# Ionomic differences between tomato introgression line IL8-3 and its parent cultivar M82 with different trends to the incidence of blossom-end rot

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**Abstract**

Fruit blossom-end rot (BER) is a serious physiological disorder that can cause significant yield losses in tomato (*Solanum lycopersicum*). Although many studies have suggested that calcium (Ca) deficiency in tomato fruits is a major factor for BER, its onset mechanism has not been fully elucidated. Ionomics is a high-throughput elemental profiling of living organisms that can be applied to understand how differences in plant's physiological status involving inorganic elements. In this study, we examined ionomic differences between the tomato cultivar M82 and its introgression line IL8-3, which contains a short chromosome segment from its wild relative *Solanum pennellii* on chromosome 8 of M82, and has a low incidence of fruit BER. Among the essential elements, Ca showed marked different behavior between the two lines. IL8-3 showed preferential Ca partitioning to fruits compared with M82. The slow growth rate and high Ca concentration observed in IL8-3 fruit during the early growth stages may also be responsible for the low BER incidence in this line. Although Ca ions bind to cell wall pectin and membrane phospholipids, and contribute to cell structure stability, these components showed no

21 significant differences between fruits of the two lines. The fruit ionome differed considerably between M82 and
22 IL8-3, and was not affected by available Ca status in the field. The M82 fruit had higher concentrations of many
23 elements such as magnesium, potassium, boron, and sulfur than did IL8-3, and this trend was also observed in
24 rotten fruit. This suggests that the influence of the leaf (source), rather than the fruit (sink), could be involved in
25 the onset mechanism of BER.
26
27 Key word: blossom-end rot, calcium, introgression line, ionome, *Solanum lycopersicum*, *Solanum pennellii*
28
29 **1. Introduction**
30 Plants are known to use at least 17 essential elements; however, plants also absorb and accumulate various
31 nonessential elements (Watanabe and Azuma, 2021). Ionomics is the study of all essential and nonessential
32 elements that accumulate in living organisms; these are quantified using high-throughput elemental analysis
33 technologies (Salt *et al.*, 2008), and can be applied to various types of plant science studies (Neugebauer *et al.*,
34 2018; Norton *et al.*, 2010; Watanabe *et al.*, 2015). Examining a plant's ionome (mineral profile) provides a
35 comprehensive view of the plant's temporal and spatial mineral dynamics. Moreover, a plant's ionome can be
36 considered an inorganic subset of its metabolome (Broadley *et al.*, 2010). Therefore, changes in a plant's
37 physiological status can be detected by changes in its ionome (Baxter *et al.*, 2008).
38 Generally, fruits and leaves have different ionomes (Watanabe *et al.*, 2016). This is because mineral
39 accumulation in the fruits (sink) largely depends on retranslocation from the leaves (source); however, the
40 efficiency of mineral accumulation may differ by both element and plant species (Brown and Hu, 1998;

41 Marschner, 2012). Retranslocation trends can be estimated by comparing the elemental concentrations of
42 between the leaves and fruits. Broad ionomic surveys of the edible portions of different vegetables showed that
43 tomato (*Solanum lycopersicum* L.) and eggplant (*Solanum melongena* L.) had very low concentrations of several
44 elements in their fruits compared with their leaves, as well as with edible parts of other vegetables (Watanabe *et*
45 *al.*, 2016). In particular, tomato fruits had less than 1/50 of the calcium (Ca) concentration of tomato leaves.
46 Blossom-end rot (BER) is a common physiological disorder in Solanaceae fruit crops. BER occurs in all of the
47 tomato-producing areas of the world, and has been shown to create losses up to 50% (Taylor and Locascio,
48 2004). BER produces a visible necrotic lesion, which is presumed to be a consequence of cell death and the
49 subsequent leakage of solutes into the extracellular space (Ho and White, 2005). The incidence of BER is
50 generally thought to be associated with a Ca deficiency in the distal portion of tomato fruits, where the Ca
51 concentration is generally lower than in the proximal portion of the fruit (Ehret and Ho, 1986). Calcium plays
52 an important role in the stabilization of cell wall pectin and plasma membranes in plants (Marschner, 2012). In
53 pectic structures, Ca ions crosslink homogalacturonan by binding to the non-esterified carboxyl groups of
54 galacturonic acid residues, thereby stabilizing cell walls (Marschner, 2012). The plasma membrane is also
55 usually stabilized when Ca ions bind to phospholipid phosphate groups (Shoemaker and Vanderlick, 2003).
56 Therefore, Ca-deficient fruits often have weak cellular structure. de Freitas *et al.* (2012) suggested that
57 increasing the amount of Ca-ion binding in fruit cell wall pectin reduces the concentration of free Ca ions in the
58 apoplast, which leads to disorders in membrane function and induces leakage.
59 Thus, a number of studies have shown that Ca deficiency in tomato fruits is a major factor for BER. By contrast,
60 however, it was also suggested that a relationship between Ca deficiency and the occurrence of BER is not

always obvious (Saure, 2014). This may be due to the combined involvement of a variety of other factors, such as salinity, other nutrient status, temperature, and humidity, in BER's onset (Ikeda and Kanayama, 2015; Taylor and Locascio, 2004). These abiotic stresses can induce oxidative stress, which is also considered a possible factor for BER (Saure, 2014). In bell pepper (*Capsicum annuum* L.), which belongs to the same family as tomato, Silber *et al.* (2005) reported a negative correlation between fruit manganese (Mn) concentration and BER incidence. This was considered to be a function of Mn-mediated inhibition of reactive oxygen species (ROS) production (Aktas *et al.*, 2005). Thus, it is possible that elements other than Ca are involved in the onset of BER, but these have not yet been investigated.

Tomato introgression lines (ILs) have been produced by crossing *S. lycopersicum* cv. M82 with its wild relative *Solanum pennellii* (Eshed and Zamir, 1995), with each IL containing only a single short chromosome segment from *S. pennellii* in the background of the *S. lycopersicum* genome. These interspecific tomato ILs have revealed thousands of quantitative trait loci (QTL) that affect plant adaptation, morphology, yield, metabolism, and gene expression, and have been widely used in various studies (Gur *et al.*, 2011; Lippman *et al.*, 2007; Yang *et al.*, 2016). Among the ILs, IL8-3, which carries an *S. pennellii* chromosome segment on chromosome 8 of M82, has been shown to have a lower incidence of BER than its parent, M82 (Uozumi *et al.*, 2012). To obtain new insights into the mechanisms of BER onset in tomato, we cultivated the tomato cultivars M82 and IL8-3 in the field and compared their ionomic characteristics.

## 2. Materials and Methods

### *2.1. Field cultivation*

The *S. pennellii* introgression line IL8-3 and its *S. lycopersicum* parent, M82, were cultivated in 2016, 2017, and 2018 in an experimental field at Hokkaido University, Sapporo, Japan (43°04′ N, 141°20′ E). The experimental field had a Gleyic Fluvisol soil type. The general chemical properties of the field soil were described in our previous study (Watanabe *et al.*, 2016). Seeds were germinated in 50-cell plug trays containing commercial nursery soil (Takii & Co., Kyoto, Japan) and grown in a greenhouse at Hokkaido University. When the seedlings reached adequate height (ca. 15 cm), they were transplanted into the field in three replicates (5 plants per replicate). Rows and intra-rows were spaced at 50 cm × 90 cm. Chemical fertilizers were applied to the field at rates of 100 kg·ha$^{-1}$ N (urea:ammonium sulfate = 3:2); 260 kg·ha$^{-1}$ $P_2O_5$ (superphosphate); 200 kg·ha$^{-1}$ $K_2O$ (potassium sulfate); and 0.5 kg·ha$^{-1}$ MgO (magnesium sulfate). Calcium treatment was applied with (+Ca) or without (−Ca) 1750 kg·ha$^{-1}$ CaO (as calcium carbonate) during the 2016 cultivation season only. A Ca treatment was not supplied in 2017 or 2018.

*2.2. Fruit size measurement*

During the 2018 cultivation season, we measured the vertical and horizontal lengths of fruits in the second fruit cluster with 10 replicates at 15, 25, and 45 day after flowering (DAF).

*2.3. Determining the incidence of BER*

The incidence of BER was recorded for each fruit in the second, third, and fourth fruit clusters occurring prior to 15 DAF. The BER incidence rate was calculated as the percentage of plants having one or more BER fruits (all replicates were combined for 15 plants).

101

102 *2.4. Sampling*

103 Fruits were sampled from the second and third fruit clusters at 10, 15, and 25 DAF with three experimental
104 replicates (3–5 fruits per replicate). After washing with deionized water, each fruit was divided equally into
105 proximal and distal halves. The pericarp tissues were frozen in liquid nitrogen and lyophilized. The lyophilized
106 samples were then ground and used for each analysis. Fruits with BER (rotten fruits) were sampled separately
107 from fruits without BER (unrotten fruits). The BER fruits were used only for mineral analysis of the distal fruit
108 halves. Leaves between the second and fourth fruit clusters were sampled nine weeks after transplant. Leaf
109 samples were washed with deionized water, dried in an oven at 70°C for 7 days, weighed, and ground for mineral
110 analysis. At the same time that the leaves were sampled, soil from unplanted rows was sampled with six
111 replicates taken from four points each (0–15 cm depth) and mixed together in equal proportions.

112

113 *2.5. Mineral analysis*

114 Plant samples (leaf plus the distal half of the fruit) in 2016 were digested in 2 mL of 61% (w/v) $HNO_3$ (electronic
115 grade; Kanto Chemical, Tokyo, Japan) in a DigiPREP apparatus (SCP Science, Quebec, Canada) for
116 approximately 2 h at 110°C, until the solution had almost disappeared. When the samples had cooled, 0.5 mL
117 $H_2O_2$ (semiconductor grade; Santoku Chemical Industries, Tokyo, Japan) was added and the samples were
118 heated at 110°C for another 20 min. Once digestion was complete, the tubes were cooled and brought to a volume
119 of 10 mL by adding 2% (w/v) $HNO_3$ in ultrapure water. The concentrations of the following elements were
120 measured using inductively coupled plasma mass spectrometry (ICP-MS: ELAN DRC-e; PerkinElmer, Waltham,

121 MA, USA): lithium (Li), boron (B), sodium (Na), magnesium (Mg), aluminum (Al), phosphorus (P), sulfur (S),
122 potassium (K), Ca, vanadium (V), chromium (Cr), Mn, iron (Fe), cobalt (Co), nickel (Ni), copper (Cu), zinc
123 (Zn), arsenic (As), selenium (Se), rubidium (Rb), strontium (Sr), molybdenum (Mo), cadmium (Cd), cesium
124 (Cs), and barium (Ba). Air-dried soil samples were extracted with 1 M ammonium acetate (soil:ammonium
125 acetate = 1:5, w/v)] for mineral analysis. Mineral concentrations in the ammonium acetate extracts were
126 determined using ICP-MS after digestion with $HNO_3$, as described above.
127
128 *2.6. Cell wall isolation*
129 Crude cell walls were prepared from fruit samples in 2016 and 2017 as described by Zhong and Lauchli (1993),
130 with minor modifications. In brief, an approximately 20-mg sample of the distal half of a lyophilized fruit was
131 obtained from the second fruit cluster and homogenized on ice with a mortar and pestle in 75% ethanol. The
132 homogenate was centrifuged at 10,000 × g for 10 min, and the supernatant was discarded. The pellet was washed
133 three times with an ice-cold acetone and methanol:chloroform mixture (1:1, v/v), followed by methanol. The
134 supernatant was discarded, and the final pellet was dried under vacuum. The dried cell wall material was then
135 treated with 20 units of α-amylase, rinsed with ultrapure water, and lyophilized. Concentrations of K, Ca, Mg,
136 and B in crude cell walls obtained from the samples in 2017 were analyzed by ICP-MS as described above.
137 Crude cell walls obtained from the samples in 2016 were used for pectin analysis.
138
139 *2.7. Pectin analysis*
140 Pectin extraction was conducted according to Zhu *et al.* (2012), with minor modifications. In brief, pectin was

extracted from the crude cell walls obtained from the samples in 2016 by incubating three times with 1 mL of 20 mM ammonium oxalate at 70°C for 1 h. The supernatant containing the oxalate−soluble pectin was collected after centrifugation at 14,000 × g for 10 min. The pectin fraction's uronic acid concentration was assayed using galacturonic acid as the standard, according to the method of Blumenkrantz and Asboe-Hansen (1973). Briefly, 200 µL of pectin extract was incubated with 1.2 mL of 98% (w/v) $H_2SO_4$ (containing 12.5 mM $Na_2B_4O_7 \cdot 10H_2O$) at 100°C for 5 min. After cooling, 20 µL of 0.15% (w/v) m−hydroxydiphenyl in 0.5% (w/v) NaOH was added to the solution. The sample was allowed to stand at room temperature for 20 min, then the absorbance was spectrophotometrically measured at 492 nm. Because carbohydrates produce a pinkish chromogen with sulfuric acid/tetraborate at 100°C, the absorbance of a blank sample was measured with 20 µL 0.5% NaOH in place of the m−hydroxydiphenyl. The blank sample's absorbance was subtracted from the total absorbance. To determine the degree of pectin methylation, crude cell walls obtained from the samples in 2016 were saponified, and the amount of cell wall polymer-bound methyl-esters in the released methanol fraction was measured colorimetrically (Hermans *et al.*, 2011). The degree of methylation was calculated as the molar ratio between the methanol and uronic acids, as described above.

*2.8. Phospholipid analysis*

Phospholipids were extracted from the distal halves of lyophilized fruit samples in 2017 using the method described by Bligh and Dyer (1959) and modified by Uemura and Yoshida (1984). A 50-mg lyophilized sample was homogenized three times using a mortar and pestle with a mixture with 2.5 mL 2-propanol, 2.5 mL chloroform, and 1.25 mL $H_2O$. The homogenized sample was centrifuged at 1,000 × g for 5 min, and the

chloroform layer was separated. Next, 5 mL of chloroform was added to the residue, shaken for 5 min, and centrifuged, and the chloroform layer was removed. This layer was then filtered through No. 6 filter paper (Advantec, Tokyo, Japan) and shaken several times with the same volume of 0.1 M KCl to remove protein and water−soluble molecules (e.g., ATP). The chloroform layer then was dehydrated with $Na_2SO_4$, evaporated at 40°C, and resolubilized in 1 mL of chloroform:methanol (2:1, v/v). Phospholipids were quantified by measuring the phosphorus concentration in the lipid extract using the malachite green spectrophotometric method (Van Veldhoven and Mannaerts, 1987) after wet digestion with sulfuric acid.

### *2.9. Statistical analyses*

The mineral concentration data were analyzed on a dry-weight basis. All statistical analyses were performed using Sigmaplot 14.0 (Systat Software, Inc., San Jose, CA, USA) and Excel for Microsoft 365 (Microsoft, Redmond, WA, USA). In order to compare plant ionomes, we conducted a principal component analysis (PCA) using the concentrations of all sample elements after standardizing the variables to a mean of zero and variance of one.

## **3. Results**

### *3.1. Incidence of BER*

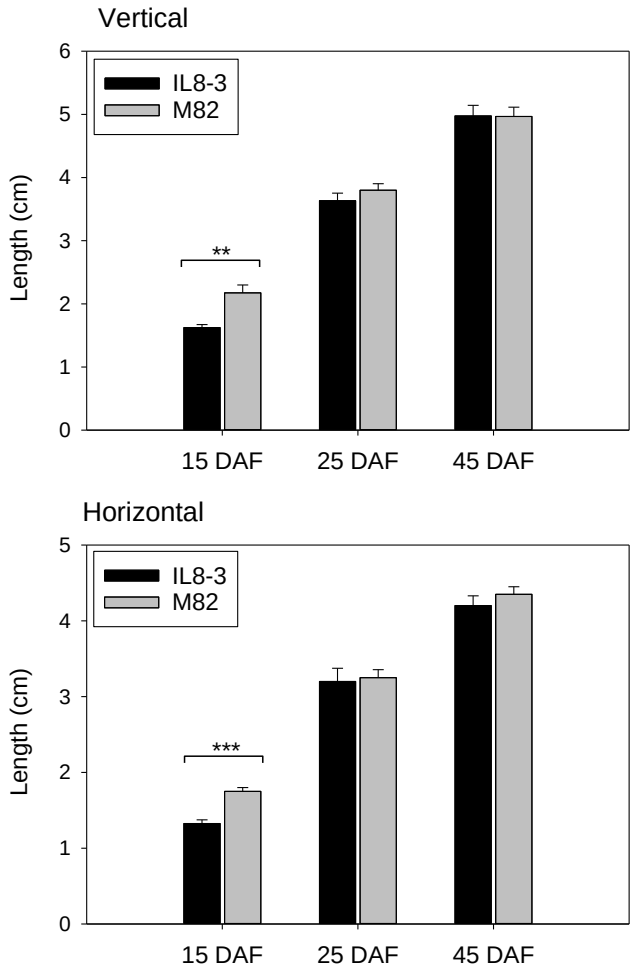
In all years of the study, IL8-3 showed a lower incidence of BER compared with its parent cultivar, M82 (Fig. S1). Calcium treatment did not affect the incidence of BER (Fig. S1).

181 *3.2. Fruit growth*

182 Vertical and horizontal lengths of fruits at 15, 25, and 45 DAF are shown in Fig. 1. Although M82 had

183 significantly larger fruits than did IL8-3 at 15 DAF, no differences between lines were observed after this point.

184




185 Fig. 1. Vertical and horizontal lengths of fruits in IL8-3 and M82 at 15, 25, and 45 days after

186 flowering (DAF). Values represent the means of 3–6 replicates, and bars indicate ± standard errors.

187 Significant differences between lines, as determined by Student's t-test, are indicated by ** and ***

188 at $P < 0.01$ and $P < 0.001$, respectively.

189

190 *3.3. Soil mineral concentrations*

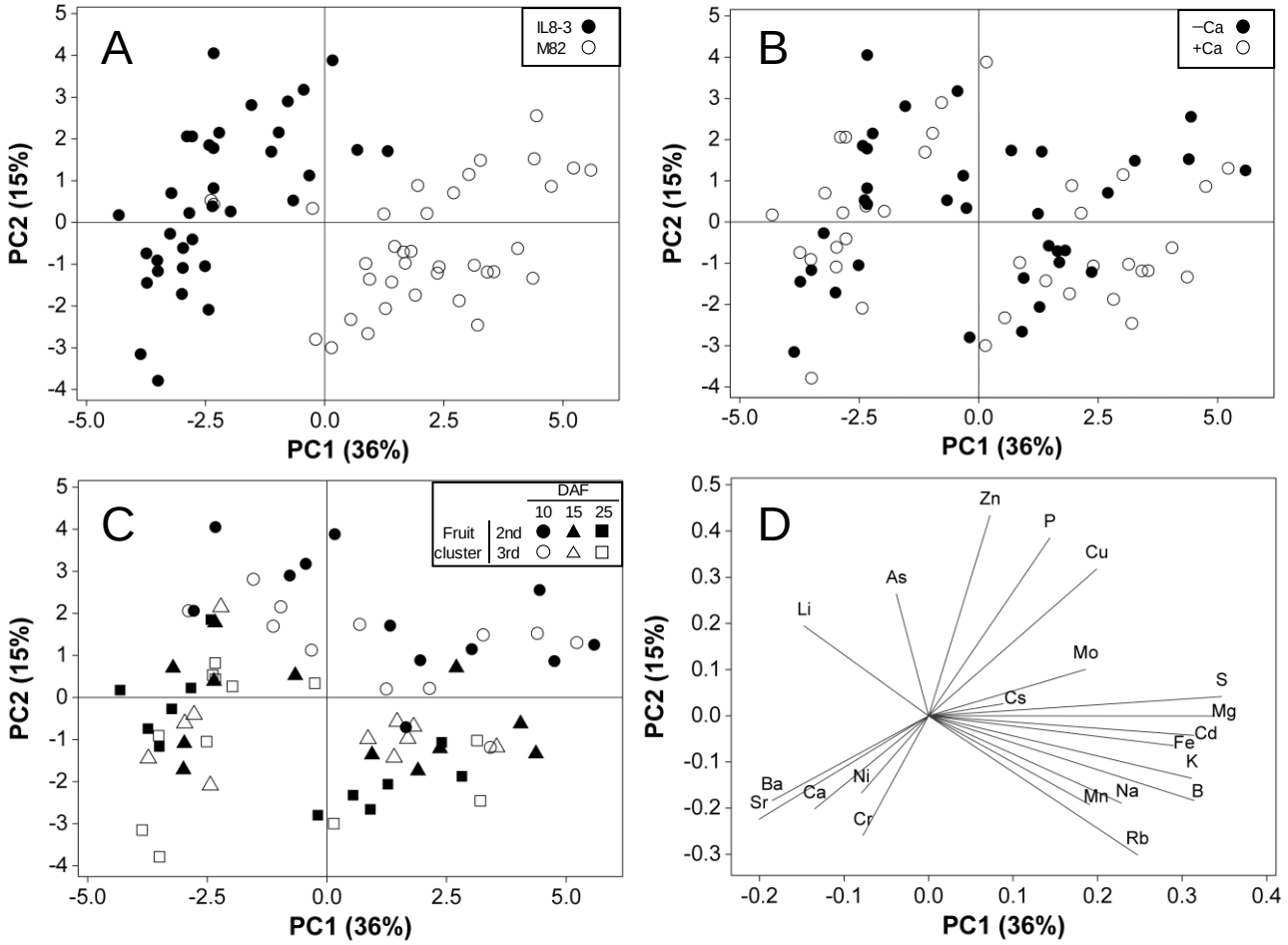
191 The ammonium acetate-extractable concentrations of each soil element are shown in Table S1. Calcium
192 fertilization did not affect most elements' soluble concentrations in soil (Table S1). However, Ca concentration
193 was significantly higher in the +Ca treatment, and the concentrations of some trace elements were significantly
194 higher in the −Ca treatment.

195

196 *3.4. Mineral profile comparison*

197 Using PCA, we compared fruit ionomes between lines (Fig. 2A), Ca treatments (Fig. 2B), and by DAF and
198 between different fruit clusters (Fig. 2C) based on each mineral's concentration in the non-BER (unrotten) fruits.
199 The PCA scores were presented by combining the first (PC1) and second (PC2) principal components. The
200 ionome in the distal part of non-BER fruit differed between IL8-3 and M82 (Fig. 2A); however, the ionome was
201 not affected by Ca treatment or by fruit cluster (Figs. 2B and 2C). The ionome at 10 DAF showed a different
202 trend compared to that at 15 and 25 DAF (Fig. 2C). The PCA showed a large ionomic difference between BER
203 and non-BER fruits, though there was not a clear difference between IL8-3 and M82 in BER fruits (Fig. S2).
204 Leaf ionome was not significantly affected by the Ca treatment, though there were different ionomic trends
205 between the IL8-3 and M82 lines (Fig. 3).

206




208 Fig. 2. Principal component analysis (PCA) of elements in the distal part of non-BER fruit in two
209 tomato lines. Element concentration values were used after standardizing the variables to a mean of
210 zero and a variance of one. PC1 and PC2 scores were plotted to indicate differences by plant line
211 (A), calcium (Ca) treatment (B), and fruit cluster and days after flowering (DAF) (C). The loading plot
212 is shown in (D).

213

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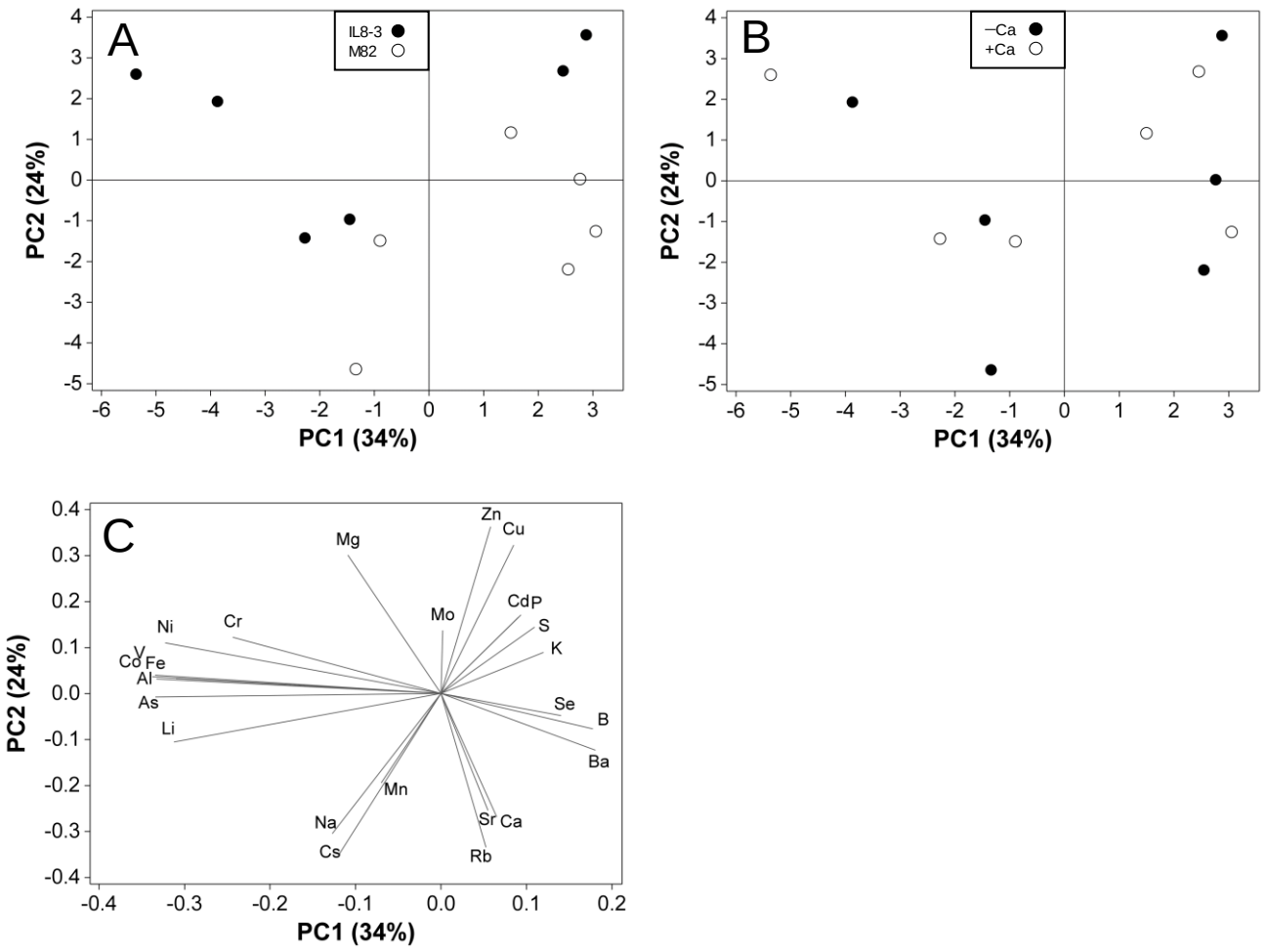



Fig. 3. Principal component analysis (PCA) of elements in the leaves from two tomato lines. Element concentration values were used after standardizing the variables to a mean of zero and a variance of one. PC1 and PC2 scores were plotted to indicate different plant lines (A) and calcium (Ca) treatment (B). The loading plot is shown in (C).

Significant differences in elemental concentrations between M82 and IL8-3 were tested using Student's t-test and shown as a heat map (Fig. 4). Because there was no obvious difference in the ionome between fruit clusters and Ca treatments (Fig. 2), the concentration data for all fruit clusters and Ca treatments were pooled. Both BER (rotten) and non-BER (unrotten/healthy) fruits were compared. In unrotten fruits, many elements had

significantly higher concentrations in M82 than in IL8-3; however, some elements, including Li, Ni, Zn, Ca, Sr, and Ba, showed lower concentrations in M82 in the early stages of fruit growth (Fig. 4). In contrast, the leaf concentrations of Ca, Sr, and Ba were significantly higher in M82 than in IL8-3 (Fig. 4). The BER fruits showed a similar trend to that of the non-BER fruits, but the difference between the lines was smaller (Fig. 4). Furthermore, many elements had higher concentrations in the BER fruits, regardless of the line; this difference was especially large in IL8-3 (Fig. S3).

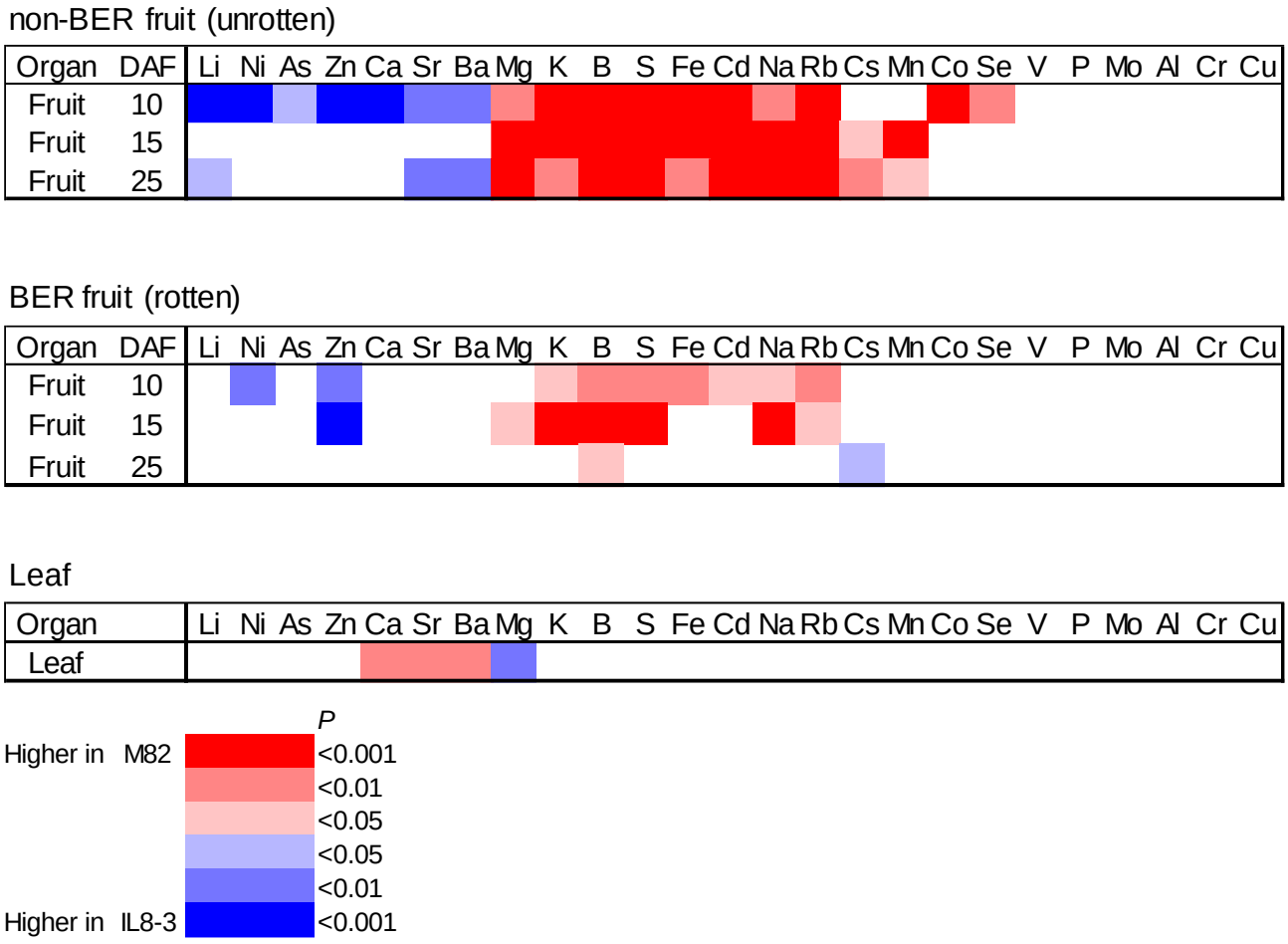



Fig. 4. Heat map analysis showing differences in elemental concentrations between the tomato lines IL8-3 and M82 in the distal part of non-BER (unrotten) and BER (rotten) fruits, and leaves (Student's t-test).

Finally, we determined the correlation between Ca and B, both of which are constituents of pectin. We observed a significant positive correlation between Ca and B in IL8-3, while no such correlation was observed in M82 (Fig. 5).

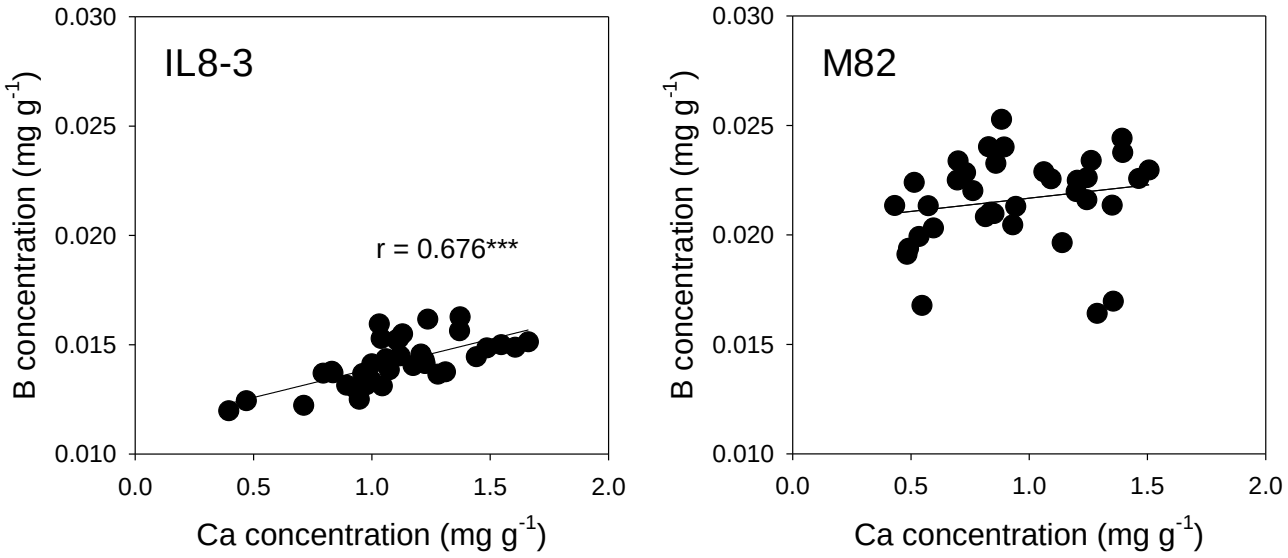



Fig. 5. Correlation between calcium (Ca) and boron (B) concentration in the distal halves of fruit in the tomato lines IL8-3 and M82. *** indicates a significant correlation ($P < 0.001$).

*3.5. Mineral concentration in fruit cell walls*

Cell walls were isolated from the distal halves of fruit to determine the concentrations of K, Ca, Mg, and B. Similar to the fruit (Fig. 4), Ca concentration in fruit cell walls was higher in IL8-3 than in M82 at 10 DAF (Fig. 6). The fruit cell wall concentrations of K, Mg, and B did not differ significantly between the two lines at 10, 15, or 25 DAF.

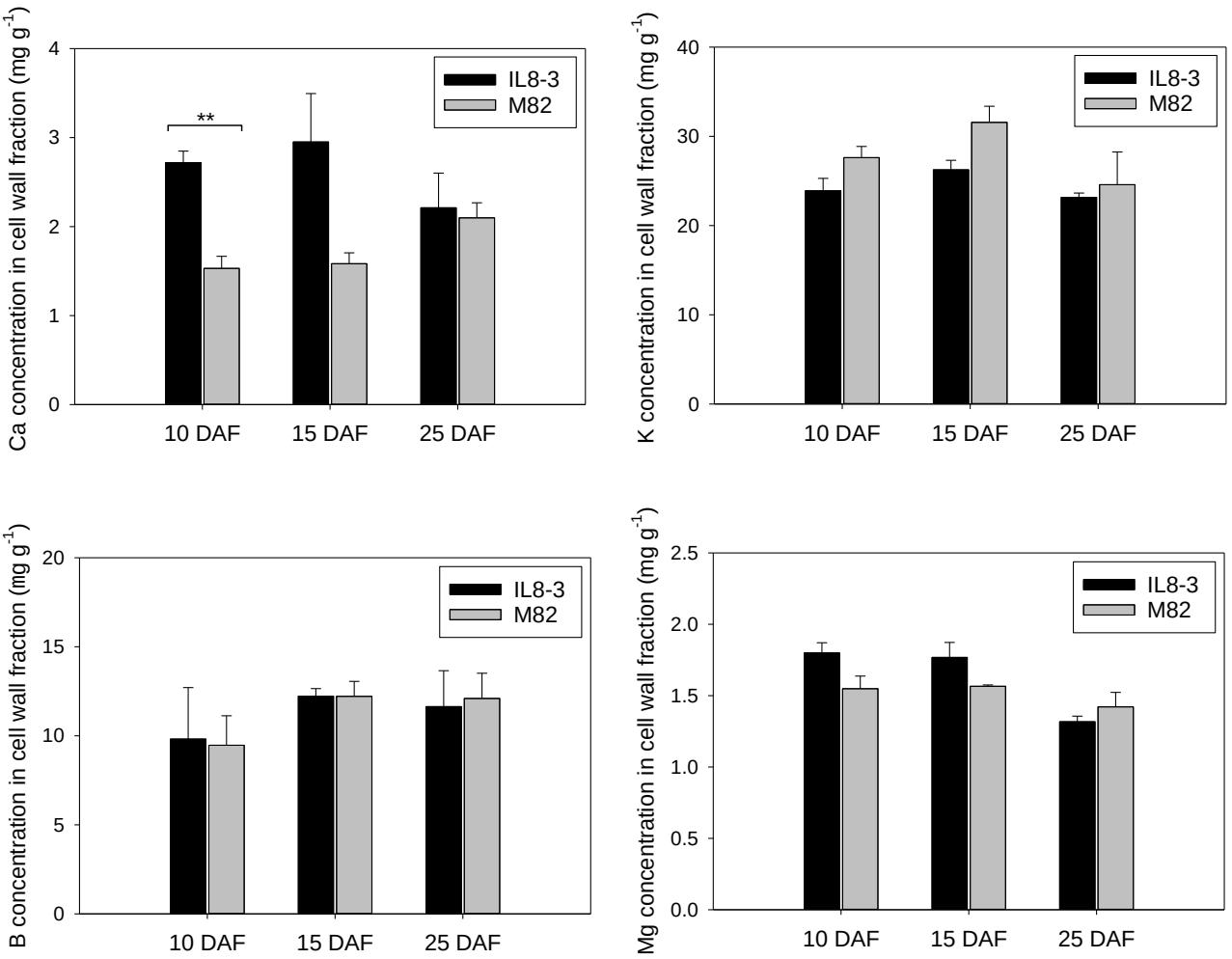
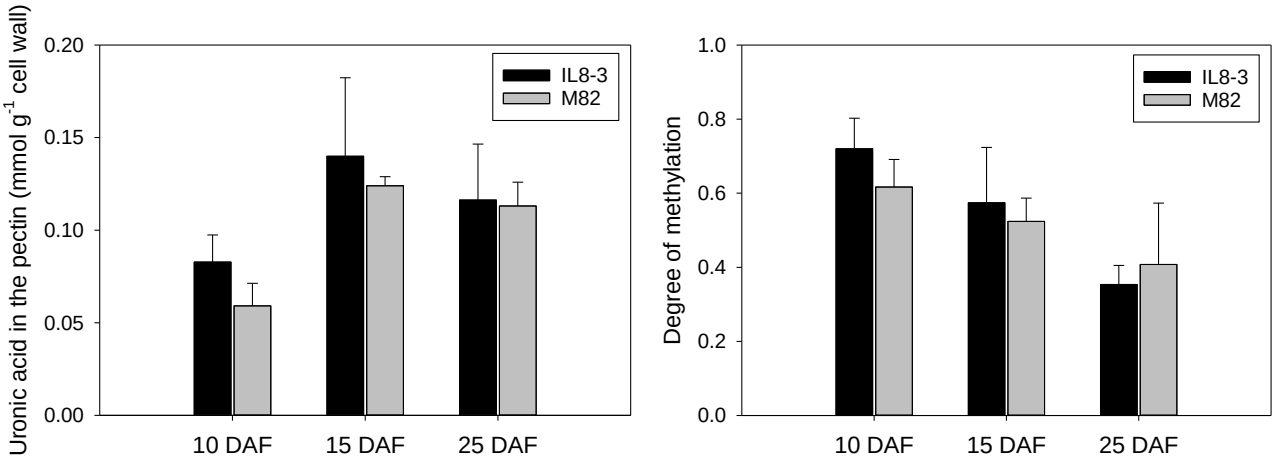



Fig. 6. Concentration of Ca, K, B, and Mg in cell walls of the distal halves of fruit in the tomato lines IL8-3 and M82 at 10, 15, and 25 days after flowering (DAF). ** indicates a significant difference between lines, as determined by Student's t-test *(P* < 0.01).

### *3.6. Pectin in distal part of fruits*

Neither pectin concentration, nor the degree of pectin methylation, differed significantly between IL8-3 and M82 at 10, 15, or 25 DAF (Fig. 7). The degree of pectin methylation tended to decrease with fruit development.

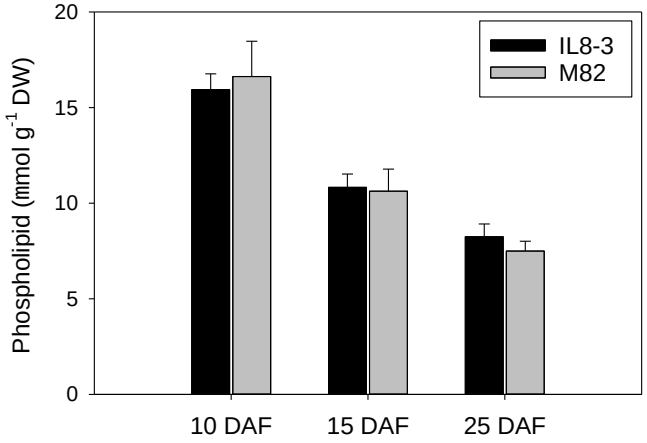



256 Fig. 7. Pectin concentration and degree of methylation in the distal halves of fruit from the tomato lines
257 IL8-3 and M82 at 10, 15, and 25 days after flowering (DAF). Results were considered to be not
258 significant when $P < 0.05$ (Student's t-test).

259

260 *3.7. Phospholipid concentration in distal part of fruits*

261 Phospholipid concentration in the distal halves of fruit decreased with fruit development, and no significant
262 differences were found between the two lines at 10, 15, or 25 DAF (Fig. 8).




263 Fig. 8. Phospholipid concentration in the distal halves of fruit from the tomato lines IL8-3 and M82 at
264 10, 15, and 25 days after flowering (DAF). Results were considered to be not significant when $P <$

0.05 (Student's t-test).

## 4. Discussion

BER is an important physiological disorder in tomato fruits. Although Ca deficiency is known to be one of the major factors for BER in tomato fruits, its onset mechanism is not fully understood. Ionomics is the study of the ionome, which is defined as the total mineral nutrient and trace element composition of an organism or tissue, and represents the inorganic components of the organism's cellular and other systems (Salt, 2004). The ionome is closely related to metabolic reactions, and ionomic differences may indirectly reflect metabolic differences. In this study, we investigated the ionomic difference between the tomato lines IL8-3 and M82, which have different susceptibilities to BER, to obtain further insight on the mechanistic factors involved in BER onset. As previously reported by Uozumi *et al.* (2012), IL8-3, which carries an *S. pennellii* chromosome segment on chromosome 8 of M82, has a lower incidence of BER than does its parent line, M82 (Fig. S1). In the present study, Ca fertilization did not affect the incidence of BER, possibly due to a high enough concentration of available Ca already present in the field soils (Table S1).

PCA is a common method used for comparing ionomes. In this study, PCA was first performed on fruits and leaves using measured element concentration data. Interestingly, the ionome in the distal half of the unrotten (healthy) fruit was completely different between IL8-3 and M82 (Fig. 2A). Although Ca fertilization significantly affected the ammonium acetate-extractable concentrations of several soil elements, including Ca (Table S1), the Ca fertilization treatment did not significantly affect the ionome; this suggests that the tomato fruit ionome is less sensitive to the cultivated soil environment, and is more genetically influenced. Similarly,

285 the tomato leaf ionome did differ between the two lines, but was not affected by the Ca fertilization treatment
286 (Fig. 3).
287 To thoroughly examine the differences between IL8-3 and M82 in the elemental accumulation characteristics of
288 the fruits and leaves, each element's concentration was compared using the combined data for all fruit clusters
289 and Ca treatments (Fig. 4). The concentration of alkaline earth metal elements (Ca, Sr, and Ba) in M82 tended
290 to be lower in fruits and higher in leaves than in IL8-3. The fruit Ca concentration was significantly lower in
291 M82 than in IL8-3 during the early stage of fruit growth (10 DAF). Conversely, the fruit concentrations of
292 various essential elements, such as Mg, K, B, S, Fe, and Mn, as well as the nonessential alkaline metal elements
293 Na and Rb, were higher in M82 than in IL8-3.
294 Since it is known that Ca, an essential element, has similar uptake and transport trends to those of the
295 nonessential elements Sr and Ba (Watanabe *et al.*, 2016; Watanabe *et al.*, 2015), it is understandable that these
296 elements show similar accumulation trends. It is well known that Ca is the most important element contributing
297 to the incidence of BER in fruits of Solanaceae crops, including tomato. Calcium accumulation in fruits is
298 reportedly low during the early stage of fruit development in M82 (Ikeda *et al.*, 2017; Uozumi *et al.*, 2012). One
299 of the most important roles of Ca in plant cells is the stabilization of cell wall pectin. Pectin is understood to be
300 synthesized in the Golgi apparatus, and is secreted into the cell walls in a highly methylated form (Atmodjo *et*
301 *al.*, 2013; Micheli, 2001). The methylated pectin is subsequently demethylated by the enzyme pectin
302 methylesterase, and free carboxyl groups are produced in the galacturonic acid residues (Micheli, 2001). In
303 highly methylated pectin, junction zones are formed by the crosslinking of homogalacturonan by hydrogen
304 bonds and hydrophobic interactions (Oakenfull and Scott, 1984). In many plant species, including tomato,

progressive demethylation of homogalacturonan by pectin methylesterase commonly occurs during fruit development (Paniagua *et al.*, 2014) and generates carboxyl groups. Then, Ca mediates the crosslinking of homogalacturonan chains by binding to these carboxyl groups. Therefore, an insufficient concentration of Ca ions supplied to the fruit will be unable to accommodate the increasing carboxyl groups occurring with fruit growth and pectin demethylation; as a result, the stability of the cell wall pectin will decrease, which can increase the incidence of BER (Marschner, 2012). This indicates that both the pectin content and its degree of methylation, in addition to Ca concentration, are involved in the development of BER in fruits. de Freitas *et al.* (2012) suggested that lower levels of free apoplastic Ca ions lead to impaired plasma membrane stabilization, inducing membrane leakage and finally resulting in BER incidence; this occurs because the Ca ions bridge the phospholipid phosphate groups at the membrane's surface (Legge *et al.*, 1982). The concentration of free apoplastic Ca ions required for membrane stabilization depends on the amount of demethylated pectin in the cell wall, as described above, as well as the proportion of Ca ions that is transported to the fruit (de Freitas *et al.*, 2012). Therefore, we analyzed the concentration of cell wall pectin, its degree of methylation, and the phospholipid concentration in fruits of the tomato lines IL8-3 and M82. We found no significant differences in any of these variables between the two lines, although some changes were observed on the basis of DAF (Fig. 7). These results suggest that the low Ca accumulation in the distal part of the fruit itself is one of the factors related to the difference in BER incidence between M82 and IL8-3. Interestingly, the Ca, Sr, and Ba concentrations of leaves collected from between the second and fourth fruit clusters were higher in M82 than in IL8-3 (Fig. 3). This indicates that these elements are partitioned differently between leaves and fruits in the two lines, with IL8-3 distributing more Ca to fruits than does M82.

325 Several elements were shown to have high concentrations in M82 fruit. Of these elements, K is known to be
326 involved in fruit growth and the transport of photosynthetic assimilates to the fruit (Memgel and Viro, 1974;
327 Tavallali *et al.*, 2018). Marcelis and Ho (1999) reported that fruit size was positively correlated with BER
328 incidence and negatively correlated with Ca concentration in sweet pepper, which belongs to the same family
329 (Solanaceae) as tomato. In fact, M82 had larger fruits than those of IL8-3 at 15 DAF (Fig. 1), and a significantly
330 lower Ca concentration in both the fruit and fruit cell wall compared with IL8-3 at 10 DAF (Figs. 4 and 6). Ikeda
331 *et al.* (2017) also reported that the rate of fruit enlargement was slower in IL8-3 than in M82, and that IL8-3 had
332 a higher fruit Ca concentration in the early fruit growth stage (11–15 DAF). Tomato fruit enlargement, i.e., the
333 accumulation of photosynthates in the fruit, is thought to occur mainly via phloem transport, whereas Ca
334 accumulation occurs mainly via xylem transport (Ho *et al.*, 1987). Marcelis and Ho (1999) stated that BER can
335 occur when the balance between photosynthate and Ca accumulation is upset. Ho and White (2005) suggested
336 that BER is initiated by a cellular dysfunction during fruit cell expansion when there is a local, transient Ca
337 deficiency. From the above, we considered that two of the main factors contributing to the difference in BER
338 incidence between M82 and IL8-3 are the rate of fruit growth enlargement, and the supply of Ca to the fruit
339 during the early stages of fruit growth.
340 Among the other elements with higher concentrations in M82 fruit, B is one that could be involved in BER. Like
341 Ca, B is required for crosslinking of the pectic polysaccharide rhamnogalacturonan II, and is essential for
342 maintaining cell wall structure (Miwa *et al.*, 2013). Therefore, B and Ca together have been spray-applied on
343 tomato buds, flowers, and fruits to ameliorate the incidence of BER (Liebisch *et al.*, 2009). However, our results
344 showed M82 fruits to have a higher B concentration than IL8-3 fruits, despite M82 having a higher incidence of

345 BER (Fig. 4). This implies that the proportion of rhamnogalacturonan II in pectin could be negatively related to
346 BER development. However, when we determined the B concentration in isolated cell walls, we found no
347 difference between M82 and IL8-3 (Fig. 6). Furthermore, Ca and B concentrations in the distal parts of IL8-3
348 fruits were found to have a significantly positive correlation, suggesting that both Ca and B are used for pectin
349 structure; in contrast, no such correlation was found for M82 (Fig. 5). These results suggest that the high B
350 concentration in M82 fruit was not due to an increase in its binding to rhamnogalacturonan II, but due to an
351 increase in the concentration of soluble B.
352 Despite showing significant concentration differences between lines, other elements' involvement in BER is not
353 clear. Manganese has been previously reported to inhibit ROS production, and may be involved in BER
354 incidence (Aktas *et al.*, 2005). In the present study, Mn had a higher concentration in M82 than in IL8-3 (Fig.
355 4), and was not correlated with BER susceptibility. Among the nonessential elements that had higher
356 concentrations in M82, Na and Rb are known to behave similarly to K in plants, and Cd is known to behave
357 similarly to Fe (Watanabe and Azuma, 2021; Watanabe *et al.*, 2016). These nonessential elements may not have
358 any physiological significance in the onset of BER. However, it is very possible that regardless of their
359 essentiality, differences in the accumulation of these elements may directly or indirectly relate to metabolic
360 differences in plants. As for the elements that showed similar accumulation trends in both rotten and unrotten
361 fruits (Fig. 4), the influence of the leaf (source) rather than the fruit (sink) may be significant for their
362 accumulation in the fruits, due to rotten tissue being less physiologically active than healthy tissue. The
363 involvement of leaves (e.g., photosynthesis) in the development of BER will also need to be studied.
364

## 5. Conclusions

The results obtained in this study indicate that the low incidence of BER in IL8-3 is due to a slower fruit growth rate and higher Ca concentration during the early stages of fruit growth, and a preferential Ca distribution to the fruit over the leaves. This study confirmed that Ca deficiency is involved in the development of BER, as has been reported in previous studies, but also implicated the involvement of many other elements, including K and B, in the development of BER. In genes whose expression differed significantly between IL8-3 and M82 fruits, as shown by the transcriptome analysis performed by Ikeda *et al.* (2016), no genes were found that could be involved in the transport or accumulation of these other elements in genes showing different expression levels between M82 and IL8-3 fruits. This suggests that the ionomic differences between IL8-3 and M82 may be secondary to metabolic differences. Future studies should aim to comprehensively investigate the relationship between ionomic, metabolomic, and transcriptomic changes in BER incidence. Furthermore, we would like to investigate whether the relationship between ionomic trends and BER susceptibility that was observed between IL8-3 and M82 is also observed among other tomato varieties.

## CRediT authorship contribution statement

**Toshihiro Watanabe**: conceptualization, methodology, data curation, formal analysis, writing - original draft, funding acquisition. **Ryota Tomizaki**: investigation, data curation. **Ryotaro Watanabe**: investigation, data curation. **Hayato Maruyama**: investigation. **Takuro Shianno**: supervision. **Masaru Urayama**: investigation, supervision. **Yoshinori Kanayama**: conceptualization, writing - review & editing, supervision, funding acquisition.

## Declaration of competing interest

The authors declare that they have no competing interests.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## Appendix A. Supplementary data

The following data are supplemental to this article:

FigsS1-S3.pptx

TableS1.pptx

## References


Aktas, H., Karni, L., Chang, D.-C., Turhan, E., Bar-Tal, A., Aloni, B., 2005. The suppression of salinity-associated oxygen radicals production, in pepper (*Capsicum annuum*) fruit, by manganese, zinc and calcium in relation to its sensitivity to blossom-end rot. Physiol. Plant. 123, 67-74.

Atmodjo, M.A., Hao, Z., Mohnen, D., 2013. Evolving views of pectin biosynthesis. Annu. Rev. Plant Biol. 64,

747-779.

Baxter, I.R., Vitek, O., Lahner, B., Muthukumar, B., Borghi, M., Morrissey, J., Guerinot, M.L., Salt, D.E., 2008. The leaf ionome as a multivariable system to detect a plant's physiological status. Proc. Natl. Acad. Sci. USA 105, 12081-12086.

Bligh, E.G., Dyer, W.J., 1959. A rapid method of total lipid extraction and purification. Can. J. Biochem. Physiol. 37, 911-917.

Blumenkrantz, N., Asboe-Hansen, G., 1973. New method for quantitative determination of uronic acids. Anal. Biochem. 54, 484-498.

Broadley, M.R., Hammond, J.P., White, P.J., Salt, D.E., 2010. An efficient procedure for normalizing ionomics data for *Arabidopsis thaliana*. New Phytol. 186, 270-274.

Brown, P.H., Hu, H., 1998. Phloem Boron Mobility in Diverse Plant Species. Bot. Acta 111, 331-335.

de Freitas, S.T., Handa, A.K., Wu, Q., Park, S., Mitcham, E.J., 2012. Role of pectin methylesterases in cellular calcium distribution and blossom-end rot development in tomato fruit. Plant J. 71, 824-835.

Ehret, D.L., Ho, L.C., 1986. Translocation of calcium in relation to tomato fruit growth. Ann. Bot. 58, 679-688.

Eshed, Y., Zamir, D., 1995. An introgression line population of Lycopersicon pennellii in the cultivated tomato enables the identification and fine mapping of yield-associated QTL. Genetics 141, 1147-1162.

Gur, A., Semel, Y., Osorio, S., Friedmann, M., Seekh, S., Ghareeb, B., Mohammad, A., Pleban, T., Gera, G., Fernie, A.R., Zamir, D., 2011. Yield quantitative trait loci from wild tomato are predominately expressed by the shoot. Theor. Appl. Genet. 122, 405-420.

Hermans, C., Porco, S., Vandenbussche, F., Gille, S., De Pessemier, J., Van Der Straeten, D., Verbruggen, N.,

425 Bush, D.R., 2011. Dissecting the role of CHITINASE-LIKE1 in nitrate-dependent changes in root
426 architecture. Plant Physiol. 157, 1313-1326.
427 Ho, L.C., Grange, R.I., Picken, A.J., 1987. An analysis of the accumulation of water and dry matter in tomato
428 fruit. Plant Cell Environ. 10, 157-162.
429 Ho, L.C., White, P.J., 2005. A cellular hypothesis for the induction of blossom-end rot in tomato fruit. Ann. Bot.
430 95, 571-581.
431 Ikeda, H., Kanayama, Y., 2015. Blossom-End Rot in Fruit Vegetables, In: Kanayama, Y., Kochetov, A. (Eds.),
432 Abiotic Stress Biology in Horticultural Plants. Springer, Tokyo, pp. 117-126.
433 Ikeda, H., Shibuya, T., Imanishi, S., Aso, H., Nishiyama, M., Kanayama, Y., 2016. Dynamic metabolic regulation
434 by a chromosome segment from a wild relative during fruit development in a tomato introgression line, IL8-
435 3. Plant Cell Physiol. 57, 1257-1270.
436 Ikeda, H., Shibuya, T., Nishiyama, M., Nakata, Y., Kanayama, Y., 2017. Physiological mechanisms accounting
437 for the lower incidence of blossom-end rot in tomato introgression line IL8-3 fruit. Hortic. J. 86, 327-333.
438 Legge, R.L., Thompson, J.E., Baker, J.E., Lieberman, M., 1982. The effect of calcium on the fluidity and phase
439 properties of microsomal membranes isolated from postclimacteric golden delicious apples. Plant Cell
440 Physiol. 23, 161-169.
441 Liebisch, F., Max, J.F.J., Heine, G., Horst, W.J., 2009. Blossom-end rot and fruit cracking of tomato grown in
442 net-covered greenhouses in Central Thailand can partly be corrected by calcium and boron sprays. J. Plant
443 Nutr. Soil Sci. 172, 140-150.
444 Lippman, Z.B., Semel, Y., Zamir, D., 2007. An integrated view of quantitative trait variation using tomato

445 interspecific introgression lines. Curr. Opin. Genet. Dev. 17, 545-552.

446 Marcelis, L.F.M., Ho, L.C., 1999. Blossom-end rot in relation to growth rate and calcium content in fruits of

447 sweet pepper (*Capsicum annuum* L.). J. Exp. Bot. 50, 357-363.

448 Marschner, P., 2012. Mineral Nutrition of Higher Plants (Third Edition). Academic Press, San Diego, p. 672.

449 Memgel, K., Viro, M., 1974. Effect of potassium supply on the transport of photosynthates to the fruits of

450 tomatoes (*Lycopersicon esculentum*). Physiol. Plant. 30, 295-300.

451 Micheli, F., 2001. Pectin methylesterases: cell wall enzymes with important roles in plant physiology. TRENDS

452 Plant Sci. 6, 414-419.

453 Miwa, K., Wakuta, S., Takada, S., Ide, K., Takano, J., Naito, S., Omori, H., Matsunaga, T., Fujiwara, T., 2013.

454 Roles of BOR2, a boron exporter, in cross linking of rhamnogalacturonan II and root elongation under boron

455 limitation in Arabidopsis. Plant Physiol. 163, 1699-1709.

456 Neugebauer, K., Broadley, M.R., El‐Serehy, H.A., George, T.S., McNicol, J.W., Moraes, M.F., White, P.J.,

457 2018. Variation in the angiosperm ionome. Physiol. Plant..

458 Norton, G., Deacon, C., Xiong, L., Huang, S., Meharg, A., Price, A., 2010. Genetic mapping of the rice ionome

459 in leaves and grain: identification of QTLs for 17 elements including arsenic, cadmium, iron and selenium.

460 Plant Soil 329, 139-153.

461 Oakenfull, D., Scott, A., 1984. Hydrophobic interaction in the gelation of high methoxyl pectins. J. Food Sci.

462 49, 1093-1098.

463 Paniagua, C., Posé, S., Morris, V.J., Kirby, A.R., Quesada, M.A., Mercado, J.A., 2014. Fruit softening and pectin

464 disassembly: an overview of nanostructural pectin modifications assessed by atomic force microscopy. Ann.

Bot. 114, 1375-1383.

Salt, D.E., 2004. Update on plant ionomics. Plant Physiol. 136, 2451-2456.

Salt, D.E., Baxter, I., Lahner, B., 2008. Ionomics and the study of the plant ionome. Ann. Rev. Plant Biol. 59, 709-733.

Saure, M.C., 2014. Why calcium deficiency is not the cause of blossom-end rot in tomato and pepper fruit – a reappraisal. Sci. Hortic. 174, 151-154.

Shoemaker, S.D., Vanderlick, T.K., 2003. Calcium modulates the mechanical properties of anionic phospholipid membranes. J. Colloid and Interface Sci. 266, 314-321.

Silber, A., Bruner, M., Kenig, E., Reshef, G., Zohar, H., Posalski, I., Yehezkel, H., Shmuel, D., Cohen, S., Dinar, M., Matan, E., Dinkin, I., Cohen, Y., Karni, L., Aloni, B., Assouline, S., 2005. High fertigation frequency and phosphorus level: Effects on summer-grown bell pepper growth and blossom-end rot incidence. Plant Soil 270, 135-146.

Tavallali, V., Esmaili, S., Karimi, S., 2018. Nitrogen and potassium requirements of tomato plants for the optimization of fruit quality and antioxidative capacity during storage. J. Food Meas. Charact. 12, 755-762.

Taylor, M.D., Locascio, S.J., 2004. Blossom-end rot: a calcium deficiency. J. Plant Nutr. 27, 123-139.

Uemura, M., Yoshida, S., 1984. Involvement of plasma membrane alterations in cold acclimation of winter rye seedlings (*Secale cereale* L. cv Puma). Plant Physiol. 75, 818-826.

Uozumi, A., Ikeda, H., Hiraga, M., Kanno, H., Nanzyo, M., Nishiyama, M., Kanahama, K., Kanayama, Y., 2012. Tolerance to salt stress and blossom-end rot in an introgression line, IL8-3, of tomato. Sci. Hortic. 138, 1-6.

Van Veldhoven, P.P., Mannaerts, G.P., 1987. Inorganic and organic phosphate measurements in the nanomolar

485 range. Anal. Biochem. 161, 45-48.

486 Watanabe, T., Azuma, T., 2021. Ionomic variation in leaves of 819 plant species growing in the botanical garden
487 of Hokkaido University, Japan. J. Plant Res. 134, 291-304.

488 Watanabe, T., Maejima, E., Yoshimura, T., Urayama, M., Yamauchi, A., Owadano, M., Okada, R., Osaki, M.,
489 Kanayama, Y., Shinano, T., 2016. The ionomic study of vegetable crops. PLoS ONE 11, e0160273.

490 Watanabe, T., Urayama, M., Shinano, T., Okada, R., Osaki, M., 2015. Application of ionomics to plant and soil
491 in fields under long-term fertilizer trials. SpringerPlus 4, 781.

492 Yang, S., Yu, Q., Wang, B., Yang, T., Li, N., Tang, Y., Aisimutuola, P., Wang, Q., Xu, J., Gao, J., 2016.
493 Identification of QTLs for red fruit firmness using the wild tomato species *Solanum pennellii* LA716
494 introgression lines. Plant Breed. 135, 728-734.

495 Zhong, H., Lauchli, A., 1993. Changes of cell wall composition and polymer size in primary roots of cotton
496 seedlings under high salinity. J. Exp. Bot. 44, 773-778.

497 Zhu, X., Lei, G., Jiang, T., Liu, Y., Li, G., Zheng, S., 2012. Cell wall polysaccharides are involved in P-
498 deficiency-induced Cd exclusion in *Arabidopsis thaliana*. Planta 236, 989-997.

499