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.

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 color, development of aroma, fruit softening and a short postharvest 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 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 transcription factors 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 color 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 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 1,131 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 5 annotated genes in the reference genome v3.5 (Garcia-Mas *et al.*, 2012) (Table S3), two of which are transcription factors of the NAC-domain family, *MELO3C016536* and *MELO3C016540*. Recombinants R24, R25 and R26 were genotyped with 6 additional SNPs between SNP-2691690 and SNP-2826073 (SEQ-1 to 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-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 transcription factor 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 (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 transcription factor 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 1,771 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 (Figure 1, Figure 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* were assayed.

A non-synonymous SNP G411T in *CmNAC-NOR* (Table S6) was previously identified after re-sequencing 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 association with ripening related traits was assessed (Table S7). SNP G411T was found to be highly associated with ripening type ($p= 4.35x10^{-5}$) and abscission layer formation ($p= 6.48x10^{-4}$), 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 5 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 9 alleles, structured in 4 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 6,200 M2 families with two overlapping amplicons, A1 and A2 of 920 and 807 bp, respectively, which covered the 1,334 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, 3 in introns and 2 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.

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, made that the number of replicates were 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 color change as a more robust measure of ripening. The external color 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" x 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-value=4.4 x e-8) and 432 (P129L; p-

value=1.6 x 10^{-6}), with 7.2 and 5.6 additional days to external color change, respectively, compared to the controls (Table S10; Figure 2b).

We applied a method for measuring ethylene fruit production, based in non-invasive ethylene quantification in attached fruit with chromatography-mass spectrometry (Pereira *et al*., in press), 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 vs. 45.7 days M in family 246; 38 days W vs. 42 days M in family 432) (Figure 4a, Table 1), coinciding with 6.1 and 5.7 additional days to external color 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 it is also expressed in other organs, we performed qPCR in the non-climacteric PS, and in the 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 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-c-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) x "Piel de Sapo" (PS) genetic background, revealed that the underlying gene is *CmNAC-NOR*, which encodes a transcription factor 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" x "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 color change and presence of an ethylene peak, when compared to 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).

The NAC domain transcription factors (TF) 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 color 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 nor 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 7 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 where clustered together. Nine out of 14 *cantalupensis* and *reticulatus* climacteric accessions were in the climacteric *cantalupensis* cluster but 5 (Can-Pres, Can-Y, Can-PS, Can-CA and Can-Ef) were included in 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 than *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 classifying melon accessions according to their ripening behaviour in a more precise manner (Pereira *et al.*, in press).

Our current data does not allow the identification of the causal polymorphism of the climacteric phenotype among the 7 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 around 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 x PS (Figure S1) was used for the positional cloning of *ETHQV6.3*. SC3-5-1 (GF31) is a near-isogenic line (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).

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 x 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/hr) 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 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 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) with an initial cycle at 95ºC for 1 min, 10 cycles of temperature gradient consisting in 90ºC for 20 s and 61ºC for 1 min diminishing the temperature from 61ºC to 57ºC at 0.4ºC per cycle, and 26 cycles at 95ºC for 20 s and 57ºC for 1 min. Fluorescence was measured at 37ºC. Twenty-four SNPs were genotyped using KASP chemistry (LGC, Teddington, UK) in a Biomark^{TM} 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 x 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 color change was evaluated visually in fruit after abscission and harvested fruits. Days from pollination to external color 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 in non-invasive ethylene quantification in attached fruit headspace by gas chromatography-mass spectrometry (Pereira *et al*., in press).

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 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).

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 analysis were performed using the Neighbor-joining method in MEGA 6.06 (Tamura *et al*., 2013) with 1,000 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 Leida et *al*. (2015). Mixed Linear Models (MLM) 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 t-Student 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 Inc., NC).

Identification of TILLING mutants in CmNAC-NOR

Mutant identification in *CmNAC-NOR* consisted on the screening of 6,200 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, Inc.). RNA samples were purified with RNeasy® spin columns (Qiagen, Hilden, Germany) and treated with RNAse free TURBO-DNase I (Turbo DNA-free™ Kit; Applied Biosystems, Ambion®, USA) for 60 min at 37ºC. 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, USA). 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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Conflicts of interest:

The authors declare no conflicts of interest

Short legends for supporting information

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" x "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" x "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 color 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*.

References

Abeles, F.B., Morgan, P.W. and Saltveit, M.E. (1992) *Ethylene in plant biology*. San Diego: Academic Press.

Adams-Phillips, L., Barry, C. and Giovannoni, J. (2004) Signal transduction systems regulating fruit ripening. *Trends Plant Sci*, **9**, 331-338.

Alexander, L. and Grierson, D. (2002) Ethylene biosynthesis and action in tomato: a model for climacteric fruit ripening. *J Exp Bot*, **53**, 2039-2055.

Argyris, J.M., Ruiz-Herrera, A., Madriz-Masis, P., Sanseverino, W., Morata, J., Pujol, M., Ramos-Onsins, S.E.E. and Garcia-Mas, J. (2015) Use of targeted

SNP selection for an improved anchoring of the melon (*Cucumis melo* L.) scaffold genome assembly. *BMC Genomics*, **16**, 4.

Ayub, R., Guis, M., Ben Amor, M., Gillot, L., Roustan, J.P., Latche, A., Bouzayen, M. and Pech, J.C. (1996) Expression of ACC oxidase antisense gene inhibits ripening of cantaloupe melon fruits. *Nat Biotechnol*, **14**, 862-866.

Blanca, J., Esteras, C., Ziarsolo, P., Perez, D., Ferna Ndez-Pedrosa, V., Collado, C., Rodra Guez de Pablos, R., Ballester, A., Roig, C., Canizares, J. and Pico, B. (2012) Transcriptome sequencing for SNP discovery across *Cucumis melo*. *BMC Genomics*, **13**, 280.

Bradbury, P.J., Zhang, Z., Kroon, D.E., Casstevens, T.M., Ramdoss, Y. and Buckler, E.S. (2007) TASSEL: Software for association mapping of complex traits in diverse samples. *Bioinformatics*, **23**, 2633-2635.

Casals, J., Pascual, L., Cañizares, J., Cebolla-Cornejo, J., Casañas, F. and Nuez, F. (2012) Genetic basis of long shelf life and variability into Penjar tomato. *Genet Resour Crop Ev*, **59**, 219-229.

Choi, Y., Sims, G.E., Murphy, S., Miller, J.R. and Chan, A.P. (2012) Predicting the functional effect of amino acid substitutions and indels. *PLoS one*, **7**.

Chung, M.Y., Vrebalov, J., Alba, R., Lee, J., McQuinn, R., Chung, J.D., Klein, P. and Giovannoni, J. (2010) A tomato (*Solanum lycopersicum*) APETALA2/ERF gene, SlAP2a, is a negative regulator of fruit ripening. *Plant J*, **64**, 936-947.

Dahmani-Mardas, F., Troadec, C., Boualem, A., Leveque, S., Alsadon, A.A., Aldoss, A.A., Dogimont, C. and Bendahmane, A. (2010) Engineering melon plants with improved fruit shelf life using the TILLING approach. *PLoS One*, **5**, e15776.

Eduardo, I., Arus, P. and Monforte, A.J. (2005) Development of a genomic library of near isogenic lines (NILs) in melon (*Cucumis melo* L.) from the exotic accession PI161375. *Theor Appl Genet*, **112**, 139-148.

Esteras, C., Formisano, G., Roig, C., Diaz, A., Blanca, J., Garcia-Mas, J., Gomez-Guillamon, M.L., Lopez-Sese, A.I., Lazaro, A., Monforte, A.J. and Pico, B. (2013) SNP genotyping in melons: genetic variation, population structure, and linkage disequilibrium. *Theor Appl Genet*, **126**, 1285-1303.

Ezura, H. and Owino, W.O. (2008) Melon, an alternative model plant for elucidating fruit ripening. *Plant Sci*, **175**, 121-129.

Gao, C., Ju, Z., Cao, D., Zhai, B., Qin, G., Zhu, H., Fu, D., Luo, Y. and Zhu, B. (2015) MicroRNA profiling analysis throughout tomato fruit development and ripening reveals potential regulatory role of RIN on microRNAs accumulation. *Plant Biotech J*, **13**, 370-382.

Garcia-Mas, J., Benjak, A., Sanseverino, W., Bourgeois, M., Mir, G., Gonzalez, V.M., Henaff, E., Camara, F., Cozzuto, L., Lowy, E., Alioto, T., Capella-Gutierrez, S., Blanca, J., Canizares, J., Ziarsolo, P., Gonzalez-Ibeas, D., Rodriguez-Moreno, L., Droege, M., Du, L., Alvarez-Tejado, M., Lorente-Galdos, B., Mele, M., Yang, L., Weng, Y., Navarro, A., Marques-Bonet, T., Aranda, M.A., Nuez, F., Pico, B., Gabaldon, T., Roma, G., Guigo, R., Casacuberta, J.M., Arus, P. and Puigdomenech, P. (2012) The genome of melon (*Cucumis melo* L.). *Proc Natl Acad Sci USA*, **109**, 11872-11877.

Garcia-Mas, J., Oliver, M., Gomez-Paniagua, H. and de Vicente, M.C. (2000) Comparing AFLP, RAPD and RFLP markers for measuring genetic diversity in melon. *Theor Appl Genet*, **101**, 860-864.

Giovannoni, J.J. (2001) Molecular biology of fruit maturation and ripening. *Annu Rev Plant Phys*, **52**, 725-749.

Giovannoni, J.J. (2004) Genetic regulation of fruit development and ripening. *Plant Cell*, **16 Suppl**, S170-180.

Giovannoni, J.J. (2007) Fruit ripening mutants yield insights into ripening control. *Curr Opin Plant Biol*, **10**, 283-289.

Handa, A.K., Anwar, R. and Mattoo, A.K. (2014) Biotechnology of fruit quality. In *Fruit Ripening: Physiology, Signalling and Genomics* (Nath, P., Bouzayen, M., Mattoo, A. and Pech, J.C. eds). Boston, MA: CABI, pp. 259-290.

Harel-Beja, R., Tzuri, G., Portnoy, V., Lotan-Pompan, M., Lev, S., Cohen, S., Dai, N., Yeselson, L., Meir, A., Libhaber, S.E., Avisar, E., Melame, T., van Koert, P., Verbakel, H., Hofstede, R., Volpin, H., Oliver, M., Fougedoire, A., Stalh, C., Fauve, J., Copes, B., Fei, Z., Giovannoni, J., Ori, N., Lewinsohn, E., Sherman, A., Burger, J., Tadmor, Y., Schaffer, A.A. and Katzir, N. (2010) A genetic map of melon highly enriched with fruit quality QTLs and EST markers, including sugar and carotenoid metabolism genes. *Theor Appl Genet*, **121**, 511-533.

Itkin, M., Seybold, H., Breitel, D., Rogachev, I., Meir, S. and Aharoni, A. (2009) TOMATO AGAMOUS-LIKE 1 is a component of the fruit ripening regulatory network. *Plant J*, **60**, 1081-1095.

Klee, H.J. (2004) Ethylene signal transduction. Moving beyond Arabidopsis. *Plant Physiol*, **135**, 660-667.

Klee, H.J. and Giovannoni, J.J. (2011) Genetics and control of tomato fruit ripening and quality attributes. *Annu Rev Genet*, **45**, 41-59.

Kovács, K., Fray, R.G., Tikunov, Y., Graham, N., Bradley, G., Seymour, G.B., Bovy, A.G. and Grierson, D. (2009) Effect of tomato pleiotropic ripening mutations on flavour volatile biosynthesis. *Phytochemistry*, **70**, 1003-1008.

Lee, J.M., Joung, J.-G.G., McQuinn, R., Chung, M.-Y.Y., Fei, Z., Tieman, D., Klee, H. and Giovannoni, J. (2012) Combined transcriptome, genetic diversity and metabolite profiling in tomato fruit reveals that the ethylene response factor SlERF6 plays an important role in ripening and carotenoid accumulation. *Plant J*, **70**, 191-204.

Leida, C., Moser, C., Esteras, C., Sulpice, R., Lunn, J.E., de Langen, F., Monforte, A.J. and Picó, B. (2015) Variability of candidate genes, genetic structure and association with sugar accumulation and climacteric behavior in a broad germplasm collection of melon (*Cucumis melo* L.). *BMC Genetics*, **16**, 28.

Lelièvre, J.-M., Latchè, A., Jones, B., Bouzayen, M. and Pech, J.-C. (1997) Ethylene and fruit ripening. *Physiol Plantarum*, **101**, 727-739.

Lin, Z., Zhong, S. and Grierson, D. (2009) Recent advances in ethylene research. *J Exp Bot*, **60**, 3311-3336.

Liu, R., How-Kit, A., Stammitti, L., Teyssier, E., Rolin, D., Mortain-Bertrand, A., Halle, S., Liu, M., Kong, J., Wu, C., Degraeve-Guibault, C., Chapman, N.H., Maucourt, M., Hodgman, T.C., Tost, J., Bouzayen, M., Hong, Y., Seymour, G.B., Giovannoni, J.J. and Gallusci, P. (2015) A DEMETER-like DNA demethylase governs tomato fruit ripening. *Proc Natl Acad Sci USA*, **112**, 10804-10809.

Manning, K., Tor, M., Poole, M., Hong, Y., Thompson, A.J., King, G.J., Giovannoni, J.J. and Seymour, G.B. (2006) A naturally occurring epigenetic

mutation in a gene encoding an SBP-box transcription factor inhibits tomato fruit ripening. *Nat Genet*, **38**, 948-952.

McMurchie, E.J., McGlasson, W.B. and Eaks, I.L. (1972) Treatment of fruit with propylene gives information about the biogenesis of ethylene. *Nature*, **237**, 235-236.

Miller, M.R., Dunham, J.P., Amores, A., Cresko, W.A. and Johnson, E.A. (2007) Rapid and cost-effective polymorphism identification and genotyping using restriction site associated DNA (RAD) markers. *Genome Res*, **17**, 240-248.

Moreno, E., Obando, J.M., Dos-Santos, N., Fernandez-Trujillo, J.P., Monforte, A.J. and Garcia-Mas, J. (2008) Candidate genes and QTLs for fruit ripening and softening in melon. *Theor Appl Genet*, **116**, 589-602.

Osorio, S., Alba, R., Damasceno, C., Lopez-Casado, G., Lohse, M., Zanor, M., Tohge, T., Usadel, B., Rose, J., Fei, Z., Giovannoni, J.J. and Fernie, A.R. (2011) Systems biology of tomato Fruit development: combined transcript, protein, and metabolite analysis of tomato transcription factor (*nor*, *rin*) and ethylene receptor (*Nr*) mutants reveals novel regulatory interactions. *Plant Physiol*, **157**, 405-425.

Paradis. E., Claude. J. and Strimmer, K. (2004) APE: analyses of phylogenetics and evolution in R language. *Bioinformatics*, **20**, 289-90.

Pech, J.C., Bouzayen, M. and Latche, A. (2008) Climacteric fruit ripening: Ethylene-dependent and independent regulation of ripening pathways in melon fruit. *Plant Sci*, **175**, 114-120.

Pereira, L., Pujol, M., Garcia-Mas, J. and Phillips, M.A. Non-invasive ethylene quantification in attached fruit headspace at 1 ppb by gas chromatography – mass spectrometry. *Plant J*, in press.

Perin, C., Gomez-Jimenez, M., Hagen, L., Dogimont, C., Pech, J.C., Latche, A., Pitrat, M. and Lelievre, J.M. (2002) Molecular and genetic characterization of a non-climacteric phenotype in melon reveals two loci conferring altered ethylene response in fruit. *Plant Physiol*, **129**, 300-309.

Perpiñá, G., Esteras, C., Gibon, Y., Monforte, A.J. and Picó, B. (2016) A new genomic library of melon introgression lines in a cantaloupe genetic background for dissecting desirable agronomical traits. *BMC Plant Biol*, **16**, 154.

Pirona, R., Eduardo, I., Pacheco, I., Da Silva Linge, C., Miculan, M., Verde, I., Tartarini, S., Dondini, L., Pea, G., Bassi, D. and Rossini, L. (2013) Fine mapping and identification of a candidate gene for a major locus controlling maturity date in peach. *BMC Plant Biol*, **13**, 166.

Pitrat, M. (2008) Melon (*Cucumis melo* L.). In *Handbook of Crop Breeding Vol I: Vegetables* (Prohens, J. and Nuez, F. eds). New York: Springer, pp. 283–315.

Puranik, S., Sahu, P.P., Srivastava, P.S. and Prasad, M. (2012) NAC proteins: regulation and role in stress tolerance. *Trends Plant Sci*, **17**, 369-381.

R Development Core Team (2016) R: A language and environment for statistical computing. Vienna, Austria: R Foundation for Statistical Computing.

Robinson, R. and Tomes, M. (1968) Ripening inhibitor: a gene with multiple effects on ripening. *Rep Tomato Genet Coop*, **18**, 36-37.

Saladié, M., Cañizares, J., Phillips, M.A., Rodriguez-Concepcion, M., Larrigaudière, C., Gibon, Y., Stitt, M., Lunn, J.E. and Garcia-Mas, J. (2015) Comparative transcriptional profiling analysis of developing melon (*Cucumis melo* L.) fruit from climacteric and non-climacteric varieties. *BMC Genomics*, **16**, 440.

Sanseverino, W., Hénaff, E., Vives, C., Pinosio, S., Burgos-Paz, W., Morgante, M., Ramos-Onsins, S.E.E., Garcia-Mas, J. and Casacuberta, J.M. (2015) Transposon insertions, structural variations, and SNPs contribute to the evolution of the melon genome. *Molecular Biol Evol*, **32**, 2760-2774.

Shan, W., Kuang, J.-f.F., Chen, L., Xie, H., Peng, H.-h.H., Xiao, Y.-y.Y., Li, X.-p.P., Chen, W.-x.X., He, Q.-g.G., Chen, J.-y.Y. and Lu, W.-j.J. (2012) Molecular characterization of banana NAC transcription factors and their interactions with ethylene signalling component EIL during fruit ripening. *J Exp Bot*, **63**, 5171-5187.

Sievers, F., Wilm, A., Dineen, D., Gibson, T.J., Karplus, K., Li, W., Lopez, R., McWilliam, H., Remmert, M., Soding, J., Thompson, J.D. and Higgins, D.G. (2011) Fast, scalable generation of high-quality protein multiple sequence alignments using Clustal Omega. *Mol Syst Biol*, **7**, 539.

Thompson, A.J., Tor, M., Barry, C.S., Vrebalov, J., Orfila, C., Jarvis, M.C., Giovannoni, J.J., Grierson, D. and Seymour, G.B. (1999) Molecular and genetic characterization of a novel pleiotropic tomato-ripening mutant. *Plant Physiol*, **120**, 383-390.

Tamura, K., Stecher, G., Peterson, D., Filipski, A. and Kumar, S. (2013) MEGA6: Molecular evolutionary genetics analysis version 6.0. *Mol Biol Evol*, **30**, 2725-9.

Tigchelaar, E., Tomes, M., Kerr, E. and Barman, R. (1973) A new fruit ripening mutant, nonripening (*nor*). *Rep Tomato Genet Coop*, **23**, 33-34.

Vegas, J., Garcia-Mas, J. and Monforte, A.J. (2013) Interaction between QTLs induces an advance in ethylene biosynthesis during melon fruit ripening. *Theor Appl Genet*, **126**, 1531-1544.

Vrebalov, J., Ruezinsky, D., Padmanabhan, V., White, R., Medrano, D., Drake, R., Schuch, W. and Giovannoni, J. (2002) A MADS-Box Gene Necessary for Fruit Ripening at the Tomato Ripening-Inhibitor (*Rin*) Locus. *Science*, **296**, 343-346.

Waterhouse, A.M., Procter, J.B., Martin, D.M., Clamp, M. and Barton, G.J. (2009) Jalview Version 2-a multiple sequence alignment editor and analysis workbench. *Bioinformatics*, **25**, 1189-91.

Zhong, S., Fei, Z., Chen, Y.-R.R., Zheng, Y., Huang, M., Vrebalov, J., McQuinn, R., Gapper, N., Liu, B., Xiang, J., Shao, Y. and Giovannoni, J.J. (2013) Single-base resolution methylomes of tomato fruit development reveal epigenome modifications associated with ripening. *Nature Biotechnol*, **31**, 154-159.

Zhu, M., Chen, G., Zhou, S., Tu, Y., Wang, Y., Dong, T. and Hu, Z. (2014) A new tomato NAC (NAM/ATAF1/2/CUC2) transcription factor, SlNAC4, functions as a positive regulator of fruit ripening and carotenoid accumulation. *Plant Cell Physiol*, **55**, 119-135.

Tables

Table 1. Phenotyping of ethylene production in the mutant families 246 and 432. Ethylene production during fruit ripening was measured in mutant families 246 and 432 and the CharMono 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 Tukey HSD test. *: p-value < 0.05; **: p-value < 0.01; ***: p-value < 0.001.

		mean ± SD (DAP)		Mean differences in days		
	M2 Family	**W**	**M**	**M-W**	**W-CharMono**	**M-Char Mono**
Days after pollination 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 color 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	-	-	-	-

Figure legends

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. Colors 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 7 variations significantly associated with the type of fruit ripening. Colors 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 colors 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.

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 color change in M2 families of mutant families 246, 432, 4933, 3717, 2503 and 502. In the Y-axis, days between pollination and external color change are represented. W (red) is

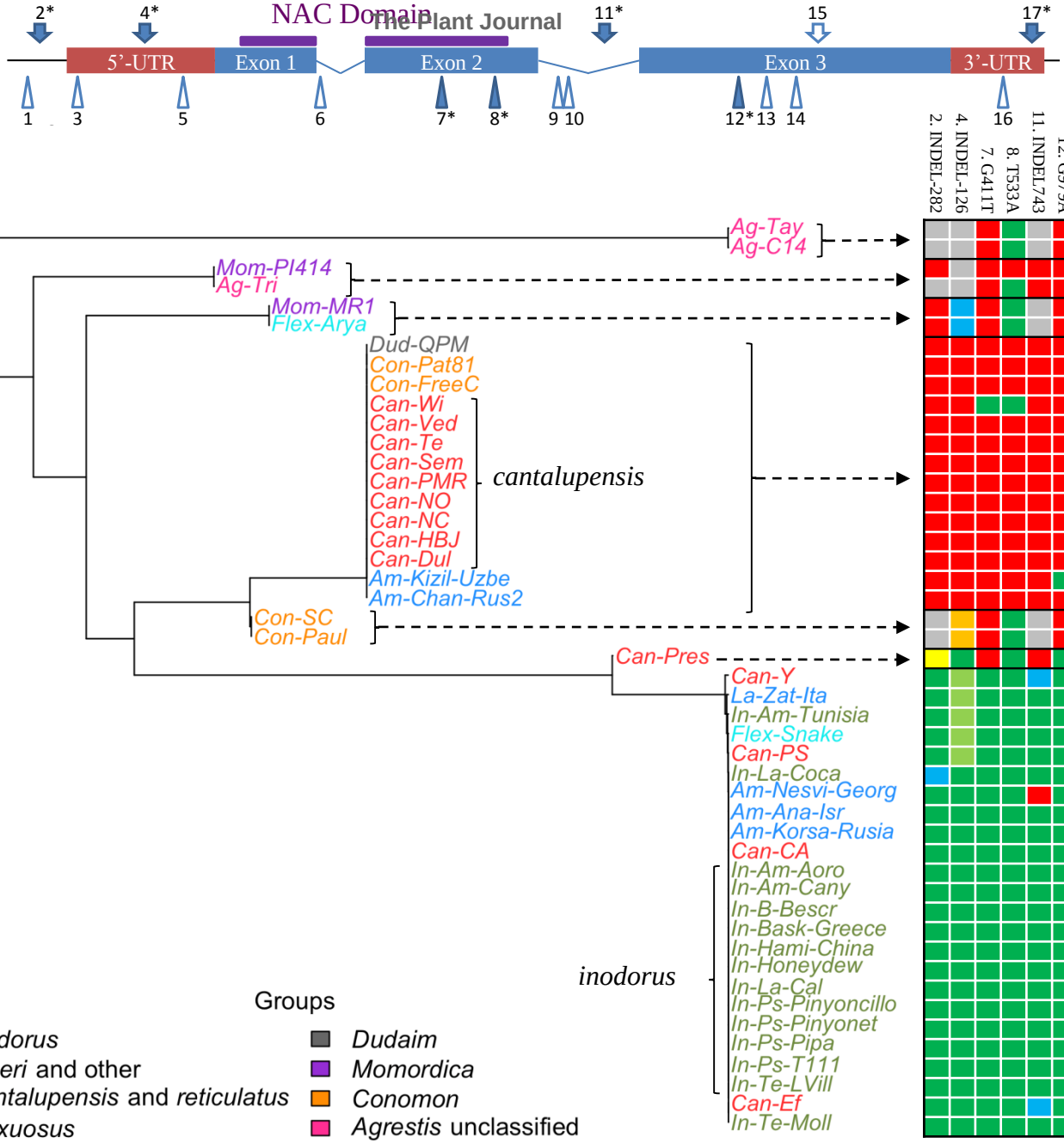
homozygous for the wild type allele; M (green) is homozygous for the mutated allele. Asterisks indicate statistically significant differences between each group after a t-Student test. Significance level ***: p-value < 0.001.

Figure 3. 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 SlNAC-NOR and SlNAC4. The prefix for each protein sequence indicates the plant species (Table S5). Colors 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.

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

Figure 5. *CmNAC-NOR* 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).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4*
11*
15
17*
5'-UTR
Exon 1
Exon 2
Exon 3
3'-UTR
1
3
5
6
7*
8*
9 10
12*
13
14
16
2. INDEL-282
4. INDEL-126
7. G411T
8. T533A
11. INDEL743
12. G979A
17. INDEL*173
Ripening type
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Ag-C14
Mom-PI414
Ag-Tri
Mom-MR1
Flex-Arya
Dud-QPM
Con-Pat81
Con-FreeC
Can-Wi
Can-Ved
Can-Te
Can-Sem
Can-PMR
Can-NO
Can-NC
Can-HBJ
Can-Dul
cantalupensis
Am-Kizil-Uzbe
Am-Chan-Rus2
Con-SC
Con-Paul
Can-Pres
Can-Y
La-Zat-Ita
In-Am-Tunisia
Flex-Snake
Can-PS
In-La-Coca
Am-Nesvi-Georg
Am-Ana-Isr
Am-Korsa-Rusia
Can-CA
In-Am-Aoro
In-Am-Cany
In-B-Bescr
In-Bask-Greece
In-Hami-China
In-Honeydew
In-La-Cal
In-Ps-Pinyoncillo
In-Ps-Pinyonet
In-Ps-Pipa
In-Ps-T111
In-Te-LVill
Can-Ef
In-Te-Moll
inodorus
Groups
Inodorus
Ameri and other
Cantalupensis and reticulatus
Flexuosus
Dudaim
Momordica
Conomon
Agrestis unclassified
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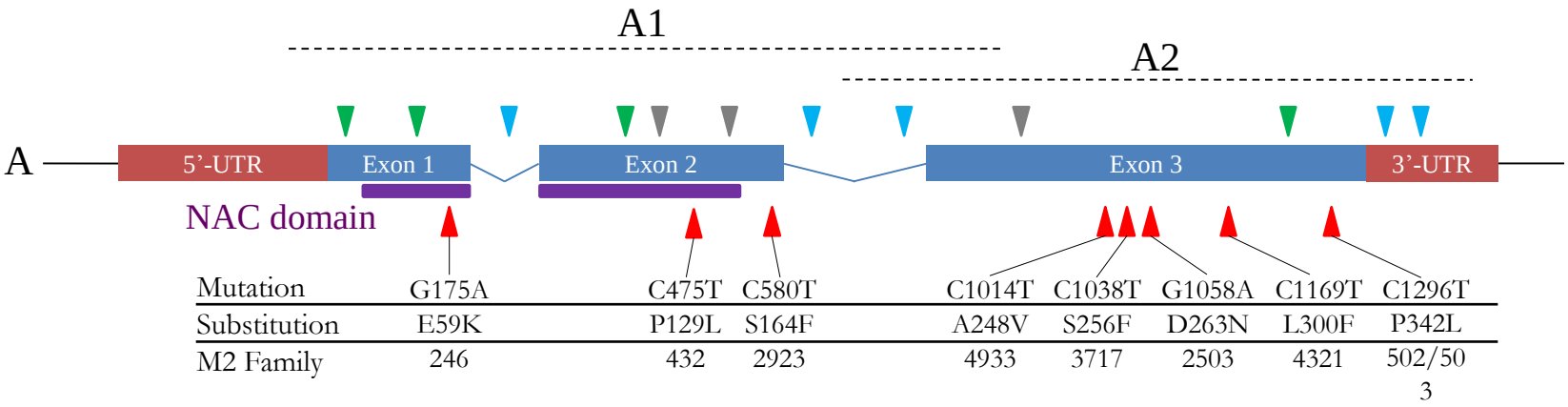
0.001
0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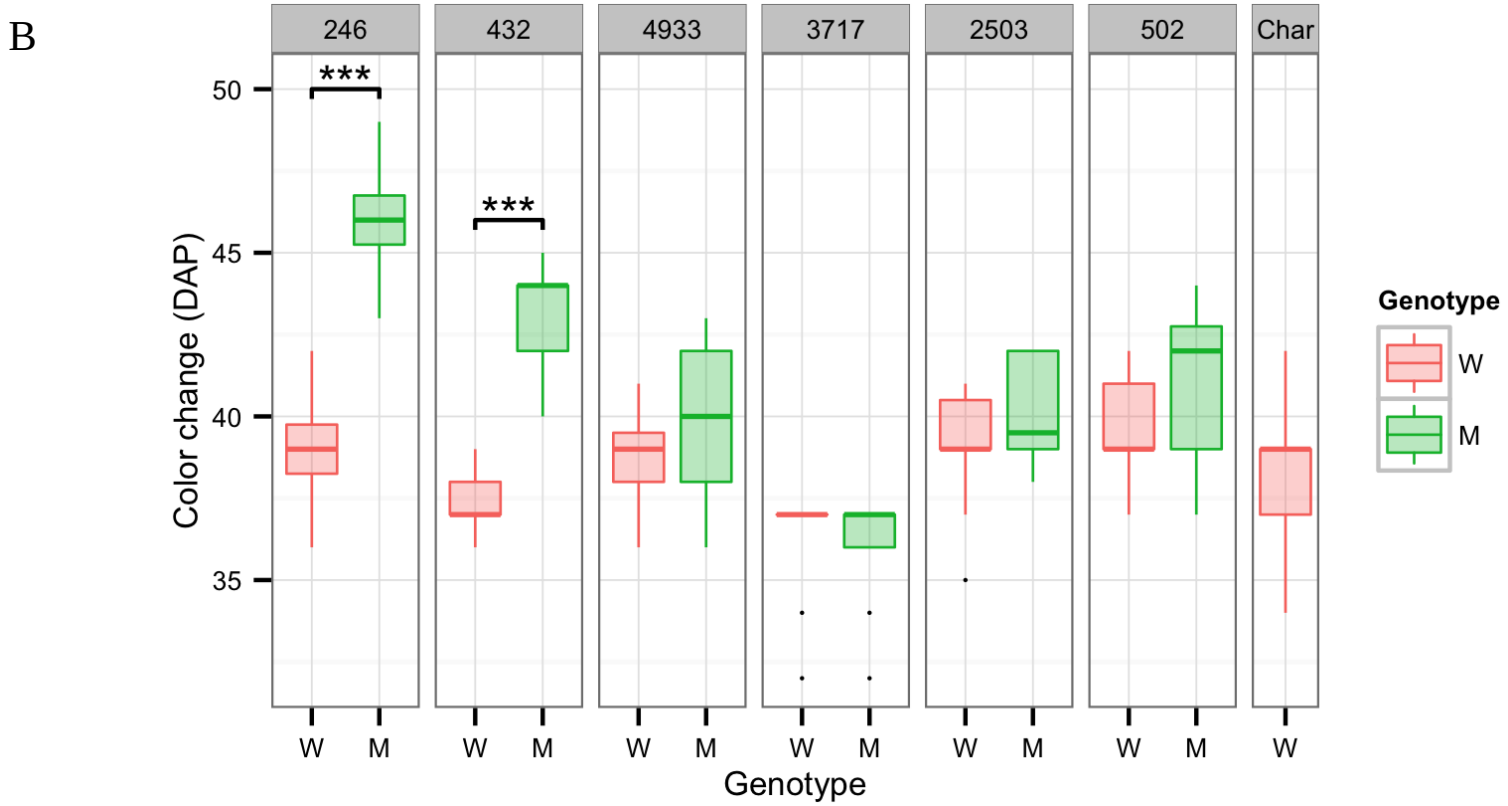
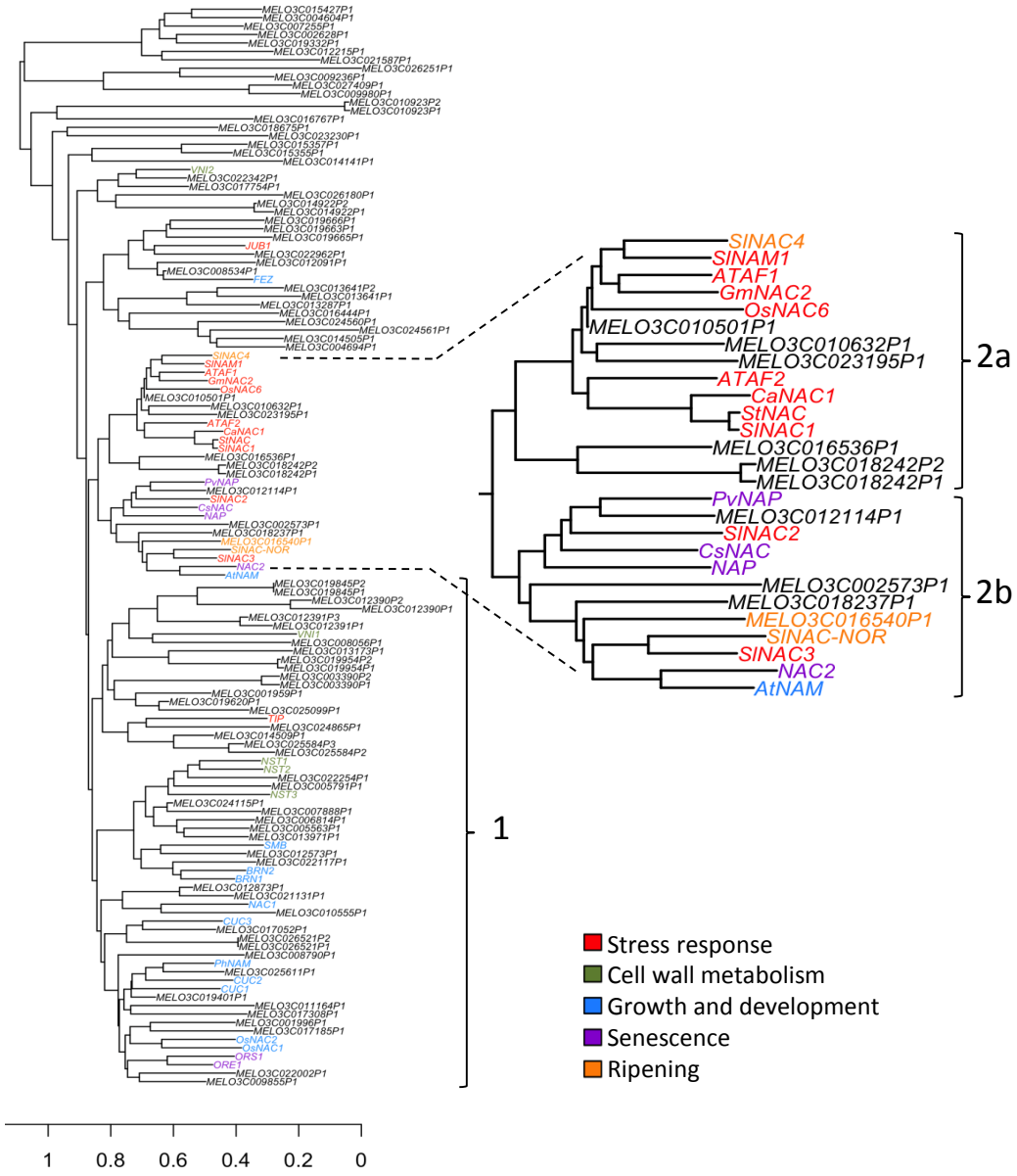

B
246
432
4933
3717
2503
502
Char
***
***
50
45
40
35
Color change (DAP)
W
M
Genotype
Genotype
W
M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StNAC
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CsNAC
NAP
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SlNAC3
NAC2
AtNAM
2b
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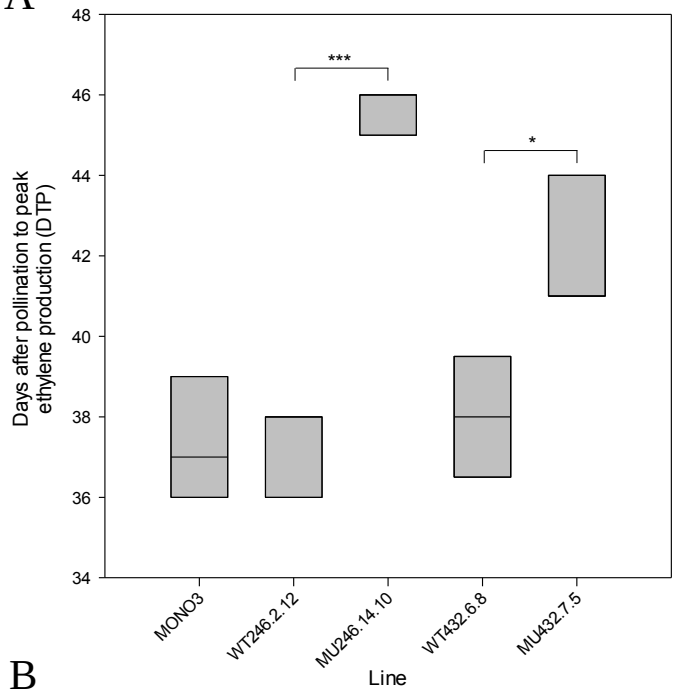
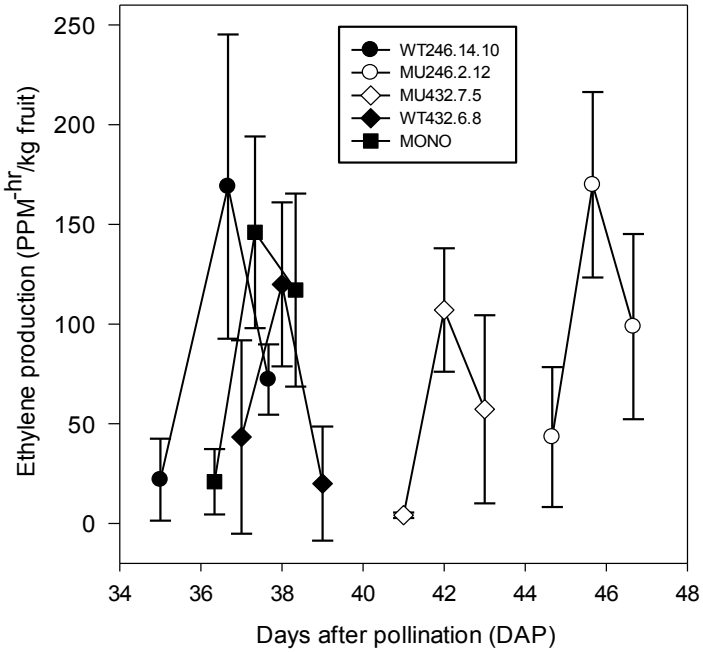
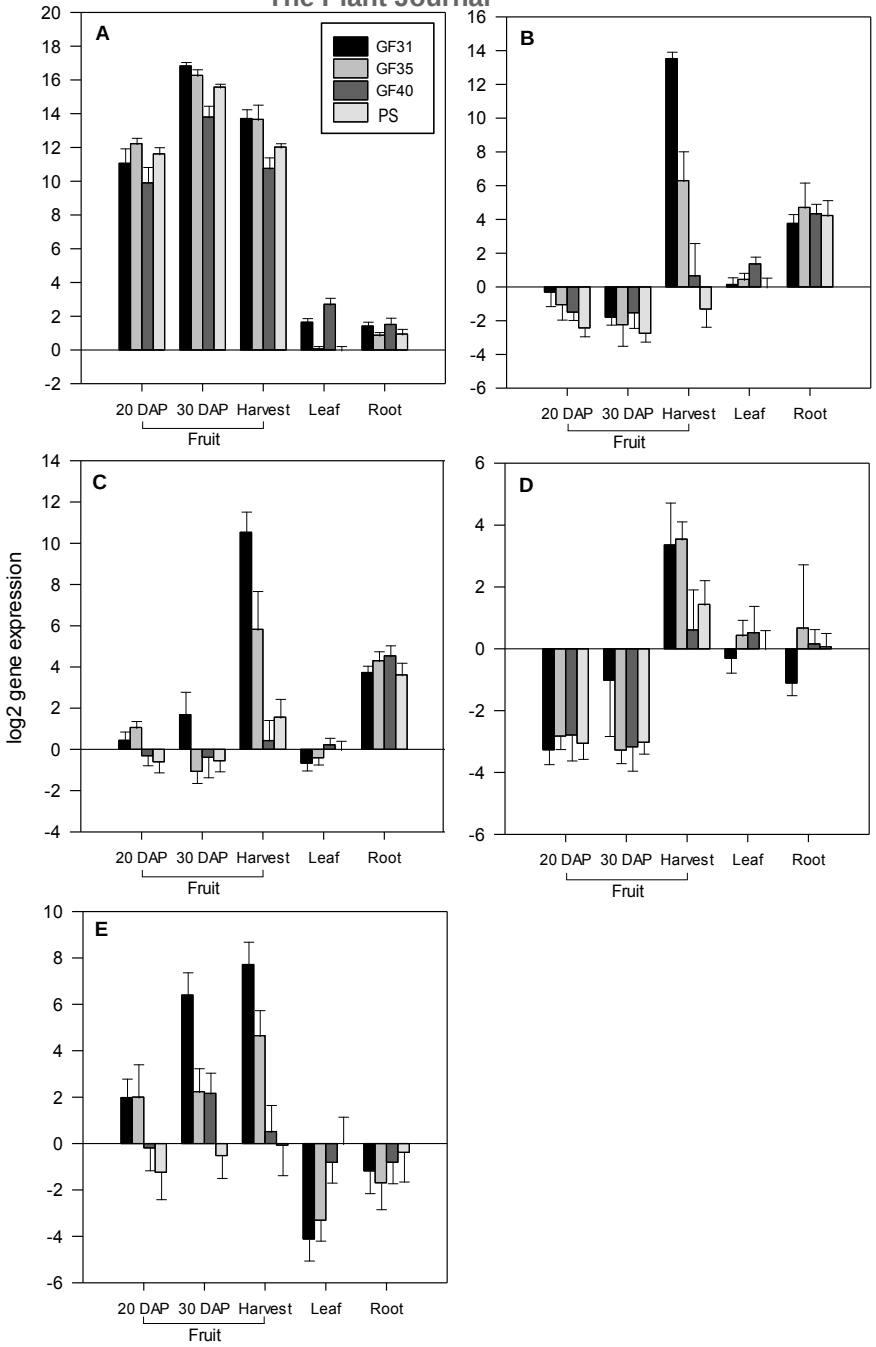
0.4
0.2
0
Stress response
Cell wall metabolism
Growth and development
Senescence
Ripening

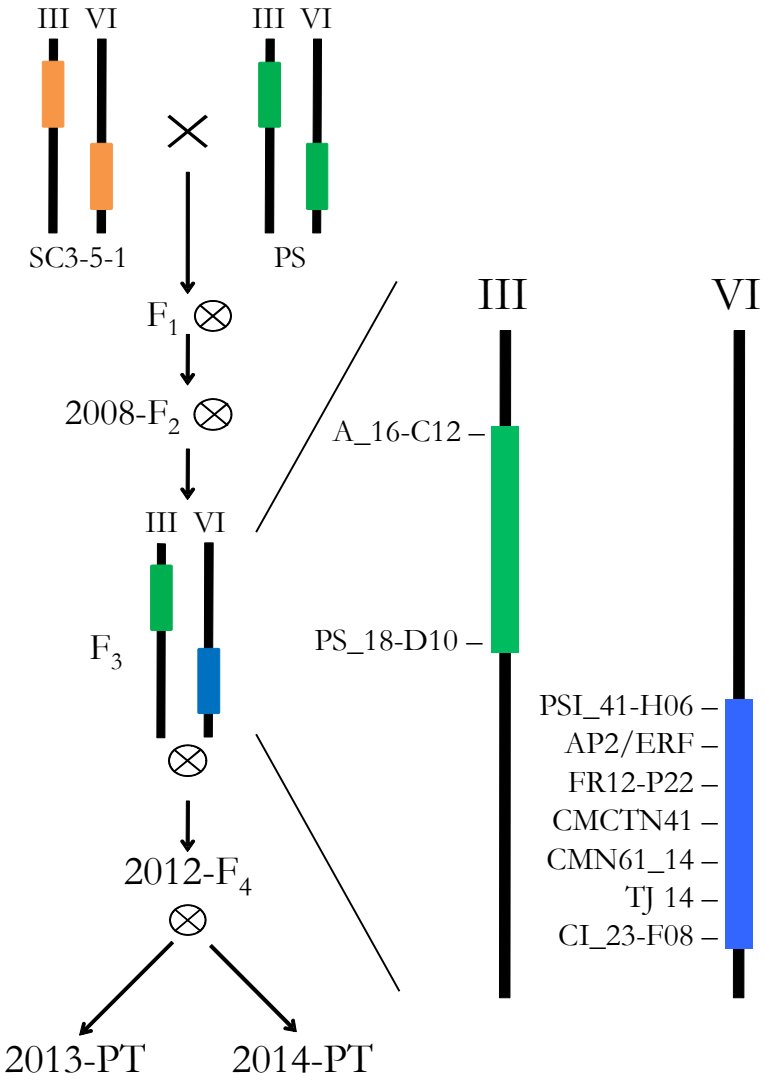
A


48
46
44
42
40
38
36
34
***
*
Days after pollination to peak ethylene production (DTP)
MONO3
WT246.2.12
MU246.14.10
WT432.6.8
MU432.7.5
Line

B


250
200
150
100
50
0
WT246.14.10
MU246.2.12
MU432.7.5
WT432.6.8
MONO
Ethylene production (PPM-hr/kg fruit)
34
36
38
40
42
44
46
48
Days after pollination (DAP)


A
B
C
D
E
GF31
GF35
GF40
PS
log2 gene expression
20 DAP
30 DAP
Harvest
Leaf
Root
Fruit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PS
F1
2008-F2
III VI
F3
2012-F4
2013-PT
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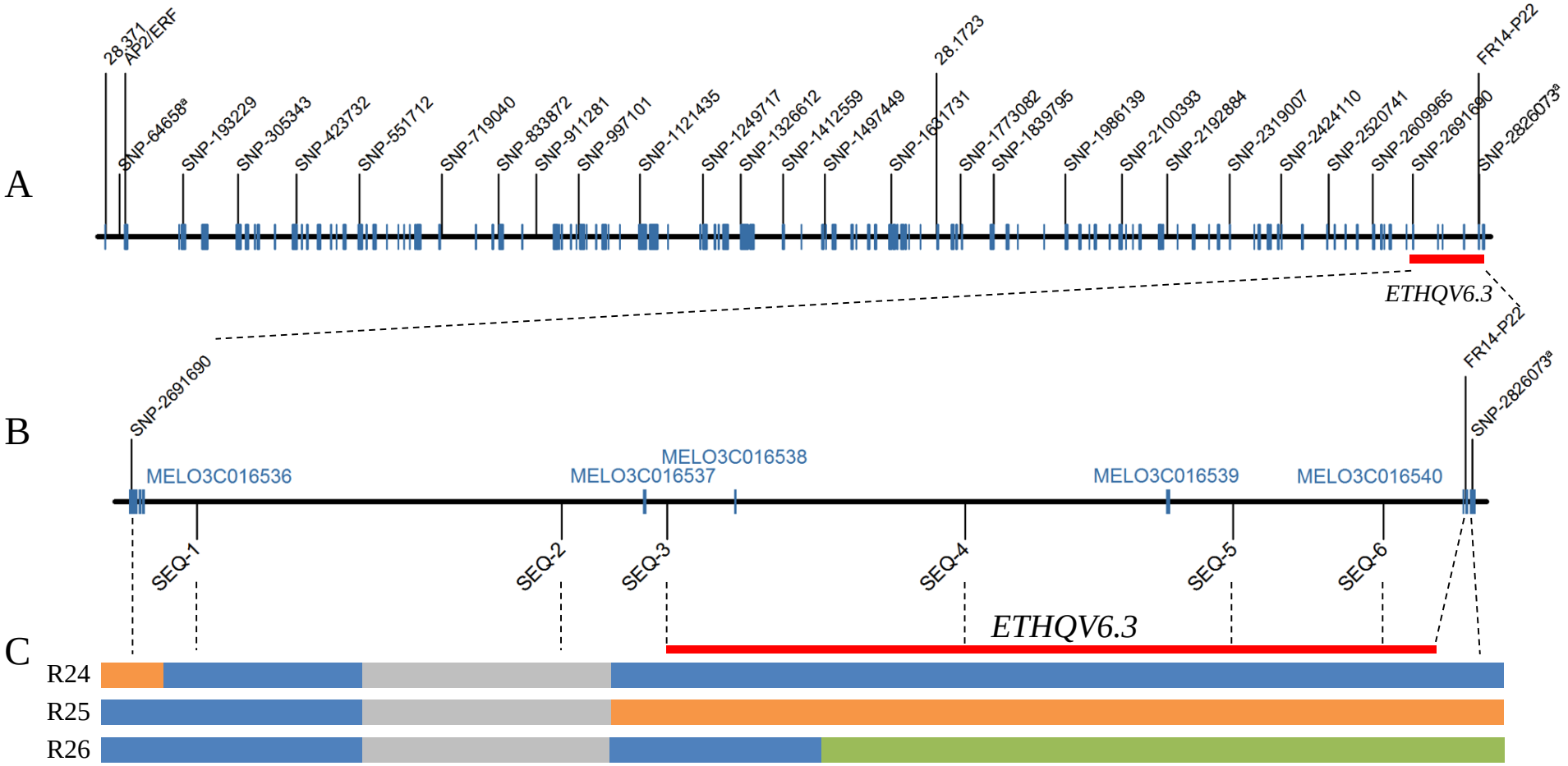
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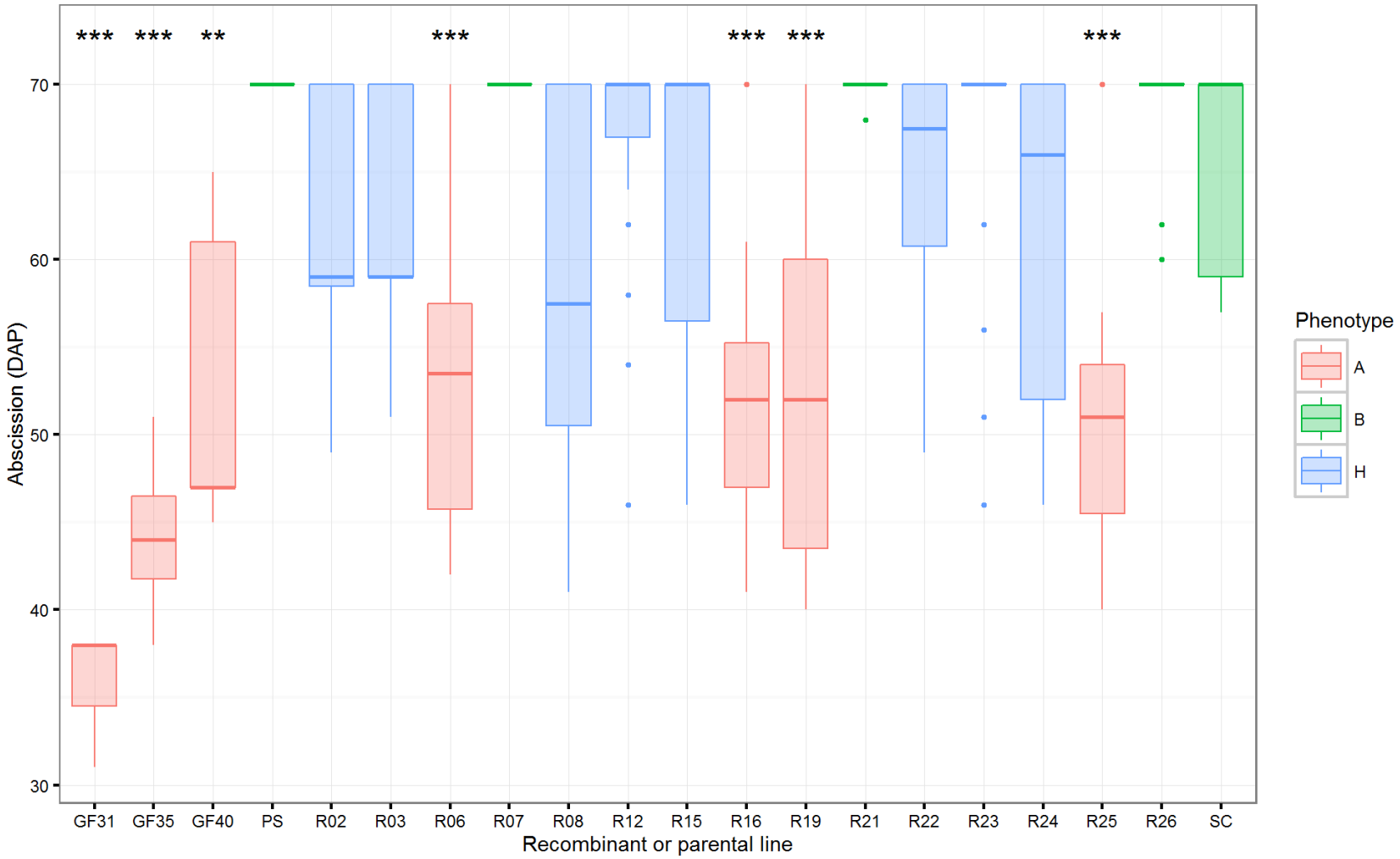
R26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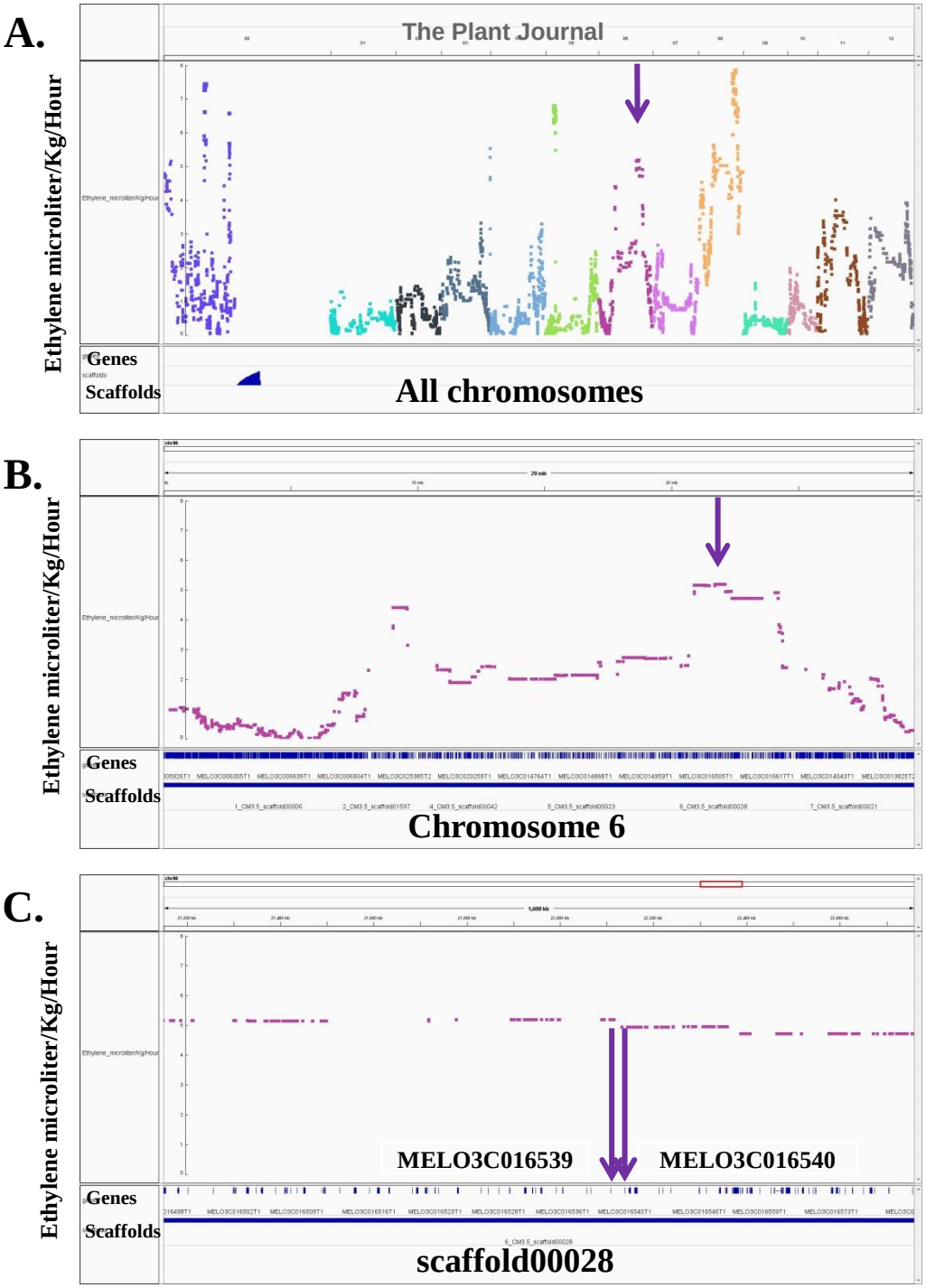
Abscission (DAP)
GF31
GF35
GF40
PS
R02
R03
R06
R07
R08
R12
R15
R16
R19
R21
R22
R23
R24
R25
R26
SC
Recombinant or parental line
Phenotype
A
B
H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The Plant Journal
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Genes
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All chromosomes
B.
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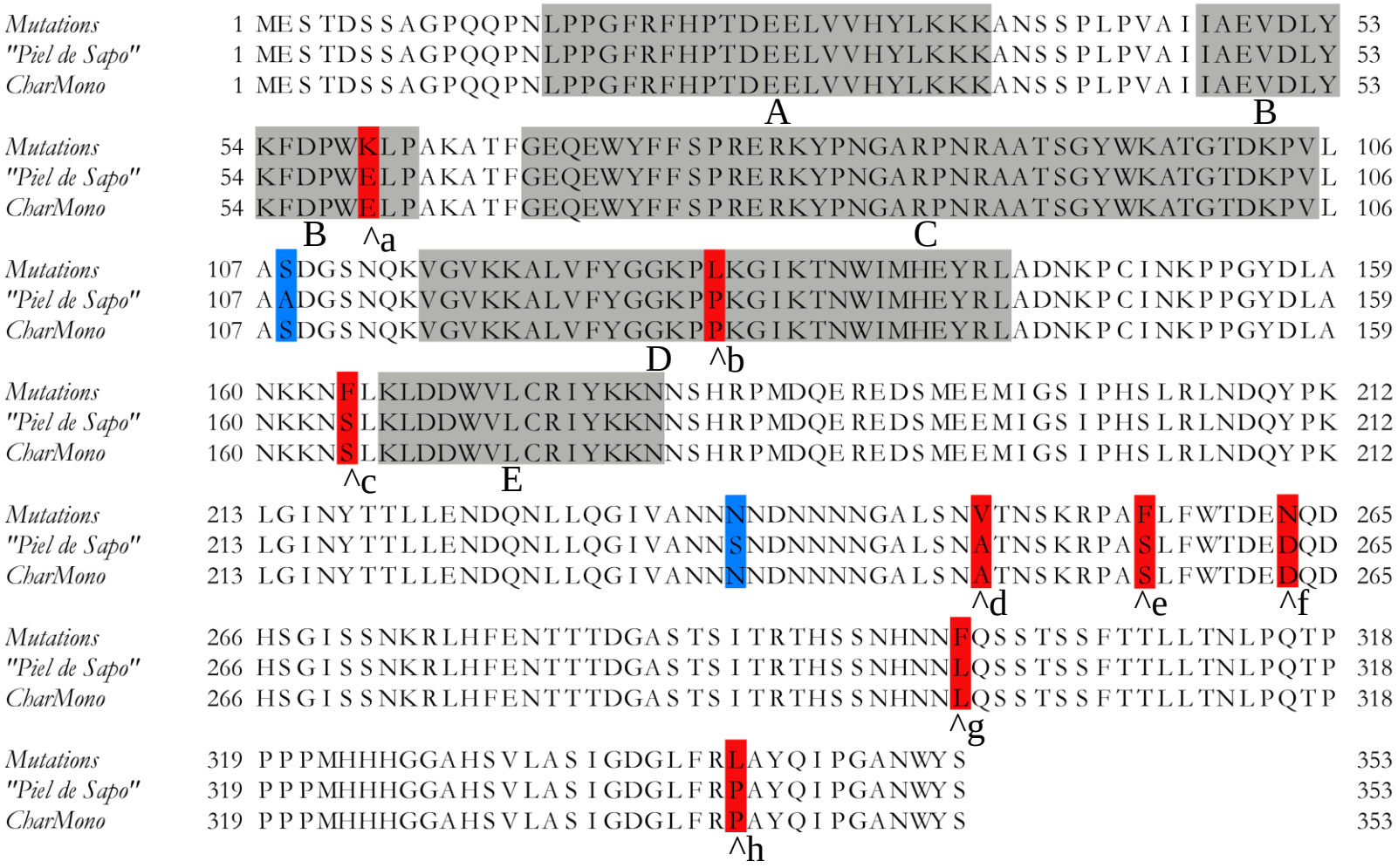
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CharMono 1 MESTDSSAGPQQPNLPPGFRFHPTDEELVVHYLKKKANSSPLPVAIIAEVDLY 53
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B
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CharMono 107 ASDGSNQKVGVKKALVFYGGKPPKGIKTNWIMHEYRLADNKPCINKPPGYDLA 159
D
^b
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^d
^e
^f
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CharMono 266 HSGISSNKRLHFENTTTDGASTSITRTHSSNHNNLQSSTSSFTTLLTNLPQTP 318
^g
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"Piel de Sapo" 319 PPPMHHHGGAHSVLASIGDGLFRPAYQIPGANWYS 353
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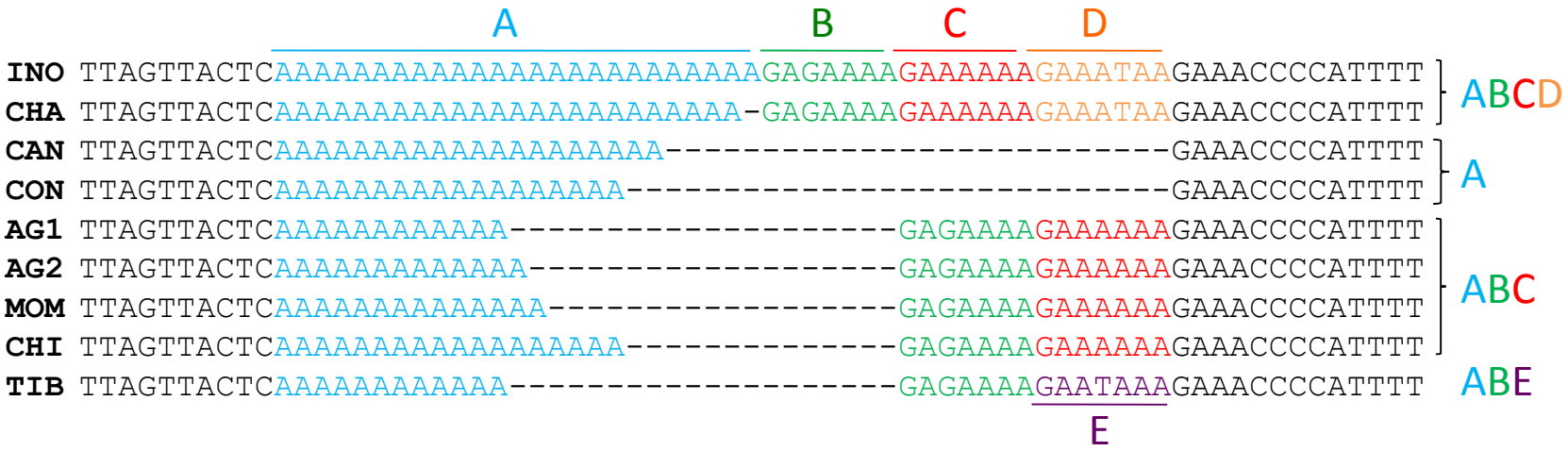
^h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B
C
D
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AG2 TTAGTTACTCAAAAAAAAAAAAA----------GAGAAAAGAAAAAAGAAACCCCATTTT
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CHI TTAGTTACTCAAAAAAAAAAAAAAAAA----------GAGAAAAGAAAAAAGAAACCCCATTTT
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ABC
ABE

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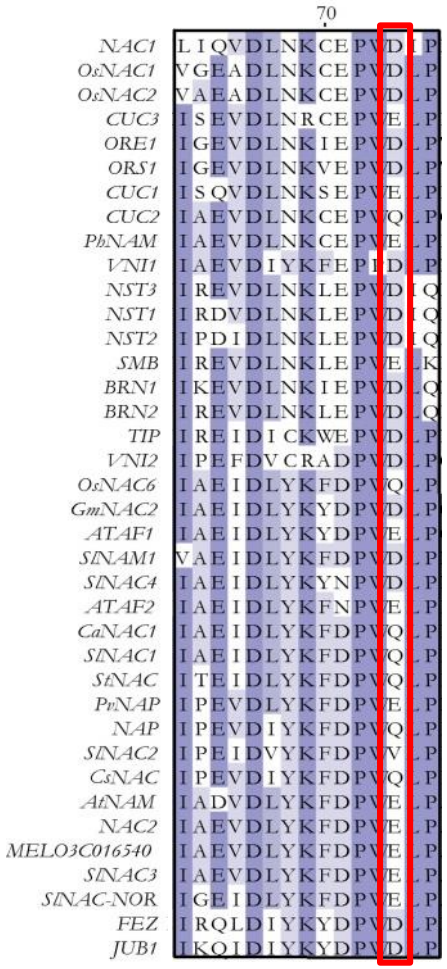

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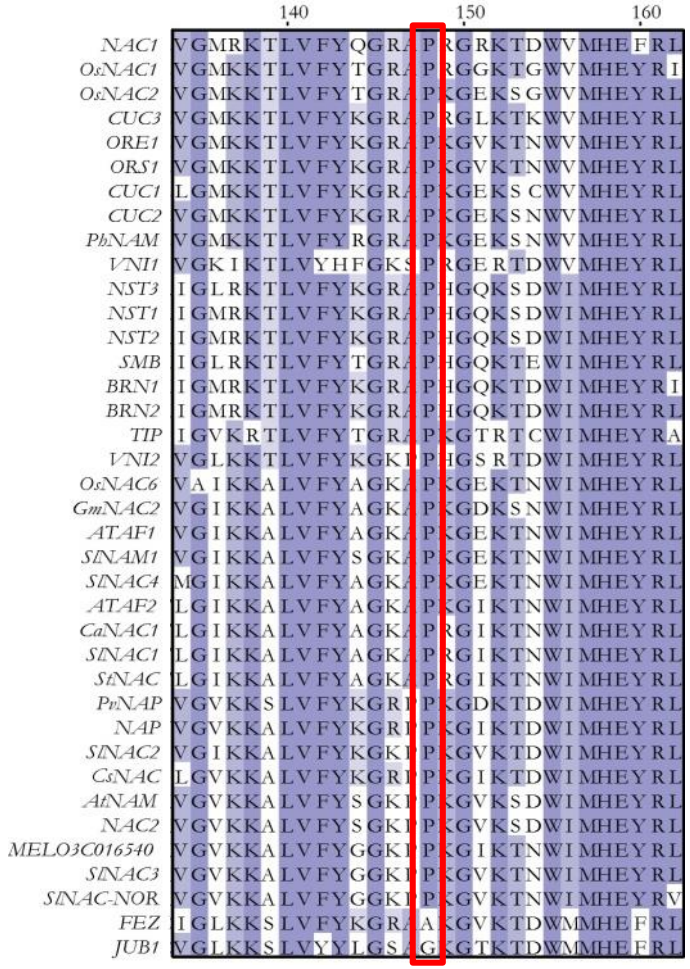

Subdomain D

Table S1

Markers used to genotype the 2008-F2 population

Marker	Type	LG	Primer F (5'-3')	Primer R (5'-3')	Tm (ºC)	[$MgCl_2$] (mM)	Citation
A_16-C12	SSR	III	ATAAATGGGTCATCGGAGGAG	GGTGGTGAATTAATGGAAGC	51	2	Fernandez *et al*. 2010
PS_18-D10	SSR	III	GATTCCTTGGGCTTGTACCTC	GCTAAGGAAAGGGTTTGTTCG	51	2	Fernandez *et al.* 2010
PSI_41-H06	SSR	VI	CAACCATTCCTCCCATTCAT	CACCACCTGTGACATTGTACG	59	2	Fernandez *et al.* 2010
AP2/ERF	SSR	VI	GCTGCTGTCAAAGATGACCA	GTCGGTTTGACTGTCGGAAT	45	2	Vegas *et al*. 2013
FR14-P22	CAPS	VI	AGGGAAAGGAAGTACCCAAATG	TTCGGCATAATACCCAATCATC	60	1,5	Deleu *et al.*2009
CMCTN41	SSR	VI	CCCCAAGATTCGTATTAATC	TGGTAGTAGAGATGATATAC	51	2	Gonzalo *et al*. 2005
CMN61_14	SSR	VI	TGCAGGATCAAGAATCAAGTTC	ACGAACTCCGGCATAATCAC	56	2	Fukino *et al*. 2008
TJ 14	SSR	VI	TTCCAATGCCCTAAAGTTGC	CAAGCAAACCAAAGACATGC	56	2	Morales *et al*. 2004
CI_23-F08	SSR	VI	CATAGAGCATTTGCCGGAGT	TGAAAAGCTAGCATGGATTGG	59	2	Fernandez *et al*. 2010

SNPs used for fine mapping *ETHQV6.3*

Marker	Type	LG	Position (bp)	Alleles (PS/SC)	Primers A1, A2 and C
SNP-64658	TaqMan	VI	24.226.183	GG/AA	-
SNP-193229	KASP	VI	24.354.754	CC/TT	GAAGGTGACCAAGTTCATGCTACAGGTTAGTGTGAGCTCATCCA GAAGGTCGGAGTCAACGGATTATCTAAAAGCTCTAAGCATGCTAAAATGTA GGGTGATATTTATAAACTCTGCGACTGAA
SNP-305343	KASP	VI	24.466.868	CC/TT	GAAGGTGACCAAGTTCATGCTGATATTGCAGCCCTTCTAAATTGCCA

					GAAGGTCGGAGTCAACGGATTGATGGCGATAATGAAGGAGATGACA
					CATCTGACAACTGTTGAGATTGTGTACAA
SNP-423732	KASP	VI	24.585.257	AA/GG	GAAGGTGACCAAGTTCATGCTGCGCCAAAATCCCGGCAAATCT
					GAAGGTCGGAGTCAACGGATTCCACATTTTATTATCGTTATTTTACTAAAATG
					GCAGAAAAGTGAAATTGAGGGTTTTATTTA
SNP-551712	KASP	VI	24.713.237	AA/GG	GAAGGTGACCAAGTTCATGCTAGTACCTTCTTAAGATGCAAACAATCG
					GAAGGTCGGAGTCAACGGATTGTTTCCAATTTTAGTCAAATTAAAATGTGTAC
					CCACAAGGAATAGCTTAAACTTGACTTATT
SNP-719040	KASP	VI	24.880.565	GG/AA	GAAGGTGACCAAGTTCATGCTCTTTATAATACAAAGAAGCCCTTACATTC
					GAAGGTCGGAGTCAACGGATTCTACATAAATATCATAAATGTTTTATTGAGAAA
					CTTTTTGTTTAGCATCTATATATATTCTCA
SNP-833872	KASP	VI	24.995.397	CC/GG	GAAGGTGACCAAGTTCATGCTACCTGCTTTGCTGAAGTTTCTTCG
					GAAGGTCGGAGTCAACGGATTCTCGATATGAGGAAGGTCAAGTAC
					CCTATTTGATCTTCACCATTTTCGACAAAA
SNP-911281	KASP	VI	25.072.806	CC/AA	GAAGGTGACCAAGTTCATGCTGTTGTGGAGGTCAATCTGATAGAAA
					GAAGGTCGGAGTCAACGGATTCAAAATTCCTATGAAGGATTGGTTTCA
					TATGATGTGGGAAGACAAACTAGTAGCTT
SNP-997101	KASP	VI	25.158.626	TT/CC	GAAGGTGACCAAGTTCATGCTAGAATGGGACATTAAACTTGTTAGAGAATT
					GAAGGTCGGAGTCAACGGATTACGGACCGTAAATATTTTATCCGTTTG
					GTACAAATATCAGTCTCACAAACATATTAA
SNP-1121435	KASP	VI	25.282.960	TT/CC	GAAGGTGACCAAGTTCATGCTAAAAGCTCTAAGCATGCTAAAATGTG
					GAAGGTCGGAGTCAACGGATTCAGGTTAGTGTGAGCTCATCCG
					GAGCCAATTCTCCTATTCAAGAGTGAATA
SNP-1249717	KASP	VI	25.411.242	GG/CC	GAAGGTGACCAAGTTCATGCTATGGCGATAATGAAGGAGATGACG
					GAAGGTCGGAGTCAACGGATTGGATCCTGACAAAGAATATTGTAACAAG
					GGATCCCATCGACCTTCATTGTCAA

SNP-1326612	KASP	VI	25.488.137	GG/TT	GAAGGTGACCAAGTTCATGCTCCACATTTTATTATCGTTATTTTACTAAAATA GAAGGTCGGAGTCAACGGATTCCTCTTCTACTCTTTAGATTTCGATTT AAACTGTTGAAGACAAAAGGGTGCAGTA
SNP-1412559	KASP	VI	25.574.084	TT/CC	GAAGGTGACCAAGTTCATGCTGTTTCCAATTTTAGTCAAATTAAAATGTGTAT GAAGGTCGGAGTCAACGGATTGACTGAAGGATTTTGATGTGAATATGTAC GGCGGACAAAAGAAACATCACCCAT
SNP-1497449	KASP	VI	25.658.974	AA/CC	GAAGGTGACCAAGTTCATGCTACATAAATATCATAAATGTTTTATTGAGAAG GAAGGTCGGAGTCAACGGATTGAGTGTTGTCTATAAAGCCTCAGTG GTGCAGTTTACTGTAGGAGATCAAGTTTA
SNP-1631731	KASP	VI	25.793.256	AA/GG	GAAGGTGACCAAGTTCATGCTCTCGATATGAGGAAGGTCAAGTAG GAAGGTCGGAGTCAACGGATTGCTTGGATATTCTAAAATATTGTTGTTTGG GGTGTTTCCAGAACAGGAACCTACAA
SNP-1773082	KASP	VI	25.934.607	TT/CC	GAAGGTGACCAAGTTCATGCTCAAAATTCCTATGAAGGATTGGTTTCC GAAGGTCGGAGTCAACGGATTACACGCTTTATCCCTAGTTACGG CCATAAAGGCACAAATCTACATTGCAGAA
SNP-1839795	KASP	VI	26.001.320	AA/TT	GAAGGTGACCAAGTTCATGCTCACGGACCGTAAATATTTTATCCGTTTA GAAGGTCGGAGTCAACGGATTGTTTCTACGACTTCGCTTCTCTTCT CCTGCCTTACAACAGTTGCTCCAAA
SNP-1986139	KASP	VI	26.147.664	TT/CC	GAAGGTGACCAAGTTCATGCTGGATCCTGACAAAGAATATTGTAACAAC GAAGGTCGGAGTCAACGGATTATATTGCAGCCCTTCTAAATTGCCG CTTTTTTCATGGAAAAATCCCTGAAAGCTA
SNP-2100393	KASP	VI	26.261.918	TT/GG	GAAGGTGACCAAGTTCATGCTCCTCTTCTACTCTTTAGATTTCGATTG GAAGGTCGGAGTCAACGGATTCGCCAAAATCCCGGCAAATCG CAATGCAAAAGGATTTGAGGGATTTGCAA
SNP-2192884	KASP	VI	26.354.409	CC/TT	GAAGGTGACCAAGTTCATGCTAGACTGAAGGATTTTGATGTGAATATGTAT GAAGGTCGGAGTCAACGGATTAAAAGTACCTTCTTAAGATGCAAACAATCA

					CGCCACTTTTCCTTAATACCGAGATTAAA
SNP-2319007	KASP	VI	26.480.532	CC/AA	GAAGGTGACCAAGTTCATGCTAGAGTGTTGTCTATAAAGCCTCAGTT
					GAAGGTCGGAGTCAACGGATTCTCTTTATAATACAAAGAAGCCCTTACATTA
					TTTTTCGCCCTCTTGTTGGAAACCTATT
SNP-2424110	KASP	VI	26.585.635	GG/AA	GAAGGTGACCAAGTTCATGCTCGCTTGGATATTCTAAAATATTGTTGTTTGA
					GAAGGTCGGAGTCAACGGATTCACCTGCTTTGCTGAAGTTTCTTCA
					TGAACCTTCATTTGCAGGAAGTCTACTT
SNP-2520741	KASP	VI	26.682.266	TT/AA	GAAGGTGACCAAGTTCATGCTGACACGCTTTATCCCTAGTTACGA
					GAAGGTCGGAGTCAACGGATTGTTGTGGAGGTCAATCTGATAGAAT
					GCTCCAGTAATGAAAATCTAGTCAGTCTT
SNP-2609965	KASP	VI	26.771.490	TT/CC	GAAGGTGACCAAGTTCATGCTGACCAACATCTCTGCCGGACA
					GAAGGTCGGAGTCAACGGATTGACCAACATCTCTGCCGGACG
					ACTCAAAGGACTAAGCGGGTTGGTT
SNP-2691690	KASP	VI	26.853.215	TT/CC	GAAGGTGACCAAGTTCATGCTGTTTCTACGACTTCGCTTCTCTTCA
					GAAGGTCGGAGTCAACGGATTGAATGGGACATTAAACTTGTTAGAGAATC
					CAGAGCATACCCTGTTTTTGACCATAAAT
SNP-2826073	TaqMan	VI	26.987.598	GG/AA	-

Primers used for DNA sequencing

Amplicon	Size (bp)	Primer F (5'-3')	Primer R (5'-3')	Tm (ºC)	[$MgCl_2$] (mM)
PRO40.1[a]	613	TACTTTGAGTTCACACGCGG	GGAAGCTCCCAAGGATCGAA	60	2
CDS40.1[a,b]	665	ATGGAGAGCACCGACTCATC	AGGTGTATTCAGAGCCGTAAAA	60	2

CDS40.2[a,c]	591	CTCCTGGCTATGATTTGGCC	TGGAAGTAGAAGCCCCATCA	60	2
CDS40.3[a,d]	603	TCAAAACGTCCTGCCTCTCT	TGGCCTCCTTTCAAATGGGT	60	2
SEQ-1[e]	394	TCCTTACTGAAGTTCACCCAAA	CACCCTTCACGTGTCAACC	60	2
SEQ-2[e]	240	GCAGGAAATGGTATCTTAGCCA	ACAACAAAATGGCATAGCAGAACG	60	2
SEQ-3[e]	392	TGCCCAACCTTATCCCATGT	GGTATGAGGCGTTTTGAGTGG	60	2
SEQ-4[e]	356	TCACGTTGGGAAACTTTGGAC	CTCAGCAAGTAATTCTCCTACCA	60	2
SEQ-5[e]	249	AGCTTTTCATTCGGCAGGTG	TGCGAGTCATTCAATCTAACCT	60	2
SEQ-6[e]	300	CGATCCTATTTCAACACACAACT	ATCTCCAACCGCGATTCTTG	60	2

[a]used for sequencing *ETHQV6.3* in the germplasm collection

[b]used for genotyping TILLING families 5388, 246, 432 and 2923

[c]used for genotyping TILLING families 3717, 4933 and 2503

[d]used for genotyping TILLING families 502, 503 and 4321

[e]used for *ETHQV6.3* fine mapping

Primers used to amplify amplicons A1 and A2 in *CmNAC-NOR* for searching TILLING mutants

Amplicon	Primer name	PCR Name	Sequence (5'-3')	Tm (ºC)
A1	A1-2F	PCR N1	GATCAGCTTTGCCTGTTTTGTGCAA	55
A1	A1-3R	PCR N1	ATTGTTGGCCACTATTCCTTGAAGC	55
A1	A1-6F	PCR N2 & seq.	CACGACGTTGTAAAACGACCTTCCTCCTCTTCTTC	50/60
A1	A1-8R	PCR N2	TAACAATTTCACACAGGCTTCCATTGAATCTTCCC	50/60

A1	A1-9R	Sequencing	GAAATAATATTGAAATAGGGATTTA	46
A2	A2-11F	PCR N1	AATATAGGTTGGCGGATAATAAGCC	55
A2	A2-12R	PCR N1	TACCAAACTAAAACCCCTCAATACC	55
A2	A2-14F	PCR N2	ACGACGTTGTAAAACGACCGGCTCTGAATACACCT	50/60
A2	A2-17R	PCR N2 & seq.	TAACAATTTCACACAGGCTAAAACCCCTCAATACC	50/60
A2	A2-16F	Sequencing	ATAACAATTTCACACAGGGGGTTGAATTGAACTGG	62

Table S2

Recombinant	SNP-64658	SNP-193229	SNP-305343	SNP-423732	SNP-551712	SNP-719040	SNP-833872	SNP-911281	SNP-997101	SNP-1121435	SNP-1249717	SNP-1326612	SNP-1412559	SNP-1497449	SNP-1631731	SNP-1773082	SNP-1839795	SNP-1986139	SNP-2100393	SNP-2192884	SNP-2319007	SNP-2424110	SNP-2520741	SNP-2609965	SNP-2691690	SNP-2826073	Phenotype (*ETHQV6.3*)
R02	A	A	A	A	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H
R03	A	A	A	A	A	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H
R06	H	H	H	H	H	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A
R07	H	H	H	H	H	H	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B
R08	B	B	B	B	B	B	B	B	B	B	B	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H
R12	B	B	B	B	B	B	B	B	B	B	B	B	B	B	H	H	H	H	H	H	H	H	H	H	H	H	H
R15	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	H	H	H	H	H	H	H	H	H	H	H	H
R16	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	A	A	A	A	A	A	A	A	A	A	A	A
R19	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	A	A	A	A	A	A	A	A	A	A	A
R21	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	B	B	B	B	B	B	B	B	B	B
R22	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	H	H	H	H	H	H	H
R23	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	H	H	H	H	H	H
R24	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	H	H
R25	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	A	A
R26	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	B	B	B
																									*	*	

Table S3

Gene	Start position (bp)	End position (bp)	Orientation	Exons	mRNA size (bp)	Protein size (aa)	Annotation
MELO3C016536	26.852.979	26.854.555	-	3	1.576	296	Similar to NAC domain-containing protein 72 (*Arabidopsis thaliana*)
MELO3C016537	26.904.508	26.904.804	-	1	296	98	Similar to Putative uncharacterized protein (*Vitis vinifera*)
MELO3C016538	26.913.606	26.913.853	-	1	247	81	-
MELO3C016539	26.956.908	26.957.304	+	1	396	67	-
MELO3C016540	26.986.354	26.988.122	+	3	1.768	353	Similar to NAC domain-containing protein 18 (*Arabidopsis thaliana*)

Table S5

Species	Protein	Biological function	UniProt code	Reference
Oryza sativa	OsNAC1	Growth and development	Q8H0I5_ORYSA	Hu *et al.* 2006
Oryza sativa	OsNAC2	Growth and development	Q8H0I4_ORYSA	Chen *et al.* 2015b
Oryza sativa	OsNAC6	Stress response	NAC48_ORYSJ	Nakashima *et al.* 2007
Solanum lycopersicum	SlNAC1	Stress response	Q6RH27_SOLLC	Selth *et al.* 2005
Solanum lycopersicum	SlNAM1	Stress response	B8XS01_SOLLC	Yang *et al.* 2011a
Solanum lycopersicum	SlNAC2	Stress response	K4BWV2_SOLLC	Uppalapati *et al.* 2008
Solanum lycopersicum	SlNAC3	Stress response	K4CH25_SOLLC	Han *et al.* 2012
Solanum lycopersicum	SlNAC4	Ripening	K4D6Q0_SOLLC	Zhu *et al.* 2014
Solanum lycopersicum	SlNAC-NOR	Ripening	Q56UP7_SOLLC	Patent US 6.762.347 B1
Glycine max	GmNAC2	Stress response	Q52QR4_SOYBN	Jin *et al.* 2013
Capsicum annuum	CaNAC1	Stress response	Q3ZN85_CAPAN	Oh *et al.* 2005
Solanum tuberosum	StNAC	Stress response	Q948Z2_SOLTU	Collinge & Boller 2001
Citrus sinensis	CsNAC	Senescence	A2IB55_CITSI	Liu *et al.* 2009b
Petunia hybrida	PhNAM	Growth and development	Q40880_PETHY	Souer *et al.* 1996
Phaseolus vulgaris	PvNAP	Senescence	Q93XA6_PHAVU	Tucker *et al.* 2002
Arabidopsis thaliana	ATAF1	Stress response	Q2HIR8_ARATH	Wu *et al.* 2009
Arabidopsis thaliana	ATAF2	Stress response	NAC81_ARATH	Delessert *et al.* 2005
Arabidopsis thaliana	CUC1	Growth and development	NAC54_ARATH	Takada *et al.* 2001
Arabidopsis thaliana	CUC2	Growth and development	NAC98_ARATH	Aida *et al.* 1997
Arabidopsis thaliana	CUC3	Growth and development	NAC31_ARATH	Vroemen *et al.* 2003
Arabidopsis thaliana	NAC1	Growth and development	B2CUT4_ARATH	Xie *et al.* 2000
Arabidopsis thaliana	NAC2	Senescence	NAC56_ARATH	Balazadeh *et al.* 2010
Arabidopsis thaliana	NST1	Cell wall metabolism	NAC43_ARATH	Mitsuda *et al.* 2005
Arabidopsis	NST2	Cell wall	NAC66_ARATH	Mitsuda *et al.* 2005

thaliana		metabolism		
Arabidopsis thaliana	NST3	Cell wall metabolism	NAC12_ARATH	Hussey *et al.* 2011
Arabidopsis thaliana	NAP	Senescence	NAC29_ARATH	Guo & Gan 2006
Arabidopsis thaliana	TIP	Stress response	Q9LKG8_ARATH	Ren *et al.* 2000
Arabidopsis thaliana	AtNAM	Growth and development	NAC18_ARATH	Duval *et al.* 2002
Arabidopsis thaliana	VNI1	Cell wall metabolism	NAC82_ARATH	Kubo *et al.* 2005
Arabidopsis thaliana	VNI2	Cell wall metabolism	NAC83_ARATH	Yamaguchi *et al.* 2010
Arabidopsis thaliana	FEZ	Growth and development	FEZ_ARATH	Willemsen *et al.* 2008
Arabidopsis thaliana	BRN1	Growth and development	BRN1_ARATH	Bennett *et al.* 2010
Arabidopsis thaliana	BRN2	Growth and development	BRN2_ARATH	Bennett *et al.* 2010
Arabidopsis thaliana	SMB	Growth and development	SMB_ARATH	Bennett *et al.* 2010
Arabidopsis thaliana	ORE1	Senescence	NAC92_ARATH	Qiu *et al.* 2015
Arabidopsis thaliana	ORS1	Senescence	NAC59_ARATH	Balazadeh *et al.* 2011
Arabidopsis thaliana	JUB1	Stress response	NAC42_ARATH	Wu *et al.* 2012

Table S6

Polymorphism	P-value
C-296T	0,328
INDEL-282[1]	0,002 (**)
G-263T	0,115
INDEL-126[1]	0.003 (**)
A-22G	0,328
C222T	0,44
G411T[1]	0,015 (*)
T533A	0,029 (*)
G656T	0,34
T670G	N.D.
INDEL743[1]	0,020 (*)
G979A[1]	0,041 (*)
A1073C	0,283
INDEL1113	0,289
A1206T	0,619
A*121G	0,467
INDEL*173[1]	0,017 (*)

[1]polymorphisms observed between SC and PS
hyphen indicates the position before the ATG
asterisk in left column indicates the position after the 3'UTR start

Table S9

M2 Family	Amplicon	Mutation	Region	Substitution	Predicted effect
4831	A1	G36A	Exon 1	Q12Q	-
82	A1	G147A	Exon 1	E49E	-
246	A1	G175A	Exon 1	E59K	Deleterious
5970	A1	G265A	Intron 1	-	-
1301	A1	G371A	Exon 2	G94G	-
5388[1]	A1	T411G[5]	Exon 2	S108A	-
432	A1	C475T	Exon 2	P129L	Deleterious
5388[1]	A1	A533T[5]	Exon 2	P148P	-
2923[2]	A1	C580T	Exon 2	S164F	Deleterious
228[2]	A1	C580T	Exon 2	S164F	Deleterious
1784	A1	C634T	Intron 2	-	-
1725	A1	C724T	Intron 2	-	-
5388[1]	A2	A978G[5]	Exon 3	N236S	-
4933	A2	C1014T	Exon 3	A248V	Neutral
3717	A2	C1038T	Exon 3	S256F	Neutral
2503	A2	G1058A	Exon 3	D263N	Neutral
4321[3]	A2	C1169T	Exon 3	L300F	Neutral
4978[3]	A2	C1169T	Exon 3	L300F	Neutral
244	A2	C1264T	Exon 3	V331V	-
502[4]	A2	C1296T	Exon 3	P342L	Neutral
503[4]	A2	C1296T	Exon 3	P342L	Neutral
5264	A2	C1307T	3'-UTR	-	-
317	A2	G1337A	3'-UTR	-	-

[1]Discarded mutant family.

[2,3,4]Pairs of mutant families with the same mutation.

[5]Mutations probably not caused by EMS.

Table S10

		External color change mean ± SD (DAP)		Mean differences (DAP)		
M2 Family	**Substitution**	**W**	**M**	**M-W**	**W-CharMono**	**M-CharMono**
246	E59K	39 ± 1,8	46,2 ± 1,8	7,2 ***	0,4	7,6 ***
432	P129L	37,4 ± 1,1	43 ± 1,7	5,6 ***	-1,1	4,4 ***
4933	A248V	38,7 ± 1,6	39,8 ± 2,6	1,1	0,1	1,2
3717	S256F	36,1 ± 1,8	35,9 ± 1,8	-0,2	-2,4 *	-2,7 *
2503	D263N	39 ± 1,9	40,2 ± 1,6	1,2	0,4	1,6
502	P342L	39,7± 1,7	41,1 ± 2,3	1,4	1,2	2,6 *
CharMono	-	38,6 ± 2,3	-	-	-	-

Table S11

Gene	Name	Forward primer sequence 5'3'	Reverse primer sequence 5'3'
MELO3C025848	Cyclophilin (*CmCYP7*)	CGATGTGGAAATTGACGGAA	CGGTGCATAATGCTCGGAA (3'5')
MELO3C021182	1-aminocyclopropane-1-carboxylate synthase *(CmACS1)*	GATTGATCATAAGCTAAGGGTTTGGT	GGATAGCTAACCTTTGGGAACACTT
MELO3C010779	1-aminocyclopropane-1-carboxylate synthase *(CmACS5)*	TAGCCCTATATACCAACCCCTAGAATATAT	TCTCCTATTATCTACCTACATACTGTACACTCAT
MELO3C014437	1-aminocyclopropane-1-carboxylate oxidase (*CmACO1*)	AGAGGGCTTGTCTTTGTGTTTG	ATTTAGTTGAAAAGTCAAAACCAAA
MELO3C016540	NAM like protein (*CmNAC-NOR*)	TACCAAACTAAAACCCCTCAATACCT	CCAAATAACATTTCTCTAAAATCTAAATCA
MELO3C016536	Similar to NAC domain-containing protein 72 (*Arabidopsis thaliana*) (uniprot_sprot:sp|Q93VY3|NAC72_ARATH)	TTTCTTTTCTCTGCCCATCC	GAGGAAAAGAAAAAGAAAAAGGAA

genotype	code	*CmNAC-NOR* haplotype [5]	varietal type	phenotype											reference
				abscission	days to abscission (DAP)	ethylene production	days to peak of ethylene	external color change	days to external color change	flesh firmness (Kg/0.5 cm2) [1]	climacteric aroma	response to external ethylene treatment	flesh colour	ripening type	
Piel de Sapo (PS)	In-Ps-T111 [1]	INO	*inodorus*	no	-	no	-	no	-	2.2	no	no	white	non-climacteric	Saladié *et al.* 2015
PI 161375 (SC)	Con-SC [1]	CON	*conomon*	no	-	no	-	no	-	1.8	no	no	green	non-climacteric	Saladié *et al.* 2015
Védrantais	Can-Ved [1]	CAN	*cantalupensis*	yes	45	yes	37	yes	37	1.45	yes	yes	orange	climacteric	Saladié *et al.* 2015
Charentais Mono	CharMono	CAN	*cantalupensis*	yes	44	yes	37	yes	37	n.d.	yes	n.d.	orange	climacteric	this work
246 [2]	E59K	CAN	*cantalupensis*	yes	47	yes	46	yes	45	2	yes	n.d.	orange	climacteric	this work
432 [2]	P232L	CAN	*cantalupensis*	yes	44	yes	42	yes	42	1.5	yes	n.d.	orange	climacteric	this work
SC3-5-1 [3]	8M31	CON	introgression line in PS background	yes	35-36	yes	35	yes	35	n.d.	yes	n.d.	white	climacteric	Vegas *et al.* 2013
GF35 [3,4]	8M35	INO	introgression line in PS background	yes	44-48	yes	40	yes	40	1.25	yes	n.d.	white	climacteric	Vegas *et al.* 2013
GF40 [3,4]	8M40	CON	introgression line in PS background	yes	> 50	yes	43	yes	43	2.15	yes	n.d.	white	climacteric	Vegas *et al.* 2013

[1] data according to Table S8

[2] TILLING mutants of Charentais Mono

[3] SC3-5-1 contains the SC alleles for *ETHQB3.5* and *ETHQV6.3*; GF35 contains the SC alleles for *ETHQB3.5*; GF40 contains the SC alleles for *ETHQV6.3*

[4] these two lines had a lower peak of ethylene compared to SC3-5-1. For abscission and external fruit color, the phenotype of GF35 and GF40 was sometimes less clear and dependant on the environmental conditions

[5] haplotype using the 7 polymorphisms significantly related to ripening type

n.d.: not determined

Significance statement

Regulatory mechanisms common to climacteric and non-climacteric fruit ripening are not fully understood. Melon is a unique model species presenting both climacteric and non-climacteric types. ETHQV6.3 QTL allele introgressed into a non-climacteric background partially restores climacteric ripening. We show that ETHQV6.3 is encoded by the NAC-domain transcription factor MELO3C016540 (CmNAC-NOR). Mutations in CmNAC-NOR in a climacteric genetic background delay fruit ripening and biosynthesis of ethylene, confirming its role in this process.