Increase in tomato locule number is controlled by two single-nucleotide polymorphisms located near WUSCHEL

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Running head: Two SNPs control locule number in tomato fruits.

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Abstract:

In tomato fruit, the number of locules (cavities containing seeds that are derived from carpels) varies from two to up to 10 or more. Locule number affects fruit shape and size, and is controlled by several Quantitative Trait Loci (QTLs). The large majority of the phenotypic variation is explained by two of these QTLs, *fasciated* and *locule number* (*lc*) that interact epistatically with one another. *FASCIATED* has been cloned, and mutations in the gene are described as key factors leading to the increase in fruit size in modern varieties. Here, we report the map-based cloning of *lc*. The *lc* QTL includes a 1600 bp region that is located 1080 bp from the 3' end of *WUSCHEL*, which encodes a homeodomain protein that regulates stem cell fate in plants. The molecular evolution of *lc* showed a reduction in diversity in cultivated accessions with the exception of two SNPs. These two SNPs were shown to be responsible for the increase in locule number. An evolutionary model of locule number is proposed herein, suggesting that the *fas* mutation appeared after the mutation in the *lc* locus to confer the extreme high locule number phenotype.

INTRODUCTION

The domestication of wild plant species has altered particular sets of traits, such as the size and the diversity of the consumed parts (Frary and Doganlar, 2003). During this process, genetic diversity decreased, not only in those genes controlling domestication traits as a result of artificial selection, but also at the whole genome level as a result of genetic bottlenecks. Improvements of cultivars and production systems have increased crops yield. At the genetic level, the yield of domesticated plants has increased considerably compared to their wild ancestors (Buckler et al., 2001), particularly over the last 40 years (http://faostat.fao.org). However, this increase will probably reach its limit due to the increase of the world human population and the decrease in arable lands. The loss of genetic diversity at important loci that accompanied the yield increase could limit improvements in the future. The identification of new alleles or new allelic combinations from wild species or tomato accessions that did not experience an erosion of their genetic diversity is essential but requires an understanding of crop domestication. Linking the evolution of genotypes and phenotypes to understand domestication is one of the main challenges of modern genetics.

The tomato clade, *Solanum* section *Lycopersicum*, is composed of several wild species and the cultivated tomato (*Solanum lycopersicum*). The wild species are distributed along the Andean coast from Ecuador to Chile, with two species (*S. cheesmaniae* and *S. galapagense*) endemic to the Galapagos Islands. The cultivated tomato is a self-pollinating species that is thought to be derived from its closest wild ancestor *S. pimpinellifolium* (Nesbitt and Tanksley, 2002). Cherry tomato accessions (*S. lycopersicum* var. *cerasiforme*) have an intermediate position between these two species, as their genomes are mosaics of those from *S. lycopersicum* and *S. pimpinellifolium* (Ranc et al., 2008).

Compared to wild species, domesticated accessions show a range of morphological diversity, particularly with respect to fruit shape and size (Paran and van der Knaap, 2007) (Supplemental Photo 1), despite the strong loss of molecular diversity throughout the whole genome (Van Deynze et al., 2007).

A number of genes controlling tomato fruit morphology have been cloned: *FW2.2* (Frary et al., 2000) controls fruit weight; *SUN* (Xiao et al., 2008) and *OVATE* (Liu et al., 2002) control elongated fruit shape; and *FASCIATED* (Cong et al., 2008) controls locule number, fruit size and flat fruit shape. *fw2.2* controls up to 30% of fruit weight variation, and was the first gene underlying a QTL that was identified by a positional cloning approach (Frary et al., 2000). The gene is differentially expressed in the carpel resulting in increased cell number in large

fruit. *FASCIATED* encodes a YABBY-like transcription factor that is expressed early in the development of stamen and carpels. The characterization of both *FW2.2* and *FASCIATED* illustrate the link between fruit size and genes involved in developmental processes.

In tomato, locule number influences fruit shape and size. The locules are directly derived from the carpels in the flower. In addition to *fasciated*, several QTLs controlling locule number have been mapped and a candidate gene approach has been used to map genes regulating floral meristem development which might colocalize with known QTLs for locule number (Barrero et al., 2006). Although none of the genes responsible for these QTLs was successfully identified, the results of this study were used to develop several interesting hypotheses.

Two QTLs, *fasciated* and *locule number* (*lc*, also named *lcn2.1*) have a major effect on the phenotype. *FASCIATED*, which could be considered a major gene, has the strongest effect by increasing the number of locules from two to more than six. *lc* has a weaker effect by increasing the number of locules from two to three or four. An epistatic interaction between the two QTLs influences the phenotype with both *lc* and *fasciated* synergistically contributing to extremely high locule number (Barrero and Tanksley, 2004; Lippman and Tanksley, 2001). *WUSCHEL*, a gene controlling stem cell fate in the apical meristem, was mapped to the same region as *lc* (Barrero et al., 2006). The lack of polymorphisms in the gene and its promoter and the similar expression level of the gene in both wild type and mutant accessions led the authors to conclude that the DNA region which has been analyzed did not correspond to *lc*. The gene responsible for *lc* and its role during domestication thus remains unknown.

We report herein the map-based cloning and the identification of two SNPs immediately downstream of *WUSCHEL* that control the trait. The characterization of the *lc* locus shows a remarkable pattern of diversity. In addition, we demonstrate that the selection of this locus has been necessary to increase locule number during the domestication of tomato.

RESULTS

Physical mapping of lc

The *lc* locus, which was first described in 1937 (Yeager, 1937), was mapped close to the ovate region (Butler, 1952). This QTL is a major locus known to modify the number of locules in tomato fruits, with a phenotypic effect that varies according to the genetic background. The QTL location on chromosome 2 was more accurately defined using molecular mapping with polymorphic markers (Lippman and Tanksley, 2001; van der Knaap and Tanksley, 2003).

Our study exploited a recombinant inbred line population obtained from an intraspecific cross between two cultivated tomato accessions (Causse et al., 2002), Cervil, a cherry tomato line, and Levovil, a classical French fresh market line carrying round fruits with many locules weighing approximately 100g. The QTL was initially mapped to the TG463 region (Lecomte et al., 2004) using two near-isogenic lines (CF12-C and CF13-L) that were genetically identical except for a 30 cM region containing *lc*. CF12-C contains the low locule number allele from Cervil (C), and CF13-L contains the high locule number allele from Levovil (L) (Fig. 1A).

An F2 segregating population (2688 plants) derived from the cross between CF12-C and CF13-L was used to identify 215 plants with a recombination between the markers T1555 and TG191 surrounding *lc* in a 4.8 cM region. The segregation of the phenotype was tested in the progeny of each recombinant line (Supplemental Table S1), allowing the mendelization of the QTL (Fig. 1B). Heterozygous plants have an intermediate phenotype (2.7 locules ± 0.50 SD) that was between homozygous plants with low locule number (2.4 locules ± 0.51 SD) and homozygous plants with high locule number (3.5 locules ± 0.75 SD). This indicated that the mutant allele was partially recessive to the wild type allele. Homozygous plants carrying both the Cervil wild type alleles produced fruits with mostly two locules. In contrast, homozygous plants carrying both the Levovil mutant alleles produced fruits mostly containing three or more locules. Given the semidominant nature of the QTL and the limited phenotypic difference between the two extreme values of the two alleles, heterozygous plants could not be differentiated from either homozygous plant unless the recombinant plant was subjected to progeny testing.

The sequence analysis of the region around the closest linked marker TG463, indicated that the *lc* region was partially syntenic to a region in *A. thaliana* surrounding *At2g18000* (Fig. 2A). Sequence analysis of TG463 showed homology with the BAC end of the tomato clone

Le_HBa0139K19. The mapping of an insertion-deletion marker found at the other BAC end confirmed that this clone originated from the *lc* region. The ctof-13-f10 marker sequence was present on this BAC as confirmed by PCR amplification. Two recombinant plants, PA205-2 and JB149-9, showed a recombination event in the Le_HBa0139K19 BAC. The PA205-2 line produced fruits with few locules (2.3). The JB149-9 line produced fruits with a higher number of locules (3.8). Taken together, these observations indicated that the Le_HBa0139K19 BAC covered the *lc* QTL.

Sequence analysis of the BAC clone Le_HBa0139K19 (109.5kb) showed that it contained 11 putative Open Reading Frames (ORF). Polymorphisms found in the BAC sequence were used to identify the recombinant breakpoints in PA205-2 and JB149-9 which allowed us to narrow down the locus to 26.6 kb (Fig. 2A). This region contained three ORFs encoding a transducin WD40 repeat regulatory protein (similar to *At5g66240*), an unknown protein and *LeWUSCHEL*.

WUSCHEL (Laux et al., 1996; Reinhardt et al., 2003) was a likely candidate for the *lc* QTL because its function is associated with floral organ number (Mayer et al., 1998) and meristem size via the regulation of another key regulator of apical meristem development, Clavata3 (Brand et al., 2000; Schoof et al., 2000). The WD40 repeat protein was also a likely candidate because these proteins are linked to several important functions in eukaryotes (Smith et al., 1999), such as cell division (Feldman et al., 1997) and chromatin remodeling (Vitaliano-Prunier et al., 2008).

To further refine the recombination map, an additional 6768 F2 plants from the same cross between CF12-C and CF13-L were genotyped with the z1416 and z1420 markers that flanked the *lc* locus. This experiment led to an additional 52 recombinant lines. Newly designed markers, located every 2 kb within this region, restricted *lc* to a 3 kb region (Fig. 2B). Final sequence analysis of the region restricted *lc* to a 1608-bp region which showed 14 polymorphic sites (13 SNPs and a 1 bp indel) between the two alleles (Fig. 2C).

Functional analysis of lc

The 1608-bp region to which *lc* was fine mapped, corresponded to a non-coding region located 1080 bp downstream of the stop codon of *WUSCHEL*. None of the *in silico* analyses of the region provided any information about its putative function. In addition, this region did not show any homology with known miRNAs nor did its sequence have a clear secondary structure that would predict a novel miRNA. Using northern blots and RT-PCR did not reveal any possible expression of this locus (data not shown).

We also examined the effect of the *lc* locus on the expression of the two adjacent genes (Fig. 3). *WUSCHEL* expression was restricted to flower buds, whereas the WD40 repeat protein was expressed in all tissues examined. There was no significant expression difference between the wild type and mutant alleles of *lc*. Therefore, these results could not discriminate conclusively which of the two candidate genes underlies *lc*.

Given that *WUSCHEL* expression was restricted to floral buds, the effect of *lc* on floral development was evaluated by determining floral organ number in several representative near-isogenic lines (Fig. 4). The increase in locule number was positively correlated with an increase in petal number but was not associated with an increase in flower size.

Sequence polymorphisms among different tomato accessions validated lc

To further characterize *lc*, the 1608 bp region was sequenced in a set of 88 accessions composed of 16 *S. lycopersicum*, 62 *S. lycopersicum* var. *cerasiforme* and 10 *S. pimpinellifolium* (Supplemental File 1). This panel of varieties was chosen to represent a large spectrum of tomato diversity (Ranc et al., 2008). Sequence analysis revealed 21 new polymorphic sites, with the majority present in the wild species. Two SNPs were found to be associated with locule number (p-value $< 1 \times 10^{-6}$), and these two SNPs were in almost complete linkage disequilibrium, as shown by the existence of only one accession (LA2402) that showed a recombination event between the two SNPs. Together, the two SNPs were thus considered a unique haplotype. The correlation between the number of locules and the *lc* haplotypes was consistent except for three lines. The Pescio, Muchamiel and Stupicke Polni Rane cultivars produced fruits with high locules (3.8, 5.5 and 4.2, respectively) and contained the low locule number haplotypes, but the mutant genotype of these three lines at the *fasciated* locus explained their phenotype.

To validate the functional effect of the two SNPs linked to *lc* by association genetics, a 235 bp region containing the two SNPs was sequenced in 92 additional lines (Supplemental Table S2). The statistical association between the genotypes at the two SNPs and the number of locules was highly significant (p-value $< 1.30 \times 10^{-12}$), demonstrating that the two SNPs located in the non-coding sequence were responsible for *lc* and could thus be considered Quantitative Trait Nucleotides (QTNs) (Fridman et al., 2004). None of the other SNPs in the region was associated with fruit locule number. The potential effect of *lc* on fruit weight was also examined. The two SNPs explained more than 12 % of fruit weight variation (p-value of association $< 3.5 \times 10^{-6}$) in the core collection of 88 accessions. In the same set, one SNP in the 5'-UTR of *fw2.2* explained the same range of variation in fruit weight but with a weaker

significance level (p-value < 3 x 10^{-4}) and no association with locule number. The mutations responsible for *fw2.2* remain unknown, and thus another SNP may be more significantly associated with fruit weight at this locus.

Molecular evolution of lc during domestication

Detailed sequence analysis revealed that the diversity of the locus was drastically reduced in the cultivated species with the exception of the two SNPs responsible for *lc* (Fig. 5A and 5C). This observation was particularly evident for a 311 bp window surrounding the two SNPs in which no sequence differences were observed in the cultivated accessions. These results indicate that *lc* is under high selective pressure, probably due to the functional importance of the locus. An analysis of 8 additional loci located on the Le_HBa0139K19 BAC and 16 molecular markers located along the entire length of chromosome 2 indicated that the two SNPs responsible for *lc* (positive Tajima's D) evolved differently from other loci on the chromosome (negative Tajima's D for the 23 other loci). The negative Tajima's D values for the 23 loci located along the entire chromosome 2 indicated an increase of the population size in contrast to *lc* with a positive value. The two SNPs were under balancing selection (Fig 5B, Tajima's D = 2.227, p-value < 0.05), indicating that both alleles were selected during the domestication process, in contrast to other loci selected during domestication such as *tb1* in maize (Wang et al., 1999) or *fw2.2* in tomato (Nesbitt and Tanksley, 2002) that were under positive selection.

The tomato accessions studied for association mapping were not selected based on fruit locule number but rather their molecular diversity at neutral markers. They were mostly composed of *S. lycopersicum* var *cerasiforme* accessions producing fruits with low locules. The fasciated phenotype was under represented in all tested accessions (5.6%). Because *lc* and *fasciated* are known to interact epistatically, another collection of accessions was used to determine whether *lc* was necessary for the phenotypic expression of the fasciated phenotype. Thus, a complementary set of 87 modern cultivars producing fruits with more than six locules and considered fasciated were analyzed. Among these 87 cultivars, only three carried the low locule number allele of *lc* (Supplemental Table S3). Approximately 97% of the cultivars carried the allele of *lc* producing a high locule number. These results suggested that *lc* is necessary for the expression of a clear fasciated phenotype. Surprisingly, only 39 cultivars (44%) among the 87 had the mutant allele of *fasciated*, confirming that *fas* is not the only locus that produces fasciated fruits.

DISCUSSION

An intraspecific cross between two cultivated tomato lines allowed a map-based cloning of the lc QTL

Almost all wild tomato species produce bi-locular fruits. During domestication, the number of locules in fruits has increased together with fruit weight, which ranges from a few grams in wild species to up to 1000 grams in some cultivated varieties. Compared to other plant species, tomato fruit size is an excellent model for understanding the molecular basis underlying domestication of fruit bearing crops. The molecular characterization of the loci that control fruit size also provides insight into fleshy fruit development. *fw2.2*, the first QTL controlling fruit weight to be cloned, was identified via a map-based cloning strategy using an interspecific cross. The same strategy was employed to clone the gene responsible for the *fasciated* QTL, which determines the multi-locular phenotype in beefsteak type tomatoes. In both examples, the wild type allele came from *S. pennellii*. This distant wild relative was used because it had high polymorphism levels compared to *S. lycopersicum* varieties. The population of introgression lines from *S. pennellii* (Eshed et al., 1992) could not be used in this study because both parents have the same haplotype at the two SNPs and the same low locule number phenotype. Although *lc* is described as a major QTL, its effect on the number of fruit locules is weak, compared to that of *fasciated*. Thus, all genetic background effects needed to be overcome to determine which phenotypic variations could be attributed to *lc* alone, without any segregation of other minor QTLs. The best method to ensure this requirement was the use of near isogenic lines. An intraspecific cross between two cultivated accessions was selected to clone the *lc* QTL, which had been previously mapped in our segregating population (Lecomte et al., 2004). The two near isogenic lines only differed from each other in the region containing *lc*. A 1608 bp region responsible for the phenotype was identified based on the genotyping of 9456 F2 plants from this cross and the phenotyping of 267 recombinant sub-NILs lines. The success of a positional cloning approach is based on reliable phenotyping. Because *lc* is semidominant, heterozygous plants have an intermediate phenotype between that associated with the two homozygous alleles. The difference between the two extreme values of the two alleles was too close to distinguish the heterozygous plants from either homozygous plants. Thus, the segregation of the phenotype was systematically

studied by self-pollinating the F2 recombinant plants and measuring the phenotype in the resulting homozygous F3 plants.

Association mapping permitted the identification of two SNPs responsible for lc but alone would not have identified the QTL

Linkage disequilibrium mapping allows the identification of the genes responsible for complex traits (Long and Langley, 1999). This approach was first developed to overcome the impossibility of obtaining the large segregating populations necessary for QTL mapping in humans (Spielman et al., 1993). The principle is simple, involving the comparison of two groups of genetically unlinked individuals that differ for a particular trait. The two groups are then genotyped with molecular markers, and statistical associations between the phenotype and the genotype at the markers are determined.

Association mapping is based on linkage disequilibrium (LD), which varies significantly between species. In maize, linkage disequilibrium is low within distances ranging from 200 to 1500 bp (Remington et al., 2001). In tomato, it remains high within distances of 20 cM (van Berloo et al., 2008). Because high LD increases the risk of detecting false positive associations, this study used a combination of map-based cloning to identify the locus region and association mapping to refine its molecular characterization. The map-based cloning step identified a 1608 bp region. For the association mapping step, this region was sequenced in a core collection composed of 88 accessions selected from a larger population that was designed to maximize the molecular diversity. In addition to the 14 polymorphic sites found between both parental lines, 21 polymorphisms were identified from this core collection. As expected, more than 81% of the new polymorphic sites were found in *S. pimpinellifolium* wild accessions. Association mapping identified two SNPs that had an almost perfect association with the locule number phenotype. This association was then validated using a larger set of tomato accessions. The same result was obtained, confirming that the two SNPs were responsible for the phenotype.

An additional 24 DNA fragments located on chromosome 2 were sequenced in the core collection (Ranc, unpublished data). The two SNPs responsible for *lc*, however, were not in linkage disequilibrium with any other polymorphic site adjacent to the locus or distantly located on chromosome 2. Given this observation, the identification of the molecular region corresponding to *lc* using association mapping alone would have been impossible except by sequencing the region by chance. New high throughput sequencing tools allow genomes to be

re-sequenced more quickly and cheaply. Because the tomato genome is now sequenced (http://solgenomics.net/genomes/Solanum_lycopersicum/index.pl, Mueller et al., 2009), such an approach involving the comparison of a large number of full genomes could be used in the near future to identify an increasing number of genes for complex traits.

A model for locule number evolution in tomato fruits

Using SSR markers, the genome of cherry tomato accessions (*S. lycopersicum* var. *cerasiforme*) has been shown to be a mosaic of the genomes from *S. pimpinellifolium* and *S. lycopersicum* (Ranc et al., 2008). Cherry tomatoes are thus a useful tool for association mapping because of their intermediate molecular diversity level and admixture genome structure. The 180 accessions used for association mapping were mostly composed of *S. lycopersicum* var. *cerasiforme* tomatoes. Wild tomato species, however, mainly produce fruits with a low locule number. Among the 180 accessions, 81.1% produced fruits with two to four locules, and 18.9% produced fruits with more than four locules. This collection allowed the identification of the two SNPs responsible for *lc* but could not be used to determine the evolutionary history of locule number during tomato domestication. The mechanism by which *fasciated* could have evolved compared to *lc* was studied using a third set of tomato accessions that only included accessions producing fruits with more than six locules. Interestingly, only 44% of the accessions carried the *fasciated* high locule number allele, but 97% carried the high locule number allele of *lc*. This result indicates that the high locule number allele of *lc* is necessary to express the fasciated phenotype in combination with the *fas* locus and that it is also required to produce the beefsteak tomato phenotype in combination with one or more other loci in addition to *fasciated*.

Additionally, all wild species tested contained the *lc* haplotypes that produces fruits with a low number of locules, suggesting that the two SNPs responsible for the increase of locule number appeared during tomato domestication in a *S. lycopersicum* var. *cerasiforme* cultivar and then spread among these cultivars. *fasciated* appeared later, and the combination of the two loci produced the fasciated phenotype exhibited by the first cultivars introduced in Europe (Daunay et al., 2007). A model is thus proposed to explain the evolution of locule number during tomato domestication and breeding (Fig. 6).

The average value of Tajima's D for the entire chromosome 2 is -0.633, suggesting a recent demographic expansion that followed the bottleneck of domestication (Fig. 5B). The two SNPs responsible for *lc* exhibit a significantly different pattern. The evolutionary history of *lc*

is more likely explained by a particular pattern of evolution rather than by the demographic evolution of the sample used to validate the QTL. The positive Tajima's D value for the two SNPs indicates a balancing selection. This type of selection is explained by the maintenance of the two alleles at the locus, with an equilibrated frequency.

In the proposed model, *lc* existed as a polymorphic locus in the wild species, and all alleles resulted in the production of fruits with two locules. Then, the two SNPs responsible for the increase in the number of locules appeared in a particular allele of *lc*, which has subsequently expanded in *S. lycopersicum* accessions because it conferred larger fruits. Later the *fas* mutation allowed a further increase in locule number when expressed in the *lc* background. In our hypothesis, we proposed that *lc* appeared prior to other loci responsible for the increase of locule number. The wild type allele of *lc* has been recently reintroduced in *S. lycopersicum* by modern breeding to diversify fruit shape.

How to characterize the molecular function of lc?

Interestingly, the genomic region of *lc* is located in a noncoding region between two putative candidate genes, *WUSCHEL* and a gene encoding a WD40 repeat protein. *WUSCHEL*, which was previously proposed as a candidate gene for *lc* (Barrero et al., 2006), has a central role in apical meristem development, being responsible for stem cell fate and affecting meristem size. No differences in the expression of the two genes could be detected in a set of sub-NILs. To determine whether the *lc* region could be expressed, quantitative RT-PCR assays using several primer pairs inside and surrounding the locus were performed. These assays did not reveal any expression of this region. Instead of using transformation to try to validate the QTL, we preferred to use a combination of fine mapping and association mapping. This method successfully identified the two SNPs responsible for *lc*. Although transgenic plants are used to help understand the function of a gene, this method would have been difficult for *lc* due to the unclear limits of the functional region necessary to express the phenotype. Indeed, the locus did not show any homology with a known coding sequence. Even though we concluded that the 1608 bp region contains the polymorphic sites responsible for the phenotype, we cannot exclude that the functional region could be larger than 1608 bp.

lc had previously been shown to be necessary to obtain a *fasciated* phenotype (Lippman and Tanksley, 2001). The results presented herein further the understanding of the interaction between *lc* and *fasciated*. First, *fasciated* was not the only locus producing tomato fruits with a high number of locules. Second, high locule number allele at *lc* was necessary for the

fasciated phenotype. At the molecular level, *fasciated* encodes a YABBY-like transcription factor (Cong et al., 2008). Interestingly, a YABBY transcription factor acts on the partitioning of shoot apical meristem in Arabidopsis (Goldshmidt et al., 2008). This observation could favor *WUSCHEL* as the best candidate interacting with *fas*. *WUSCHEL* expression is regulated by several factors (Dodsworth, 2009). Given that the *lc* SNPs are located 1080 bp from the stop codon of *WUSCHEL*, they could act as post-transcriptional regulators.

WD40 repeat proteins play important roles in eukaryotes. Interestingly, the WD40 protein near *lc* is orthologous to *At5g66240* in *Arabidopsis*. *At5g66240* is itself homologous to the *At5g66430* gene that encodes the FAS2 protein (Kaya et al., 2001). FAS1 and FAS2 are two subunits of the CAF1 complex (Chromatin remodeling factor-1). The *FAS* genes are described as regulating the maintenance of the expression state of *WUSCHEL* in the shoot apical meristem. Mutations in *fas1* and *fas2* were also described as causing stem fasciation together with altered floral development (Leyser and Furner, 1992). Stem fasciation is often linked to fruit fasciation in tomato, which is due to the high increase of locule number.

All together, these observations do not allow discrimination between *WUSCHEL* and *FAS2-like* as the gene that is modified by the two SNPs and thus is responsible for the phenotype. *lc* could act on shoot apical meristem development by regulating both or either gene in a complex way. The two SNPs could also act on the regulation of one or several genes elsewhere in the genome.

CONCLUSION

Although we were unable to identify the function of the two polymorphic nucleotides responsible for *lc*, we can assume that they play an important role in meristem development. Although the *lc* region is not expressed, the two SNPs have a pleiotropic effect on locule number, floral organ number and fruit weight and might act by regulating meristem size. These QTN are flanked by two genes that could affect tomato fruit development. Thus, these two SNPs may regulate either or both of these two genes, but at a very specific stage.

The two QTLs *fasciated* and *lc* make tomato an excellent model to study floral meristem and fleshy fruit development. Herein, two SNPs responsible for the increase of locule number were identified and used to develop a model of the evolutionary history of tomato domestication. An understanding of the mechanism by which this non-coding region affects locule number in the fruits and floral organ number in the flowers still remains of great interest and will require additional experiments. Although this study did not reveal any evidence for the expression of the *lc* region, it might be expressed in specific cells or at a precise time of development. *In situ* hybridization experiments should be performed to determine whether the *lc* region is expressed at the cellular level in meristems, where it should act. Similar experiments using WUSCHEL and the FAS2-like protein as probes may also reveal differential expression between lines not identified by RT-PCR on floral buds.

MATERIALS AND METHODS

Plant material and phenotyping

All accessions are maintained in the GAFL research unit (INRA Avignon, France) and are available upon request (helene.burck@avignon.inra.fr, mathilde.causse@avignon.inra.fr).

All plants were grown in greenhouses in Montfavet (south of France) between 2004 and 2008. Fine mapping and ultra-high resolution mapping were performed on a F2 population derived from a cross between the two near isogenic lines CF12-C and CF13-L (described as F8-V-C and F8-V-L, respectively (Lecomte et al., 2004)). Plants were sown in 96-well plates and transferred to 3-L containers after genotype selection. The locule number of each plant was determined by phenotyping 20 mature fruits (10 from the second truss and 5 each from the third and fourth trusses). The petal number and flower diameter of each plant was determined by analyzing 20 flowers.

For the diversity analysis, 10 fruits per accession were phenotyped.

Genotyping

The z100_CAPS and z274 primers were used to amplify the T1555 and TG191 markers, and the resulting PCR products were digested with *BamHI* and *NdeI*, respectively, and mapped on the F6 RIL population derived from the cross between Cervil and Levovil (Causse et al. 2002). Taqman markers developed from the associated SNPs were used to screen 2688 F2 plants for the fine mapping analysis. Those plants with different genotypes at T1555 and TG191 (i.e. recombination between the two markers) were selected for fine mapping. The same markers were also used to screen 8 F3 plants from the self-progeny of each F2 recombinant plant. Two plants homozygous for the segregating markers were then selected for phenotyping.

Polymorphisms in the z1416 and z1420 markers were used to develop Taqman markers that were used to screen 6768 F2 plants for the ultra-high resolution mapping analysis. As above, these markers were also used to genotype 8 F3 plants from the self-progeny of each recombinant F2 plant.

Alleles and BAC sequencing

All sequencing reactions were performed either manually on the ABI3730XL (Philippe et al., 2010) or by Genome Express (Meylan, France), GATC (http://www.gatc-biotech.com) or MWG (http://www.eurofinsdna.com). The TG463 sequence was obtained by sequencing the

plasmid. All other sequencing reactions were performed using PCR products. All primers used for PCR amplification are described in Supplemental Table S4.

The LeHBa0139K19 BAC sequencing was performed by Genome Express.

Sequence and polymorphism analysis

Sequence alignment and SNP detection were performed manually using the Genalys software (Takahashi et al., 2003), which is available at http://software.cng.fr.

Accession numbers

All sequences have been submitted to Genebank database (http://www.ncbi.nlm.nih.gov/genbank) with the Sequin v10.3 software (http://www.ncbi.nlm.nih.gov/projects/Sequin). The accession numbers are listed in Supplemental Table S5.

Expression analysis

Samples from leaves, flower buds or fruits at 7 or 14 days after anthesis were randomly harvested (4 plants/line) and immediately frozen in liquid nitrogen. Total RNA were extracted from 3 different pools with TRI Reagent Solution (Ambion, Austin,TX,USA) following the procedure described by the manufacturer.

RT-qPCR were performed and analyzed has described by Prudent et al. (2010). The genes of interest coding for *WUSCHEL* (AJ538329), the WD40 protein (SGN-U585584) and the eIF-4A-2 gene used as an internal control (SGN-U593757) were amplified; a specific PCR product was obtained with the corresponding efficiency: 100.8%, 108.1%, 110.8% respectively.

RT-PCR were performed in the same conditions with the same samples but amplified without SYBR Green on a classic thermal cycler.

Northern Blots were performed using *lc* locus as a probe on the same RNA samples as described by Muños et al. (2004).

Diversity analysis

A total of 180 accessions (37 *S. lycopersicum*, 128 *S. lycopersicum var. cerasiforme*, 15 *S. pimpinellifolium*) were phenotyped during the summers of 2007 and 2008. Part of this sample represents a core collection of 88 individuals selected to maximize the diversity of the whole collection (Ranc et al., 2008). A total of 24 genomic 500 bp fragments (8 on the BAC

identified by positional cloning and 16 on the rest of the chromosome 2) were used to sequence the plants in this core collection. A mixed linear model (Yu et al., 2006) implemented using Tassel software was used for the association tests of the core collection. The genetic structure of each sample was taken into account and calculated using Structure2.0 software (http://pritch.bsd.uchicago.edu/structure.html) based on microsatellite genotyping information (Ranc et al., 2008). The genetic background interaction was also taken into account using an estimation of kinship matrix following the recommendations of Yu et al. (2006). To validate any associations, fruit weight was used as a covariate in the model. A 200 bp window surrounding the two SNPs was sequenced on an additional set of 92 accessions, and the association between the two SNPs and fruit locule number was then validated.

DNAsp v.4 software was used to compare the molecular diversity (π) between *S. lycopersicum* (17 accessions) and *S. pimpinellifolium* (11 accessions) using a sliding window analysis method. Sequences obtained from the BAC clone and whole chromosome 2 were used to estimate Tajima's D and to test for evidence of selection pressures applied to the *lc* locus or to the entire chromosome for cultivated accessions (65 accessions). The *lc* fragment was restricted to a 500 bp region surrounding the SNPs to maintain the same length as the other amplicons.

ACKNOWLEDGMENTS

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Aurélie Chauveau and Rémi Bounon helped in the allele sequencing experiments.

Florent Leydet and Laure David participated in the fine mapping experiment and in the association mapping, respectively.

Hélène Burck manages and characterizes all the accessions of the tomato collection at INRA in Avignon and provided the seeds.

We thank the greenhouse experimental team of the GAFL research unit for growing all plants.

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FIGURE LEGENDS

Figure 1. Phenotypic analysis and mendelization of the locule number QTL (*lc*).

A. Fruit morphology of lines used in the study.

The parental lines Cervil and Levovil were crossed to obtain the F1 hybrid and the near isogenic recombinant F8 lines CF12-C and CF13-L. The L2 line was derived from Levovil, and its *lc* region of chromosome 2 containing the Cervil allele has been introgressed by marker-assisted selection.

B. Phenotypic effect of the QTL.

The average locule number of F2 heterozygous plants (H) or F3 plants homozygous for either the Cervil (C) or the Levovil (L) allele was determined. The plants were selected based on genotyping data, and heterozygous plants were selected by analyzing genotypic and phenotypic segregation in their corresponding F3 progeny. Each histogram, with its standard deviation, is partitioned according to the proportion of fruits with two locules (white), three locules (black) and four or more locules (gray). A Student's t-test indicated that the means are significantly different between C and H ($p<0.001$), between H and L ($p<0.001$) and between H and C ($p<0.01$).

Figure 2. Fine mapping (A), ultra-high resolution mapping (B) and polymorphism (C) of *lc* QTL.

A. All sequences highlighted in green represent tomato genes syntenic with the *At2g18000* region in *Arabidopsis thaliana*. Markers highlighted in yellow were used to screen for recombinant plants. The PA205-2 and JB149-9 recombinant lines allowed the identification of the BAC clone containing the QTL. This BAC, LeHBa139K19, contains 11 putative ORFs, with some showing homology to known genes or expressed sequences. *lc* is located in the region between z1416 and z1420.

B. The z1416 and z1420 markers were used to screen for recombinant plants. Newly designed markers finally localized *lc* between z1497 and z1499. Seven plants with recombination events between these two markers were identified. The molecular characterization of these 7 lines restricted *lc* to a 1608 bp non-coding DNA region. The orientation (from ATG to stop codon) and the exons (in blue) of the cDNAs coding for *WUSCHEL* and the WD40 protein are shown.

C. Sequence alignment of the two alleles of *lc* from Cervil and Levovil. The 14 polymorphic sites are highlighted in gray. The two underlined SNPs are responsible for *lc* (Fig. 5).

Figure 3. Expression analysis of the two candidate genes

RT-PCRs were performed on total RNA extracted from either floral buds, leaves or fruits. Primers were designed on *WUSCHEL* (AJ538329 accession number), the WD40 repeat protein (SGN-U585584 tomato unigene). eIF4A2 (SGN-U593757 tomato unigene) was used as a reference gene.

Figure 4. Effect of lc on locule number (A), flower size (B) and petal number (C).

Cervil and Levovil are the parental lines. L2 and L4 have been obtained by marker assisted selection from Levovil; they are both identical to Levovil except for a region of chromosome 4 from Cervil (L4) and the region of the chromosome 2 from Cervil which contains *lc* (L2). L2 and L4 must be compared to Levovil. CF12-C and CF13-L are a pair of identical isogenic lines except for a 30 cM region which contains *lc*. JB1538 and JB1546 are identical to CF12-C and CF13-L respectively but differ for less than 30cM. PA205-2 and JB149-9, on the same principle, differ from the one another by a 28kb region surrounding *lc*.

The lines are all homozygous at the *lc* locus and contain either the alleles from Cervil (C) which produce fruits with low locule number or from Levovil (L) which produce fruits with high locule number. The genetic background is either Cervil (C) or Levovil (L); it is recombinant (R) between Cervil and Levovil for the isogenic lines.

The mean of each line has been compared to Cervil. Statistical significances of the t-test are indicated as ns (not significant) or *** ($P<0.001$).

Figure 5. Molecular diversity of the *lc* locus

A. Tomato haplotypic structure of *lc* and other loci on chromosome 2

Each block corresponds to one of the 24 amplicons (average length of 500 bp). Columns and rows represent individuals and SNPs, respectively. With Heinz1706 used as reference, polymorphisms are indicated either in gray (identical allele) or in black (different allele).

B. Tajima test over the whole chromosome and within *lc*.

Stars indicate a significant departure from the neutrality hypothesis. A significant positive value of Tajima's D for *lc* indicated either a balancing selection of the locus or a population

decrease. An analysis of the entire chromosome showed that *lc* evolved differently from the entire chromosome: *lc* underwent a balancing selection, whereas chromosome 2 evolved following a population expansion. Dashed lines represent the mean and standard error for the whole chromosome.

C. Molecular diversity of *lc* in wild and cultivated lines.

The diversity (π) of the locus was drastically reduced in cultivated lines (black lines) except for two SNPs (arrow) in comparison with wild accessions (gray lines). A total of 17 cultivated and 11 wild lines were used in this analysis.

Figure 6. Model of locule number evolution in tomato fruits during domestication

Solanum pimpinellifolium is considered the wild ancestor of the cultivated tomato *Solanum lycopersicum.* Based on the analysis of 267 tomato accessions, we propose a model that could explain the history of locule number evolution during tomato domestication. In our study, only 4.6% of the high locule number accessions (i.e., those with >3 locules) had the low locule allele of *lc* and 96.9% of the fasciated accessions (i.e., those with >6 locules) had the high locule allele of *lc*, but only 49.4% of them had the *fas* allele. These results indicate that *lc* was required for the increase in locule number in tomato fruits during domestication. The *lc* locus could have appeared before the *fasciated* locus. These two QTLs are the major loci controlling locule number. Modern breeding has used other loci to expand phenotypic diversity.

Supplemental Photo 1. Photo of fruits from some varieties used in the study.

Fruits illustrate morphological diversity.

Supplemental File 1. Locule number phenotype and genotype of *fas* and *lc* loci for association studies in 88 tomato accessions.

These data are from of 88 tomato accessions composed of 16 *S. lycopersicum*, 62 *S. lycopersicum* var. *cerasiforme* and 10 *Solanum pimpinellifolium.* These accessions were grown and phenotyped for locule number in 2007 and 2008. 10 fruits were measured for each accession. All accessions were genotyped for *fasciated* and *lc* by sequence analysis. The genotype of *lc* is detailed for each of the 35 polymorphic sites with M82 as a reference. Each site is noted 0 if homozygous and identical to M82, 1 if homozygous and polymorphic

compared to M82, 2 if heterozygous and -1 if lacking. The data highlighted in red are those of the 3 lines containing the low locule allele of *lc* and high locule allele of *fas*.

Supplemental Table S1. Genotype and phenotype of the 215 recombinant F2 plants and their F3 progeny used for *lc* fine mapping.

These data show genotypes for T1555 and TG191 markers and phenotypes for locule number. For each F2 recombinant plant, 8 F3 plants from the selfed progeny were genotyped in order to remove heterozygosity (H). Two F3 plants were selected: one with the two alleles from Cervil (C), the other with the two alleles from Levovil (L). Locule number corresponds to the average of 20 measured fruits. Data highlighted in purple are those of the two lines which allowed the identification of the BAC clone containing *lc*. For each pair of F3 lines, locule number averages have been compared by a Student's t-test, statistical significance are noted ns (not significant), *** ($P<0.001$), ** ($P<0.01$) and * ($P<0.05$).

Supplemental Table S2. Validation of the association by genotyping the two SNPs in 92 tomato accessions.

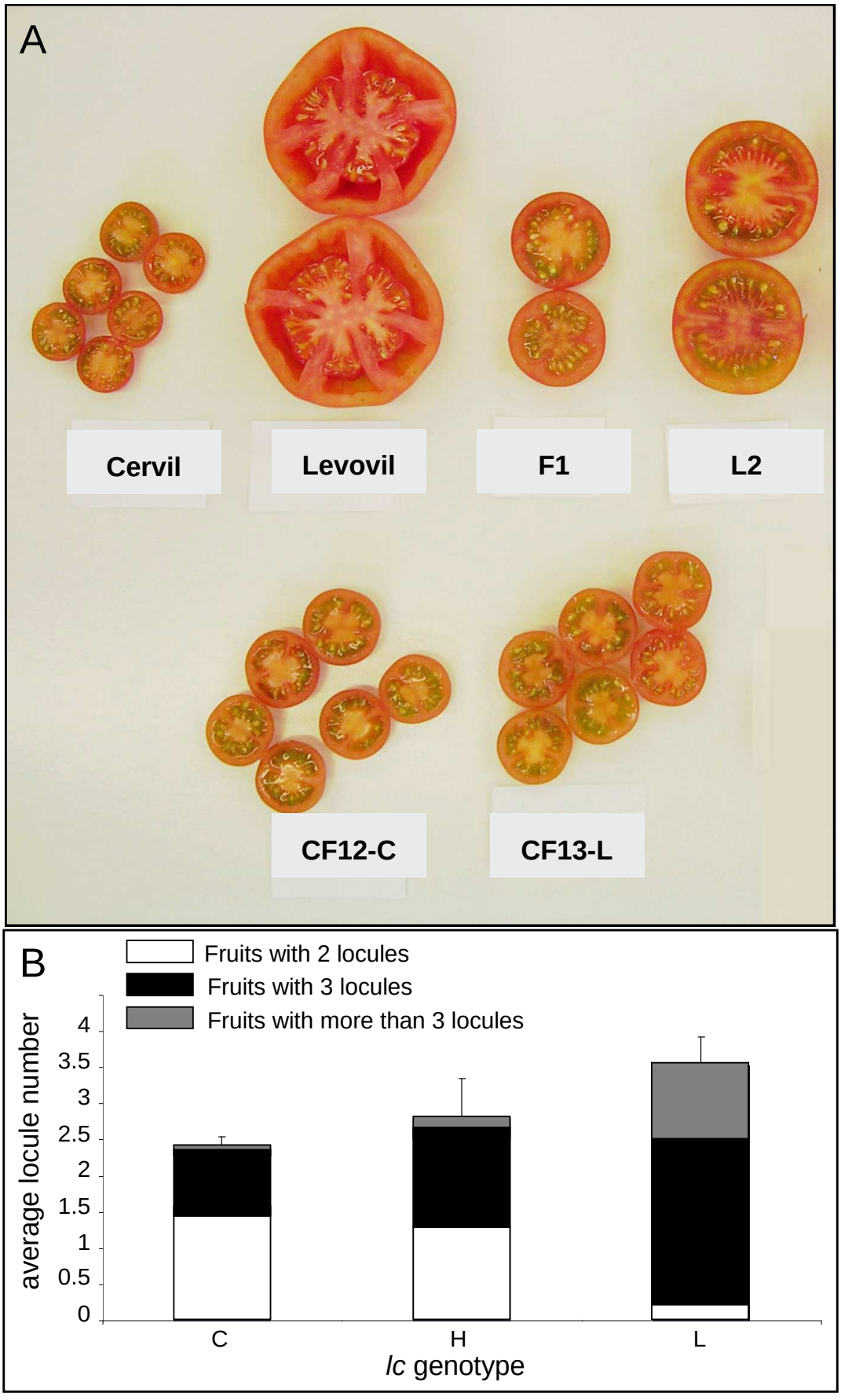
The data were obtained using 92 tomato accessions composed of 21 *S. lycopersicum*, 66 *S. lycopersicum* var. *cerasiforme* and 5 *Solanum pimpinellifolium.* These accessions have been grown and phenotyped for locule number in 2007 and 2008. 10 fruits have been measured for each accession. All accessions were genotyped by sequencing the 235 bp region containing the two SNPs responsible for *lc*. Genotype is noted 0 if homozygous for the low locule allele, 1 if homozygous for the high locule allele and 2 if heterozygous.

Supplemental Table S3. Analysis of *lc* and *fas* loci in tomato accessions producing fasciated fruits with more than 6 locules.

The data were obtained using 87 tomato accessions producing fasciated fruits with more than 6 locules. These accessions are a part of the collection maintained in the GAFL research unit (INRA, France). All accessions have been genotyped for the two SNPs responsible for *lc* and for *fas*. The genotype at the *lc* locus is noted 0 if homozygous for the low locule allele, 1 if homozygous for the high locule allele and 2 if heterozygous. The genotype at the *fasciated* locus is noted *fas* for the alleles producing fasciated fruits and *wt* for the alleles producing unfasciated fruits.

Supplemental Table S4. Primer sequences used in the study.

Supplemental Table S5. Accession numbers of the sequences.



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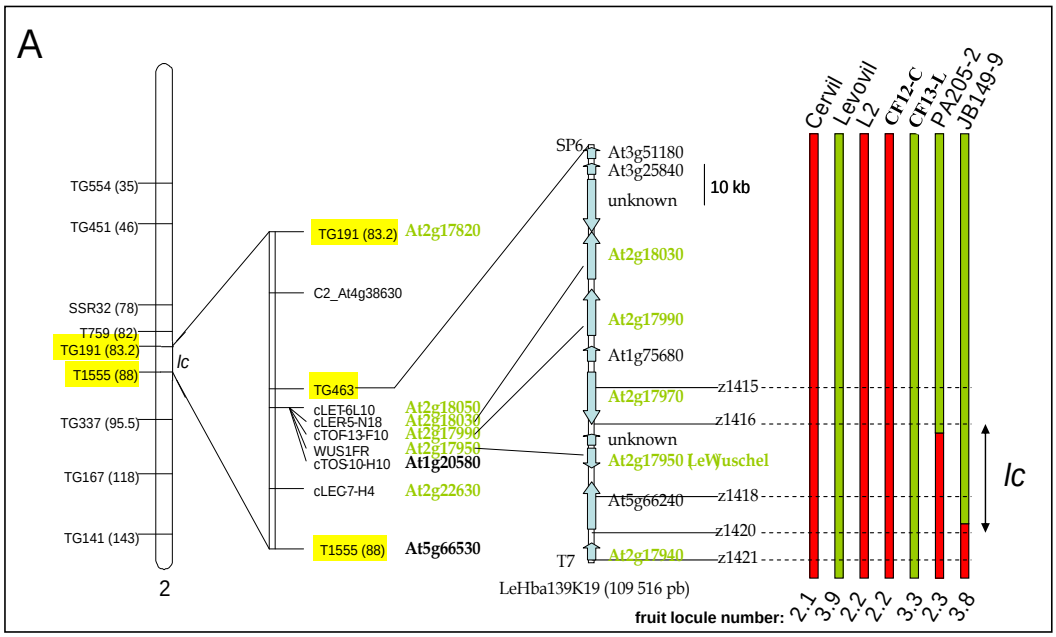

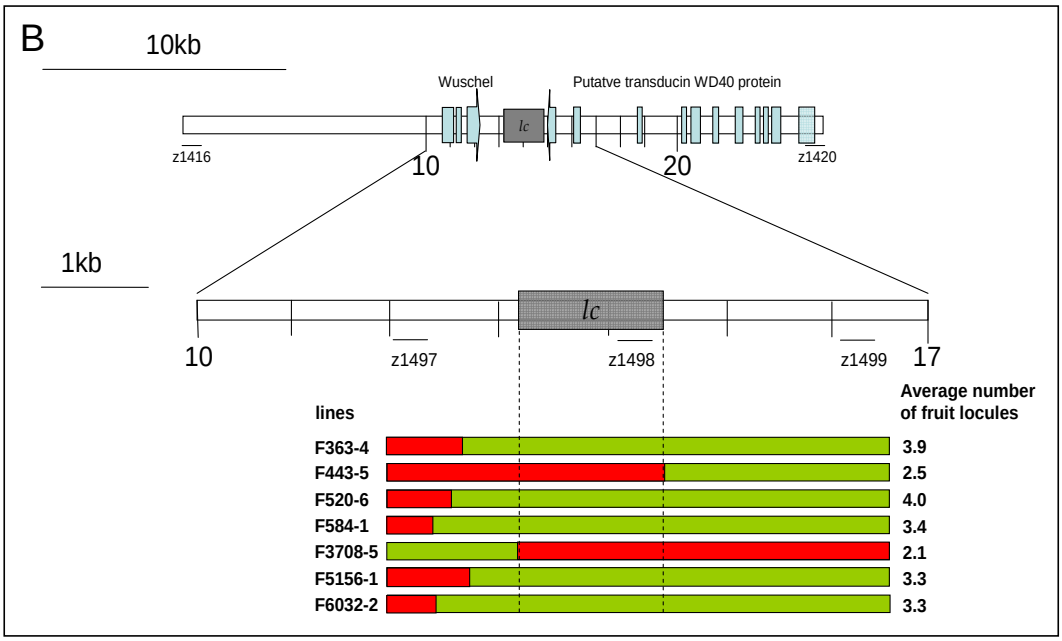

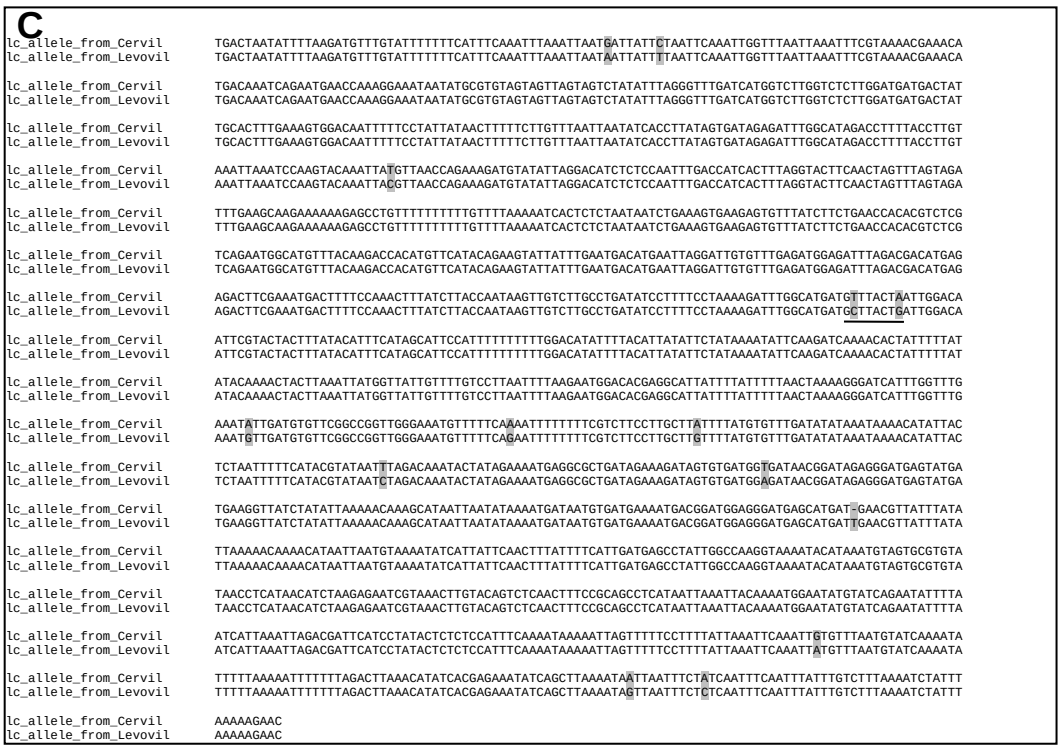

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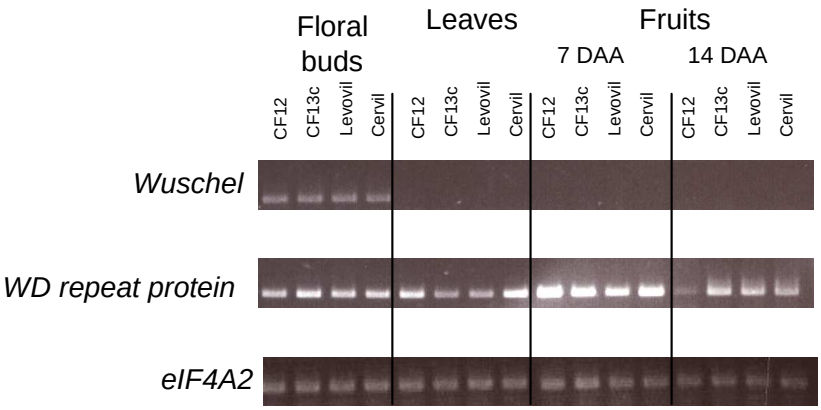

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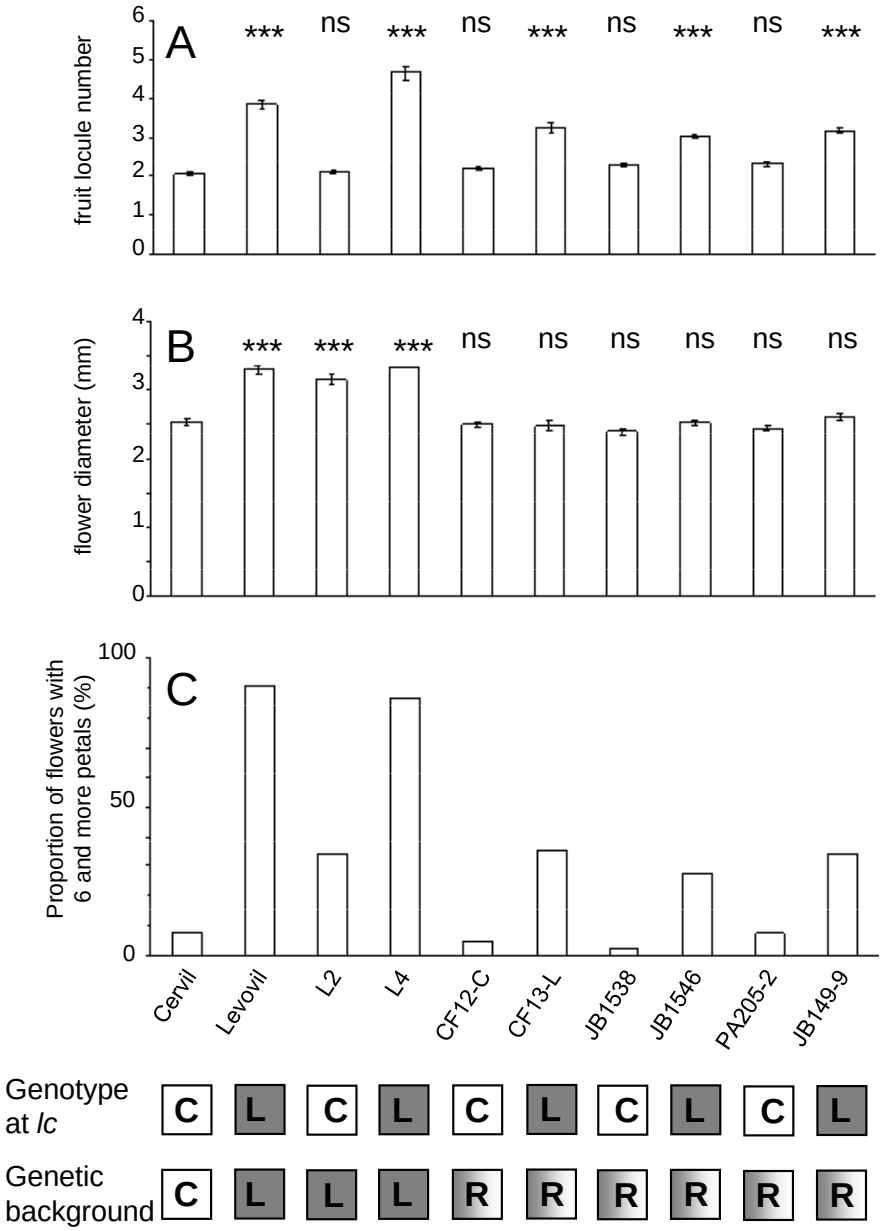

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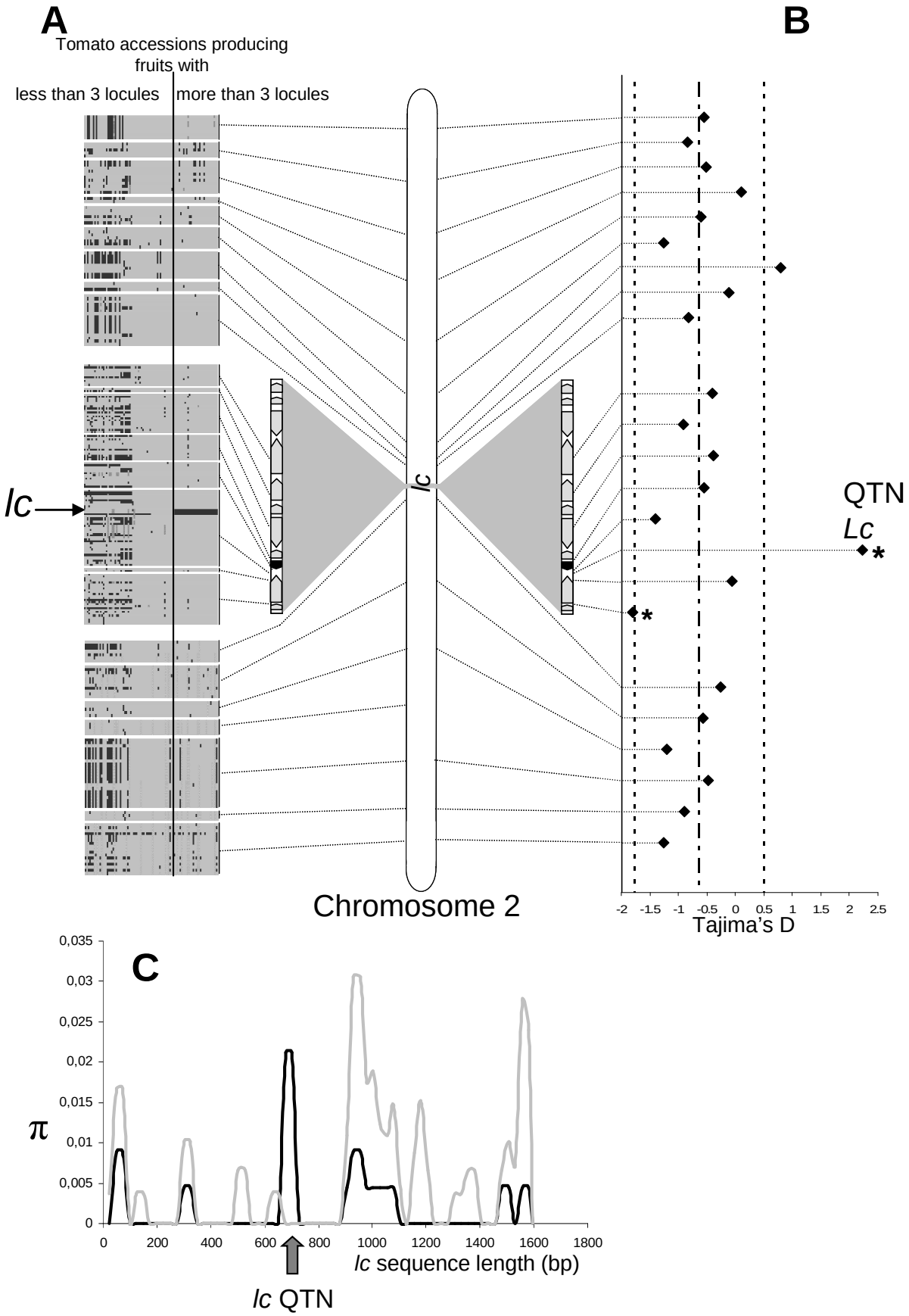

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Each block corresponds to one of the 24 amplicons (average length of 500 bp). Columns and rows represent individuals and SNPs, respectively. With Heinz1706 used as reference, polymorphisms are indicated either in gray (identical allele) or in black (different allele).

B. Tajima test over the whole chromosome and within *lc*.

Stars indicate a significant departure from the neutrality hypothesis. A significant positive value of Tajima's D for *lc* indicated either a balancing selection of the locus or a population decrease. An analysis of the entire chromosome showed that *lc* evolved differently from the entire chromosome: *lc* underwent a balancing selection, whereas chromosome 2 evolved following a population expansion. Dashed lines represent the mean and standard error for the whole chromosome.

C. Molecular diversity of *lc* in wild and cultivated lines.

The diversity (π) of the locus was drastically reduced in cultivated lines (black lines) except for two SNPs (arrow) in comparison with wild accessions (gray lines). A total of 17 cultivated and 11 wild lines were used in this analysis.

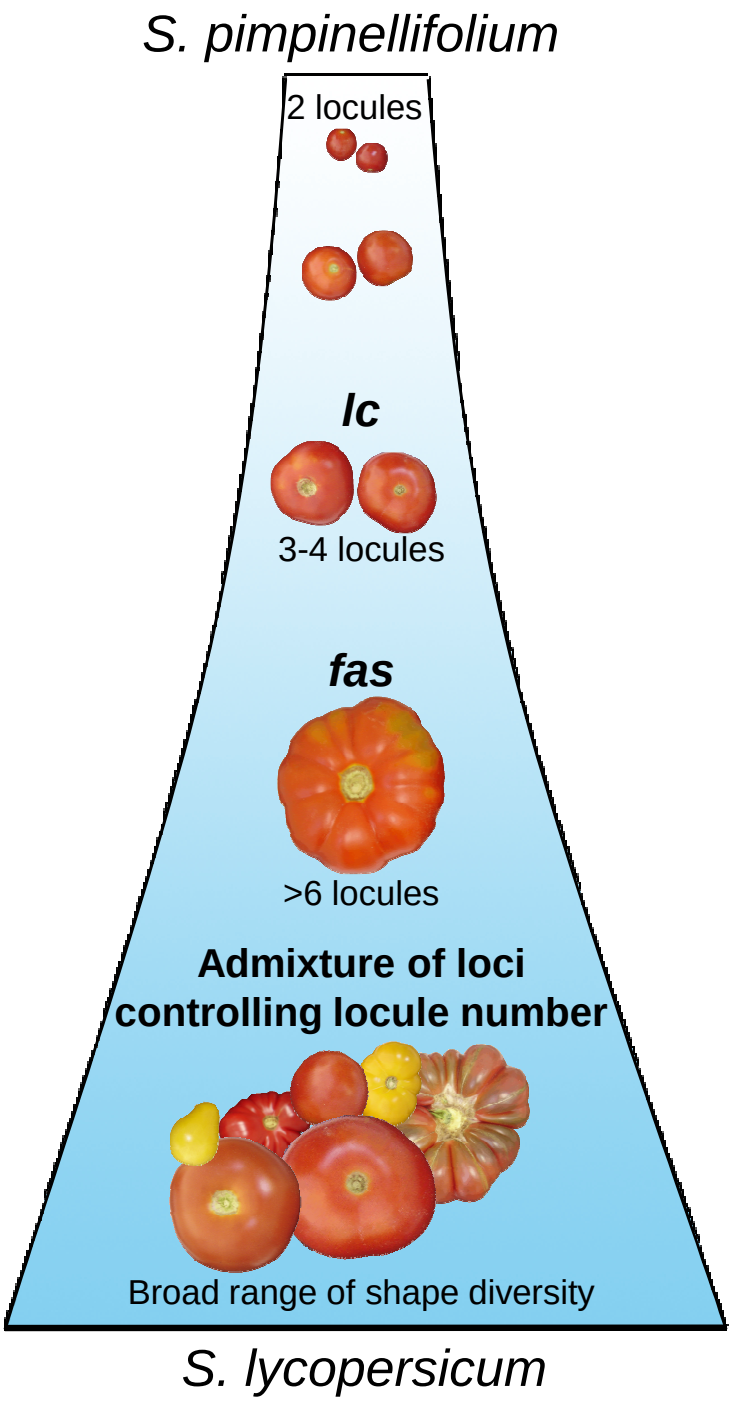

Figure 6. Model of locule number evolution in tomato fruits during domestication

Solanum pimpinellifolium is considered the wild ancestor of the cultivated tomato *Solanum lycopersicum*. Based on the analysis of 267 tomato accessions, we propose a model that could explain the history of locule number evolution during tomato domestication. In our study, only 4.6% of the high locule number accessions (i.e., those with >3 locules) had the low locule allele of *lc* and 96.9% of the fasciated accessions (i.e., those with >6 locules) had the high locule allele of *lc*, but only 49.4% of them had the *fas* allele. These results indicate that *lc* was required for the increase in locule number in tomato fruits during domestication. The *lc* locus could have appeared before the *fasciated* locus. These two QTLs are the major loci controlling locule number. Modern breeding has used other loci to expand phenotypic diversity.