Physiological Investigation of Quantitative Traits for Fruit Mass Assessment Using a Tomato Introgression Line

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Fruit mass is an important factor for determining the yield of tomatoes (Solanum lycopersicum), with higher mass being an important objective. Most fruit traits, including fruit mass, are quantitative, and numerous quantitative trait loci (QTL) control these traits. Previous studies investigating tomato introgression lines (ILs) revealed several QTLs for fruit yield, and suggested that IL12-1 is a potential line to increase fruit mass. Our aim was to facilitate genetic studies of the diverse characteristics of wild relatives of tomato. Therefore, tomato ILs from a cross between Solanum pennellii and the cultivar S. lycopersicum ‘M82’ were used. ILs that carry a S. pennellii chromosome segment on chromosome 12 of ‘M82’ were evaluated further for fruit mass expansion and regulation. IL12-1-1, a subline of IL12-1, was found to produce large ripening fruits compared with ‘M82’, a phenotype that resulted in increased pericarp thicknesses. To investigate the physiological mechanisms contributing to the increased fruit mass of IL12-1-1, the cell counts of fruit pericarp tissues during fruit development were evaluated. Cell numbers of IL12-1-1 fruit pericarp at 20 days after flowering were higher than those of ‘M82’, a difference that most likely occurred during the cell division phase. In addition, the levels of the phytohormones auxin and cytokinin, which are known to be related to cell division of the fruit tissue, were higher in IL12-1-1 compared with ‘M82’. Therefore, differences in these phytohormones between ‘M82’ and IL12-1-1 may be affected by the number of cells in the pericarp tissues. Expression analysis of Solyc12g005250 (SLKLP) and Solyc12g005310 (SLGH3-15), which are located in the IL12-1-1 region of the S. pennellii chromosome, showed significant differences between ‘M82’ and IL12-1-1 during the cell division phase; a better understanding of IL12-1-1 cellular and molecular features can contribute to the breeding and increased production of tomato crops.

Key Words: auxin, cell number, cytokinin, Solanum pennellii.

Introduction

Tomatoes (Solanum lycopersicum) are one of the most produced and consumed vegetables worldwide. In 2019, the total global production was approximately 180.7 million tons, mainly in China, India, Turkey, and the United States, according to data from the Food and Agriculture Organization of the United Nations (<http://faostat.fao.org/>, Accessed: October 29, 2021). Most fruit quality traits, including fruit yield, are quantitative, and numerous interacting quantitative trait loci (QTL) control these traits (Matsumoto et al., 2021). Plant and organ size, including fruit mass, are determined by the overall cell number and size. Hence, changes in either one of these features may affect their sizes (Guo and Simmons, 2011). Of these, fruit mass (i.e., fruit size and fresh weight) is an important factor for determining the productivity of fruit crops; thus, numerous studies have been conducted to identify QTLs that regulate fruit mass. For example, fw2.2, fw3.2, and fw11.3 were identified as QTLs for fruit size (Frary et al., 2000; Huang and van der Knaap, 2011). In particular, fw3.2 is one of the major QTLs for fruit mass that was detected on chromosome 3 (van der Knaap and Tanksley, 2003) and...
further described at the gene level (Zhang et al., 2012), while fw11.3, which was mapped to the bottom of chromosome 11, was shown to influence fruit mass by controlling carpel and locule numbers (Lippman and Tanksley, 2001). Moreover, subsequent studies identified candidate genes and mechanisms while considering the related phenotypes (Huang and van der Knaap, 2011; Mu et al., 2017). Among these QTLs, fw2.2, another major QTL that is related to fruit mass, changes fruit weight by up to 30% (Frary et al., 2000).

Some wild relatives of tomatoes, including Solanum pennellii, have diverse characteristics. A total of 76 introgression lines (ILs) were developed by crossing the cultivated species S. lycopersicum ‘M82’ with the wild relative S. pennellii to use the wild relatives of tomatoes for genetic studies (Eshed and Zamir, 1995). In particular, fw2.2 was identified on chromosome 2 of S. pennellii using the IL2-5 tomato IL (Alpert et al., 1996). Cell division to enhance cell number and expand cell size are believed to occur in two phases during tomato fruit development, with cell division often occurring within few weeks after pollination (Cong et al., 2002). Subsequent studies demonstrated that fw2.2 transcription is correlated with reduced cell division (Frary et al., 2000) and is inversely correlated with the mitotic activity of pericarp and placental cells in the early stages of fruit development; thus, fw2.2 negatively controls cell division in developing fruits (Cong et al., 2002). Moreover, fw2.2 was shown to induce these regulatory effects on cell division by interacting with the regulatory subunit of casein kinase II at or near the plasma membrane (Cong and Tanksley, 2006). Moreover, analysis of the tomato ILs revealed several QTLs for fruit yield, with IL12-1 being suggested as a potential line to increase fruit mass (Eshed and Zamir, 1995). However, there are no reports on QTLs for fruit yield on chromosome 12 of S. pennellii. Therefore, in the present study, our aim was to further assess ILs that carry an S. pennellii chromosome segment on chromosome 12 of ‘M82’. Additional experiments were performed to help clarify the physiological mechanisms that contribute to this phenotypic variation.

Materials and Methods

Plant materials

Tomato ILs (IL12-1, IL12-1-1, IL12-2, IL12-3, and IL12-3-1), which contained an S. pennellii chromosome segment on chromosome 12, were used along with the S. lycopersicum parent ‘M82’. The plants were grown from 2019–2021 at the University Farm of Utsunomiya University in Tochigi, Moka, Japan (36.49° N, 139.98° E). These ILs and ‘M82’ seeds were sown on March 1, 2019, February 25, 2020, and March 5, 2021, in plug trays with 128 cells filled with nursery soil (Super Mix A; Sakata Seed Corporation, Yokohama, Japan) and cultivated in a field as described by Matsumoto et al. (2021). Fruit samples were collected at 10, 20, and 30 days after flowering (DAF) and at the breaker and ripening stages.

Fruit mass and Brix of tomato ILs

Fruit size (diameter and length) and weight of ‘M82’ and ILs were measured with calipers at the ripening stage and during fruit development. To analyze changes in fruit mass and Brix during fruit development, ‘M82’ and IL12-1-1 fruits were cultivated in the field in 2020 and 2021 and harvested at 10, 20, and 30 DAF and at the breaker and ripening stages. Pericarp thickness was measured in 2021 during fruit development. To analyze changes in Brix during fruit development, five fruits that were harvested on the same day were mixed and freshly squeezed juice from the mixed pericarp tissues was used to measure Brix using a PAL-UX|ACID F5 (Atago Co., Ltd., Tokyo, Japan).

Assessment of pericarp tissue cell counts

To analyze the number of pericarp tissue cells, ‘M82’ and IL12-1-1 tomato lines were grown in a field in 2021, and fruits were harvested at 10, 20, and 30 DAF, and at ripening stages. Formalin fixation, paraffin embedding, and cell number counting of pericarp tissues were performed as previously described (Brukhin et al., 2003; Cheniclet et al., 2005; de Jong et al., 2015; Ogata et al., 2012; Zhang et al., 2013; Zhang et al., 2016). Briefly, pericarp tissues fixed in 10% formalin neutral buffer solution were washed and dehydrated in serial ethanol dilutions (50%, 70%, 80%, 90%, 99.5%, and 100%). These pericarp tissues were then soaked in ethanol/limonene (1:1) and limonene/paraffin (1:1) solutions for 2–6 h and 100% paraffin at 58°C overnight. Limonene was completely volatilized, and pericarp tissues were embedded in paraffin (Paraplast; Leica Biosystems, Wetzlar, Germany). Sections were prepared using a microtome (RM2255; Leica Biosystems) mounted onto glass slides, deparaffinized with limonene and ethanol solutions, and stained with 0.05% (w/v) toluidine blue solution (pH 7.0). Then, cells were counted using a microscope (BX-60; Olympus Corp., Tokyo, Japan).

Detection and quantification of auxin and cytokinin

Ten and 20 DAF fruits were harvested in 2021 to determine phytohormones by liquid chromatography with tandem mass spectrometry. In each analysis, the fruit was sliced at the equatorial area and chopped into 3–5 mm cubes, and 2–3 fruits harvested on the same day were mixed. Three mixed samples were prepared for each analysis. Briefly, 0.2–0.5 g of homogenized samples were soaked in 0.1 mL of [13C6]-indole-3-acetic acid (IAA) and [13C6]-trans-Zeatin (10 ng·mL−1) acetone solution. The vials were sealed and sonicated at 5°C for 20 min and stored at 4°C in the dark for at least four days. The solutions were filtered, and the supernatant was injected into an ultra-performance liquid
chromatography (UPLC)-connected tandem mass spectrometer (Nexera X2; Shimadzu Corp., Kyoto, Japan). Chromatographic separation was achieved using a C$_{18}$ column (Kinetex C18; Phenomenex Inc., Torrance, CA, USA). Mass spectrometry was performed using a triple quadrupole/linear ion trap instrument (QTRAP5500; AB Sciex K.K. AB Sciex, Tokyo, Japan) with an electrospray ionization source system. To quantify auxin (IAA) and cytokinin (trans-zeatin), multiple reaction monitoring was performed to monitor transitions of m/z 176–130 for IAA, 220–136 for trans-Zeatin, and 182–136 and 225–137 for the internal standard, [13C$_6$]-IAA and [2H$_5$]-trans-Zeatin, respectively. Data acquisition and analyses were performed using Analyst and Multi Quant software (version 3.31; AB Sciex).

Gene expression analysis

‘M82’ and IL12-1-1 were grown in a field in 2020, and fruits from five plants were harvested at 10 and 20 DAF. Samples were composited, homogenized, and stored at −80°C before use. Total RNA was extracted using a Plant Total RNA Mini Kit (FA VORGEN Biotech Corp., Ping-Tung, Taiwan), and reverse transcription was performed using a ReverTra Ace qPCR RT Kit (Toyobo Co., Ltd., Osaka, Japan). Quantitative real-time polymerase chain reaction was performed using a LightCycler 96 Real-Time PCR System (Roche, Basel, Switzerland) with a Thunderbird SYBR qPCR Mix (Toyobo). The following PCR cycle conditions were used: 95°C for 60 s; followed by 40 cycles at 95°C for 12 s, 57°C for 15 s, and 72°C for 30 s for Solyc12g005250, and 95°C for 60 s; followed by 40 cycles at 95°C for 10 s, 59°C for 20 s, and 72°C for 30 s for Solyc12g005310. A melting curve was used to confirm the presence of a single product. Primer sequences for Solyc12g005250 and Solyc12g005310 were designed using sequences derived from the latest version of the tomato genome (SL4.0) and gene annotation data (ITA4.0) obtained from the Sol Genomics Network database (<https://solgenomics.net/>, Accessed: August 5, 2021) and Primer 3 (version 4.1.0) software (<https://bioinfo.ut.ee/primer3/>). Primer sequences for gene expression analysis were 5’-GCTAA AAGAAACTCAAGCCATC-3’ and 5’-GGGCTGGAG AAGGTATGTGA-3’ for Solyc12g005250, and 5’-CTT GACAAGTTCTGGTACGTCA-3’ and 5’-AGCAACG ATCGCTTCCAAAG-3’ for Solyc12g005310. The fold changes in gene expression between ‘M82’ and IL12-1-1 were determined using the ΔΔCt method of relative quantification. The gene-specific primer sets for SlActin, which were used as a reference for expression normalization, were obtained from Ikeda et al. (2017).

Statistical analysis

Statistical analyses were performed using Excel (Microsoft Corp., Redmond, WA, USA) and Bell Curve for Excel Version 3.20 (Social Survey Research Information Co., Ltd., Tokyo, Japan). The statistical significance of the results was determined using Welch’s t-test at the 5% and 1% levels.

Results and Discussion

Fruit mass and Brix of tomato ILs

The fruit mass (mean fruit diameter, length, and weight) measured at the ripening stage was found to be greater in IL12-1-1 than in ‘M82’ specimens, whereas no differences were observed between the other ILs (except fruit diameter and length in IL12-2) and ‘M82’ in the field (Fig. 1). Eshed and Zamir (1995) reported that fruit mass is greater in IL12-1 than in ‘M82’. However, in our study, the fruit masses of IL12-1 and ‘M82’ were similar. This could be because fruit mass is a quantitative trait and its variation is affected by various environmental factors. Additionally, our results indicate...
that IL12-1-1, a subline of IL12-1, is a promising line for genetically increasing tomato fruit mass. According to the latest version of the genome and gene annotation data of the Sol Genomics Network database, IL12-1-1 contains approximately 0.18 Mbp of an *S. pennellii* chromosome segment. IL12-1 and IL12-2, that are reported to have increased fruit size, also possess a shared *S. pennellii* chromosomal region from IL12-1-1 (Fig. 2). Thus, the subsequent investigations were focused on IL12-1-1 and its changes in fruit mass during development (Figs. 3 and S1).

Overall, in 2021, significant differences in fruit diameter were observed between ‘M82’ and IL12-1-1 from 20 DAF to ripening stages (Fig. 3A) and in length and weight in the breaker and ripening stages (Fig. 3B, C). A similar trend was observed in 2020 (Fig. S1), and differences in fruit mass were apparent at later developmental stages. Since the fruit mass of IL12-1-1 at the ripening stage was higher than that of ‘M82’ in different years, it is reasonable to hypothesize that the *S. pennellii* chromosome segment inserted in the modified tomato lines contains a gene that promotes mass increase during fruit ripening. However, there is believed to be a trade-off between fruit sugar content and yield (Kanayama, 2017). Therefore, we measured the fruit Brix of ‘M82’ and IL12-1-1 for two years. The results indicated that fruit Brix values in ‘M82’ and IL12-1-1 were similar or sometimes high in IL12-1-1 (Fig. S2). These results suggest that IL12-1-1 has a potential gene that can increase fruit mass without decreasing the sugar content.

Fruit development is divided into three phases in most plants, including tomato fruit: the fruit set, cell division, and cell expansion phases (Gillaspy et al., 1993; Srivastava and Handa, 2005). The fruit set phase involves the development of the ovary and influences cell division and fruit development (Gillaspy et al., 1993). In the second phase, cell division and final cell numbers that constitute the harvested fruits are established (Mapelli et al., 1978; Srivastava and Handa, 2005). Lastly, during the cell expansion phase, the fruit reaches its maximum size (Srivastava and Handa, 2005). The cell division and expansion phases are considered critical to establish the final fruit size of tomato fruit, so we investigated pericarp thickness during fruit development. In this study, significant differences in

![Fig. 2. Schematic diagrams of chromosome 12 in ‘M82’, IL12-1, IL12-1-1, and IL12-2. IL12-1, IL12-1-1, and IL12-2 contain approximately 1.01 Mbp (147 genes), 0.18 Mbp (39 genes), and 61.9 Mbp (5089 genes) of an *S. pennellii* chromosome segment, respectively, according to the latest version of the tomato genome (SL4.0) data from the Sol Genomics Network database.](image)

![Fig. 3. Fruit diameter (A), length (B), weight (C), and pericarp thickness (D) during fruit development. Fruit samples were harvested in 2021. Values indicate means ± SE (n = 24–32). Significant differences between ‘M82’ and IL12-1-1 at *P* < 0.01 and 0.05, calculated using Welch’s *t*-test, are indicated by ** and *, respectively.](image)
pericarp thickness between ‘M82’ and IL12-1-1 during fruit development were noted from 30 DAF (Fig. 3D). Therefore, we focused on pericarp tissues and investigated the cell numbers during fruit development.

**Cell counts of pericarp tissues**

The number of fruit pericarp tissue cells in ‘M82’ and IL12-1-1 were investigated (Fig. 4). Significant differences in cell numbers between ‘M82’ and IL12-1-1 were observed at 20 DAF, whereas no differences were found at other developmental stages (Fig. 4A). The structure of pericarp tissues at 20 DAF was observed using paraffin sections of ‘M82’ and IL12-1-1 fruit (Fig. 4B, C). During the cell division phase, cell layer formation occurs within 5–8 DAF, and randomly oriented cell divisions last for 10–18 DAF (Azzi et al., 2015; Cheniclet et al., 2005), with cell division being completed within 20 DAF. In the present study, cell counts of IL12-1-1 fruit pericarp at 20 DAF were approximately 46.3% higher than those of ‘M82’ (Fig. 4A), a difference that potentially occurred during the cell division phase. Ho (1996) reported that the final fruit size is dependent on the number of cells established in the second fruit developmental phase. Accordingly, the differences in fruit size between ‘M82’ and IL12-1-1 at the ripening stage may have been promoted during the cell division phase. Cell division in fruit tissue is known to involve phytohormones; thus, the levels of phytohormones in the tomato fruit lines were evaluated to elucidate any physiological differences in the cell division phase.

**Detection and quantification of auxin and cytokinin**

Phytohormones, such as auxins, gibberellins, cytokinins, and ethylene, significantly affect plant growth and maturation (Gray, 2004; Srivastava and Henda, 2005). Among these phytohormones, auxin plays a key role in both fruit set and development (Kanayama, 2017; Nishio et al., 2010; Vogel, 2006). The auxin-responsive *SIARF9* is highly expressed in the ovary during pollination and negatively regulates cell division in the early stages of fruit development (de Jong et al., 2015; Vriezen et al., 2008). Similarly, *SIARF5* and *SIARF7* play important roles in fruit development (de Jong et al., 2011; Liu et al., 2018). In addition, cytokinin has been reported to induce cell division after anthesis (Ariizumi et al., 2013), and cytokinin levels increase after flowering and reach a peak at 5 DAF (Matsuo et al., 2012). Therefore, auxin and cytokinin may be closely related to the cell division phase. As a result, the following experiments focused on these phytohormones.

Auxin levels show a constant increase in the first 30 days of fruit development (Srivastava and Henda, 2005), and they induce fruit growth by activating cell division (Ariizumi et al., 2013). Auxin levels in 10 and 20 DAF fruits were determined and were found to be higher in IL12-1-1 than in ‘M82’ fruits at both time points (Fig. 5A). Thus, auxin level differences between ‘M82’ and IL12-1-1 may affect the number of cells in the pericarp tissues. Cytokinin levels were also found to be higher in IL12-1-1 fruit at 20 DAF compared with that in the other samples (Fig. 5B). However, no signifi-
Significant differences were observed in either auxin or cytokinin levels because of the large differences in the measurement values. Cytokinin also induces cell division after anthesis, as described above (Ariizumi et al., 2013); however, further investigations are necessary to further elucidate the mechanisms involved in hormonal regulation of fruit mass.

Gene expression analysis
According to the latest version of the genome and gene annotation data of the Sol Genomics Network database, IL12-1-1 contains approximately 0.18 Mbp of an S. pennellii chromosome segment, which comprises 39 genes (Table S1). Among these genes, Solyc12g005250 and Solyc12g005310 were identified as candidate genes for regulating fruit mass according to the gene annotation data of the Sol Genomics Network database. Solyc12g005250 (SIKLP) encodes a kinesin-like protein that may be responsible for modulating cell division and enlargement in fruit crops (Yang et al., 2013). Expression of SIKLP in IL12-1-1 was higher than in 'M82' at both 10 and 20 DAF (Fig. 6A). In cucumber (Cucumis sativus L.) fruit, kinesin genes are positively correlated with rapid cell division and expansion during early fruit development (Yang et al., 2013). Thus, higher expression of SIKLP in IL12-1-1 fruit may be related to its higher fruit mass.

It was reported that Solyc12g005310 may function as GH3 and is therefore related to auxin regulation (Sravankumar et al., 2018). In tomato, a total of 24 GH3s were identified, and Solyc12g005310 was named SIGH3-15 (Kumar et al., 2012; Sravankumar et al., 2018). SIGH3-15 encodes a group II GH3 enzyme that may function as an IAA-amido synthetase, and this enzyme maintains auxin homeostasis by conjugating excess IAA to amino acids (Sravankumar et al., 2018; Staswick et al., 2005). A previous study investigated the tissue-specific expression of SIGH3 genes, revealing that the expression of SIGH3-15 is barely detectable in fruit tissues compared with levels in leaves, shoots, cotyledons, and flower buds (Kumar et al., 2012). However, in the present study, SIGH3-15 was detectable, and significant differences in its expression were observed at 10 DAF (Fig. 6B). Since auxin levels could be related to the differences in fruit mass between 'M82' and IL12-1-1 (Fig. 5A), SIGH3-15 may contribute to this difference and positively control cell division, unlike fw2.2.

In a series of studies on fw2.2, fine mapping and a detailed physiological analysis revealed further insights into the hypertrophy of fruit mass induced by fw2.2. Therefore, further studies (for example, fine mapping and transformation) are needed to clarify whether Solyc12g005250 and Solyc12g005310 are responsible for increasing the fruit mass in IL12-1-1 and elucidate the function of these genes in fruit tissue.

Conclusion
This study demonstrated that IL12-1-1, a tomato introgression line containing the S. pennellii chromosome segment on chromosome 12 of ‘M82’, produces large ripening fruit compared with the parental ‘M82’. We counted the cell number in the pericarp tissue to reveal the physiological mechanisms accounting for the increase in fruit mass of IL12-1-1, and observed that the IL12-1-1 cell number increased at 20 DAF. The concentrations of auxin and cytokinin in IL12-1-1 fruits were higher than those in ‘M82’. This difference may have affected the cell number in the pericarp tissue. Additionally, expression analysis of Solyc12g005250 (SIKLP) and Solyc12g005310 (SIGH3-15) showed significant differences between ‘M82’ and IL12-1-1 during the cell division phase. Since we have confirmed that IL12-1-1 is a promising line that can increase fruit mass without decreasing sugar content, additional mapping and further studies on the mechanisms leading to increased fruit mass in IL12-1-1 will contribute to the breeding and increased production of tomato crops.

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