Molecular Components of Arabidopsis Intact Vacuoles Clarified with Metabolomic and Proteomic Analyses

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We analyzed the metabolites and proteins contained in pure intact vacuoles isolated from Arabidopsis suspension-cultured cells using capillary electrophoresis–mass spectrometry (CE-MS), Fourier transform–ion cyclotron resonance (FT–ICR)-MS and liquid chromatography (LC)–MS. We identified 21 amino acids and five organic acids as expected substances (e.g. organic phosphate compounds). Further, we identified small amounts of 27 substances including well-known vacuolar molecules, but also some unexpected substances (e.g. organic phosphate compounds). Non-target analysis of the vacuolar sample with FT–ICR-MS suggested that there are 1,106 m/z peaks that could predict the 5,090 molecular formulae, and we have annotated 34 compounds in these peaks using the KNapSack database. By conducting proteomic analysis of vacuolar sap, we found 186 proteins in the same vacuole samples. Since the vacuole is known as a major degradative compartment, many of these were hydrolases, but we also found various oxidoreductases and transferases. The relationships between the proteins and metabolites in the vacuole are discussed.

Keywords: Arabidopsis • CE-MS • FT-ICR-MS • Metabolome • Proteome • Vacuole.

Abbreviations: CE, capillary electrophoresis; F6P, fructose 6-phosphate; FT–ICR, Fourier transform–ion cyclotron resonance; G6P, glucose 6-phosphate; MS, mass spectrometry; PEP, phosphoenolpyruvate; pNPP, p-nitrophenyl phosphate; QQQ, triple quadrupole; QTOF, quadrupole time of flight.

Introduction

The vacuole is the largest organelle in plant cells, occupying >80% of the cell volume. Apart from its space-filling role, the vacuole has many other important functions including the accumulation of inorganic ions and metabolites, the sequestration of toxic substance such as salts and heavy metals, and the degradation of discarded macromolecules or organelles (Martinoia et al. 2012). In order to clarify the functions of vacuoles in detail, it is important to analyze the molecular components forming the vacuole, such as the vacuolar membrane proteins and other proteins and constituents of the vacuolar sap. There have been a number of studies that have examined vacuolar proteins and other substances contained in vacuoles. In a previous study, we developed an isolation method for intact vacuoles from Arabidopsis suspension-cultured cells and reported the proteomic analysis of the vacuolar membrane (Shimaoka et al. 2004). Subsequently, other groups have also reported proteomic data for the vacuolar membrane of not only Arabidopsis, but also other seed plants (barley, rice, etc.). Carter et al. (2004) have characterized some of the proteins in the vacuolar sap. We also discovered that there are detergent-resistant membrane domains in the vacuolar membrane and that different kinds of membrane protein preferentially distribute between detergent-resistant and -soluble membrane domains (Yoshida et al. 2013). Although many new proteins were found in the vacuolar membrane and in the vacuolar sap, it was difficult to clarify the physiological functions of those proteins purely by proteomic analyses.
To understand the biological functions of those vacuolar proteins, it is important also to know the status of metabolites and inorganic ions in the vacuoles. Thus, metabolome and ionome analyses of the vacuoles have been conducted. Krueger et al. (2011) reported the metabolomic analyses of organelles of Arabidopsis leaf cells, using a non-aqueous method to isolate the organelle fractions. Tohge et al. (2011) published metabolomic analyses of intact vacuoles isolated from barley leaves, while Oikawa et al. (2011) conducted comprehensive analyses of metabolites of the vacuole and extracellular spaces of a single Chara internode.

A problem with studying organelle proteomes and metabolomes is the potential for contamination from other cellular fractions. It is quite likely that significant contamination occurs during non-aqueous fractionation of cells (Klie et al. 2011, Heing et al. 2013). Isolation of intact vacuoles has less contamination, but it takes a longer time to isolate the intact organelles from cells or protoplasts. Characean cells have a large central vacuole, and it is easy to isolate the vacuolar contents quickly from other metabolites. Unfortunately, since there are no genome data for characean cells, it is difficult to compare the metabolome data with the proteomic data.

In the present study, we used intact vacuoles isolated from Arabidopsis suspension-cultured cells for metabolomic analyses of the vacuolar sap. Although we expect less contamination, the longer time needed to isolate vacuoles carries with it the risk of alteration of vacuolar contents during the isolation procedure. We have previously reported the proteomic analyses of the vacuolar membrane isolated with the same method as used here (Shimaoka et al. 2004, Yoshida et al. 2013, Ohnishi et al. 2018), but in this study we report both proteomic and metabolomic analyses in the vacuolar sap of the same material.

It is well recognized that for metabolomic analysis, the selection of analytical methods is very important. Until now, gas chromatography–mass spectrometry (GC-MS) has been the most popular method. It can analyze a range of biological molecules, but usually only low molecular weight and non-polar substances. We have also used derivatization of major biological materials to make them volatile. Liquid chromatography (LC)–MS is also useful for lipids, carbohydrates or secondary metabolites, but not for ionic substances, which include many of the more important metabolites. In order to study these ionic species, we have used a combination of capillary electrophoresis (CE) and various mass spectrometries [quadrupole time of flight (QTOF) and triple quadrupole (QQQ) with ion trap]. CE-MS is also a useful analytical method for the detection of ionic metabolites such as amino acids, organic acids, nucleotides and sugar-phosphates (Monton and Soga 2007). CE-MS-based metabolomics were used in studies that led to the discovery of oxidative stress biomarkers in mammals (Soga et al. 2006), as well as for the evaluation of metabolic diversity in transgenic rice (Sato et al. 2004, Takahashi et al. 2006), the identification of unknown gene functions (Watanabe et al. 2008) and the analysis of novel metabolic pathways in Arabidopsis (Ohkama-Ohtsu et al. 2008). Recently, we also reported analysis of auxin-induced changes in primary metabolites of Arabidopsis shoots and roots using CE-MS (Anegawa et al. 2015). Thus, CE-MS is a powerful technique for metabolomic studies of organisms, but these MS methodologies have a limit of mass detection. Although it is possible to identify known substances with standards, it is difficult to identify unknown substances. For more accurate mass detection, we also used 12 Tesla (T) Fourier transform–ion cyclotron resonance–MS (FT-ICR-MS) for metabolomic analyses (Mesfioui et al. 2012, Xu et al. 2013).

We attempted a comprehensive analysis of the vacuolar components, both proteins and metabolites, of intact vacuoles isolated from Arabidopsis suspension-cultured cells using LC-MS, CE-MS and FT-ICR-MS. Our measurements confirmed the presence of well-known substances but also that of many new compounds that were found in the vacuole. We discuss the possible functions of these compounds in vacuoles of plant cells.

**Results**

**Identification of metabolites in the intact vacuolar sap isolated from Arabidopsis suspension-cultured cells**

In previous studies, we established a method for the isolation of intact vacuoles from Arabidopsis suspension-cultured cells and reported the proteomic analysis of the vacuolar membrane proteins (Shimaoka et al. 2004, Yoshida et al. 2013, Ohnishi et al. 2018). Purity of the vacuolar membrane fraction was confirmed by the measurement of enzymatic activity and Western blotting of organelar marker proteins (Shimaoka et al. 2004). From these tests, it was suggested that the contamination from other organelles was very low, and we proceeded to use the isolated intact vacuoles for metabolite analysis.

**Quantitative analyses of the metabolites in the vacuolar sap**

Using standards, vacuolar metabolites were quantitatively identified by CE-QTOF. Twenty-one cationic compounds (amino acids) and 10 anionic compounds (organic acids and organic phosphate compounds) were detected in the vacuolar sap (Fig. 1; Supplementary Table S1). To validate the concentration in the vacuole, the activity of α-mannosidase was used as a vacuolar marker for each analysis.

Among the amino acids, basic amino acids such as lysine, arginine and glutamine were the major constituents in the vacuoles. In the organic acid fraction, malate, citrate, iso-citrate, succinate and fumarate were found abundantly (Supplementary Table S1). The level of malate was quite high, while citrate was also prominent. Organic phosphates were also detected, although their levels were much lower.

**Fig. 2** shows the distribution of metabolites among cells, protoplasts and vacuoles on the primary metabolic map. Levels of each compound are shown in Supplementary Table S1. When we compared levels of metabolites between cells and protoplasts, we found that during protoplast preparation, the levels of some metabolites increased while those of organic...
acids of the tricarboxylic acid (TCA) cycle largely decreased. These changes might be caused by the degradation of the cell wall or possibly by low oxygen conditions during treatment in the enzyme solution to release the protoplasts. Additionally, it takes a further 30 min to isolate intact vacuoles from protoplasts, but it seems less likely that major metabolic changes would occur in the vacuolar sap during this time. This is supported by the fact that metabolites occurring in the vacuoles at high levels, such as lysine, arginine and malate, showed the same levels between protoplasts and vacuoles. This suggests that these metabolites are located in the vacuoles without being significantly metabolized. On the other hand, some metabolites such as citrate or iso-citrate showed higher levels in the vacuoles than in protoplasts, which may indicate unknown reactions during vacuole isolation.

Non-target analysis of vacuolar metabolites with CE-MS and FT-ICR-MS

Apart from the metabolites annotated with CE-MS, we also detected many unannotated peaks in the vacuolar sap. Owing to the detection limit of QTOF, it was difficult to annotate such peaks. Thus we attempted to measure the vacuolar metabolites using FT-ICR-MS with ultra-high resolution (12 T) (Mesfioui et al. 2012, Xu et al. 2013). Using this method, we detected a total of 3,526 m/z peaks in cells and 1,106 in vacuoles; there were 23,738 predicted molecular formulae in cells and 5,090 in vacuoles. The number of common formulae between cells and vacuoles was 254, and 355 annotated peaks were found in cells and 34 in vacuoles (Table 1; Supplementary Tables S2, S3). Compounds whose m/z values permitted a prediction of a molecular formula using the AutoMF Calculator (Nakamura et al. 2008), and which existed in more than two different samples, were judged as real substances contained in the cell and vacuole. In some cases, multiple molecular formulae were predicted from a single m/z value. We counted those as different molecular formulae.

Proteomic analysis of vacuolar sap by chipLC-MS

We performed a proteomic analysis of the vacuolar membrane and the vacuolar sap together with a metabolomic analysis. In the vacuolar membrane, we found that there were several unknown proteins besides proton pumps and transporters (Shimaoka et al. 2004, Yoshida et al. 2013). In this study, we have extended the data by adding an analysis of the proteins contained in the vacuolar sap. Thus far, we have identified 474 proteins in the vacuolar membrane (Yoshida et al. 2013) and 186 proteins in the vacuolar sap (Fig. 3; Table 2;
Supplementary Table S4). **Fig. 3** shows the classification of vacuolar sap proteins based on their enzymatic functions. Since the vacuole is important in degradation, it was not surprising to find a large number of hydrolases. However, in addition to hydrolases, we found various oxidoreductases and transferases. **Table 2** shows the ranking of the top 20 vacuolar sap proteins. None of these proteins contained transmembrane domains. We compared our data with those of Carter et al. (2004) and Jaquinod et al. (2007) who have also reported some vacuolar sap proteins.
Possible existence of organic phosphate compounds in the vacuole

In contrast to the analyses of vacuolar metabolites in barley mesophyll cells reported by Tohge et al. (2011), we found various organic phosphate compounds in the vacuoles isolated from Arabidopsis suspension-cultured cells (Fig. 1; Supplementary Table S1). This was somewhat surprising, given that vacuolar sap is thought to have strong phosphatase activity. Further evidence for the presence of phosphate compounds in the vacuole was obtained by using other analytical methods, and by analyzing phosphate compounds in the vacuole of Chara cells. Supplementary Table S5 and S6 summarizes organic phosphate compounds in Arabidopsis and Chara vacuoles detected by CE-QQQ and CE-TOF. In these measurements, we could detect >30 organic phosphates in the vacuolar sap. The purity of intact vacuoles isolated from Arabidopsis suspension-cultured cells is quite high (Shimaoka et al. 2004), but there remains the possibility of very slight contamination from the cytosol and other organelles. A greater certainty that organic phosphates do in fact exist in plant vacuoles was obtained using vacuolar sap isolated from Chara intermodal cells. Chara vacuole has also shown phosphatase activity [(85.4 ± 16.2) × 10^{-6} µmol Pi min^{-1} μg protein^{-1} as a substrate of pNPP]. This value was almost in the same range of phosphatase activity in Arabidopsis vacuoles (Table 3).

Table 1 Number of total peaks of m/z, predicted molecular formulae and annotated peaks detected in the cell and vacuolar samples measured with FT-ICR-MS

<table>
<thead>
<tr>
<th></th>
<th>Cell (n = 5)</th>
<th>Vacuole (n = 3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of m/z peaks</td>
<td>3,526</td>
<td>1,106</td>
</tr>
<tr>
<td>Number of predicted molecular formulae</td>
<td>23,738</td>
<td>5,090</td>
</tr>
<tr>
<td>Number of common formulae between cells and vacuoles</td>
<td>254</td>
<td></td>
</tr>
<tr>
<td>Number of annotated peaks</td>
<td>355</td>
<td>34</td>
</tr>
<tr>
<td>Number of common substances between cells and vacuoles</td>
<td>6</td>
<td></td>
</tr>
</tbody>
</table>

1 m/z peaks which predicted a molecular formula by AutoMF Calculator.
2 In some case, multiple molecular formulae were predicted from a single m/z value.
3 Predicted formulae were annotated by the KNapSAck database.

Fig. 3 Classification of proteins in the vacuolar sap.

Phosphatase activity in the vacuole

In our proteomic measurement of the vacuolar sap, we found many real and putative phosphatases (Supplementary Table S4). We were able to confirm phosphatase activity for a range of substrates with breakdown rates in the order of phosphoenolpyruvate (PEP) > ATP > glucose 6-phosphate (G6P) > fructose 6-phosphate (F6P) > p-nitrophenyl phosphate (pNPP; artificial substrate) (Table 3). In order to confirm the co-existence of organic phosphates and phosphatase in the same vacuole, we also measured phosphatase activities in the vacuolar sap isolated from Chara intermodal cells. Chara vacuole has also shown phosphatase activity [(85.4 ± 16.2) × 10^{-6} µmol Pi min^{-1} μg protein^{-1} as a substrate of pNPP]. This value was almost in the same range of phosphatase activity in Arabidopsis vacuoles (Table 3).

Discussion

Isolated vacuoles for metabolomic and proteomic analyses

For metabolomic and proteomic analyses of vacuoles, the use of isolated intact vacuoles avoids many of the drawbacks associated with cross-contamination from the cytoplasm and other organelles. While the membranes of organelles can be purified by density gradient centrifugation, the subcellular distribution of metabolites has been commonly investigated using non-aqueous methods (Klie et al. 2011, Heinig et al. 2013), but cross-contamination among organelles is difficult to avoid with this procedure. Such cross-contamination can be largely avoided if vacuoles are initially separated intact from the rest of the cell components. Thus far, intact vacuoles have been used for the proteomic analyses of the vacuolar membrane (Carter et al. 2004, Shimaoka et al. 2004, Jaquind et al. 2007, Yoshida et al. 2013), of vacuolar sap (Carter et al. 2004) in Arabidopsis or for the metabolomic analysis of vacuolar sap isolated from barley mesophyll protoplasts (Tohge et al. 2011).
Table 2  Protein ranking detected in the vacuolar sap

<table>
<thead>
<tr>
<th></th>
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<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>AT1G11580</td>
<td>494</td>
<td>EC3.1.1.11</td>
<td>AtPMEPCRA; pectinesterase</td>
<td>×</td>
<td>○</td>
</tr>
<tr>
<td>2</td>
<td>At2g36530</td>
<td>431</td>
<td>EC4.2.1.11</td>
<td>Enolase</td>
<td>×</td>
<td>×</td>
</tr>
<tr>
<td>3</td>
<td>At3g02230</td>
<td>244</td>
<td>EC2.4.1.-5.4.99.30</td>
<td>Glycosylated polypeptide</td>
<td>×</td>
<td>×</td>
</tr>
<tr>
<td>4</td>
<td>At3g55260</td>
<td>208</td>
<td>EC3.2.1.52</td>
<td>β-hexosaminidase</td>
<td>○</td>
<td>×</td>
</tr>
<tr>
<td>5</td>
<td>At4g08770</td>
<td>189</td>
<td>EC1.11.1.7</td>
<td>Peroxidase</td>
<td>○</td>
<td>×</td>
</tr>
<tr>
<td>6</td>
<td>At3g26720</td>
<td>184</td>
<td>EC3.2.1.24</td>
<td>Glycosyl hydrolase</td>
<td>○</td>
<td>○</td>
</tr>
<tr>
<td>7</td>
<td>At2g24200</td>
<td>169</td>
<td>EC3.4.11.1</td>
<td>Leucine aminopeptidase 1</td>
<td>×</td>
<td>×</td>
</tr>
<tr>
<td>8</td>
<td>At5g15650</td>
<td>155</td>
<td>EC3.4.11.1</td>
<td>Glycosylated polypeptide-2</td>
<td>×</td>
<td>×</td>
</tr>
<tr>
<td>9</td>
<td>At4g30920</td>
<td>149</td>
<td>EC3.4.11.1</td>
<td>Aminopeptidase family protein</td>
<td>×</td>
<td>×</td>
</tr>
<tr>
<td>10</td>
<td>At1g79690</td>
<td>122</td>
<td>EC3</td>
<td>Nudix hydrolase homolog</td>
<td>○</td>
<td>×</td>
</tr>
<tr>
<td>11</td>
<td>At4g20850</td>
<td>148</td>
<td>EC3.1.1.11</td>
<td>Cytosolic thioredoxin</td>
<td>○</td>
<td>○</td>
</tr>
<tr>
<td>12</td>
<td>At2g18170</td>
<td>149</td>
<td>EC1.4.1.3</td>
<td>β-Subunit of the glutamate dehydrogenase</td>
<td>×</td>
<td>×</td>
</tr>
<tr>
<td>13</td>
<td>At5g19550</td>
<td>148</td>
<td>EC2.6.1.1</td>
<td>Nitrogen metabolism. Major cystolic isoenzyme controlling aspartate biosynthesis in the light.</td>
<td>×</td>
<td>○</td>
</tr>
<tr>
<td>14</td>
<td>At5g17920</td>
<td>146</td>
<td>EC2.1.1.14</td>
<td>Cytosolic cobalamin-independent methionine synthase</td>
<td>×</td>
<td>○</td>
</tr>
<tr>
<td>15</td>
<td>At3g20370</td>
<td>142</td>
<td>–</td>
<td>Meprin and TRAF homology domain-containing protein</td>
<td>×</td>
<td>×</td>
</tr>
<tr>
<td>16</td>
<td>At1g54010</td>
<td>116</td>
<td>EC3</td>
<td>Myrosinase-associated protein, putative</td>
<td>○</td>
<td>○</td>
</tr>
<tr>
<td>17</td>
<td>At2g05710</td>
<td>113</td>
<td>EC4.2.1.3</td>
<td>Tyrosine-phosphorylated protein</td>
<td>○</td>
<td>×</td>
</tr>
<tr>
<td>18</td>
<td>At4g08780</td>
<td>108</td>
<td>EC1.11.1.7</td>
<td>Peroxidase</td>
<td>○</td>
<td>×</td>
</tr>
<tr>
<td>19</td>
<td>At3g56310</td>
<td>94</td>
<td>EC3.2.1.22</td>
<td>Γ-Galactosidase</td>
<td>○</td>
<td>×</td>
</tr>
<tr>
<td>20</td>
<td>At5g42980</td>
<td>92</td>
<td>EC1</td>
<td>Cytosolic thioredoxin</td>
<td>○</td>
<td>×</td>
</tr>
</tbody>
</table>

All values are the means of n = 3 independent determinations.

Table 3 Phosphatase activities in isolated vacuoles of Arabidopsis suspension-cultured cells

<table>
<thead>
<tr>
<th>Substrate</th>
<th>μmol Pi min⁻¹ µg protein⁻¹</th>
<th>Relative activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PEP</td>
<td>(767 ± 107) × 10⁻⁶</td>
<td>100</td>
</tr>
<tr>
<td>ATP</td>
<td>(621 ± 125) × 10⁻⁶</td>
<td>81</td>
</tr>
<tr>
<td>G6P</td>
<td>(593 ± 101) × 10⁻⁶</td>
<td>77</td>
</tr>
<tr>
<td>F6P</td>
<td>(180 ± 31) × 10⁻⁶</td>
<td>23</td>
</tr>
<tr>
<td>pNPP</td>
<td>(36 ± 4) × 10⁻⁶</td>
<td>5</td>
</tr>
</tbody>
</table>

Even when pure intact vacuoles are used, a small amount of contamination is still possible, and metabolite levels may be altered during the time it takes for the vacuoles to be isolated. Comparisons between the vacuum contents of Arabidopsis and Chara were used to validate the results. Access to the vacuum contents of the huge cylindrical intermodal cells of Chara can be obtained simply by excising the ends of the cells. This avoids both contamination and delay in isolating the sap (Oikawa et al. 2011). This procedure showed that many similar compounds occurred in both Arabidopsis and Chara vacuoles. However, the pattern of amino acids in the vacuole of Chara internodes reported previously (Sakano and Tazawa 1984, Mimura et al. 1990) was very different from that found in Arabidopsis in the present study.

We recently reported a metabolomic analysis of alkaloids obtained from single cells of Catharanthus roseus (Yamamoto et al. 2016). For that study, we sampled the vacuole contents with a micropipette and analysed them with nanospray MS. Hopefully, this technique can be adapted for use with other cell types in the future.

Known metabolites in the vacuolar sap measured with CE-MS

Fig. 1 and Supplementary Table S1 show the levels of amino acids and organic acids in the isolated vacuoles measured with CE-MS. Many of these metabolites were already known to exist in the vacuole (MacLennan et al. 1963). Lysine, arginine, malate, citrate and fumarate enter into the vacuole via specific transporter proteins (Emmerlich et al. 2003, Shimada et al. 2006, Kovemann et al. 2007). As shown in Fig. 2, the levels of some metabolites in the vacuoles were equal to or more than those in the protoplasts. This inconsistency could be due to an underestimate of the volume of protoplasts if α-mannosidase activity exists other than in the vacuole. The second possibility is ion suppression in the measurement with MS, due to high concentrations of osmotica such as sorbitol in the vacuole isolation medium. Also some metabolites such as glucose 1-phosphate (G1P), F6P or dihydroxyacetone phosphate (DHAP) in the vacuoles could not be found in cells and protoplasts. Ion suppression from high levels of cellular contents may cause such discrepancies.

In our measurements, we could not detect sugars and sugar alcohols, although these were reported by Tohge et al. (2011). This was most probably due to the difficulty in detecting such
metabolites with CE-MS and we also could not distinguish original vacuolar contents from sugars in the isolating medium.

**New metabolites in the vacuolar sap measured with CE-MS**

It is well known that amino acids and organic acids exist in the vacuole, but we have shown that many different organic phosphates, including sugar phosphates and nucleotides, also occur in the vacuole, at concentrations in several tens of micromolar. This was not expected given the known presence of high levels of phosphatase activity in vacuoles (Veljanovski et al. 2006), which we were able to confirm in our own extracts (Table 3).

The finding of organic phosphates in the vacuole of Chara serves to increase confidence in the Arabidopsis data (Supplementary Table S6). Oikawa et al. (2011) have also reported daily changes in the vacuolar organic phosphate levels of Chara, and Tohge et al. (2011) recorded the presence of glycerol-3-phosphate in the barley vacuole. Thus, it is probable that organic phosphates are transported into the vacuole.

Theodorou and Plaxton (1993) suggested that the vacuoles are used to recycle phosphate, using a combination of PEP transport into the vacuole and PEP-dependent phosphatase. Our results support this possibility, as does the report of a phosphatase exporter in the vacuolar membrane in rice (Wang et al. 2015), which may function in the export of inorganic phosphates following the degradation of organic phosphates. The existence of other specific enzymes such as phospholipase or nudix hydrolase found in the vacuolar sap may also involve such a process (Supplementary Table S4).

The vacuole has a complex form in the cell. Even if organic phosphates and phosphatases seem to be co-localizing in the same vacuole, they may be separately sublocalized in the same vacuole. However, by using Chara internodal cells, which have simpler vacuolar form, we confirmed the presence of phosphatase activities in the central vacuole. At present, we have no answer to explain such discrepancies. More than 30 years ago, Kaiser et al. (1982) showed a rapid appearance of photosynthetic products in the vacuole. Although they did not show the presence of organic phosphates in the vacuole, a closer relationship existing between carbon metabolism in the chloroplasts and vacuoles may cause dynamic changes of the photosynthetic products depending on the enzymes in the vacuole.

**Annotated and non-annotated metabolites in the vacuole measured with CE-MS and FT-ICR-MS**

We used standard compounds to identify metabolite peaks in measurements with CE-MS, but many peaks remain unidentified. It is difficult to annotate these peaks using only mass values measured with QTOF-MS, so we also attempted to identify the vacuolar contents with FT-ICR-MS (12 T) which allows us to narrow the range of mass value to within ±0.5 p.p.m. Using the same vacuolar sample, we detected 1,106 peaks of m/z values and annotated 34 peaks by using molecular formulae predicted from AutoMF Calculator (Supplementary Tables S2, S3).

**Degrading activity and reuse of metabolites**

Macromolecules, such as proteins and nucleic acids, or organelles are known to be imported into the vacuoles by autophagy (Masclaux-Daubresse et al. 2017). Some of the metabolites detected here must be degradation products from such macromolecules. For reuse of these degraded products, it is necessary for them to be exported from the vacuolar sap to the cytosol via membrane proteins. To date, vacuolar exporting systems have not been well described. However, both a sugar exporter and an inorganic phosphate exporter have been reported (Martinoia et al. 2012, Wang et al. 2015).

**Proteins in the vacuolar sap**

Carter et al. (2004) and Jaquind et al. (2007) have provided some proteomic data for vacuolar sap. Most of their work, however, has focused on the proteomic analysis of the vacuolar membrane. Here, we have described in greater detail the proteome of the vacuolar sap rather than the membrane fraction (Fig. 3; Table 2: Supplementary Table S4). As expected, many hydrolases such as esterase, aminopeptidase or galactosidase were detected, as expected for an organelle that plays a major role in degrading and recycling cellular substances.

**Materials and Methods**

**Plant material and culture**

*Arabidopsis thaliana* suspension-cultured cells (supplied courtesy of Dr. Umeda, Nara Advanced Institute of Science and Technology) were cultured in modified Murashige and Skoog medium supplemented with 4.5 μM 2,4-D and 3% sucrose. An aliquot of 2–3 ml of cell suspension was transferred to 20 ml of fresh medium every 7 d. Cells were cultured with shaking (125 r.p.m.) at around 23 °C in the dark.

*Chara australis* (formerly called Chara corallina) cells were cultured in a bucket filled with tap water supplemented with an extract of rotten leaves under 14 h/10 h light/dark cycles at 25 °C.

**Isolation of intact protoplasts and vacuoles from Arabidopsis suspension-cultured cells**

Protoplasts and vacuoles were isolated from Arabidopsis suspension-cultured cells by modifying a method described by Shimaoka et al. (2004). After the cell wall digestion, the protoplasts were washed twice by density gradient centrifugation of isolation media. The washed protoplasts were used for analyses of the metabolites.

For the metabolomic analyses of the isolated vacuoles, they were also washed and concentrated after isolation of the intact vacuoles. Percoll is
essential for the isolation and purification of vacuoles, but it interferes with the metabolic analyses. Once purified, vacuoles were washed and concentrated by density gradient centrifugation with 500 mM sucrose and 500 mM betaine monohydrate to remove Percoll from the isolation medium.

**Preparation of Arabidopsis vacuolar sap for metabolic analysis**

The washed protoplasts and vacuoles were disrupted by freezing, then 100% methanol was added to the vacuolar medium to a final concentration of 70%. The vacuoles were incubated in the methanol solution on ice for several minutes and then centrifuged (120,000 g, 75 min). The supernatant, which contained the vacuolar sap, was frozen with liquid nitrogen and dried with a freeze-dryer (FreeZone 1 L, LABCONCO).

**Isolation of vacuolar sap from an intermodal cell of Chara australis**

In order to avoid possible complications associated with isolation of vacuoles from complex tissue such as Arabidopsis (e.g. metabolic changes, contamination with other organelles or cytoplasm), a more rapid and direct technique was used to sample vacuolar sap from a single internode of the giant-celled alga *C. australis*. This involved isolating a single intermodal cell from neighboring cells, allowing it to lose turgor in air, then excising both ends on a polycarbonate board. By inclining the board, vacuolar sap flowed from a cut end and was collected for analysis (Mimura et al. 1990, Okawa et al. 2011).

**Calculation of vacuolar volumes and contents**

α-Mannosidase is known to be a marker enzyme for vacuoles (Kaiser et al. 1986). To quantify the vacuolar contents, α-mannosidase activities in the vacuoles, protoplasts and cells were measured in individual experiments according to Mimura et al. (1990). The fresh weight of cells equivalent to α-mannosidase activity OD₄₅₀ = 1.2 was 143 mg. We have assumed that 10% of the total cell weight is cell wall, and vacuoles occupy 80% of the rest of cells. Thus, the vacuolar volume of cells equivalent to OD₄₅₀ = 1.2 was estimated as 104 μl. The volume of vacuoles equivalent to α-mannosidase activity OD₄₅₀ = 0.4, 0.6 and 1.2 was subjected to CE-MS analyses of metabolites. ‘OD₄₅₀ = 1’ corresponds to 1.3 × 10⁻³ μmol p-nitrophenol (p-PNP) min⁻¹. In the case of proplasts, the equivalent α-mannosidase activity was OD₄₅₀ = 0.24.

**Sample preparation for CE-MS analysis**

For the CE-MS analyses, the method reported by Harada et al. (2006, 2008) and Okawa et al. (2011) was modified. The cultured cells were collected on filter paper and washed with milli-Q water. A 143 mg aliquot of fresh cells having α-mannosidase activity equal to a sample of the vacuolar sap were transferred into a 2 ml plastic tube. The cells were frozen in liquid nitrogen, homogenized with zirconia beads in a tissue grinder (SH-48, KURABO) at 1,200 r.p.m. for 3 min. Freeze-dried vacuolar sap, proplasts and homogenized cells were re-suspended in a mixture of 500 μl of methanol, 500 μl of chloroform and 200 μl of Milli-Q water containing internal standards (8 μM methionine sulfone for cation analyses, 8 μM PIPES and 8 μM camphor-10-sulfonic acid for anion analyses). The extracted solution was thoroughly stirred with a vortex mixer for 5 min then centrifuged at 15,000 × g at 4 °C for 5 min. Then 500 μl of supernatant was transferred to a new 1.5 ml plastic tube; 100 μl of distilled water was added to the samples, then centrifuged again. The supernatant was centrifugally filtered through an Ultrafree MC, 5 kDa cut-off filter (Millipore) at 10,000 × g for 60 min. The filtrate was dried with a centrifugal concentrator (PV-1200, Wakenyaku Co. Ltd.) then the residue was dissolved in 20 μl (vacuole) or 50 μl (whole cell) of distilled water and used for analyses with CE-QTOF.

**Measurement of metabolites by CE-MS**

Analysis of vacuolar metabolites was performed by the Agilent CE system coupled with the Agilent 6520 accurate-Mass QTOF system (Agilent Technologies) (Sawada and Nogami 2004). Both systems were controlled by a MassHunter Workstation Data Acquisition for QTOF (Version B. 04). MS/MS spectra were analyzed by MassHunter Workstation Qualitative Analysis (Version B. 04). CE separations were carried out with a fused silica capillary (TS5050375, Picospray). The capillary dimensions were 50 μm i.d. and 100 cm in length. The capillary temperature was maintained at 20 °C. For cationic compounds, the electrolyte for the CE separation was 1 M formic acid (pH 1.5). For anionic compounds, the electrolyte for the CE separation was 30 mM ammonium formate adjusted to pH 10 with ammonium hydroxide. The sample solutions were injected at 50 nM bar for 3.5 s (± 4 nl) for cation analyses and 10 s (± 10 nl) for anion analyses. Prior to each run, the capillary was flushed with electrolyte for 5 min. The applied voltage was set at 25 kV for cations and 30 kV for anions. The sample tray was cooled below 4 °C. Fifty percent (v/v) methanol–water was delivered as the sheath liquid at 8 μl min⁻¹. QTOF measurement was conducted in the positive ion mode and the negative ion mode, with the capillary voltage set at 4 kV for cations and 3.5 kV for anions. The flow rate of heated dry nitrogen gas (heater temperature 300 °C) was maintained at 10 p.s.i.g. In QTOF, the fragmentor, skimmer and Oct RFV voltage were set at 100, 50 and 160 V, respectively. Exact mass data were acquired at a rate of 1.5 cycles s⁻¹ over a 60–1,000 m/z range.

For some measurements, another CE-MS/MS system was used as described by Harada et al. (2008). For the measurements of *Chara* vacuolar contents, the CE-TOF was performed as described by Watanabe et al. (2008) and Okawa et al. (2011).

MS/MS spectra were analyzed by MassHunter Workstation Qualitative Analysis. The metabolite levels were calculated as peak area ratios to methionine sulfone for cation analyses and to PIPES or camphor-10-sulfonic acid for anion analyses.

**Reagents for CE-MS analysis**

For the analysis of cationic metabolites, a 17 amino acid standard mixture ampule (Agilent Technologies) containing glycine, alanine, serine, proline, valine, threonine, isoleucine, leucine, aspartate, lysine, glutamate, methionine, histidine, phenylalanine, arginine, tyrosine and cysteine was used to prepare concentrations of 100 μM by diluting with pure water before use. Other cationic compounds were prepared at a concentration of 100 μg ml⁻¹ each in 0.1 N HCl or pure water. All anionic metabolites were prepared at a concentration of 100 μg ml⁻¹ each in pure water. All standard solutions were stored at −30 °C until use.

Methionine sulfone and camphor-10-sulfonic acid were purchased from Wako Pure Chemical Industries Ltd. and Tokyo Chemical Industry Co., Ltd., respectively. Stock solutions were prepared in pure water (methionine sulfone) or 0.1 N HCl (camphor-10-sulfonic acid). All chemicals were analytical or LC-MS grade. Water was purified with a Milli-Q purification system (Millipore). Aspartate, adenosine, adenosine triphosphate, glutathione, tryptophan and uridine were purchased from Wako Pure Chemical Industries Ltd. Cysteine, γ-aminobutyric acid and ornithine were purchased from Sigma-Aldrich, Tokyo Chemical Industry Co. and Nacalai Tesque Inc., respectively. Citrate, malate, 2-oxoglutarate, oxaloacetate, G6P, 6-phosphogluconate (6PG) were purchased from Wako Pure Chemical Industries Ltd. Pyruvate, cisaconitrate, isocitrate, ribulose 5-phosphate (Ru5P), glucose 1-6-phosphate (G1P), fructose 1, 6-bisphosphate (FBP) and 3-phosphoglycerate (3PG) were purchased from Sigma-Aldrich, Succinate, fumarate, F6P, glyceraldehyde phosphate (GAP) and dihydroxycetone phosphate (DHAP) were purchased from Nacalai Tesque Inc. Ribose 5-phosphate (R5P) was purchased from MP Biomedical Inc.

**Measurement of metabolites by FT-ICR-MS**

The FT-ICR-MS system and conditions were as described by Mesfioui et al. (2012). Samples were introduced under both negative and positive ion modes using an Apollo II ESI ion source of a Bruker Daltonics 12 Tesla Apex Qe ESI FT-ICR-MS, at the College of Science Major Instrumentation Cluster (COSMIC), Old Dominion University, Virginia, USA.

In the negative ion mode, just before measurements, samples were diluted in 1:1 (v/v) H₂O/methanol solution with 0.1% ammonium hydroxide (pH 9). The addition of NH₄OH was to improve the ionization efficiency of natural organic matter (Sleghter and Hatcher 2007). In the positive ion mode, samples were redissolved in 1:1 (v/v) H₂O/methanol solution with 0.1% formic acid. The addition of formic acid was to increase the ionization efficiency. Samples were continuously introduced by a syringe pump with an infusion rate of 120 μl h⁻¹.
and electrospray voltages were optimized for each sample. Ions were accumulated in a hexapole for 1.0 s before being transferred to the ion cyclotron resonance (ICR) cell. Two to three hundred transients were co-added for each sample and digitized with a 4 M Word data acquisition size. Fourier transformation and calculation of the magnitude of the free induction decay (FID) signal was accomplished using the Bruker Daltonics Data Analysis software.

Vacuole- and cell-specific peak lists were made as follows. Using the m/z of all peaks, we made a list of candidates for a molecular formula using AutoMF Calculator (Nakamura et al. 2008) under the following calculating conditions: C (min 1, max 95), H (min 0, max 15), N (min 1, max 45), S (min 0, max 5), monophosphate (min 0, max 6), diphosphate (min 0, max 6) and triphosphate (min 0, max 6). From all of the m/z peaks which predicted a molecular formula, we made an m/z list of each sample by removing blank peaks, then we selected molecular formulae for compounds that were found in more than two different samples. We identified metabolite candidates by searching the metabolite database KNAPSAcK, concentrating on plant taxa (Shinbo et al. 2006).

Measurement of enzyme activities

After centrifugation of isolated vacuoles (8,000 × g, 15 min at 4 °C), the supernatant was desalted by NAP-SH columns (Sephadex TM g-25 DNA Grade, GE Healthcare). Enzyme reactions were conducted in 100 mM citrate buffer pH 5.6 containing 5 mM MgCl$_2$ and 5 mM substrate (PEP, ATP, NADP, G6P, F6P, PPI and pNPP) for 30 min. In all measurements except for pNPP, inorganic phosphates liberated from substrates were measured by the Bencini method (Bencini et al. 1983). For pNPP phosphatase, the enzyme activity was evaluated by detecting the product, p-NP, at 405 nm. The protein content of vacuoles was measured by the Bradford protein assay method. In the case of Chara vacuolar sap, we measured phosphate activity using pNPP as a substrate.

Preparation of vacuolar sap samples for proteomic analysis

Isolated vacuoles were treated with 150 mM KCl and 0.05% deoxycholate to remove surface proteins from the vacuolar membrane, and then frozen at −80 °C to rupture the vacuoles. The thawed sample was then centrifuged at 120,000 × g at 4 °C for 75 min. The supernatant was used as the vacuolar sap fraction. A 150 μg aliquot of vacuolar sap proteins was precipitated by trichloroacetic acid and washed twice with 0.25 M Tris–HCl (pH 8.8) in 70% acetone and finally washed with 0.1 M Tris–HCl (pH 6.8). The proteins were then reduced with 6 M urea and 5 mM iodoacetamide at 37 °C for 30 min. After dialysis of the urea concentration by adding 20 mM ammonium bicarbonate, the protein samples were digested with trypsin (Promega) for 12 h at 37 °C for 60 min and alkylated with 32 mM iodoacetamide at 37 °C in the dark for 60 min. After dilution of the urea concentration by adding 20 mM ammonium bicarbonate, the protein samples were digested with trypsin (Promega) for 12 h at 37 °C. Peptide solutions were dried in an evaporator and stored at −20 °C before being analyzed by MS.

Proteomic analysis

Analysis of vacuolar proteins was performed by the Agilent 1200 HPLC-Chip system coupled with the Agilent 6520 accurate-Mass QTof system (Agilent Technologies). The chip consisted of a 40 nl enrichment column (Zorbax 300SB-C18 5 μ) and a 75 μm × 43 mm analytical column driven by the Agilent Technologies 1200 series nano-capillary LC system. Both systems were controlled by MassHunter Workstation Data Acquisition for QTof. MS/MS spectra were analyzed by MassHunter Workstation Qualitative Analysis. Peptides were re-suspended in water (0.5 μg μl$^{-1}$) and 2 μl was injected by a micro-well plate sampler and loaded into the enrichment column at 4 μl min$^{-1}$ in 0.1% (v/v) formic acid by the capillary pump. After the peptides were concentrated in the enrichment column, the chip was then switched to separation mode and peptides were eluted from the enrichment column and run through the separation column during 30 min and 1 h gradients [0–60% (v/v) acetonitrile] directly into the mass spectrometer at a flow rate 0.3 μl min$^{-1}$ by the nano pump.

QTof measurement was conducted in the positive ion mode. A flow rate of dry nitrogen gas (heater temperature 300 °C) was 51 min$^{-1}$ at 300 °C. The Vcap, fragmentor and skimmer were set at 1870, 175 and 65 V, respectively. Collision energy, slope 3.7 V, offset 2.5 V; MS scan range and rate, 300–2,400 at 1 Hz; MS/MS scan range and rate, 50–3,000 at 1 Hz.

Database searching was carried out using Mascot (Matrix Science). Proteins with a minimum of one peptide with a score more than the threshold level (significant score) were listed.

Supplementary Data

Supplementary data are available at PCP online.

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Disclosures

The authors have no conflicts of interest to declare.

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