Silencing GRAS2 reduces fruit weight in tomato

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Abstract GRAS family transcription factors are involved in multiple biological processes in plants. Here, we report that GRAS2 plays a vital role in regulating fruit weight in tomato (Solanum lycopersicum). We establish that the expression of GRAS2 was elevated in ovaries and maintained at a constant level in fertilized ovules. Reduction of GRAS2 expression in transgenic plants reduced fruit weight through modulating ovary growth and cell size. At the metabolic level, downregulation of GRAS2 decreased activities of the gibberellic acid biosynthesis and signal transduction pathways, leading to insufficient levels of active gibberellic acid during the initial ovary development of tomato. Moreover, genotypic diversity of GRAS2 was consistent with the molecular basis of fruit weight evolution, suggesting that GRAS2 contributes to the molecular basis of the evolution of fruit weight in tomato. Collectively, these findings enhance our understanding of GRAS2 functions, in fruit development of tomato, and demonstrate a strong association between the GRAS gene family and fruit development.

INTRODUCTION

Tomato (Solanum lycopersicum) is one of the most important crops, worldwide, because of its nutritive and commercial value. The stable inheritance, self-pollination, short growth cycle and small genome of tomato have made it the model plant of choice to study fleshy fruit development (Sato et al. 2012). Tomato fruit weight, in particular, has been studied as a key character in tomato evolution. Cultivated tomato evolved from wild relatives over a long period and has undergone complex patterns of migration from the Andean regions of South America to the rest of the world (Jenkins 1948; Bergougnoux 2014). Overall, the evolution of cultivated tomato can be considered to have two stages: an early domestication stage and a later improvement stage (Lin et al. 2014).

Many of the genetic and molecular mechanisms responsible for fruit development in tomato have been characterized, and nearly 30 tomato fruit weight quantitative trait loci (QTLs) have been identified by the classical bi-parental linkage mapping approach (Grandillo and Tanksley 1999). However, only a few of the genes, corresponding to these QTLs, have been cloned, including fruit weight (fw) 2.2 (Frary et al. 2000), fw3.2 (Chakrabarti et al. 2013), locule (lc) (Munos et al. 2011), fasciated (fas) (Barrero and Tanksley 2004; Cong et al. 2008) and fw11.3 (Mu et al. 2017). Among those cloned genes, fw2.2 and fw3.2 are required for modulating cell division, fw11.3 plays a role in regulating cell size, and both fas and lc are responsible for fruit size changes, via modifying flower carpel number.

The GRAS family has a wide distribution in plant species and is characterized by several conserved motifs in the C-terminus, including LHR I, VHIID, LHR II, PFYRE and SAW motifs (Pysh et al. 1999). Members of the GRAS gene family have been identified in the genomes of several plants, including Populus, Arabidopsis thaliana, Prunus mume, rice (Oryza sativa) and tomato (Liu and Windmer 2014; Huang et al. 2015; Lu et al. 2015). Sequence diversity of GRAS family members underlies their roles in different fundamental processes of plant growth and development. Based on amino acid sequence, the GRAS family can be divided into eight distinct subfamilies: DELLA, SHORT-ROOT (SHR), PHYTOCHROME A SIGNAL TRANSDUCTION 1 (PAT1), SCARECROW-LIKE 3 (SCL3), SCARECROW (SCR),...
L. longiflorum SCARECROW-like (LISCL), LATERAL SUPPRESSOR (LS), and HAIRY MERISTEM (HAM) (Tian et al. 2004).

Among other developmental events mediated by GRAS proteins, some GRAS transcription factors are involved in the gibberellic acid (GA)-dependent regulatory network. In Arabidopsis, the SCL3 subfamily gene SCL3 integrates and maintains the functional GA pathway (Heo et al. 2011; Zhang et al. 2011). In addition, five identified DELLA genes serve as repressors in the GA signaling pathway (Sanchez-Fernandez et al. 1998; Silverstone et al. 1998; Lee et al. 2002; Wen and Chang 2002). In Arabidopsis, SCR and SHR form a SCR/SHR complex to regulate radial organization of the root (Cui et al. 2007). Light signaling pathways include four PAT1 subfamily genes: PAT1, SCL5 and SCL13, involved in phytochrome A signal transduction, and SCL21, involved in phytochrome B signal transduction (Bolle et al. 2000; Torres-Galea et al. 2006a, 2013b). The LS subfamily gene MOC1 is essential for the tillering process in rice, and LAS, a homolog of MOC1, plays an important role in the axillary meristem initiation in Arabidopsis (Greb et al. 2003; Li et al. 2003). In petunia (Petunia hybrida Vilm), HAM controls shoot meristem maintenance by regulating signals from differentiating cells (Stuurman et al. 2002).

Fifty-three GRAS family members have been identified in tomato (Huang et al. 2015), but only a few have been functionally characterized. Although the functions of GRAS family genes in tomato are not fully understood, there is increasing evidence that they are involved in fruit development. For instance, DELLA, a negative regulator of GA signaling, was shown to restrain fruit growth (Marti et al. 2007). In addition, GRAS24 regulates fruit development through modulating GA and auxin signaling (Huang et al. 2016).

In this study, we functionally characterized GRAS2, a PAT1 subfamily gene, during tomato fruit development. Knockdown of GRAS2 significantly reduced fruit weight. In addition, our analysis of genetic variants in the coding region of GRAS2, in a tomato resequencing population (Lin et al. 2014), revealed that the genotypes of GRAS2 were associated with the fruit-weight trait, highlighting GRAS2 as a candidate for further research into the molecular basis of fruit evolution. These findings thus reveal the functions of GRAS2, enhancing our understanding of the GRAS gene family function during tomato fruit development.

RESULTS

Phenotypic characterization of GRAS2-RNAi lines
To functionally characterize GRAS2 in tomato, 16 independent GRAS2 RNA interference (RNAi) transgenic lines and 18 GRAS2 overexpression (OE) transgenic lines were generated. Compared with wild type (WT), GRAS2-OE lines (T₁) showed a normal phenotype. However, nine of 16 GRAS2-RNAi lines (T₁) had smaller fruit than WT, with fruit weight only approximately 60% of WT (Figure 1A; Table 1). Three GRAS2-RNAi lines (5, 12 and 16)
with lower transcript accumulation of GRAS2 in fruits compared to WT were selected for further analysis (Figure 1B). In GRAS2-RNAi line 5, final pericarp thickness was only 69% of WT (Figure 1C; Table 1). However, there were no differences in locule numbers (Figure 1C; Table 1), indicating that GRAS2 was not involved in regulating this trait. In a pollen viability assay, the light-yellow pollen of GRAS2-RNAi 5 reflected decreased viability compared to WT (Figure 1D), leading to a reduction in the fruit-setting ratio of GRAS2-RNAi 5 (Table 1). Because of the smaller fruit size and reduction in fruit set, fruit yields of the GRAS2-RNAi lines were reduced to 50% of WT (Table 1). In the floral organs, GRAS2-RNAi lines had shorter petals, sepals, and stamens (Figure 1E; Table 1). In addition, seed numbers per fruit of GRAS2-RNAi lines were reduced to 80% of WT (Figure 1F), though seed weights were not significantly different from WT (Table 1). GRAS2-RNAi lines were always shorter than WT (Table 1). Taken together, these results show that suppression of GRAS2 caused a series of developmental alterations in tomato, especially in fruit development.

### GRAS2 is actively expressed during early development of tomato fruit

We next characterized the temporal expression pattern of GRAS2. GRAS2 was specifically expressed in the ovary within 10 days post anthesis (dpa), a period when the ovule had been fertilized and the ovary initiated a fast-growing stage. However, GRAS2 expression declined dramatically after 10 dpa and remained low through the fruit-ripening stage (Figure 2A).

The GRAS2 spatiotemporal expression was further studied by monitoring GUS activity in the developing ovary using ProGRAS2:GUS transgenic lines within 10 dpa. Though GUS was rarely detected in the style tissue, high

### Table 1. Phenotypes of wild type and GRAS2-RNAi lines

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Wild-type</th>
<th>RNAi-5</th>
<th>RNAi-12</th>
<th>RNAi-16</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plant height (7-d-old, cm)</td>
<td>2.95 ± 0.18</td>
<td>1.82 ± 0.15**</td>
<td>1.11 ± 0.16**</td>
<td>1.01 ± 0.07**</td>
</tr>
<tr>
<td>Plant height (40-d-old, cm)</td>
<td>35.72 ± 2.83</td>
<td>27.33 ± 2.71**</td>
<td>24.81 ± 2.64**</td>
<td>22.73 ± 1.91**</td>
</tr>
<tr>
<td>Plant height (90-d-old, cm)</td>
<td>124.16 ± 6.67</td>
<td>107.16 ± 5.21**</td>
<td>98.16 ± 7.64**</td>
<td>95.83 ± 7.23**</td>
</tr>
<tr>
<td>Fruit per plant (n)</td>
<td>26.83 ± 3.31</td>
<td>19.16 ± 1.16**</td>
<td>17.50 ± 2.42**</td>
<td>23.33 ± 1.97</td>
</tr>
<tr>
<td>Fruit-set (%)</td>
<td>92.80 ± 4.65</td>
<td>75.31 ± 6.42**</td>
<td>72.7 ± 3.47**</td>
<td>68.4 ± 3.76**</td>
</tr>
<tr>
<td>Petal length (0 dpa, mm)</td>
<td>13.41 ± 1.07</td>
<td>11.01 ± 0.65**</td>
<td>10.69 ± 0.54**</td>
<td>11.03 ± 0.66**</td>
</tr>
<tr>
<td>Stamen length (0 dpa, mm)</td>
<td>10.38 ± 1.05</td>
<td>8.19 ± 0.44*</td>
<td>8.53 ± 0.46*</td>
<td>7.95 ± 0.61*</td>
</tr>
<tr>
<td>Style length (0 dpa, mm)</td>
<td>7.54 ± 0.51</td>
<td>7.42 ± 0.40</td>
<td>7.54 ± 0.32</td>
<td>7.43 ± 0.37</td>
</tr>
<tr>
<td>Sepal length (0 dpa, mm)</td>
<td>11.44 ± 0.80</td>
<td>9.73 ± 0.68**</td>
<td>9.59 ± 0.75**</td>
<td>9.96 ± 0.56**</td>
</tr>
<tr>
<td>Ovary diameter (0 dpa, mm)</td>
<td>1.99 ± 0.11</td>
<td>1.62 ± 0.10**</td>
<td>1.60 ± 0.13**</td>
<td>1.57 ± 0.12**</td>
</tr>
<tr>
<td>Locule number (n)</td>
<td>2.30 ± 0.48</td>
<td>2.40 ± 0.52</td>
<td>2.20 ± 0.42</td>
<td>2.30 ± 0.48</td>
</tr>
<tr>
<td>Pericarp thickness (20 dpa, mm)</td>
<td>2.98 ± 0.24</td>
<td>1.97 ± 0.18**</td>
<td>1.84 ± 0.23**</td>
<td>1.75 ± 0.12**</td>
</tr>
<tr>
<td>Pericarp thickness (40 dpa, mm)</td>
<td>5.08 ± 0.31</td>
<td>3.54 ± 0.22**</td>
<td>3.38 ± 0.13**</td>
<td>3.21 ± 0.14**</td>
</tr>
<tr>
<td>Cell length (20 dpa, mm)</td>
<td>0.174 ± 0.008</td>
<td>0.118 ± 0.006**</td>
<td>0.112 ± 0.008*</td>
<td>0.105 ± 0.007**</td>
</tr>
<tr>
<td>Cell size (20 dpa, mm³)</td>
<td>0.019 ± 0.003</td>
<td>0.009 ± 0.002**</td>
<td>0.007 ± 0.003**</td>
<td>0.007 ± 0.002**</td>
</tr>
<tr>
<td>Cell layer number (20 dpa, n)</td>
<td>16.71 ± 1.95</td>
<td>16.28 ± 1.74</td>
<td>16.14 ± 1.89</td>
<td>15.85 ± 2.07</td>
</tr>
<tr>
<td>Mature fruit transverse diameter (mm)</td>
<td>41.77 ± 0.91</td>
<td>35.65 ± 1.12**</td>
<td>33.57 ± 1.01**</td>
<td>31.43 ± 2.03**</td>
</tr>
<tr>
<td>Mature fruit longitudinal diameter (mm)</td>
<td>39.37 ± 1.09</td>
<td>33.81 ± 0.82**</td>
<td>33.79 ± 0.92**</td>
<td>30.76 ± 1.66**</td>
</tr>
<tr>
<td>Mature fruit weight (g)</td>
<td>40.10 ± 3.23</td>
<td>25.93 ± 2.21**</td>
<td>22.48 ± 1.73**</td>
<td>17.86 ± 3.14**</td>
</tr>
<tr>
<td>Fruit yield per plant (g)</td>
<td>935.84 ± 68.68</td>
<td>508.66 ± 38.27**</td>
<td>450.01 ± 51.72**</td>
<td>360.09 ± 29.29**</td>
</tr>
<tr>
<td>Weight per seed (mg)</td>
<td>2.49 ± 0.12</td>
<td>2.55 ± 0.14</td>
<td>2.45 ± 0.08</td>
<td>2.47 ± 0.07</td>
</tr>
<tr>
<td>Seeds per fruit (n)</td>
<td>128.10 ± 9.41</td>
<td>104.70 ± 9.20**</td>
<td>101.5 ± 8.09**</td>
<td>98.01 ± 8.25**</td>
</tr>
</tbody>
</table>

Values are means ± SD. Ten flowers or fruits from six plants were used for statistical analysis. The statistical significance of differences from the wild type values was analyzed using a t-test: *P < 0.05, **P < 0.01
GUS activity was observed in the ovary wall (0 dpa) (Figure 2B). Subsequently, the GUS activity spread into the placenta and the young seeds at 5 dpa (Figure 2C). GUS activity disappeared from the placenta but remained in the peripheral integument layers of seeds at 7 dpa (Figure 2D). GUS activity was mostly absent from the young seeds at 10 dpa (Figure 2E). Overall, our analysis of GUS activity showed that GRAS2 expression varied during the early stage of fruit development.

The above analyses pointed to strong GRAS2 expression in young seeds (fertilized ovules) throughout the three selected time points (5, 7, and 10 dpa). In situ hybridization analysis of GRAS2 transcripts showed the green fluorescence-labeled probe targeted to GRAS2 specifically at the peripheral integument layers and embryos of young seeds at 7 dpa (Figure 3), which was consistent with GUS-staining results in the developing ovary at the same time point. Collectively, these results indicate that GRAS2 is actively expressed during early development of tomato fruit.

Silencing GRAS2 restrains ovary growth and cell expansion

To further investigate fruit development, we recorded kinematic growth during the early fruit development stages in GRAS2-RNAi lines. Fruit growth was suppressed from the fruit-setting through fruit-ripening stage (45 dpa); (Figure 4A, B), suggesting that the smaller sizes of GRAS2-RNAi ripening fruits were attributable to restricted ovary growth.

To explore further the differences in developing ovaries between GRAS2-RNAi lines and WT, we carried out histological analysis of ovaries at 0 dpa and pericarps at 20 dpa. In ovaries, smaller placenta cells and thinner ovary walls were observed in GRAS2-RNAi 5 compared to WT (Figure 4C, D). Pericarps had shorter cell lengths in GRAS2-RNAi 5 than in WT (Figure 4E; Table 1). A similar difference was observed in cell widths between GRAS2-RNAi lines and WT (Table 1). These results indicate a reduction of pericarp cell size in GRAS2-RNAi lines (Table 1). There were no obvious differences between the number of cell layers in pericarps of GRAS2-RNAi 5 and WT (Figure 4E; Table 1). This lack of change in cell layer number suggests that suppression of GRAS2 leads to an inhibition of cell expansion, but not cell division, in early fruit development.

We also analyzed the transcript levels of the five genes (XTH1, XTH7, EXPA5, PEC, and Cel1) related with cell expansion in the developing ovaries of GRAS2-RNAi lines and WT. Except for Cel1 expression at 15 dpa, the expression of five genes (XTH1, XTH7, EXPA5, PEC, and Cel1) in GRAS2-RNAi lines was significantly reduced compared with WT ovaries during ovary development.
(P < 0.05; Figure S1). These findings demonstrate that GRAS2 promotes the expression of genes related to cell expansion during development.

**GRAS2 is a nuclear-localized protein and has transcriptional activity**

The tomato GRAS2 gene contains a 1,752 bp open reading frame (ORF), which encodes a putative protein of 584 amino acids. To explore the subcellular localization of GRAS2 protein, the vector pCAMBIA1302-GRAS2-GFP and the nuclear marker GHD7-CFP were transiently co-expressed in Arabidopsis protoplasts. Confocal imaging showed the green fluorescence signal of pCAMBIA1302-GRAS2-GFP to be completely merged with the blue fluorescence signal of the nuclear marker GHD7-CFP, indicating that GRAS2 localized to the nucleus (Figure 5A).

GRAS2 and another three GRAS members are well conserved in the C-terminal region, including in the LHRI, LHRII, VHIID, PFYRE and SAW motifs, which are typical conserved motifs of GRAS family transcription factors (Figure S2). To assess whether GRAS2 has transcriptional activation activity, a yeast two-hybrid assay was carried out. A GAL4 DNA-binding domain GRAS2 fusion protein (GRAS2-BD) was transferred into yeast cells AH109. GRAS2-BD promoted yeast cell growth in the Synthetic Dropout (SD) medium lacking histidine and adenine, while the negative control BD did not (Figure 5B), indicating that GRAS2 has transcriptional activation activity. To further analyze which part of GRAS2 determines this activity, GRAS2 was divided into two fragments, the non-conserved region (GRAS2A) and the conserved region (GRAS2B). In the transactivation assay, GRAS2A-BD promoted yeast cell growth in the SD medium lacking histidine and adenine, whereas GRAS2B-BD did not (Figure 5B). Thus, the transcriptional activation activity of GRAS2 is determined by its non-conserved region.

**Downregulation of GRAS2 represses GA biosynthesis and signal transduction pathways**

Tomato fruit weight is reduced by application of LAB19899 (an inhibitor of GA biosynthesis) during the early stage of ovary development, but fruit weight is recovered upon treatment with exogenous GA3 (Serrani et al. 2007), showing that bioactive GA3 was essential for early development of tomato fruit (Fos et al. 2001; Mariotti et al. 2011; Shinozaki et al. 2015). Given that the
shrunked cells, decreased growth of floral organs and fruit, poor fruit-setting and reduced number of seeds in GRAS2-RNAi lines were similar to GA-deficient phenotypes, we hypothesized that the reduction in GRAS2 expression disrupted the GA-mediated stimulation of ovary growth normally observed in tomato. We quantified endogenous GA3 from ovaries of GRAS2-RNAi lines and WT at 3 dpa using HPLC-MS/MS. The concentrations of active GA3 in GRAS2-RNAi lines were significantly decreased compared to WT (P < 0.01; Figure S3). This result implies that decreased expression of GRAS2 is associated with a reduction in active GA3, to cause a deficiency of positive growth signal during ovary development.

To investigate the role of GRAS2 in GA biosynthesis and deactivation, the expression levels of six GA biosynthetic enzyme genes (GA20ox1, GA20ox2, GA20ox3, GA20ox4, GA3ox1, and GA3ox2) and five GA-inactivation genes (GA2ox1, GA2ox2, GA2ox3, GA2ox4, and GA2ox5) were measured in the ovaries of GRAS2-RNAi lines and WT at 3, 7, and 10 dpa. Although there were no changes in the expression of GA20ox3, GA2ox1, GA2ox3, and GA2ox4, the expression levels of other seven genes (GA20ox1, GA20ox2, GA20ox4, GA3ox1, GA3ox2, GA2ox2, and GA2ox5) were decreased in GRAS2-RNAi lines compared to WT during ovary development (excluding GA20ox2 expression at 10 dpa and GA20ox4 expression at 7 and 10 dpa; Figure S4). These findings indicate that GRAS2 silencing causes a reduction in both GA biosynthesis and deactivation.

To explore the effect of GRAS2 silencing on GA signal transduction, the expression levels of two genes (GID1, and GAST1) related with GA signal transduction were analyzed in the ovaries of GRAS2-RNAi lines and WT at 3, 7, and 10 dpa. GID1 and GAST1 expression in the GRAS2-RNAi lines was significantly decreased compared to WT during ovary development (P < 0.05; Figure S5). The decreased expression of GID1 and GAST1 would be predicted to cause a reduction in

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**Figure 4. The effect of GRAS2 silencing on ovary growth and cell expansion**

(A) A kinematic growth record for the early stage of tomato fruit development (from 0 to 45 dpa) in GRAS2-RNAi 5, 12, 16, and WT. Data presented are the means ± SD of three biological replicates. Each replicate contains ten individuals. (B) Representative ovaries (0 dpa) from WT and GRAS2-RNAi line 5. O, ovary; S, style. Placenta cells (C) and ovary wall (D) of ovaries at 0 dpa from WT and GRAS2-RNAi 5. (E) Cell sizes of pericarp in WT and GRAS2-RNAi 5 at 20 dpa. (B) Scale bar = 5 mm. (C), (D) Scale bar = 100 μm. (E) Scale bar = 200 μm.
the activity of GA signal transduction in the GRAS2-RNAi lines.

To further investigate whether the GRAS2-RNAi lines remained sensitive to active GA, ovaries of GRAS2-RNAi lines and WT at 3 dpa were treated with exogenous 50 μM GA₃. The final fruit weights (45 dpa) of GRAS2-RNAi fruits with GA₃ treatment were significantly increased compared with those of untreated fruits (P < 0.05; Figure S6), revealing responsiveness to GA application in the GRAS2-RNAi lines. This responsiveness indicates that the activity of GA signal transduction in the GRAS2-RNAi lines is reduced rather than completely disrupted.

Diversity of GRAS2 genes is consistent with the genetic and molecular basis of fruit weight evolution

We used a resequencing tomato population to analyze the evolutionary history of GRAS2. A population of 288 accessions with known fruit sizes comprised three established evolutionary subgroups (PIM, wild species S. pimpinellifolium; CER, cherry species S. lycopersicum var. cerasiforme; BIG, cultivated species S. lycopersicum) (Lin et al. 2014). Four single nucleotide polymorphisms (SNPs) and one insertion/deletion (InDel) were detected in the coding region of GRAS2 among these accessions (Figure S6; Table 2).

These genetic variants altered the structure of GRAS2 during its evolution, and the observed pattern of variation was consistent with the known evolutionary history of tomato fruit weight. For instance, the genotype of the InDel, which caused a change in the primary structure of GRAS2, was distinct between the PIM group and the BIG group (Figure 6B). At SNP 6348936, SNP 63489682, SNP 63489849, SNP 63489970, and the InDel, big-fruited accessions had the genotypes T, C, T, T, and GAA, respectively. Notably, the big-fruited genotype ratios (number of big-fruited genotype/number of group individuals) sharply increased in the course of transition from the PIM to the CER group (Table 2), showing a strong selection just like TaGS5-3A (Ma et al. 2016).

A phylogenetic analysis of GRAS2 in the 288 accessions showed a branch composed of major wild tomatoes and several cherry tomatoes distinct from those comprising mostly CER and BIG tomatoes (Figure 6C), indicating clear genetic variants generated in the course of transition from the PIM to the CER group. By contrast, the branch differences between the CER and BIG group were not distinct in the phylogenetic analysis of GRAS2, indicating that the CER group was not an outgroup of the BIG group. This finding indicated that the major genetic variants of GRAS2 arose in the early stage of tomato evolution.

Given these findings, we further tested whether GRAS2 contributed to the evolution of fruit weight. We performed a Genome-Wide Association Study (GWAS) on fruit weight in the resequencing population to identify fruit weight genetic loci resulting from the domestication or improvement stages of cultivated tomato evolution. A significant peak signal (P < 9.09 × 10⁻⁹) arose at the end of chromosome 7. This signal included SNPs that had significant linkage disequilibrium (r² > 0.6), with the most significant
Figure 6. GRAS2 has a genetic basis of the evolution of fruit weight and underlies the locus fw7.3

(A) The relative distribution of four SNPs and one InDel in the coding region of GRAS2. Black box represents the coding region of GRAS2; red star represents SNP; red triangle represents InDel. The physical position of GRAS2 on chromosome 7 is indicated from 63,490,264 bp to 63,488,513 bp (Tomato WGS Chromosomes, SL2.40). (B) A glutamic acid insertion caused by the GAA insertion of the InDel site in the coding sequence of GRAS2 distinctly distinguishes between cultivated and wild accessions. PIM, wild tomato group; BIG, cultivated tomato group.
SNP63621877 ($P = 1.64 \times 10^{-9}$) mainly distributed from 63.40 to 63.90 Mb (Tomato WGS Chromosomes, SL2.40). This distribution overlapped with the GRAS2 location (Figure 6D, E), indicating that GRAS2 is a strong candidate to underlie the molecular basis of fruit weight evolution in the resequencing population.

**DISCUSSION**

In this study, we demonstrate that GRAS2 is involved in development in tomato, especially in fruit development. GRAS2 was actively expressed during the early stage of fruit growth, highlighting its importance in regulating ovary growth. We report that GRAS2 is expressed in young seeds, serves as a transcriptional activator, regulates cell expansion and is involved in active GA biosynthesis and signal transduction, providing details of its regulation of ovary growth. The consistency between GRAS2 genotypes and fruit weight in the resequencing population provided further evidence that GRAS2 is tightly related with fruit development.

Derived from fertilized ovules, the young seed serves as a source to continuously generate the required positive growth signals, such as auxins and GAs, throughout seed and fruit formation (Sjut and Bangerth 1982). Both GUS staining and in situ hybridization showed that GRAS2 expression is located in the young seed, pointing to an association between GRAS2 and positive growth signals.

Previous research has shown that the transcription activation domain of a transcription factor affects the transcriptional efficiency of the transcription complex, determining the expression of downstream gene...
The transcriptional activation activity of GRAS2 that we observed implies that GRAS2 may regulate the expression of downstream genes related to fruit development. In addition, the decreased expression of five GA biosynthetic enzyme genes (GA20ox1, GA20ox2, GA20ox4, GA3ox1, and GA3ox2) and two GA-inactivation enzyme genes (GA2ox2 and GA2ox5) in GRAS2-RNAi lines illustrate the influence of GRAS2 on the GA biosynthesis and deactivation activity.

Some GA-deficient mutants, such as Arabidopsis sleepy1 (sly1) and potato photoperiod-responsive 1 (phor1), are insensitive to exogenous GA treatment because of deficiency in the GA signal transduction pathway (Amador et al. 2001; McGinnis et al. 2003). However, other GA-deficient mutants associated with the inhibition of the GA biosynthesis pathway, such as rice semi-dwarf 1 (sdl), can be rescued by an application of GA (Neeraja et al. 2009). We found that the GRAS2-RNAi fruits remained sensitive to GA3 application on ovaries, showing that decreasing the GRAS2 does not completely disrupt the GA signal transduction pathway, and that the reduced activity of GA signal transduction was probably attributable to insufficient levels of active GAs (Shi et al. 1992).

In contrast to the involvement of GRAS24 in the downstream signaling of GA (Huang et al. 2016), GRAS2 likely functions in the upstream biosynthesis of GA. The differential roles of GRAS2 and GRAS24 in the GA pathway further illustrate the importance of GRAS genes for the stability of the GA pathway. In addition, the lack of full recovery of GRAS2-RNAi fruit weight under GA3 treatment suggested that the smaller sizes of GRAS2-RNAi fruits were not merely due to a deficiency in active GAs, and thus that GRAS2 might either directly or indirectly influence metabolic pathways that cannot be repaired by exogenous GA3 treatment to regulate fruit development.

Since knockdown of GRAS2 caused a significant decrease in cell size, rather than in cell number, GRAS2 appears to be more closely related to cell expansion. Xyloglucan endotransglucosylase/hydrolases (XTHs) are key factors in restructuring the existing cell wall for cell expansion during developmental processes (Van-Sandt et al. 2006; Miedes and Lorences 2009). A previous study has shown that XTH8 expression is upregulated by gibberellin in rice (Jan et al. 2004). Similarly, we infer that the deficiency of active GAs caused by knockdown of GRAS2 likely leads to the reduction of XTH expressions, which would decrease the activity of XTH in the construction and restructuring of cell walls, thereby suppressing cell expansion. In addition, PEC, EXPAs, and Cel1 play positive roles in fruit cell expansion (Lashbrook et al. 1994; Vriezen et al. 2008; Glass et al. 2015). The reduced expression of these three genes in GRAS2-RNAi lines might decrease cell wall loosening, making further expansion difficult. Altogether, compared to WT, the downregulated expression of cell expansion-related genes in GRAS2-RNAi lines supports the findings of histological analysis, indicating that GRAS2 is closely associated with cell expansion.

Previous research has placed GRAS2 in the PAT1 subfamily of GRAS genes. However, the GRAS2 sequence shares similarity with the PAT1 sequence merely in a 47 bp sequence encoding a C-terminal conserved region of GRAS2 (Figure S9). Our characterization of a novel function for GRAS2 in fruit development thus contributes to a more comprehensive understanding of GRAS proteins.

It is possible that GRAS2 homologs perform similar functions in plant growth and development, especially in fruit development. For instance, CA07g18600 (version: 1.55), the homologous gene of GRAS2 in pepper, shows 88% similarity with GRAS2 in amino acid sequence and is aligned in the pepper chromosome 7 from 226,087,613 bp to 226,089,361 bp. The pepper locus fruit length (fl) 7.1 is mapped to the long arm of chromosome 7, between the signature marker TG272 (position: 193,763,538 bp) and TG216 (position: 255,954,214 bp), which includes CA07g18600 (Rao et al. 2003). Moreover, CA07g18600 expression can be detected in young pepper fruits. Based on the function of GRAS2 in tomato fruit, we hypothesize that CA07g18600 is a candidate gene underlying the locus fl7.1 to regulate pepper fruit growth. Therefore, more studies on the role of GRAS2 in regulating fruit development are worth conducting in pepper, eggplant, and other Solanaceae species.

In addition, fw7.3, which explains almost 10% of the phenotypic variation in fruit weight, mapped with signature markers TG20A and TG499 from 63.02 Mb to 65.21 Mb (Tanksley and Nelson 1996). This locus has also been named fruit diameter (fd) 7.1, which accounts for approximately 13% of the phenotypic variation in a different bi-parental linkage mapping population and maps between TA20A (63.02 Mb) and the end of the long arm of chromosome 7 (65.26 Mb) (Lippman and Tanksley 2001). Both fw7.3 and fd7.1 overlap the GRAS2
location (63.49 Mb). Based on the association between fruit weight and the properties of GRAS2, GRAS2 appears to have a tight association with the locus fw7.3, and should serve as a candidate gene for fw7.3 in further research.

In summary, we have demonstrated that GRAS2, a novel GRAS family member, exhibits active expression in the ovary, regulates pericarp cell size, and is involved in GA biosynthesis and signal transduction. We further established that GRAS2 has transcriptional activation activity in yeast, and demonstrated the consistency of GRAS2 genotypes with fruit weight evolution in a resequencing population. These findings emphasize the importance of further studies to explore the roles of GRAS2 in the development of tomato.

**MATERIALS AND METHODS**

**Plant material and growth condition**
The tomato (*Solanum lycopersicum*) cultivar “Ailsa Craig” was used in all experiments. All plants were grown in a greenhouse under conditions of 24°C (day)/20°C (night), 60% relative humidity. For gene expression analysis, tissues including roots, stems and leaves were collected from 2-month-old plants. Sepal, flower and ovary samples were taken when flowers were at anthesis. Fruit samples at various developmental stages (5, 10, 15, 20, 30, and 40 dpa) were collected from five healthy plants.

**Construct recombination and tomato transformation**
For the GRAS2-RNAi construct, a unique 441 bp fragment of GRAS2 ORF (Figure S10) was amplified using sequence-specific primers fused with attB1 and attB2 sites. The amplified fragment was recombined into pDONR221 by a BP reaction, and then exchanged into the pHELLSGATE8 vector by a LR reaction.

For the ProGRAS2::GUS construct, an upstream genomic DNA sequence (from –3,000 bp to −1 bp) of the GRAS2 ORF was amplified using sequence-specific primers fused with attB1 and attB2 sites. The product was recombined into pDONR221 by a BP reaction, and then exchanged into the pV3P vector (modified from pHELLSGATE2) containing the GUS coding sequence.

The GRAS2-RNAi and ProGRAS2::GUS constructs were transformed into the *Agrobacterium* strain C58 and then tomato cotyledon explants.

**Gene expression analysis**
Total RNAs from different tissues of WT and GRAS2-RNAi lines were extracted using Trizol reagent (Invitrogen Inc. USA). According to the manufacturer’s instructions, extracted RNA was used as template with M-MLV reverse transcriptase (Toyobo, Japan) to synthesize complementary DNA (cDNA). The template (cDNA) concentrations were normalized with actin expression levels in quantitative PCR (qPCR) analysis. The qPCR was carried out using the Power SYBR Premix Ex Taq kit and the TakaRa two-step method (Takara, Japan). The qPCR programs were carried out using the Roche LightCycler 480 Real-Time PCR Detection System (Roche, Switzerland). Each sample of WT and GRAS2-RNAi lines was averaged by three biological replicates.

**Fluorescence in situ hybridization (FISH)**
A unique sequence of the GRAS2 ORF from 482 bp to 510 bp was used as a template to synthesize a fluorescent probe (5’ AACCUGGCCAUUGUGUGCCACUTACCGGU 3’). For in situ hybridization, tomato fruit slices were sampled and processed as described by Sunde et al. (2003).

**GUS staining**
A GUS staining solution (100 mmol/L sodium phosphate buffer) was used to evaluate the GUS activity of GRAS2 promoter. The GUS staining process was performed as described by Wang et al. (2014).

**Subcellular localization**
The GRAS2 ORF without its terminator (TGA) was amplified and recombined into pCAMBIA1302. The vector pCAMBIA1302-GRAS2-GFP and nuclear marker GHD7-CFP were mixed, then transformed into Arabidopsis protoplasts according to the protocol of Yoo et al. (2007). Images of protein fluorescence were acquired by confocal microscopy (Leica, Germany).

**Pollen viability assay**
Pollen viability was evaluated by soaking pollen grains in I-KI solution (1% KI and 0.5% I2). Viable pollen stains dark blue because it contains more starch granules, whereas weak pollen stains light yellow because it contains less starch.

**Transcriptional activation activity of GRAS2**
The coding region, non-conserved domain (fragment A) and conserved domain (fragment B) of GRAS2 were
amplified and recombined into the yeast expression vector pGBK7 (Clontech, USA). According to the manufacturer’s instructions, constructs GRAS2-BD, GRAS2A-BD, GRAS2B-BD, negative control pGBK7-Lam, positive control pGBK7-53+pGADT7-RecT were transformed into the yeast strain AH109, respectively. Transformed AH109 cells were selected on SD/-Trp medium and SD/-Ade/-His/-Trp medium (Clontech, USA). Based on growth of the corresponding cells, the transcriptional activation activity of each protein was evaluated.

Determinant of GA3 concentrations
Fifteen young fruits at 3 dpa of each line (WT, GRAS2-RNAi 5, 12, and 16) were mixed together and samples (0.1–0.5 g fresh weight) of each line were used for further analysis. GA3 concentrations were measured by High Performance Liquid Chromatography–tandem Mass Spectrometry (HPLC-MS), as described by Liu et al. (2014).

GA treatment
For the exogenous GA3 treatment, ovaries of GRAS2-RNAi 5, 12, 16, and WT at 3 dpa were directly treated with 15 μL GA3 solution. The GA3 solution was made of 50 μM GA3 (Sigma-Aldrich, USA), 1% ethanol, 0.1% Tween 20. Ten ovaries in each line were used in this experiment.

GWAS on fruit weight and phylogenetic analysis
A total of 288 accessions including 28 S. pimpinellifolium accessions, 103 S. lycopersicum var. cerasiforme accessions and 157 S. lycopersicum accessions from the resequencing population (Lin et al. 2014) were used. Tomato plants were grown in the open field in the Experimental Station of Huazhong Agricultural University, Wuhan, China in the springs of 2012 and 2013. Each accession included 20 plants in a plot and all of accessions were grown in a randomized design in each replicate. Average fruit weight was based on a random sample of at least 10 ripe fruits per plot. Two-year data for fruit weight in each accession were averaged and then log, transformed to serve as the final trait data in the association panel (Figure S11–13; Table S1–3).

The 5.5 million high-quality SNPs (Ye et al. 2017) selected from more than 11.6 million SNPs (Lin et al. 2014) were used as the genotypic panel for GWAS analysis.

Genome-Wide Association Study analysis on fruit weight of the resequencing population was performed using the Genome-wide Efficient Mixed Model Association algorithm (GEMMA, v0.94) (Zhou and Stephens 2012), which accounted for population stratification and sample structure.

To identify the significant signals of GWAS, a Bonferroni corrected threshold was used (Li et al. 2012b). In our association study, the Bonferroni threshold was \( P = 9.09 \times 10^{-9} \) \((-\log_{10} P = 8.04\) for the whole population. The phylogenetic tree was constructed using MEGA5 with 100 bootstrap replicates (Tamura et al. 2007). The regional plot of fw7.3 to show the linkage disequilibrium of SNPs with the most significant SNP63621877 was made by LocusZoom (v1.3; Pruim et al. 2010).

Statistical analyses
Two-tailed student’s t-test was used to analyze the significance differences. All of the statistical analyses were carried out using the software Statistical Product and Service Solutions (SPSS) version 10.0 (Link and Pachaly 1975).

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AUTHOR CONTRIBUTIONS
Z.Y. and J.Z. planned and designed the research. M.L., X.W., C.L. and H.L performed experiments, conducted fieldwork, analyzed data and made conclusions based on results. Z.Y., J.Z. and M.L. wrote the manuscript.

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SUPPORTING INFORMATION

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Figure S1. Relative expression analysis of genes related with cell expansion

Figure S2. Alignment of GRAS2, GRAS3 and GRAS7

Five conserved domains of the GRAS family (LHRI, VHIID, LHRII, PFYRE and SAW) are underlined. Conserved amino acids are displayed in black boxes.

Figure S3. GA3 concentration in ovaries of the WT and GRAS2-RNAi lines

Figure S4. Downregulation of GRAS2 suppresses the GA biosynthesis pathway

Figure S5. Effect of GRAS2 silencing on the GA signal transduction pathway

Figure S6. Ovaries (3 dpa) of WT and RNAi lines are treated with 50 μM GA3, and their final fruit weights (45 dpa) are underlined. Conserved amino acids are displayed in black boxes.

Figure S7. Multiple amino acid sequence alignment of GRAS2 and its homologs in other Solanaceae species (pepper, potato, eggplant and tobacco)

Figure S8. GRAS2 and homologous genes could be cloned in the cDNA templates of tomato, pepper, and eggplant

Figure S9. Model of the coding region of GRAS2 and its fragments, conserved region and non-conserved region

Figure S10. The position of 441 bp sequence located in the nucleotide sequence of GRAS2 and expression analysis of genes homologous with 441 bp sequence in GRAS2-RNAi lines
**Figure S11.** Linear regression analysis of fruit weight over the two years of accessions in the resequencing population

**Figure S12.** Diversity of fruit weight in the three groups of the resequencing population

**Figure S13.** QQ plot for associations with fruit weight in the resequencing population

**Table S1.** Data for fruit weight of the resequencing population used in the GWAS analysis (available in separate file.xls)

**Table S2.** Details of four SNPs and one InDel in the coding region of GRAS2 (available in separate file.xls)

**Table S3.** Details of significant signals in the Manhattan plot of fruit weight

**Table S4.** Details of primer sequences used in this study