Title	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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1   **Ionomic differences between tomato introgression line IL8-3 and its parent cultivar M82 with different**

2   **trends to the incidence of blossom-end rot**

3

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8

9   **Abstract**

10   Fruit blossom-end rot (BER) is a serious physiological disorder that can cause significant yield losses in tomato

11   (*Solanum lycopersicum*). Although many studies have suggested that calcium (Ca) deficiency in tomato fruits is

12   a major factor for BER, its onset mechanism has not been fully elucidated. Ionomics is a high-throughput

13   elemental profiling of living organisms that can be applied to understand how differences in plant's physiological

14   status involving inorganic elements. In this study, we examined ionomic differences between the tomato cultivar

15   M82 and its introgression line IL8-3, which contains a short chromosome segment from its wild relative *Solanum*

16   *pennellii* on chromosome 8 of M82, and has a low incidence of fruit BER. Among the essential elements, Ca

17   showed marked different behavior between the two lines. IL8-3 showed preferential Ca partitioning to fruits

18   compared with M82. The slow growth rate and high Ca concentration observed in IL8-3 fruit during the early

19   growth stages may also be responsible for the low BER incidence in this line. Although Ca ions bind to cell wall

20   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

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

2.4. Sampling

Fruits were sampled from the second and third fruit clusters at 10, 15, and 25 DAF with three experimental

replicates (3–5 fruits per replicate). After washing with deionized water, each fruit was divided equally into

proximal and distal halves. The pericarp tissues were frozen in liquid nitrogen and lyophilized. The lyophilized

samples were then ground and used for each analysis. Fruits with BER (rotten fruits) were sampled separately

from fruits without BER (unrotten fruits). The BER fruits were used only for mineral analysis of the distal fruit

halves. Leaves between the second and fourth fruit clusters were sampled nine weeks after transplant. Leaf

samples were washed with deionized water, dried in an oven at 70°C for 7 days, weighed, and ground for mineral

analysis. At the same time that the leaves were sampled, soil from unplanted rows was sampled with six

replicates taken from four points each (0–15 cm depth) and mixed together in equal proportions.

2.5. Mineral analysis

Plant samples (leaf plus the distal half of the fruit) in 2016 were digested in 2 mL of 61% (w/v) HNO_3 (electronic

grade; Kanto Chemical, Tokyo, Japan) in a DigiPREP apparatus (SCP Science, Quebec, Canada) for

approximately 2 h at 110°C, until the solution had almost disappeared. When the samples had cooled, 0.5 mL

H_2O_2 (semiconductor grade; Santoku Chemical Industries, Tokyo, Japan) was added and the samples were

heated at 110°C for another 20 min. Once digestion was complete, the tubes were cooled and brought to a volume

of 10 mL by adding 2% (w/v) HNO_3 in ultrapure water. The concentrations of the following elements were

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

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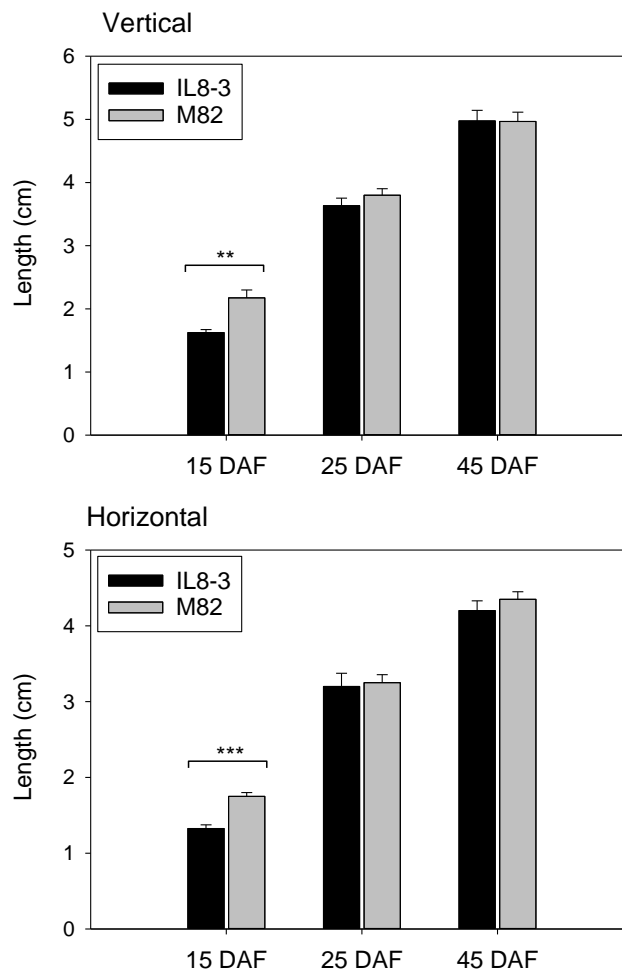
185   Fig. 1. Vertical and horizontal lengths of fruits in IL8-3 and M82 at 15, 25, and 45 days after

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

207
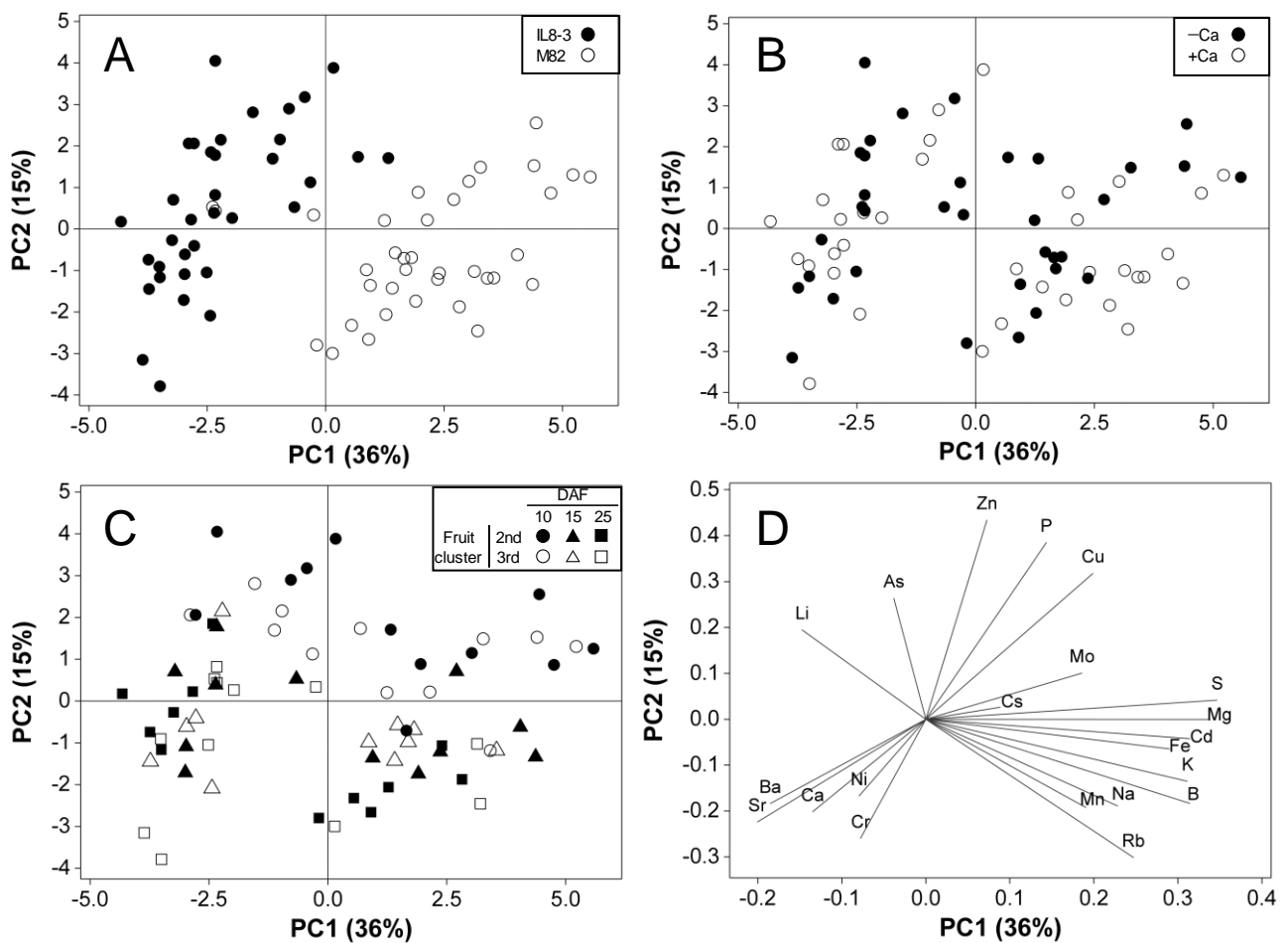
Fig. 2. Principal component analysis (PCA) of elements in the distal part of non-BER fruit in two tomato lines. Element concentration values were used after standardizing the variables to a mean of zero and a variance of one. PC1 and PC2 scores were plotted to indicate differences by plant line (A), calcium (Ca) treatment (B), and fruit cluster and days after flowering (DAF) (C). The loading plot is shown in (D).

215
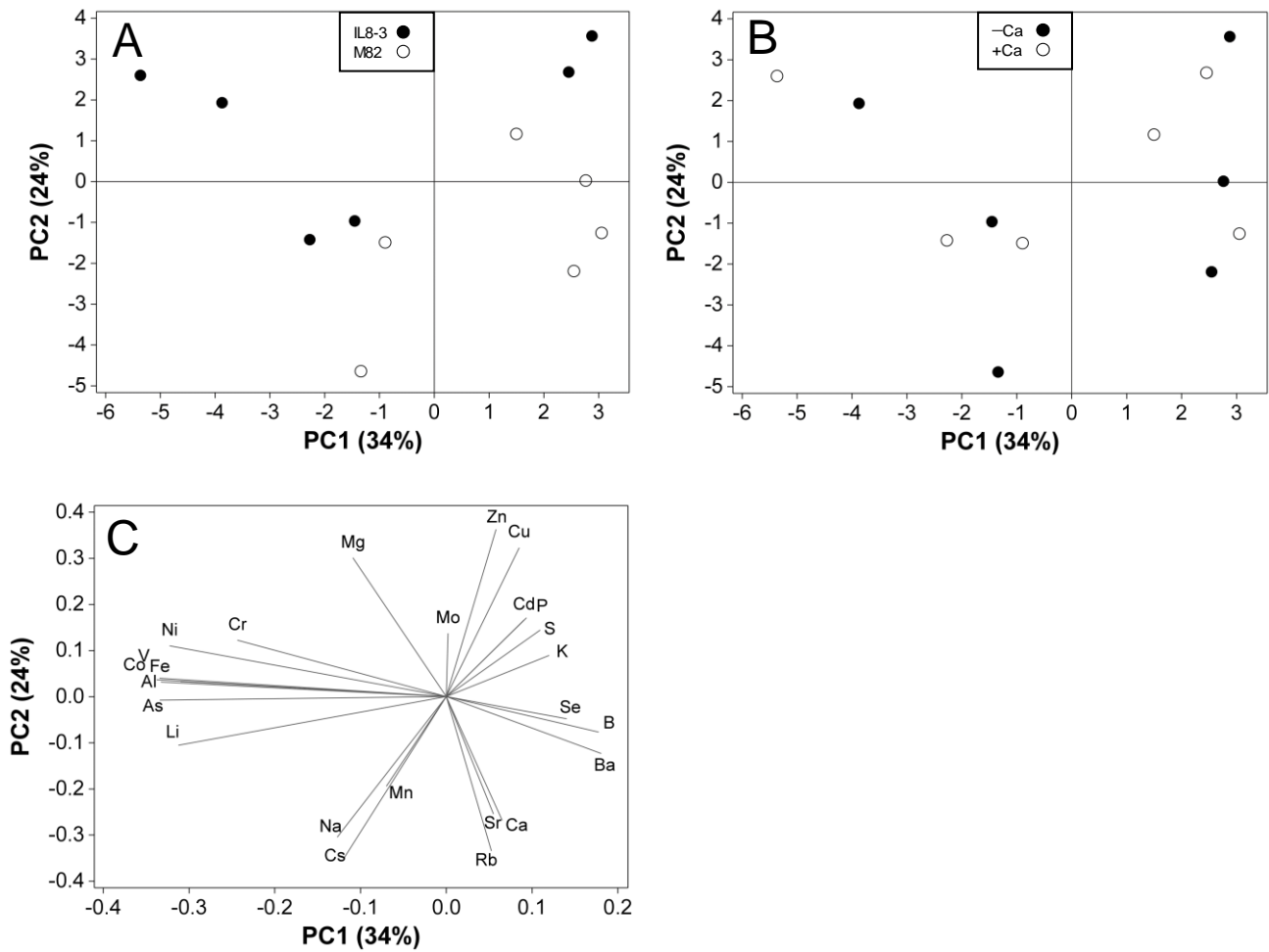
Fig. 3. Principal component analysis (PCA) of elements in the leaves from two tomato lines. Element

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

225 significantly higher concentrations in M82 than in IL8-3; however, some elements, including Li, Ni, Zn, Ca, Sr,

226 and Ba, showed lower concentrations in M82 in the early stages of fruit growth (Fig. 4). In contrast, the leaf

227 concentrations of Ca, Sr, and Ba were significantly higher in M82 than in IL8-3 (Fig. 4). The BER fruits showed

228 a similar trend to that of the non-BER fruits, but the difference between the lines was smaller (Fig. 4).

229 Furthermore, many elements had higher concentrations in the BER fruits, regardless of the line; this difference

230 was especially large in IL8-3 (Fig. S3).
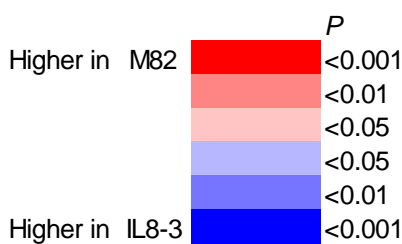
non-BER fruit (unrotten)

Organ	DAF	Li	Ni	As	Zn	Ca	Sr	Ba	Mg	K	B	S	Fe	Cd	Na	Rb	Cs	Mn	Co	Se	V	P	Mo	Al	Cr	Cu
Fruit	10																									
Fruit	15																									
Fruit	25																									

BER fruit (rotten)

Organ	DAF	Li	Ni	As	Zn	Ca	Sr	Ba	Mg	K	B	S	Fe	Cd	Na	Rb	Cs	Mn	Co	Se	V	P	Mo	Al	Cr	Cu
Fruit	10																									
Fruit	15																									
Fruit	25																									

Leaf

Organ	Li	Ni	As	Zn	Ca	Sr	Ba	Mg	K	B	S	Fe	Cd	Na	Rb	Cs	Mn	Co	Se	V	P	Mo	Al	Cr	Cu
Leaf																									

P

Higher in M82
<0.001
<0.01
<0.05
<0.05
<0.01
Higher in IL8-3   <0.001

231 Fig. 4. Heat map analysis showing differences in elemental concentrations between the tomato lines

232 IL8-3 and M82 in the distal part of non-BER (unrotten) and BER (rotten) fruits, and leaves (Student's

233 t-test).

234   Finally, we determined the correlation between Ca and B, both of which are constituents of pectin. We

235   observed a significant positive correlation between Ca and B in IL8-3, while no such correlation was observed

236   in M82 (Fig. 5).

237
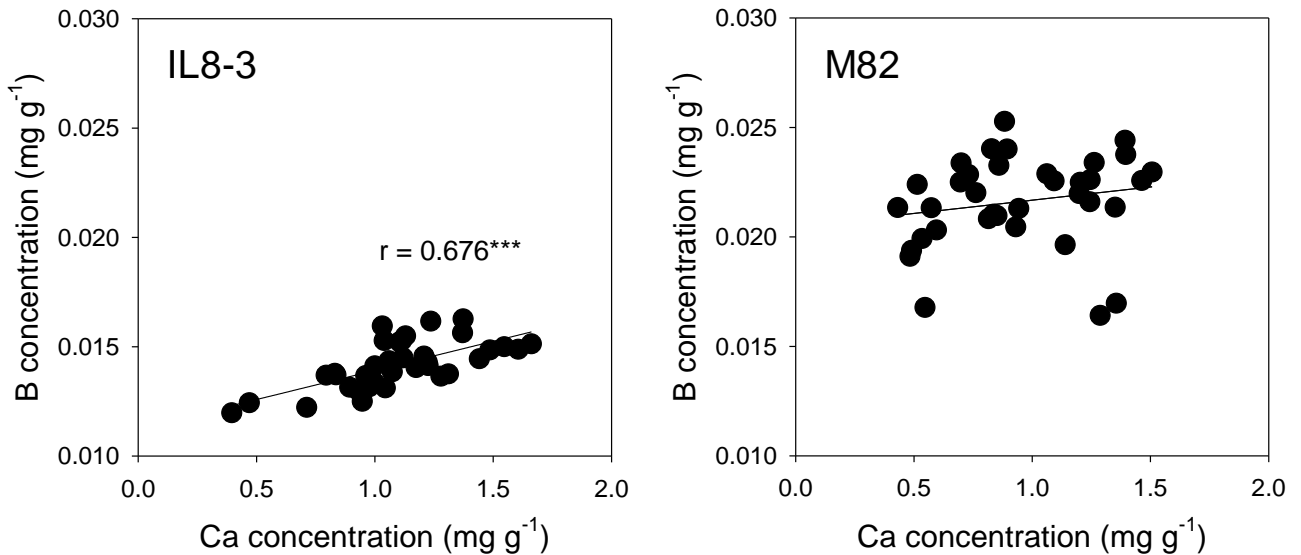
238   Fig. 5. Correlation between calcium (Ca) and boron (B) concentration in the distal halves of fruit in the

239   tomato lines IL8-3 and M82. *** indicates a significant correlation ($P < 0.001$).

240

241   *3.5. Mineral concentration in fruit cell walls*
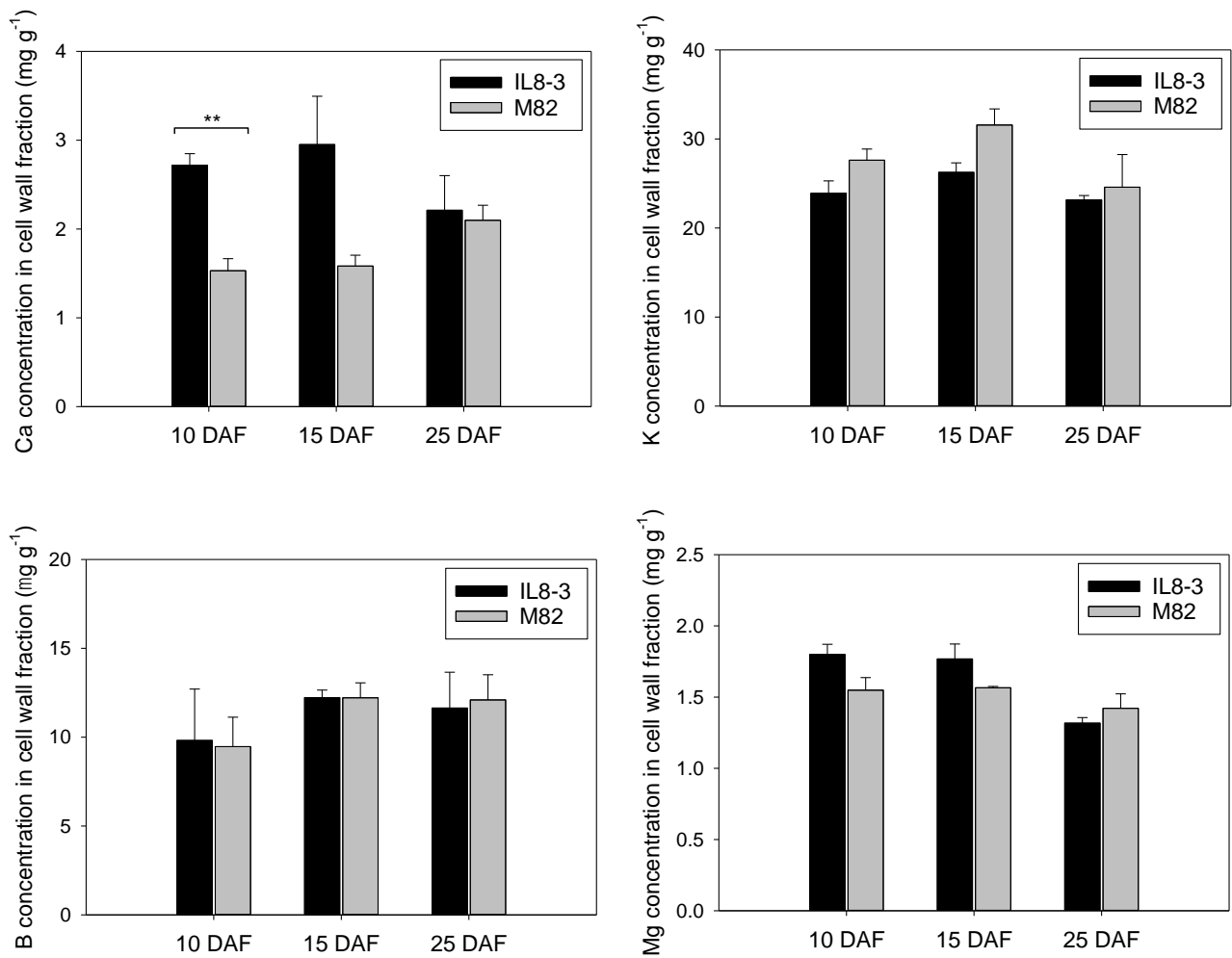
242   Cell walls were isolated from the distal halves of fruit to determine the concentrations of K, Ca, Mg, and B.

243   Similar to the fruit (Fig. 4), Ca concentration in fruit cell walls was higher in IL8-3 than in M82 at 10 DAF (Fig.

244   6). The fruit cell wall concentrations of K, Mg, and B did not differ significantly between the two lines at 10,
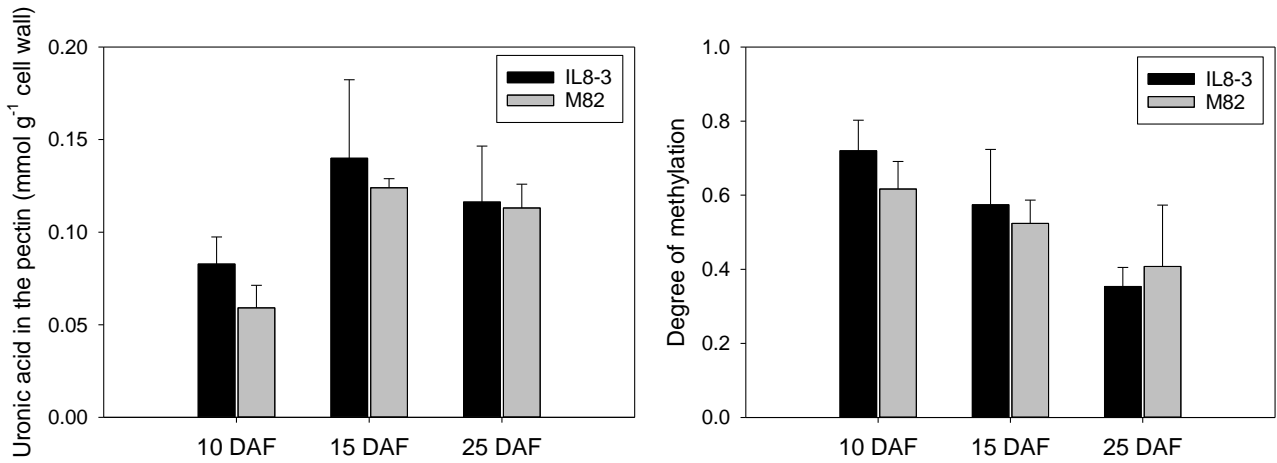
245   15, or 25 DAF.

246

247

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.
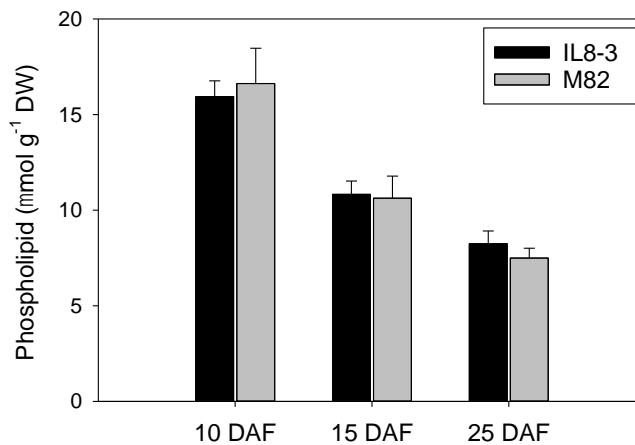
256 Fig. 7. Pectin concentration and degree of methylation in the distal halves of fruit from the tomato lines

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 $P <$

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,

19

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.

385

Declaration of competing interest

The authors declare that they have no competing interests.

388

Acknowledgment

394

Appendix A. Supplementary data

The following data are supplemental to this article:

FigsS1-S3.pptx

TableS1.pptx

399

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