Theor Appl Genet (2012) 125:273–284
DOI 10.1007/s00122-012-1832-8

ORIGINAL PAPER

Fine mapping of *fw3.2* controlling fruit weight in tomato

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Received: 23 September 2011 / Accepted: 14 February 2012 / Published online: 10 March 2012

Abstract Tomato (*Solanum lycopersicum*) is an important crop in the Solanaceae family. One of the key traits selected during domestication is fruit mass which is controlled by many quantitative trait loci. The fruit weight locus *fw3.2* is one of the major loci responsible for fruit mass in tomato. Identification of the underlying gene will improve our understanding of the molecular mechanism of fruit development while also providing insights into genes that were selected during domestication. We fine mapped *fw3.2* to a 51.4-kb interval corresponding to a region comprising seven candidate genes. Gene action showed that the allele from cultivated tomato was additive to dominant in giving rise to an enlarged fruit. Fruit shape analysis indicated that *fw3.2* primarily played a role in controlling fruit weight, with a minor effect on fruit shape. Gene expression and nucleotide diversity were investigated and the likelihood of the genes control fruit mass is discussed.

Communicated by I. Paran.

Electronic supplementary material The online version of this article (doi:10.1007/s00122-012-1832-8) contains supplementary material, which is available to authorized users.

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Introduction

Fruit mass, measured by its weight, is an important agricultural trait and major feature of domestication. To identify loci controlling fruit mass, a number of quantitative trait loci (QTL) analyses have been conducted. The identification of loci controlling fruit weight gives us insights into domestication and fruit development, as well as providing useful markers for marker-assisted selection in breeding programs. In tomato (*Solanum lycopersicum*), up to 28 loci accounting for fruit mass have been identified (Grandillo et al. 1999; Paran and van der Knaap 2007). Among these, each of the six loci: *fw1.1, fw2.2, fw2.3, fw3.1, fw3.2, fw4.1,* and *fw9.1*, exerts a major effect by accounting for more than 20% phenotypic variance in at least one independent study. In addition, *fw1.1, fw2.1, fw2.2, fw3.1, fw3.2,* and *fw11.3* have been identified in at least four independent studies (Grandillo et al. 1999). Many of the fruit weight QTL detected in the tomato and other Solanaceous crops appear to colocalize, suggesting that the same genes were selected in the independently domesticated crops (Rao et al. 2003; Paran and van der Knaap 2007).

The fine mapping and cloning of the underlying fruit weight QTL has advanced the most in tomato. Positional cloning of tomato *fw2.2* resulted in the identification of the underlying gene, *FW2.2* (Frary et al. 2000). The mutation in the promoter has been proposed to result in fruit enlargement via heterochronic changes in gene expression (Frary et al. 2000; Cong et al. 2002; Liu et al. 2003). *FW2.2*-related genes are found to control organ size and number in other plants. *Cell Number Regulator 1 (CNR1)*, an ortholog of *FW2.2* in maize, controls plant and organ mass by regulating cell number (Guo et al. 2010). Transcript levels of *Pafw2.2*, a *FW2.2*-like gene in avocado, are

negatively correlated with organ cell number (Dahan et al. 2010). In soybean, an *FW2.2*-like gene named *GmFWL1* is involved in nodule initiation and nuclear size (Libault et al. 2010). Based on structural modeling and location of the protein, *FW2.2*-like genes are hypothesized to be involved in the cell cycle pathway by regulating metal transportation (such as cadmium) across the plasma membrane (Cong and Tanksley 2006; Guo et al. 2010; Libault et al. 2010). Two other genes that control tomato fruit mass in addition to fruit shape are *FASCIATED (FAS)* and *LOCULE NUMBER (LC)* (Cong et al. 2008; Munos et al. 2011). *LC* is most likely encoded by the tomato ortholog of *WUSCHEL*, a gene encoding a homeodomain protein regulating stem cell fate in plants (Munos et al. 2011). *FAS* encodes a YABBY-like transcription factor controlling organ polarity, and the mutation that increases locule number and fruit weight is caused by a 249-kb inversion knocking out the gene (Cong et al. 2008; Huang and van der Knaap 2011). Very close to *FAS*, another fruit weight locus has been identified. The fine mapping of this locus, *fw11.3*, has narrowed down the region to 22 candidate genes (Huang and van der Knaap 2011).

fw3.2 is a major tomato fruit weight QTL explaining 19% of the fruit mass variance in an F_2 population derived from a cross between the heirloom tomato cultivar Yellow Stuffer (YS) and the wild species LA1589 (Fig. 1) (Van der Knaap and Tanksley 2003). This locus also controls fruit shape in other tomato varieties (Van der Knaap et al. 2002; Brewer et al. 2007; Gonzalo and van der Knaap 2008), suggesting that the underlying gene has pleiotropic effects on fruit development. The objective of this study is to confirm the *fw3.2* locus by progeny testing of recombinant plants of partially backcrossed lines and to delineate the locus to a smaller region for candidate gene identification.

Materials and methods

Plant materials

One F_2 plant (99T190-94) derived from a cross between *S. lycopersicum* cultivar, YS, and a wild species *S. pimpinellifolium* accession LA1589 (Fig. 1) was backcrossed to YS three times by marker-assisted selection. Four fruit weight loci, *fw1.1*, *fw2.2*, *fw5.2* and *fw7.2* (Van der Knaap and Tanksley 2003), were selected to be homozygous for the YS allele, while the *fw3.2* locus was maintained in the heterozygous state during the backcrosses. Recombinant plants were selected from the selfed advanced backcrossed lines that showed robust segregation for the fruit mass the previous year. To avoid outcrossing, recombinant plant seed increases were performed in the greenhouse at the Ohio Agricultural Research and Development Center (OARDC, Wooster, OH, USA). Fruit weight evaluations of all selected progeny materials were performed in the field at the OARDC in the summers from 2005 to 2010.

Map construction and marker development

Based on the genetic and physical maps available at the Sol Genomic Network (SGN, http://solgenomics.net/), 10 PCR-based markers (SSR111, TG222, T1659, TG246, SSR22, T1130, TG134B3, TG134B5, TG242, and TG284) were developed and mapped to the *fw3.2* locus using MAPMAKER v 2.0 (Lander et al. 1987) (Supplemental Table 1). For fine mapping of the locus, 12 additional markers developed mostly from BAC end sequences were developed (H203B2, H36B1, P26, H291G05B2, H51B2, H51G24B2, H51B2, M44B2, H51G24B2, H233B1, H51G24B1, and H51B1). The remaining markers (P2A,

Fig. 1 Fruit mass comparison between yellow stuffer (*left*) and LA1589 (*right*). *Bar* 2 cm

P3, COBRA2, P5, P7 and P9) were developed from the sequence of the BAC HBa0051C24 encompassing the *fw3.2* locus (Supplemental Table 1).

All primers were designed with the Primer 3 program (http://frodo.wi.mit.edu/primer3/). PCR was conducted to amplify fragments from YS and LA1589 genomic DNA in 60 µl for purification and sequencing. The PCR mixture contains 50 mM KCl, 10 mM Tris–Cl (pH8.3), 0.1 mM dNTP, 0.1 µM forward primer, 0.1 µM reverse primer and 2–3 mM $MgCl_2$. PCR was carried out for 30 cycles (denaturing at 94°C for 30 s, primer-specific annealing at 52–58°C for 30 s, and extending at 72°C for 30 s, plus denaturing at 94°C for 1 additional minute at the beginning and extending at 72°C for 2 additional minutes at the end). PCR products were loaded onto agarose gels stained with ethidium bromide for DNA visualization. PCR product purification was conducted with QIAquick PCR purification kit (QIAGEN Inc, USA). Sequencing of PCR products were conducted with the ABI Prism 3100 × l genetic analyzer (Applied Biosystems, USA) using the 3′-BigDye dideoxynucleotide triphosphates labeling chemistry at the Molecular and Cellular Imaging Center (MCIC) of OARDC, Wooster, OH.

After sequencing of PCR products, polymorphisms between two parents (YS and LA1589) were found by aligning the sequences manually with the software Sequencher® 4.10.1 (Gene Codes Corporation, USA). SNPs were developed into CAPS markers or sequence-based SNP markers. For CAPS markers, 9 µl of buffer-restriction enzyme mixtures (3 µl of 10× reaction buffer, 0.3 µl of restriction enzyme and 6 µl of ddH2O) was added to 20 µl PCR product for digestion. For InDel markers and fragment size differences of more than 15 bp, DNA was visualized by running PCR product on agarose gels stained with ethidium bromide. For fragment size differences of less than 15 bp, samples were run on the Beckman CEQ8800 (Beckman Coulter, USA). Prior to amplification, an M13 tail (5′-CACGACGTTGTAAAACGAC-3′) was added to forward primers and a fluorescently labeled M13 tail primer was added to the PCR mixture. The data generated by the Beckman CEQ8800 instrument was imported into The CEQ8800 Series Fragment Analysis Software for genotyping analysis.

Recombinant plant selection

YS carries fruit that is hollow on the inside and displays inconsistent seed set. Therefore for fine mapping, the aim was to continue with plants carrying solid fruit, those that had good fruit and seed set, and those that clearly segregated for fruit mass at *fw3.2*. After three backcrosses at which stage the other major fruit size QTL were fixed, recombinant seedlings were selected based on marker scores from parents that were heterozygous at the *fw3.2* locus and those that segregated clearly in progeny testing (Supplemental Figure 1). In 2004, four BC_3 advanced backcross lines (04S64-4, 04S-19, 04S-51 and 04S-64) were selfed to generate BC_3S_1 lines (04S335, 04S336, 04S337 and 04S338). In 2005, two plants (04S337-7 and 04S338-5) were selfed to generate progeny seedlings (05S42 and 05S43) for field tests. Ninety-eight plants (46 from the 04S337-7 and 52 from the 04S338-5) containing one or two recombination events at the *fw3.2* locus were selected and transplanted to the field for fruit weight analysis. In the following years, unique recombinant plants were grown in the greenhouse to collect selfed seeds. The progeny testing of the recombinant plants was conducted in the field which allowed us to narrow down the region more precisely. In 2006, recombinant plants were selected from family 05S322 and 05S323 (derived from selfed 04S338-5). From 411 seedlings, 208 recombinant plants were identified based on the SSR111, T1659 and TG242 markers (176 single and 32 double recombinants). Fifteen of these recombinant plants were progeny tested in the field and evaluated for segregation of fruit weight. An additional two recombinant plants (from 05S43) were progeny tested as well. In 2007, 48 recombinants out of 682 seedlings from 06S522-8 (30 out of 317) and its parent 05S322-201 (18 out of 365) were identified. Forty-two recombinant plants produced sufficient seed in the greenhouse, and 39 of them germinated well for progeny testing in the field. In 2008, 19 recombinant plants (one of them with a double crossover and one of them with a quadruple crossover) were selected from 829 seedlings and grown in the greenhouse for seed increase. After sowing, 17 recombinant families generated sufficient germination for progeny tests. In 2009, no additional seedling screenings were conducted. Five recombinant plants (two of them were single recombinant plants, and three were double recombinant plants) identified during the 2008 progeny seedlings selection were progeny tested in 2009. In 2010, two recombinant plants (09S69-52 and 09S164-62) were selected and progeny tested in the field. One (09S164-62) was identified after screening 374 progeny seedlings using 6 markers developed from the BAC HBa0051C24 sequence; another (09S69-52) was identified during the 2009 progeny testing (see details of the pedigrees in Supplemental Figure 1).

Progeny seedling selection and phenotypic analysis

A selfed recombinant plant segregates at the heterozygous locus by producing approximately 25% progeny with homozygous YS cultivar alleles (represented by the score of 1), 50% with heterozygous alleles (represented by the score of 2) and 25% with homozygous LA1589 wild-type alleles (represented by the score of 3). In 2005, 98 recombinant plants were treated as two families (05S42 and 05S43,

derived from 04S337-7 and 04S338-5, respectively). In each family, association between each marker and fruit weight in the field was analyzed by one-way ANOVA. From 2006 to 2010, progenies from a single recombinant plant segregating at the heterozygous region (represented by the score of 2) was treated as a family (a plot) consisting of up to 13 progeny plants that were homozygous for the YS and up to 13 that were homozygous for the LA1589 allele (represented by the score of 1 and 3, respectively). Seedlings were transplanted in the field in numerical order based on seedling numbers prior to marker-assisted selection at a 5-feet spacing. Thirty ripe fruits (but not over-ripe) were randomly harvested from each plant, and 20 of them were weighted for average fruit mass calculation. For each plant, the total weight of 20 fruits was divided by 20.

DNA extraction

For recombinant plants and their progeny seedlings selection, DNA was extracted by Geno/Grinder method. After placing 1 cm^2 leaves from seedling plants in cluster tubes in a rack, 350 µl DNA extraction-lysis mixture buffer [1 part DNA extraction buffer (0.35 M sorbitol, 0.1 M Tris, 5 mM EDTA, pH 8.25, and the addition of 30 mM sodium bisulfite before use), 1 part nuclei lysis buffer (0.2 M Tris, 0.05 M EDTA, 2 M NaCl, 2% CTAB, pH 7.5–8) and 0.4 part 5% sarkosyl] was added to ground the leaf tissue with the 2000 Geno/Grinder (SPEX CertiPrep, USA) at 500 strokes per minute for 2 min. Tubes were centrifuged for 2 min at 3,400*g* to reduce foam and incubated at 65°C for 20 min. In the hood, 350 µl of chloroform:isoamyl alcohol (24:1) was added and samples were shaken several times to mix the phases. The aqueous phase was separated following a centrifugation step at 5,000*g* for 10 min (Sorvall Legend RT, Thermo Fisher Scientific, USA). DNA was precipitated by adding 100 µl of isopropanol to 140 µl aqueous phase followed by mixing and centrifugation. The DNA pellet was washed with 200 µl 70% ethanol, dried and re-suspended in 200 µl TE, from which 2 µl was used for each PCR. Selected plants were confirmed by re-extracting DNA followed by PCR analysis. To extract DNA from preselected plants, the micro-prep method was used to avoid possible contamination. Leaf tissues (2 cm^2) were placed in 1.5 ml Eppendorf tubes and ground manually with pestles attached to drills. DNA extraction steps following the tissue grinding were similar to the Geno/Grinder method mentioned above.

BAC clone sequence analysis

Four overlapping BAC clones derived from tomato variety Heinz1706 (C03Hba291A23, C03HBa51G24, C03HBa 291G05 and C03HBa51C24) were ordered from Clemson University Genomics Institute (http://www.genome.clemson.edu, GUCI). Plasmids of BAC clones were extracted and purified with QIAGEN plasmid Midi Kit (QIAGEN Inc, USA). For shotgun sequencing, BAC C03Hba0051C24 clone was sent to The Genome Center at Washington University, and raw sequencing data of shotgun fragments were assembled manually with the Sequencher® 4.10.1 program.

RNA extraction

Flower buds, flowers at anthesis and young fruit were collected in the morning from ten plants of the *fw3.2* near isogenic lines (NILs, pedigree 10S189 and 10S190 Supplemental Figure 1). Tissues were harvested and immediately frozen in liquid nitrogen. Tissues were ground in liquid nitrogen using a mortar and pestle, 50 mg of the powder was transferred to an Eppendorf tube with Trizol® Reagent (Invitrogen, US) and RNA was extracted following the manufacturer's specifications. Total RNA was precipitated in isopropanol and pellets were washed twice with 1 ml of 70% ethanol. Pellets were dissolved in 50 µl RNase-free water and incubated at 65°C for 3–5 min before the RNA concentration was measured. RNA samples were stored at −80°C until further analysis.

Northern blotting and probe labeling

Ten mg of total RNA per sample was loaded onto 1.2% agarose gel containing 0.5 M formaldehyde and run in 1× MOPS buffer for 2 h. RNA was transferred to Hybond N membranes with 10× SSC buffer and crosslinked using the UV Stratalinker 2400 (Stratagene). To amplify the template for probe labeling, reverse transcription was carried out with the superscript first-strand synthesis system (Invitrogen, USA). RT-PCR was performed using primers located in the exons of the genes. Fifty nanogram of the PCR product was used as template for labeling. Labeling of the probe was conducted in 25 µl reaction mixture containing 50 mM KCl, 10 mM Tris–Cl (pH 8.3), 0.12 mM dXTP (A, G, T), 0.2 µM forward primer, 0.2 µM reverse primer, 3 mM $MgCl_2$, and 25 µCi P32 α-dCTP. The amplification was carried out for three cycles (denaturing at 94°C for 1 min, primer-specific annealing at 50°C for 10 min, and extending at 72°C for 2 min). The probe was denatured by adding 35 µl of 0.4 N NaOH to the sample followed by addition of the denatured probe to the hybridization solution. The expression of *eIF-4a* was used as control.

Probe hybridization and phosphorimage analysis

Membranes were incubated with 10 ml of pre-hybridization mixture containing 5 ml formamide, 2 ml 25× saline-sodium

citrate (SSC) stock solution (3.75 M sodium chloride and 375 mM trisodium citrate, adjusted to pH 7.0 with HCl), 0.25 ml 2.15 M sodium phosphate buffer (PB, 1.15 M NaH_2PO_4 and 1 M Na_2HPO_4), 1 ml 100× Denhardt's solution (2% Ficoll, 2% polyvinylpyrrolidone and 2% bovine serum albumin) and 0.2 ml 10% SDS for 4 h at 42°C. Hybridization was conducted overnight at the same temperature with 5 ml hybridization mixture containing 2.5 ml formamide, 1 ml 25× SSC buffer, 0.05 ml 2.15 M PB, 0.1 ml 100× Denhardt's buffer, 0.05 ml 10% SDS and 1 ml 50% dextran sulfate. After hybridization, the membranes are washed with 2× SSC buffer at 42°C, followed by two washes with low stringency buffer (2× SSC, 0.05% SDS and 0.02% sodium pyrophosphate (PPi) buffer) for 15 min each at 65°C, and two washes with high stringency buffer (0.2× SSC, 0.05% SDS and 0.01% PPi) for 15 min each at 65°C. The membranes were dried on Whatman 3 M paper for 2 min and exposed to phosphorscreens. Digital images of radioactive blots were produced by the Molecular Dynamics Storm840 imaging system (GE Healthcare).

Results

Confirming the map position of *fw3.2*

Fruit mass is an important agricultural trait controlled by QTL. *fw3.2* is a major QTL that explains 19% of fruit weight variation in the F_2 population between YS and LA1589 (Van der Knaap and Tanksley 2003). To confirm the location of *fw3.2*, 10 PCR-based markers (SSR111, TG222, T1659, TG246, SSR22, T1130, TG134B3, TG134B5, TG242 and TG284) were developed and mapped to the locus (Fig. 2). Ninety-eight recombinant plants in the *fw3.2* locus were selected from BC_3S_2 lines and transplanted to the field in the summer of 2005 for fruit weight measurement and QTL analysis (Supplemental Table 2). Fruit weight was significantly associated with eight markers in the 05S43 family (progeny plants derived from 04S338-5) and with three markers in 05S42 (derived from 04S337-7). The analysis in 05S43 suggested the possibility of two QTL controlling fruit weight. One QTL might overlap with the *fw3.1* (Grandillo et al. 1999) and another with *fw3.2* (Van der Knaap and Tanksley 2003).

Fine mapping of *fw3.2*

Large-scale progeny tests were conducted in the field in 2006 to map the *fw3.2* to a smaller interval. Since the 05S43 family showed higher marker association with the *fw3.2* locus, progeny seedlings of 04S338-5 (parent of 05S43) were screened and 15 selected recombinant plants were progeny tested in the field. The results further demonstrated the likelihood of two fruit weight QTL on chromosome 3. One locus corresponding to *fw3.1* mapped near SSR111, and the other to *fw3.2* located between the T1130 and TG134B5 markers 31 cM apart. This result was consistent with that of the 05S43 population that was evaluated in 2005 (Supplemental Table 2).

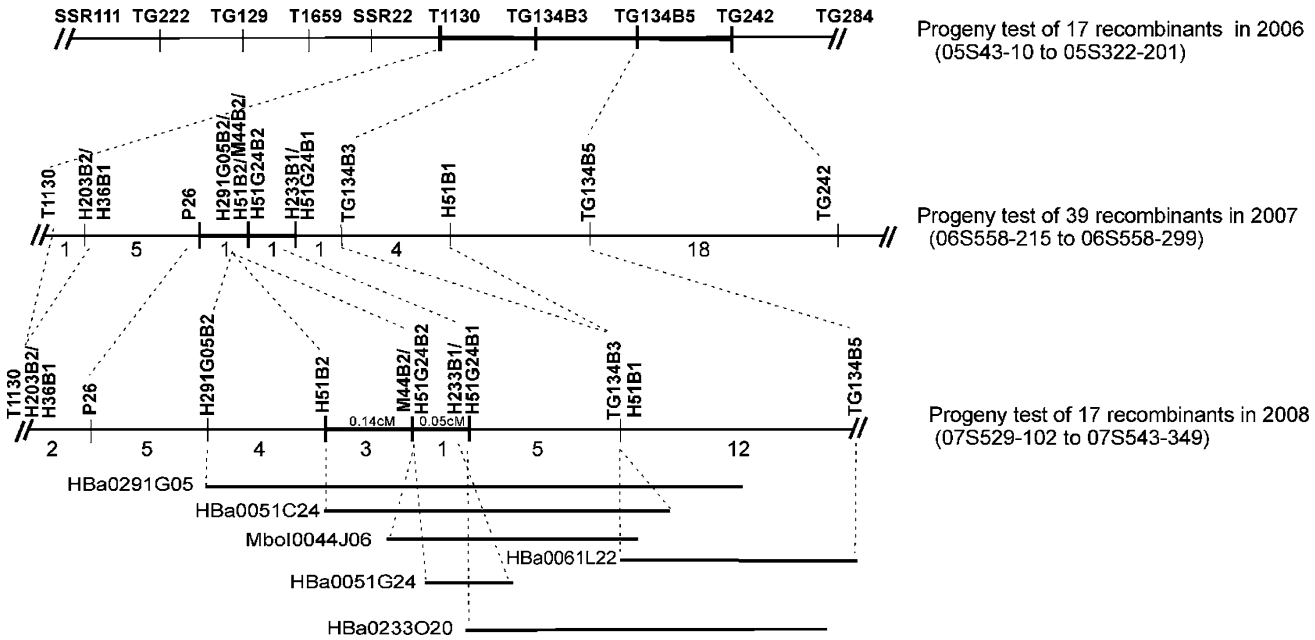

Fig. 2 Mapping and fine mapping of *fw3.2* to BAC HBa0051C24. Numbers below the chromosome indicates number of recombinant plants in the interval that were progeny tested;highlighted region on the chromosome delimits the region of the locus in each year

Compared to *fw3.2*, the *fw3.1* region near SSR111 is close to the centromere, which typically exhibits lower recombination frequency. In addition, segregation of *fw3.1* was not always clear (Supplemental Table 2, families in the bottom part of progeny test in 2006). Therefore, we focused on *fw3.2* region (T1130-TG134B5 interval) for fine mapping. To eliminate interference from the *fw3.1* locus, 06S522-8 and its parent 05S322-201 that were fixed for the *fw3.1* locus were used for recombinant plant identification in the fall of 2006 and progeny testing in 2007. Progeny testing of the recombinant plants in 2007 placed *fw3.2* at a 1.8-cM interval flanked by markers T1130 and TG134B3.

No additional molecular markers were available from the tomato genetic map (Tanksley et al. 1992) and thus for additional marker development, we used sequences from the tomato physical map (Sol Genomics Network at http://solgenomics.net/). On the 2007 tomato FPC contig map, five contigs were located at the *fw3.2* locus anchored by markers T1130, T0781, AscI, C2_Atlg61620 and TG134B3 on the SGN genetic map. Three markers (H203B2, H36B1 and H24B2) were developed from the middle three contigs, respectively. The first two markers mapped to *fw3.2*, but the third marker mapped outside the locus, indicating that at that time not every contig was assigned to the correct location in the genome. One marker was derived from a MADS box gene (P26) and nine markers from the TG134B3-anchored contig (H291G05B2, H51B2, H51G 24B2, H51B2, M44B2, H233B1, H51G24B1, H51B1 and H233B2) were developed and mapped to the *fw3.2* region for further analyses. Based on the recombinant plants tested in 2007, the locus was resolved between the marker P26 and H51G24B1 (Supplemental Table 2, Fig. 2). To further fine map the locus, additional recombinant plants (17 families) were selected and progeny tested in 2008. The *fw3.2* was delimited to a 0.19-cM interval between the marker H51B2 and H233B1 (Supplemental Table 2, Fig. 2), a 60.5-kb region covered by the BAC HBa0051C24. With six new markers (P2A, P3, COBRA2, P5, P7 and P9) developed from the BAC sequence, progeny tests conducted in 2009 and 2010 confirmed and delimited the locus to a 51.4-kb region (Supplemental Table 2, Fig. 3).

Gene action calculation

Gene action (*D*/*A* value) indicates which allele is completely or partially dominant over the other allele. The *D*/*A* value of the *fw3.2* locus was 0.08 in the F_2 progeny population between YS and LA 1589 (Van der Knaap and Tanksley 2003), which suggests that the alleles act in an additive manner with a dosage effect. To better understand the gene action of *fw3.2*, *D*/*A* was calculated using the BC_3S_4, BC_3S_5 and BC_3S_8 plants that have homozygous genetic background with the exception of the *fw3.2* locus (Table 1). In 2008, four families (one BC_3S_4 and three BC_3S_5) were analyzed and the *D*/*A* ranged from −0.16 to 0.57. In 2010, two families (BC_3S_8) were analyzed and the *D*/*A* was 1 and 0.45, respectively. The families showed an additive to dominant gene action of the YS allele. Because it is unlikely that *fw3.2* allele is a loss of function in wild type, we hypothesize that the YS allele might be the result of a gain-of-function mutation that occurred during the domestication of tomato.

Pleiotropic effect of the *fw3.2*

The *fw3.2* locus was found to control fruit shape in YS and other cultivars such as Rio Grande and Long John (Van der Knaap et al. 2002; Van der Knaap and Tanksley 2003; Brewer et al. 2007; Gonzalo and van der Knaap 2008). To test for pleiotropic effect of the *fw3.2*, fruit shape analysis was conducted in 2008 (Table 2). The analysis showed that the fruit weight traits measured as fruit perimeter, area, width and height consistently segregate between NILs in the four families. Other traits such as blockiness of the proximal end at 20% (pblk20%), proximal end angle at 10% (pan10%) and proximal indentation area (piar) also segregated between NILs in all of the families albeit with less significance. The remaining fruit shape traits did not consistently segregate. The results indicated a major effect of *fw3.2* on fruit mass and a minor effect on some fruit shape traits.

Predicted genes at the *fw3.2* locus

The sequence of the BAC C03Hba0051C24 encompassing *fw3.2* was analyzed with the ab initio gene prediction programs FGENESH (http://www.softberry.com/berry.phtml) (Salamov and Solovyev 2000) and is consistent with the recently released annotation of the tomato genome (http://solgenomics.net/). Seven putative genes (ORF2 to ORF8) were present in this region (Table 3; Fig. 3). ORF2 had 80% protein sequence identity with the COBRA-like protein 4 (*COBL4/IRX6*), which is one of several genes involved in secondary cell wall formation in *Arabidopsis* (Brown et al. 2005). ORF3 also encoded a COBRA family protein and had 60% identity to the COBRA-like protein 2 that was predicted to be one of GPI-anchored proteins in *Arabidopsis* (Borner et al. 2003). ORF4 encodes a protein with 62% protein sequence identity to *PNM1* in *Arabidopsis* that localizes to the nucleus and mitochondria, and coordinates the expression of the two genomes (Hammani et al. 2011). The *PNM1* belongs to the pentatricopeptide repeat-containing protein (PPR) family playing a role in RNA binding (Aubourg et al. 2000; Small and Peeters 2000). ORF5 encoded a protein that had 44% identity with the PsbP [an extrinsic subunit of photosystem II (PSII)]-like

Table 1 Gene action at the *fw3.2* locus

Parent	Progeny	Genotype of parent[a]						Fruit weight (g) progeny test						*P* value[e]			Gene action[f]		
		T1130	P26	M44B2	TG134B3	H51B1	TG134B5	Ave1[b]	SD	*N*	Ave2[c]	SD	*N*	Ave3[d]	SD	*N*	*P* (1 vs. 2)	*P* (2 vs. 3)	*P* (1 vs. 3)	*D*	*A*	*D/A*
06S560-162	08S82	1	2	2	2	2	2	96.52	5.95	10	74.65	6.22	10	58.72	6.51	10	***	****	****	−2.97	18.90	−0.16
06S558-244	08S83	2	2	2	2	2	2	68.85	7.94	10	62.94	5.37	10	52.37	7.19	10	0.07	**	***	2.33	8.24	0.28
06S558-249	08S84	2	2	2	2	2	2	83.12	10.05	10	71.36	5.40	10	50.53	5.99	10	**	****	****	4.53	16.29	0.28
06S558-208	08S85	2	2	2	2	2	2	51.49	6.02	10	48.73	4.49	10	38.63	2.97	10	0.26	****	****	3.67	6.43	0.57
Parent	Progeny	M44B2	P3	COBRA2	P5	P9	H233B1	Ave1[b]	SD	*N*	Ave2[c]	SD	*N*	Ave3[d]	SD	*N*	*P* (1 vs. 2)	*P* (2 vs. 3)	*P* (1 vs. 3)	*D*	*A*	*D/A*
09S69-52	10S61	1	1	2	2	2	2	60.47	7.74	10	60.44	4.33	10	45.99	4.06	10	0.99	****	***	7.21	7.24	1
09S69-68	10S63	2	2	2	2	2	2	65.39	3.92	12	60.20	4.29	22	46.43	3.67	12	*	****	****	4.29	9.48	0.45

[a] Marker score on parent plant: 1, homozygous *S.lycopersicum*; 2, heterozygous; 3, homozygous *S.pimpinellifolium*

[b] Average fruit weight (g) for homozygous *S. lycopersicum* class (20 fruits per plant)

[c] Average fruit weight (g) for heterozygous *S. lycopersicum* class (20 fruits per plant)

[d] Average fruit weight (g) for homozygous *S. pimpinellifolium* class (20 fruits per plant)

[e] * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$

[f] The gene action (degree of dominance) = D/A, where D = Aa-(AA+aa)/2, and A = (AA-aa)/2

protein 1 (PPL1) in *Arabidopsis*, which is required for efficient repair of photodamaged PSII (Ishihara et al. 2007). ORF6 encoded a likely ortholog of KLUH/CYP78A5 (66% identity in protein sequence), a member of P450 family. KLUH controls organ size in *Arabidopsis* through regulating cell proliferation by generating a novel mobile growth signal distinct from the classical phytohormones in a non-cell-autonomous manner (Anastasiou et al. 2007). ORF7 had 62% protein sequence identity with *ALS1* gene in *Arabidopsis*, which encodes a novel ABC transporter that may facilitate vacuolar sequestration of aluminum in Al-sensitive tissue such as the root tip (Larsen et al. 2007). ORF8 encoded a galactose oxidase/kelch repeat domain-containing protein (with 51% protein sequence identity), which has unknown function and may be involved in protein–protein interactions (Shchelkunov 2010).

Nucleotide polymorphisms between the alleles

Many SNP, indels (smaller than 25 bp) and gaps (larger than 24 bp) were found between the BAC Hba0051C24 sequence from Heinz1706 and the draft genome sequence of LA1589 available on SGN (Fig. 4). A few regions, notably around ORF6, were resequenced to resolve a few erroneous SNPs and indels in the draft LA1589 genome assembly. Other regions, for example at the 5′ end of ORF5, were missing in the LA1589 genome assembly and were not investigated further. Many SNP, indels and gaps were found in the intergenic region and introns. ORF2 and ORF7 had no SNPs or indels in the coding region. ORF3 had three SNPs and all were silent. ORF4 had 6 SNPs in the coding region of which five were predicted to change the corresponding amino acid. ORF5 had one SNP in the coding region, resulting in a silent mutation. ORF6 has seven SNPs of which two were predicted to change the amino acid. ORF8 had one SNP that was predicted to result in an amino acid change. These results will have to be taken with caution since the LA1589 genome sequence has not been validated and may contain errors in addition to a few gaps in the assembly. Moreover, although it is likely that Heinz1706 carries the same allele as YS, nucleotide polymorphisms may exist between these two cultivated parents and *fw3.2* may not be segregating in Heinz1706. Regardless, based on nucleotide polymorphisms, it is difficult to predict which gene is more likely to encode *FW*3.2. Promoter polymorphisms are equally likely to be associated with crop domestication phenotypes (Doebley et al. 2006).

Expression of the candidate genes

The candidate genes were compared to the tomato EST database (Table 3). No ESTs corresponding to ORF2 and ORF3 were found. Expression of ORF5 was only found in

Table 2 Fruit shape analysis of the *fw3.2* NILs

Trait[a]	08S82					08S83					08S84					08S85				
	Ave1[b]	Ave2[c]	Ave3[d]	*P* value[e]	*D*/*A*[f]	Ave1[b]	Ave2[c]	Ave3[d]	*P* value[e]	*D*/*A*[f]	Ave1[b]	Ave2[c]	Ave3[d]	*P* value[e]	*D*/*A*[f]	Ave1[b]	Ave2[c]	Ave3[d]	*P* value[e]	*D*/*A*[f]
per	18.25	16.94	15.11	****	0.16	15.99	15.35	14.55	****	0.11	17.63	16.53	15.07	****	0.14	14.95	14.59	13.20	****	0.60
ar	21.26	18.72	15.14	****	0.17	16.41	15.59	13.98	***	0.32	19.69	17.53	14.63	****	0.15	14.71	13.97	11.68	****	0.51
fd	5.92	5.37	4.76	****	0.07	5.12	4.89	4.61	****	0.10	5.71	5.31	4.85	***	0.07	4.69	4.60	4.16	***	0.63
fl	4.38	4.23	3.87	****	0.42	3.92	3.87	3.68	***	0.58	4.19	4.02	3.66	****	0.34	3.80	3.69	3.41	****	0.45
pblk20%	0.91	0.90	0.88	****	0.51	0.90	0.89	0.89	*	−0.25	0.90	0.90	0.89	*	1.20	0.90	0.89	0.89	*	−1.09
pan10%	210.75	202.39	195.18	***	−0.07	203.94	198.14	195.14	*	−0.32	217.53	216.38	211.83	*	0.60	201.08	198.15	195.00	*	0.04
piar	0.18	0.13	0.09	***	−0.22	0.13	0.10	0.09	*	−0.48	0.22	0.21	0.17	*	0.80	0.11	0.10	0.08	*	0.24
dan2%	188.36	187.64	169.97	0.06	0.92	187.55	183.85	185.58	0.83	−2.76	188.77	197.46	170.05	0.10	1.93	188.42	173.16	182.16	0.50	−3.88
dan10%	165.34	159.04	168.37	0.47	5.15	168.01	168.70	165.11	0.51	1.47	163.64	174.51	162.65	0.89	22.97	164.83	162.88	166.86	0.56	2.92
diar	0.03	0.02	0.02	0.05	−0.37	0.02	0.02	0.01	0.14	0.42	0.04	0.02	0.01	***	−0.38	0.01	0.01	0.01	0.17	0.80
pblk10%	0.77	0.77	0.74	*	1.20	0.77	0.76	0.75	0.09	0.02	0.77	0.78	0.76	0.42	2.24	0.77	0.75	0.76	0.05	−1.46
dblk10%	0.67	0.66	0.65	*	−0.22	0.66	0.66	0.66	0.46	2.17	0.68	0.68	0.67	0.32	0.99	0.66	0.66	0.64	*	1.56
tri10%	1.15	1.17	1.14	0.19	3.09	1.17	1.15	1.15	0.17	−1.34	1.14	1.15	1.13	0.83	4.20	1.17	1.14	1.18	0.72	11.64
psh10%	0.08	0.06	0.04	**	−0.17	0.06	0.05	0.04	*	−0.26	0.09	0.08	0.07	**	0.30	0.05	0.05	0.04	0.25	0.89
pan5%	227.50	222.63	215.80	**	0.17	224.47	218.74	215.75	*	−0.31	234.98	232.46	227.42	**	0.33	218.08	215.98	216.24	0.49	−1.28
pan20%	156.11	149.37	141.49	***	0.08	149.90	146.30	144.09	*	−0.24	157.16	158.23	152.86	0.18	1.50	146.03	144.30	141.53	*	0.23
dan5%	179.30	177.73	182.67	0.62	1.93	174.64	180.32	174.59	0.99	208.70	190.62	185.88	175.34	*	0.38	169.00	181.44	161.22	0.42	4.19
dan20%	133.59	127.24	124.86	**	−0.45	128.30	128.38	126.44	0.34	1.08	135.60	132.62	131.98	0.16	−0.65	123.43	125.60	123.34	0.95	48.46
fs	0.74	0.79	0.82	****	−0.23	0.77	0.79	0.80	**	−0.51	0.74	0.76	0.76	0.16	−0.76	0.81	0.81	0.82	0.30	1.73
dblk20%	0.83	0.82	0.82	0.15	−1.28	0.82	0.83	0.82	0.59	4.22	0.83	0.83	0.83	0.51	1.38	0.82	0.82	0.81	0.27	3.33
Tri20%	1.10	1.10	1.07	**	1.03	1.09	1.08	1.08	0.09	−1.11	1.09	1.09	1.08	0.28	1.04	1.10	1.08	1.09	0.25	−3.95
hrt20%	0.86	0.96	0.79	0.56	3.98	0.90	0.99	0.90	0.97	42.91	0.72	0.80	0.96	*	0.37	0.97	0.92	0.90	0.54	−0.35
psh	0.08	0.06	0.04	**	−0.17	0.06	0.05	0.04	*	−0.26	0.09	0.08	0.07	**	0.30	0.05	0.05	0.04	0.25	0.89
pan2%	203.21	204.05	200.49	0.44	1.62	198.47	202.45	199.39	0.82	−7.59	206.93	205.71	199.96	***	0.65	201.53	201.88	201.68	0.93	−3.62

[a] Fruit shape trait analyzed between NILs in the field

"per" represents the perimeter;

"ar" represents the fruit area;

"fl" represents the maximum fruit height;

"fd" represents the fruit width;

"fs" reprents the fruit shape index;

"dan 5%" represents the distal end angle measured at 5% from the distal end;

"pan 5%" represents the proximal end angle measured at 5% from the proximal end;

"dblk 10%" represents the blochness of the distal end (the ratio of the width at 10% of the height from the end of the fruit to the middle-position width)

"dblk 20%" represents the blochness of the distal end (the ratio of the width at 20% of the height from the end of the fruit to the middle-position width)

"pblk 10%" represents the blochness of the proximal end (the ratio of the width at 10% of the height from the top of the fruit to the middle-position width)

"pblk 20%" represents the blochness of the proximal end (the ratio of the width at 20% of the height from the top of the fruit to the middle-position width)

"diar" represents the distal indentation area;

"piar" represents the proximal indentation area; "hrt" represents the fruit heart shape,

"tri" represents the fruit shape triangle, "psh" represents the proximal shoulder height

[b] Average fruit weight (gram) for homozygous *S. fycopersicum* class (20 fruit per plant)

[c] Average fruit weight (gram) for heterozygous *S. fycopersicum* class (20 fruit per plant)

[d] Average fruit weight (gram) for homozygous *S. pimpinellifolium* class (20 fruit per plant)

[e] * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$;

[f] The gene action (degree of dominance) = *D*/*A*, where D = Aa-(AA+aa)/2, and A = (AA-aa)/2

Table 3 Candidate genes at *fw3.2*

ORFs	Names	Putative protein function	Number of exons	Gene length (bp)	EST source tissue
ORF2	Solyc03g114900	A member of the COBRA family	6	1,930	NA
ORF3	Solyc03g114910	A member of the COBRA family	5	2,799	NA
ORF4	Solyc03g114920	Pentatricopeptide (PPR) repeat-containing protein	1	1,569	Leaf, fruit, fruit pericarp, root, whole seedlings
ORF5	Solyc03g114930	Photosystem II reaction center PsbP family protein	8	5,125	Leaf
ORF6	Solyc03g114940	Cytochrome P450 family protein	2	2,342	Roots, fruit, floral bud, flower
ORF7	Solyc03g114950	ABC transporter	17	5,302	Leaf, floral bud, flower
ORF8	Solyc03g114960	Kelch repeat-containing protein	8	7,444	Leaf, fruit, floral bud, flower

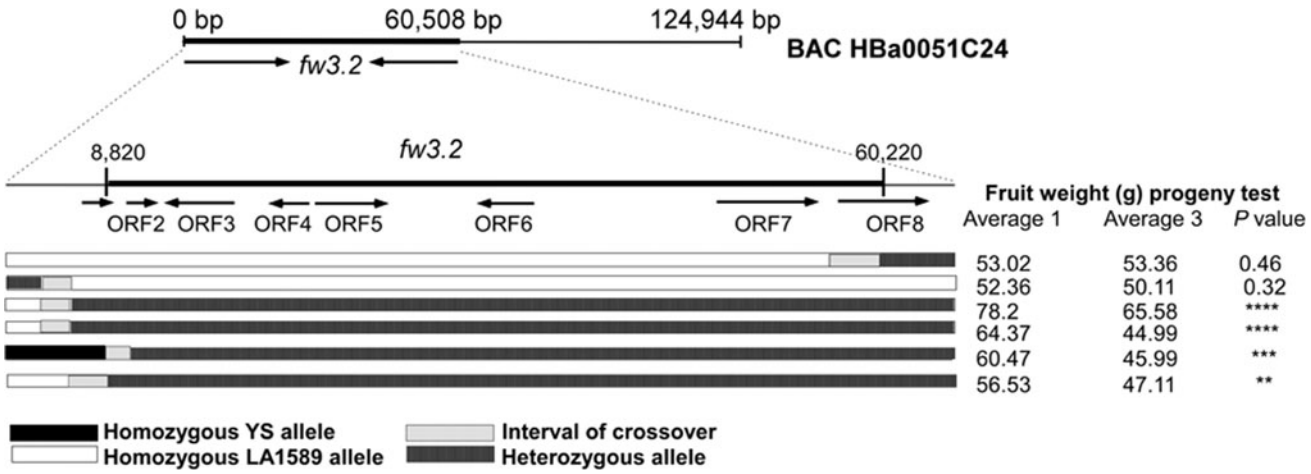

Fig. 3 Fine mapping of *fw3.2* to a 51.4-kb region. The region carries seven candidate genes, ORF2–ORF8, and the direction of transcription is indicated by the *arrow* for each ORF. Below the location of the genes, each *bar* represents the genome structure at the locus and location of the crossover of the recombinant plant. Average weight for the YS allele (indicated by *1*) or the LA1589 allele (indicated by *3*) is shown for each progeny of the recombinant plant, including whether the weights differ significantly between genotypes. The progenies evaluated were: 08S74, 08S89, 08S77, 08S456, 10S61 and 10S62, respectively

leaf. ORF4, ORF6, ORF7 and ORF8 were expressed in many tissues, including floral buds, flowers and fruit tissues. Northern blot analysis of the NILs showed that the expression of ORF6 and ORF8 was higher in the flower buds and young fruit from the NIL carrying the cultivated allele compared to the wild allele (Fig. 5). On the other hand, ORF3 is expressed lower in the lines carrying the cultivated allele. The expression pattern of the other genes is inconclusive. Based on the gene expression findings, this result suggests that the two genes that are higher expressed in cultivated tomato might underlie *fw3.2*.

Discussion

Fine mapping of *fw3.2* and the effect on fruit morphology

Among the seven loci that control fruit mass in the YS variety, *fw3.2* is the locus that contributes the most to the genetic variation (19%) in the F_2 population derived from a cross with LA1589 (Van der Knaap and Tanksley 2003). ANOVA analysis in 2005 and progeny testing in 2006 showed that chromosome 3 contained two fruit weight QTLs. One QTL around marker SSR111 overlaps with the previously identified *fw3.1* locus, and the second is *fw3.2* that was fine mapped in this study. Both QTL overlap with previously detected major loci (Grandillo et al. 1999), suggesting that the large fruited allele of *fw3.2* is found in many varieties. Interestingly, tomato *fw3.2* may overlap with pepper *fw3.1* (Rao et al. 2003), suggesting that the same gene was selected in these independently domesticated crops. However, a caution for other QTL fine mapping projects is that even though the QTL was the most significant and explained the highest amount of variation in the F_2 population, we nearly lost the locus in one of the backcrosses. While progeny 05S43 clearly segregated for *fw3.2*, progeny 05S42 did not (Supplemental Table 2). If we would have only worked with one backcross population, it would

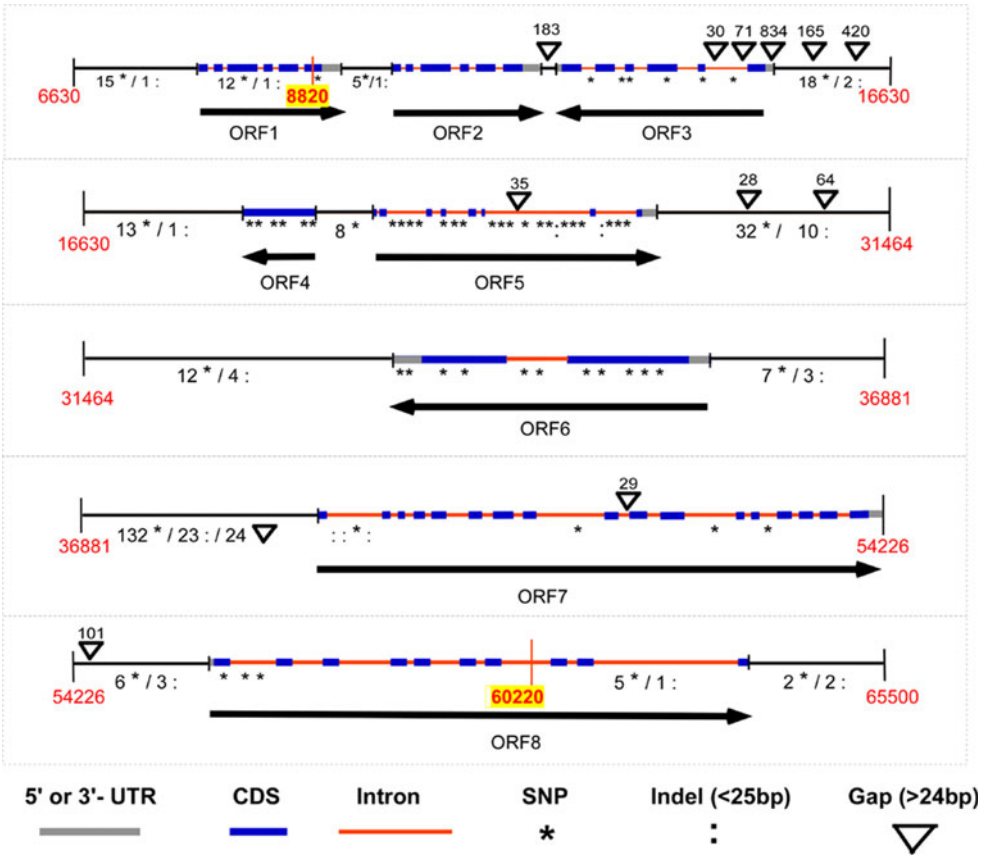

Fig. 4 Nucleotide polymorphisms between the BAC sequence from tomato cultivar Heinz1706 and the draft genome sequence of LA1589. Each SNP, indel and gap is indicated in the coding regions. In the intergenic regions, the polymorphisms are indicated as follows: 15*/1: means 15 SNP and 1 indel. The size of the gap >24 nucleotides is indicated above the gap

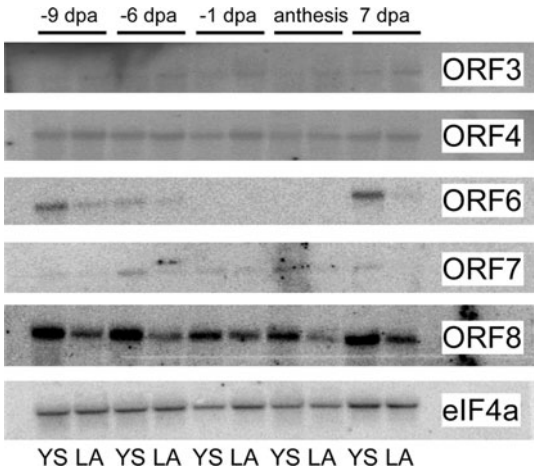

Fig. 5 Expression of *FW3.2* candidate genes in tomato floral buds and young fruit. RNA was isolated from five tissue types indicated above the lanes. Each tissue type was collected from NIL carrying the YS or the LA1589 allele, respectively

have been likely that the locus would not have been fine mapped.

In addition to fruit mass, F_2 analyses showed that *fw3.2* controlled fruit shape in YS as well (Van der Knaap and Tanksley 2003). Interestingly based on other F_2 studies, the same locus also controlled fruit shape in varieties such as Howard German, Banana Legs, Rio Grande and Long John (Van der Knaap et al. 2002; Brewer et al. 2007; Gonzalo and van der Knaap 2008). In this study, we demonstrate with NIL plants (one family of BC_3S_4 and three families of BC_3S_5 lines) that *fw3.2* has a major effect on fruit weight and only minor effects on fruit shape. Very few of the shape QTL that were found in the F_2 studies were found with the NILs. In the NILs, only proximal fruit shape traits such as blockiness, proximal indentation area and proximal end angle were associated with *fw3.2* such that the YS allele resulted in a more indented fruit and a less round (more blocky) shape at the stem end of the fruit.

FW3.2 candidate genes

In this study, we fine mapped *fw3.2* to a 51.4-kb region containing seven candidate genes (ORF2–ORF8). Among these, ORF6 stands out because it is the likely ortholog of the *Arabidopsis KLUH* gene that has been shown to control organ size (Anastasiou et al. 2007; Adamski et al. 2009; Kazama et al. 2010). Another member of this P450 78A subfamily, *CYP78A9*, also controls organ size in *Arabidopsis* and its overexpression leads to large seedless siliques (Ito and Meyerowitz 2000). One of the *CYP78A* members in rice, *PLASTOCRON1* (*PLA1*)/*CYP78A11*, promotes leaf

and stem growth and regulates leaf initiation and termination of vegetative growth (Miyoshi et al. 2004). In tomato, the likely *KLUH* ortholog is differentially expressed in NILs, suggesting that this gene may encode *FW3.2*.

Other genes are also plausible candidates for *FW3.2*. ORF2 and ORF3 encode putative COBRA-like family genes. COBRA (COB) family controls anisotropic expansion and cell wall biosynthesis in *Arabidopsis* (Schindelman et al. 2001; Roudier et al. 2002). A mutation in a COB-like gene, *IRX6*, reduces the level of cellulose in secondary walls in *Arabidopsis* (Brown et al. 2005). COBRA-like proteins were predicted or confirmed to be GPI-anchored proteins in *Arabidopsis* (Borner et al. 2003). *BRITTLE CULM 1* (*BC1*) encodes a COB-like protein that controls cell wall biosynthesis in rice (Li et al. 2003). *ROOTHAIRLESS 3* (*RTH3*) and *BRITTLE STALK 2* (*BK2*), two genes of COB-like family, control grain yield and cellulose biosynthesis in maize, respectively (Ching et al. 2006; Sindhu et al. 2007; Hochholdinger et al. 2008). Furthermore, *BRITTLE STALK-2-LIKE PROTEIN 3* (*ZmBk2L3*) in maize is one of the nine members of the *ZmBk2L* family containing most motifs of GPI-anchored proteins, which is a putative ortholog of *COBRA* (*AtCOB)* gene in *Arabidopsis* (Brady et al. 2007). *AtCOB*s were found to be co-expressed with *CESA* genes encoding cellulose synthases (Brown et al. 2005; Persson et al. 2005). Therefore, based on their role in cell wall biosynthesis which relates to cell expansion, ORF2 and ORF3 are also viable candidates for *FW3.2*. Yet, based on expression patterns, these genes are not likely to encode *FW3.2*.

The other candidate genes are less likely to control fruit weight. The likely ortholog of ORF4 in *Arabidopsis*, *PNM1,* is involved in RNA binding in mitochondria (Aubourg et al. 2000; Small and Peeters 2000). The likely ORF5 ortholog functions in photosynthesis (Alexandrov et al. 2009). The likely ORF7 ortholog, *ALS1,* encodes a novel ABC transporter that is involved in the aluminum stress resistance pathway (Larsen et al. 2007). ORF8 is differentially expressed and encodes a member of the kelch repeat domain-containing proteins that may be involved in protein–protein interactions (Shchelkunov 2010). It is not known whether this protein is also involved in organ size. Hence, based on their orthologs' function, ORF4, ORF5, ORF7 and ORF8 are less likely candidates for *FW3.2*.

Acknowledgments We thank the members of the Van der Knaap laboratory, in particular Jenny Moyseenko for assistance with greenhouse and field experiments. We thank John Elliot and Lee Duncan for field and greenhouse plant care, respectively. We also thank Dr. Sandy Clifton from Washington University Genome Center for an excellent job on the sequencing of the tomato BAC clone. The research was supported by National Science Foundation grants DBI 0227541 and IOS 0922661.

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