Natural Variation in an ABC Transporter Gene Associated with Seed Size Evolution in Tomato Species

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Abstract

Seed size is a key determinant of evolutionary fitness in plants and is a trait that often undergoes tremendous changes during crop domestication. Seed size is most often quantitatively inherited, and it has been shown that Sw4.1 is one of the most significant quantitative trait loci (QTLs) underlying the evolution of seed size in the genus Solanum—especially in species related to the cultivated tomato. Using a combination of genetic, developmental, molecular, and transgenic techniques, we have pinpointed the cause of the Sw4.1 QTL to a gene encoding an ABC transporter gene. This gene exerts its control on seed size, not through the maternal plant, but rather via gene expression in the developing zygote. Phenotypic effects of allelic variation at Sw4.1 are manifested early in seed development at stages corresponding to the rapid deposition of starch and lipids into the endospermic cells. Through synteny, we have identified the Arabidopsis Sw4.1 ortholog. Mutagenesis has revealed that this ortholog is associated with seed length variation and fatty acid deposition in seeds, raising the possibility that the ABC transporter gene may modulate seed size variation in other species. Transcription studies show that the ABC transporter gene is expressed not only in seeds, but also in other tissues (leaves and roots) and, thus, may perform functions in parts of the plants other than developing seeds. Cloning and characterization of the Sw4.1 QTL gives new insight into how plants change seed during evolution and may open future opportunities for modulating seed size in crop plants for human purposes.

Introduction

Seeds represent the vehicle by which plants vie for evolutionary success. A key feature of seeds is their size, which in turn is one of the most variable traits in the plant kingdom. Seeds range in weight from less than 1 microgram in the Coral-root orchid (Corallorhiza maculate) to more than 10 kg in the Coco-de-mer palm (Lodoicea maldivica). This large range can be observed not only among taxa, but also within taxa. For example, contained in the genus Solanum are a set of 9 cross compatible species closely related to the tomato. Despite their close taxonomic affinities, these species show a 10-fold range in seed size suggesting a rapid rate of evolutionary change (Figure 1).

Why plants vary so much in seed size is not known. However, evolutionists and ecologists have long noted this great variation and hypothesized its importance in adaptation. In terms of survival, there are both risks and benefits for a species to increase (or decrease) seed size. Because maternal resources are limited, a species has to “decide” whether to invest energy into a few large seeds or many small seeds [1,2]. Intra- and interspecific studies of offsprings fitness in plant communities have demonstrated that plants producing a small number of large seeds often have higher tolerance to drought [3], herbivory [4], shading [5], and nutrient-deficient soils [6]. However, plants producing a large number of small seeds exhibit superior colonizing abilities with the advantage of dispersal due to the abundance of seeds and higher likelihood to escape from predation [7,8].

Scientific interest in seed size relates not only to its importance in evolution, but also to crop domestication. Crops domesticated for consumption of their seeds (e.g. soybean, wheat, sunflower) often produce seeds significantly larger than their wild ancestors [9–12]. It is likely that early humans consciously selected for larger seeds in an effort to increase yield and improve harvest efficiency. However, seed size also increased during domestication in crops not harvested for their edible seed. For example, domesticated tomatoes produce seeds up to several fold larger than their wild ancestors (Figure 1) [13]. Likewise cultivated squash (Cucurbita pepo) produce seed more than two fold larger than their wild counter parts [14]. Why seed size increased during domestication in crops not consumed for their seeds is unclear. However, it has been conjectured that seed size increased in these species due to indirect selection for greater seedling vigor and germination uniformity under field production [15].

Despite the importance of seed size in plant evolution and crop domestication, relatively little is known about the genetic and molecular processes underlying natural variation in seed size. Most of our knowledge comes from quantitative trait mapping studies which have revealed a fairly large number of QTL affecting seed size in a variety of plants – e.g. Arabidopsis [16], rice [17–22], soybean [23,24], sunflower [11,25–28]. However, most of these
Author Summary

Given fixed resources, plants have a choice whether to produce many small seeds or a few large seeds. In terms of reproductive fitness, there are costs and benefits to both strategies. As a result, plant species vary more than 100,000-fold in both seed size and seed output. The current study focuses on understanding the molecular and developmental basis of a single genetic locus (or quantitative trait locus) that determines seed size between the cultivated tomato and its wild relatives. We show that the cause of size variation can be traced to a gene encoding an ABC transporter protein. The gene apparently exerts its control on seed size through expression in the developing seeds and not the mother plant that nurtures those seeds. A comparison with the model plant Arabidopsis thaliana suggests that the ABC transporter identified in tomato may also control seed size in other plants, opening research opportunities for understanding plant adaptation and for potentially modulating seed size in crop plants for human purposes.

studies have not gone beyond the mapping stage and hence provide little insight into the developmental and molecular mechanisms underpinning seed size variation. The exception is rice where three seed size QTLs have been recently cloned. These encode a previously unknown RING-type E3 ubiquitin ligase [29], a putative transmembrane protein [30], and deletion of a gene of unknown function [17].

Tomato is one of the few species not domesticated for edible seeds, where extensive QTL mapping for seed size has been conducted. Over the past 25 years, mapping studies involving crosses between the cultivated tomato and related wild species have revealed approximately 20 QTLs which account for most seed size variation [13,31–34]. Different subsets of these QTLs were identified in different studies. However, a common feature of all studies was that a major QTL on chromosome 4 (referred to as Seed weight 4.1 or Sw4.1) invariably accounted for a large portion of the genetic variation for seed size. Sw4.1 is responsible for up to 23% of the total phenotypic variation in segregating populations and up to 54% of the seed weight variation in crosses between nearly isogenic lines [13]. The conservation of Sw4.1 across tomato species, and its potential role in the evolution and domestication of cultivated tomato, makes Sw4.1 a prime candidate for characterization and cloning. Thus the objective of this study was to uncover the genetic, developmental and molecular mechanisms underlying modulation of seed size by the Sw4.1 QTL.

Results/Discussion

Sw4.1 Controls Seed Weight through Zygotic Effects

The size or weight of a seed can potentially be affected by the genotype of three different plant parts/tissues: a) the female plant bearing the fruit which contains the developing seed and contributes the testa; b) the triploid endosperm which nourishes the developing embryo and c) the diploid embryo. A maternal effect would be caused by a substantial contribution of the maternal genotype from the testa (a) and/or endosperm (b) to the seed development, while a zygotic effect would be attributed to the equal contribution of maternal and paternal genotypes from the zygote (c).

To differentiate maternal from zygotic effects, a reciprocal cross experiment was conducted using a pair of nearly isogenic lines (NILs) (see Material and Methods). In Cross 1, an inbred line homozygous for a “large-seed” (L/L) allele from S. lycopersicum was used as the female in a cross with a nearly isogenic line (NIL) homozygous for the “small-seed” (S/S) allele from S. pimpinellifolium. In Cross 2, the reciprocal cross was performed using the S/S as the female parent. For Cross 1, the F1 seed from Cross 1 should be significantly larger than F1 seed from Cross 2. The results from these experiments revealed that reciprocal crosses result in seed indistinguishable in weight: 3.07 mg (Cross 1) versus 3.10 mg (Cross 2) (P = 0.99). Whereas self-pollination of the same parents, L/L and S/S, resulted in seeds weighting 3.34 mg and 2.63 mg respectively (P<0.005).

The equivalency in seed weight for the reciprocal F1s suggests that Sw4.1 does not influence seed weight through any significant effect exerted by the genotype of the maternal environment (i.e., fruit). It should be noted that reciprocal crosses would result in triploid endosperm with different parental allelic dosage (L/L/S versus L/S/S). Such differences in allelic dosage might also cause differences in seed weight between seed produced from reciprocal crosses. The fact that no such differences were observed suggests that the genotype of the developing embryo is most likely the major point of control in the differential seed weight associated with Sw4.1 alleles.

Additive Interaction of the L and S Alleles

By comparing the seed weight values of the self-pollinated L/L and S/S NILs, it was estimated that the additive gene effect of Sw4.1 is approximately 0.36 mg ([L/L-S/S]/2). As a result, it is estimated that the genetic effect of a single “large-seed” (L) allele is to increase seed weight by 14% – a value very similar to what was reported previously [33]. Using these same parental values, as well as data from the F1 seed lots, it was also possible to calculate the degree of dominance (d/a or k) for interaction of the L and S alleles. It is thus estimated that

Figure 1. Phylogenetic relationships of species in the genus Solanum most closely related to the cultivated tomato. Numbers at nodes indicates bootstrap values. Modified from Spooner et al. [55] and Peralta et al. [56].

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the alleles interact in a largely additive manner (d/a = −0.21). This result suggests that the change in seed size affected by the two alleles is not due to a loss-of-function (e.g. deletion) at the locus, in which case a dominant-recessive gene action would be observed. This result is consistent with results from the gene expression experiments to be presented later.

Timing of Sw4.1 Effects during Seed Development

In an effort to identify the time during development at which Sw4.1 alleles modulate seed size, a comparative developmental study was conducted on the large-seeded (L/L) NIL and small-seeded (S/S) NIL. Fruit size was also measured to determine whether Sw4.1 may also affect this character. Developmental plots for the two NILs are shown in Figure 2. No differences were observed for the NILs with respect to fruit size at any time during development. It is therefore concluded that Sw4.1 alleles specifically modulate changes in seed size, and that these changes are not the indirect effect of modulations in fruit size. These results are consistent with the earlier showing that the Sw4.1 effect on seed size is exerted largely through the genotype of the zygote and not the maternal plant.

Beginning at 10 days after pollination (DAP), seed size (as measured by width and length) was consistently greater for the L/L NIL than for the S/S NIL. For example, seeds from the L/L NIL were on average 23% longer than those of the S/S NIL at 15 DAP ($P=0.008$, Figure 2). At maturity, seeds of the L/L NIL were 17% longer than seeds of the S/S NIL ($P=0.001$). Overall, L/L NIL showed the greatest change in seed size, relative to the S/S NIL, in the period from 10 DAP to 15 DAP, suggesting that Sw4.1 exerts its largest differentiating effect during this early period of seed development. In an effort to determine the stage of embryo development corresponding to this critical period, paraffin cross sections were prepared from seeds at 10, 15 and 20 DAP. Microscopic examinations of the sections revealed that the embryos are globular at 10 DAP and torpedo-shape to curving at 15 DAP. These stages are similar to those previously reported by Lersten [35] and correspond to the initiation of rapid deposition of starch and lipids into the endospermic cells.

Sw4.1 Exerts Equal Effects on the Size of Both the Embryo and Endosperm but Does Not Affect Seed Viability or Germination Rate

An examination of sections of mature seed from the L/L and S/S NILs revealed that the L/L NIL produces seed that are increased with respect to the size of both the embryo ($P=0.014$) and endosperm ($P=0.002$) relative to those of the S/S NIL. However, no difference was observed between the two NILs with respect to the ratio of endosperm to embryo ($P=0.956$). In both NILs the embryo accounts for the largest portion of the total seed with an embryo:endosperm ratio of 4:1. These results suggest that Sw4.1 modulates changes in the amount of both embryo and endosperm tissue during seed development.

Germination tests of the L/L and S/S NILs revealed that both give rise to highly viable seed (germination rates of 97.6±3.1 versus 96.3±2.1, respectively). Therefore, while Sw4.1 affects seed size, it appears to have no detectable affect on seed viability as measured by germination percentage. Another way to characterize

Figure 2. Plots for developmental changes in fruit length, seed width and seed length for the L/L and S/S NILs. Bars indicate the standard errors.

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Testing the Effects of the ABC Transporter Gene on Seed Size via RNAI Transformation Experiments

Transformation experiments were used to test whether the ABC transporter gene has the ability to modulate seed size. This gene is
Figure 3. High resolution mapping of Sw4.1 QTL on tomato chromosome 4. (A) TA2080 S/S NIL showing the introgression region from S. pimpinellifolium containing Sw4.1 QTL (shaded). (B) Sw4.1 mapping of F2-population of 150 individuals (TA209 – L/L NIL×TA2080 – S/S NIL) within a 48 cM region. (C) Sw4.1 mapping within a 11 cM region from 140 individuals from a F2 heterozygous individual (98T342-95). (D) Sw4.1 mapping within a 2.4 cM region from recombinants selected from 1,000 seeds from F2 heterozygous individuals. The S1 marker was then used to isolate and sequence the 130 kb BAC clone LE_HBa0077005. (E) Sw4.1 mapping within the ~130 kb BAC LE_HBa0077005 from recombinants selected from 9,000 seeds from F2 heterozygous individuals. The numbers between markers represent the number of crossover events in each interval.

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Table 1. Progeny analysis of 10 heterozygous individuals with recombination within the BAC.

| F3 recombinant | pedigree | Recombinant/non-recombinant | A B C D E F G H I J K L M N O P Q R S T U V W X n | SWE (mg) | One tail t-test | P value |
|----------------|----------|-----------------------------|---------------------------------|--------|----------------|--------|
| 05T321-31     | 07T542   | R                           | 3 3 3 3 3 3 3 3 3 3 3 3 3 3 3 3 3 3 3 3 3 3 3 | 2.338 | NR>R           | 0.105 |
| NR            |          | 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 | 2.552 |                   |        |
| 05T324-484    | 07T543   | R                           | 3 3 3 3 3 3 3 3 3 3 3 3 3 3 3 3 3 3 3 3 3 3 3 | 2.419 | R>NR           | 0.039 |
| NR            |          | 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 | 1.992 |                   |        |
| 05T620-215    | 07T544   | R                           | 3 3 3 3 3 3 3 3 3 3 3 3 3 3 3 3 3 3 3 3 3 3 3 | 3.121 | R>NR           | 0.011 |
| NR            |          | 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 | 2.5   |                   |        |
| 06T661-255    | 07T540   | R                           | 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 | 3.075 |                   |        |
| NR            |          | 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 | 3.264 |                   |        |
| 05T439-150    | 07T541   | R                           | 3 3 3 3 3 3 3 3 3 3 3 3 3 3 3 3 3 3 3 3 3 3 3 | 2.664 | NR>R           | 0.21  |
| NR            |          | 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 | 2.795 |                   |        |
| 06T642-57     | 07T539   | R                           | 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 | 2.725 | NR>R           | 0.01  |
| NR            |          | 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 | 3.072 |                   |        |
| 05T410-349    | 07T545   | R                           | 3 3 3 3 3 3 3 3 3 3 3 3 3 3 3 3 3 3 3 3 3 3 3 | 2.919 | NR>R           | 0.42  |
| NR            |          | 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 | 2.956 |                   |        |
| 04T952-307    | 07T546   | R                           | 3 3 3 3 3 3 3 3 3 3 3 3 3 3 3 3 3 3 3 3 3 3 3 | 2.776 | R>NR           | 0.001 |
| NR            |          | 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 | 1.891 |                   |        |
| 05T322-13     | 07T547   | R                           | 3 3 3 3 3 3 3 3 3 3 3 3 3 3 3 3 3 3 3 3 3 3 3 | 2.942 | R>NR           | 0.003 |
| NR            |          | 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 | 2.497 |                   |        |
| 05T427-200    | 07T548   | R                           | 3 3 3 3 3 3 3 3 3 3 3 3 3 3 3 3 3 3 3 3 3 3 3 | 2.84  | R>NR           | 0.001 |
| NR            |          | 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 | 2.095 |                   |        |

**1** indicates homozygous for corresponding marker from L/L NIL. **3** indicates homozygous for corresponding marker from S/S NIL. SWE = seed weight. In italic and bold is the region indicated by the one-tail t-test of either recombinant versus non-recombinant (R>NR) or non-recombinant versus recombinant (NR>R) for the positioning of the Sw4.1 QTL. Shown at bottom (X) is the consensus region for the location of Sw4.1 based on progeny tests from all 10 recombinants. Marker A = ST4; B = G1; C = dS4220; D = SSR152; E = S1; F = 3UTR; G = UNK1; H = S27; I = S38; J = S41; K = SSR252; L = S27; M = ABC21375; N = dABC20665; O = S51; P = G13-2; Q = G14-1; R = G16; S = S106indel1; T = S106indel2; U = G18; V = S54; W = S57; X = T725.

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The large size of the gene, absence of efficient enzymatic sites for cloning and lack of a full-length cDNA, precluded complementation analysis with the full-length genomic copy of the gene. Therefore, a gene silencing approach, via RNAi hairpin formation, was employed as an alternative strategy for testing the potential role of the ABC transporter gene in determining seed size. Efficient post-transcriptional silencing has been reported when 3′ UTR regions are targeted for RNAi machinery [48,49]. Hence, a 278 bp fragment from the 3′ UTR of the ABC transporter gene was inserted in the pHELLSGATE2 (Invitrogen) binary vector, the pSP13-1 construct (Figure S1). Sequence specificity was assessed by blasting the 278 bp fragment against the tomato unigene database in the SGN website (www.sgn.cornell.edu). The retrieval of a unique unigene that corresponds to this ABC transporter gene suggests this sequence is specific to this gene and therefore there would present a low risk to silence other genes in the same ABC transporter family. This construct was transformed into both the L/L and S/S NILs. Multiple independent T₀ and non-transgenic controls were then analyzed for both NIL sets. L/L transformants were highly fertile, yet produced seeds weighing on average 38% less than those from the non-transgenic controls (P = 0.03, Table 2). S/S transformants also produced smaller seed (11% less heavy) than the non-transgenic controls, however the statistical difference did not quite reach statistical significance (P = 0.09, Table 2). It is worth noting that transformation/complementation with two other genes from the BAC (annexin and a gene of unknown function – genes number 6 and 7, respectively, in Figure 4) did not show statistical difference between transgenic and non-transgenic plants (data not shown). The transformation experiments thus appear to corroborate the results from high-resolution mapping – both pointing to the ABC transporter gene as the cause of the Sw4.1 QTL.

**Allelic Polymorphisms in the ABC Transporter Gene**

Comparing the sequence of the L and S allele, within the 23 kb Sw4.1 interval, revealed 79 SNPs and single indels located in introns, 10 in the promoter region and 6 in exons (Figure S2). In the exons, 2 non-synonymous changes were observed (Figure 6). Either of these might be causal to the phenotypic effects rendered by the L and S alleles. However, neither substitution is located in a conserved functional domain (e.g. nucleotide binding folds or hydrophobic integral membrane domains) (Figure 6). Among the
polymorphisms in non-coding regions, a few are worth mentioning as possible causal candidates for the Sw4.1 QTL. One is a 12 nt indel approximately 1.5 kb upstream in the 5’ promoter and the second, a 24 nt indel in the first intron (Figure 6). Either of these indels might cause a change in expression – as could the many other small nucleotide differences observed in non-coding regions of the two alleles.

Expression of ABC Transporter Gene during Seed Development

In an effort to determine the expression pattern of the ABC transporter gene, and especially whether the L and S allele differ in regulation/expression during seed development, a set of semi-

Figure 5. Relationship of genes in tomato BAC containing Sw4.1 and corresponding syntenic regions in Arabidopsis genome. doi:10.1371/journal.pgen.1000347.g005

Figure 6. Annotation of tomato ABC transporter gene associated with Sw4.1 QTL. Exons are shown in red. Conserved functional domains are shown in blue, ATP-binding cassette (ABC) or nucleotide binding folds (NBFs), and green, hydrophobic integral membrane domains (TMDs). doi:10.1371/journal.pgen.1000347.g006

Table 2. Summary of transgenic silencing of ABC transporter gene in L/L and S/S NILs based on RNA interference.

| NIL Plant | Transgenic Status | N  | Seed Weight (mean ± SE – mg) | P value |
|-----------|-------------------|----|----------------------------|---------|
| L/L       | Transgenic        | 10 | 2.016 ± 0.345               | 0.031   |
|           | Non-transgenic    | 3  | 3.235 ± 0.522               |         |
| S/S       | Transgenic        | 10 | 2.262 ± 0.495               | 0.093   |
|           | Non-transgenic    | 2  | 2.529 ± 0.132               |         |

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quantitative RT-PCR experiments were conducted. The first experiment revealed that this gene is expressed at high levels in seeds, and lower levels in shoots and roots (Figure 7A). High expression of the ABC transporter gene in developing seeds is further evidence that this gene is the cause of the Sw4.1 QTL. The subsequent experiment compared expression of the ABC transporter gene in both the L and S NILs at 10, 15 and 20 DAP – the time when the major change in seed size is observed (see previous section) (Figure 7B). Two conclusions can be drawn from this second experiment. First, the ABC gene is expressed in both the L and S NILs – ruling out a loss-of-function as the cause of seed size variation associated with Sw4.1. Second, the failure to detect any major change in expression of the ABC transporter gene between the L and S NILs during seed development would seem to rule out a gross change in the regulation as the cause of the Sw4.1 QTL (Figure 7B). However, we cannot exclude the possibility of a difference in translational/post-translational regulation or small changes in spatial or temporal regulation as the cause of QTL effect. For example, in tomato it was previously shown that a modest change in the timing of allele expression can cause major QTL effects on fruit size [50].

Conclusions

The results presented herein point to natural variation in an ABC transporter gene as a major cause of the change in seed size that differentiates the cultivated tomato from related wild species. While prior studies in a number of plants have reported the effects of induced mutations on seed size, this example is among the few in which the cause of changes in seed size in nature populations has been pinpointed. Further, the ortholog to this gene in Arabidopsis has been identified through homology/synteny. Results from QTL mapping and mutagenesis studies are consistent with this gene also playing a role in determining seed size in Arabidopsis. However, further studies are required to clearly establish whether this ABC transporter gene operates in a similar manner in both tomato and Arabidopsis. Also, it remains to be established whether variation in ABC transporter genes is a major cause of seed size variation in natural populations of Arabidopsis or other plant species. Having identified the cause of the Sw4.1 QTL in tomato may open the door to addressing these questions in the future.

Materials and Methods

Plant Material and Genetic Markers

Two Solanum lycopersicum nearly isogenic lines (NILs), with alternative alleles at the Sw4.1 locus, were the origin of all genetic stocks used in this study. TA209 carries the large-seeded (S. lycopersicum) allele at Sw4.1 locus. TA2080 is isogenic with TA209, but carries a 55–84 CM segment of chromosome 4 of S. pimpinellifolium LA1589 (Figure 3A) containing the small-seeded allele of Sw4.1 [13,33]. TA2080 was developed via marker assisted selection during 5 sequential backcrosses of LA1589 into TA209 followed by a single selfing generation (BC3S1). An F2 population, segregating for Sw4.1, was then developed from a cross between TA209 and TA2080. A derived, shorter introgression NIL, derived from TA2080, was used for the reciprocal cross
experiments. Likewise, the S/S NIL (TA3820) used for transformation experiments was also a shorter derivative of TA2000. Detailed description of the construction of both derived subNILs can be found in Ors [46]. The sequence of all markers used in this study can be found in Table S2.

Reciprocal Cross and Gene Action Experiments

Plants were grown in pots in the greenhouse in 12 randomized blocks. Each block was comprised of the following six A1 genotypes and type of pollination: L/L: selfing; S/S: selfing; L/S: selling; L/L: used as the female in crosses to S/S; S/L: used the female in crosses to L/L. Each plant was either selfed or crossed manually. At maturity, 5 normal fruit (no blossom end rot, non-parthenocarpic) were harvested from each plant and the seed extracted. The fruit were weighed and the average weight per fruit was recorded. From a pool of seeds from 5 fruits, 50 healthy seeds were randomly sampled. From these, an average seed weight was calculated for each plant. A comparison of the least squares means was performed using the adjustment for multiple comparisons Tukey-Kramer (SAS enterprise guide 3.0).

Developmental Analyses

Effects of Six4.1 on seed development and fruit size: Ten pairs of L/L and S/S NIL plants were grown in the greenhouse. Two to three fruits were harvested from each plant at each of the following stages: anthesis (0 days after pollination – DAP), 5 DAP, 10 DAP, 15 DAP, 20 DAP, 25 DAP, 30 DAP, 35 DAP and 40 DAP. Because of the microscopic size of ovule and developing seeds, it was not possible to collect mass (weight) data. Instead, all traits were recorded as spatial metrics (e.g., length, width). Both fruit and seed were scanned using a HP ScanJet (1200 dpi). Length and width measurements were then extracted from the images using the software Tomato Analyzer Version v.1.2 [51]. A second independent experiment, of identical design, was then conducted. However, based on results from the first experiment, fruit were collected only at 10 DAP, 15 DAP and 20 DAP – periods associated with most rapid changes in seed development. For the verification of seed developmental stages, seeds from 10, 15 and 20 DAP were fixed, dehydrated and embedded in paraplast (Sigma) as described by [52].

Role of Six4.1 in determining the proportion of embryo to endosperm in mature seed: Five pairs of L/L and S/S individuals were grown in the greenhouse. Five mature fruits were harvested from each individual and the seeds extracted. A random sample of 10 normal seeds was then drawn from each individual. Each individual seed was then dissected longitudinally into halves and the images digitalized under a dissecting scope (ZeissStemi 2000-CS attached to 3CCD camera MTI) using the software Scion Image (www.scioncorp.com). The areas of the entire seed, embryo and endosperm were manually delineated and measured using the software ImageJ 1.31 v (http://rsb.info.nih.gov/ij/).

Seed Germination and Viability Experiments

Ten pairs of L/L and S/S individuals were grown in the greenhouse. One hundred normal seeds from a pool of 10 fruits of each genotype were germinated in Petri dishes on filter paper saturated with distilled water. The seeds were scanned prior to the germination process for seed length measurement (Tomato Analyzer v.1.2). The number of germinated seed was recorded on a daily basis until no additional seed germination was obtained. For these experiments, a seed was considered germinated once the root tip had emerged. Tests of heterogeneity on the number of germinated seeds at days 3, 4 and 5 (when the majority of seed germination was observed for all the genotypes) were performed for the detection of possible differences in germination rate between NILs (Minitab 15).

Progeny Testing of Selected Recombinants

The genetic stocks used for the progeny analysis were derived from sub-NILs selected from each selected recombinant within the BAC (Figure 4). Ten homozygous recombinant and ten homozygous non-recombinant individuals were selected, via marker analysis, from selfed seed of selected recombinants (Table 1, Figure 4). The selected progeny were grown in a completely randomized design in the greenhouse. From each plant, seeds were extracted from 5 fruits and pooled. From each pool, 50 normal seeds were randomly selected and weighed. For each family, statistical comparisons for seed weight were made between the 10 recombinant and 10 non-recombinant progeny from each family using a one-tailed t-test (Minitab 15) (Table 1).

BAC Annotation and Comparison with the Arabidopsis Genome

BAC LE_HBa0077O05, isolated with marker probe S1, was annotated using the automated annotation tools developed by SGN and refined manually through BLAST searches against EST libraries (Solanaceae, coffee, Arabidopsis). Arabidopsis genes, putatively orthologous to genes in the tomato BACs, were identified using the Best Reciprocal Matches (RBM) in BLAST comparisons [45]. Based on these putative orthologs, it was possible to identify region in the Arabidopsis genome showing conserved microsynteny with the Six4.1 region of tomato chromosome 4.

Sequencing of Six4.1 Interval from S. pimpinellifolium and Annotation of Polymorphisms between S. lycopersicum and S. pimpinellifolium

A 38 kb segment, delimited by markers dABC20665 and St106indel1 and known from progeny analyses to encompass the Six4.1 QTL, was also sequenced via PCR from the genome of S. pimpinellifolium LA1589 – the small-seeded parent of the original mapping population. The objective of the sequencing was to identify polymorphisms that might be causal to the Six4.1 QTL. Sequence analysis was performed with DNASTAR Lasergene software and alignments with BioEdit version 7.0.9.0 using ClustalW.

Sequencing Tecombinants in the 38 kb Six4.1 Interval to Pinpoint the Exact Crossover Points

In order to further narrow the Six4.1 interval, PCR-based sequencing was performed on the two recombinant individuals that define the left (05T427-200) and right (06T661-255) side of the 38 kb interval. By sequencing across the exact crossover point in each recombinant, it was possible to narrow the location of the Six4.1 QTL to a 23 kb interval (Figure 4).

Transformation Experiments

RNAi transgene construction. A 278 bp fragment from the 3’UTR of the ABC transporter gene orthologous to Atg39850 was inserted in the binary vector pHeDsGate 2 (Invitrogen) [53] for the generation of a hairpin construct for the RNAi mechanism induction and gene silencing in L/L and S/S NILs. The schematic representation of plasmid construction is shown in Figure S1. The pHELLSGATE vector was designed such that a single PCR product from primers with the appropriate attB1 and attB2 sites would be recombined into it simultaneously to form the two arms of the hairpin [53]. The recovery of successful recombination (insertion) of both arms of the hairpin was ensured by ccdB genes, which were replaced by the arm sequences (ABC transporter fragment). CcdB gene is lethal in standard E. coli strains such as DH5a, strain that was used for plasmid cloning. The intron retention was ensured by the chloramphenicol-resistance gene.
ABC Transporter Gene Modulates Tomato Seed Size

(DC) within the intron. The successful insertion for both arms of the hairpin was confirmed by sequencing analysis (data not shown). Recombinant pHELLSGATE constructs, called pSP13-1, were sent for transformation (Plant Science Initiative, University of Nebraska) for the direct transformation into Agrobacterium for transformation into the L/L and S/S NILs.

Experimental design and statistics. Transgenic analysis of the T0 generation: 10 transgenic L/L NILs, 3 non-transgenic L/L NILs, 10 transgenic S/S NILs and 2 non-transgenic S/S NILs were grown in greenhouse in a random design. Classification of plants as transgenic or non-transgenic was based on the amplification of the insert-vector using the primers Act1-ABC and Xh0l insert-vector for the sense amplification (403 bp) and Act1B-ABC and XbaI insert-vector for the anti-sense amplification (400 bp). RNA levels for the ABC transporter gene were not measured in seeds from T0 plants since this would have precluded seed size measurements from the same plants. For efficient RNAi mechanism induction, both arms of the hairpin must be present as well as the intronic sequence. Therefore, amplification of both XbaI and Xh0l were required for the assignment of transgenic plants. As a positive control for PCR amplification, S85 primers were included in each reaction. S85 amplifies 800 bp from the ABC transporter promoter region, which would be present in transgenic and non-transgenic plants. Five fruits of each plant were harvested and the seeds were extracted. One pool of seeds was randomly selected from each plant and weighed. One tail t-tests, comparing transgenic and non-transgenic plants were then performed using Minitab 15.

Analysis of ABC Transport Gene Transcript

Experimental design. Ten L/L NIL and ten S/S NIL individuals were paired and grown in greenhouse. Each flower was manually pollinated and the date of pollination noted. Seeds from fruits 10, 15 and 20 DAP were removed and immediately frozen in liquid nitrogen. Seeds at these stages were pooled from 5 healthy plants for the RNA extraction. Frozen seeds were ground to a fine powder in liquid nitrogen and total RNA was isolated using Trizol (Invitrogen). First-strand cDNA was synthesized by reverse transcription with oligo(dT)16 primer following manufacturer's protocol (Invitrogen). Another set of plants was grown and the date of pollination noted. Seeds from individuals were paired and grown in greenhouse. Each flower was manually pollinated and the date of pollination noted. Seeds from these stages were pooled from 5 healthy plants since this would have precluded seed size measurements from the same plants.

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Semi-quantitative RT-PCR. ABC transporter transcript levels were detected by using semi-quantitative RT-PCR. The primers used for the amplification are S53R (5′ GGGAAGA- CGAACCAAATGAA 3′) and ABCp35R (5′ CGGGAACTAG-GGCCGATATCA 3′). These primers were designed based on the 3′ end and 5′ UTR of the gene. BLAST search against A. thaliana non-redundant database in NCBI and tomato EST database in SGN (www.sgn.cornell.edu) confirmed the uniqueness of this sequence and specificity to this ABC transporter gene. The internal control was actin TOM52 [54]. For the semi-quantitative approach, from a total reaction of 100 ul, 10 ul were collected at the end of each one of these cycles: 25, 29, 30, 32, 34 and 35.

Supporting Information

Figure S1 Diagram of pSP13-1 construct used for RNAi based gene silencing of ABC transporter gene in transgenic experiments. Found at: doi:10.1371/journal.pgen.1000347.s001 (0.75 MB TIF)

Figure S2 Detailed alignment of L and S alleles showing all polymorphisms in the 23 kb region encompassing the Sc4.1 QTL. Found at: doi:10.1371/journal.pgen.1000347.s002 (7.55 MB TIF)

Table S1 Annotation of the 12 genes contained in the tomato BAC L. HBa0077005. Found at: doi:10.1371/journal.pgen.1000347.s003 (0.04 MB DOC)

Table S2 Markers for S. lycopersicum and S. pennellifolium; marker type, primer sequence, restriction enzyme and fragment size. Found at: doi:10.1371/journal.pgen.1000347.s004 (0.15 MB DOC)

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Author Contributions

Conceived and designed the experiments: CHO SDT. Performed the experiments: CHO SDT. Analyzed the data: CHO SDT. Contributed reagents/materials/analysis tools: CHO SDT. Wrote the paper: CHO SDT.
