped with 6 additional SNP markers between SNP-2691690 and SNP-2826073 (SEQ-1 to SEQ-6, Table S2), allowing to further reduce the interval to 80.7 kb between markers SEQ-3 and SNP-2826073 and containing three genes, *MELO3C016538*, *MELO3C016539* and *MELO3C016540* (Figure 1b and 1c). *MELO3C016538* and *MELO3C016539* encode short mRNAs of 247 and 396 bp, respectively, with no homologies in sequence databases. The third gene in the interval, *MELO3C016540*, a member of the NAC-domain transcription factor family, was considered as a good candidate for *eth6.3*.

A climacteric ripening QTL from a different genetic background maps in the same interval as MELO3C16540

An F3 population obtained from the cross between the Noy-Amid cultivar (non-climacteric, *inodorous* type) and the Dulce cultivar (climacteric, *reticulatus* type) was

phenotyped for ethylene emission at harvest. Parental lines, F1 and 131 F3 individual plants representing the whole scale of ethylene emission were selected for genotyping from 700 F3 plants previously evaluated for ethylene emission in 2013. The same individuals were genotyped with 76,988 SNPs identified by GBS and used to map QTL for ethylene emission at harvest. One of the QTL (LOD 5.3, $r^2 = 0.16$) was found in chromosome 6 in the same region where *eth6.3* is located, with a QTL interval including 160 annotated genes and a bin of 17 genes at the peak (genomic location 21718892-22116151; *MELO3C016523* to *MELO3C016539*) (Table S5). *MELO3C016540* was located adjacent to the bin at the peak, with only one SNP in the intergenic region separating between them, strengthening the findings presented above and suggesting that *eth6.3* may be common in melon climacteric/non-climacteric germplasm.

MELO3C016540 belongs to the melon NAC domain transcription factor family and is phylogenetically related to the tomato SlNAC-NOR

Transcription factors of the NAC family are plant specific and contain a conserved domain NAC (<u>N</u>am, <u>A</u>TAF1/2, <u>C</u>UC2) in the N-terminal region, which is involved in DNA binding an contains 5 conserved subdomains A-E (Puranik *et al.*, 2012). We identified 81 genes of the NAC-domain family in the melon genome, putatively encoding 92 proteins (Figure S3) and evenly distributed in the 12 chromosomes, with some members arranged in clusters. *MELO3C016540* has a length of 1,771 bp, contains three exons (184, 314 and 564 bp) and two introns (89 and 183 bp) and encodes a 352 aa protein (Figure 2a).

MELO3C016540 was aligned with 37 NAC-domain proteins from different plant species and with known function (Figure S4; Table S6). The alignment showed a highly conserved N-terminal region of approximately 200 aa that contains the NAC domain. The cladogram suggested the clustering of proteins according to their biological function: group 1 mainly includes NAC proteins involved in growth and development, but also cell wall metabolism and senescence; group 2 contains NAC proteins involved in stress response, and group 3 contains NAC proteins involved in senescence, but is more heterogeneous. Group 3 also contains a protein involved in fruit ripening, the tomato SlNAC-NOR, which clusters close to MELO3C016540. A second tomato NAC protein also involved in fruit ripening, SlNAC4, is clustered in group 2. The phylogenetic analysis suggests that *MELO3C016540* is the melon orthologue of the tomato *SlNAC-NOR*, a

regulator of climacteric fruit ripening (Giovannoni, 2004), thus being a solid candidate gene for *eth6.3*.

Functional validation of MELO3C016540 through the identification of TILLING mutants

In order to validate *MELO3C016540* as the candidate gene for *eth6.3*, we screened for mutants using a melon TILLING platform established in the climacteric "Charentais Mono" genetic background (Dahmani-Mardas *et al.*, 2010). We used two overlapping amplicons, A1 and A2 of 920 and 807 bp, respectively, which covered the 1,334 bp ORF of *MELO3C016540*, and screened 6,200 M2 families. We identified 21 families containing 20 mutations (Table 2), however 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, 3 in introns and 2 in the 3'UTR, with eight of them producing non-synonymous amino acid changes (Figure 2a; Table 2). Three of the non-synonymous mutations in the MELO3C016540 protein were located between residues 15 and 178, corresponding to the NAC domain (Figure S5). 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.

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 2). 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 dehiscence to phenotype the mutant families, and families 246 (E59K) and 502 (P342L) significantly increased the time to dehiscence in M plants in 6,4 and 11,7 days, respectively (not shown). 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 dehiscence in some control "Charentais Mono" plants, resulted in the re-design of the phenotyping assay in a second season, where we chose to phenotype days from pollination to external color change as a more robust measure of ripening (Figure 2b and 2c). The external color change is a good measure of ethylene production in climacteric fruits, as we have observed in a RIL population from the cross

of "Védrantais" x "Piel de Sapo" (Pereira, personal communication). The second year, six mutant families were phenotyped (246, 432, 4933, 3717, 2503 and 502), using a higher number of W and M fruits per family (between n=7 and n=18). Two families showed significant differences between W and M fruits: 246 (E59K; p-value=4.4 x e-8) and 432 (P129L; p-value=1.6 x e-6), with 7.2 and 5.6 additional days to external color change, respectively, compared to the controls (Table 3; Figure 2d). Regarding days from pollination to dehiscence, although not significant, the values were also increased in families 246 (46.4 days W vs. 51.9 days M) and 432 (47 days W vs. 54.9 days M).

The availability of a novel method for measuring ethylene fruit production based in non-invasive ethylene quantification in attached fruit headspace by gas chromatography-mass spectrometry (Pereira et al, under review), allowed to measure the production of ethylene in the fruits of the TILLING mutant families 246 and 432, in the 2016 season. We observed a significant increase in the days from pollination to the production of the ethylene peak in both families (37.3 days W vs. 45.7 days M in family 246; 38 days W vs. 42 days M in family 432) (Figure 3a, Table 4), coinciding with 6.1 and 5.7 additional days to external color change. Again, the values for days from pollination to dehiscence, although not significant, were also increased in both families (Table 4). However, we could not see a significant difference in the amount of ethylene produced in both mutant families (Figure 3b).

The phenotypes of the TILLING mutant families E59K and P342L in the climacteric line "Charentais Mono" suggest that *MELO3C016540* is involved in the control of climacteric fruit ripening, as two mutations in the NAC region produce a significant delay in the process of climacteric ripening. The mutations E59K and P129L are located in subdomains B and D of the NAC domain, in residues that are conserved in a set of NAC proteins of known function (Figure S6).

MELO3C016540 sequence diversity in melon germplasm

We selected a panel of 54 melon accessions to characterize the genetic variability of *MELO3C016540*, which contained lines from 11 of the 16 botanical groups that have been described in melon (Pitrat, 2008) and representing the two subspecies *melo* and *agrestis* (Table S1). These accessions were previously phenotyped for fruit ripening behaviour (Table S1). We identified 17 polymorphisms, both SNP (12) and indels (5), distributed in the promoter region (2), 5'UTR (3), exons (6), introns (4), 3'UTR (1) and

the terminator region (1) (Figure 4; Table S1). We constructed a cladogram using the sequence multiple alignment, which showed a clear separation of the *cantalupensis* and *inodorus* groups, although five *cantalupensis* accessions were clustered in the *inodorus* group (Figure 4). Interestingly, the allele of SC clustered close to the climacteric *cantalupensis* group. We observed a strong correlation between the three variables that were phenotyped in the accessions panel, climacteric behaviour with abscission zone (0.78), and with fruit firmness (-0.41), which resulted in using only climacteric behaviour for an association analysis. Seven polymorphisms in *MELO3C016540* were significantly associated with "climacteric behaviour": INDEL-282, INDEL-126, G411T, T533A, INDEL743, G979A and INDEL*173 (Figure 4; Tables S1 and S7). G411T and T533A produced non-synonymous amino acid changes A108S and S236N, respectively, although located outside the NAC domain region and predicted as neutral according to PROVEAN (Figure S5). The polymorphisms showing the strongest association with the ripening type 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 that may have an effect in the protein translation (Figure 5). The INDEL-126 analysis in the accessions resulted in the identification of 9 alleles, structured in 4 blocks with a polyA track (A), and three repeats GAGAAAA (B), GAAAAAA (C) and GAAATAA (D). The SC allele is similar to the *cantalupensis* one, containing only block A, whereas PS contains the block structure ABCD.

Association of MELO3C016540 with the climacteric behaviour

In a previous work, Leida et al. (2015) studied the association of candidate genes with climacteric behaviour in a panel of 175 melon accessions of diverse origins that included wild relatives, feral types, landraces and breeding lines, representing the diversity of the species. The accessions had been 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. However, SNPs nearby *MELO3C016540* were not assayed.

The non-synonymous SNP G411T (A108S) in *MELO3C016540* (Table S7) had been previously identified in a SNP database generated after resequencing eight pools of accessions that represented the main melon botanical groups (snv26555 available at

) (Blanca *et al.*, 2012). SNP G411T was polymorphic between climacteric and non-climacteric groups of melons in the previous resequencing assay (Blanca *et al.*, 2012). In fact the full climacteric *cantalupensis* and *momordica* melons had the T allele, the non-climacteric *inodorus* Piel de sapo melons had the G allele, and both alleles were present in the group of African *agrestis*, in which a variable climacteric behaviour has been reported (Leida *et al.*, 2015). We genotyped SNP G411T in the panel of 175 melon accessions used in Leida et al (2015) and its association with ripening related traits was assessed with Mixed Linear Models (MLM) using the full SNP data set as cofactors. SNP G411T was found to be highly associated with climacteric behaviour ($p= 4.35 \times 10^{-5}$) and abscission layer formation ($p= 6.48 \times 10^{-4}$), further supporting the implication of *MELO3C016540* in climacteric fruit ripening.

MELO3C016540 expression in fruit

In order to know if the expression of *MELO3C016540* is fruit specific or it is also expressed in other organs as leaf and root, we performed qPCR in the non-climacteric Piel de Sapo T111 line (PS), and in the NILs containing *eth3.5* (GF35), *eth6.3* (GF40) or both of them (GF31) (Figure 6a). *MELO3C016540* is highly expressed in fruit tissue of all four genotypes, either climacteric or non-climacteric, during fruit development at 20 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 *eth6.3* interval (Figure 6e). *MELO3C016536* is highly expressed in fruit tissue in GF31, GF35 and GF40, but it is not expressed in fruit of the non-climacteric PS. *MELO3C016536* is not expressed in leaves and roots in all four genotypes. As controls, 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 6b, c and d). 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 *eth6.3* has revealed that the underlying gene is *MELO3C016540*, which encodes a transcription factor of the NAC (NAM, ATAF1,2 and CUC2) family. *eth6.3* was identified in the PI 161375 (SC) x "Piel de Sapo" (PS) genetic background. We show evidence that another QTL involved in ethylene emission, identified in the "Noy Amid" x "Dulce" genetic background, maps in the same genomic interval than *MELO3C016540*. Moreover, a high association was found between SNP G4111T present in *MELO3C016540* with different ripening-related traits that were phenotyped in a panel of 175 melon accessions. All together, these findings strongly suggest the involvement of *MELO3C016540* in the climacteric ripening process in melon.

NAC transcription factors (TF) constitute one of the largest families of plant TFs (Puranik *et al.*, 2012). A phylogenetic analysis of the melon NAC-domain transcription factor gene family including NAC proteins of known function from different plant species suggests that *MELO3C016540* is the orthologue of the tomato *Nor* gene, which is involved in fruit ripening (*SlNAC*-NOR, Figure 7). Both proteins are included in a clade that contains other NAC members mainly 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 in *nor* mutants (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). In this respect, the *inodorus* melon type PS resembles the *nor* tomato mutant, as it does not show a peak of ethylene during ripening, it lacks fruit abscission, the exocarp color remains green through maturation and a reduction in fruit softening is observed. 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). It has been suggested that in species containing both climacteric and non-climacteric genotypes, like melon, the latter may be attributed to mutations related with ethylene synthesis genes (Giovannoni, 2004). The SC allele of *eth6.3* introgressed into the PS non-climacteric type in line GF40 shows a moderate climacteric type. The SC allele of *eth3.5*, another QTL in chromosome 3, is independently rescuing the climacteric ripening capacity of PS in line GF35, suggesting that at least two

genes may be impaired in the non-climacteric phenotype of PS. The NAC-domain 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 studies have also reported the involvement of other members of the NAC family 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 along different plant clades.

The involvement of *MELO3C016540* in the control of fruit ripening was demonstrated after characterizing several TILLING mutant phenotypes in a high-climacteric "Charentais Mono" genetic background. Two mutant families containing the non-synonymous mutations E59K and P232L in *MELO3C016540* showed a significant delay in the onset of the climacteric fruit ripening when compared to controls, both at the level of external color change and presence of an ethylene peak. "Charentais Mono" is a highly climacteric line belonging to the *cantalupensis* type (Dahmani-Mardas *et al.*, 2010). The effect of both mutants in the climacteric phenotype resulted in a delay of the ripening process, which is compatible with both mutations being non-synonymous amino acid changes in subdomains B and D of the NAC domain region (Figure S6). Interestingly, the tomato long shelf life "de Penjar" varietal type, which is cultivated in North-east Spain, is due 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. Contrary, the *nor* mutant is due to a 2-bp deletion in the third exon, producing an extreme non-ripening phenotype (Casals *et al.*, 2012).

The sequence of *MELO3C016540* in a panel 54 melon accession belonging to different botanical groups revealed 17 polymorphisms (SNP and indel), of which 7 were strongly associated with the climacteric phenotype (Figure 4). Two features of the sequence diversity analysis of *MELO3C016540* deserve further attention. First, the *MELO3C016540* alleles 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, whereas all 15 *inodorus* and other non-climacteric accessions where clustered together, 9 out of 14 *cantalupensis* and *reticulatus* climacteric accessions were in the same cluster, and 5 (Can-Pres, Can-Y, Can-PS, Can-CA and Can-

Ef) were included in the *inodorus* group. A genetic analysis of non-climacteric ripening using a RIL population obtained from the cross between the climacteric variety "Védrantais" (*cantalupensis*) and the non-climacteric SC (Perin *et al.*, 2002a), 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. Interestingly none of these QTL map in the same genomic intervals than *eth3.5* and *eth6.3* (data not shown), suggesting that the non-climacteric phenotype of SC may be attributed to mutations in the above mentioned QTLs. This would also explain why the *eth3.5* and *eth6.3* alleles of SC, which are almost identical to those of the climacteric *cantalupensis* accessions, are able to partially rescue the climacteric phenotype when introgressed into the non-climacteric PS. It has been described that the non-climacteric phenotypes of SC and PS are different, as the 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). QTL 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 QTL reported in melon so far, suggests that the classification of ripening behaviour into just two classes may be reviewed into a more complex scenario contemplating a continuous ripening spectrum (Saladié *et al.*, 2015). A group of climacteric *cantalupensis* accessions, which contain the PS allele of *MELO3C016540*, still show the climacteric behaviour, probably due to the presence of the "climacteric alleles" for other QTLs involved in ripening. The delayed ripening phenotype observed in the "Charentais Mono" TILLING mutants E59K and P232L would also support this hypothesis. The recent availability of a novel non-invasive method for the ethylene quantification in attached fruits will help classifying melon accessions according to their ripening behaviour in a more precise manner (Pereira et al, under review).

Our current data does not allow identifying which is the causal polymorphism of the climacteric phenotype among the 7 polymorphisms identified in *MELO3C016540* that are highly associated with ripening behaviour. *MELO3C016540* is expressed in flesh at different stages of fruit development in both climacteric and non-climacteric types, peaking around 30 DAP (Figure 6a) 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 *MELO3C016540* suggests that the differential ripening phenotypes may occur post-transcriptionally through other mechanisms. Two of the natural polymorphisms found in *MELO3C016540* produce non-synonymous changes A108S and S236N, which are located outside the NAC subdomains. 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 *MELO3C016540* 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, showing effects in 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 and still poorly understood regulatory network of fruit ripening in tomato (Zhu *et al.*, 2014). Among the cluster of NAC proteins that includes MELO3C016540 and tomato NOR, other melon NAC proteins are also found (Figure 7). Interestingly MELO3C016536, which is in the same genomic interval than MELO3C016540, is phylogenetically related to SlNAC4 and other NAC proteins involved in stress responses and shows a clear differential expression between climacteric and non-climacteric lines in fruit flesh (Figure 6e). It would not be surprising that, as in tomato, other NAC genes are also involved in regulating fruit ripening, which remains to be tested in future studies.

Melon, a species that contains both climacteric and non-climacteric genotypes, may help in understanding the differences between these two types of ripening behaviours and the common mechanisms shared with tomato, the classical climacteric model species for studying fruit ripening. Current genetic data suggest that several factors are involved in the regulation of fruit ripening in melon, and *eth6.3*, 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.

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Figure legends

Figure 1. High-resolution physical map of the *eth6.3* interval. **A**. Original interval of *eth6.3* in melon chromosome 6 (Vegas *et al.*, 2013). Blue bars indicate annotated genes in the melon genome v3.5.1 (Argyris *et al.*, 2015). Two flanking SNPs[a] were genotyped using TaqMan probes, and twenty-four SNPs evenly distributed in the interval were used to genotype 16 recombinant F2 progenies using KASP chemistry (represented as SNP-with their physical position). Additional markers in the interval are from Vegas et al. (2013). The 139 Kb interval containing *eth6.3* is represented with a red bar. **B**. Reduced interval of 139 kb containing 5 annotated genes (blue bars). Six additional SNPs (SEQ-1 to SEQ-6) were used to reduce the interval of the QTL to 80.7 Kb (red bar). **C**. Graphical representation of informative recombinants R24, R25 and R26 used to define the final position of *eth63*. The genotype of the recombinants is represented in green (homozygous for PS), orange (homozygous for SC) and blue (heterozygous). Missing genotypes are represented in grey.

Figure 2. Mutants identified for *MELO3C016540* and phenotyping. **A**. Structure of *MELO3C016540*. A1 and A2 represent the regions amplified for searching mutants in the "Charentais Mono" TILLING population. Red boxes represent UTRs, blue boxes represent exons and blue lines represent introns. The NAC domain in 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**. Unripe fruit of family 246. **C**. Ripe fruit of family 246. **D**. Phenotypic differences according to external color change in M2 families of mutants 246, 432, 4933, 3717, 2503 and 502 observed in the second year. In the Y-axis, days between pollination and external color 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 t-Student test. Significance level ***: p-value < 0.001.

Figure 3. (A) Box plots for days after pollination to peak ethylene production (DTP) 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 *MELO3C016540*

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 days after pollination (DAP). Means are plotted ± SD (n=4) for MONO and (n=3) for WT and MU families.

Figure 4. Sequence diversity of *MELO3C016540* in a collection of 54 melon accessions. Left panel: Cladogram obtained with NJ with bootstrap and 1,000 iterations in MEGA 6, and represented with R from the multiple alignment obtained with Clustal Omega. The scale indicates the genetic distance. Colors for each accession represent the melon botanical classification after Esteras et al. (2013): 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*. Right panel: genotyping of the collection. Colors indicate the observed genotypes for each SNP/indel. The structure of *MELO3C016540* with the position of SNPs (diamonds) and indels (triangles) is represented above. Black diamonds and triangles indicate a significant association of each variation with the type of fruit ripening.

Figure 5. Sequence of INDEL-126 in the collection of melon accessions. The represented sequence corresponds to the interval between nucleotides -140 and -84 in the 5'-UTR region of *MELO3C016540*. Groups ABCD, A, ABC and ABE (right) are defined based on the presence of blocks A (blue, polyA track), B (green, "GAGAAAA"), C (red, "GAAAAAA"), D (orange, "GAAATAAA") and E (purple, "GAATAAA"). INO: *inodorus*; CHA: various *melo*; CAN: *cantalupensis*; CON: *conomon*; AG1 and AG2: *agrestis*; MOM: *momordica*; CHI: *chito*; TIB: *tibish*.

Figure 6. *MELO3C016540* expression. Gene expression was measured by qPCR in 3 climacteric NILs (GF31, GF35, and GF40) and the non-climacteric line Piel de Sapo (PS) for *MELO3C016540* (A), *CmACO1* (B), *CmACS1* (C), *CmACS5* (D), and *MELO3C016536* (E). Gene expression was plotted relative PS expression in leaves and measured in developing fruit at 20 and 30 days after pollination (DAP), fruit at harvest, leaf and root tissue. Means are plotted ± SE (n=3).

Figure 7. Multiple alignment of the melon NAC domain family with NAC domain containing proteins of known function of other plant species. The cladogram was obtained after the multiple alignment of 128 protein sequences with the NJ method in MEGA 6 and represented with R. The zoom shows the clade that contains MELO3C016540 and the tomato SlNAC-NOR and SlNAC4 proteins. The prefix for each protein sequence indicates the plant species: Sl, *Solanum lycopersicum*; Os, *Oryza sativa*; Gm, *Glycine max*; Ca, *Capsicum annum*; St, *Solanum tuberosum*; Cs, *Citrus sinensis*; Ph, *Petunia hybrida*; Pv, *Phaseolus vulgaris*. Arabidopsis proteins do not contain prefix except AtNAM. Sequences are described in Table S6. Colors indicate protein function according to literature: red, stress response; green, cell wall metabolism; blue, plant growth and development; purple, senescence; orange, fruit ripening.

Tables

Table 1. Candidate genes annotated in the 139 kb interval between SNP-2691690 and SNP-2826073. Start and end positions according to the melon genome v3.5.1 pseudomolecules (Argyris *et al.*, 2015). +/- represents the strand where the gene is annotated. For each gene, the number of exons, mRNA size, protein size and the functional annotation are given.

Table 2. Mutations identified in *MELO3C016540*. For each mutant, the amplicon used to screen the TILLING population (A1 or A2), the nucleotide position and the location in the gene sequence are listed. The predicted amino acid substitution for non-synonymous mutations and the predicted effect according to PROVEAN are also included. [1]Discarded mutant family. [2,3,4]Pairs of mutant families with the same mutation. [5]Mutations probably not caused by EMS.

Table 3. Phenotyping of external color change in the TILLING mutants. Six mutant families and the "Charentais Mono" line were genotyped and phenotyped for external color change, measuring the days from pollination to the color change (DAP). W: homozygote for the wild type allele, M: homozygote for the mutant allele. SD: standard deviation. Asterisks indicate the level of significance after a t-Student test. *: p-value < 0.05; **: p-value < 0.01***: p-value < 0.001.

Table 4. Phenotyping of ethylene production in the TILLING mutant families 246 and 432. The ethylene production during fruit ripening was measured in the mutant families 246 and 432 and the "Charentais Mono" line. The external color change and the abscission dates were also recorded. W: homozygote for the wild type allele, M: homozygote for the mutant allele. SD: standard deviation. Asterisks indicate the level of significance after a t-Student test. *: p-value < 0.05; **: p-value < 0.01***: p-value < 0.001.

Supplementary material

Figure S1. Scheme with the plant material used to identify *eth6*.3. Black vertical bars represent chromosomes III and VI. Colored boxes represent *eth3.5* and *eth6.3* QTLs in chromosomes III and VI, respectively. In green, PS allele in homozygosis; orange, SC allele in homozygosis; blue, heterozygous. On the right, markers used to select the 7M80-11.4 individual, from which the 2012-F4 population was obtained.

Figure S2. Distribution of the fruit abscission dates of the progenies of 16 recombinants, expressed in days after pollination (DAP). The color of the boxes represents the assigned phenotype to each recombinant: A (red): climacteric; B (green): non-climacteric; H (blue): heterozygote. PS: "Piel de Sapo"; SC: "Songwhan Charmi"; GF31: NIL containing *eth3.5* and *eth6.3*; GF35: NIL containing *eth3.5*; GF40: NIL containing *eth6.3*. Asterisks indicate the level of significance after a Dunnett test using PS as control: ***: p-value < 0.001; **: p-value < 0.01.

Figure S3. Phylogenetic analysis of the melon NAC domain family. The cladogram was obtained after the multiple alignment of 92 protein sequences (corresponding to 81 annotated genes) with the NJ method in MEGA 6 and represented with R. Numbers indicate the node support with bootstrap with 1,000 iterations. The lower scale represents the relative genetic distance. MELO3C016540 and MELO3C016536 are represented in red.

Figure S4. Multiple alignment of MELO3C016540 with NAC domain containing proteins of known function of other plant species. The cladogram was obtained after the multiple alignment with the NJ method in MEGA 6 and represented with R. Numbers indicate the node support with bootstrap with 1,000 iterations. The lower scale represents

the relative genetic distance. Colors indicate protein function according to literature: red, stress response; green, cell wall metabolism; blue, plant growth and development; purple, senescence; orange, fruit ripening. MELO3C016540 in black. Group 1 contains proteins involved in growth, development and cell wall metabolism. Group 2 contains proteins involved in stress response. Group 3 contains proteins involved in senescence and fruit ripening. The prefix for each protein sequence indicates the plant species: Sl, *Solanum lycopersicum*; Os, *Oryza sativa*; Gm, *Glycine max*; Ca, *Capsicum annum*; St, *Solanum tuberosum*; Cs, *Citrus sinensis*; Ph, *Petunia hybrida*; Pv, *Phaseolus vulgaris*. Arabidopsis proteins do not contain prefix except AtNAM. Sequences are described in Table S6.

Figure S5. Mutations in the *MELO3C016540* sequence. The alignment performed with Clustal Omega and represented with Jalview shows the MELO3C016540 sequences of "Piel de Sapo" line T111 (PS), "Charentais Mono" (CharMono) and the artificial sequence "Mutations" that includes all non-synonymous mutations (in red). The residues highlighted in blue correspond to the polymorphisms between PS and CharMono S108A and N236S. Domains in grey boxes represent the NAC subdomains A to E. a: E59K (mutant 246); b: P129L (mutant 432); c: S164F (mutant 2923); d: A248V (mutant 4933); e: S256F (mutant 3717); f: D263N (mutant 2503); g: L300F (mutant 4321); h: P342L (mutant 502)

Figure S6. Mutations E59K (family 246) and P129L (family 432) in the NAC domain region of MELO3C016540 and other NAC domain containing proteins. Alignment performed with Clustal Omega and represented with Jalview of 38 NAC domain proteins from different plant species. Red squares represent the mutations. Blue color indicates amino acid conservation higher than 75 %. The prefix for each protein sequence indicates the plant species: Sl, *Solanum lycopersicum*; Os, *Oryza sativa*; Gm, *Glycine max*; Ca, *Capsicum annum*; St, *Solanum tuberosum*; Cs, *Citrus sinensis*; Ph, *Petunia hybrida*; Pv, *Phaseolus vulgaris*. Arabidopsis proteins do not contain prefix except AtNAM. Sequences are described in Table S6.

Table S1. Melon germplasm used for assessing the variation analysis of *MELO3C016540*. Fifty-four melon accessions described in Esteras et al. (2013) are indicated, with information about the subspecies, botanical group, name, origin and market class. The table includes the phenotype for each accession for type of ripening (0

to 4, 0: non-climacteric as PS, 4: climacteric as Védrantais), fruit flesh firmness expressed in Kg/0.5 cm^2, and abscission (1 to 4, 1: no slip, 2: half-slip, 3: full-slip, 4: complete dehiscence. The alleles of 17 polymorphisms found in the sequence of *MELO3C016540* with their location are also shown. For heterozygotes, both alleles are separated by "x". The parental lines of the NILs SC and PS are in grey.

Table S2. Sequences of the markers and primers used during the high-resolution mapping of *eth6.3* and for the TILLING screening.

Table S3. Primer sequences of the genes used for qPCR.

Table S4. Phenotyping and genotyping of 16 informative recombinants and fine mapping of *eth6.3*. Plant R27 is not included as it was later identified as a false recombinant. Two flanking SNPs and 24 SNPs in the interval are represented according to their physical position in the melon genome. The phenotype after the progeny test is represented in the right column. A (orange): homozygous SC, B (green): homozygous for PS, H (blue): heterozygote.

Table S5. QTL peak for ethylene measured at harvest in the RIL population of Noy-Amid x Dulce.

Table S6. NAC-domain containing proteins of different plant species with known function. Their biological function, UniProt code and reference are provided.

Table S7. Polymorphisms in *MELO3C016540* associated with the climacteric phenotype. Seventeen polymorphisms found in the *MELO3C016540* sequence are listed. The association of each polymorphism with the "type of ripening" score in Supplementary Table 1 was calculated with ANOVA-GLM. Asterisks indicate the significance level: *: p-value < 0.05; **: p-value < 0.01. [1]polymorphisms observed between SC and PS. The hyphen indicates the position before the ATG start codon. The asterisk in the left column indicates the position after the 3'UTR start.