Physiological Characterization of Tomato Introgession Line IL5-4 That Increases Brix and Blossom-end Rot in Ripening Fruit

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Fruit Brix is an important indicator in determining the quality of tomatoes (Solanum lycopersicum), and increasing it is an important objective. The production of high Brix tomatoes requires breeding and genetic studies of fruit. During domestication S. lycopersicum lost genetic variation of some wild tomato relative that could be useful for breeding. In this study, we investigated introgression lines (ILs) from a cross between the wild relative Solanum pennellii and the cultivated tomato S. lycopersicum ‘M82’. While there are many genetic and physiological studies that demonstrate the usefulness of tomato S. pennellii ILs, few have investigated the high Brix values of IL fruit. Accordingly, we attempted to detect tomato ILs that resulted in high Brix ripening fruit, in order to obtain valuable genetic and genomic resources for the investigation of phenotypes originating in the S. pennellii genome. IL5-4 may be a line that carries an S. pennellii chromosome segment on chromosome 5 of ‘M82’. Previous research indicated that IL5-4 fruit have higher Brix levels than ‘M82’ fruit. Our results corroborated these findings and revealed Brix changes in fruit during development. We also found that IL5-4 plants showed a higher incidence of blossom-end rot (BER), a major physiological disorder in tomatoes. Therefore, we investigated the physiological mechanism responsible for the higher incidence of BER in IL5-4, by focusing on calcium content, which may be related to BER occurrence. The total and water-soluble Ca contents of fruit tissues were significantly lower in IL5-4 than in ‘M82’ in the proximal part, while no differences were observed in the distal part. Thus, our results suggested that a higher incidence of BER in IL5-4 fruit may not be related to both total and water-soluble Ca contents in the distal fruit tissue, and genetic factors originating in the S. pennellii chromosome may induce high BER incidence in IL5-4. The characterization of IL5-4 in this study showed that it is a valuable genetic and genomic resource for high-Brix breeding stock and for the investigation of novel BER mechanisms.

Key Words: calcium, physiological disorder, Solanum pennellii.

Introduction

Tomatoes (Solanum lycopersicum) are among the most widely produced and consumed vegetable crops worldwide and are valuable sources of micronutrients, including amino acids, vitamins, and antioxidants (Liu et al., 2016; Tieman et al., 2017). According to data from the Food and Agriculture Organization of the United Nations (<http://faostat.fao.org/>, Accessed: November 16, 2020), total global tomato production in 2018 was approximately 182 million tons. Brix in ripening fruit is an important factor in determining fruit quality, and increasing it is one of the priority objectives in tomato cultivation and breeding. Salinity and water stress in the root zone are known to increase fruit Brix by influencing the content of soluble sugars (Adams, 1991; Saito et al., 2008a, b). However, increasing salinity progressively reduces fruit yield (Adams, 1991).

For the production of tomatoes with high Brix val-
ues, without the use of salinity and water stress in the root zone, breeding and genetic studies of tomato fruit are necessary. It is known that the fruit of some wild tomato species have high Brix values; this genetic variation may be useful for the investigation and isolation of quantitative trait loci (QTL) that control fruit Brix (Kanayama, 2017). At the same time, the genetic base of cultivated tomatoes has narrowed, while flavor and nutrient contents were reduced by breeding and domestication (Klee and Tieman, 2013; Overy et al., 2005). However, most fruit quality traits (e.g., Brix and fruit yield) are quantitative, and numerous interacting QTL control these traits, while wild relatives have unfavorable traits (Ikeda et al., 2013). In tomato, a set of 76 introgression lines (ILs) was developed by crossing the cultivated species S. lycopersicum ‘M82’ with the wild relative Solanum pennellii (Eshed and Zamir, 1995). These ILs contain a single short chromosome segment from S. pennellii in the background of the S. lycopersicum genome, while unfavorable S. pennellii traits were mostly removed, thereby enabling QTL analysis on the S. pennellii chromosome segment as a single genetic factor (Kanayama, 2017).

Some examples and studies demonstrate the usefulness of tomato S. pennellii ILs. A tomato QTL for Brix named Brix 9-2-5 was found on chromosome 9, and identified as a cell wall invertase gene (LIN5) using IL9-2-5 (Fridman et al., 2002, 2004). The physiological mechanisms for the high Brix values of IL9-2-5 fruit were investigated and reverse genetic studies of LIN5 conducted (Baxter et al., 2005; Zanor et al., 2009). In addition to these studies, QTL related to harvest index and Brix in ripening fruit were investigated using IL2-1 and IL8-3, respectively (Gur et al., 2010; Ikeda et al., 2013). Calafiore et al. (2019) investigated the fruit yield and quality traits of IL7-3 and identified three candidates, which may increase the level of ascobic acid in the fruit. D’Amelia et al. (2019) used IL12-4 and found a basic Helix-Loop-Helix transcription factor (SlAR), whose silencing influences carotenoid accumulation in tomato fruit, and carried out a reverse genetic approach to gain insight into the function of SlAR. Moreover, large-scale metabolic analyses were performed on ILs to investigate QTL related to metabolites in tomato fruit and seeds (Alseekh et al., 2015, 2017, 2020). Although Eshed and Zamir (1995) investigated and identified several ILs that increase Brix in fruit, few studies focused on and attempted to investigate high Brix values in IL fruit.

Therefore, in this study, we sought tomato ILs with high Brix values in ripening fruit; consequently, we focused on IL5-4, which carries a S. pennellii chromosome segment on chromosome 5 of ‘M82’. Previous studies suggest that the Brix of IL5-4 fruit is higher than that of ‘M82’ fruit (Eshed and Zamir, 1995; Gur and Zamir, 2004; Overy et al., 2005). Our study confirmed these results, and investigated the Brix changes occurring in fruit during development. Furthermore, during our experiment, we found that IL5-4 plants showed a higher incidence of blossom-end rot (BER), a major physiological disorder in tomatoes. Therefore, we also investigated the physiological mechanism responsible for the higher incidence of BER in IL5-4.

Materials and Methods

Plant materials

The experiments were conducted from 2018 to 2020 at the university farm of Utsunomiya University in Tochigi, Moka, Japan (36.49° N, 139.98° E). IL5-4 and ‘M82’ seeds were sown on March 15, 2018, March 1, 2019, and February 25, 2020 in plug trays with 128 cells filled with nursery soil (Super Mix A; Sakata Seed Corporation, Yokohama, Japan), and cultivated in a greenhouse at the university farm of Utsunomiya University before transplanting. The seedlings were transplanted to the field and pots when the first flower opened. The field was fertilized with 0.01 kg·m⁻², 0.06 kg·m⁻², and 0.05 kg·m⁻² of nitrogen, phosphate, and potassium, respectively, and covered with white horticultural mulch cover. The IL5-4 and ‘M82’ plants transplanted to the 24-cm diameter plastic pots (CSM-240; Kaneya Co., Ltd., Minami-Chita, Japan) filled with culture soil (Prime Mix TKS-2; Sakata Seed) were grown in a greenhouse, and lateral buds were removed during growth. We measured the fruit weight and size (diameter and length) during fruit development. The fresh and dry weights of the vegetative organs (i.e., stems, leaves, and roots) were measured immediately after the flowering period and after the first 15 days after flowering (DAF) when the fruit were harvested.

Fruit Brix, acidity, and carbohydrate content

A total of 50 tomato ILs were grown in the field in 2018, and the normal fruit were harvested at the ripening stage for Brix analysis (Fig. S1), while IL5-4 was selected as the target line. For the analysis of changes in Brix and acidity during fruit development, the IL5-4 and ‘M82’ plants were grown in the field in 2019 as described above. Fruits were sampled at 10, 20, and 30 DAF, breaker and ripening stages. Five fruit that were harvested on the same day were mixed, and fresh squeezed juice from mixed pericarp tissues was used for measuring Brix and acidity using PAL-BX|ACID F5 (Atago Co., Ltd., Tokyo, Japan).

Incidence of BER and calcium assay of fruit and vegetative tissues

For the determination and analysis of BER in IL5-4 and ‘M82’ fruit, the plants were grown in plastic pots as described above. The incidence of BER was determined for three years (2018, 2019, and 2020), and fruit that showed BER symptoms were counted. Experiments to determine the calcium (Ca) contents of 15 DAF fruit
and vegetative organs were conducted in 2020. Vegetative organs (stems, leaves, and roots) were sampled just after the flowering period and the first 15 DAF fruit were harvested. Fruit samples were sliced at the equatorial plane and divided into proximal (peduncle side) and distal (style side) parts. A total of 30 fruit were sampled and 10 fruit samples were mixed and constituted the replicates. The samples were dried at 80°C for more than a week and digested with nitric acid and perchloric acid to determine total Ca content using a polarized Zeeman atomic absorption spectrophotometer (Z-2310; Hitachi High-Tech Science Corporation, Tokyo, Japan). The water-soluble Ca content of 15 DAF fruit was extracted from fresh tissue according to the method of Yoshida et al. (2014), and determined using a polarized Zeeman atomic absorption spectrophotometer.

Statistical analysis

All statistical analyses were performed using Excel (Microsoft Corporation, 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 analyzed with Welch’s t-test at the 5% and 1% levels using Bell Curve for Excel Version 3.20 software.

Results

Fruit and plant sizes

The mean fruit fresh weight, diameter, and length were measured from 10 DAF to the ripening stage. All factors that determine fruit size were greater at early developmental stages (10 and 20 DAF) in IL5-4 than in ‘M82’ fruit, whereas no significant differences in fruit size were observed from 30 DAF to the ripening stage (Fig. 1). The fresh weights of the vegetative organs were also investigated (Fig. 2). Significant differences were found between the shoots (stems and leaves) of IL5-4 and ‘M82’ (Fig. 2A, B), whereas no differences were observed between the roots (Fig. 2C). Similar results were observed for the dry weights of these organs (Fig. S2).

Fruit Brix and acidity during development

The Brix values of IL5-4 fruit at the ripening stage were approximately 24.2% and 19.7% higher than in ‘M82’ fruit in 2018 and 2019, respectively (Fig. 3). Changes in Brix and acidity in IL5-4 and ‘M82’ fruit during development were measured in 2019 (Fig. 4). Significant differences in Brix were found between IL5-4 and ‘M82’ fruit at later developmental stages (breaker and ripening stages), while no differences were found between the two lines at other stages (Fig. 4A). The acidity of IL5-4 and ‘M82’ fruit differed 10 DAF and at the breaker stage, whereas no significant differences were observed at other stages, including the ripening stage (Fig. 4B).

Incidence of BER and Ca content

The incidence of BER was higher in IL5-4 than in ‘M82’ for three years (Fig. 5). Ca contents, which may be related to the incidence of BER, were determined for 15 DAF fruit and vegetative organs. The total and water-soluble Ca contents were significantly lower in IL5-4 than in ‘M82’ fruit (Fig. 6). The water-soluble Ca contents of fruit tissues were significantly lower in IL5-4 than in ‘M82’ in the proximal part, while no differences were observed in the distal part. Additionally, we determined the total Ca contents of vegetative organs (Fig. 7). Ca content differences were observed between the shoots of IL5-4 and ‘M82’ when the first 15 DAF fruit were harvested (Fig. 7A, B), whereas no differences were observed among the roots, stems, and leaves sampled just after the flowering period.

Discussion

The agricultural productivity of cultivated tomatoes has increased owing to breeding and domestication; these processes, however, have also narrowed the genetic basis of tomatoes and, consequently, reduced their
flavor and nutrient contents (Gur and Zamir, 2004; Klee and Tieman, 2013; Overy et al., 2005). Additionally, cultivated tomatoes contain only a small fraction of the genetic variation of their wild relatives (Eshed and Zamir, 1994). Therefore, utilizing the diversity of wild tomato relatives lost during domestication is an important objective in breeding (Gur and Zamir, 2004; Tanksley and McCouch, 1997). Some wild relatives of tomatoes have diverse characteristics and may be useful for breeding; however, the genomes of wild relatives also contain some unfavorable traits and have many interacting QTLs (Kanayama, 2017). In order to use the wild relatives of tomatoes for breeding and genetic studies, ILs from a cross between *S. pennellii* and the cultivated *S. lycopersicum* ‘M82’ were developed (Eshed and Zamir, 1994). A previous study investigated 50 ILs that contain a single short *S. pennellii* chromosome segment and revealed several QTL for total soluble solid contents (Eshed and Zamir, 1995). Our study verified this result in terms of finding a promising line, and the fruit Brix values of two ILs (IL5-4 and IL9-3) were observed to be higher than ‘M82’ both in the field and in the greenhouse (Fig. S1). These results are in accordance with the results reported by Eshed and Zamir (1995). Data reported previously were validated by our study. The two aforementioned lines certainly have genes for high Brix values in the *S. pennellii* chromo-

**Fig. 2.** Fresh weights of stems (A), leaves (B), and roots (C). Research was conducted just after the flowering period [0 days after flowering (DAF)], and when the first 15 DAF fruit were harvested (15 DAF). Values indicate means ± SE (n = 5–10). Significant differences between IL5-4 and ‘M82’ at \( P < 0.05 \) and 0.01, calculated using Welch’s \( t \)-test, are indicated by * and **, respectively.

**Fig. 3.** Fruit Brix values at the ripening stage in 2018 (A) and 2019 (B). Values indicate means ± SE (n = 7–15). Significant differences between IL5-4 and ‘M82’ at \( P < 0.01 \) and 0.05, calculated using Welch’s \( t \)-test, are indicated by * and **, respectively.

**Fig. 4.** Fruit Brix values (A) and acidity (B) in 2019 during fruit development. Values indicate means ± SE (n = 6–10). Significant differences between IL5-4 and ‘M82’ at \( P < 0.05 \) and 0.01, calculated using Welch’s \( t \)-test, are indicated by * and **, respectively.
some segment. Among these lines, we focused on IL5-4, as there are currently no detailed studies regarding the high Brix values of IL5-4 fruit and because a higher incidence of BER was observed in those fruit.

Fruit yield and plant growth possibly affect the Brix values of fruit (Ikeda et al., 2013); therefore, we measured the fruit sizes and plant mass. In our study, the IL5-4 and ‘M82’ fruit size were almost the same starting from 30 DAF until the ripening stage (Fig. 1). We also measured the fresh weights of vegetative organs and found significant differences between the shoots of IL5-4 and ‘M82’ at $P < 0.05$ and 0.01, calculated using Welch’s t-test, are indicated by * and **, respectively.

Fig. 5. Incidence of blossom-end rot in 2018 (A), 2019 (B), and 2020 (C). Values indicate means ± SE ($n = 31–248$). Significant differences between IL5-4 and ‘M82’ at $P < 0.05$ and 0.01, calculated using Welch’s t-test, are indicated by * and **, respectively.

Fruit of IL8-3 and IL9-2-5, which carry a S. pennellii chromosome segment on chromosomes 8 and 9 of ‘M82’, also have high Brix values at later developmental stages; these lines accumulate starch at earlier stages of fruit metabolism, when tomato fruit are generally high in starch (Baxter et al., 2005; Ikeda et al., 2013). Therefore, it is possible that the high starch content at the immature fruit stage may account for the high sugar content in ripe IL5-4 fruit. The higher relative mass of shoots in IL5-4 plants supports this hypothesis; however, further investigation is needed to clarify the reasons for the high Brix values of IL5-4 fruit. Sugar composition and starch accumulation analysis, such as those conducted by Baxter et al. (2005) and Ikeda et al. (2013), are necessary to further investigate the high Brix values of IL5-4 fruit.

Additionally, we found that the incidence of BER was higher in IL5-4 than in ‘M82’ for three years (Fig. 5). BER is a physiological disorder that can negatively affect the production of many fruit vegetables, including tomatoes (de Freitas et al., 2011a; Saure, 2001; Taylor and Locascio, 2004; White and Broadley, 2003). It was reported that the fruit yield of IL5-4 was lower than that of ‘M82’ (Eshed and Zamir, 1995), and the number of fruitages in our study were almost the same for three years (data not shown). Therefore, the results of our study suggested that this decrease was possibly induced by a high incidence of BER. BER is believed to be a symptom of a local Ca deficiency physiological disorder in the distal fruit tissue (de Freitas et al., 2012; Ho and White, 2005; Uozumi et al., 2012; White and
Significant differences between IL5-4 and ‘M82’ at \( P < 0.01 \), calculated using Welch’s \( t \)-test, are indicated by **.

![Graphs showing Ca contents in stems (A), leaves (B), and roots (C).](image)

**Fig. 7.** Total Ca contents of stems (A), leaves (B), and roots (C). Research was conducted just after the flowering period [0 days after flowering (DAF)], and when the first 15 DAF fruit were harvested (15 DAF). Values indicate means ± SE (n = 5–10). Significant differences between IL5-4 and ‘M82’ at \( P < 0.01 \), calculated using Welch’s \( t \)-test, are indicated by **.

Broadley, 2003), and is induced within 15 DAF, when fruit growth is most rapid (de Freitas et al., 2011a; Ikeda et al., 2017; Saure, 2001). We then focused on the Ca contents of 15 DAF fruit, and compared IL5-4 with ‘M82’. The total Ca contents were lower in the whole fruit tissue of IL5-4 than in ‘M82’; however, the total Ca contents in the distal fruit tissue were similar in IL5-4 and ‘M82’ (Fig. 6A).

A previous study demonstrated that pectin methylesterase-silenced fruit, which have lower BER incidences, have similar total tissue, cytosol, and vacuolar Ca concentrations; the wild-type fruit, which have higher BER incidences, have lower water-soluble apoplastic Ca contents and higher membrane leakage, which is the first symptom of BER (de Freitas et al., 2012). Another study also suggested that water-soluble apoplastic Ca content is related to BER incidence (Ho and White, 2005), so we investigated the water-soluble Ca contents of IL5-4 and ‘M82’ fruit. However, the water-soluble Ca content was lower in the proximal part of IL5-4 than ‘M82’ fruit tissues, while no differences were observed in the distal part (Fig. 6B). The total Ca contents of vegetative organs were also determined, and differences between IL5-4 and ‘M82’ were observed only in the shoots when the first 15 DAF fruit were harvested (Fig. 7A, B); no differences were observed in roots and the other stages of shoots. It is known that most of the xylemic water and Ca flow towards the leaves and away from the fruit under high leaf transpiration conditions (de Freitas et al., 2011b), and our results showed that the shoot mass of IL5-4 was larger than that of ‘M82’ (Fig. 2). Therefore, high leaf transpiration of IL5-4 may adversely affect the Ca contents in its fruits.

Many studies suggested that BER is a symptom of Ca deficiency physiological disorder in the distal fruit tissue. However, Saure (2014) implied that Ca deficiency is not the cause but a result of BER in tomato fruit because depletion of the apoplastic water-soluble Ca contents in fruit was observed only after BER symptoms were visible. In this study, the total and water-soluble Ca content in the distal fruit tissue were similar in IL5-4 and ‘M82’ (Fig. 6). These results were in agreement with the suggestion of Saure (2014), and our results indicated that a higher incidence of BER in IL5-4 fruit might not be related to both total and water-soluble Ca contents in distal fruit tissue. However, to elucidate this, it would be necessary to investigate the localization of water-soluble Ca because it is suggested that water-soluble apoplastic Ca content is related to BER incidence (Ho and White, 2005). The development of BER requires several steps; abiotic stress increases the production of reactive oxygen species (ROS) and causes lipid peroxidation with an increase in membrane leakiness (Saure, 2014). In our study, the high incidence of BER in IL5-4 fruit may not have been caused by abiotic stress, as both IL5-4 and ‘M82’ plants were grown under the same conditions. Therefore, novel genetic factors originating in the \( S.\ pennellii \) chromosome may induce high BER incidences in IL5-4 and IL5-4 is a valuable resource for unraveling the mechanisms of BER incidence.

In this study, we partially characterized IL5-4, which produces fruit with high Brix values and BER incidence in the ripening stage. The high-quality genome sequence of domesticated tomato cultivar ‘Heinz 1706’ was sequenced (The Tomato Genome Consortium, 2012). Genome and gene annotation data updates, as well as the latest version (SL4.0 of the tomato genome and ITAG4.0 of the annotation) of the Sol Genomics Network database are available in (<https://solgenomics.net/>), Accessed: November 16, 2020). According to SL4.0 and ITAG4.0, the \( S.\ pennellii \) chromosome segment of IL5-4 is approximately 1.25 Mbp, and 156 genes are located in this region. Fine-mapping of IL9-2-5 revealed that high Brix QTL \( (Brix9-2-5) \) encodes a cell wall invertase gene (Fridman and Zamir, 2003; Fridman et al., 2000, 2004), and many genetic
and genomic tomato resources will allow genes affecting the occurrence of BER to be identified and to inform breeding strategies for the elimination of BER (Ho and White, 2005). IL5-4 is a valuable genetic and genomic resource and mapping studies will reveal the primary genes and a comprehensive mechanism for high Brix values and incidence of BER in IL5-4 fruit. In addition, more detailed physiological study is also of interest. In IL8-3 fruit, which shows high Brix value and low BER incidence, the expression of Ca transport genes is enhanced and some of these gene expressions are induced by sugar (Amagaya et al., 2020; Ikeda et al., 2017). Therefore, further studies on the mechanisms leading to high Brix value and low BER incidence in IL5-4 may reveal new insights into sugar metabolism and BER incidence in fruit crops.

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