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

significant differences between fruits of the two lines. The fruit ionome differed considerably between M82 and IL8-3, and was not affected by available Ca status in the field. The M82 fruit had higher concentrations of many elements such as magnesium, potassium, boron, and sulfur than did IL8-3, and this trend was also observed in rotten fruit. This suggests that the influence of the leaf (source), rather than the fruit (sink), could be involved in the onset mechanism of BER.

Key word: blossom-end rot, calcium, introgression line, ionome, *Solanum lycopersicum*, *Solanum pennellii*

1. Introduction

Plants are known to use at least 17 essential elements; however, plants also absorb and accumulate various nonessential elements (Watanabe and Azuma, 2021). Ionomics is the study of all essential and nonessential elements that accumulate in living organisms; these are quantified using high-throughput elemental analysis technologies (Salt *et al.*, 2008), and can be applied to various types of plant science studies (Neugebauer *et al.*, 2018; Norton *et al.*, 2010; Watanabe *et al.*, 2015). Examining a plant's ionome (mineral profile) provides a comprehensive view of the plant's temporal and spatial mineral dynamics. Moreover, a plant's ionome can be considered an inorganic subset of its metabolome (Broadley *et al.*, 2010). Therefore, changes in a plant's physiological status can be detected by changes in its ionome (Baxter *et al.*, 2008).

Generally, fruits and leaves have different ionomes (Watanabe *et al.*, 2016). This is because mineral accumulation in the fruits (sink) largely depends on retranslocation from the leaves (source); however, the 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

81 The *S. pennellii* introgression line IL8-3 and its *S. lycopersicum* parent, M82, were cultivated in 2016, 2017, and
82 2018 in an experimental field at Hokkaido University, Sapporo, Japan (43°04′ N, 141°20′ E). The experimental
83 field had a Gleyic Fluvisol soil type. The general chemical properties of the field soil were described in our
84 previous study (Watanabe *et al.*, 2016). Seeds were germinated in 50-cell plug trays containing commercial
85 nursery soil (Takii & Co., Kyoto, Japan) and grown in a greenhouse at Hokkaido University. When the seedlings
86 reached adequate height (ca. 15 cm), they were transplanted into the field in three replicates (5 plants per
87 replicate). Rows and intra-rows were spaced at 50 cm × 90 cm. Chemical fertilizers were applied to the field at
88 rates of 100 $kg \cdot ha^{-1}$ N (urea:ammonium sulfate = 3:2); 260 $kg \cdot ha^{-1}$ P_2O_5 (superphosphate); 200 $kg \cdot ha^{-1}$ K_2O
89 (potassium sulfate); and 0.5 $kg \cdot ha^{-1}$ MgO (magnesium sulfate). Calcium treatment was applied with (+Ca) or
90 without (−Ca) 1750 $kg \cdot ha^{-1}$ CaO (as calcium carbonate) during the 2016 cultivation season only. A Ca treatment
91 was not supplied in 2017 or 2018.
92
93 *2.2. Fruit size measurement*
94 During the 2018 cultivation season, we measured the vertical and horizontal lengths of fruits in the second fruit
95 cluster with 10 replicates at 15, 25, and 45 day after flowering (DAF).
96
97 *2.3. Determining the incidence of BER*
98 The incidence of BER was recorded for each fruit in the second, third, and fourth fruit clusters occurring prior
99 to 15 DAF. The BER incidence rate was calculated as the percentage of plants having one or more BER fruits
100 (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

141 extracted from the crude cell walls obtained from the samples in 2016 by incubating three times with 1 mL of
142 20 mM ammonium oxalate at 70°C for 1 h. The supernatant containing the oxalate−soluble pectin was collected
143 after centrifugation at 14,000 × g for 10 min. The pectin fraction's uronic acid concentration was assayed using
144 galacturonic acid as the standard, according to the method of Blumenkrantz and Asboe-Hansen (1973). Briefly,
145 200 μL of pectin extract was incubated with 1.2 mL of 98% (w/v) H_2SO_4 (containing 12.5 mM $Na_2B_4O_7 \cdot 10H_2O$)
146 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
147 to the solution. The sample was allowed to stand at room temperature for 20 min, then the absorbance was
148 spectrophotometrically measured at 492 nm. Because carbohydrates produce a pinkish chromogen with sulfuric
149 acid/tetraborate at 100°C, the absorbance of a blank sample was measured with 20 μL 0.5% NaOH in place of
150 the m−hydroxydiphenyl. The blank sample's absorbance was subtracted from the total absorbance. To determine
151 the degree of pectin methylation, crude cell walls obtained from the samples in 2016 were saponified, and the
152 amount of cell wall polymer-bound methyl-esters in the released methanol fraction was measured
153 colorimetrically (Hermans *et al.*, 2011). The degree of methylation was calculated as the molar ratio between
154 the methanol and uronic acids, as described above.
155
156 *2.8. Phospholipid analysis*
157 Phospholipids were extracted from the distal halves of lyophilized fruit samples in 2017 using the method
158 described by Bligh and Dyer (1959) and modified by Uemura and Yoshida (1984). A 50-mg lyophilized sample
159 was homogenized three times using a mortar and pestle with a mixture with 2.5 mL 2-propanol, 2.5 mL
160 chloroform, and 1.25 mL H_2O. The homogenized sample was centrifuged at 1,000 × g for 5 min, and the

161 chloroform layer was separated. Next, 5 mL of chloroform was added to the residue, shaken for 5 min, and
162 centrifuged, and the chloroform layer was removed. This layer was then filtered through No. 6 filter paper
163 (Advantec, Tokyo, Japan) and shaken several times with the same volume of 0.1 M KCl to remove protein and
164 water−soluble molecules (e.g., ATP). The chloroform layer then was dehydrated with Na_2SO_4, evaporated at
165 40°C, and resolubilized in 1 mL of chloroform:methanol (2:1, v/v). Phospholipids were quantified by measuring
166 the phosphorus concentration in the lipid extract using the malachite green spectrophotometric method (Van
167 Veldhoven and Mannaerts, 1987) after wet digestion with sulfuric acid.
168
169 *2.9. Statistical analyses*
170 The mineral concentration data were analyzed on a dry-weight basis. All statistical analyses were performed
171 using Sigmaplot 14.0 (Systat Software, Inc., San Jose, CA, USA) and Excel for Microsoft 365 (Microsoft,
172 Redmond, WA, USA). In order to compare plant ionomes, we conducted a principal component analysis (PCA)
173 using the concentrations of all sample elements after standardizing the variables to a mean of zero and variance
174 of one.
175
176 **3. Results**
177 *3.1. Incidence of BER*
178 In all years of the study, IL8-3 showed a lower incidence of BER compared with its parent cultivar, M82 (Fig.
179 S1). Calcium treatment did not affect the incidence of BER (Fig. S1).
180

3.2. Fruit growth

Vertical and horizontal lengths of fruits at 15, 25, and 45 DAF are shown in Fig. 1. Although M82 had significantly larger fruits than did IL8-3 at 15 DAF, no differences between lines were observed after this point.

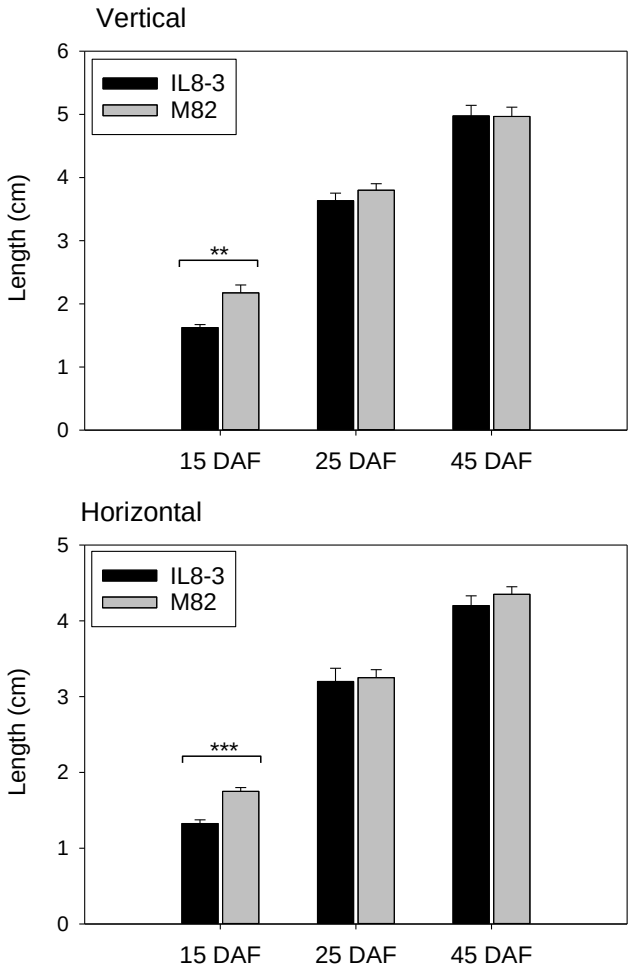

Fig. 1. Vertical and horizontal lengths of fruits in IL8-3 and M82 at 15, 25, and 45 days after flowering (DAF). Values represent the means of 3–6 replicates, and bars indicate ± standard errors. Significant differences between lines, as determined by Student's t-test, are indicated by ** and *** at $P < 0.01$ and $P < 0.001$, respectively.

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

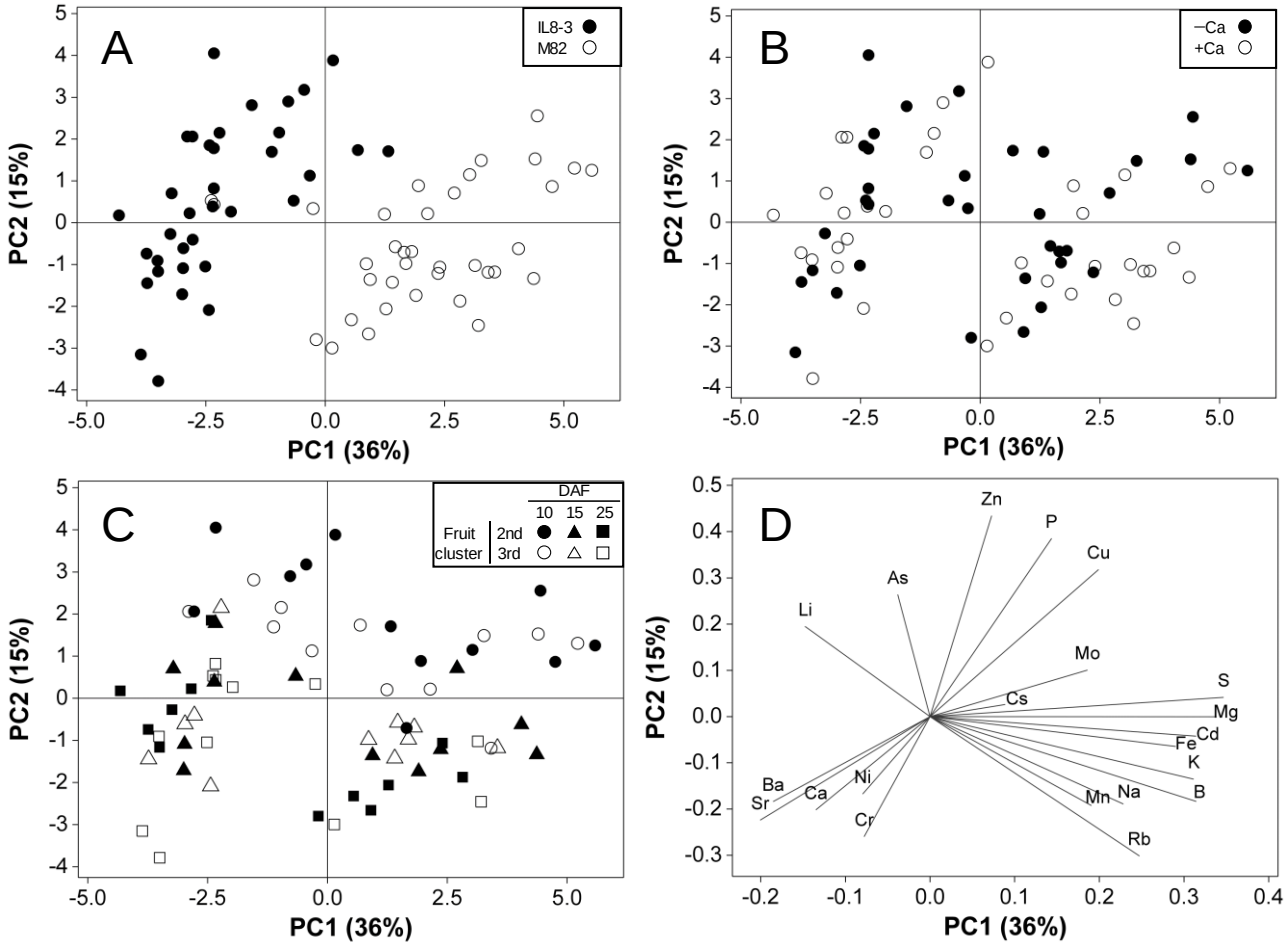

Fig. 2. Principal component analysis (PCA) of elements in the distal part of non-BER fruit in two tomato lines. Element concentration values were used after standardizing the variables to a mean of zero and a variance of one. PC1 and PC2 scores were plotted to indicate differences by plant line (A),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

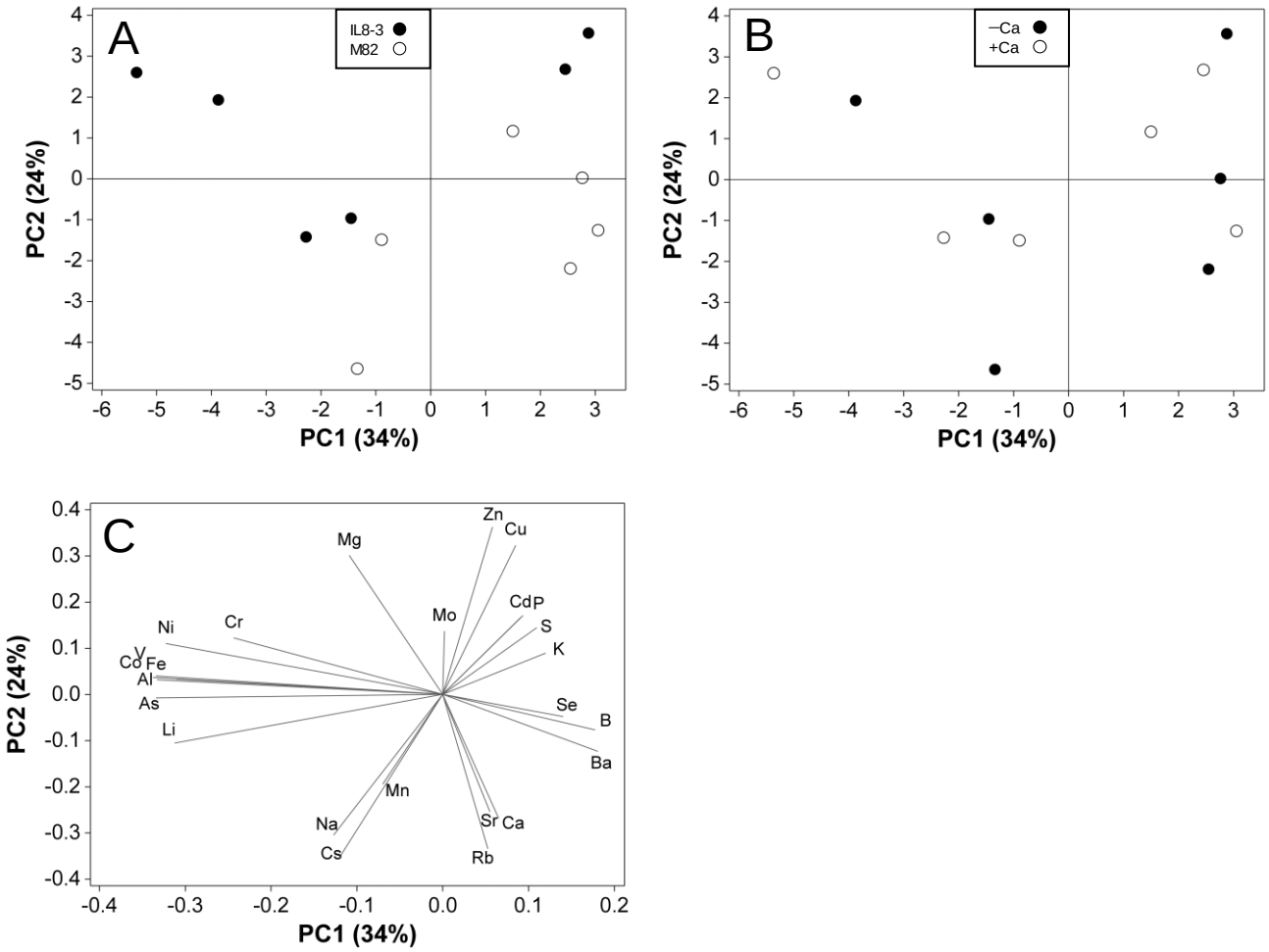

Fig. 3. Principal component analysis (PCA) of elements in the leaves from two tomato lines. Element concentration values were used after standardizing the variables to a mean of zero and a variance of one. PC1 and PC2 scores were plotted to indicate different plant lines (A) and calcium (Ca) treatment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

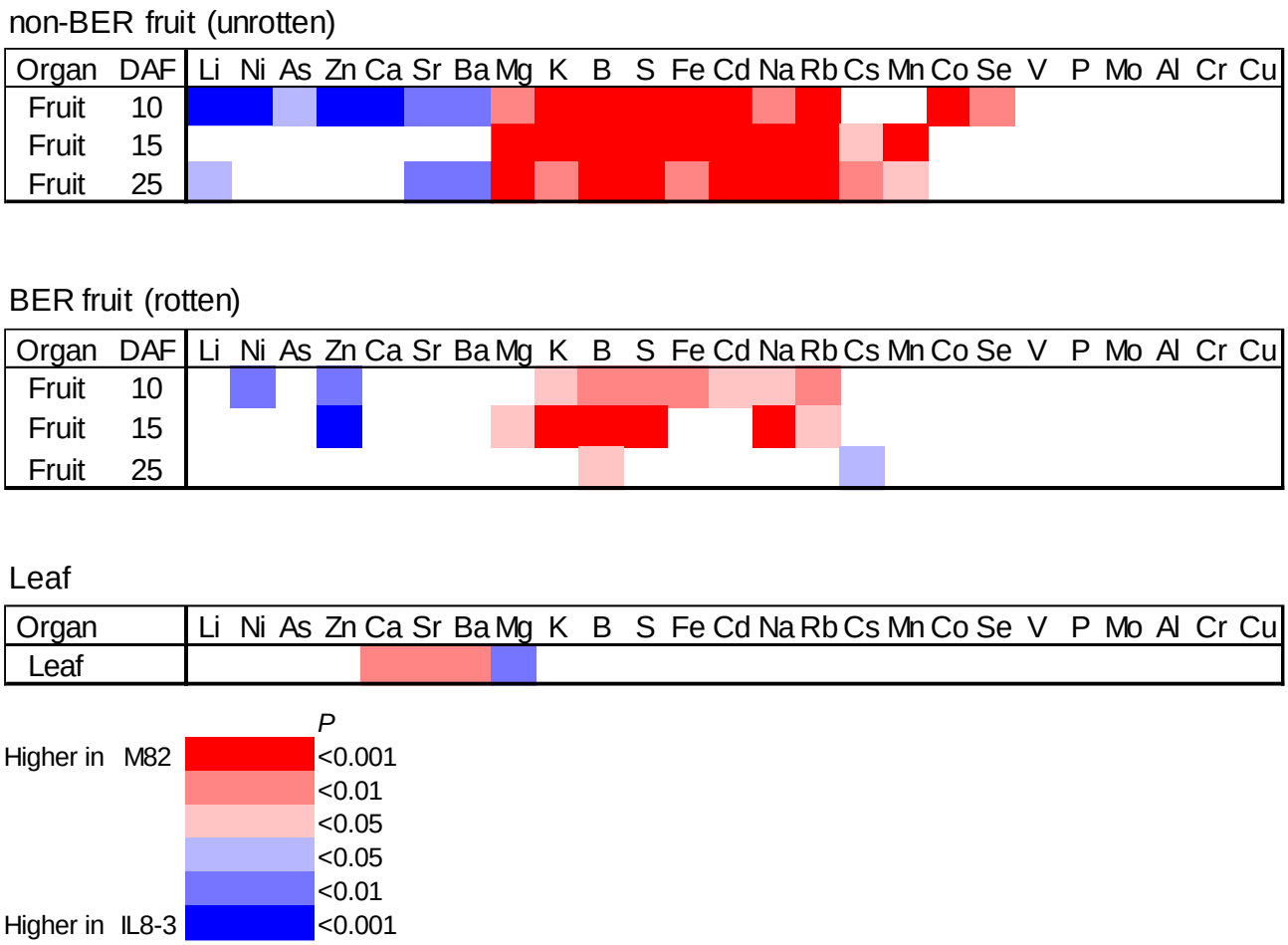

Fig. 4. Heat map analysis showing differences in elemental concentrations between the tomato lines IL8-3 and M82 in the distal part of non-BER (unrotten) and BER (rotten) fruits, and leaves (Student's t-test).

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).

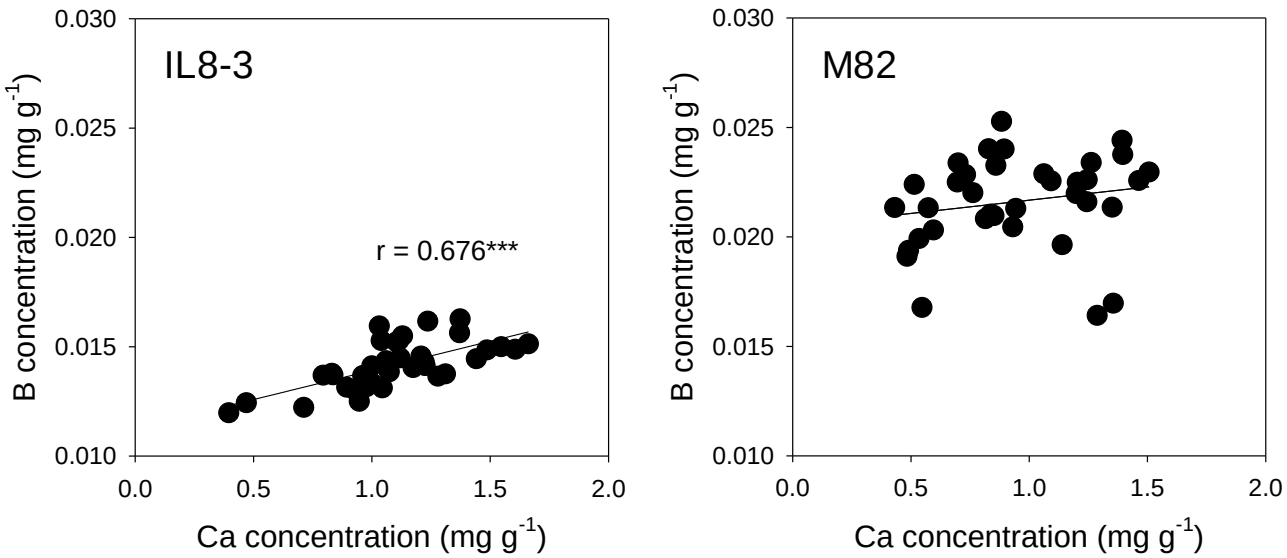

Fig. 5. Correlation between calcium (Ca) and boron (B) concentration in the distal halves of fruit in the tomato lines IL8-3 and M82. *** indicates a significant correlation ($P < 0.001$).

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.

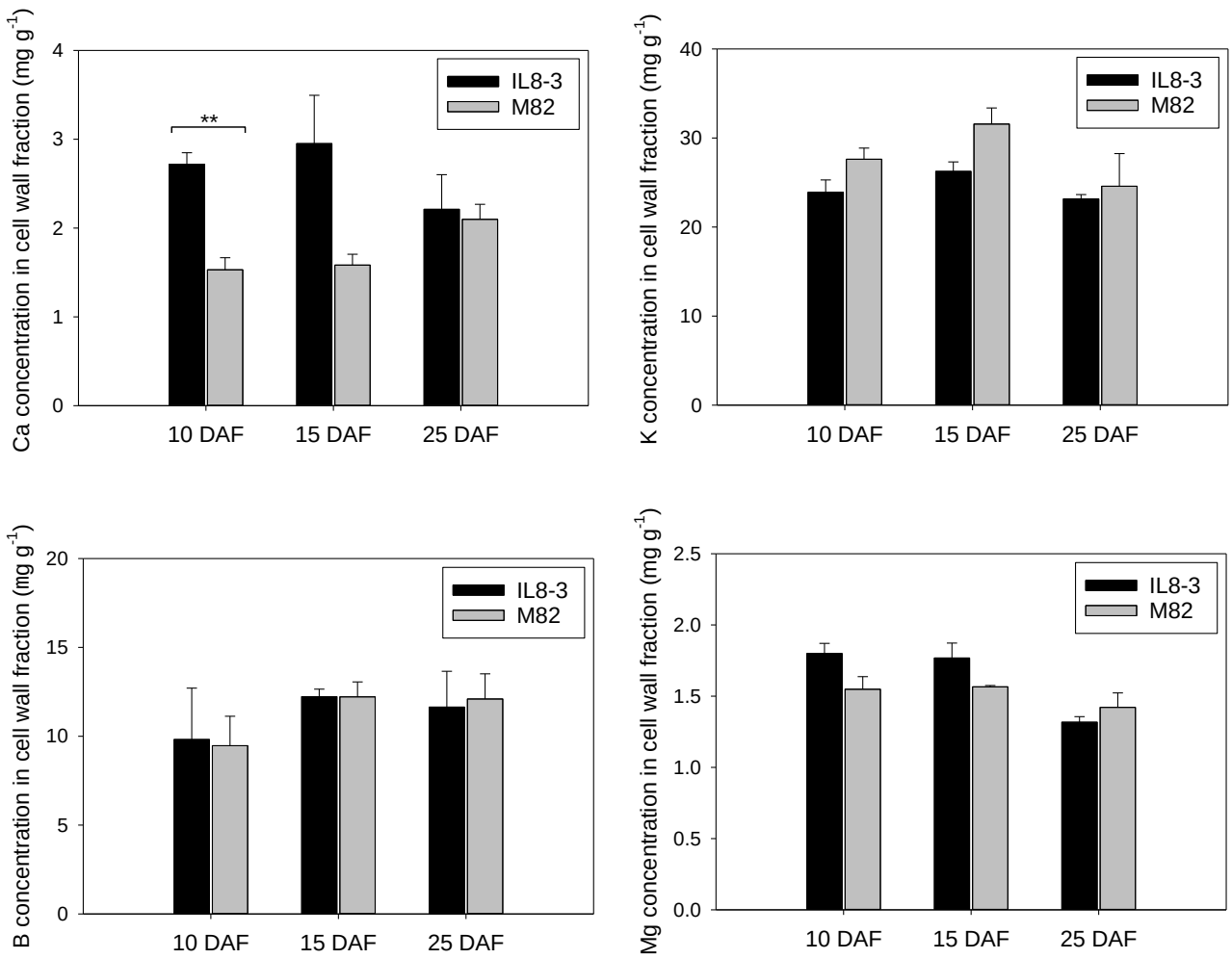

Fig. 6. Concentration of Ca, K, B, and Mg in cell walls of the distal halves of fruit in the tomato lines IL8-3 and M82 at 10, 15, and 25 days after flowering (DAF). ** indicates a significant difference between lines, as determined by Student's t-test *(P* < 0.01).

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.

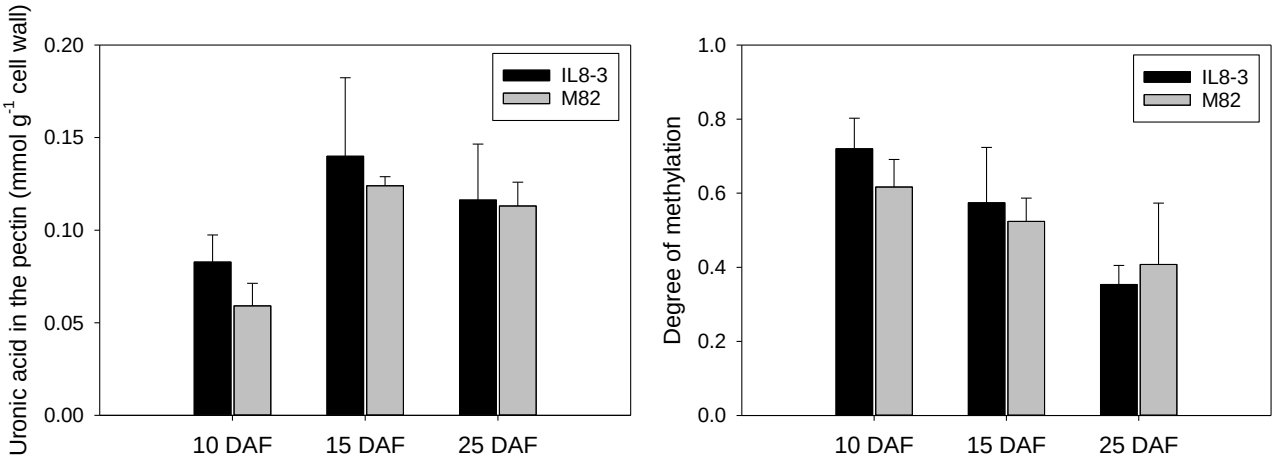

Fig. 7. Pectin concentration and degree of methylation in the distal halves of fruit from the tomato lines IL8-3 and M82 at 10, 15, and 25 days after flowering (DAF). Results were considered to be not significant when $P < 0.05$ (Student's t-test).

3.7. Phospholipid concentration in distal part of fruits

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

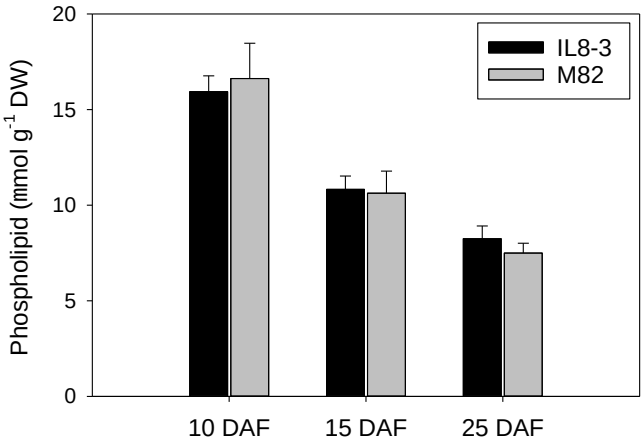

Fig. 8. Phospholipid concentration in the distal halves of fruit from the tomato lines IL8-3 and M82 at 10, 15, and 25 days after flowering (DAF). Results were considered to be not significant when $P <$

265 0.05 (Student's t-test).

266

267 **4. Discussion**

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

279 PCA is a common method used for comparing ionomes. In this study, PCA was first performed on fruits and
280 leaves using measured element concentration data. Interestingly, the ionome in the distal half of the unrotten
281 (healthy) fruit was completely different between IL8-3 and M82 (Fig. 2A). Although Ca fertilization
282 significantly affected the ammonium acetate-extractable concentrations of several soil elements, including Ca
283 (Table S1), the Ca fertilization treatment did not significantly affect the ionome; this suggests that the tomato
284 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,

305 progressive demethylation of homogalacturonan by pectin methylesterase commonly occurs during fruit
306 development (Paniagua *et al.*, 2014) and generates carboxyl groups. Then, Ca mediates the crosslinking of
307 homogalacturonan chains by binding to these carboxyl groups. Therefore, an insufficient concentration of Ca
308 ions supplied to the fruit will be unable to accommodate the increasing carboxyl groups occurring with fruit
309 growth and pectin demethylation; as a result, the stability of the cell wall pectin will decrease, which can increase
310 the incidence of BER (Marschner, 2012). This indicates that both the pectin content and its degree of methylation,
311 in addition to Ca concentration, are involved in the development of BER in fruits. de Freitas *et al.* (2012)
312 suggested that lower levels of free apoplastic Ca ions lead to impaired plasma membrane stabilization, inducing
313 membrane leakage and finally resulting in BER incidence; this occurs because the Ca ions bridge the
314 phospholipid phosphate groups at the membrane's surface (Legge *et al.*, 1982). The concentration of free
315 apoplastic Ca ions required for membrane stabilization depends on the amount of demethylated pectin in the cell
316 wall, as described above, as well as the proportion of Ca ions that is transported to the fruit (de Freitas *et al.*,
317 2012). Therefore, we analyzed the concentration of cell wall pectin, its degree of methylation, and the
318 phospholipid concentration in fruits of the tomato lines IL8-3 and M82. We found no significant differences in
319 any of these variables between the two lines, although some changes were observed on the basis of DAF (Fig.
320 7). These results suggest that the low Ca accumulation in the distal part of the fruit itself is one of the factors
321 related to the difference in BER incidence between M82 and IL8-3. Interestingly, the Ca, Sr, and Ba
322 concentrations of leaves collected from between the second and fourth fruit clusters were higher in M82 than in
323 IL8-3 (Fig. 3). This indicates that these elements are partitioned differently between leaves and fruits in the two
324 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.

385

386 **Declaration of competing interest**

387 The authors declare that they have no competing interests.

388

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394

395 **Appendix A. Supplementary data**

396 The following data are supplemental to this article:

397 FigsS1-S3.pptx

398 TableS1.pptx

399

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